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Automated generation of immature dendritic cells in a single-use system

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Abstract

Dendritic cells (DCs) are an indispensable part of studying human responses that are important for protective immunity against cancer and infectious diseases as well as prevention of autoimmunity and transplant rejection. These cells are also key elements of personalized vaccines for cancer and infectious diseases. Despite the vital role of DCs in both clinical and basic research contexts, methods for obtaining these cells from individuals remains a comparatively under-developed and inefficient process. DCs are present in very low concentrations (<1%) in blood, thus they must be generated from monocytes and the current methodology in DC generation involves a laborious process of static culture and stimulation with cytokines contained in culture medium. Herein, we describe an automated fluidic system, MicroDEN, that allows for differentiation of monocytes into immature-DCs (iDCs) utilizing continuous perfusion of differentiation media. Manual steps associated with current *ex vivo* monocyte differentiation are vastly reduced and an aseptic environment is ensured by the use of an enclosed cartridge and tubing network. Benchmark phenotyping was performed on the generated iDCs along with allogeneic T-cell proliferation and syngeneic antigen-specific functional assays. MicroDEN generated iDCs were phenotypically and functionally similar to well plate generated iDCs, thereby demonstrating the feasibility of utilizing MicroDEN in the broad range of applications requiring DCs.

Keywords

Dendritic cell; Monocyte; Vaccine; Cancer vaccinology; Antigen-presenting cell; Personalized medicine

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Declaration of Interest

SKM is a founder of Flaskworks LLC which aims to commercialize the MicroDEN and related systems.

1. Introduction

Dendritic cells (DCs) are an indispensable component of the mammalian immune system that present antigens to T-cells within the body and aid in development of adaptive immunity towards future infection.¹ DCs are also fundamental in developing personalized vaccines for refractory patients related to transplant rejection,^{2–4} cancer treatment,^{5–13} and infectious viral diseases.^{9, 14–17} DCs are the most potent antigen presenting cells (APCs) capable of inducing naïve T-cells.^{18, 19} They play a crucial role in the *in vivo* expansion of T-cells and can be used to expand T-cells *in vitro*, e.g., for use as T-cell therapies targeting a panel of neoantigens against a specific tumor in an individual.^{5, 20, 21} From a mechanistic standpoint, DCs are an indispensable part of studying human responses that are important for protective immunity against cancer and infectious diseases as well as prevention of autoimmunity and transplant rejection.

Despite the vital role of DCs in both clinical and basic research contexts, methods for obtaining these cells from individuals remains a comparatively under-developed and inefficient process. DCs are present in very low concentrations (<1%) in blood, thus they must be generated from monocytes (MOs) and the state-of-the-art in DC generation involves a laborious process of static culture and stimulation with cytokines contained in culture medium.^{22, 23} Numerous manual steps are required to differentiate a sample of patient-derived blood or peripheral blood mononuclear cells (PBMCs) into sufficient numbers of DCs that can be utilized for vaccine development, T-cell therapy, or mechanistic studies. When scaled even to the level of tens of samples for a study involving one or two conditions or separate blood draws, the resource requirements in terms of personnel hours and number of manual steps becomes significant. Considering the existing and projected use of these cells at much larger scale, such as in Phase I – III clinical trials of vaccines and personalized cell therapy regimens, the current approach to DC generation poses an unusually large burden, most significantly in terms of cost, but also in terms of time required to perform comprehensive studies and trials and increased potential for error (e.g., contamination, patient cell misidentification, etc.).

Monocytes constitute 10–40% of PBMCs and differentiate into either macrophages (M ϕ) or DCs *in vivo*.^{24–26} This differentiation is leveraged in current state-of-the-art DC generation that involves (a) collection of peripheral blood, (b) extraction of peripheral blood mononuclear cells (PBMCs) through Ficoll density gradient separation, (c) plastic adhesion or magnetic separation of MOs from PBMCs, and 6–7 day static culture of MOs with the differentiation cytokines IL-4 and GM-CSF.²⁶ Peripheral blood is easily collected through venipuncture or leukapheresis and the subsequent isolation and differentiation of MOs is well understood and routinely performed. The static culture is currently performed in either well plates or cell culture flasks (T-flasks) for both research and therapeutic purposes and requires several manually performed steps where the culture vessel is (a) open to surrounding air within an aseptic environment (e.g., laminar flow hood) and (b) transferred from an aseptic environment to an incubator. These aforementioned steps introduce potential for contamination when the incubator and laminar flow hood are not within the same aseptic environment (i.e., advanced research labs) and rely on the manual manipulation of the culture flask/cells by lab personnel.

A 6-well well plate and a T-175 flask typically generate *ca.* 1.5–2.5 million and 6–20 million DCs, respectively, depending on donor, prior therapy received by the donor, isolation method, and differentiation procedure.^{27, 28} While the number of DCs generated in static culture varies widely, obtaining therapeutically relevant numbers of DCs requires tens of well plates or flasks even with the best DC yield. Furthermore, these protocols require cytokine replenishment every 2–3 days of culture;^{28, 29} this means removing the culture flask from the incubator, manually adding DC differentiation medium to each well or flask, and returning the flask to the incubator. While possible, this process becomes overly laborious when cells from multiple patients are being cultured simultaneously, placing a strain on laboratory resources and personnel time.

