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Manuscript submission: JOVE

Dear Vineeta,

Thank you for your recent correspondence in regards to an opportunity to revise our manuscript titled *"Stimulation and visualization of extracellular trap release in differentiated human monocyte-derived macrophages in vitro."*, for publication in JoVE. We have revised the manuscript carefully according to the editorial and reviewers comments, as detailed in the attached response letter. All changes made to the manuscript are shown in tracked changes.

We hope that the filming for the article can be completed by the end of September 2019, as the first author, who will be demonstrating the protocol is a final year PhD student, and will be submitting her thesis and completing her studies by this date.

In addition, would it be possible to copy all correspondence in relation to this manuscript to Dr Benjamin Rayner (Benjamin.rayner@hri.org.au) who is the co-corresponding author for this manuscript. This is particularly important for coordination of the filming, which will be undertaken at the Heart Research Institute in Sydney, and not at my location in Denmark.

Thank you again for your kind invitation and this publication opportunity,

Yours sincerely,

Professor Clare L. Hawkins

TITLE:

In Vitro Stimulation and Visualization of Extracellular Trap Release in Differentiated Human Monocyte-Derived Macrophages

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KEYWORDS:

extracellular trap, macrophage, MET, inflammation, SYTOX green, fluorescence microscopy

SUMMARY:

Presented here is a protocol to detect macrophage extracellular trap (MET) production in live cell culture using microscopy and fluorescence staining. This protocol can be further extended to examine specific MET protein markers by immunofluorescence staining.

ABSTRACT:

The release of extracellular traps (ETs) by neutrophils has been identified as a contributing factor to the development of diseases related to chronic inflammation. Neutrophil ETs (NETs) consist of a mesh of DNA, histone proteins, and various granule proteins (i.e., myeloperoxidase, elastase, and cathepsin G). Other immune cells, including macrophages, can also produce ETs; however, to what extent this occurs in vivo and whether macrophage extracellular traps (METs) play a role in pathological mechanisms has not been examined in detail. To better understand the role of METs in inflammatory pathologies, a protocol was developed for visualizing MET release from primary human macrophages in vitro, which can also be exploited in immunofluorescence experiments. This allows further characterization of these structures and their comparison to ETs released from neutrophils. Human monocyte-derived macrophages (HMDM) produce METs upon exposure to different inflammatory stimuli following differentiation to the M1 pro-inflammatory phenotype. The release of METs can be visualized by microscopy using a green fluorescent nucleic acid stain that is impermeant to live cells (e.g., SYTOX green). Use of freshly isolated primary macrophages, such as HMDM, is advantageous in modeling in vivo inflammatory events that are relevant to potential clinical applications. This protocol can also be used to study MET release from human monocyte cell lines (e.g., THP-1)

following differentiation into macrophages with phorbol myristate acetate or other macrophage cell lines (e.g., the murine macrophage-like J774A.1 cells).

INTRODUCTION:

The release of ETs from neutrophils was first identified as an innate immune response triggered by bacterial infection[1]. They consist of a DNA backbone to which various granule proteins with anti-bacterial properties are bound, including neutrophil elastase and myeloperoxidase[2]. The primary role of neutrophil ETs (NETs) is to capture pathogens and facilitate their elimination[3]. However, in addition to the protective role of ETs in immune defense, an increasing number of studies have also discovered a role in disease pathogenesis, particularly during the development of inflammation-driven diseases (i.e., rheumatoid arthritis and atherosclerosis[4]). The release of ETs can be triggered by various pro-inflammatory cytokines including interleukin 8 (IL-8) and tumor necrosis factor alpha (TNFα)[5,6], and the localized accumulation of ETs can increase tissue damage and evoke a pro-inflammatory response[7]. For example, ETs have been implicated as playing a causal role in the development of atherosclerosis[8], promoting thrombosis[9], and predicting cardiovascular risk[10].

It is now recognized that in addition to neutrophils, other immune cells (i.e., mast cells, eosinophils, and macrophages) can also release ETs on exposure to the microbial or pro-inflammatory stimulation[11,12]. This may be particularly significant in the case of macrophages, considering their key role in the development, regulation, and resolution of chronic inflammatory diseases. Therefore, it is important to gain a greater understanding of the potential relationship between ET release from macrophages and inflammation-related disease development. Recent studies have shown the presence of METs and NETs in intact human atherosclerotic plaques and organized thrombi[13]. Similarly, METs have been implicated in driving kidney injury through the regulation of inflammatory responses[14]. However, in contrast to neutrophils, there are limited data on the mechanisms of MET formation from macrophages. Recent studies using human in vitro models of MET formation show some differences in the pathways involved in each cell type (i.e., regarding the absence of histone citrullination with macrophages)[6]. However, some have shown that NET release can also occur in the absence of histone citrullination[15].

The overall goal of this protocol is to provide a simple and direct method to assess MET release in a clinically relevant macrophage model. There are a number of different in vitro macrophage cell models that have been used to study METs (i.e., the THP-1 human monocyte cell line and various murine macrophage cell lines)[16]. There are some limitations associated with these models. For example, the differentiation of THP-1 monocytes to macrophages usually requires a priming step, such as the addition of phorbol myristate acetate (PMA), which itself activates protein kinase C (PKC)-dependent pathways. This process is known to trigger ET release[4] and results in a low basal MET release from THP-1 cells. Other studies have highlighted some differences in bioactivity and inflammatory responses mounted by macrophages in vivo compared to PMA-treated THP-1 cells[17].

Similarly, the behavior and inflammatory responses of different murine macrophage-like cell lines do not completely represent the response spectrum of primary human macrophages[18]. Therefore, for the purpose of investigating macrophage ET formation in the clinical setting, primary human monocyte-derived macrophages (HMDMs) are believed to be a more relevant model rather than monocytic or murine macrophage-like cell lines.

