Reports

A real-time assay for neutrophil chemotaxis

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Neutrophils are the predominant cells during acute phases of inflammation, and it is now recognized that these leukocytes play an important role in modulation of the immune response. Directed migration of these cells to the sites of injury, known as chemotaxis, is driven by chemoattractants present at the endothelial cell surface or in the extracellular matrix (ECM). Since uncontrolled or excessive neutrophil chemotaxis is involved in pathological conditions such as atherosclerosis and severe asthma, studying the chemical cues triggering neutrophil migration is essential for understanding the biology of these cells and developing new anti-inflammatory therapies. Although several methods have been developed to evaluate neutrophil chemotaxis, these techniques are generally labor-intensive or alter the native form of these cells and their physiological response. Here we report a rapid, non-invasive, impedance-based, and label-free assay for real-time assessment of neutrophil chemotaxis.

Leukocyte migration toward infected or injured tissues is vital for host defense response and wound healing. However, leukocyte migration may also lead to pathological conditions such as cancer and chronic inflammatory diseases by triggering or prolonging tissue injury and remodeling. As first responders in acute phases of inflammation, neutrophils rapidly migrate from the blood stream to the sites of injury. Recent discoveries have revealed the ability of these cells to shape the immune response by playing an important role at the interface between innate and adaptive immunity (1). It has also been demonstrated that uncontrolled neutrophil migration may contribute to pathological conditions such as atherosclerosis, metabolic syndrome, severe asthma, and tumorigenesis (2–5).

Cell migration requires adhesive receptor–ligand interactions between leukocytes and either endothelial cells or the extracellular matrix (ECM) (6). Many chemical factors have been found to stimulate neutrophil migration. Interleukin 8 (IL-8) is one of the most potent chemoattractant in humans and several animal species including rabbits, cows, and horses (6,7). Different types of migration have been defined according to whether migration occurs directionally or randomly (8). Directed migration occurs toward increasing concentrations of a soluble attractor. This form of movement is known as chemotaxis, and it is essential for leukocyte trafficking as well as the initiation and persistence of the immune response. Leukocyte migration may also be actively directed against the source of the gradient, which is known as fugetaxis. This type of movement has been suggested as a mechanism for down-regulation of inflammation (9). Random migration, referred to as chemokinesis, occurs when the attractor is present uniformly, and it is likely involved in immune surveillance. In vitro studies have shown that a chemoattractant such as Interleukin 8 (IL-8) can induce chemotaxis or chemokinesis according to the presence or absence of a gradient (10) or fugetaxis when it is present at higher concentrations (11).

Given the implication of neutrophils in several human and animal diseases, studying the signals that trigger excessive migration of these cells offers great possibilities for developing new strategies to modulate neutrophil chemotaxis. Thus, the development of technologies that provide reliable and prognosticative information regarding drug–cell interactions is key to discarding ineffective candidates early on and improving the success rate of biomedical research (12).

The Boyden chamber assay was the first reference method available for the study of cell migration. It is based on the measurement of cell transmigration from one compartment to another across a porous filter membrane in response to the presence of a chemotactic agent (13). In order to improve migration quantification and allow cell observation, other techniques, such as those using the Zigmond chamber (14), hydrogel (15), or the Dunn chamber (16), were subsequently developed. However, these methods are limited in terms of reproducibility given the difficulty in creating stable and robust gradients. To overcome these shortcomings, other approaches, such as TAXIScan (17), microfluidic-based systems (e.g., IBIDI and blood drop assays) (18–20), and 3-D collagen gels (21) have been developed in recent years. Despite improvements in gradient stability, measurement accuracy, and physiological relevance, most of these newer assays

METHOD SUMMARY

Here we present a novel method for real-time assessment of neutrophil chemotaxis. Our assay is based on the measurement of variations in electrical impedance resulting from cell adherence to the underside of a membrane as neutrophils migrate toward a chemoattractant.

remain expensive, technically challenging, and require experienced users for experimental set-up and data analysis.