Upon identifying the aforementioned deficiencies in the current DC generation process, we developed an automated fluidic system that allows for differentiation of monocytes into iDCs utilizing continuous perfusion of differentiation media within an enclosed aseptic environment called MicroDEN. Manual steps associated with current *ex vivo* DC generation are vastly reduced with MicroDEN and an aseptic environment is ensured by the use of an enclosed DC generation cartridge and tubing network that supplies fresh cytokines and media to the cells while concurrently removing spent media from the cartridge, all of which is an advancement from current static culture techniques. Furthermore, the automated perfusion system requires no user intervention after setup and can be left to run until harvest. Benchmark phenotyping was performed on the generated iDCs along with allogeneic PBMC and syngeneic antigen functional assays. MicroDEN generated iDCs were phenotypically similar to well plate generated iDCs and there were no salient differences between MicroDEN and well plate generated iDCs in functional assays developed to study DC-dependent T-cell induction.

2. Materials

2.1 MicroDEN console

The first generation MicroDEN console is shown in Figure 1a. The console has a 12×10 inch footprint and contains two peristaltic pumps, an LED display, button, electronics to run the pump, an inclined cell culture cartridge table with clips to secure the cartridges, and media reservoir holders. This configuration allows the console and assembly to be moved as a single unit.

The inlet medium reservoir holds fresh DC differentiation media (media + cytokines) and is capped with a 0.2 micron sterile filter to allow gas exchange as the media is depleted. A PTFE tube draws fluid from the reservoir, through Pumpsil tubing, through silicone tubing which allows gas exchange with the ambient environment, and into the cell culture cartridge. Fresh DC differentiation media flows through the cartridge and spent media flows out through a silicone tube and into the waste reservoir where effluent is collected. The entire assembly is closed and remains aseptic. At harvest, the tubing is disconnected from the cartridge and iDCs are aspirated from the cartridge. The first generation MicroDEN console holds two cell culture cartridges. Figure 1b shows two MicroDEN consoles inside an incubator during an experiment.

2.2 MicroDEN cartridge

The MicroDEN cell culture cartridge has a polystyrene base that facilitates cell adhesion and is completely closed to the outside environment. The assembly remains sterile when the tubing is connected to the cartridge. Silicone tubing is connected at both the inlet and outlet to facilitate gas exchange between the media and ambient environment. When placed inside an incubator, the CO₂ concentration within the cartridge is maintained at 5% (incubator setting). The cartridge has a cell culture surface area of 39.7 cm² and the seeding density was 690,000 PBMCs per cm², thus 27.4 million PBMCs were seeded into each MicroDEN cartridge.

The cartridge is fabricated from commercially available poly(methyl methacrylate) (PMMA) and polystyrene (PS) that are cut using an Epilog Zing 16 laser system and adhered using 3M 966 Adhesive Transfer Tape. Internal dimensions of the cartridge (length × width × height) are 75.00 mm × 60.00 mm × 3.17 mm. The wall shear stress is 0.25 μPa at 2.2 μL/min perfusion and the pressure drop (ΔP) is 89.5 mPa, calculated from Navier-Stokes for laminar flow between static parallel plates.

Due to perfusion, there is potential for cells, particularly iDCs, to be washed out of the cartridge. To investigate this, effluent in the waste reservoir was centrifuged. Each time this was performed, there were no noticeable cells through visible inspection using a microscope and hemocytometer. This confirms that cells are not removed from the cartridge during perfusion. The 2.2 μL perfusion flow rate is low enough to enable sufficient perfusion of fresh differentiation media and cellular waste without removal of non-adherent cells. The low perfusion flow rate also reduces the possibility of bubbles forming within the peristaltic pump tubing. During all experiments, no bubbles were observed entering the MicroDEN cartridge.

2.3 Media and cytokines

2.3.1 Northeastern University (NEU)—RPMI 1640 with phenol red (Gibco 11875119) was used for all experiments. The base medium was supplemented with 10% fetal bovine serum (FBS; not heat inactivated; Sigma Aldrich F2442) and 1% penicillin-streptomycin (P/S; Gibco 15140122). Experiments conducted at Northeastern University (NEU) used as-supplied FBS (not heat inactivated). IL-4 (500 U/mL media; R&D Systems 204IL) and GM-CSF (800 U/mL media; R&D Systems 215GM) were added to the complete media (CM; RPMI 1640 + FBS + P/S) to make up the DC differentiation medium.

2.3.2 Saint Louis University (SLU)—RPMI 1640 with phenol red (Gibco 11875119) was used for all experiments. The base medium was supplemented with 10% fetal bovine serum (FBS; heat inactivated; Sigma Aldrich F2442), 1% L-glutamine (Lonza BioWhittaker BW17–605E), and 1% penicillin-streptomycin (P/S; Gibco 15140122). IL-4 (500 U/mL media; R&D Systems 204IL) and GM-CSF (800 U/mL media; Leukine®) were added to the complete medium (CM; RPMI 1640 + FBS + L-glutamine + P/S) to make up the DC differentiation medium.

2.4 Peripheral blood mononuclear cells (PBMCs)

2.4.1 Northeastern University—Experiments at NEU were conducted using PBMCs from a single donor. A unit of whole blood (500 mL) was purchased from StemExpress. The venipuncture occurred in the early morning and the blood was delivered within 4 hours. The blood was then immediately separated using Ficoll density gradient media to extract the PBMCs. The isolated PBMCs were washed 2x in DPBS (HyClone SH30028.LS) and suspended in CryoStor CS10 cryopreservation medium. The aliquots were placed in a Mr. Frosty container then placed in a -80°C freezer overnight then transferred to storage in a vapor-phase nitrogen (LN_2) storage system. The cells remained in LN_2 storage for at least one week before being resuscitated for an experiment. Experiments 1, 2, and 3 were performed at NEU using these PBMCs. Each experiment was conducted independently. All cell counts were conducted using Trypan Blue staining and an Invitrogen Countess II Automated Cell Counter.

