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Supplemental Information

Dopamine induces functional extracellular traps in microglia

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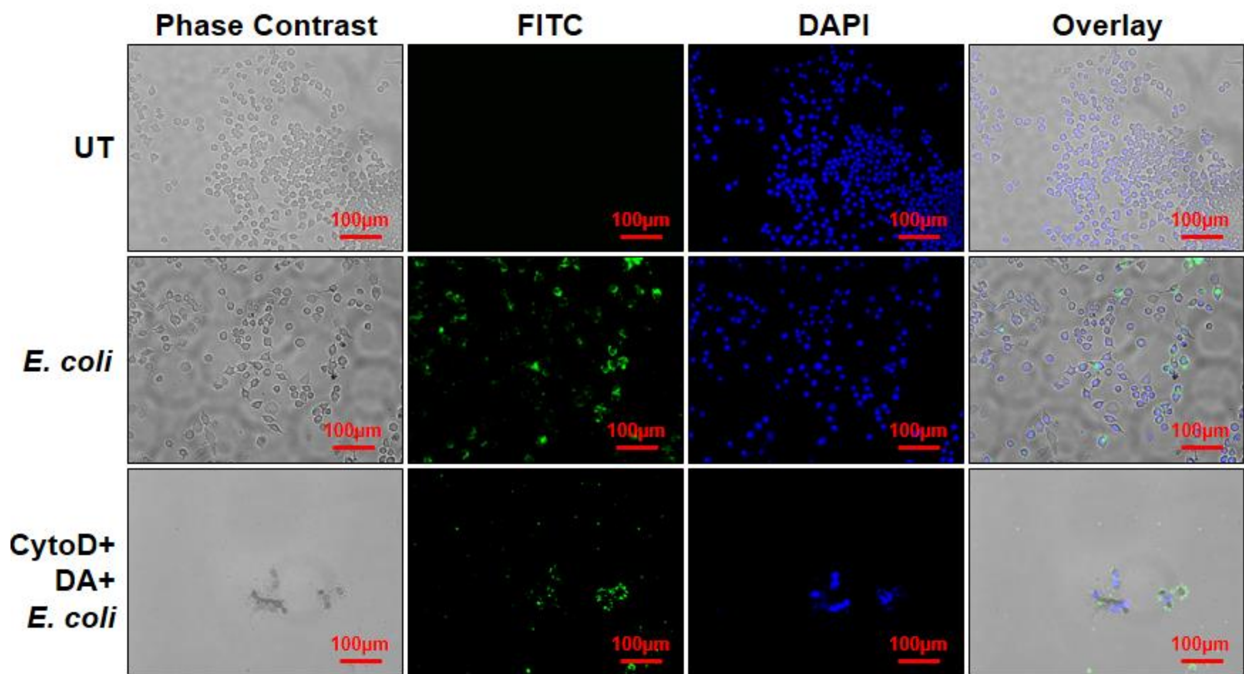

Figure S1. Dopamine Induces Formation of Functional Extracellular Traps Independent of Actin Polymerization, Related to Figure 5. BV2 microglia were pretreated with Cytochalasin D (CytoD) and were incubated with DA for 3 hours. FITC tagged *E. coli* (green) was added followed by DA incubation and cells were further incubated for 21 hours. ETs were stained with DAPI (blue). In the merged image green dots are overlapping with the blue ET suggesting that ETs are trapping *E. coli*. The images are the representative of two experiments. At least 7 frames were imaged per well of the two well chamber slide. Scale bars, 100µm.

