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Human primary neutrophil mRNA does not contaminate human resolving macrophage mRNA after efferocytosis

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Abstract

The ingestion of apoptotic corpses by macrophages, a process called efferocytosis, is a crucial step in inflammation resolution since it alters macrophage phenotype toward a pro-resolving profile to foil inflammation and to favor tissue repair. Up to now, the resolving macrophages remain poorly characterized, especially in humans. Global investigations, like RNA sequencing, would be very helpful to unravel some features of these elusive cells. Nonetheless, these inquiries may be challenging in a single-specie model since the fate of ingested mRNA remains unknown and may hinder any subsequent mRNA investigations in the phagocyte. A full human model consisting of primary human neutrophil and primary human monocyte-derived macrophage co-culture was set up several decades ago to mimic *in vitro* the efferocytosis process. However, to our knowledge, this model has not been characterized as a suitable model to perform global mRNA investigations. Indeed, the extent of ingested neutrophil mRNA contamination has not been assessed in resolving macrophages. This work answers to this crucial question. Indeed, based on the protocols presented in this article, we demonstrate that neutrophil mRNA is severely degraded and is not able to cross-contaminate resolving macrophage mRNA, contrary to apoptotic human peripheral blood derived mononuclear cell (PBMC) or apoptotic leukemic Jurkat cell mRNA. Moreover, this allogenic co-culture system does not favor neither neutrophil activation nor macrophage pro-inflammatory cytokine release. Collectively, we highlight that this model of primary human neutrophil and primary human monocyte-derived macrophage co-culture is the best model for mRNA investigations in human resolving macrophages to help improving our knowledge on these crucial cells.

Key words: human, resolving macrophage, apoptotic neutrophils, efferocytosis, inflammation resolution, RNA sequencing

List of abbreviations: resolving macrophage (M^R), apoptotic neutrophils (AN), monocyte-derived macrophages (MDM), Carboxyfluorescein succinimidyl ester (CFSE), interferon (IFN), interleukin (IL), Macrophage colony-stimulating factor (M-CSF), phorbol 12-myristate 13-acetate (PMA), tumor necrosis factor (PMA), peripheral blood-derived mononuclear cells (PBMC).

1. Introduction

Macrophages constitute a heterogeneous population of innate immune cells. They are present in all tissues and play a role in development, tissue and metabolic homeostasis, tissue repair and host defence (1). They are able to phagocytose pathogens, infected and dead cells and to display processed antigens in association with major histocompatibility class II complex (2). They also produce various mediators like cytokines, chemokines, enzymes, growth factors, reactive oxygen intermediates or lipid mediators. During the past several decades, many macrophage subtypes have been identified and characterized based on their biological functions, phenotypes, secretomes, and metabolomes. So far, two major distinct states have been described (3, 4):

- Classically activated macrophages (also called M1-like) that exhibits powerful anti-microbial and anti-tumoral activity. They are induced by inflammatory stimuli, such as lipopolysaccharide, TNF α and IFN- γ .

- Alternatively activated macrophages (also called M2-like) that have enhanced phagocytic abilities, can promote tissue repair and possess pro-angiogenic and pro-fibrotic properties. They are generated in response to certain cytokines, such as IL-4, IL-10 or IL-13. Thereafter the M2 state has been subdivided into four categories depending on the inducing stimuli (3): M2a-like generated with IL-4 and IL-13, M2b-like induced by stimulation with immune complexes and TLR-agonists, M2c-like generated with glucocorticoids, TGF- β and IL-10, and M2d-like (or tumor-associated macrophage [TAM]-like) induced by TLR agonists through the adenosine receptor.

As illustrated above, various stimuli can induce a plethora of phenotypes and functions. Moreover, macrophages are highly plastic cells and these stimuli can reprogram them by leading to phenotype and function switches. "M1-like" macrophages can adopt an "M2-like" phenotype with the appropriate stimuli and *vice versa* (5). Despite considerable advances, some macrophage phenotypes remain poorly characterized like resolution-phase macrophages (M^R).

Phagocytosis of apoptotic corpses, a process called efferocytosis, is a stimulus inducing profound phenotypical changes in macrophages. Efferocytosis gives birth to a new type of macrophage: the M^R (6–8). Efferocytosis is therefore considered as a major trigger of inflammation resolution, since it allows inflammatory macrophage reprogramming toward a

1 pro-resolving profile (8). Since the end of the 90s, several papers have shed light on some
2 critical features of M^R (9–15). For example, M^R have been demonstrated to regulate several
3 innate and adaptive immune cells (16, 17). However, most of these studies were conducted in
4 various mouse models. Up to now, data about human M^R are quite sparse, and these cells
5 remain overlooked. Global approaches like RNA sequencing may provide crucial
6 information on these cells.

7 An *in vitro* full human system based on the co-culture of human primary apoptotic
8 neutrophils (AN) and primary monocyte-derived macrophages (MDM) was set up several
9 decades ago and provided some features on human M^R (9). However, we do not hitherto
10 know if this single-specie model is suitable for mRNA investigations like RNA sequencing.
11 Indeed, mRNA arisen from human primary apoptotic neutrophils may contaminate M^R
12 mRNA. To alleviate this issue, macrophages and apoptotic bodies from different species can
13 be mixed to induce efferocytosis (10). Nonetheless this method may lead to some flaws since
14 interspecies sequence similarities can be high. For example, mouse and human genomes
15 share 40% of nucleotide alignment (18). A study has shown that the mapping of 60 RNA-
16 Sequencing data sets performed in Chinese hamster *versus* the human genome has a total
17 mapped rate of 22.8% (19). Moreover, ingestion of apoptotic bodies from another specie
18 may preclude some physiological mechanisms. For these reasons, a multi-species system,
19 despite presenting some advantages for RNA investigations, may not represent an optimal
20 model.

21 With this work, we answer to the crucial question: can ingested apoptotic neutrophil mRNA
22 contaminate macrophage mRNA in a single-specie model, thus precluding further
23 transcriptomic investigation in human M^R? To achieve this goal, we have reconstituted a
24 long been used model by co-culturing primary human AN with human primary MDM to
25 induce efferocytosis and human M^R generation. We show here that human primary
26 neutrophils, whether apoptotic or not, exhibit degraded mRNA after using a specific cell
27 purification protocol. By Q-PCR, we show that the remaining amplifiable neutrophil mRNA
28 are degraded and non-detectable, contrary to PBMC or Jurkat cell mRNA, after ingestion by
29 MDM. Moreover, we confirmed that macrophages are efficiently reprogramed by
30 efferocytosis and that purifying and mixing apoptotic neutrophils with allogenic MDM did
31 not induce neutrophil activation, nor inflammatory macrophage responses, as previously

1 shown (9, 20, 21). Together our data establish that this well-known co-culture system allows
2 global mRNA approaches to expand our knowledge of the elusive human M^R.

3

2. Material and Methods

2.1. Cell purification and culture

Leukocyte-platelet concentrates and cytopheresis kits were obtained from healthy volunteers after informed consent signature (authorization number #AC-2015-2408). Samples were collected from the French Blood Transfusion Center (EFS Bourgogne Franche-Comté, Besançon, France) and processed the same day. Several blood donors were used during this study.