2.4.2 Saint Louis University—Two experiment sets were conducted at SLU. The first set included three experiments conducted simultaneously using PBMCs from three donors. The venipuncture occurred the same day as Ficoll density gradient separation. The isolated PBMCs were washed 2x in DPBS (Lonza) and suspended in cryopreservation medium containing 90% HI-FBS and 10% DMSO (Sigma). The aliquots were placed in a Mr. Frosty container then placed in a -80°C freezer overnight then transferred to storage in a vapor-phase nitrogen (LN_2) storage system. The cells remained in LN_2 storage for at least one week before being resuscitated for an experiment. Experiments 4, 5, and 6 were performed at SLU using these frozen PBMCs. The second set included two experiments using fresh (not frozen) PBMCs that were isolated from whole blood collected by venipuncture on the same day as Ficoll density gradient separation. Experiment 7 was performed with PBMCs from a PPD+ (tuberculin purified protein derivative) donor using two MicroDEN cartridges (denoted “a” and “b”) and three wells (denoted “a,” “b,” and “c”). Experiment 8 was carried out with PBMCs from a PPD- donor. Experiments 7 and 8 were conducted separately. All cell counts were conducted using Trypan Blue staining and a hemocytometer.

2.5 Immunophenotyping

Flow cytometry was used to characterize the phenotype of input PBMCs (*i.e.*, MO concentration of input PBMCs) and output iDCs.

2.5.1 Northeastern University—Flow cytometry data was collected on a Beckman-Coulter Cell Lab Quanta SC benchtop flow cytometer with a 488 nm laser line and three fluorescence channels. Cells were stained with Fc Block (BD Biosciences 564220) for 10 minutes prior to antibody staining. Panel A tested viability (Live/Dead Fixable Green; Invitrogen L34970), CD209 (R&D Systems FAB161P100), and CD14 (Abcam ab157312). Panel B tested CD80 (BD Biosciences 557226), CD83 (BD Biosciences 556855), and CD86 (BD Biosciences 561128). See Section S1.1 for gating strategy.

2.5.2 Saint Louis University—Flow cytometry data was collected on a BD LSR II flow cytometer. The staining panel tested viability (Live/Dead Fixable Aqua; Invitrogen L34966), CD45 (BD Biosciences 347463), CD209 (BD Biosciences 551545), CD14 (BD Biosciences

561390), CD80 (BD Biosciences 564158), CD83 (BD Biosciences 561132), and CD86 (BD Biosciences 561124). See Section S1.2 for gating strategy.

2.6 Functional assays

All functional assays were performed concurrently at SLU using cells from Experiments 4, 5, and 6 (different blood donor for each experiment). As culture medium, these assays utilized 10% human AB serum (Sigma) in RPMI 1640 with 1% P/S and 1% l-glutamine.

2.6.1 Allogeneic T-cell proliferation assay—Harvested iDCs were counted and suspended at 20,000 to 0 iDCs in 0.1 mL complete media and added to 96-well round-bottom cell culture plates. 200,000 allogeneic PBMCs suspended in 0.1 mL medium were added to each well and allowed to co-culture at 37°C/5% CO₂/85% relative humidity (RH). After 3 days, supernatants were collected and IFN- γ production was measured via ELISA. To measure cellular proliferation, 1 Ci ³H-thymidine was added to each well and cells cultured at 37°C/5% CO₂/85% RH. After 18 hours, the cells were harvested using a Connectorate Cell Harvester System, filter mats scintillated with Ultima Gold F (Perkin Elmer), and ³H-thymidine incorporation measured using a Microbeta 1450 microplate scintillation counter.

2.6.2 Syngeneic antigen-specific assay—Harvested iDCs from PPD⁺ donors were counted and suspended at 20,000 to 0 iDCs in 0.1 mL complete media per well of 96-well round-bottom plates. 100,000 syngeneic CD3⁺ T-cells isolated with Miltenyi Pan T-cell isolation kits were added to each well along with *M. tuberculosis* (Mtb) culture filtrate (10 μ g/mL; BEI resources). After 6 days of culture at 37°C/5% CO₂/85% RH, the supernatants were collected and IFN- γ production measured via ELISA. 1 μ Ci ³H-thymidine was added to each well and cells cultured for an additional 18 hours. Cells were then harvested and ³H-thymidine incorporation was measured as in Section 2.6.1.

2.7 Controls

2.7.1 Northeastern University—Well plate controls at NEU were performed in 4 wells of a Corning Costar 6-well plate for each experiment. The cell culture area of each well was 9.5 cm² and the seeding density was 651,000 PBMCs per cm². Thus 6.25 million PBMCs were suspended in 2.5 mL complete media (RPMI 1640 + FBS + P/S) per well.

2.7.2 Saint Louis University—Well plate controls at SLU were performed in 2 wells of a Corning Costar 24-well plate. The cell culture area of each well was 1.9 cm² and the seeding density was 526,300 PBMCs per cm². Thus 1.0 million PBMCs were suspended in 1.0 mL complete media (RPMI 1640 + FBS + P/S) per well.