ET release from M1 polarized HMDMs has been demonstrated following exposure of these cells to a number of different inflammatory stimuli, including the myeloperoxidase-derived oxidant hypochlorous acid (HOCl), PMA, TNFα, and IL-8[6]. Described here is a protocol to polarize HMDMs to the M1 phenotype and visualize subsequent MET release upon exposure to these inflammatory stimuli. PMA is used as a stimulus of MET release to facilitate comparisons to previous studies that have used neutrophils. Importantly, HOCl, IL-8, and TNFα are also used to stimulate MET release, which are believed to be better models of the inflammatory environment in vivo. The microscopic method for visualization of ET release involves staining the extracellular DNA in live cell cultures using SYTOX green, an impermeable fluorescent green nucleic acid stain that has been successfully applied in previous neutrophil studies. This method allows for rapid and qualitative assessment of ET release, but it is not appropriate as a stand-alone method for the quantification of ET release extent. Alternative methodology should be used if quantification is required to compare the extent of ET release resulting from different treatment conditions or interventions.

PROTOCOL:

The HMDM were isolated from human buffy coat preparations supplied by the blood bank with ethics approval from the Sydney Local Health District.

1. HMDM culture

1.1. Isolate the monocytes from buffy coat preparations prepared from the peripheral blood of healthy human donors using a commercially available preparation to isolate lymphocytes, followed by countercurrent centrifugal elutriation[19,20].

1.2. Confirm the presence of monocytes by cytospinning and staining with modified Giemsa stain for monocyte characterization[19].

1.3. Under sterile conditions, adjust the density of monocytes to 1 x 10^6 cells/mL using RPMI-1640 media without serum. Add 1 mL of this cell suspension to each well of a 12 well tissue culture plate. Culture in a cell incubator at 37 ˚C in the presence of 5% CO_2 for 2 h to promote adherence to the tissue culture plate.

1.4. Under sterile conditions, remove the cell media and replace with complete RPMI-1640 culture media containing 10% (v/v) pooled human serum and 20 mM L-glutamine.

1.5. Culture the cells at 37 ˚C in the presence of 5% CO_2 in a cell incubator for 8 days,

changing the media every 2 days.

2. Polarization of HMDM

2.1. Under sterile conditions, prepare the M1 priming media by adding interferon γ (IFNγ; 20 ng/mL) and lipopolysaccharide (LPS; 1 µg/mL) to the complete RPMI-1640 culture media. Prepare the M2 priming media by adding interleukin 4 (IL-4; 20 ng/mL) to the complete RPMI-1640 culture media.

2.2. Under sterile conditions, aspirate media from the tissue culture plate wells that contain the HMDM, which have been seeded and cultured as described in section 1.

2.3. Carefully wash the wells containing the cells 3x with sterile PBS (pre-warmed to 37 ˚C), using 1 mL aliquots of PBS.

2.4. Add 1 mL of either the M1 or M2 priming media to each well containing the HMDM (whichever is appropriate for the experiment).

2.5. Incubate the cells for 48 h at 37 ˚C in the presence of 5% CO_2 in a cell incubator.

3. Stimulation of HMDM to induce MET release

3.1. Under sterile conditions, prepare the culture media containing different stimulators of MET release (whichever is appropriate for the experiment) to the complete RPMI-1640 media: PMA (25 nM), human recombinant TNFα (25 ng/mL), or human recombinant IL-8 (50 ng/mL).

3.2. For experiments with HOCl stimulation, prepare HOCl (200 µM) in HBSS (pre-warmed to 37 ˚C), immediately before the addition to the cells. Ensure that the HOCl is not prepared in complete cell media.

NOTE: The concentration of the stock solution of HOCl is quantified by measuring the UV absorbance of the solution at 292 nm and pH = 11[6] and using an extinction coefficient of 350 $M^{-1}cm^{-1}$ [21].

3.3. After the polarization treatment described in section 2, aspirate the cell media from each well and carefully wash the cells 3x with 1 mL aliquots of either: sterile PBS (for PMA, TNFα and IL-8) or HBSS (for HOCl), which have been pre-warmed to 37 ˚C.

3.4. For experiments with PMA, TNFα, or IL-8: add 1 mL of the complete media containing PMA, TNFα, or IL-8 after removing the PBS in the final washing step.

3.5. For experiments with TNFα, incubate the cells for 6 h at 37 ˚C in the presence of 5% CO_2 in a cell incubator. For experiments with PMA and IL-8, incubate the cells for 24 h at 37 ˚C in the presence of 5% CO_2 in a cell incubator.

3.6. For experiments with HOCl, add 1 mL of HOCl in HBSS after removing the HBSS in the final washing step. Then, incubate the cells for 15 min at 37 °C in the presence of 5% CO_2 in a cell incubator.

3.6.1. Carefully aspirate the cell supernatant and wash the cells 3x with 1 mL aliquots of HBSS as described in step 3.3.

3.6.2. After removing the HBSS from the final wash step, add 1 mL of complete RPMI-1640 culture media. Then, incubate the cells for 24 h at 37 ˚C in the presence of 5% CO_2 in a cell incubator.

4. Visualization of MET in live cell culture

4.1. Prepare SYTOX green dye in HBSS at a concentration of 40 µM.

4.2. At the end of treatments described in section 3 to induce MET release, directly add 25 µL of 40 µM of the dye to each well containing HMDM.

4.3. Incubate cells at room temperature (RT) for 5 min in the dark.

4.4. Place the HMDM in tissue culture wells on the microscope stage of an inverted fluorescent microscope for imaging.

4.5. Microscope procedures

4.5.1. Turn on a broad-spectrum fluorescent light source, brightfield light source, and inverted microscope installed with a high-resolution color digital camera (see **Table of Materials**).

4.5.2. Rotate the filter wheel to the “number 2” position for green fluorescence (excitation = 504 nm, emission = 523 nm) for imaging of the green stained samples contained within the tissue culture wells.

4.5.3. Using the 5x objective, focus the image with the coarse focus, then the fine focus knobs on the microscope, until the image appears sharp, clear, and focused when viewed through the microscope eyepiece.

4.5.4. Switch the microscope to the camera mode.

4.5.5. Start the associated software.

4.5.6. Select the **Capture** tab on the software.

4.5.7. Click the **Play** button to preview the image and adjust the fine focus knob on the

microscope until the image appears sharp, clear, and focused in the software preview window.