Human primary neutrophils were isolated at room temperature from 50 mL of fresh leukocyte-platelet concentrates using a Ficoll-Paque density gradient ($\rho = 1.077$ g/mL, Eurobio) followed by sedimentation in a 3% dextran solution (Sigma). 25 mL of undiluted concentrate were layered over 15 mL of Ficoll-Paque in a 50 mL conical tube. After centrifugation at 900g, 20 min, the red blood cell's containing layer was collected and washed twice in PBS (centrifugation at 300g, 5min) and resuspended in 15 mL of PBS. Cell suspension was then diluted in a 3% dextran solution at a 1:3 ratio. After gentle homogenization, neutrophils were allowed to sediment for one hour. The upper layer, containing neutrophils, was collected and washed once in PBS. Red blood cells were lysed by incubation of the neutrophil pellet in a hypertonic solution (NH₄Cl (0.077 M), EDTA (0.633 mM), KHCO₃ (5 mM)). After an incubation of 5 min, PBS was added (to achieve 50 mL) in order to stop the reaction. Cells were centrifuged (300g, 3 min), and purified neutrophils were resuspended in complete medium (RPMI 1640-GlutaMAX-HEPES (Gibco), 10% fetal bovine serum (Gibco), 1X penicillin/streptomycin (Thermo-Fisher) , 1X nonessential amino acid (Lonza), 1X Sodium Pyruvate (Lonza)) and incubated at 37°C, 5% CO₂. Neutrophils were further handled carefully to avoid any mechanical stress.

Human monocytes were isolated from fresh cytopheresis kit using a Ficoll-density gradient followed by positive CD14⁺ cell selection (human CD14⁺ MicroBeads, Miltenyi). CD14⁺ magnetic separation was performed according the manufacturer recommendations. Purity was assessed by CD14 labelling by flow cytometry and was of at least 98%. CD14⁺ monocytes were resuspended in complete medium (RPMI 1640-GlutaMAX-HEPES (Gibco), 10% fetal bovine serum (Gibco), 1X penicillin/streptomycin (Thermo-Fisher), 1X nonessential amino acid (Lonza), 1X Sodium Pyruvate (Lonza)) with M-CSF at 50 ng/mL (Miltenyi) at a concentration of one million cells per mL. Cells were kept at 37°C, 5% CO₂ for 7 days to allow differentiation into macrophages and one volume of medium was added at day 3.

1 Peripheral blood-derived mononuclear cells (PBMC), isolated from cytopheresis kit by Ficoll-
2 Paque separation, and Jurkat cells were cultured in complete medium (RPMI 1640-GlutaMAX-
3 HEPES (Gibco), 10% fetal bovine serum (Gibco), 1X penicillin/streptomycin (Thermo-Fisher),
4 1X nonessential amino acid (Lonza), 1X Sodium Pyruvate (Lonza)). Apoptosis was induced in
5 Jurkat cells by incubation with 10 μ M of Camptothecin (Sigma, C9911) for 6 h and in PBMC by
6 X-ray irradiation (35 Gy, 8 min) using a RayCell CE X-ray irradiator (Best Theratronics).

7
8 For mixing experiments, apoptotic cells were harvested, centrifuged at 300g, 5 min, resuspended
9 in X-Vivo15 (Gibco) and mixed with macrophages at a 5:1 ratio for the indicated period. MDM,
10 cultured in a dedicated well, were previously harvested and counted to evaluate the number of
11 apoptotic cells required for each experiment. After 6 h or 24 h, the non-engulfed apoptotic cells
12 were then removed by extensive PBS washes, and macrophages were harvested by scrapping in a
13 PBS-10 mM EDTA solution and further processed. For LPS stimulation experiments, apoptotic
14 neutrophils were removed after 24 h and LPS (Lipopolysaccharides from *Escherichia coli*
15 O55:B5, Sigma) was added in X-vivo15 medium to MDM at 100 ng/mL for 24 h.

16

17 **2.2. Phenotyping and phagocytosis test**

18 For phenotyping experiments, CD14⁺ cells were seeded in a 6-well plate at a concentration of 1
19 million cells per mL in 2.5mL. After 7 days of differentiation, MDM were harvested, washed in
20 PBS and 100 000 cells were resuspended in 100 μ L PBS-3% FCS with 2 μ L of the appropriate
21 antibodies (see table 1). After 15 min incubation at 4°C in dark, cells were washed one time with
22 PBS, and resuspended in PBS before flow cytometry analysis (SP6800 Spectral Analyzer, Sony
23 or LSR Fortessa, Becton Dickinson).

24 For phagocytosis experiments, CD14⁺ cells were seeded in a 12-well plate at a concentration of 1
25 million cells per mL in 1mL. After 7 days of differentiation, MDM, cultured in a dedicated well,
26 were counted to evaluate the number of neutrophils required for each mixing experiment.
27 Apoptotic cells were labelled with 10 μ M CFSE (Invitrogen, C34554) for 10 min at 37°C or with
28 pHrodo (Thermo-Fisher, P35372). Reaction was stopped by complete medium addition (to
29 achieve 50mL), and cells were centrifuged at 300g, 3 min, resuspended in X-vivo15 and mixed at
30 a 5:1 ratio with MDM. MDM were harvested 24 h later and labelled, as described above with a
31 CD206-BV421 antibody. Gating strategy was specified in each appropriate experiment.

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2.3. Viability assessment

Cells were harvested at the indicated time point, and 100 000 cells were resuspended in 100 μ L of Annexin-V Binding Buffer (BD, 556454), incubated with 5 μ L of FITC-Annexin-V (BD, 556419) and 5 μ L of 7AAD (BD, 559925), for 15 min at room temperature in the dark. Cells were centrifuged at 300g, 5 min and resuspended in 100 μ L of Annexin-V Binding Buffer. Viability was assessed by flow cytometry within one hour (SP6800 Spectral Analyzer, Sony or LSR Fortessa, Becton Dickinson). Gating strategy was: definition of a SSC vs. FSC gate > doublet discrimination > Annexin V-FITC vs. 7AAD dot plot.

2.4. Cytokine measurement by ELISA

Experiments were conducted in a 6-well plate containing a final volume of 2.5 mL of X-vivo15. Two mL of supernatants were collected at the indicated time points and centrifuged 5 min at 300g to remove any cells. Samples were stored at -80°C while awaiting cytokine measurement. ELISA were conducted according to manufacturer's instructions on 100 μ L of supernatant. IL-6, TNF α , IL-10 and IL1- β kits were purchased from Biolegend and TGF- β kit from R&D Systems. Microplates were read with a Sunrise absorbance microplate reader (Tecan) at 450 nm and results were normalized to cell number.

2.5. RNA quality and Q-PCR experiments

Experiments were conducted in a 6-well plate. At the indicated time points, cells were harvested, centrifuged 5 min at 300g and the dry pellets were stored at -80°C, while awaiting RNA extraction. RNA was extracted with NucleoSpin® RNA Plus kit (Macherey-Nagel) according to manufacturer's instructions. RNA quantity was measured using a Nanodrop spectrophotometer (Thermo-Fisher). RNA quality was assessed using an Experion™ Automated Electrophoresis System according to manufacturer's instructions.