2.8 Statistics

Welch's t-test (heteroscedastic Student's t-test) was used to compare means of two samples. This was used for (a) NEU data comparing MicroDEN and well plate iDC yield and (b) comparing NEU and SLU iDC yield. The null hypothesis is that there is no difference between MicroDEN and well plate iDC yield. The threshold for rejection was $p < 5\%$. Wilcoxon signed ranks test was used to compare the three SLU experiments. This was

deemed appropriate since each experiment used a different donor. The null hypothesis is that there is no difference between MicroDEN and well plate iDC yield. The threshold for rejection is $p < 5\%$.

3. Experimental Protocol

The experimental protocol is analogous for experiments at NEU and SLU. All work was performed within a sterile laminar flow hood. All incubators were maintained at 37°C and 5% CO₂ throughout all entire experiments.

3.1 Setup: Day 0

PBMCs – fresh or frozen – were suspended in complete medium at the appropriate concentration and input into the MicroDEN cell culture cartridge or well plate (see Tables 1–3). Each port of the MicroDEN cartridge was capped with a 0.2 micron sterile filter (EMD Millipore SLGP033RS). The cartridge and well plate were placed inside an incubator for 1 hour to induce monocyte plastic adherence. After 1 hour, the cartridge and well plate were removed from the incubator and the medium/cell suspension was removed using a pipette. Remaining non-adherent and loosely adherent cells were removed by two subsequent washing steps using warm (37°C) complete medium. At this point, MOs and some lymphocytes were retained within the cartridge/well plate and most non-adherent lymphocytes were removed. Retention of MOs was investigated by running flow cytometry on the non-adherent cell population removed from the cartridge after washing. The data indicated that 90–95% of the input MOs were retained within the MicroDEN cartridge and was similar between the cartridge and well plate.

After the washes, DC differentiation medium was added to the cartridge and well plate. The well plate was wrapped in aluminum foil to minimize fluid evaporation and placed inside an incubator. The cartridge was connected to the silicone tubing and placed on the MicroDEN console. The console was transferred to the incubator, turned ON, and DC differentiation medium was perfused at 2.2 µL/min for the duration of the experiment. Prior to connecting the cartridge, DC differentiation medium was placed inside the inlet medium reservoir and the tubing was primed so that fresh DC medium began to flow into the cartridge once the console began pumping. The well plates were removed from the incubator on day 3 and day 5 in order to replenish cytokines.

3.2 Harvest: Day 7

After running for 7 days, the console was turned off and removed from the incubator. The cartridge was disconnected from the tubing and the media/cell suspension was aspirated using a pipette. Remaining non-adherent and loosely adherent cells were removed by two subsequent washing steps using cold DPBS (4°C); strongly adhered cells remaining in the cartridge after 2 washes were not collected. The same washing procedure was used for the well plate. Nearly all of the cells within the cartridges and well plates were non-adhered or loosely adhered. Very few cells remained within the cartridges and well plates after washing. Once the cells were harvested, they were spun down, counted, and used in subsequent flow cytometry or functional assay tests.

4. Results

4.1 Immunophenotyping

Flow cytometric immunophenotyping of Experiment 2 (NEU) and Experiment 5 (SLU) using frozen PBMCs is shown in Figures 2 and 3, respectively. Immunophenotyping of Experiment 7a (SLU) using fresh PBMCs is shown in Figure 4. Immunophenotyping results of the remaining experiments are shown in Section S2.1

4.1.1 Frozen PBMCs: Northeastern University—The initial input PBMC population for Experiment 2 using frozen PBMCs contained 36.1% MOs. After 7 days of perfusion, 52.7% of harvested cells were CD209⁺ CD14[−] indicative of an iDC subtype.^{30, 31} 5.5% of cells were CD209⁺ CD14⁺ while the remaining cells were not considered iDCs. The iDCs were CD80⁺ CD83[−] CD86[−] which is consistent with the expected surface marker expression for immature DCs.^{30, 31} Comparatively, 83.4% of cells harvested from the well plate were CD209⁺ CD14[−], 6.7% were CD209⁺ CD14⁺, and the rest were not considered iDCs. Only CD209⁺ CD14[−] iDCs were considered when calculating iDC yield in Table 1.

MicroDEN iDCs were phenotypically similar to well plate iDCs with the only salient difference being degree of CD209 expression. Well plate iDCs showed greater expression of CD209 compared to MicroDEN iDCs, indicated by the forward shift along the CD209 axis in Figure 2. Side scatter (SS) vs. electronic volume (EV) plots, measuring cell granularity (SS) and size (EV), show that MicroDEN iDCs are smaller than well plate iDCs (*i.e.*, EV for well plate iDCs is shifted right). The Coulter Principle is used for determining EV and higher EV correlates to larger cells.³² Larger cells are expected to exhibit increased antibody conjugation, thus well plate iDCs have stronger CD209 expression.

4.1.2 Frozen and Fresh PBMCs: Saint Louis University—The initial input PBMC population for Experiment 5 using frozen PBMCs contained 25.5% MOs. After 7 days of perfusion, 90.4% of harvested cells were CD209⁺ CD14[−] indicative of iDCs.^{30, 31} 0.1% of cells were CD209⁺ CD14⁺ while the remaining cells were not considered iDCs. The iDCs were CD80⁺ CD83[−] CD86[−] which is consistent with MicroDEN iDCs generated at NEU. The initial input PBMC population for Experiment 7 using fresh PBMCs contained 25.6% MOs. After 7 days of perfusion, 93.5% and 93.1% of harvested cells were CD209⁺ CD14[−], from two cartridges running concurrently. Only CD209⁺ and CD14[−] iDCs were considered when calculating iDC yield in Tables 2–3. The fresh MO derived iDCs were phenotypically similar to the frozen MO derived iDCs. This finding is critical for broad applicability of MicroDEN and its integration into the user's iDC generation protocol.

