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Label-free leukocyte sorting and impedance-based profiling for diabetes testing

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Abstract

Circulating leukocytes comprise of approximately 1% of all blood cells and efficient enrichment of these cells from whole blood is critical for understanding cellular heterogeneity and biological significance in health and diseases. In this work, we report a novel microfluidic strategy for rapid (< 1 hour) label-free leukocyte sorting and impedance-based profiling to determine cell activation in type 2 diabetes mellitus (T2DM) using whole blood. Leukocytes were first size-fractionated into different subtypes (neutrophils, monocytes, lymphocytes) using an inertial spiral sorter prior to single-cell impedance measurement in a microfluidic device with coplanar electrode design. Significant changes in membrane dielectric properties (size and opacity) were detected between healthy and activated leukocytes (TNF- α /LPS stimulated), during monocyte differentiation and among different monocyte subsets (classical, intermediate, non-classical). As proof-of-concept for diabetes testing, neutrophil/monocyte dielectric properties in T2DM subjects (n=8) were quantified which were associated with cardiovascular risk factors including lipid levels, C-reactive protein (CRP) and vascular functions (LnRHI) ($P < 0.05$) were observed. Overall, these results clearly showed that T2DM subjects have pro-inflammatory leukocyte phenotypes and suggest leukocyte impedance signature as a novel surrogate biomarker for inflammation.

Keywords: Impedance cytometry; Dean flow fractionation; Leukocyte phenotyping; Diabetes mellitus

1. Introduction

Circulating leukocytes are key components of our immune system against infections and are involved in the pathogenesis of major diseases such as cancer (Rüegg 2006), metabolic disorders including diabetes (Gordon and Taylor 2005; Sprangers et al. 2016), and neurological diseases (Wilson et al. 2010). The study of leukocyte heterogeneity and functions is becoming increasingly important in cell biology research to elucidate disease pathobiology and mechanisms (Beyrau et al. 2012), and can also serve as intrinsic biomarkers for cell-based diagnostics and therapeutics (Hou et al. 2016; Tay et al. 2018). Impedance cytometry is a high throughput and label-free single cell analytical tool for electrical characterization of cell size and intrinsic dielectric properties of cellular components such cell membrane and cytoplasm (Chen et al. 2015; Cheung et al. 2010; Petchakup et al. 2017; Sun and Morgan 2010). The technology has been widely exploited for cell type identification (Hassan et al. 2017; Simon et al. 2016; Spencer et al. 2014a), platelet activation testing (Evander et al. 2013), cancer cell discrimination (Spencer et al. 2014b), as well as detection of parasite-infected cells for disease diagnostics (Du et al. 2013; Küttel et al. 2007). However, electrical characterization of circulating leukocytes in whole blood remains a technical challenge as conventional leukocyte isolation methods require affinity-based antibodies binding steps which will attenuate membrane properties (Fukuda and Schmid-Schönbein 2002; Jensen 2012). Rollo *et al.* have recently developed an impedance sensor for activated T lymphocytes but it requires negative fluorescence selection of target cells (using antibodies) by flow cytometry which is expensive and time-consuming. Hence there exists a critical need to develop microfluidic label-free leukocyte sorting technologies with minimal perturbation and manipulation and can be easily combined with impedance cytometry to study leukocytes in their native state. This strategy is more cost-effective and can greatly facilitate automation and assay robustness with minimal user sample manipulation for point-of-care testing.

In this work, we report a novel microfluidic strategy for rapid and label-free leukocyte sorting and impedance profiling from whole blood. Peripheral blood mononuclear cells were obtained by density gradient (Ficoll) centrifugation and size-fractionated into different subtypes (neutrophils, monocytes, lymphocytes) using Dean Flow Fractionation (DFF), a inertial microfluidics cell separation technique previously developed by our group (Hou et al. 2016). Enriched leukocytes were simultaneously washed and eluted off-chip for impedance measurement in a microfluidic impedance flow cytometer with coplanar electrodes. We demonstrated that the developed platform enables identification of different leukocyte subtypes, leukocyte activation profiling, monitoring of monocyte differentiation and characterization of monocyte subtypes. As proof-of-concept for diabetes testing, DFF-isolated neutrophils and monocytes obtained from patients with type 2 diabetes mellitus (T2DM, n=8) were characterized. Diabetic neutrophils exhibited higher opacity as compared to monocytes ($P < 0.05$), and the impedance of both leukocyte subtypes were strongly correlated to each other ($P < 0.001$). Interestingly, neutrophil/monocyte size and impedance were associated with established cardiovascular risk factors including lipid levels, and vascular function (LnRHI) ($P < 0.05$). Taken together, these results clearly suggest that T2DM subjects have a pro-inflammatory (activated) neutrophil/monocyte phenotype and leukocyte impedance signature is a novel intrinsic surrogate biomarker of inflammation. The sensitivity of the impedance flow cytometry is significantly enhanced when combined with a label-free leukocyte purification step. To the best of our knowledge, this is the first work demonstrating leukocyte electrical characterization in diabetes for immune health profiling. We envision that this microfluidic strategy can be further developed into an integrated “sample-in answer out” impedance-based diagnostics tool for rapid risk stratification in patients with inflammatory diseases such as diabetes and cardiovascular diseases.