For Q-PCR experiments, RNA was first retrotranscribed using RevertAid H Minus First Strand cDNA Synthesis Kit with random primers, according to manufacturer's instructions (Thermo-Fisher, K1632). 12 μ L of RNA template was engaged in each reverse transcription reaction. Q-PCR experiments were then conducted on a CFX96 real-time detection system (Biorad) using either Taqman probes (*Elane* probes, Hs00236952_m1 (Applied Biosystem) ; for one reaction:

1 mastermix: 1µL of fluo mix probe, 10 µL of TaqMan Universal Master Mix II with UNG
 2 (Applied Biosystem), 5 µL H₂O. DNA template: 4 µL) or Sybr Green (SsoAdvanced™ Universal
 3 SYBR® Green Supermix, (BioRad) based probes (*CD66b*, *GAPDH*, *MT-ND6*, *IL-6*, *TNFα*,
 4 *CD3E* and *TRAC* probes, see table 2) (for one reaction: mastermix: 10µL of syber green
 5 Supermix, 0,06 µL of each primer at 100 µM, 6µL H₂O and 4µL of DNA template). *GAPDH*
 6 gene expression was used to normalize results when necessary.

7

8 **2.6. Elastase assay**

9 Elastase activity was assessed by Neutrophil Elastase (NE) Activity Assay Kit (BioVision, K383-
 10 100) according to manufacturer’s instructions. Briefly, supernatants were harvested at the
 11 indicated time points and centrifuged at 300g, 5 min to remove any cells. 50 µL of cell culture
 12 supernatant was mixed with 50 µL of NE substrate mix. Fluorescence was read at 380 nm in
 13 kinetic mode for 10 min using EnVision® Xcite Multilabel Reader (PerkinElmer).

14

15 **2.7. Statistical analysis**

16 Unless stated otherwise, graph values are presented as means ± standard deviations, calculated on
 17 at least three independent experiments. Depending on the results of normality tests, statistical
 18 significance was determined using two-tailed Student’s t-test, one-sample t-test or one-way
 19 ANOVA test with a Tukey's adjustment.

20

Table 1: list of antibodies

| Antibodies | Supplier | Cat No |
|-------------------|-----------------|---------------|
| CD16-APC | Miltenyi Biotec | 130-106-763 |
| CD15-FITC | Sony | 2109520 |
| PE-CF594-CD14 | BD Biosciences | 562335 |
| Alexa647-CD163 | BD Biosciences | 562669 |
| BV421-CD80 | Sony | 2126110 |
| BB515-CD206 | BD Biosciences | 564668 |
| PE-hMER | Sony | 2438040 |
| BV421-CD206 | BD Biosciences | 564062 |

Table 2: list of primers used in Q-PCR experiments

| Target | Sequence | specificity |
|----------------|----------------------------|--------------------|
| CD66b forward | TCAAAGCATTTGCAATCAGC | human |
| CD66b reverse | GTGGGCAACTTCACAAAGGT | human |
| GAPDH forward | GAA GGT GAA GGT CGG AGT C | human |
| GAPDH reverse | GAA GAT GGT GAT GGG ATT TC | human |
| TNF forward | AGGACCAGCTAAGAGGGAGA | human |
| TNF reverse | CCCGGATCATGCTTTCAGTG | human |
| IL6 forward | CACTGGCAGAAAACAACCTG | human |
| IL6 reverse | TGGCTTGTTCCCTCACTACTCT | human |
| MT-ND6 forward | TGATTGTTAGCGGTGTGGTC | human |
| MT-ND6 reverse | CCACAGCACCAATCCTACCT | human |
| TRAC forward | ATTGCCCTCTTCTCCCTCT | human |
| TRAC reverse | GCAGTGTTTGGCAGCTCTTC | human |
| CD3E forward | GTTGTCCCCATCCCAAAGT | human |
| CD3E reverse | GGTGGAGGGAGTAGGGGATT | human |

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3. Results

3.1. Efferocytosis is induced in a co-culture of primary human apoptotic neutrophils and primary human monocyte-derived macrophages.

The co-culture of AN and MDM was set up several decades ago (9). Nonetheless, some protocols, used here, may slightly differ from those used in the original articles. First, we have to determine if, in our hand and with our protocols, this model still recapitulate key features. Since human neutrophils enter spontaneous apoptosis *in vitro* (22), we first determined the optimal time point for the further efferocytosis experiments. Neutrophils were isolated from leukocyte-platelet concentrates using a Ficoll-density gradient followed by sedimentation in a 3% dextran solution. A purity of nearly 92% was achieved using the method (Supplementary Fig. 1A). We then analysed their viability in a time course by flow cytometry after Annexin-V/7AAD staining (Fig. 1A). About 30% of freshly isolated neutrophils are apoptotic. After 13 h in culture, this percentage raise up to 77% and it slightly decreases at the later time points. On the other hand, the percentage of necrotic cells starts to increase at 24 h. The optimal time point for adding neutrophils to MDM culture to trigger efferocytosis is at 13 h after isolation, since it exhibits the highest percentage of apoptotic neutrophils (AN) and the lowest percentage of necrotic cells.

We then determined the efferocytosis efficiency in our co-culture system. Human macrophages were generated by M-CSF induced differentiation of human primary CD14⁺ cells during 7 days. MDM phenotype was verified by flow cytometry using CD14, CD163, MERTK, CD80 and CD206 labelling to assess differentiation efficiency (Supplementary Fig. 1B). Generated MDM were subsequently co-cultured with CFSE-labelled AN for 6 or 24 h and labelled with CD206-BV421 antibody. Phagocytosis efficiency was assessed by flow cytometry (Fig. 1B). MDM containing at least one neutrophil was defined as CD206⁺/CFSE⁺ cells. After 6 h of co-culture, about 64% ($\pm 16.7\%$) of MDM have ingested at least one neutrophil and after 24 h, about 61% ($\pm 18\%$) (Fig. 1C-D). We checked that MDM viability is not impacted in this co-culture system (supplementary Fig. 1C). We also verified that AN are efficiently engulfed and do not just stick to MDM surface by labelling AN with pHrodo dye, a pH-sensitive dye non-fluorescent at neutral pH and brightly fluorescent in acidic environments. This labelling allows to discriminate internalized AN from AN bound to the cell surface. We obtain no statistical difference when AN are labelled with CFSE or with pHrodo (68.1% ± 10.21 at 6 h and 80.03% ± 6.43 at 24 h of MDM

1 were CD206⁺/pHrodo⁺ ; 80.13% ±3.8 at 6 h and 81.5% ±6.63 at 24 h of MDM were
2 CD206⁺/CFSE⁺), indicating that AN were engulfed and processed into phagosomes
3 (Supplementary Fig. 2A, 2B and 2C). Moreover, these results indicate that CFSE labelling of AN
4 allows the accurate assessment of phagocytosis efficiency. These data collectively show that
5 efferocytosis is efficiently triggered in this co-culture.