MicroDEN iDCs were phenotypically similar to well plate iDCs with the only salient difference being cell size. Interestingly, CD209 expression is similar for MicroDEN and well plate iDCs but MicroDEN iDCs are larger as shown in the SS/FSC plot in Figure 3 for iDCs derived from frozen PBMCs. This finding is opposite of what was observed in the NEU experiments (Section 4.1.1). Contrarily, there is no noticeable size difference for iDCs derived from fresh PBMCs as shown in Figure 4. Figure 5 shows SS vs. EV/FSC plots for all nine experiments. MicroDEN iDCs are consistently smaller than well plate iDCs at NEU; whereas, MicroDEN iDCs are consistently larger than well plate iDCs at SLU for iDCs

derived from frozen PBMCs. Potential reasons for this difference are donor variability, well plate size (6-well or 24-well), measurement type (EV or FSC), cryopreservation protocol (CS-10 or FBS/media), and testing site (NEU or SLU). See Section 5.0 for further discussion.

Harvested cells at both test sites are primarily CD209⁺ iDCs; however, the harvest includes cells that are not iDCs. These contaminating cells are likely T-cells, B-cells, and NK cells that were adherent and not washed away after plastic adherence, though the majority of contaminating cells were removed by the washing steps. Note that this heterogeneity in the standard well plate technique in addition to the MicroDEN cartridges. Contaminating lymphocytes in the iDC product can be removed by seeding enriched MOs into the cartridge (thereby eliminating the plastic adherence and washing steps) or by iDC enrichment after harvesting, for example using magnetic bead based negative selection.”

4.2 Functional Assays

Functional assays were performed on cells harvested from MicroDEN cartridges and well plates. Figure 6 shows allogeneic T-cell proliferation assays for iDCs from Experiments 4 – 7. For each experiment, ³H-thymidine incorporation was nearly analogous between MicroDEN and well plate generated iDCs. ³H-thymidine incorporation scaled proportionally with the number of iDCs per well, indicating increased T-cell proliferation proportional to input iDCs. IFN- γ production was also similar between MicroDEN and well plate generated iDCs. This measurement showed substantial variability that appears to be donor dependent since each experiment was performed using cells from a different donor but the MicroDEN and well plate lines closely resemble each other. Figure S8 shows allogeneic functional assays for iDCs generated in Experiment 8 using fresh PBMCs. These iDCs were co-cultured with allogeneic T-cells from two different donors and T-cell proliferation remains consistent between the different donor cells. Overall, both ³H-thymidine incorporation and IFN- γ production results demonstrate functionally active and viable iDCs.

Figure 7 shows syngeneic antigen-specific assays for iDCs from Experiments 4 – 7 using *M. tuberculosis* culture filtrate. For each experiment, ³H-thymidine incorporation was greater when the cells were exposed to Mtb culture filtrate compared to plain media, indicating iDCs were successfully processing and presenting mycobacterial proteins. Furthermore, MicroDEN iDCs showed ³Hthymidine incorporation levels similar to well plate iDCs and these results scaled proportionally with the number of iDCs per well. IFN- γ production also scaled proportionally with iDCs per well and there was no significant difference between MicroDEN and well plate generated iDCs. Overall, iDCs generated in MicroDEN are functionally similar to well plate generated iDCs when exposed to allogeneic T-cells (Figure 6) and syngeneic T-cells and *M. tuberculosis* proteins (Figure 7).

4.3 Immature Dendritic Cell Yield

IDC yield at NEU was calculated by counting the harvested cells and multiplying by the percentage that were viable and the percentage that were CD209⁺ CD14[–] from immunophenotyping (Section 4.1.1 and Table 1). IDC yield at SLU was calculated by counting the viable cells and multiplying by the percentage that were CD209⁺ CD14[–] from

immunophenotyping (Section 4.1.2 and Tables 2–3). Three independent experiments with frozen PBMCs were conducted at both test sites (NEU and SLU) and two independent experiments with fresh PBMCs were conducted at SLU. Experiment 7 used two MicroDEN cartridges. Each experiment had its own well plate control.

4.3.1 Frozen PBMCs—Yield of microDEN generated iDCs from frozen PBMCs was $6.6\% \pm 3.5\%$ and $3.1\% \pm 0.8\%$ at NEU and SLU, respectively, as shown in Figure 8. Comparatively, yield of well plate generated iDCs was $6.8\% \pm 3.4\%$ and $3.6\% \pm 0.3\%$, respectively. IDC yield was similar at NEU and SLU indicating that MicroDEN is not operator or site dependent. The difference between sites is likely attributable to blood donor-to-donor differences which can be expected whenever working with biological specimens. Other possible causes of this difference includes cryopreservation protocol and resuscitation technique although donor-to-donor variability is likely the largest factor. Importantly, iDC yield was similar between MicroDEN and the well plate control at each test site indicating that MicroDEN performed similarly to the well plates for iDC generation.

4.3.2 Fresh PBMCs—iDC yield was $8.6\% \pm 1.0\%$ and $5.6\% \pm 0.5\%$ when generated from fresh PBMCs in MicroDEN and well plates, respectively, as shown in Figure 8. This data is similar to the data collected in Experiments 1–6 indicating MicroDEN efficacy using both frozen and fresh PBMCs. Furthermore, the data shows that iDC yield is greatest when fresh PBMCs are used in MicroDEN (see Figure S12). This is an encouraging finding that suggests MicroDEN may improve yields over well plates; however, more experiments are necessary to make definitive conclusions.