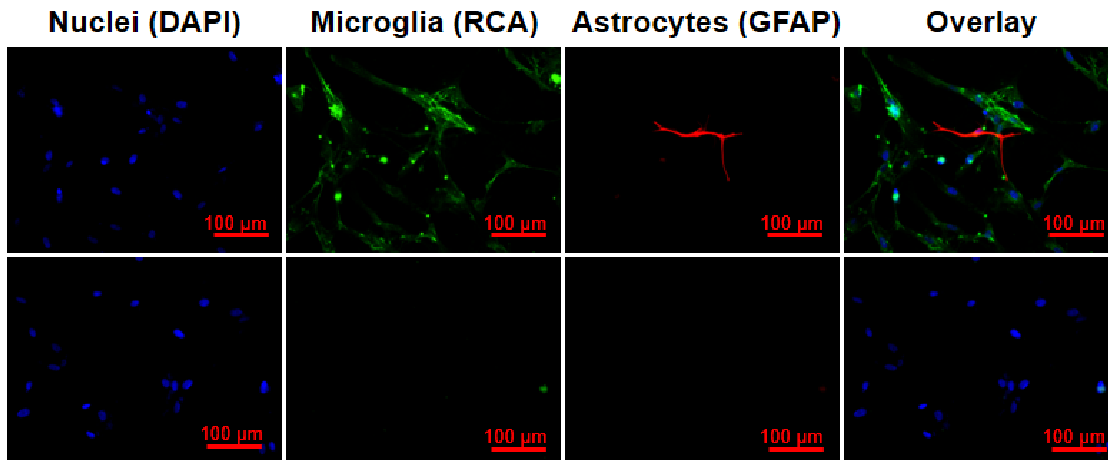

Figure S2. Primary Microglia Isolation from Adult Human Brain Tissue, Related to Figure 6. Primary human microglial isolated from adult human brain tissues. RCA (Green) was used as microglia marker and GFAP (Red) was used as astrocyte marker. About 80% of the cells isolated were microglia. Scale bars, 100μm.

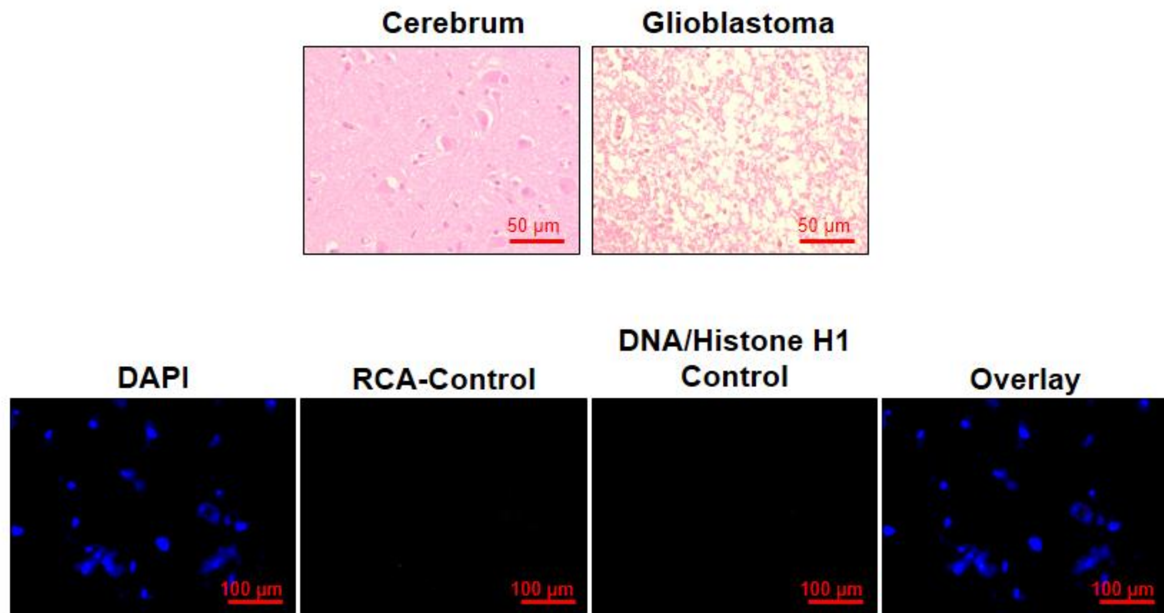

Figure S3. H & E Image, RCA Control and Secondary Antibody Control for DNA/Histone H1, Related to Figure 7. Scale bars, 50µm and 100µm.

Table S1. Patient information for the tissues used for IHC. Related to Figure 7.

Serial no.	Dianosis	Age/Gender	Mutations
1	GBM	70/M	IDHwt - MGMT low methylation
2	GBM	66/F	IDHwt - MGMT methylated

Transparent Methods

Cell culture

The murine microglial cell line BV2 were a kind gift from Dr. Anirban Basu (National Brain Research Centre, Gurgaon). The cells were cultured in DMEM, high glucose medium, supplemented with 10% FBS and 1 % antibiotic antimycotic stabilized solution (Himedia). The cells were grown in 96-well plate or in chamber slides as required. They were incubated at 37°C with 5% CO_2 for 24 hours (Eppendorf-170 S, Incubator). The confluent microplate or chamber slides were then used for experiments.

