HHS Public Access

Author manuscript

Lab Invest. Author manuscript; available in PMC 2014 March 18.

Published in final edited form as:

Lab Invest. 2011 December ; 91(12): 1787–1795. doi:10.1038/labinvest.2011.94.

Microfluidics-Based Capture of Human Neutrophils for Expression Analysis in Blood and Bronchoalveolar Lavage

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Abstract

Gene expression analysis can be a powerful tool in predicting patient outcomes and identifying patients who may benefit from targeted therapies. However, isolating human blood neutrophils (PMNs) for genomic analysis has been challenging. We employed a novel microfluidic technique that isolates PMNs by capturing $CD66b^+$ cells and compared it to dextran-Ficoll gradient isolation. We also employed microfluidic isolation techniques to blood and bronchoalveolar lavage (BAL) samples of patients with ARDS to evaluate PMN genomic alterations secondary to pulmonary sequestration. PMNs obtained from *ex vivo* lipopolysaccharide (LPS)-stimulated or unstimulated whole blood from five healthy volunteers were isolated by either dextran-Ficoll gradient, microfluidics capture, or a combination of the two techniques. Blood and BAL fluid PMNs were also isolated using microfluidics from seven hospitalized patients with ARDS. Gene expression was inferred from extracted RNA using Affymetrix U133 Plus 2.0 GeneChips™. All methods of PMN isolation produced similar quantities of high-quality RNA, when adjusted for recovered cell number. Unsupervised analysis and hierarchal clustering indicated that LPS stimulation was the primary factor affecting gene expression patterns among all *ex vivo* samples. Patterns of gene expression from blood and BAL PMNs differed significantly from each other in the patients with ARDS. Isolation of PMNs by microfluidics can be applied to both blood and BAL specimens from critically ill, hospitalized patients. Unique genomic expression patterns are obtained from the blood and BAL fluid of critically ill patients with ARDS, and these differ significantly from genomic patterns seen after ex vivo LPS stimulation.

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Financial Conflict of Interest Statement: The studies were funded by the National Institute of General Medical Sciences, and the authors have no financial conflicts of interest.

Keywords

Neutrophil isolation; microfluidics; genomics; dextran; ficoll

Introduction

Still a developing technology, genome-wide expression has emerged as a robust and sensitive tool to study genomic variation among different clinical states, tissues, and cellular mechanisms. To date, wide-spread use of genome-wide analyses has demonstrated both prognostic capabilities in both solid and disseminated neoplasms, and predictive utility for populations at risk for various diseases. More recently, we have shown that unique genome-wide patterns of expression from whole blood are strongly associated with sepsis and multiorgan dysfunction syndrome (1, 2), and these patterns can be used to identify previously unappreciated signaling pathways important to inflammation (3-9). Due to the relative ease and minimal morbidity of obtaining blood samples from a human subject, peripheral blood leukocytes and various subpopulations have taken center stage as an optimal tissue source for genomic analysis in the field of inflammation (10-12).

Pioneering the field of genomics in trauma has been the *'Inflammation and Host Response to Injury'* Large-Scale Collaborative Research Program seeking to understand and possibly predict the heterogeneous outcomes associated with major trauma and burn injuries. Through this effort, it has now become clear that the sensitivity of genomic analysis is most powerful when highly enriched cell populations are the source of genomic input rather than mixed cell populations (10, 13). Whole blood and solid tissues contain heterogeneous cell populations and therefore meaningful changes in gene expression patterns can be obscured by conflicting shifts in leukocyte sub-populations present in the same sample. For this reason, most investigations have evolved towards genomic analyses using enriched leukocyte subpopulations.

Though unintended, cellular enrichment has the potential to introduce genomic artifacts as a result of the isolation procedure. Until recently, the predominant method used to isolate leukocyte populations for genomic analysis has utilized either antibody precipitation and/or sequential centrifugation over discontinuous density gradients (*e.g*. Percoll, Ficoll, Dextran), separating cell populations based on differential densities. Unfortunately, these methods are laborious and protracted (>2 hours), and subjects the sample to considerable *ex vivo* perturbations resulting in documented phenotypic and functional changes (14-16).

In the present report, we employed a microfluidic cassette capable of rapidly isolating polymorphonuclear leukocytes or neutrophils (PMNs) from biological fluids by antibody capture. This cassette contains a series of branched channels 50 μm in height that are coated with a monoclonal antibody to human CD66b (a cell surface marker specific to granulocytes). Biological fluids are passed through the cassette inlet at optimized flow rates and unbound cells are washed away through a single device outlet, leaving only the adherent $CD66b^+$ cell populations. These adherent cells are then lysed with a chaotrope and nucleic acids are extracted *en bloc*.