5.0 Discussion/Impact

Current iDC generation techniques involve static culture using well plates and T-flasks and generally fresh cytokines are added manually every 2–3 days to maintain the differentiation medium. To alleviate this constraint, we developed MicroDEN which continuously perfuses fresh cytokines and medium into the cell culture chamber while concurrently removing depleted medium. Automated perfusion is a substantial improvement over current static cell culture techniques that require cells to differentiate in nutrient and cytokine depleted media. Toxic byproducts of cellular respiration such as lactate are also concomitantly removed from the cell culture cartridge which ensures that the cells are constantly exposed to fresh media. MicroDEN also allows for easy pipetting of a cell suspension into the cartridge which has more surface area than an entire 6-well plate. Additionally, MicroDEN eliminates the need to remove well plates and flasks from the incubator which minimizes potential sources of contamination and cell misidentification when working with cells from multiple donors.

Reduction of manual steps is a significant advantage of MicroDEN over static culture techniques and this becomes increasingly evident when generating iDCs from multiple patients or donors. Instead of pipetting fresh differentiation media into each well (and removing supernatant from each well as in some protocols), MicroDEN requires only a single reservoir change; this considerably simplifies the overall protocol and reduces inter-operator variability as the enclosure holding the cells is not directly handled as it would be if using well plates or flasks. MicroDEN also has the same advantage at harvest since many

wells do not need to be individually washed and pipetted as several wells are combined into one cartridge, essentially allowing multiple wells – a single cartridge – to be washed/harvested simultaneously.

Experiments at both NEU and SLU show that MicroDEN generated iDCs are phenotypically similar to well plate generated iDCs and iDC yield in MicroDEN is comparable to well plates. The majority of cells harvested from MicroDEN after 7 days of perfusion 2.2 $\mu\text{L}/\text{min}$ are CD209⁺ CD14⁻ CD80⁺ CD83⁻ CD86⁺ which definitively indicates that these harvested cells are iDCs.^{30, 31} Three independent experiments at NEU and three independent experiments at SLU all yielded similar iDC yields showing that MicroDEN is neither user dependent or blood donor dependent, using resuscitated PBMCs from healthy donors. Experiments using fresh PBMCs yielded similar results which shows that MicroDEN is useful for a wide array of iDC generation protocols. iDC yield is an important parameter for characterizing success of an differentiation and typically 20–30% of input MOs will differentiate into iDCs in a standard well plate. Since MOs constitute 10–40% of PBMCs, *ca.* 5–15% of input PBMCs will differentiate into iDCs, which is consistent with previously reported data.^{22, 33} Our experiments – including both fresh and frozen PBMC derived iDCs – had a yield of $6.1\% \pm 3.1\%$ in MicroDEN with perfusion and $5.3\% \pm 2.4\%$ in well plates in which the difference is insignificant (Welch's t-test; $p = 0.59$).

Yield and purity of iDCs in MicroDEN can likely be further improved by seeding enriched MOs into MicroDEN opposed to total PBMCs. Operational parameters such as seeding density and cytokine concentration would need to be optimized though the exact parameters can be tailored to the user's need. Differentiation medium and perfusion flow rate can likely remain the same between PBMC and MO culture as long as the medium is appropriate for MO differentiation.

There are two possibilities for the size dichotomy between MicroDEN and well plate iDCs. The first considers shear stresses on the cells during static and perfusion culture. In static culture, the cells reside in stagnant media and external shear stress on the cells is negligible. The lack of shear stress causes the cells to expand outward resulting in a larger cell diameter. Cells in perfusion culture are continuously subjected to external shear stress due to fluid flow and consequently maintain a confined orientation (*i.e.*, smaller cell diameter)^{34–36} While the MicroDEN perfusion flow rate used in this study (2.2 $\mu\text{L}/\text{min}$) is much slower than blood flow within the body,^{37, 38} cells are still subjected to shear stresses due to fluid flow and this could result in MicroDEN iDCs being smaller than well plate iDCs. Increased antibody conjugation could be a consequence of larger cells and Figure 2 depicts the larger diameter iDCs from well plate having stronger CD209 expression than the smaller diameter MicroDEN iDCs.

Creating structures such as pillars and wells within the cartridge can possibly reduce the effects of shear stress that cause smaller iDCs in MicroDEN. However, such structures would also introduce turbulence and flow heterogeneity which, in our view, would increase yield variability and operator dependence. Macroscopically flat polystyrene has proven for decades to be ideal for iDC generation from MOs. Considering this and the variability of

iDC size in MicroDEN, we believe that creating artificial structures, such as wells or pillars, on the cell culture surface will negatively impact iDC generation.