Primary human microglia isolation

Ethical clearance for acquiring tissues was taken from the Institute ethics committees of Indian Institute of Technology Jodhpur and All India Institute of Medical Science (AIIMS) Jodhpur. Informed consent was acquired for the use of tissue samples for experiments from human participants. A protocol for isolating primary microglia from adult human brain tissues was developed (Agrawal et al., 2020). Brain tissue was transported to lab in artificial cerebrospinal fluid (acsf) (2mM – $CaCl_2$-$2H_2O$, 10mM – Glucose, 3mM – KCl, 26mM – $NaHCO_3$, 2.5mM – NaH_2PO_4, 1mM – $MgCl_2$-$6H_2O$, 202mM – Sucrose) on ice. Tissue was washed for 5 minutes with acsf and then 5 minutes with PBS. Tissue was minced into small pieces and was incubated in 10ml trypsin for 25 minutes at 37°C. 10 ml media (DMEM/F12 with glutamine, 1% penicillin-streptomycin, 20% L929 supernatant, 10% FBS) was added and tissue was centrifuged at 2000xg for 10 minutes at 4°C. Supernatant was removed and the pellet was dissolved in media, plated and incubated at 37°C with 5% CO_2. On the 2nd day, considering the processing day as day 0, supernatant from the flask was collected and centrifuged at 1466xg for 4 minutes at 4°C. Supernatant was discarded and the pellet was plated in a fresh flask. Fresh media was added to the day 0 flask. Media of both flasks were changed again on the fourth and sixth day. The population of microglial cell were confirmed by staining them with *Ricinus communis* agglutinin-1 (RCA-1) lectin (Vector labs, FL-1081) (Jha et al., 2010). RCA stained cells were counted by blinded observers.

MTT assay

10,000 BV2 microglia cell were seeded per well in a 96 well plate. Cells were pretreated with either 10mM NAC for 3 hours or 10µM Cytochalasin D (CytoD) for 20 minutes and were incubated at 37°C with 5% CO_2. After pretreatment cells were treated with 250µM, 500µM, 750µM, 1mM of DA for 24 hours. DA containing media was removed carefully from the microplate. 100µl of fresh serum free media and 10µl of MTT solution (Sigma) was added to each well. The plate was kept in incubator (Model 170S, Eppendorf) at 37°C with 5% CO_2 in dark for 2 hours. After incubation, 100µl of acidic isopropanol solution was added to each well and mixed thoroughly using pipette. Absorbance at 570nm was measured using a multi-mode microplate reader (Synergy H1 Hybrid, Biotek Instruments Inc).

Immunocytochemistry

15,000 BV2 microglia cell seeded in each well of 2 well culture slides. Cells were pretreated with either 10mM NAC for 3 hours or 10µM CytoD for 20 minutes and were treated with mentioned concentration of DA or 24 hours. After treatment, cells were washed twice with 1X PBS for 5 minutes and were fixed for 10 minutes with 4% PFA. BV2 microglia cell were washed again twice and mounted with Fluoroshield with DAPI (Sigma-F6057). 15,000 primary adult human microglia cells were seeded in 2 well culture slides and treated with 2.5µM DA, LPS or 25nM PMA for 12 hours. 15,000 BV2 microglia cell were seeded in 2 well culture slides and treated with 250µM DA for 24 hours. After treatment, cells were washed twice with 1X PBS for 5 minutes and were fixed for 10 minutes with 4% PFA. Cells were washed again with 1X PBS and permeabilized with 0.1% TritonX-PBS for 15 minutes at room temperature. Further cells were blocked with 5% FBS in 0.1% TritonX-PBS for 1 hour in humidified chamber at 4°C and stained overnight in humidified chamber at 4°C with 1:1500 dilution of Anti-DNA/Histone H1 primary antibody (Merck, MAB3864) for primary cells and with 1:250 dilution of anti-Neutrophil Myeloperoxidase antibody (Sigma-Aldrich, N5787) for BV2 cells. After primary incubation, cells were washed and incubated for 1 hour with 1:500 dilution of anti-mouse or 1:1000 dilution of anti-rabbit secondary antibody. Cells were further washed and mounted with Fluoroshield with DAPI (Sigma-F6057). Images were taken using a fluorescence microscope (Leica Systems). Bright field images at 20X and 40X were taken on Nikon or Leica Microscope. Images were analyzed using ImageJ (Schneider et al., 2012).