PMNs from unstimulated and *ex vivo* LPS-stimulated whole blood obtained from healthy human subjects were processed in parallel by either dextran-Ficoll sedimentation or through the anti-CD66b$^+$ coated microfluidics device. Additionally, an aliquot of PMNs isolated by the dextran-Ficoll method was then subjected to further enrichment using the microfluidic cassette. The extracted RNA from the enriched PMNs obtained from the different isolation procedures was then further processed in parallel for genome-wide expression analysis. In addition, PMNs from whole blood and waste bronchoalveolar lavage (BAL) fluid were also isolated from seven hospitalized patients with Acute Lung Injury (ALI) and/or Acute Respiratory Distress Syndrome (ARDS). We demonstrate that PMNs from healthy control subjects have similar genome-wide expression patterns despite the different isolation methods. However, the expression of blood and BAL neutrophils from patients with ALI/ARDS is markedly different from the patterns obtained from unstimulated and *ex vivo* LPS stimulated blood PMNs from healthy subjects. These results indicate that the microfluidic isolation procedure appears equivalent to the gold-standard method of PMN isolation for genomic analysis, and can identify differences in gene expression secondary to the source of the PMNs and their *in vivo* or *ex vivo* stimuli.

Materials and Methods

Subjects

Peripheral venous blood was obtained from five healthy volunteers after obtaining signed, informed consent. Venous blood and waste BAL fluid (obtained during a diagnostic procedure) were also obtained from seven critically-ill patients with ALI/ARDS, after signed informed consent was provided by their legal representatives. All protocols were approved by the University of Florida, Institutional Review Board prior to their initiation.

Ex Vivo Studies

Whole blood samples from the healthy control subjects were each divided into two aliquots (see Figure 1). The first aliquot, representing the unstimulated arm, was processed immediately for PMN enrichment. In the second aliquot, the stimulated arm, *E. coli* (0111:B4) lipopolysaccharide (LPS; Sigma Fine Chemicals, St. Louis) was added at physiological concentrations (100 ng/ml) and the whole blood placed in a 5% CO_2 37° C incubator for two hours prior to PMN isolation. Venous blood (21 ml) from the healthy subjects was collected into Vacutainer tubes containing sodium EDTA (Becton Dickinson, Franklin Lakes, NJ). A 4 ml aliquot of either LPS stimulated or unstimulated whole blood was processed by dextran-Ficoll for neutrophil isolation, a 0.35 ml aliquot of whole blood was processed in parallel using the microfluidics cassette, and a 4 ml aliquot of whole blood was processed first by dextran-Ficoll, followed by the microfluidics cassette.

For the ALI/ARDS patients, a single 4 ml blood sample was collected and PMNs were isolated using the microfluidics cassette. In addition, waste BAL fluid (from a diagnostic BAL) was first gravity filtered through a sterile cell strainer (BD Falcon™, Bedford MA) to eliminate particulate debris prior to PMN isolation using the microfluidics cassette.

Neutrophil Isolation by Dextran-Ficoll Gradients

Peripheral blood PMNs were isolated by a modified dextran-Ficoll gradient as previously described by Nauseef, *et al*(17). Briefly, the whole blood was diluted with phosphate-buffered saline (PBS) mixed with 2% fetal calf serum & 100 U/ml polymixin B. Subsequently, this was layered on top of Ficoll-Paque Plus™ (Amersham Pharmacia Biotech) and centrifuged at 500 × *g* for 20 minutes at room temperature. The supernatant, interface cells, and Ficoll were then discarded and the remaining neutrophil/erythrocyte suspension was mixed with a 6% dextran solution and allowed to sediment in a 5% CO_2 incubator at 37° C for 30 minutes. The PMN enriched clear portion was then transferred to a new tube, resuspended in an erythrocyte lysis buffer (EL buffer, Qiagen, Valencia, CA) and kept on ice for 10 minutes. The sample was then washed with sterile PBS. The PMN pellet was resuspended in RLT buffer (Qiagen) and placed in a QIAshredder™ spin column (Qiagen) to collect nucleic acids according to manufacturer's instructions from the lysed PMNs. The RNA was stored at -70°C.

PMN Isolation by Microfluidic Cassette

Microfluidic isolation of PMNs was carried out as previously described (18-20). In short, a syringe loaded with 0.35 mL of whole blood or the strained BAL fluid were connected to the inlet and were continuously infused into the cassette (Figure 2a) by an automated pump over five minutes (30 μl/min). During this time, whole blood flowed through the cassette channels while $CD66b^+$ cells (PMNs and eosinophils) were bound to the channel walls through anti-CD66b antibody interactions and other non-$CD66b^+$ cells (erythrocytes and other leukocytes) remained unbound. After five minutes, a new syringe filled with PBS, was connected and infused through the cassette for five minutes at 90 μl/min. During this time, unbound cells were washed out of the channels via the outlet port and were discarded while $CD66b^+$ cells (>95% PMNs) remained bound to the channel walls. RLT buffer was then infused into the cassette to lyse the adherent cells and then ejected through the outlet channel for nucleic acid collection. The cell lysate was then centrifuged over a QIA shredder spin column and the nucleic acids were stored at -70°C.

PMN Isolation by Dextran-Ficoll followed by Microfluidics Cassette

Following isolation of PMNs via dextran-Ficoll sedimentation, the PMN pellet was resuspended in 2 ml sterile PBS. 350 μl of this sample was subsequently subjected to microfluidic processing as described above. Harvested nucleic acids were similarly stored at -70°C.

Assessment of Isolated PMN Purity

The resulting PMN purity from each method was assessed by light microscopy and Wright-Giemsa staining of cytospin preparations from the dextran-Ficoll isolated cells, and cassettes fixed and stained following PMN capture.