An alternative to these cell migration assays based on the measurement of electrical impedance has also emerged recently. Electric cell–substrate impedance sensing (ECIS) systems such as ECIS/Taxis (22) and xCELLigence act by applying a harmless electrical current on gold electrodes that is impeded by the adherence of cells to the electrodes (23,24). The degree of impedance reflects the number and the morphology of adhered cells (12). Thus, adherence of live cells in their native form can be monitored in real time, giving better insights into cell function kinetics. Unlike other methods, ECIS assays have multiple applications as changes in cell adhesion and morphology may be used to analyze cell proliferation, cytotoxicity, and migration (Roche Application Note. Real-time, label-free monitoring of cellular invasion and migration with the xCELLigence system. Nat. Methods. 2002) (25–28). Compared to other ECIS systems, xCELLigence, developed by ACEA Biosciences in partnership with Roche Applied Science, allows easier and faster experimental analysis with user-friendly software without the need for lengthy preparation and stabilization steps. This system has been validated for analysis of macrophage and monocyte chemotaxis (28), but has yet to be adapted for neutrophils.

Here we present a reproducible, rapid, and easy impedance-based assay to specifically measure neutrophil chemotaxis in real time with the xCELLigence system. Unlike

macrophages and monocytes, neutrophils are non-adherent cells; therefore, the direct use of the commercial material is not possible (24,25-28), and several optimization steps are required. Given the difficulty in obtaining large amounts of human neutrophils, our experiments were performed using equine neutrophils. These cells show great similarities with human and murine neutrophils, and they are used to investigate neutrophil-associated respiratory diseases in humans (29–32).

Materials and methods

Ethics statement

All experiments were performed with healthy mixed-breed mares belonging to the research herd of the Faculty of Veterinary Medicine of the University of Montreal. All animal experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the Faculty of Veterinary Medicine of the University of Montreal (Rech-1716).

Neutrophil isolation

Thirty-five milliliters of equine blood was collected from a jugular vein into heparinized sterile tubes. Peripheral neutrophils were isolated by Ficoll gradient centrifugation according to the manufacturer's instructions (Ficoll-Paque PREMIUM 1084; GE Healthcare Biosciences, Mississauga, Canada). The high-density layer containing granulocytes and erythrocytes was recovered, and erythrocytes were lysed

with distilled water (Invitrogen/Thermo Fisher Scientific, Burlington Canada). Cells were then suspended in PBS (Life Technologies, Burlington, Canada) supplemented with 2 mM EDTA (Life Technologies) and 0.5% BSA (Sigma-Aldrich, Oakville, Canada) before cell count and viability were measured with an ADAM Cell Counter (Montreal Biotech Inc., Montreal, Canada). Following this protocol, neutrophil purity was >99%, and viability was >98%. Finally, neutrophils were suspended at a suitable concentration in complete medium made with RPMI 1640 (Life Technologies) supplemented with 200 mM L-glutamine (Life Technologies), 100 U/mL penicillin–streptomycin (Pen Strep) (Life Technologies), and 10% low-endotoxin, heat-inactivated FBS (Life Technologies) before the chemotaxis assay.