Immunohistochemistry

We used 5-μm sections embedded in paraffin that were deparaffinized and rehydrated through alcohols (Jha et al., 2010). The paraffin embedded paraformaldehyde fixed glioma (grade 4, Glioblastoma) and normal brain tissue were obtained with approval from the Internal Review Board and the Ethics Committees of AIIMS, Jodhpur and Tata Memorial Cancer Hospitals. Informed consent was acquired for the use of tissue samples for experiments from human participants. We have performed all experiments in accordance with the ethical guidelines and regulations of All India Institute of Medical Sciences Jodhpur and Indian Institute of Technology Jodhpur. For the detection of microglia, tissues were stained with 1:500 *Ricinus communis agglutinin-1* (RCA-1) lectin (Vector labs, FL-1081) (Jha et al., 2010). Glioma (paraformaldehyde fixed paraffin embedded grade IV glioblastoma) and normal brain (paraformaldehyde fixed paraffin embedded) tissue sections were stained for DNA/Histone H1 using 1:300 of anti- DNA/Histone H1 antibody (Merck) primary antibody and 1:500 anti-mouse secondary antibody. Nuclei were stained blue with DAPI. Immunofluorescence was observed using fluorescence microscope (Leica Systems) and analyzed using ImageJ (Schneider et al., 2012).

Quantification of extracellular traps in tissues

5-μm embedded paraffin sections of GBM and cerebrum were stained as described in immunohistochemistry section. The number of punctate structure with RCA, for microglia, and DNA/Histone H1, for ETs, overlap were quantified by the algorithm developed by us. The average of the quantified value was represented on the graph. Error bars represent standard error. The data is representative of two experiments.

Quantification of extracellular traps in culture supernatant

The ETs in supernatant was quantified by measuring the fluorescence of digested traps (Robledo-Avila et al., 2018, Yoo et al., 2014, Joshi et al., 2013). 2,00,000 cells were seeded in 6 well plate. Cells were treated with 250μM of dopamine and were incubated at 37°C with 5% CO_2 for 24 hours. Following incubation 10U/ml DNase I Solution (Himedia) was added to the wells and the plate was incubated at room temperature for 15 minutes. 5mM EDTA was added to the wells to stop the reaction. Supernatant was collected and centrifuged at 300g at room temperature. After

centrifugation supernatant was transferred to clean centrifuge tubes. 200µl of supernatant was added to wells in a 96 well plate in duplicates. 5µM SYTOX™ Green Nucleic Acid Stain (Invitrogen) was added to the wells and the plate was incubated in dark for 15 minutes. The fluorescence was measured at excitation/emission = 485/530.

Extracellular traps functional assay

15,000 BV2 microglia cell seeded in 2 well chamber slides. After pretreatment with NAC or CytoD, as mentioned above, cells were treated with 250µM DA and incubated at 37°C with 5% CO_2. FITC tagged *E. coli* taken from Vybrant Phagocytosis Assay Kit (V-6694), was added after 3 hours of addition of DA. Cells were further incubated at 37°C with 5% CO_2 for 21 hours. Cells were washed twice with 1X PBS for 5 minutes and were fixed with 4% PFA. Then they were washed again twice with 1X PBS for 5 minutes and were mounted with Fluoroshield with DAPI. Images were taken using a fluorescence microscope (Leica Systems)

Detection of ROS

10,000 cells were seeded in 96 well microtiter plate and were incubated overnight at 37°C with 5% CO_2. Cells were treated with 20µM of 2′, 7′-Dichlorofluorescin diacetate (D6883 Sigma) for 30 minutes. Media was removed and the cells were washed once with 1X PBS. 100µl media was added to each well and cells were treated with 10mM NAC for 3 hours. Following this treatment cells were treated with 250µM DA for 24 hours. Fluorescence intensity indicating ROS generation was measured with a Synergy H1 Hybrid Multi-Mode reader (BioTek) at excitation and emission wavelength of 485/535 nm.

Algorithm and code for quantification of extracellular traps in tissues

There are three primary chromatic colors of light present there in the digital cell images. Those are red (R), green (G) and blue (B). As we know, any digital color image consists of those three color channels and any other color is a combination of these three with different proportion (Koschan and Abidi, 2008, Gonzalez and Woods, 2018). We have utilized this basic property of digital color images while determining the

overlapping between different regions. We have extracted out the three different color channels from the color image and processed those separately to determine the overlapped regions. That is, we perform a thresholding operation on each of the R, G and B images separately and carried out intersection operations between them.

Say, I is the colored cell image under consideration. In the other words, it can be said that I is a matrix of size $MXNX3$. Now, after extracting out three color channels there will be three different images each of size MXN. Let those be denoted as I_R, I_G and I_B. Let we set three threshold values T_R, T_G and T_B to extract out the regions with higher values of these three colors in the three images. The thresholding operation on I_R is carried out according to the equation given below.

$$B_R = 1 \; if \; I_R > T_R \\ \quad 0 \quad otherwise$$

B_R is the binarized image that we receive after performing thresholding on I_R. Please note, the pixels with value '1' in B_R represents the pixels with higher red component present in it. Similarly, we can have two more binarized images from I_G and I_B as B_G and B_B with threholds T_G and T_B respectively.