2. Experimental section

2.1 Leukocyte isolation

The experimental workflow is shown in Fig. 1A. Depending on the leukocyte of interest, the whole blood samples were pre-processed differently. To acquire white blood cells (WBCs) for neutrophil isolation, RBC lysis buffer (eBioscience) was added into whole blood (10:1 v/v) for 3 min and quenched with 0.5% bovine serum albumin (BSA, Biowest) in phosphate buffered saline (PBS, Lonza). To acquire peripheral blood mononuclear cells (PBMCs) for monocytes or lymphocytes isolation, whole blood was subjected to Ficoll-Paque™ Plus (GE Healthcare) density centrifugation according to manufacturer's protocol. After sample pre-processing, leukocyte subtypes were then sorted using Dean flow fractionation with our previously developed microchip (Hou et al. 2016). Monocytes or neutrophils were collected from outlet 2 while lymphocytes were collected from outlet 3 of the device. The sorted cells were washed and resuspended to 1×10^6 cells/mL in 0.1% BSA for downstream processing.

2.2 Leukocyte impedance measurement

Prior to sample perfusion into the device, the device was flushed with 70% ethanol followed by deionized water and primed with sheath (0.1% BSA in PBS) for 30 min to prevent non-specific binding. 5 μ m polystyrene beads (Nalgene) were added into the sample as an internal reference. The experimental setup and schematic of microfluidic device is shown in Fig. 1B. The sample and sheath solution were loaded into 1 mL syringes and perfused into the device at 5 μ L/min using syringe pumps (Chemyx). The device was allowed to run for 1 min to stabilize the flow prior to electrical measurement. To establish electrical measurement, the middle electrode was supplied with 3V at two different frequencies (0.3MHz and 1.72MHz) by a lock-in amplifier (HF2LI, Zurich instrument). When a single cell passes through the electrodes (Fig. 1C), differential current responses were recorded from the side electrodes by

1 using transimpedance amplifiers (DHPCA-100, FEMTO®) with transimpedance gain of
2 10kV/A. Then output signal were sent to the lock-in amplifier to extract a magnitude and phase
3 of the signal of interest at a sampling rate of 112k sample/s. The recorded sequential data in
4 Fig. 1D was processed to extract to peaks corresponding to single cell events using a custom-
5 made program written in MATLAB (MathWorks). The theory and simulation can be found in
6 Fig. S1.

7 **2.3 Flow cytometry analysis**

8 To determine the leukocyte composition, PBMCs and DFF-sorted samples were stained for 30
9 min at 4°C with allophycocyanin (APC)-labeled anti-human CD66b, phycoerythrin (PE)-
10 labeled anti-human CD14 and fluorescein isothiocyanate (FITC)-labeled anti-human
11 CD3/CD19 antibodies to identify neutrophils, monocytes and T/ B-lymphocytes respectively.
12 To characterize monocyte activation after TNF- α and LPS treatment, DFF-sorted monocytes
13 were co-stained with PE-labeled anti-human CD14 and FITC-labeled CD11b antibodies. Non-
14 specific antibody binding was examined using corresponding isotype negative control
15 antibodies. The cells were washed once prior to flow cytometry analysis. All antibodies were
16 purchased from eBioscience (1:20 dilution) and analyzed using a BD LSR Fortessa flow
17 cytometer (BD Biosciences).

18 **2.4 Statistical analysis and data presentation**

19 Mann-Whitney test was used to determine the statistical significance of the difference between
20 two sets of data. $P < 0.05$ was considered of statistical significant difference. All analysis was
21 performed using GraphPad Prism V7.0 (GraphPad software). Density scatter plots were plotted
22 in MATLAB using dscatter function from MATLAB Central File Exchange (Henson 2016).

2.5 Study approval

Written informed consent was obtained for all subjects during recruitment. All protocols were approved by the institutional review board of Nanyang Technological University (IRB-2014-04-27), Tan Tock Seng Hospital (2014/00416) and Singapore General Hospital (CIRB2017/2512), in compliance with the Human Biomedical Research Act (Ministry of Health, Singapore). Blood samples were collected either from venepuncture into a 4 mL K2 EDTA tube (BD vacutainer) of healthy and diabetics for DFF isolation or from healthy donors undergoing leukapheresis for monocyte subsets isolation.