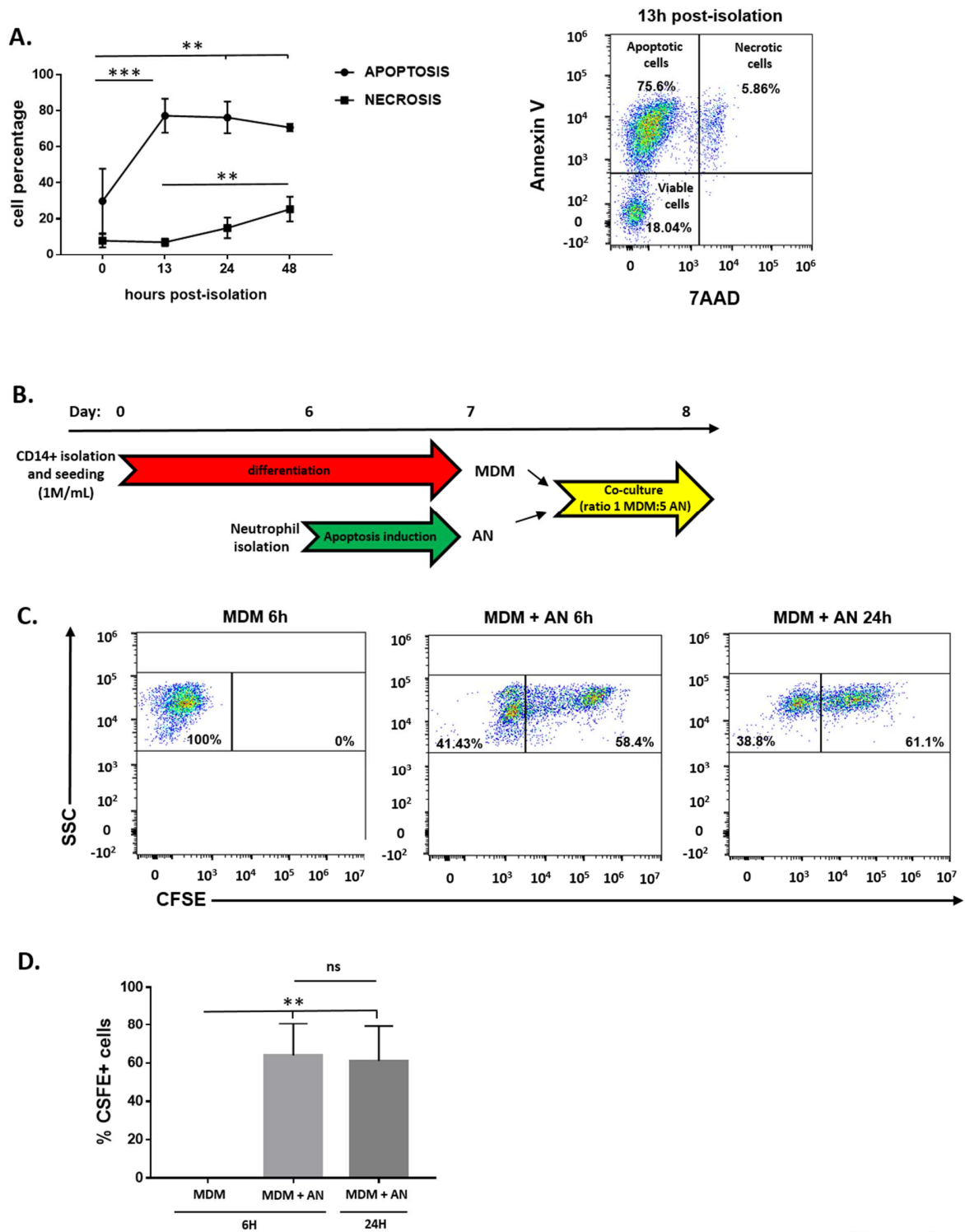


Figure1

1
 2 **Figure 1. Characterization of apoptotic neutrophil/monocyte-derived macrophage**
 3 **culture (A)** Neutrophil viability assessment. Purified primary neutrophils were cultured for the

1 indicated time. Left panel: Percent of apoptotic (●) and necrotic (■) cells were evaluated by flow
2 cytometry after Annexin-V-FITC/7AAD labelling. Annexin-V-FITC/7AAD positive cells were
3 assessed after FSC vs. SSC gating and doublet discrimination. Values are means \pm SD. n= at
4 least 3 independent experiments. One-way anova test with Tukey's post-test. **: $p \leq 0.01$, ***:
5 $p \leq 0.001$. Right panel: representative dot plot of Annexin-V-FITC vs. 7AAD fluorescence
6 intensities after FSC vs. SSC gating and doublet discrimination. Numbers indicate the
7 percentage of cells in the related quadrant. **(B)** Time line of our co-culture system. Primary
8 human CD14⁺ monocytes were purified and seeded at 1 million cells per mL at day 0 and
9 differentiated with 50 ng/mL of M-CSF into macrophage (MDM). At day 6 primary human
10 neutrophils were purified and cultured for 13 h to induce spontaneous apoptosis (AN). At day 7,
11 MDM and apoptotic neutrophils (AN) were mixed at a 1:5 ratio and macrophages were
12 harvested 6 h or 24 h later for further analysis. **(C-D)** Phagocytosis test. At day 7, AN were
13 labelled with CFSE (10 μ M) for 10 min at 37°C, mixed with MDM and incubated for the indicated
14 time. Non-engulfed neutrophils were then removed by 5 PBS washes. MDM were subsequently
15 harvested by scrapping and labelled with CD206-BV421 antibody. After FSC vs; SSC > doublet
16 discrimination > SSC vs CD206 gating, MDM CD206 positive cells that have ingested at least
17 one AN were CFSE⁺. **(C)** Representative dot plots of SSC vs. CFSE fluorescence intensity of
18 CD206 positive cells. Numbers indicate the percentage of cells in each gate, Representative of 3
19 independant experiments. **(D)** Quantification of phagocytosis tests. Values are means \pm SD. n=
20 3. One-way anova with Tukey's post-hoc test, **: $p \leq 0.01$, ns: non-significant.
21

22 **3.2. Human primary neutrophils contain less mRNA than MDM and these mRNA** 23 **are highly degraded.**

24 In order to ensure that neutrophil mRNA cannot contaminate macrophage mRNA after
25 efferocytosis, we determined first the mRNA quantity per cell in neutrophils and macrophages.
26 Freshly isolated and 13 h cultured neutrophils exhibit the same mRNA per cell ratio but
27 macrophages contain 7.31 fold more mRNA per cell (Fig. 2A). We then analysed the
28 detectability of these mRNA. We chose to quantify by Q-PCR two highly expressed mRNA
29 arisen from the nuclear gene *GAPDH* and the mitochondrial gene *MT-ND6*. When normalized to
30 the retrotranscribed mRNA quantity, *GAPDH* and *MT-ND6* mRNA are respectively about 7700
31 and 5600 fold less detectable in AN compared to MDM (Fig. 2B,C). But *GAPDH* and *MT-ND6*
32 mRNA detectability remains equivalent in freshly isolated and in 13 h post-isolation cultured
33 neutrophils (Fig. 2B,C). These experiments suggest that neutrophil mRNA are degraded even
34 right after cell isolation.