RNA isolation

Total cellular RNA was isolated using a commercially available kit (RNeasy™, Qiagen). RNA concentration and purity were confirmed by capillary electrophoresis (Agilent 2100 Bioanalyser, Agilent Inc).

cDNA Synthesis and Chip Hybridization

Amplified cDNA was prepared from approximately 1.5-5 ng of starting PMN RNA using the WT-Ovation Pico RNA Amplification System (NuGen, San Carlos, CA) per the manufacturer's protocol. Subsequently, the cDNA was fragmented and labeled using the FL-Ovation cDNA Biotin Module V2 kit (NuGen) per the manufacturer's protocol. Labeled cDNA was hybridized onto Hg-U133 Plus 2.0 GeneChip™ oligonucleotide arrays (Affymetrix, Santa Clara, CA), and processed per the manufacturer's protocol with few modifications.

Microarray Data Analysis

Chip expression and signal normalization were performed using DNA Chip Analyzer (dChip v1.0.0.1, www.dchip.org) by perfect match algorithms. BRB ArrayTools™ software was used to analyze the data for statistical inference. Differences among groups were evaluated by an adjusted F test using a false discovery adjusted probability (Q) of less than 0.001. To assess the ability of individual gene lists to predict treatment assignments, leave-one-out, cross validations were performed (one nearest neighbor, three nearest neighbor, nearest centroid and diagonal linear discriminate analyses). A Monte Carlo simulation with 1000 permutations of the dataset was run, and the probability was set at less than 0.001.

Results

RNA Quantity/Quality Isolated Using Microfluidics Isolation and Dextran-Ficoll

RNA quantity and quality were evaluated from blood PMNs isolated by either the microfluidics approach, by dextran-Ficoll density centrifugation, or by a combination of the two techniques. Both techniques generated similar high quantities of nucleic acids for genomic analysis (Table 1). The differences in absolute quantities recovered reflect the differences in starting material (4 mls of blood for the dextran-Ficoll and 0.35 mls for the microfluidics). In fact, the yields when adjusted for starting volumes were remarkably similar, with slightly greater unit yields with the microfluidics. In all cases, the two methods provided more than sufficient material for ammplifcation and subsequent hybridization to the GeneChip™ (Table 1, last column). The purity of the samples was also equivalent with greater than 95% of the captured cells identified as PMNs by morphology and immunohistochemistry staining (Figure 2b).

Genomic Analysis of Unstimulated and Ex Vivo LPS Stimulated Whole Blood PMNs from Healthy Control Subjects

The primary determinant of gene expression patterns from whole blood PMNs was exposure to *ex vivo* LPS stimulation and not the method of PMN isolation. Using an unsupervised approach, 16,190 out of the 54,645 probe sets had a coefficient of variation of > 0.5.

Hierarchical cluster analysis revealed that the primary separation between the samples was based on whether they were from LPS-stimulated or unstimulated whole blood (Figure 3). In contrast, clustering based on either the individual patient or method of isolation was less dominant. A subsequent supervised analysis of all samples revealed 7,123 probe sets differentially expressed between unstimulated and LPS-stimulated samples using a false-discovery adjusted probability of $p<0.001$. Additionally, all four leave-one-out cross validation prediction models were able to classify the samples with 100% accuracy according to whether they were ex vivo LPS-stimulated or unstimulated based on the 7,123 probe sets (*data not shown*).

However, there was some evidence that gene expression patterns could have been affected by the method of isolation, particularly after *ex vivo* LPS stimulation. As shown in Figure 4, hierarchical clustering within the LPS-stimulated group revealed that samples isolated using dextran-Ficoll (regardless of whether microfluidics was performed) clustered together, and the microfluidics isolation alone, clustered more broadly. These findings suggest that the dextran-Ficoll separation introduced some common gene expression pattern in the LPS-stimulated cells. The importance of these findings, however, is unclear because similar clustering based on isolation was not seen in the unstimulated group.

Microfluidic cassette PMN isolation was subsequently used in a clinical setting on samples from seven critically ill patients. Microfluidics isolation of enriched leukocyte populations has rarely been performed on populations obtained from non-blood sources, such as BAL, diagnostic peritoneal lavage, urine or cerebrospinal fluid. Expression patterns in BAL and blood PMNs were compared to expression patterns in whole blood from the healthy controls and the *ex vivo* LPS stimulated samples. As shown in Figure 5, a supervised hierarchical cluster analysis revealed that the patterns of gene expression in blood and BAL from ARDS patients were not only distinct from themselves, but were also distinct from PMN gene expression patterns obtained from healthy control subjects, unstimulated, or stimulated ex vivo with LPS. In fact, hierarchical clustering revealed that the gene expression patterns from ARDS patients blood and BAL PMNs were more similar to each other than they were to blood PMNs from healthy control subjects, regardless of whether they were stimulated *ex vivo* or unstimulated. More importantly, leave-one-out cross validation procedures revealed that the source of the PMNs could be identified with 100% assurance ($p<0.001$).