4.5.8. Click the **Capture** button.

NOTE: The captured image will automatically be displayed in the accompanying software.

4.5.9. Within the software, click **File** | **Save as** the required image file type.

4.5.10. On the microscope, rotate the filter wheel to the "number 5" position for brightfield imaging and repeat steps 4.5.2–4.5.9 to obtain the corresponding brightfield image.

4.5.11. Repeat the steps 4.5.2–4.5.10 as necessary for subsequent image acquisition.

REPRESENTATIVE RESULTS:

Brightfield images showing the morphological changes of HMDM in response to stimuli for cell differentiation are shown in **Figure 1**. M1 polarized macrophages from experiments with HMDM exposed to IFNγ and LPS showed an elongated and spindle-like cell shape, as indicated by the black arrows in **Figure 1** (middle panel). For comparison, the morphology of the M2 polarized macrophages after exposure of HMDM to IL-4 for 48 h were typically round and flat, as indicated by the black arrows in **Figure 1** (far right panel).

The ability of differentiated HMDM phenotypes to release METs was visualized by live cell fluorescence imaging with SYTOX green, as presented in **Figure 2**. **Figure 2A** shows the control data obtained from each HMDM phenotype incubated for 24 h in the absence of any pro-inflammatory stimuli. In this case, there was very limited green staining, as was expected, given the cell impermeant nature of this stain. **Figure 2B** showed positive staining for METs, resulting from the exposure of M1 HMDMs to HOCl, PMA, IL-8, or TNFα. The METs are indicated by the white arrows, shown as green streaks, resulting from the strands of extracellular DNA. With HOCl, in addition to staining extracellular DNA, there was some green staining apparent in the cells. This cellular staining was also observed to some extent with the other stimuli and is believed to reflect loss of membrane integrity resulting from ET-independent cell death as a result of the treatment conditions.

Figure 2B also shows the corresponding experiments performed with M2 HMDMs, which were exposed to IL-4. In this case, there was no release of DNA from the cells, as indicated by the absence of the strands/streaks of extracellular DNA; though, there was some cellular uptake of green fluorescent dye with the HOCl and TNFα. For comparative purposes, **Figure 3** shows representative data indicating MET release from THP-1 macrophages exposed to TNFα (50 ng/mL) for 4 h. In this case, it should be noted that a non-polarized population of cells was used, and the THP-1 monocytes were differentiated to macrophages by pre-treatment with PMA (50 ng/mL for 72 h) as described previously[22].

FIGURE AND TABLE LEGENDS:

Figure 1: Morphological changes of differentially polarized HMDMs. Representative brightfield images from non-differentiated and differentiated HMDMs (n ≥5). HMDMs were cultured with complete media containing human serum and glutamine for 8 days before priming to the M1 or M2 phenotype upon exposure to IFNγ and LPS or IL-4, respectively, for 48 h. Scale bar indicates 200 µm. Arrows indicate examples of cells showing morphological characteristics of M1 or M2 HMDMs.

Figure 2: METs produced by M1 HMDMs following HOCl, PMA, IL-8, and TNFα stimulation. Representative images of SYTOX green stained HMDMs from (**A**) Non-stimulated M1 and M2 HMDMs incubated for 24 h in the absence of any inflammatory stimuli were used as the control, demonstrating the absence of MET release. (**B**) M1 and M2 HMDMs were treated with 1) HOCl (200 µM, 15 min), PMA (25 nM), or IL-8 (50 ng/mL) with incubation for 24 h or 2) TNFα (25 ng/mL) with incubation for 6 h to induce MET release. METs were visualized by the addition of SYTOX green, as indicated by white arrows in the upper panel from M1 HMDMs. No METS were seen in the corresponding experiments with M2 HMDMs. Data are representative of replicate culture wells from n ≥3 individual donors. Scale bar indicates 500 µm.

Figure 3: METs produced by non-polarized THP-1 macrophages following TNFα stimulation. Representative images of SYTOX green-stained TNP-1 macrophages, which were differentiated by pre-treatment with PMA (50 ng/mL for 72 h) before further incubation for 4 h in the absence or presence of TNFα (50 ng/mL) to induce MET release. METs were visualized by addition of SYTOX green. Data are representative of replicate culture wells from n ≥3 experiments. Scale bar indicates 100 µm.

DISCUSSION:

The generation and visualization of MET formation using M1 differentiated HMDMs represents a new in vitro model that may be useful for investigating the potential pathological role of these macrophage structures, particularly under chronic inflammatory conditions. It provides a robust protocol for the stimulation of primary human macrophages to release METs, which can also be utilized in related studies with human monocyte or murine macrophage cell lines. The successful implementation of this protocol for the stimulation of MET formation by HMDM is dependent on careful cell culture and handling to maintain good cell viability. This will increase the extent and quality of METs observed. To provide better cell nutrition, the RPMI media used to maintain the HMDM should contain human serum, rather than fetal bovine serum, as well as L-glutamine. Each stock solution of this complete RPMI media should be used within 1 week. Correct storage of the media is also important. The media should be stored at 4 ˚C in the absence of light.

In addition, it is important to regularly observe and monitor the shape and morphology of the cells by microscopy during the culturing of HMDM. Any abnormal or unexpected changes in cell shape observed prior to the addition of IFNγ and LPS or IL-4 (to polarize the macrophages) may indicate a reduction in cell survival rate. HMDM should be adherent and do not proliferate. It is

important, therefore, to monitor the cells for any changes in morphology, cell density, or loss of adherence during the normal 8 day culture period. A dramatic decrease in cell density during first 2 days post-isolation suggests that the resulting HMDMs may not be sufficiently viable for subsequent experiments. A limitation of using primary human cells is the variation between donors, which can markedly influence the extent of MET release. In addition, the quality of the buffy coat preparations received for the isolation of monocytes can vary. It is important to repeat experiments with at least three individual cell donors to ensure that data are representative of larger populations of cells.