35 To test this hypothesis, we checked mRNA quality using Experion Automated Electrophoresis
36 System. Neutrophil mRNA, whatever the time point after neutrophil isolation, exhibits strong
37 smears contrary to macrophage mRNA after electrophoresis (Fig. 2D). Moreover, the RNA
38 quality indicator (RQI), measured on a scale from 1 to 10, is between 1.74 and 2.21 for
39 neutrophil mRNA depending on the time point and is above 9 for macrophage mRNA (Fig. 2E).

1 This demonstrates that human primary neutrophil mRNA are severely degraded after isolation
 2 according to our protocol.
 3 Collectively, these experiments argue that human primary neutrophils contain less mRNA than
 4 MDM, and that these neutrophil mRNA are less detectable due to extensive degradation, even in
 5 freshly isolated neutrophils, after a purification based on our protocol.

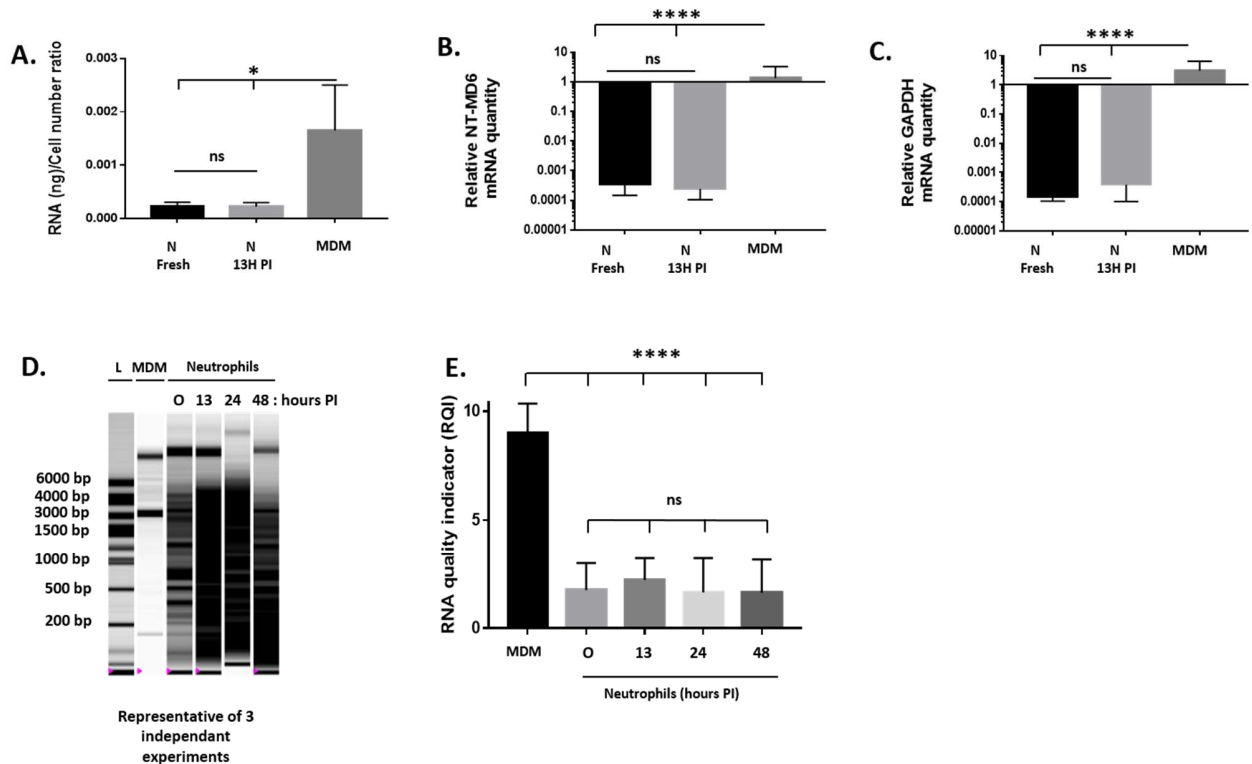


Figure2

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Figure 2. Human primary neutrophils exhibit low degraded mRNA content. (A) Human primary neutrophils contain less mRNA quantity than MDM. Freshly isolated or 13 h post-isolation (PI) human primary neutrophil and MDM mRNA is isolated and quantified with NanoDrop spectrophotometer and normalized to cell number. Values are means \pm SD. $n=3$ for neutrophils points and $n=4$ for MDM. Two-tailed unpaired t-test. *: $p \leq 0.05$, ns: non-significant. (B-C) Human primary neutrophil mRNA is less amplifiable than MDM mRNA. Freshly isolated or 13 h PI human primary neutrophil and MDM mRNAs were isolated and retrotranscribed into cDNA. *NT-ND6* (B) and *GAPDH* (C) mRNA are then amplified by Q-PCR and normalized to the mRNA quantity previously retrotranscribed. Values are means \pm SD. $n=3$ for neutrophils and $n=4$ for MDM. Two-tailed unpaired t-test. ****: $p \leq 0.0001$, ns: non-significant (D) Human primary neutrophil mRNA is degraded. Freshly isolated or 13/24/48h PI human primary neutrophil and MDM mRNAs were isolated. Ladder (L), MDM and neutrophil mRNAs were separated using Experion Automated Electrophoresis System. Picture is representative of three independent experiments. (E) Quantification of Experion Automated Electrophoresis experiments. Values are means \pm SD. $n=7$ for neutrophils and $n=4$ for MDM. One-way ANOVA with Tukey's post-hoc test. ****: $p \leq 0.0001$, ns: non-significant.

3.3. Human primary neutrophil specific mRNA are not detectable post-efferocytosis.

During efferocytosis, the engulfed corpse is trapped in a phagosome called efferosome which subsequently fuse with lysosome leading to apoptotic cell destruction (23). Nonetheless, to our knowledge, the fate of ingested mRNA during efferocytosis remains unknown. To investigate this, we performed a co-culture of MDM and AN to induce efferocytosis. Six or 24 h later, macrophage mRNA were extracted and submitted to Q-PCR analysis to detect two specific mRNAs, arisen from *Elane* and *CD66b* genes, which are highly expressed in neutrophils but not in macrophages (24, 25). As mentioned in Fig. 1C, at least 57% ($\pm 15\%$) of MDM have engulfed at least one apoptotic neutrophil at 24 h. However, *Elane* (Fig. 3A) and *CD66b* (Fig. 3B) mRNA levels are comparable in MDM co-cultured or not with neutrophils. Moreover, we also checked the *TNF α* mRNA level in MDM. MDM differentiated with M-CSF express low level of *TNF α* mRNA, whereas AN contain large amount of it (about 90 fold more compared to MDM). Nonetheless, ingestion of AN by MDM did not induce an increase of *TNF α* mRNA level in efferocytic MDM (Fig. 3C). These data indicate that neutrophil mRNA does not contaminate MDM mRNA after efferocytosis.