Much to our surprise, the differences in the pattern of gene expression between blood and BAL PMNs were not similar to the changes seen with *ex vivo* LPS stimulation of blood PMNs. As shown in Table 2, selected BAL PMN gene expression was associated with marked decreases in the expression of PMN granular proteins (myeloperoxidase, neutrophil collagenase, NGAL, and lactoferrin), increased expression of chemokines (CXCL1 (Gro-α), CXCL2 (MIP-2α), CXCL3 (MIP-2β), CXCL5 (ENA-78), CXCL16 (SRPSOX)), and decreased expression of pro-apoptotic proteins (BCL10 and BCLAF1) (all $p<0.001$) compared to blood PMNs from the same patient. In contrast, LPS stimulation of whole blood PMNs did not recapitulate the changes seen above with the exception that the expression of CXCL2 and CXCL3 were modestly increased. In contrast, LPS stimulation was more associated with increased expression of genes involved in NF-KB, MAPK and oxidative phosphorylation.

Discussion

As the field of genomics rapidly expands, it has become increasingly clear that standardization of data collection and processing is necessary to extract meaningful insights into the human transcriptome (13, 21), especially when studying diverse patient populations. The challenge has been to develop methodologies that are not only robust, but feasible within the clinical setting. Thus, techniques must be rapid, easy to perform, require small blood volumes, and produce meaningful data when performed by skilled nursing and technical staff.

Historically, the most traditional technique to isolate PMNs from whole blood has been density centrifugation using Ficoll and dextran. Unfortunately, these techniques are laborious, challenging and have the potential to stimulate cell populations through physical perturbation and exposure to multiple solutions throughout the process. We sought to compare the resulting genomic output from the dextran-Ficoll technique with a more rapid microfluidics cassette that is based on the principal of antibody capture. The former technique is characterized by large blood volumes, several hours of processing and exposure of the cells to gradients and nonphysiologic solutions, whereas the latter uses very small volumes of blood, is rapid, is processed with physiologic solutions, and is easy to perform by research nurses and technicians (20). The results suggest that the microfluidics technique can be readily used on samples from hospitalized patients and experimental laboratory blood samples. In fact, under conditions of *ex vivo* whole blood stimulation, microfluidics PMN isolation may be associated with less inter-sample variation than with the traditional dextran-Ficoll isolation. Coupled with the small volume requirements, ease of use and low variability, microfluidics methodologies are attractive for the isolation of PMNs for subsequent genome-wide analysis.

The natural question is whether the two techniques give equivalent results. The two isolation strategies are based on fundamentally different properties, and therefore, the resulting cell capture could be very different. With that said, the unsupervised analysis of unstimulated and LPS-stimulated whole blood PMNs from healthy volunteers (Figure 3) revealed that variations in gene expression patterns were dominantly affected by the presence (or absence) of LPS stimulation and not the individual subject or the method of isolation. This suggests that patterns of gene expression introduced by either dextran-Ficoll or microfluidics PMN isolation are less dramatic than the changes in gene expression evoked by *ex vivo* LPS stimulation. By analyzing the unstimulated and stimulated groups separately, the dramatic genomic variation secondary to LPS stimulation is removed, and the method of isolation effects on the overall patterns of gene expression can be examined. The clustering of samples isolated by only microfluidics in the LPS-stimulated group suggests that PMN microfluidics processing produces the least variable mRNA output of all the tested methods. Whether the outcome of a more homogenous genomic response in the microfluidics group is due to the introduction of minimal isolation artifact (i.e. a more clean method for PMN isolation) or the resulting self-selection of a more similar subset of PMNs (likely mediated by the relative abundance of $CD66b^+$ expression) is unclear. The fact that samples isolated by dextran-Ficoll followed by microfluidics capture clustered more closely with samples isolated by only dextran-Ficoll suggests at a minimum, that the biological and analytical

artifacts introduced by microfluidics processing are small, and possibly even less than the variability among subjects or due to the dextran-Ficoll. A supervised analysis of only the stimulated samples identified 387 probe sets (Q test [false discovery adjusted probability] at $p<0.001$) significantly different between all three methods which is greater than the number we would expect by chance (with Bonferroni's correction) indicating that there is likely some difference in PMN apparent gene expression due to isolation method, but that that difference is small when compared to the difference generated by *ex vivo* LPS stimulation (7,123 probe sets differentially expressed at $p<0.001$).

Previously, we have shown that total leukocytes isolated with microfluidics phenotypically appear to be much less activated than leukocytes subjected to a hypertonic erythrocyte lysis step (as required by dextran-Ficoll isolation) (18). It is still unclear whether PMN antibody binding to cassette walls via the CD66b receptor induces internal signaling that affects genomic expression. However, the time from PMN binding to cell lysis is less than 10 minutes and so any genomic changes would have to be rapid, and as our results indicate, are minimal compared to that of a known PMN stimulus (e.g. LPS).

Furthermore, the results presented here suggest that the microfluidics cassettes can be used for genomic analysis of PMN populations not only from blood, but also from BAL fluid. Not surprisingly, the patterns of gene expression from BAL PMNs differed significantly from blood, reflecting reduced apoptosis, increased chemokine expression and reduced expression of granular contents. However, the patterns of gene expression from BAL PMNs were more similar to the pattern from the blood PMNs of the same subjects, than to the patterns from the blood PMNs stimulated *ex vivo* with LPS.