For more information on device fabrication, monocyte treatment, macrophage differentiation, monocyte subsets sorting and immunofluorescent staining, please refer to supplementary information.

3. Results and discussion

3.1 Impedance-based leukocyte subtypes identification

To determine if DFF leukocyte isolation step can enhance impedance detection, the impedance profile of different blood cell samples, namely the diluted whole blood, peripheral blood mononuclear cells (PBMCs), DFF-sorted monocytes, DFF-sorted lymphocytes and DFF-sorted neutrophils **were first characterized** (refer to methods on DFF isolation of different leukocyte subtypes). **A concentration of 1×10^6 cells/mL was used to ensure that cells are flowing in single cell profile to minimize chances of particle coincidence, which may affect impedance analysis.**

Single-cell impedance measurements were plotted as a 2D scatter distribution plot based on cell size ($|Z_{LF}|$, impedance magnitude at 0.3MHz) and opacity ($|Z_{HF}|/|Z_{LF}|$, ratio of impedance magnitude at 1.72MHz to 0.3MHz) which corresponds to cell membrane property (Holmes et al. 2009; van Berkel et al. 2011). As shown in Fig. 2A, diluted whole blood sample had low impedance signal of $|Z_{LF}|$ below 0.002 V which comprised mainly of RBCs and platelets as they are the most abundant cells (~100-1000-fold more than leukocytes) in whole blood. The PBMCs impedance profile was significantly different from whole blood with the depletion of RBCs. To facilitate analysis, the impedance signal **was divided** into 3 zones based on cell size to distinguish RBCs and platelets (zone 1, RBCs: 5–6 μ m; platelets: 2–3 μ m), lymphocytes (zone 2, 7–8 μ m) and neutrophils/monocytes (zone 3, 10–15 μ m). As expected, PBMC samples consisted of ~80–85% lymphocytes and ~15–20% monocytes, which corresponded to the presence of a large and small population in zones 2 and 3, respectively. There was also a significant population in zone 1 which were the platelets as they are usually isolated in the PBMCs fraction during Ficoll centrifugation. Our impedance gating strategy was further validated on DFF-sorted lymphocytes and monocytes showing observed similar distribution patterns in zone 2 and 3 respectively. Impedance signal was clearly enhanced after DFF enrichment, and lymphocyte opacity (~1.2) was higher than monocytes (~1.1) in both PBMCs

and DFF-sorted samples, indicating distinct differences in the membrane properties of these cell types. Next, DFF-sorted neutrophils were characterized and were found to be residing in zone 3 due to their similarities in cell size as monocytes. Of note, there was a significant depletion of platelets in zone 1 for DFF-sorted samples due to the buffer exchange capabilities of DFF which enables leukocytes to be eluted in new buffer prior to analysis (Yeo et al. 2015). This is an important feature as the presence of small particles (such as a platelet or cell debris) can affect impedance measurement when pass through the detection region simultaneously. Neutrophils had a higher opacity (~1.2) than monocytes (~1.1) and was similar to previous work (Holmes et al. 2009). For analysis of cell purity after DFF, the frequency distribution of the various leukocyte subtypes of unsorted PBMCs and DFF-sorted samples were measured (Fig. 2B). Additionally, the flow cytometric analysis of sorted leukocytes (CD14 for monocytes, CD66b for neutrophils and CD3/CD19 for lymphocytes) was also performed to identify various leukocyte subtypes. (Fig. S3). The different leukocyte subtypes were sufficiently enriched (~60–80%) for downstream impedance detection. Taken together, these results clearly demonstrate the capabilities and importance of label-free DFF isolation to obtain enriched leukocyte subtypes for enhanced impedance measurement detection sensitivity and selectivity.

3.2 Impedance-based leukocyte activation profiling

Next, the effects of inflammatory stimulus (TNF- α and LPS) on the impedance profile of DFF-sorted monocytes were investigated. PBMCs were isolated and monocytes were then sorted using DFF after treatment of whole blood samples with TNF- α (10ng/mL) or LPS (10 μ g/mL) for 4 h. Enriched monocytes were then perfused through the developed microfluidic impedance device and ~1000–5000 single cell events were recorded (~5 min) for analysis. As shown on Fig. 3A, the impedance scatter distribution was higher for TNF- α -treated monocytes as compared to untreated monocytes obtained from the same individual. Interestingly, 2 distinct