It is important to note that the protocol described for HMDM differentiation has been optimized for cells isolated from human buffy coat preparations by countercurrent elutriation, which results in a high purity monocyte preparation. Validation of the polarization treatments (to confirm phenotype) by flow cytometry and assessment of the expression of M1 marker CD86 and M2 marker CD206 have been performed previously using this preparation of HMDM[6]. It is appreciated that this isolation method may be not be widely accessible, and that alternative isolation methods (i.e., magnetic bead sorting) may be preferable. If an alternative source of monocytes or different isolation protocols is used, additional flow cytometry experiments to validate phenotype change are recommended, and some optimization of treatment conditions to stimulate MET release may be required.

For optimal fluorescence images, exposure time should be carefully adjusted and kept as short as possible to avoid background fluorescence and staining artifacts from the plastic culture plates, which have adherent coatings. It is important to take multiple images from each well containing cells. It is advantageous if the samples are blinded before fluorescence microscopy analysis. In addition to staining extracellular strands of DNA, there is also evidence for the cellular uptake of SYTOX green. This is believed to reflect a loss in membrane integrity, which may be because of MET release, and it can also reveal alternative pathways of cell death (see also below). This highlights the importance of performing additional experiments to quantify and further characterize MET release.

For a quantitative comparison of the extent of MET release under different treatment conditions or following different interventions to modulate MET release, further analysis is required. This can be achieved by performing qPCR analysis of nuclear and mitochondrial DNA present in the cell supernatants, as described previously[6]. This method is usually combined with lactate dehydrogenase release assays to control for cell lysis unrelated to MET release[6]. The SYTOX green staining can also be quantified by a fluorescence plate reader following DNase treatment to partially digest the METs and release them from culture plates. This methodology has been used extensively in previous work with neutrophil NETs[1,2,23].

The future applications of this protocol relate to providing opportunities for further characterization of METs using immunohistochemistry. It will be possible to probe the protein composition of METs using this approach with targeted antibodies (i.e., against citrullinated histones or MPO) to gain additional insight into the roles of these structures in more complex biological samples. These experiments will also be important for shedding more light on the

delineation of MET release following other forms of cell death, such as pyroptosis[15]. It is worth noting that in our experience, METs from macrophages do not adhere to the plates as strongly as NETs from neutrophils, which can make immunohistochemistry analysis more challenging. Nonetheless, the data from these experiments will be of interest, given the fact that macrophages often play a dominant role in chronic inflammatory diseases. Further characterization of these structures is critical to assess their roles in vivo, which has thus far only been performed to a limited extent[16]. It is believed that the described method using HMDM to achieve this purpose may be more clinically relevant in comparison to other immortalized cell line-derived macrophage sources; although, these cell lines may have utility in providing a more stable model for MET characterization with less potential for donor variation.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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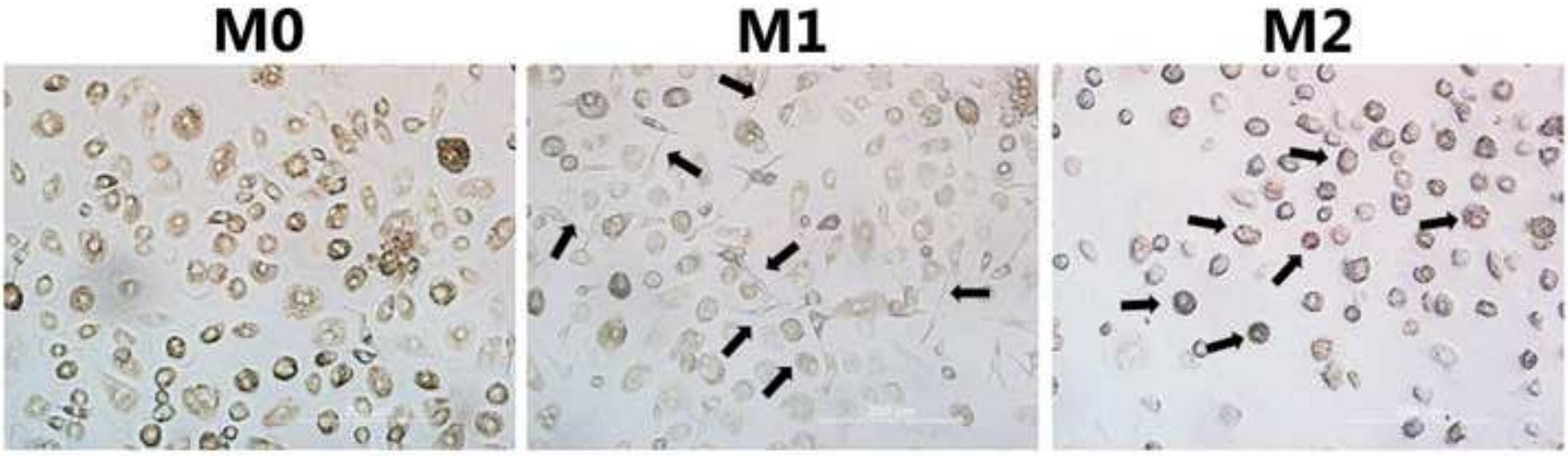