Practical Considerations

Of utmost importance when comparing procedural methods involved in scientific inquiry is the validity and accuracy of the results produced. The results presented here suggest that the two techniques, dextran-Ficoll and microfluidics cassettes are essentially equivalent in generating high quality nucleic acids by enriching populations of PMNs. However, other considerations, such as time, costs, and ease of application are important as well. After working with both macroscale (dextran-Ficoll) and microscale (microfluidics) PMN separation techniques for genomic analysis, it is clear that microfluidic technology offers multiple distinct advantages: less time required for processing, fewer steps for user error, significantly less sample volume required, and more efficient RNA recovery. Additionally, microfluidics isolation may result in the introduction of less artifactual *ex vivo* stimulation, as the procedure requires significantly fewer sample manipulations (e.g. no centrifugation spins for washing or differential density separation, lack of erythrocyte lysis step), less exposure to nonphysiologic solutions or *g* forces, and requires significantly less processing time to nucleic acid recovery.

Importantly, the microfluidics approach can be used in the clinical setting and can be applied to both blood and BAL sources of PMNs. The techniques can clearly distinguish genomic differences between blood and BAL PMNs from critically ill patients, and from blood PMNs between healthy and sick individuals.

Acknowledgments

Supported in part by grants GM-40586 and GM-81923, awarded by the National Institute of General Medical Sciences, U.S.P.H.S. KMK-S, AGC and CM were supported by a National Institute of General Medical Sciences training grant in burns and trauma (GM-008721). AGC was supported by F32 NRSA GM-093665-01, awarded by the National Institute of General Medical Sciences.

Development of the microfluidics cassette was supported by the Inflammation and Host Response to Injury Large Scale Collaborative Research Program (Glue Grant;U54 GM062119-7), awarded to Dr. Ronald G. Tompkins, Massachusetts General Hospital by the National Institute of General Medical Sciences. This work represents a secondary product of the Program, and although the completed manuscript has been seen by the leadership of the Program, the conclusions and discussion are the authors, and do not necessarily represent the views of either the Program, Massachusetts General Hospital, or the National Institute of General Medical Sciences.

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Alphabetical List of Abbreviations Used in the Text

ALI	acute lung injury
ARDS	acute respiratory distress syndrome
BAL	bronchoalveolar lavage
LPS	lipopolysaccharide
PBS	phosphate-buffered saline
PMN	polymorphonuclear

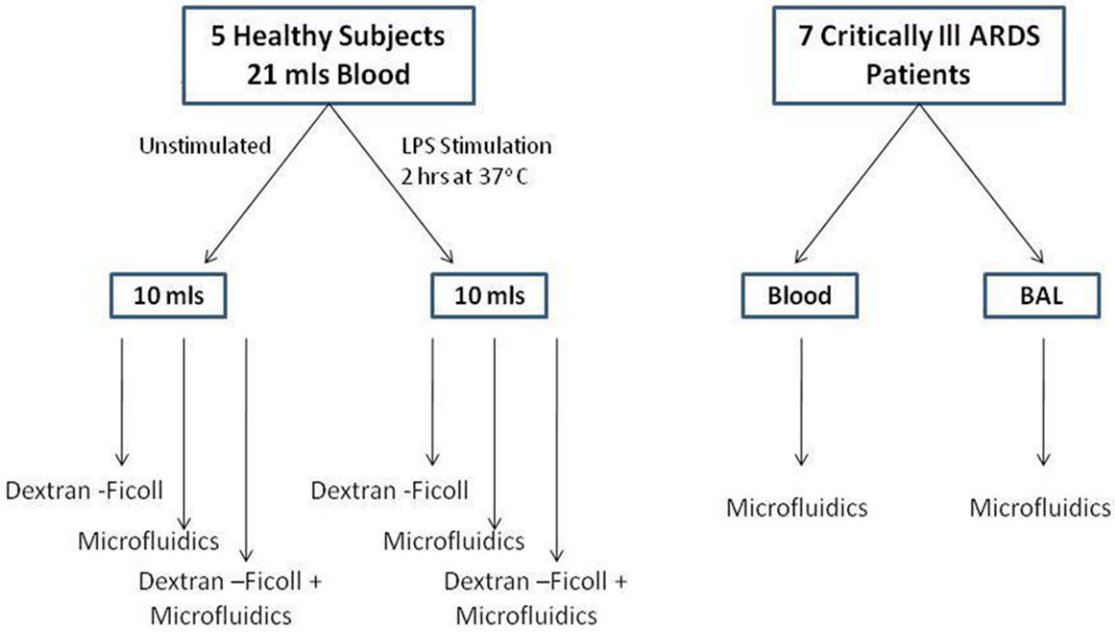

Figure 1.

Schematic Representation of Study Designs: Ex vivo Stimulation of Whole Blood from Healthy Subjects, and Blood and BAL Fluid from Hospitalized Patients with ARDS.