Real-time chemotaxis assay

Chemotaxis assays were performed in cell invasion and migration (CIM)–plates (CIM-Plate 16; ACEA Biosciences Inc., San Diego, CA) using an xCELLigence RTCA-DP instrument (ACEA Biosciences Inc.). A CIM-plate comprises two separate parts: an upper chamber with a microporous membrane (pore size = 8 μm) embedded with gold microelectrodes on its underside and a lower chamber. The plate is assembled by locking the upper chamber on top of the lower chamber. This migratory assay is based on a small and harmless electrical current applied between the electrodes. Cell adherence to this surface while migrating from the upper chamber toward a chosen chemoattractant placed
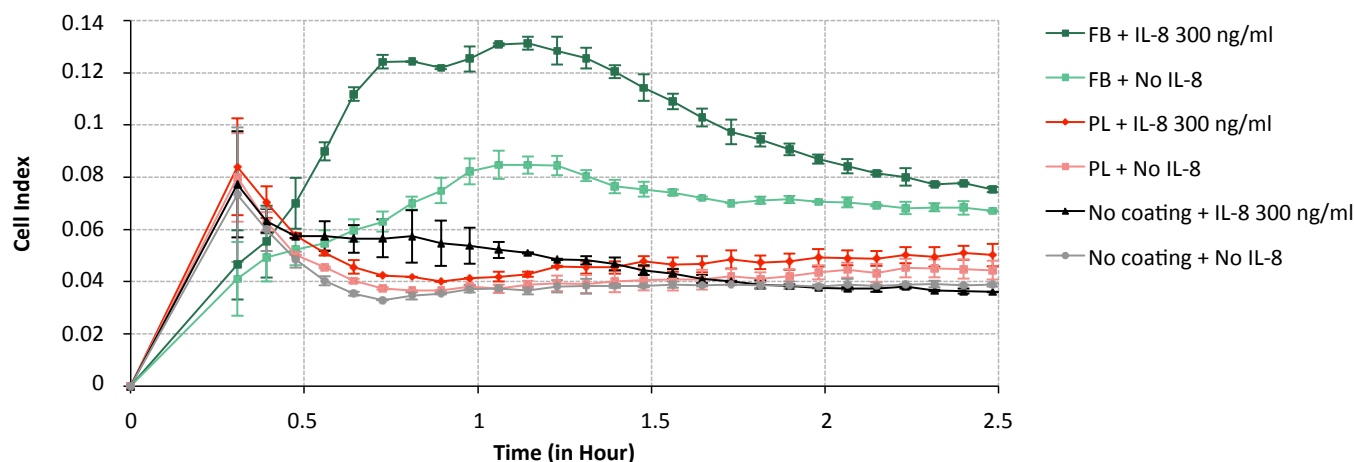
Figure 1. Effect of different coating agents on neutrophil adherence and detection. Prior to neutrophil addition to CIM-Plate 16 plates, the lower surface of the membrane of the upper chamber was coated with 50 μg/mL fibronectin (FB) (light and dark green lines) or 0.01% poly-L-lysine (PL) (light and dark red lines). The assay was then performed using 2×10^5 cells in the upper wells and in the presence of 300 ng/mL interleukin 8 (IL-8) in the lower wells (dark green and dark red lines) or in the absence of IL-8 (light green and light red lines). Two negative controls, one without both coatings but with IL-8 (in black) and another without coating and without IL-8 (in gray) were also added to the experiment. The cell index (CI) was measured as cell electrical impedance every 5 min over 2.5 h. The results are shown as the mean CI for two technical replicates ± SD.
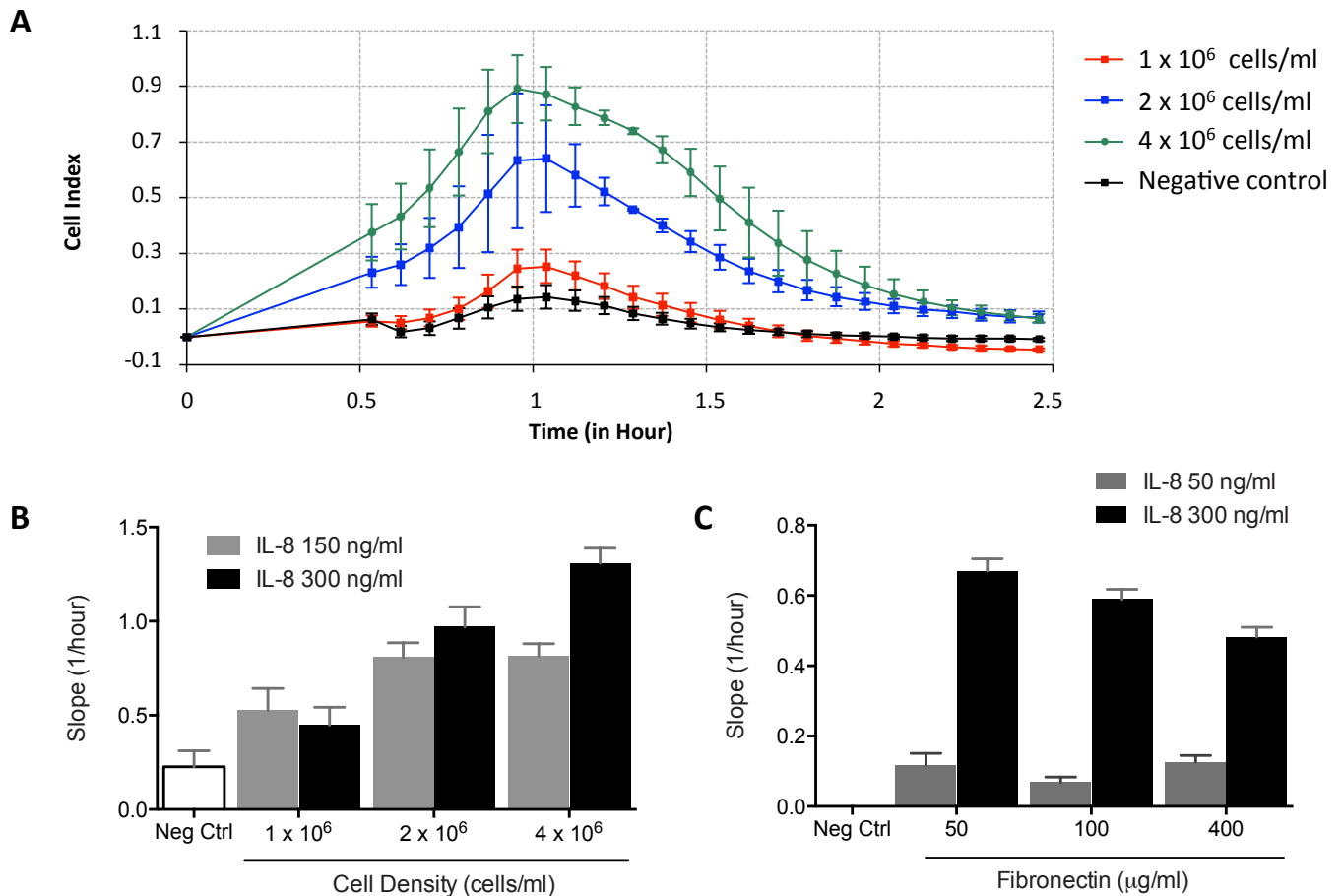