Figure 2

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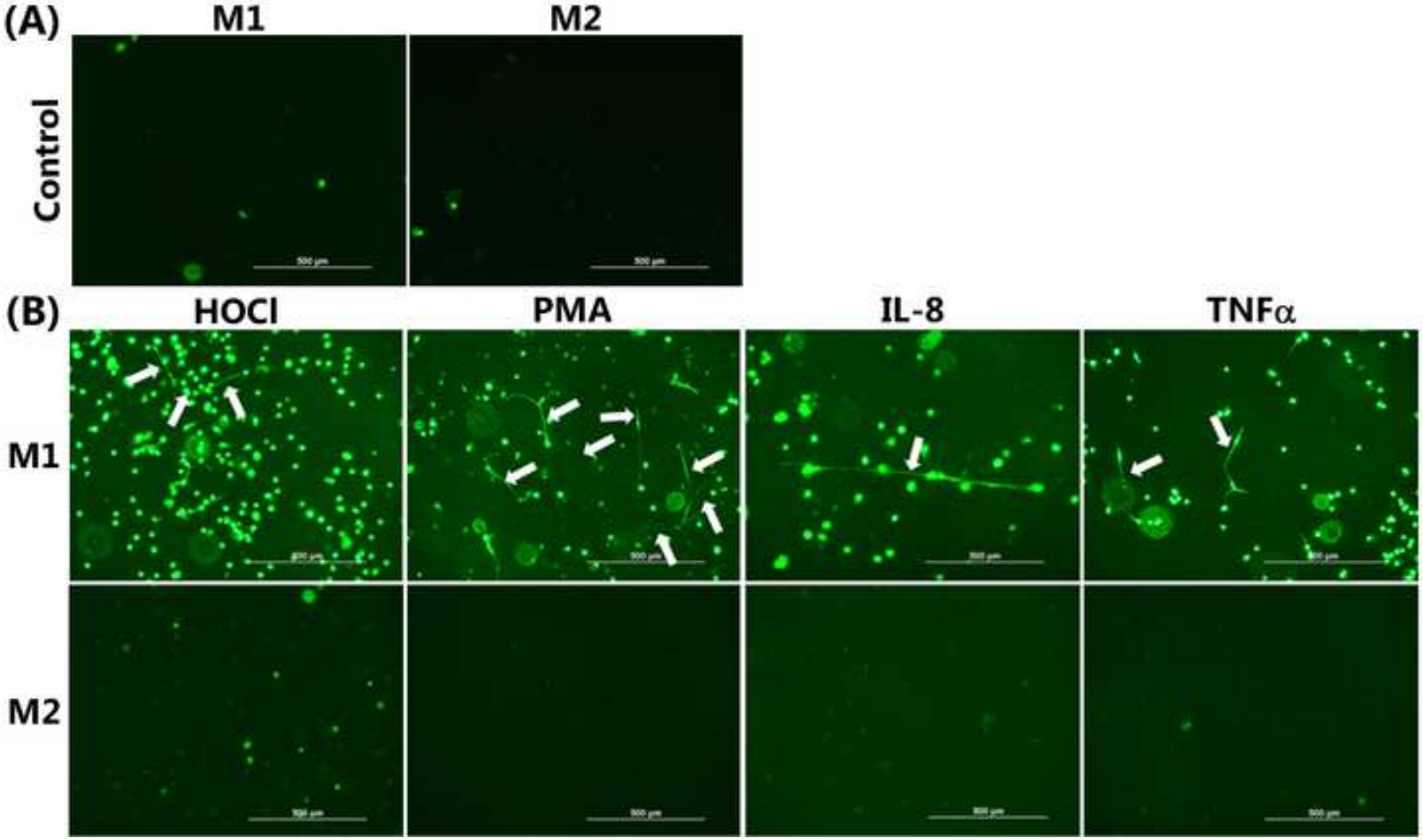
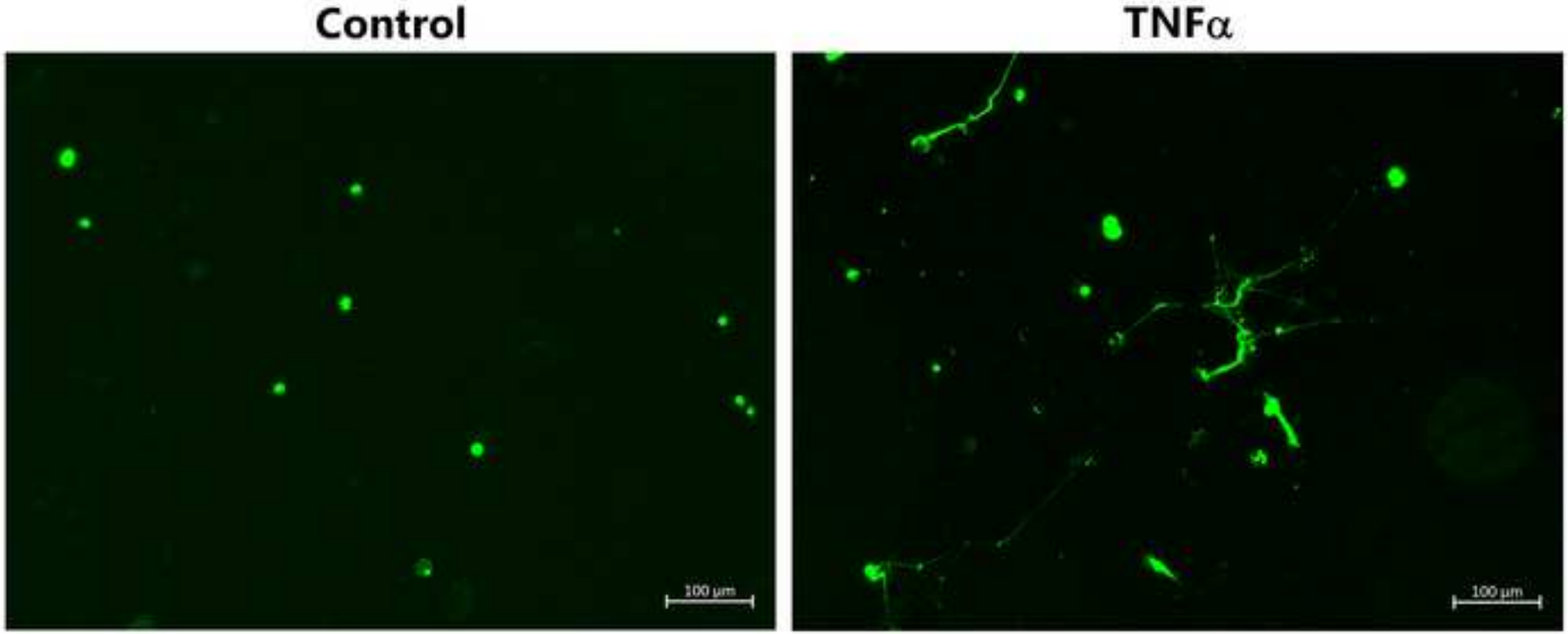