1 subpopulations were observed for LPS-treated monocytes which we defined as LPS_{low}
2 (equivalent opacity and size distribution) and LPS_{High} (higher opacity and smaller cell size) as
3 compared to untreated monocytes. The mean opacity and cell size based on the centroid of the
4 impedance distribution were further quantified. Opacity was significantly higher for both
5 TNF α -treated and LPS_{High} monocytes ($P < 0.01$) as compared to untreated monocytes, and there
6 were negligible differences in cell size among all groups except between LPS_{low} and LPS_{High}
7 monocytes ($P < 0.05$) (Fig. 3B). We then sought to determine the leukocyte composition of the
8 treated blood samples before and after DFF sorting using flow cytometry. Increased neutrophil
9 population (CD66b+) were found in the PBMCs of both TNF- α and LPS treated blood (TNF-
10 α ~28%, LPS ~17% and untreated ~3%), which was likely due to low-density neutrophils that
11 had undergone degranulation during activation (Deng et al. 2016; Nicolás-Ávila et al. 2017).
12 Their presence in PBMCs were also reported in different diseases (Carmona-Rivera and Kaplan
13 2013; Deng et al. 2016; Fu et al. 2014; Herteman et al. 2017) (Fig. 3C). As previous literatures
14 have reported differential impedance profile between monocytes and neutrophils (Hassan et
15 al. 2016; Holmes and Morgan 2010; Holmes et al. 2009; Simon et al. 2016), we hypothesize
16 that the two populations in the LPS-treated samples correspond to neutrophils (LPS_{High}) and
17 monocytes (LPS_{low}) as shown in Fig. 3A. The effects of LPS treatments (concentration and
18 duration) were further investigated and LPS_{high} and CD66b+ populations were found to
19 increase in a time and dose-dependent manner in both impedance measurements and flow
20 cytometry analysis, thus confirming that the LPS_{High} population consists of neutrophils (Fig.
21 S4). Noteworthy, flow cytometry analysis of TNF α -treated samples revealed higher neutrophil
22 population (~28%), but there was only a single population distribution in our impedance
23 measurement as opposed to LPS treatment. We hypothesized that TNF- α might induce
24 significant changes on monocyte membrane properties which resulted in similar impedance
25 characteristics similar to low-density neutrophils (shift towards higher opacity and lower cell

size). Monocyte membrane protein expression of CD14 and CD11b, which reflects monocyte activation status (Abeles et al. 2012; van Oostrom et al. 2004), were also upregulated significantly for TNF α and LPS treated conditions as compared to untreated monocytes (Fig. 3D). Overall, these results suggest the use of impedance measurements to characterize leukocytes activation and monitor membrane-associated cellular processes. The proposed strategy can also be used as a novel and label-free approach to detect low-density neutrophils for point-of-care testing.

3.3 Impedance-property of monocyte differentiation into macrophages

Monocytes differentiation into macrophages is a vital immune function and they are responsible for homeostasis, tissue repair and defence against pathogens (Wynn et al. 2013). To determine if impedance signal can be used to monitor the differentiation process, monocytes impedances were measured at day 0 (undifferentiated), day 4 (partially differentiated) and day 7 (fully differentiated) when treated with M-CSF to induce macrophages formation (Fig. 4A). Representative impedance profiles of monocytes at different differentiation stages were shown in Fig. 4B. Impedance distribution was clearly more heterogeneous and widespread during the process. As monocytes undergo differentiation, a significant increase in cell size was observed at day 4 ($P < 0.05$) and day 7 ($P < 0.01$) as compared to day 0. (Fig. 4C). Average opacity was similar at day 4 but decreased slightly at day 7 (no significant differences) probably due to the increase in surface area. These results suggest the feasibility of using impedance cytometry as a label-free approach to monitor monocyte differentiation.

3.4 Monocyte subsets impedance profiling

Monocyte is a heterogenous cell population and can be classified into classical (CD14⁺⁺/CD16, intermediate (CD14⁺⁺/CD16) and non-classical (CD14⁺/CD16⁺⁺) monocytes based on CD14 and CD16 expressions (Boyette et al. 2017; Wong et al. 2011). Previous literatures have reported the role of different monocyte subsets in the pathogenesis of cardiovascular diseases

(Shantsila et al. 2011; Wildgruber et al. 2016), and identification of specific monocyte subsets would be imperative for functional studies and clinical testing. In this study, the impedance profiles of purified monocyte subsets pre-sorted using FACS were characterized (see Methods). Both classical and non-classical monocytes exhibited a single-peak profile while intermediate monocytes had a relatively more heterogenous impedance distribution (Fig. S5A). Fig. 4D shows the normalized opacity and size of each monocyte subset from 4 healthy subjects. Intermediate monocytes were found to be the largest in cell size and non-classical monocytes were the smallest, which were consistent with prior literatures (Cros et al. 2010; Shantsila et al. 2011). Interestingly, intermediate monocytes had the lowest opacity as compared to classical monocytes ($P < 0.01$), and future investigations are warranted to study the associations between monocyte dielectric properties and their functional phenotypes. It should be noted that monocyte subsets were sorted based on antibodies labelling that can inherently affect their cellular functions and properties (Jensen 2012; Progatzky et al. 2013). Subtle differences in impedance profile between unstained and stained monocytes were also observed (Fig. S5C), which further highlight the importance of antibodies-free monocyte isolation for impedance-based assays. Nevertheless, our data clearly demonstrated subtle changes in impedance characteristics of different monocyte subsets which can be further exploited for label-free cell sorting.