To determine the overlapped regions between red and green regions we perform $B_R \cap B_G$. The intersected region will reflect the overlapped areas of red and green regions in the cell image. The same will be performed between $B_G \cap B_B$, and $B_R \cap B_B$ to determine the green-blue and red-blue overlapped regions.

Further, we have eliminated the noisy regions those were present in the intersected image. We have considered the small regions as noise and eliminated them. The small regions are chosen to be of 10% of size of the largest region that is present in the intersected image. The remaining regions will finally be labelled as the overlapped regions.

The code for quantification is as follows:

```
clear;

I1=imread('C:\Users\RISHABH\Documents\IHC-Glioma&Cerebrum-ASC-P3-CD11b-
quantificationImages\IHC-Glioma&Cerebrum-ASC-P3-CD11b-
quantificationImages\Cerebrum_CD11b&ASC\edited-Blue-35B&50C&RedGreen-
```

```
50B&40C\IHC-Glioma&Cerebrum-ASC-P3-CD11b-
quantificationImages\Cerebrum_CD11b&ASC\edited-Blue-35B&50C&RedGreen-
50B&40C/image0466.tif');

[xr yr]=find(I1(:,:,1)>100 & I1(:,:,2)>70 & I1(:,:,3)>70);

I2=uint8(zeros(size(I1)));

a1=size(xr)

for i=1:a1

I2(xr(i),yr(i),:)=255;

end


bw=im2bw(I2);

se = strel('sphere',5);

    dilatedBW = imclose(bw, se);

figure, imshow(I1)


%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%

measurements=regionprops(dilatedBW,'Area');

      %a1=measurements.Area;

      areaIm=zeros(1,length(measurements));

      if length(measurements)>1

      for k = 1 : length(measurements)

   areaIm(k) = measurements(k).Area;

      end
```

```
 sz=floor(max(areaIm)/10);

 NewBW=bwareaopen(dilatedBW,sz);

%imshow(NewBW);

%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%%
%%%%%%%%%%%%%%%


  stats = regionprops('table',NewBW,'Centroid', ...

                  'MajorAxisLength','MinorAxisLength');


  % Get centers and radii of the circles

  centers = stats.Centroid;

  c1=size(centers);

  diameters = mean([stats.MajorAxisLength stats.MinorAxisLength],2);

  radii = diameters/2;

  c11=c1(1);

  if c11<1

    c11=0;

  end

wrt1=sprintf('Regions with Red Green Blue Overlapping = %d', c11);

title(wrt1)

  % Plot the circles

  hold on

  viscircles(centers,radii);
```

```
          hold off
wrt2=sprintf('RedGreenBlueOverlap.tif');
imwrite(I1,wrt2);
        else
            print('No Overlapping RedGreenBlue');
        end
[xg yg]=find(I1(:,:,1)>100 & I1(:,:,2)<50 & I1(:,:,3)>70);
I2=uint8(zeros(size(I1)));
a1=size(xg);
for i=1:a1
I2(xg(i),yg(i),:)=255;
end
bw=im2bw(I2);


se = strel('sphere',5);
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        radii = diameters/2;
        wrt1=sprintf('Regions with Red Blue Overlapping = %d', c11);
        title(wrt1)
        % Plot the circles
        hold on
        viscircles(centers,radii);
        hold off
 else
          print('No Overlapping RedBlue');
       end
        %wrt2=sprintf('RedBlueOverlap.tif');
%imwrite(I1,wrt2);


[xg yg]=find(I1(:,:,1)<50 & I1(:,:,2)>70 & I1(:,:,3)>70);
I2=uint8(zeros(size(I1)));
a1=size(xg);
for i=1:a1
I2(xg(i),yg(i),:)=255;
end
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        c11=c1(1);
        if c11<1
            c11=0;
        end
        diameters = mean([stats.MajorAxisLength stats.MinorAxisLength],2);
        radii = diameters/2;
        wrt1=sprintf('Regions with Green Blue Overlapping = %d', c11);
        title(wrt1)
        % Plot the circles
        hold on
        viscircles(centers,radii);
        hold off
      end
[xg yg]=find(I1(:,:,1)>70 & I1(:,:,2)>50 & I1(:,:,3)<50);
I2=uint8(zeros(size(I1)));
a1=size(xg);
for i=1:a1
I2(xg(i),yg(i),:)=255;
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end

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        viscircles(centers,radii);

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end
```

Supplemental References:

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