Figure 3



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
120Q broad spectrum fluorescent	EXFO Photonic Solutions, Toronto,		x-cite series
Corning CellBIND Multiple Well Plate (12 wells)	Sigma-Aldrich	CLS3336	For cell culture
Differential Quik Stain Kit (Modified Giemsa)	Polysciences Inc.	24606	Characterisation of monocytes
Hanks balanced salt solution (HBSS)	Thermo-Fisher	14025050	For washing steps and HOCl treatment
Hypochlorous acid (HOCl)	Sigma-Aldrich	320331	For MET stimulation
Interferon gamma	Thermo-Fisher	PMC4031	For M1 priming
Interleukin 4	Integrated Sciences	rhil-4	For M2 priming
Interleukin 8	Miltenyl Biotec	130-093-943	For MET stimulation
L-Glutamine	Sigma-Aldrich	59202C	Added to culture media
Lipopolysaccharide	Integrated Sciences	tlrl-eblps	For M1 priming
Lymphoprep	Axis-Shield PoC AS	1114544	For isolation of monocytes
Olympus IX71 inverted microscope	Olympus, Tokyo, Japan		
Phorbol 12- myristate 13-acetate (PMA)	Sigma-Aldrich	P8139	For MET stimulation
Phosphate buffered saline (PBS)	Sigma-Aldrich	D5652	For washing steps
RPMI-1640 media	Sigma-Aldrich	R8758	For cell culture
SYTOX green	Life Technologies	S7020	For MET visulaization
TH4-200 brightfield light source	Olympus, Tokyo, Japan		x-cite series

Tumor necrosis factor alpha	Lonza	300-01A-50	For MET stimulation

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Author(s):	Yunjia Zhang, Benjamin S. Rayner and Clare L. Hawkins

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Date:	9th July 2019

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RESPONSE TO EDITORIAL AND REVIEWERS COMMENTS

JoVE60541 "Stimulation and visualization of extracellular trap release in differentiated human monocyte-derived macrophages in vitro"

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

This has been done.

2. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The summary has been re-worded as requested, as follows:

"Here we present a protocol to detect macrophage extracellular trap (MET) production in live cell culture using microscopy and fluorescence staining. This protocol can be further extended to examine specific MET protein markers by immunofluorescence staining."

3. Please ensure the Introduction contains all of the following with citation:

a) A clear statement of the overall goal of this method

This has been added to the introduction at line 78 as follows:

"The overall goal of this protocol is to provide a simple and direct method to assess MET release in a clinically-relevant, macrophage model."

b) The rationale behind the development and/or use of this technique

We believe this has been adequately explained in the text provided in the introduction. The first 2 paragraphs outline the relevance of extracellular trap formation in pathology and the recent studies implicating macrophages as contributing to this phenomenon.

c) The advantages over alternative techniques with applicable references to previous studies

The advantages of the use of primary human monocyte-derived macrophages compared to immortalised cell lines has been described (see lines 79-89), with appropriate supporting references. Lines 97-99 have been rephrased to better emphasise the significance of using more clinically-relevant stimuli for MET release, as follows:

"We utilize PMA as a stimulus of MET release to facilitate comparison to previous studies with neutrophils. Importantly, we also utilize HOCl, IL-8 and TNFα to stimulate MET release, which we believe better models the inflammatory environment *in vivo*."

d) A description of the context of the technique in the wider body of literature

This information has been included in the first 2 paragraphs (lines 51-77) of the introduction.

e) Information to help readers to determine whether the method is appropriate for their application

Additional text has been added to clearly state that the method is qualitative not quantitative (see lines

102-106), which should help readers to determine the applicability of this method to their studies, as follows:
"This method allows a rapid, qualitative, assessment of ET release, but it is not appropriate to be used as a stand-alone method for the quantification of the extent of ET release. Alternative methodology should be used if quantification is required to compare the extent of ET release resulting from different treatment conditions or interventions, for example."

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example Lymphoprep (Axis-Shield PoC AS), Diff Quik, SYTOX green, etc.
We have removed "Lymphoprep" and "Diff Quik" and rephrased the accompanying text accordingly (see lines 114 and 118). However, SYTOX green is the name of the stain used in this protocol. Removing this information will make it difficult to understand and reproduce this protocol as the accompanying text would become unnecessarily cumbersome to read, as SYTOX green would be replaced with "cell impermeant green fluorescent DNA binding stain". Therefore for clarity, this information has to be retained.

5. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of a .xlsx file.
This has been done.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
This information has been added (see lines 110-111) as follows:
"The HMDM were isolated from human buffy coat preparations supplied by the blood bank with ethics approval from the Sydney Local Health District."

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
The text has been checked and we believe it is written as requested.

8. The Protocol should contain only action items that direct the reader to do something.
The text has been checked and we believe it is written as requested.

9. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
The protocol text has been checked and does not contain use of personal pronouns.

10. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step.
The text has been checked and additional steps have been added for clarity.

11. Please reformat the units as mg/mL instead of mg.ml-1.
This has been done.

12. Please ensure you answer the "how" question, i.e., how is the step performed?
This has been checked.

13. 1.1, 1.2: Please briefly describe the procedure to show the actions involved.
The isolation and characterisation of the monocytes is not part of this protocol and has been described in detail in the references cited in the text should the reader require further information.

14. For all steps involving the use of instruments, please include all knob turns, etc. For software steps, please provide click by click instructions. For example, click live to view the cells and then turn the right knob to adjust the focus. Please expand step 4 accordingly.
We have expanded the protocol to include the procedure for capturing the microscopy images in Section 4.5 of the protocol, see lines 196-229.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The steps for filming have been highlighted in yellow – it is sections 2,3 and 4 of the protocol.

16. Please include all the Figure and Table Legends together at the end of the Representative Results in the manuscript text.
This has been done.

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The images provided have not been used in a previous publication.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
The text in the discussion section has been carefully checked and we believe it contains the information required.

a) Critical steps within the protocol
The importance of good culture technique and storage of the media is indicated (see lines 291-305).

b) Any modifications and troubleshooting of the technique
Troubleshooting relates to the good cell culture technique described (lines 291-305).

c) Any limitations of the technique
This is described in lines 305-309 and lines 312-322.

d) The significance with respect to existing methods
This is described in lines 282-291.

e) Any future applications of the technique
This is described in lines 313-336.