3.5 Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM)

As proof-of-concept for rapid and label-free immune health profiling, dielectric properties of neutrophils and monocytes isolated by DFF from T2DM patients ($n=8$, Table S2) were measured. T2DM was chosen as the disease model because neutrophils and monocytes are widely implicated in the disease pathogenesis (Giulietti et al. 2007; Min et al. 2012), and have been proposed as biomarkers for atherosclerosis risks in T2DM (Patkó et al. 2012). Fig. 5A

shows the impedance-based characterization of cell size and opacity of monocytes and neutrophils in T2DM patients. No associations between leukocyte size and opacity were observed (Fig. S7). While there were negligible differences in $|Z_{LF}|$ (cell size) of both populations, neutrophil opacity was significantly higher than monocytes ($P < 0.05$), and neutrophil and monocyte opacity was strongly correlated for all subjects (Fig. 5B). In general, opacity and size were higher in diabetic monocytes as compared to healthy controls (Fig. S6). This trend is indeed consistent with *in vitro* TNF- α treated monocytes, thus suggesting a pro-inflammatory signature in T2DM subjects. Monocyte-platelet aggregates (MPA) was also present in DFF-sorted monocytes based on immunofluorescence staining for platelets (CD41a), monocytes (CD14) and cell nucleus (Hoechst dye) (Fig. 5C). This is interesting as MPA is a sensitive indicator for platelet activation (Michelson et al. 2001) and can alter the monocytes towards a more atherogenic phenotype (Linden and Jackson 2010). Moreover, platelet binding to monocyte surface can also contribute to biophysical changes in monocyte size ($|Z_{LF}|$) and opacity.

To further understand the clinical significance of the leukocyte impedance measurements using the developed technology, we need to determine their associations with established cardiovascular risk factors (blood pressure, lipids, glycaemic, CRP *etc.*) and vascular functions. Our results showed that diabetic monocyte size ($|Z_{LF}|$) was associated with increasing non high-density lipoprotein (HDL) and low-density lipoprotein (LDL) cholesterol levels ($P < 0.05$) (Fig. 6A,B), which is important as LDL cholesterol accelerates monocyte-macrophage differentiation (Escate et al. 2016) and play a key role in lipid loading in monocytes (Fernandez-Ruiz et al. 2016). Similar trends were also observed for total cholesterol ($P = 0.06$) and triglyceride levels ($P = 0.08$) (Fig. S7). Monocyte opacity decreased with reactive hyperemia index (LnRHI) ($P < 0.05$), a measurement of endothelial function and a powerful surrogate marker for cardiovascular health (Rubinshtein et al. 2010). With a cutoff of < 0.51

defined as abnormal or dysfunctional endothelium, this corresponded to T2DM subjects with the highest monocyte opacity (most activated). Decreasing monocyte opacity with hyperlipidemia duration ($P < 0.05$) (Fig. 6D) **was also observed**. A possible reason is that T2DM subjects with hyperlipidemia are taking lipid-lowering medications (e.g. statins), and subjects with more recent hyperlipidemia (thus less medication) may still reflect an overall more activated monocyte phenotype. Lastly, **while** neutrophil size and opacity did not **have** any associations with lipid levels, neutrophil size was strongly correlated to C-reactive protein (CRP), a key marker for low-grade inflammatory state and cardiovascular risk ($P < 0.05$) (Fig. 6E) and duration of hyperlipidemia ($P < 0.05$) (Fig. 6F). Taken together, these results strongly support our hypothesis that T2DM subjects with severe endothelial dysfunction or higher cardiovascular risks have a more pro-inflammatory (more activated) neutrophil/monocyte phenotype as seen by their higher size and opacity. However, the sample size in this study is small, and a large-scale clinical validation is necessary to confirm our hypotheses and further develop these impedance-based biomarkers for label-free immune health profiling.

Overall, the results show the versatility of developed platform in leukocyte impedance phenotyping using coplanar electrode configuration. Although a parallel electrode configuration has a better sensitivity and detection limit as compared to the coplanar electrode configuration (adopted in this work) owing to less divergent electric field (Sun et al. 2008), the fabrication process is more complicated and more expensive. Herein, we demonstrated that the subtle changes in dielectric properties of cells can be detected even with coplanar electrode configuration after a microfluidic purification step. Additionally, the coplanar electrode configuration facilitates integration with other microfluidic modules for “sample in-answer out” platforms. In term of response time, there are minimal differences between parallel and coplanar electrode designs since they both require similar electronic setups (transimpedance amplifiers and lock-in amplifier). Lastly, the operating throughput of our developed cytometer

- 1 is in the range of 400-1000 cells/min, which is comparable to systems from other groups
- 2 (Cheung et al. 2005; Rollo et al. 2017; Zhou et al. 2018).