Since apoptotic human PBMC and apoptotic Jurkat cells are also commonly used as apoptotic corpse to trigger efferocytosis, we investigated the fate of specific mRNA expressed in these cells in efferocytic MDM. We first assessed that apoptosis is efficiently induced. Human PBMC were irradiated (X-rays) and exhibit about 29.4% \pm 8.5 of cells in apoptosis, whereas Jurkat cells were treated with camptothecin and exhibits about 40% \pm 7.4 of cells in apoptosis (Supplementary Fig.3A). These cells are efficiently engulfed by MDM: 75.1% \pm 9.4 of MDM have engulfed at least one apoptotic PBMC and 84.6% \pm 5.7 of MDM at least one apoptotic Jurkat cell (Supplementary Fig.3B). We then measured by Q-PCR the expression level of two specific T cell transcripts: *CD3E* and *TRAC* mRNA. Contrary to specific AN transcripts, specific apoptotic Jurkat cell transcripts were significantly enriched in efferocytic MDM. Specific apoptotic PBMC transcripts were also enriched in some samples although not reaching globally the significant threshold (Fig.3D). These results indicate that performing transcriptomic studies in MDM post-efferocytosis with apoptotic human PBMC or apoptotic Jurkat cells may be risky and should be avoided.

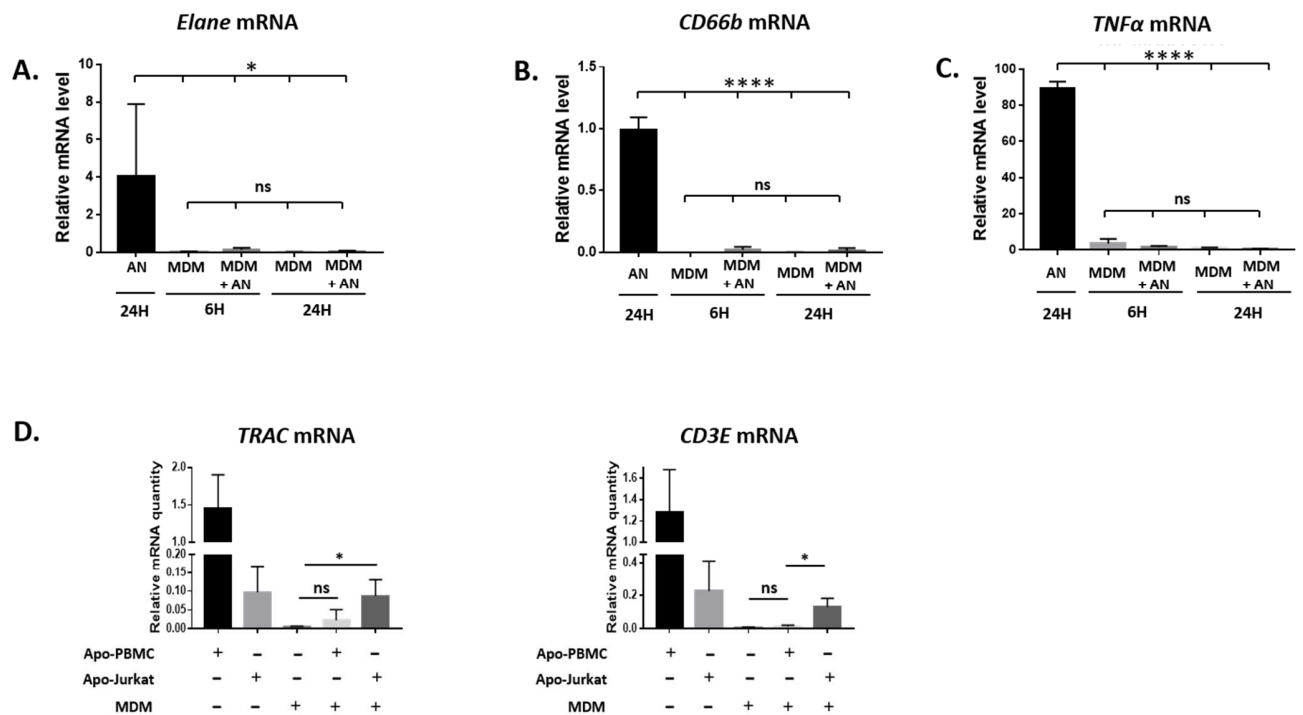


Figure3

1
2
3 **Figure 3. Human primary neutrophil mRNA are not detectable in macrophages after**
4 **efferocytosis.** MDM were co-cultured with or without AN for 6 or 24h. Non-engulfed neutrophils
5 were removed by 5 PBS washing. MDM were subsequently harvested by scrapping and mRNA
6 was extracted and retrotranscribed into cDNA. *Elane* mRNA (A), *CD66b* mRNA (B) and *TNFα*
7 mRNA (C) were quantified by Q-PCR. Results were normalized to GAPDH expression. Values
8 are means ± SD. n=at least 3 independent experiments (A), n=at least 4 independent
9 experiments (B-C), n= 3 independent experiments. One-way anova with Tukey's post-hoc test. *:
10 p≤0.05, ****: p≤0.0001, ns: non-significant. (D) **Some human primary Jurkat and PBMC**
11 **mRNA can be detected in macrophages after efferocytosis.** MDM were co-cultured with or
12 without apoptotic PBMC and apoptotic Jurkat cells for 24h. Non-engulfed apoptotic corpses
13 were removed by 5 PBS washing. MDM were subsequently harvested by scrapping and mRNA
14 was extracted and retrotranscribed into cDNA. *TRAC* mRNA (Left panel), *CD3E* mRNA (right
15 panel) were quantified by Q-PCR. Results were normalized to GAPDH expression. Values are
16 means ± SD. n= 3 independent experiments. Two-tailed unpaired t-test. *: p≤0.05, ns: non
17 significant.
18
19

20 3.4. Allogenic apoptotic neutrophil and monocyte-derived macrophage co-culture did 21 not induce cell activation

22 Since our co-culture system of MDM and neutrophils are collected from two different donors, we
23 checked if this allogenic context did not artificially influence the activation status of these cells.

1 We first measured neutrophil activation by quantifying Elastase activity in culture supernatant.
2 Elastase is a specific enzyme stocked in azurophilic granules of neutrophils released upon cell
3 activation. Extracellular Elastase activity is by the way representative of the neutrophil activation
4 state (26). When neutrophils were activated by PMA, we observed a strong Elastase activity in
5 cell culture supernatant (Fig. 4A). However, no Elastase activity was detected when MDM were
6 cultured alone neither after a 24 h incubation with neutrophils (Fig. 4A). These data demonstrate
7 that when co-cultured with MDM, activation of allogenic human primary neutrophils is not
8 triggered.

9 Then, we also monitored the impact of neutrophils on MDM activation. Allogenic co-culture may
10 induce macrophage pro-inflammatory responses. To rule out this possibility, we quantified by
11 ELISA the amount of TNF α and IL-6, two pro-inflammatory cytokines, in culture supernatant.
12 The amount of TNF α and IL6 released by MDM is comparable, whether macrophages are
13 cultured alone or with allogenic neutrophils (Fig. 4B). This imply that MDM are not activated
14 when co-incubated with primary neutrophils.

15 Collectively, these data emphasize that allogenic primary MDM and primary neutrophils co-
16 culture did not trigger an artifactual activation of any of these cells.