19. Please do not abbreviate the journal titles in the references section.
The journal names have been written in full as requested.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
In the current manuscript Zhang et al. present a protocol for induction and visualization of extracellular traps released from monocyte-derived macrophages (METs).

Major Concerns:
-) The current manuscript covers induction and visualization of METs. However, quantification of the results is important in almost any study. The authors refer to methods to quantify extracellular DNA release by other means (e.g. PCR). Instead, it should be described in the manuscript how to quantify MET release on the Sytox-stained slides.
We agree with the reviewer that a limitation of the method is its qualitative rather than quantitative nature, which we have outlined in detail in the discussion (lines 328-335). We have added an additional comment to the introduction further highlighting the qualitative not quantitative nature of the protocol. Although it is possible to use the microscopy images in a semi-quantitative manner, we do not feel that it is appropriate to do this, and to avoid confusion and misleading the reader, we have not added the information requested.

-) Fig. 1 shows different morphology of differentiated M1 and M2 macrophages. However, the brightfield images in Figure 2a do not seem to confirm these morphological differences. Please explain or use different pictures.
There is some variation in the cellular characteristics in the brightfield images on comparison between different donors. Given that Figure 2 has been included to highlight the visualisation of the METs using SYTOX green, we have removed the brightfield images for clarity.

-) In Fig. 2B high exposure times have been used to visualize the delicate DNA strands. This can be concluded from the somewhat greenish background color of the IF images, which is however more faint in Fig. 2, especially in the M2 panel. Have different exposure times been used?
There is some slight variation in the exposure times used to best visualize the METs under different stimulation conditions, which highlights the limitation of this method in terms of quantification, as detailed in the Discussion (see also further comments below).

Minor Concerns:
-) Given that a debate about the delineation of MET formation from pyroptosis is still ongoing (Böltz et al., Cell Death Differ 2019, 26: 395-408) it would be very useful if the authors could comment if any other staining than extracellular DNA would be (more) specific for METs. Could any other markers for METs (like per example histone H4cit3) be used in addition to extracellular DNA?
There is no evidence for histone citrullination on stimulation of HMDM with any of the stimuli used in this protocol (see reference 6, Rayner et al, FRBM 2018). To address this comment, we have added an extra sentence to the discussion to state that further characterization of the METs is warranted to help delineate MET release from other forms of cell death (see lines 340-341).

-) Can the authors comment on the purity of their monocyte/macrophage culture after 8 days?
The purity of the monocytes is assessed immediately following elutriation and is greater than 90% as assessment by the modified Giesma staining protocol.

-) There are some typos in the manuscript, e.g. "monocyte" instead of "monocytes" in line 110, "observed" instead of "observe" in line 244.
We apologise for these typos, which have now been fixed.

Reviewer #2:
Manuscript Summary:
This manuscript describes a protocol for analysis of extracellular release of chromatin structures (extracellular traps) from differentiated human macrophages. Neutrophil extracelular traps have attracted considerable attention since they were first reported some 15 years ago, and there are still many unanswered questions about them. There is some evidence that macrophages undergo a similar process but they have been less studied, and any pathophysiological relevance is unclear. A case could be made for further investigation, and although this manuscript describes relatively standard procedures and presents nothing new, it does describe these clearly and gives some helpful hints. However, I think there are issues if this method is relied on alone, for interpretation of results.

Major Concerns:
so I hope that the final images will be an improvement.
The final images supplied are higher resolution and an improvement on the original images supplied.

Figure 1. The labelling of some cells with arrows implies that differentiation is sporadic. What is the form of the other cells, and how does that affect interpretation of results?
We have not specifically assessed the form of the cells that have not undergone MET release, though it is an important future direction, and could be possible with additional staining. The protocol is designed to be a qualitative method to assess MET release in live cell culture, therefore the presence of cells that have not undergone MET release does not affect the interpretation of the data.

Figure 2. Please explain why the brightfield images vary so much in color.
This was to some extent a result of a change in the microscopy equipment used to image the cells. We have changed the images included in the figures to minimize this variation.

The images in Figure 2 which provide the evidence for macrophage ET formation by the described protocol, are not in all cases very convincing,
We have revised Figure 2 and replaced the images to better highlight the delicate DNA strands in the METs.

The intracellular Sytox green staining also needs a better explanation. In neutrophils, where NET formation in most cases is a form of cell death, there is associated intracellular staining. What is the evidence that it is not the same here? I think that it will be hard to interpret results from the images shown - for example can you really say that here is a difference between M1 and M2 for HOCl and TNF, based on sparse ET staining in (3?) fields? What is recommended for validation?
This manuscript is focused on the protocol used to MET visualisation using polarised HMDM as a model macrophage cell type. The comprehensive analysis and comparison of MET release in the M1 and M2 polarised cells has been reported elsewhere (reference 6, Rayner et al, FRBM, 2018). However, to address this comment, we have added additional comments to the discussion (see lines 324-335) to explain the basis of the intracellular SYTOX green staining, and need for further validation to support MET release, as opposed to other forms of cell death, as follows:
"In addition to staining extracellular strands of DNA, there is also evidence for the cellular uptake of SYTOX green. This is believed to reflect loss in membrane integrity, which may be as a consequence of MET release, or reveal alternative pathways of cell death (see also below). This highlights the importance of performing additional experiments to quantify and further characterize MET release."

Minor Concerns:
1. Line 74. The neutrophil situation is not so clear cut, as a fairly recent publication from the Zychlinsky group shows no inhibition of NET formation by PAD4 inhibitors.
To address this comment, we have added extra text to the introduction to outline that NET release can also be independent of citrullination, and an additional supporting citation (see lines 74-76), as follows:
"However, recent studies show that NET release can also occur in the absence of histone citrullination (reviewed[15])."

2. Line 81. More information is needed. Is the Jensen work from this group, or has permission to describe it been given?
The Jensen work is from the Hawkins group at the University of Copenhagen, and Jensen has now been included as an author, on the basis of the request to include some data obtained with THP-1 cells (see response to reviewer 5).

3. Line 116. Pooled or autologous serum?
Pooled human serum – this has been added to the protocol (see line 127).

4. Line 137. Should flow cytometry be performed to confirm differentiation? Step 3.2.
We agree with the reviewer that this would be helpful should the source of monocyte or isolation method for monocytes differ to the one described here. Comments to reflect this have been added to the discussion (lines 310-319).