4. Conclusions

In summary, we have developed a novel microfluidic strategy for rapid and label-free leukocyte sorting coupled with impedance profiling from whole blood. Enhanced impedance detection selectivity **was demonstrated** when DFF was used to fractionate and enrich for different leukocyte subtypes prior to analysis in a microfabricated impedance cytometer. The impedance-based monocyte quantification was successfully used for monocyte activation profiling, monitoring monocytes differentiation to macrophages, as well as biophysical characterization of monocyte subsets (classical, intermediate, non-classical). The developed technology **was also clinical validated** for label-free leukocyte impedance profiling in T2DM subjects, **in which** leukocyte dielectric properties (size and opacity) were significantly associated with cardiovascular risk factors including lipid levels, C-reactive protein (CRP) and vascular function (LnRHI). We envisage the proposed strategy will greatly facilitate immunology research, and future work includes the integration of leukocyte DFF sorting module with impedance cytometry on a single chip for rapid point-of-care testing.

Author contributions

C.P, K.H.H.L and H.W.H designed research. C.P, H.M.T, R.D. and H.W.H performed experiments and analysed the data. W.H.Y and S.C.W assisted in the isolation monocyte subsets. C.P, H.M.T and H.W.H wrote the manuscript. All authors reviewed the manuscript.

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References

- Abeles, R.D., McPhail, M.J., Sowter, D., Antoniadou, C.G., Vergis, N., Vijay, G.K., Xystrakis, E., Khamri, W., Shawcross, D.L., Ma, Y., Wendon, J.A., Vergani, D., 2012. *Cytometry. Part A* 81(10), 823-834.
- Beyrau, M., Bodkin, J.V., Nourshargh, S., 2012. *Open Biology* 2(11).
- Boyette, L.B., Macedo, C., Hadi, K., Elinoff, B.D., Walters, J.T., Ramaswami, B., Chalasani, G., Taboas, J.M., Lakkis, F.G., Metes, D.M., 2017. *PLOS ONE* 12(4), e0176460.
- Carmona-Rivera, C., Kaplan, M.J., 2013. *Semin Immunopathol* 35(4), 455-463.
- Chen, J., Xu, C., Zhao, Y., Chen, D., Wu, M.-H., Wang, J., 2015. *International Journal of Molecular Sciences* 16(5), 9804.
- Cheung, K., Gawad, S., Renaud, P., 2005. *Cytometry Part A* 65A(2), 124-132.
- Cheung, K.C., Di Berardino, M., Schade-Kampmann, G., Hebeisen, M., Pierzchalski, A., Bocsi, J., Mittag, A., Tárnok, A., 2010. *Cytometry Part A* 77(7), 648-666.
- Cros, J., Cagnard, N., Woollard, K., Patey, N., Zhang, S.Y., Senechal, B., Puel, A., Biswas, S.K., Moshous, D., Picard, C., Jais, J.P., D'Cruz, D., Casanova, J.L., Trouillet, C., Geissmann, F., 2010. *Immunity* 33(3), 375-386.
- Deng, Y., Ye, J., Luo, Q., Huang, Z., Peng, Y., Xiong, G., Guo, Y., Jiang, H., Li, J., 2016. *PLOS ONE* 11(4), e0153567.
- Du, E., Ha, S., Diez-Silva, M., Dao, M., Suresh, S., Chandrakasan, A.P., 2013. *Lab Chip* 13(19), 3903-3909.
- Escate, R., Padro, T., Badimon, L., 2016. *Atherosclerosis* 246, 177-186.
- Evander, M., Ricco, A.J., Morser, J., Kovacs, G.T.A., Leung, L.L.K., Giovannardi, L., 2013. *Lab Chip* 13(4), 722-729.
- Fernandez-Ruiz, I., Puchalska, P., Narasimhulu, C.A., Sengupta, B., Parthasarathy, S., 2016. *J. Lipid Res.* 57(4), 574-586.
- Fu, J., Tobin, M.C., Thomas, L.L., 2014. *Annals of Allergy, Asthma & Immunology* 113(6), 635-640.e632.
- Fukuda, S., Schmid-Schönbein, G.W., 2002. *J. Leukoc. Biol.* 72(1), 133-139.
- Giulietti, A., van Etten, E., Overbergh, L., Stoffels, K., Bouillon, R., Mathieu, C., 2007. *Diabetes Res Clin Pract.* 77(1), 47-57.
- Gordon, S., Taylor, P.R., 2005. *Nat Rev Immunol* 5(12), 953-964.
- Hassan, U., Ghonge, T., Reddy Jr, B., Patel, M., Rappleye, M., Taneja, I., Tanna, A., Healey, R., Manusry, N., Price, Z., Jensen, T., Berger, J., Hasnain, A., Flaughner, E., Liu, S., Davis, B., Kumar, J., White, K., Bashir, R., 2017. 8, 15949.
- Hassan, U., Watkins, N.N., Reddy Jr, B., Damhorst, G., Bashir, R., 2016. *Nat. Protocols* 11(4), 714-726.