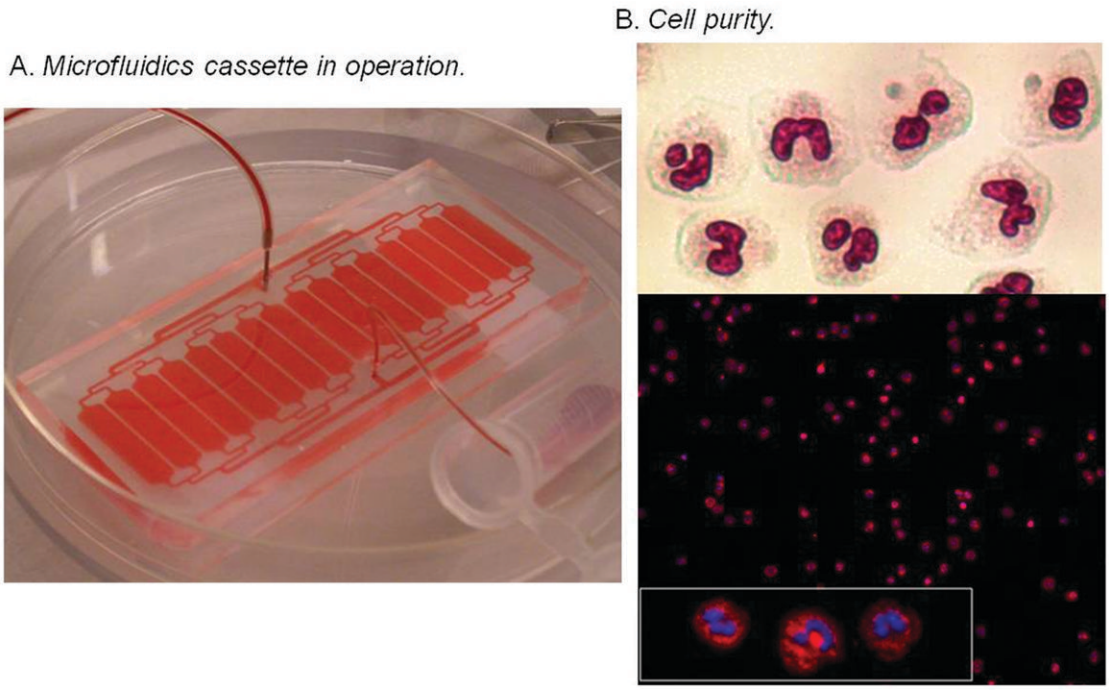

Figure 2.

Microfluidics Cassette for the Enrichment of PMNs from Whole Blood.

a.) Microfluidic cassette with whole blood filling channels between inlet and outlet ports. b.) Sample purity of captured neutrophils on cassette walls following PBS wash based on Wright-Giemsa and immunofluorescent staining. For fluorescence, cells were labeled with DAPI and PE-labeled CD66 and DAPI staining (magnification 100 x and 10 x, respectively).

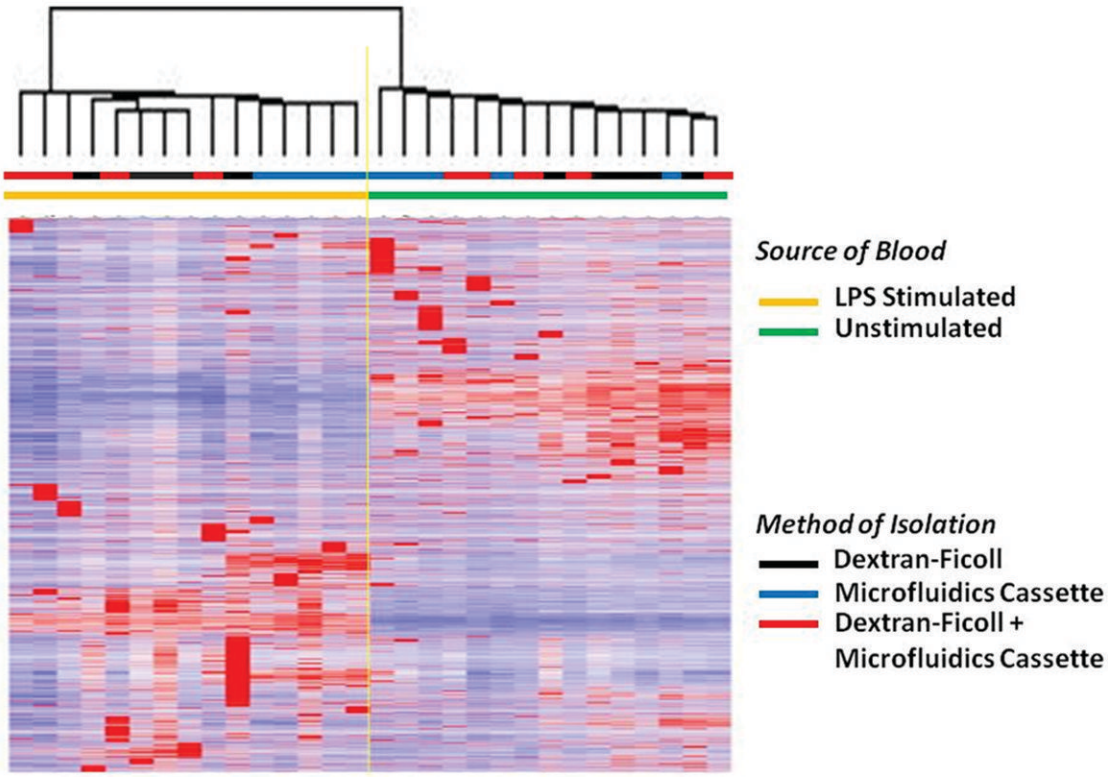

Figure 3.