5. Line 145. Include how to determine HOCl concentration.
This information has been added to step 3.2 (see lines 158-160), as follows:

Note - the concentration of the stock solution of HOCl is quantified by measuring the UV absorbance of the solution at 292 nm at pH 11[6], using an extinction coefficient of 350 M^{-1} cm^{-1} [21].

6. Line 148. Why is the vehicle sometimes PBS and other HBSS?
The HBSS is used only for the experiments with the HOCl as a substitute for cell media to help maintain cell viability during the treatment, while avoiding confounding reactions of the HOCl with media components.

7. Line 162. Why 3 images? Use more standard terminology for magnification.
The protocol has been re-written to include more specific detail as to the microscopy procedure, which is in the new section 4.5 of the protocol.

Reviewer #3:
Manuscript Summary:
The manuscript by Zhang Y, et al describes the tissue culture of human monocyte-derived macrophages, which after stimulation may release DNA. The DNA fibers are designated as extracellular traps and are visualized by Sytox Green staining of the cells. Although, these DNA fibers may not represent real extracellular traps as those described for neutrophils (NET), this manuscript provides a clear and useful protocol for preparing macrophage cultures and for visualizing external DNA. Following this protocol other aspects of these macrophage extracellular nets can be studied.

Major Concerns:
A serious limitation of this protocol is the need for elutriation for preparing monocytes. This is probably the best method for isolating a pure population of cells. However, the method requires a very sophisticated and expensive equipment. Elutriation is not easily available to many laboratories. It would be useful to provide a more accessible alternative for obtaining monocytes.
We appreciate the comment by the reviewer, but we have not specifically investigated or compared different monocyte isolation methods, therefore feel it is not appropriate without validation to include this information in the protocol. However, we have added an additional paragraph to the discussion related to this point, stressing the need for additional validation by flow cytometry of phenotype change and the possibility for additional optimisation of the treatment conditions (see lines 310-319).

Minor Concerns:
There are few errors in the text.
In line 177, it reads "Place cells under an inverted fluorescent microscope for imaging with FITC and a bright field channel" DNA fibers are stained with Sytox Green and not FITC. This is confusing and should be corrected.
This section (4.5) of the protocol has been re-written and the specific excitation and emission wavelengths have been added for clarity.

Also, in line 180, it reads "For each well containing HMDM, take 3 images randomly under 200 μm or 500 μm magnification." The numbers shown represent linear measurements, not magnification. A better description of the microscope settings would improve clarity here.
We have included a more detailed description of the microscope settings in “Microscope procedure”

outlined in Section 4.5, including information on the specific magnification and objective to use for imaging.

Reviewer #4:
Manuscript Summary:
Well-written paper, but some additional discussion concerning Mi/M2 polarity and functionality could be included, since only M1 phenotype release ET.
We believe this type of discussion is outside the scope of the current manuscript, which is a methods paper. However, point is discussed in detail in our previous paper (reference 6, Rayner et al, FRBM, 2018).

Minor Concerns:
Using PBMC for polarization into M1 and M2 phenotypes is somewhat challenging, since different biomarkers for these phenotypes shows some overlap. Are the authors convinced that only M1 cells release ET? Only a few cells seem to release ET. One could include some additional phenotypic characterization in their assay.
Additional phenotype characterisation under these treatment conditions has been performed in our previous study, where we quantified surface markers by flow cytometry and alterations in cytokine expression. We have added discussion about the previous validation experiments performed and the need for additional validation should the source of monocytes or isolation method differ from that described here (see lines 310-319 and comments above).

Reviewer #5:
Manuscript Summary:
Similar to neutrophil extracellular traps (NETs), macrophage extracellular traps (METs) can be induced with specific stimuli under certain conditions. The authors proposed a means of stimulation of HMDM for visualizing METs via a membrane-impermeable DNA dye (SYTOX green). The methods provided by the authors are straightforward, and they should be useful for studying the phenomenon of METs.

Major Concerns:
Figures 1 and 2A: In the brightfield images provided by the authors, it was difficult to discern the different phenotypes of macrophage morphology (M1 vs. M2) and therefore obtain a sense of the extent of macrophage polarization. It appears that a substantial number of cells do not undergo differentiation in these images. In particular in Figure 2A, the two panels of M1 and M2 macrophages appear undiscernable. The authors should consider adjusting or modifying the protocol to obtain more optimal results on macrophage polarization, e.g. testing different time points or adjusting protocol for macrophage priming, as suboptimal polarization may impact subsequent experimentations and results interpretation.
Figure 1 has been revised to include more representative brightfield images. The protocol to polarize the HMDM for these experiments has been optimised and validated by flow cytometry and cytokine measurements, described in our previous work (see reference 6, Rayner et al, FRBM, 2018).

Minor Concerns:
1. Please provide quantitation of cell changes in the proposed protocol, so the visualized observations in the images can be properly assessed. For example, the extent of macrophage polarization as

performed in this protocol could be quantitated to aid the readers in assessing the effectiveness of the protocol. The production of METs could also be quantitated. Accordingly, a section on image quantitation for MET staining in the protocol would also be helpful.
We have added comments to the discussion in regards to the validation of phenotype change (see lines 310-319), as indicated above in the responses to reviewers 2 and 4, as the comprehensive analysis and comparison of MET release in the M1 and M2 polarised cells has been reported elsewhere (reference 6, Rayner et al, FRBM, 2018). In terms of the quantification, we hope the additional text provided in the discussion will be helpful in this regard (see lines 327-335), as we feel this protocol is qualitative and additional quantification is required by alternative methods.

2. In the abstract, the authors mentioned that the proposed protocol was also applicable to study with cell lines such as THP-1. No data supporting the claim were provided in the manuscript, however. The authors should demonstrate with additional data that the THP-1 model is amenable to the protocol's application.
We have included an additional Figure in the manuscript to show the release of METs from THP-1 macrophages treated with TNFα. A description of these data has been provided in the Representative Results section (see lines 253-256).