The second possible cause of iDC size dichotomy could be due to the degree of differentiation the cells have undergone. Mature DCs (iDCs) are larger (i.e., greater diameter) than iDCs and the cell diameter increases as a MO differentiates into an iDC,^{39, 40} thus MicroDEN iDCs may be less differentiated than well plate iDCs. As mentioned previously, larger cells may have stronger CD209 expression due to increased antibody conjugation. CD80, CD83, and CD86 expression are similar between MicroDEN and well plate iDCs indicating that the extent of differentiation is similar; however, these three antibodies are not sufficient for fully characterizing the extent of differentiation and it is not possible to definitively determine at this point if MicroDEN iDCs are less differentiated. Extent of differentiation could be determined by expanding phenotype testing to include CD1a/b/c, CD11c, CD16, CD40, CD123, CD141, CD304, and HLA-DR expression.^{30, 41–44}

Cell functionality is a critical factor for determining efficacy of iDC generation and ensuring that the generated iDCs are therapeutically relevant. Allogeneic T-cell proliferation shows that MicroDEN iDCs behave similar to well plate iDCs in both ³H-thymidine incorporation and IFN- γ production assays. Furthermore, co-culture of syngeneic T-cells from PPD+ individuals with *M. tuberculosis* culture filtrate showed no difference between MicroDEN and well plate generated iDCs in antigen-specific induction of T-cells. These data definitively show that MicroDEN generated iDCs successfully induce T-cell proliferation and appropriately process and present antigens to T-cells. T-cell proliferation is one of the most essential steps in inducing an immune response to a foreign antigen and is necessary for the therapeutic effects produced by DCs for both DC vaccines and T-cell based therapies.

MicroDEN cartridges were also ran under static conditions in each experiment to serve as a control. The static condition is analogous to the perfusion condition except that media was not perfused through the cartridge during the experiment, i.e., media remained static within the MicroDEN cartridge. This was performed to determine the influence of perfusion on iDC yield and phenotype. Figure S9 shows the surface marker expression of cells harvested from the static MicroDEN cartridges. Immunophenotype was generally similar between static MicroDEN and well plate with a few differences. First, Experiment 1 & 3 showed few CD80⁺ cells which is contrary to MicroDEN with perfusion and well plate; however, Experiment 2 does look similar to the perfusion and well plate experiments. Second, Experiments 4–6 have a small amount of CD83⁺ and CD86⁺ cells in static MicroDEN which is similar to perfusion MicroDEN but this population is not observed in the well plates.

Functional assays were also performed on iDCs generated in static MicroDEN cartridges concurrent with Experiments 4–6. Figure S10 shows that allogeneic and syngeneic T-cell proliferation and INF- γ production for the static experiments is very similar to the well plates (also under static conditions) and MicroDEN with perfusion. This data indicates that the MicroDEN iDCs generated under static conditions remain viable and functionally active. Figure S11 shows iDC yield of static microDEN experiments and well plate controls. For experiments at both NEU and SLU, there was no difference in iDC yield between static MicroDEN and well plate (Welch's t-test; p=0.31 and 0.32, respectively).

MicroDEN has been developed to improve upon current DC generation techniques via continuous perfusion and reduction of manual steps. The broader technological framework of automation and system design employed in MicroDEN is a step towards scaled-up production of DCs for a broad range of clinical applications such as immunotherapies targeting hematological cancers, melanoma and other malignancies, infectious diseases, and the prevention of transplant rejection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The MicroDEN automated fluidic system that allows for differentiation of monocytes into immature-DCs utilizing continuous perfusion of differentiation media. Monocytes are enriched via plastic adherence from the input PBMC population within two cell culture cartridges. The cartridges are then connected to a perfusion system with tubing (a) to allow for continuous infusion of DC differentiation media (base complete media + IL-4 + GM-CSF) which is then (b) placed inside a standard cell culture incubator and allowed to run for 7 days. A 12 inch (30.5 cm) ruler is shown for size comparison.

Figure 2.

Surface marker expression profile of MicroDEN and well plate generated iDCs at NEU using frozen PBMCs from Experiment 2. Flow cytometry data of input PBMCs (top row), harvested cells from MicroDEN with 2.2 $\mu\text{L}/\text{min}$ perfusion (middle row), and Costar well plate control (bottom row). The data indicates that MicroDEN and well plate generated iDCs are phenotypically similar and the MicroDEN iDCs are smaller than well plate iDCs. See Section S1.1 for gating strategy.

Figure 3.

Surface marker expression profile of MicroDEN and well plate generated iDCs at SLU using frozen PBMCs from Experiment 5. Flow cytometry data of input PBMCs (top row), harvested cells from MicroDEN with 2.2 $\mu\text{L}/\text{min}$ perfusion (middle row), and Costar well plate control (bottom row). The data indicates that MicroDEN and well plate generated iDCs are phenotypically similar. See Section S1.2 for gating strategy.

Figure 4.

Surface marker expression profile of MicroDEN and well plate generated iDCs at SLU using fresh PBMCs from Experiment 7a. Flow cytometry data of input PBMCs (top row), harvested cells from MicroDEN with 2.2 $\mu\text{L}/\text{min}$ perfusion (middle row), and Costar well plate control (bottom row). The data indicates MicroDEN and well plate generated iDCs are phenotypically similar. See Section S1.2 for gating strategy.

Figure 5.

Electronic volume (Experiments 1–3 at NEU) and forward scatter (Experiments 4–8 at SLU) of MicroDEN generated iDCs (red histogram) and well plate generated iDCs (blue histogram). EV and FSC are measures of and positively correlated to cell size. Magnitude of the y-axis is proportional to sample size, *i.e.*, cells detected. Each experiment generated a different number of iDCs, thus the magnitude varies.

Figure 6.

iDCs generated by MicroDEN and well plates are functionally comparable in allogeneic T-cell proliferation assays measured by ^3H -thymidine incorporation (a–d) and IFN- γ production (e–h) for Experiments 4–6 (frozen PBMCs) and 7 (fresh PBMCs) at SLU. Data is presented as average $\pm$ population standard deviation for 3 tests at each iDC concentration. Note that (d) and (h) are on different scales from the other figures. The inset of (e) shows the same data but on a different y-axis scale.