Unsupervised analysis of genome-wide expression.

16,190 probe sets achieved a coefficient of variance >50% and were used to cluster samples according to similar patterns of gene expression using Pearson's Correlation Coefficient. The primary separation is distinguished by presence or absence of ex vivo stimulation by LPS, and not the individual patient or the method of cell isolation.

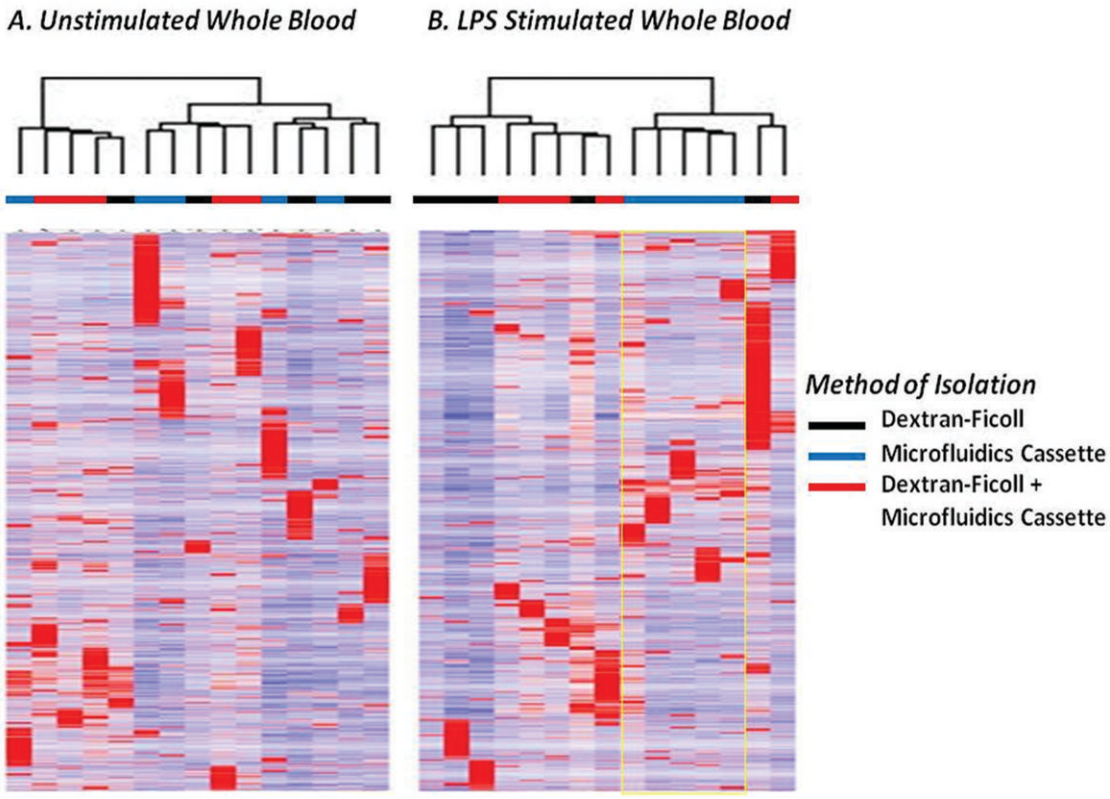

Figure 4.

Unsupervised analysis of genome wide expression of unstimulated (a) and LPS-stimulated (b) samples. Unsupervised sub-analyses of both the A.) Unstimulated samples where no clustering pattern is readily identified and B.) LPS stimulated samples where microfluidics based PMN isolation clusters together (see yellow outline).

Performance of classifiers during cross-validation.

	Array id	Class label	Mean Number of genes in classifier	Diagonal Linear Discriminant Analysis Correct?	1-Nearest Neighbor	3-Nearest Neighbors Correct?	Nearest Centroid Correct?
1	ARDS1-BAL	ARDS-BAL	19101	YES	YES	YES	YES
2	ARDS2-BAL	ARDS-BAL	17338	YES	YES	YES	YES
3	ARDS3-BAL	ARDS-BAL	17390	YES	YES	YES	YES
4	ARDS4-BAL	ARDS-BAL	17578	YES	YES	YES	YES
5	ARDS5-BAL	ARDS-BAL	17961	YES	YES	YES	YES
6	ARDS6-BAL	ARDS-BAL	18076	YES	YES	YES	YES
7	ARDS7-BAL	ARDS-BAL	18095	YES	YES	YES	YES
8	ARDS1-Blood	ARDS-Blood	17761	YES	YES	YES	YES
9	ARDS2-Blood	ARDS-Blood	17344	YES	YES	YES	YES
10	ARDS3-Blood	ARDS-Blood	17249	YES	YES	YES	YES
11	ARDS4-Blood	ARDS-Blood	17583	YES	YES	YES	YES
12	ARDS5-Blood	ARDS-Blood	17092	YES	YES	YES	YES
13	ARDS6-Blood	ARDS-Blood	17136	YES	YES	YES	YES
14	ARDS7-Blood	ARDS-Blood	17594	YES	YES	YES	YES
15	Health1-Stim	Health-Stim	17826	YES	YES	YES	YES
16	Health2-Stim	Health-Stim	16773	YES	YES	YES	YES
17	Health3-Stim	Health-Stim	16590	YES	YES	YES	YES
18	Health4-Stim	Health-Stim	16923	YES	YES	YES	YES
19	Health5-Stim	Health-Stim	16772	YES	YES	YES	YES
20	Health1-Unstim	Health-Unstim	17695	YES	YES	YES	YES
21	Health2-Unstim	Health-Unstim	16876	YES	YES	YES	YES
22	Health3-Unstim	Health-Unstim	17482	YES	YES	YES	YES
23	Health4-Unstim	Health-Unstim	17406	YES	YES	YES	YES
24	Health5-Unstim	Health-Unstim	17169	YES	YES	YES	YES
Mean percent of correct classification:				100	100	100	100