17

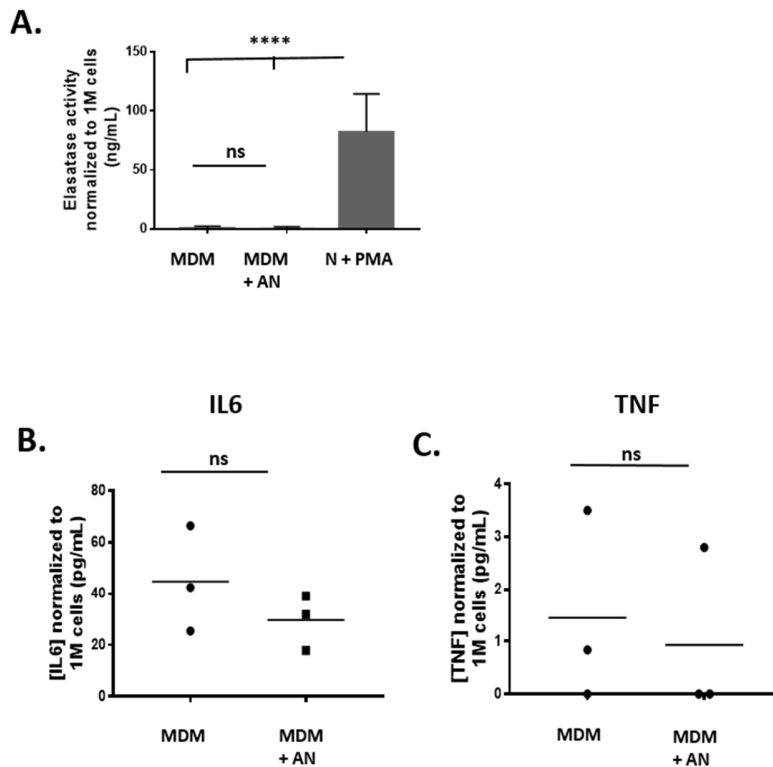


Figure 4

1
 2 **Figure 4. Human primary neutrophil and MDM co-culture does not induce allogenic**
 3 **reactions.** (A) Human primary neutrophils are not activated by co-culture with MDM. MDM are
 4 co-cultured with or without AN for 24 h. AN were stimulated with PMA (100nM, 1 h at 37°C) as a
 5 positive control. Elastase activity in cell supernatant was quantified using a fluorimetric test and
 6 normalized to 1 million cells. Values are means \pm SD. n=at least 3 independent experiments.
 7 One-way ANOVA with Tukey's post-hoc test. ****: $p \leq 0.0001$. (B-C) MDM-AN did not induce
 8 MDM pro-inflammatory responses. MDM are co-cultured with or without AN for 24 h. Cell
 9 supernatants were collected and centrifuged at 300 g 5 min to remove any cells. IL6 (B) and
 10 TNF α (C) proteins were quantified by ELISA. Results were normalized to 1 million cells. Values
 11 are means \pm SD. n=3. Two-tailed unpaired t-test. ****: $p \leq 0.0001$, ns: non-significant.
 12

13 3.5 Efferocytosis efficiently reprograms human monocyte-derived macrophages

14 Quantification of pro-inflammatory cytokine secretion after LPS stimulation is commonly
 15 performed to assess the efficiency of MDM reprogramming after efferocytosis (9). MDM were co-
 16 cultured or not with AN for 24 h, AN were subsequently removed and MDM were stimulated
 17 with LPS for 24 h. We then measured, by ELISA, the release of TNF α in cell supernatant. MDM
 18 which have previously engulfed AN secrete 2.94 times less TNF α compared to MDM cultured
 19 without AN, demonstrating that MDM are less prone to Toll like receptor 4 stimulation after

1 efferocytosis and are therefore reprogramed. We also checked the *TNF α* mRNA level to decipher
2 whether efferocytosis can affect or not *TNF α* transcription. There is no significant difference in
3 *TNF α* mRNA level whether MDM were previously co-cultured or not with AN, indicating that
4 the lower released of *TNF α* after LPS stimulation post-efferocytosis is not transcription-
5 dependent (Supplementary Fig. 5).

6

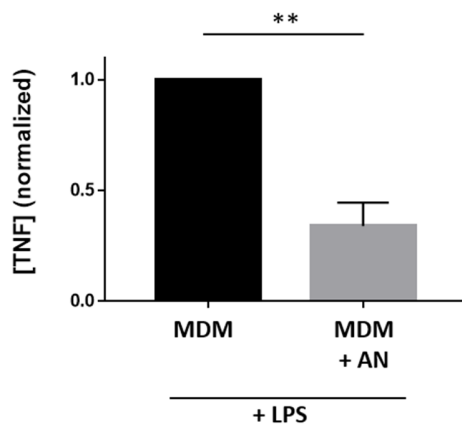


Figure 5

7 **Figure 5. MDM are efficiently reprogramed by efferocytosis.** MDM are co-cultured with or
8 without AN for 24h. AN were then removed by PBS washing and MDM were stimulated with LPS
9 (100 ng/mL for 24 hours). After LPS stimulation, cell supernatants were collected and
10 centrifuged at 300 *g*, 5 min to remove any cells. *TNF α* proteins were quantified by ELISA.
11 Results were normalized to MDM values for each donor. Values are means \pm SD. *n*=3. One-
12 sample t-test. **: *p*≤0.01.

13

14

16 4. Discussion and conclusion

17 *In vitro* systems where macrophages and apoptotic cells are co-cultured to study efferocytosis
18 allowed to unravel many features of M^R, like identification of some murine markers or secreted
19 mediators for example (9, 14, 27–29). However, up to now, involved pathways and
20 comprehensive molecular changes induced by efferocytosis remain puzzling in mice, and even
21 more in humans. Global approaches, like RNA sequencing, may help to clear up some
22 mechanistic insights. Nonetheless, these approaches may be impeded by few hurdles like
23 apoptotic corpses mRNA cross-contamination or induced allogenic immune reactions. Some
24 studies try to alleviate the risk of mRNA cross-contamination by setting up a model where
25 apoptotic corpses and macrophages are obtained from two different species (10, 12). Despite

1 being useful, in this model some but not all transcripts can be discriminated due to high sequence
2 similarity, thus precluding part of efferocytosis triggered mRNA variations (18, 19). A two-
3 species model may also hinder some physiological mechanisms since in the body, a macrophage
4 only engulfs apoptotic corpses deriving from its own specie. The nature of the apoptotic corpses
5 employed is often “non physiological”. Irradiated Jurkat T cells or thymocytes are commonly
6 used as apoptotic cell source (12, 29). In these cells, apoptosis is artificially triggered with
7 irradiation, and they do not represent the main cell type engulfed physiologically during the
8 resolution phase of inflammation, which is mainly apoptotic blood-derived neutrophils. In this
9 sense, the type of ingested corpses and the way to induce apoptosis may influence the
10 macrophage response (discussed in (30)). If we add the fact that human primary neutrophils
11 exhibit a much higher apoptotic rate, compared to apoptotic Jurkat cells or apoptotic PBMC, and
12 that apoptosis is triggered without chemical treatment or irradiation, the use of AN seems
13 preferable.