Figure 7.

iDCs generated by MicroDEN and well plates are functionally comparable in syngeneic T-cell assays using *M. tuberculosis* (Mtb) culture filtrate to induce antigen-specific T-cell activation measured by ^3H -thymidine incorporation (a–d) and IFN- γ production of the proliferated cells (e–h) for Experiments 4–6 (frozen PBMCs) and 7 (fresh PBMCs) at SLU. Data is presented as average $\pm$ population standard deviation for 3 tests at each iDC concentration. Note that (d) and (h) are on different scales from the other figures. The inset of (g) and (h) shows the same data but on a different y-axis scale to better visualize data near axis.

Figure 8.

Immature dendritic cell (iDC) yield for MicroDEN with perfusion at 2.2 $\mu\text{L}/\text{min}$ and Costar well plate control. Data are represented as average $\pm$ population standard deviation of Exp. 1–3: three independent experiments at NEU using a single blood donor (Table 1); Exp. 4–6: three independent experiments at SLU using three different blood donors (Table 2); Exp. 7–8: two independent experiments at SLU using two different blood donors (Table 3).

Differences in means according to Welch's t-test is insignificant between MicroDEN and well plate for the experiments using frozen PBMCs; however, the difference is significant for Exp. 7–8 using fresh PBMCs (p-values are shown in figure). Wilcoxon signed ranks test showed that the difference between MicroDEN and well plate iDC yield for Experiments 4–6 was insignificant ($p = 0.500$). See Table S1 for statistics.

Experiments 1–3: Data for the three independent experiments at NEU using frozen PBMCs from a single donor in both MicroDEN cartridges and well plates. Cells were counted using a Countess II Automated Cell Counter and phenotypes were obtained using flow cytometry.

Table 1

NEU	PBMCs Seeded ($\times 10^6$)	Cells Harvested ($\times 10^6$)	Total Viability	iDCs of Viable Cells	iDC Viability	Viable iDCs Harvested ($\times 10^6$)	iDC yield of seeded PBMCs
MicroDEN Perfusion 1	27.4	4.3	77.8%	66.7%	92.3%	2.242	8.2%
MicroDEN Perfusion 2	27.4	6.3	81.3%	52.7%	93.5%	2.682	9.8%
MicroDEN Perfusion 3	27.4	6.0	37.0%	21.5%	97.9%	0.477	1.7%
Well plate 1	25.0	1.9	90.5%	86.3%	92.7%	1.500	6.0%
Well plate 2	25.0	4.0	84.4%	83.4%	88.0%	2.830	11.3%
Well plate 3	25.0	2.4	82.2%	38.6%	94.6%	0.768	3.1%

Experiments 4–6: Data for the three independent experiments at SLU using frozen PBMCs from a different donor for each experiment: the same donor PBMCs were used for both MicroDEN and well plate within an experiment. Cells were counted using Trypan Blue staining and a hemocytometer. Phenotypes were obtained using flow cytometry.

Table 2

SLU	PBMCs Seeded ($\times 10^6$)	Cells Harvested ($\times 10^6$)	Total Viability	iDCs of Viable Cells	iDC Viability	Viable iDCs Harvested ($\times 10^6$)	iDC yield of seeded PBMCs
MicroDEN Perfusion 4	27.4	0.70	78.2%	79.5%	91.2%	0.557	2.0%
MicroDEN Perfusion 5	27.4	1.00	90.6%	90.4%	96.2%	0.904	3.3%
MicroDEN Perfusion 6	27.4	1.16	94.0%	91.1%	96.7%	1.057	3.9%
Well plate 4	2.0	0.08	93.8%	92.9%	96.5%	0.070	3.5%
Well plate 5	2.0	0.08	96.7%	98.6%	97.4%	0.079	3.9%
Well plate 6	2.0	0.07	97.4%	93.2%	98.2%	0.065	3.3%

Table 3

Experiment 7–8: Data for the two independent experiments at SLU using fresh PBMCs from a different donor for each experiment: the same donor PBMCs were used for both MicroDEN and well plate within an experiment. Cells were counted using Trypan Blue staining and a hemocytometer. Phenotypes were obtained using flow cytometry.

SLU	PBMCs Seeded ($\times 10^6$)	Cells Harvested ($\times 10^6$)	Total Viability	iDCs of Viable Cells	iDC Viability	Viable iDCs Harvested ($\times 10^6$)	iDC yield of seeded PBMCs
MicroDEN Perfusion 7a	27.4	2.76	90.8%	93.5%	99.8%	2.581	9.4%
MicroDEN Perfusion 7b	27.4	2.68	94.4%	93.1%	99.9%	2.495	9.1%
MicroDEN Perfusion 8	27.4	2.30	87.1%	86.3%	99.9%	1.985	7.2%
Well plate 7a	6.25	0.32	88.9%	93.7%	99.8%	0.300	4.8%
Well plate 7b	6.25	0.42	91.3%	88.3%	99.8%	0.371	5.9%
Well plate 7c	6.25	0.20	90.9%	91.5%	99.7%	0.183	2.9%
Well plate 8	9.38	0.58	93.5%	96.4%	99.6%	0.559	6.0%

Note: Experiment 7 used two MicroDEN cartridges running concurrently denoted as “a” and “b.” Experiment 7 used three wells characterized individually denoted as “a,” “b,” and “c.”