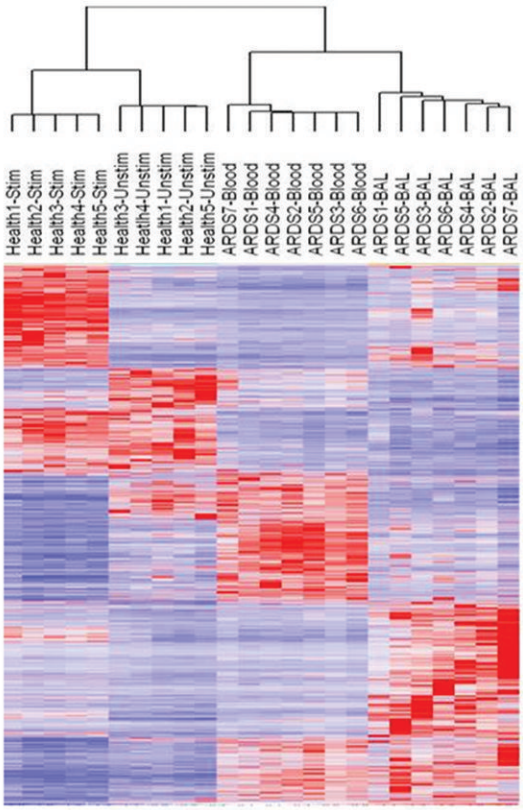

Figure 5.

A supervised analysis of gene expression from Blood and BAL PMNs Isolated from ARDS Patients compared to Unstimulated and LPS-Stimulated Whole Blood from Healthy Control Subjects. Differences in gene expression among the four groups were analyzed by F test using a false discovery adjusted (Q) probability of less than 0.001. The gene list was then used to determine whether it could accurately predict the group assignment using four different models and leave one out cross validation. Each of the four models predicted the classification with 100% accuracy. $p<0.0001$ using a Monte Carlo simulation with 1000 permutations of the dataset.

Table 1

RNA yields and quality using the different isolation procedures

Method A (dextran-Ficoll), Method B (microfluidic cassette), Method C (dextran-Ficoll followed by microfluidic cassette). The RIN number is obtained from the Agilent Bioanalyzer and is a proprietary measure used to assess the quality of the RNA in terms of degradation products. The values presented here are consistent with a high quality product suitable for microarray analyses.

	RNA Conc (pg/ul)	RNA Quantity (pg)	RIN	RNA Amp Quantity (pg)
Method A	14,270[ab]	570,800[ab]	7.78	5,017[d]
Method B	1,927[ac]	69,388[ac]	8.32	4,786[e]
Method C	597[bc]	21,496[bc]	8.5	2,970[de]

[a] 2-tail T-test: p<0.007,

[b] p<0.005,

[c] p<0.005,

[d] p<0.002,

[e] p<0.003

Table 2

Selected Gene Expression Patterns in Neutrophils obtained from Critically Ill Patients or Stimulated Ex Vivo with LPS

Selected genes from BAL were all significantly different from blood of critically ill patients, or LPS stimulated versus unstimulated, using a false discovery adjusted probability of $p<0.001$.

PMN Granule Proteins			Anti-Apoptotic Proteins		
Gene	**Fold Change in BAL**	**Fold Change due to LPS**	***Gene***	**Fold Change in BAL**	**Fold Change due to LPS**
MPO: myeloperoxidase	↓32.4	Unchanged	*BAG3:* BCL2-associated athanogene 3	↑15.2 (anti-apoptotic)	Unchanged
MMP: neutrophil collagenase	↓29.7	Unchanged	*BCL10:* CIPER	↓3.1 (pro-apoptotic	Unchanged
LCN2: lipocalin-2 (NGAL)	↓7.2	Unchanged	*BCLAF1:* BTF1	↓2.2	Unchanged
LTF: Lactoferrin	↓23.4	Unchanged			

PMN Chemokines		
Gene	**Fold Change in BAL**	**Fold Change due to LPS**
CXCL1: Groα	↑4.8	Unchanged
CXCL2: MIP2α	↑31.1	↑4.8
CXCL3: MIP2β	↑117.8	↑13.8
CXCL5: ENA-78	↑36.2	Unchanged
CXCL16: SRPSOX	↑2.5	Unchanged