14 We shed light here on the feasibility of mRNA investigations in a long been used full human
15 model of efferocytosis. Obviously, we did not claim that this system perfectly mimics the *in vivo*
16 response. But so far, regarding the complexity of *in vivo* investigations in humans, the co-culture
17 of human AN and MDM is the most accurate and the closest possible *in vitro* system to reflect
18 what occurs in human body at the resolution phase of inflammation. It has also the great
19 advantage to allow investigations in human M^R, which is an overlooked model compared to its
20 murine counterpart.

21 In this system, even in fresh neutrophils, isolated according to our experimental conditions,
22 transcript amplification is tricky, including for highly expressed genes like *GAPDH* and *MT-*
23 *ND6*. All transcripts detected exhibit a high cycle quantification value (Cq) (data not shown).
24 This is surprising since many labs performed Q-PCR experiments and RNAseq studies on fresh
25 human neutrophils (31, 32). However, our protocol is slightly different from their own. For
26 instance, we did not process neutrophils within an hour but within 4-5 hours after blood
27 collection, since we are working on fresh leukocyte-platelet concentrates. Some other subtle
28 differences in blood collection, cell purification and RNA extraction protocols may explain why
29 we observe degraded mRNA in our “fresh” neutrophils. Therefore, our results absolutely do not
30 challenge the reliability of previously published work on mRNA investigations in human
31 neutrophils. It just emphasizes that this neutrophil purification protocol is suitable for setting up a

1 model of efferocytosis to limit neutrophil mRNA contamination in M^R, but should be avoided for
2 mRNA investigations in human primary neutrophils. Indeed, we demonstrate here that AN
3 mRNA does not contaminate MDM mRNA. This absence of contamination can be due to the low
4 RNA quantity and quality in neutrophils after a purification based on our extraction protocol.
5 Even if we did not observe any statistically significant increase of *Elane* and *CD66b* mRNA in
6 MDM after efferocytosis, we still can detect an extremely low background (Figure 3). Moreover,
7 a highly expressed mRNA in neutrophils, like *TNF α* mRNA, does not lead to an increase of basal
8 *TNF α* mRNA level in MDM post-efferocytosis. Engulfed cells transit *via* efferosome were they
9 are ultimately degraded, meaning that the low neutrophil mRNA quality is probably even more
10 altered. Furthermore, even if *Elane* and *CD66b* mRNA are specific of neutrophils we can still
11 occasionally detect few amplifiable *Elane* and *CD66b* transcripts in MDM cultured alone (Figure
12 3). Collectively, considering the tremendous discrepancies in mRNA quality and quantity
13 between AN and MDM, it is very unlikely that the reminiscent *Elane* and *CD66b* mRNA levels
14 reflect a contamination with neutrophil mRNA. On the contrary, when using apoptotic Jurkat
15 cells or human apoptotic PBMC, we were able to detect some T-cell specific transcripts like
16 *CD3E* and *TRAC* mRNA in MDM after efferocytosis. Indeed, these transcripts are highly
17 expressed even in apoptotic PBMC and apoptotic Jurkat T cells, exhibiting low quantification
18 cycle values (data not shown), and are easily amplified by Q-PCR. In contrast, as stated above,
19 most neutrophil transcripts exhibit a low quantification cycle values, even with usually highly
20 expressed genes like *NT-ND6* and *GAPDH*. The discrepancies of these C_q values between PBMC
21 and Jurkat cell, on one side, and AN, on the other side, may reside in RNA quality and quantity
22 differences and most probably explain why AN mRNA are not detectable in efferocytic
23 macrophages, contrary to some PBMC and Jurkat mRNA. This clearly establishes the potential
24 risk of mRNA contamination in MDM post-efferocytosis, when using apoptotic human PBMC
25 and apoptotic Jurkat T cells. Therefore, we discourage the use of these cells as apoptotic corpse
26 in any transcriptomic studies of efferocytic MDM. The use of AN extracted from leukocyte-
27 platelet concentrate must be prioritized for this application.

28 Another pitfall in this system may be the question of DNA horizontal transfer. Indeed, several
29 manuscripts demonstrate that some portions of the apoptotic cell DNA may be transferred to the
30 phagocyte cell, leading to the expression of some apoptotic cell genes and promoting
31 tumorigenesis. These results were obtained in other cellular models with apoptotic bodies

1 expressing viral oncogenes (Epstein-Barr virus or human papillomavirus) (33, 34). Nonetheless it
2 seems that DNA transfer is lost if it does not confer a selective advantage to the recipient cell
3 (35). In our model, even if we cannot completely exclude DNA transfer during efferocytosis, AN
4 specific transcripts we studied are not expressed in MDM, suggesting that even if DNA transfer
5 occur, it does not lead to significant neutrophil gene expression. Further experiments are required
6 to investigate the potential occurrence of DNA transfer, even if it seems here unlikely.

7 The co-culture system, used here, was characterized several decades ago (9, 21). Nonetheless,
8 since we changed some parameters like cell purification methods (CD14⁺ magnetic sorting *versus*
9 selection by adherence...) or apoptosis induction (spontaneous apoptosis but not UV-irradiation),
10 we verified that in our hands, this model recapitulates some previously characterized key
11 features: 1) Efferocytosis is induced in this model. At 6 h, most of MDM uptake at least one
12 neutrophil. Although non-significant, we observed a slight decrease of CFSE positive MDM
13 between 6 and 24 h. This suggests that all MDM able to phagocyte already reached satiety at 6 h
14 (Figure 1C). Partial lysosomal destruction of CFSE may be responsible for this minor decreased
15 of CFSE positive macrophage frequency. Using pHrodo labelling we confirmed that AN are
16 internalized and not just docked at MDM surface. By the way, the use of an imaging flow
17 cytometer may bring a more direct proof of apoptotic corpse engulfment when using apoptotic
18 cell CFSE labelling.

19 2) There is no artefactual cell activation in this allogenic co-culture system. Indeed human
20 primary neutrophils are very fragile and can be activated by a simple mechanical stress for
21 example. Neutrophil granule release, containing Elastase in particular, may induce macrophage
22 activation (36). However, we did not observe any increase of Elastase activity nor macrophage
23 pro-inflammatory cytokine secretion in co-culture cell supernatants (Figure 4A-C), thus ensuring
24 that this system did not suffer from these pitfalls.

25 3) Macrophages are efficiently reprogramed by efferocytosis (Figure 5). Indeed efferocytic MDM
26 are less prone to release pro-inflammatory TNF α upon LPS stimulation. This mechanism seems
27 to be transcription-independent, since *TNF α* mRNA level is not affected by efferocytosis.
28 Alteration of TLR signalling may be involved in this phenomenon, but further experiments are
29 required to test this hypothesis.

30 With this work, we demonstrated the superiority of the AN-MDM co-culture system compared to
31 apoptotic PBMC/apoptotic Jurkat cells-MDM and we shed light on the proof-of-concept of

1 mRNA investigation feasibility in this full human model of resolving macrophage, thus opening
2 the door to a better characterization of this essential actor in human inflammation resolution.

3

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7
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11
12
13 **Competing and declaration of interests:**

14 The data presented in this manuscript are original, and have not been published or submitted
15 elsewhere. All listed authors have approved the manuscript and agreed with the submission. In
16 addition, we declare no financial interest related to this work.

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