Henson, R., 2016. Flow Cytometry Data Reader and Visualization
<http://www.mathworks.com/matlabcentral/fileexchange/8430-flow-cytometry-data-reader-and-visualization>).
 MATLAB Central File Exchange.

Herteman, N., Vargas, A., Lavoie, J.-P., 2017. *Sci. Rep.* 7(1), 7743.

Holmes, D., Morgan, H., 2010. *Anal. Chem.* 82(4), 1455-1461.

Holmes, D., Pettigrew, D., Reccius, C.H., Gwyer, J.D., van Berkel, C., Holloway, J., Davies, D.E., Morgan, H., 2009. *Lab Chip* 9(20), 2881-2889.

Hou, H.W., Petchakup, C., Tay, H.M., Tam, Z.Y., Dalan, R., Chew, D.E.K., Li, K.H.H., Boehm, B.O., 2016. *Sci. Rep.* 6, 29410.

Jensen, E.C., 2012. *Anat Rec* 295(12), 2031-2036.

Küttel, C., Nascimento, E., Demierre, N., Silva, T., Braschler, T., Renaud, P., Oliva, A.G., 2007. *Acta trop.* 102(1), 63-68.

Linden, M.D., Jackson, D.E., 2010. *Int. J. Biochem. Cell Biol.* 42(11), 1762-1766.

Michelson, A.D., Barnard, M.R., Krueger, L.A., Valeri, C.R., Furman, M.I., 2001. *Circulation* 104(13), 1533-1537.

Min, D., Brooks, B., Wong, J., Salomon, R., Bao, W., Harrisberg, B., Twigg, S.M., Yue, D.K., McLennan, S.V., 2012. *Mediators Inflamm.* 2012, 649083.

Nicolás-Ávila, J.Á., Adrover, J.M., Hidalgo, A., 2017. *Immunity* 46(1), 15-28.

Patkó, Z., Császár, A., Acsády, G., Óry, I., Takács, É., Fűrész, J., 2012. *Interv Med Appl Sci* 4(4), 181-185.

Petchakup, C., Li, K., Hou, H., 2017. *Micromachines* 8(3), 87.

Progatzky, F., Dallman, M.J., Lo Celso, C., 2013. *Interface Focus* 3(3), 20130001.

Rollo, E., Tenaglia, E., Genolet, R., Bianchi, E., Harari, A., Coukos, G., Guiducci, C., 2017. *Biosens. Bioelectron* 94, 193-199.

Rubinshtein, R., Kuvin, J.T., Soffler, M., Lennon, R.J., Lavi, S., Nelson, R.E., Pumper, G.M., Lerman, L.O., Lerman, A., 2010. *Eur. Heart J.* 31(9), 1142-1148.

Rüegg, C., 2006. *J. Leukoc. Biol.* 80(4), 682-684.

Shantsila, E., Wrigley, B., Tapp, L., Apostolakis, S., Montoro-Garcia, S., Drayson, M.T., Lip, G.Y.H., 2011. *J Thromb Haemost* 9(5), 1056-1066.

Simon, P., Frankowski, M., Bock, N., Neukammer, J., 2016. *Lab Chip* 16(12), 2326-2338.

Spencer, D., Elliott, G., Morgan, H., 2014a. *Lab Chip* 14(16), 3064-3073.

Spencer, D., Hollis, V., Morgan, H., 2014b. *Biomechanics* 8(6), 064124.

Sprangers, S., de Vries, T.J., Everts, V., 2016. *J Immunol Res* 2016, 1475435.

Sun, T., Green, N., Morgan, H., 2008. *Nano* 03(01), 55-63.

Sun, T., Morgan, H., 2010. *Microfluid and Nanofluidics* 8(4), 423-443.

Tay, H.M., Dalan, R., Li, K.H.H., Boehm, B.O., Hou, H.W., 2018. *Small* 14(6), 1702832.

van Berkel, C., Gwyer, J.D., Deane, S., Green, N., Holloway, J., Hollis, V., Morgan, H., 2011. *Lab Chip* 11(7), 1249-1255.

van Oostrom, A.J., van Wijk, J.P., Sijmonsma, T.P., Rabelink, T.J., Castro Cabezas, M., 2004. *Neth J Med.* 62(9), 320-325.

Wildgruber, M., Aschenbrenner, T., Wendorff, H., Czubba, M., Glinzer, A., Haller, B., Schiemann, M., Zimmermann, A., Berger, H., Eckstein, H.-H., Meier, R., Wohlgemuth, W.A., Libby, P., Zerneck, A., 2016. *Sci. Rep.* 6, 39483.

Wilson, E.H., Weninger, W., Hunter, C.A., 2010. *J. Clin. Investig* 120(5), 1368-1379.

Wong, K.L., Tai, J.J.-Y., Wong, W.-C., Han, H., Sem, X., Yeap, W.-H., Kourilsky, P., Wong, S.-C., 2011. *Blood* 118(5), e16-e31.

Wynn, T.A., Chawla, A., Pollard, J.W., 2013. *Nature* 496, 445.

Yeo, D.C., Wiraja, C., Zhou, Y., Tay, H.M., Xu, C., Hou, H.W., 2015. *ACS Applied Materials & Interfaces* 7(37), 20855-20864.

Zhou, Y., Yang, D., Zhou, Y., Khoo, B.L., Han, J., Ai, Y., 2018. *Anal. Chem.* 90(1), 912-919.

1 5. Figures and Figure legends

Fig. 1 Experimental setup (A) Workflow of leukocyte impedance phenotyping strategies starting with sample pre-processing (RBC lysis or Ficoll density centrifugation), DFF leukocyte sorting and impedance profiling (B) Schematic of experimental setup illustration of microfluidic chips, (C) Optical image illustrating single cell flowing through the electrodes in the detection region and (D) Time domain measurement of multiple events from single cells.

Fig. 2 Impedance profiling of different blood cell samples (A) Impedance profiles (density scatter plot of cell size ($|Z_{LF}|$, V) versus opacity) of diluted whole blood (1000 $\times$ dilution), PBMCs, DFF-sorted monocytes, DFF-sorted lymphocytes and DFF-sorted neutrophils. Impedance profile was gated into 3 different zones based on cell size to distinguish cellular debris, platelets and RBCs (zone 1: 0.0004-0.002V), lymphocytes (zone 2: 0.002-0.007V), and monocytes and neutrophils (zone 3: 0.007-0.015V). Due to high density of zone 1 population, the scatter density of zone 1 was calculated separately from zone 2 and zone 3. (B) Frequency distribution of different leukocyte subtypes in each zone for unsorted PBMCs and DFF-sorted leukocytes to determine cell purity.

Fig. 3 Impedance-based activation profiling of enriched monocytes (A) Representative impedance profile of untreated (left), TNF- α treated (center) and LPS treated monocytes (right) with number of cells (n) (B) Average monocyte opacity (left) and cell size (right) for different conditions. Values were taken from the centroid positions of impedance profile for untreated, TNF- α , LPS_{low} (blue circle) and LPS_{High} (red circle). Bars were represented as mean $\pm$ s.e.m. *P $\leq$ 0.05, **P $\leq$ 0.01 (C) Flow cytometry analysis of leukocyte composition of PBMCs and DFF-sorted samples (n=4). (D) Flow cytometry analysis of monocyte activation markers CD14 (top) and CD11b (bottom) at different conditions.

Fig. 4 Characterization of monocyte differentiation into macrophages and monocyte subsets characterization (A) 20× Brightfield images of seeded monocytes (day 0), and macrophages after 4 days 7 days of differentiation with M-CSF. Scale bar = 20 μ m (B) Impedance profile of monocytes, monocytes undergoing differentiation at day 4, and fully-differentiated macrophages at day 7 (MΦ). (C) Average monocyte size (bottom) and opacity (top) at different differentiation stages. Bars were represented as mean $\pm$ s.e.m. *P < 0.05, **P < 0.01 (D) Normalized opacity (left) and size (right) for monocyte subtypes from 4 different subjects. The normalization was acquired by dividing opacity of each subset by the average value of every subsets (same subject). Paired t-test was used to determine the statistical significance of the difference between two sets of data. *P < 0.05, **P < 0.01. For raw data please refer to Fig. S5B.

Fig. 5 Impedance-based immune health profiling in type 2 diabetes mellitus (T2DM) (A) Measurement of cell size and opacity of monocytes and neutrophils in T2DM patients. (B) Strong correlation of monocyte and neutrophil opacity in T2DM patients. (C) Representative brightfield and immunofluorescence images of T2DM monocytes with the presence of monocyte-platelet aggregates (MPAs) after DFF.

Fig. 6 Correlation of monocyte and neutrophil impedance parameters with (A) Non-HDL cholesterol, (B) LDL Cholesterol, (C) LnRHI, (D) Hyperlipidemia and correlation of neutrophil impedance parameters with (E) C-reactive protein (CRP) and (F) Hyperlipidemia