Figure 2. Assay optimization: coating and cell density. (A) Cell density was optimized, with increasing neutrophil densities in the upper wells and increasing concentrations of interleukin 8 (IL-8) in the lower wells. The membrane was coated with 50 μg/mL fibronectin. A negative control with 4×10^6 cells/mL and without IL-8 was included to this experiment (Neg Ctrl). The cell index (CI) was measured as cell electrical impedance every 5 min for 2.5 h. The results are shown as the mean of two technical replicates ± SD. For graphical clarity, only the results obtained with 300 ng/mL IL-8 are depicted here. (B) Slope analysis was performed using the RTCA software. The results obtained with both 150 ng/mL and 300 ng/mL IL-8 were analyzed. (C) Three concentrations of fibronectin were tested for coating the lower surface of the upper chamber membrane. Neutrophils (4×10^6 cells/mL) were added to the upper wells, and increasing concentrations of IL-8 were added to the lower wells. A negative control with 50 μg/mL fibronectin but without IL-8 was added to the experiment (Neg Ctrl). Slope analysis was performed using the RTCA software. The results are shown as mean of two technical replicates ± SD.

in the lower chamber is measured. The more cells that accumulate on the underside of the membrane, the more the electrical current is impeded by the insulating properties of cellular plasma membranes. The overall cell-induced impedance is measured in real time as the cell index (CI), a dimensionless parameter. More details concerning impedance measurements with the xCELLigence system can be found in Xi et al. (12).

As neutrophils are non-adherent cells, the underside of the membrane was coated with different coating agents prior to each experiment, as described below.

Equine IL-8 recombinant protein (Thermo Fisher Scientific, Rockford, IL) and leukotriene B4 (LTB4) (EMD Millipore, Etobicoke, Canada) were diluted to concentrations ranging from 50 ng/mL to 450 ng/mL and from 1×10^{-8} M to 1×10^{-6} M, respectively, in filtered 1× PBS with 10% low-endotoxin, heat-inactivated FBS. Then,

160 μl diluted IL-8 or LTB4 was added to the lower chambers in duplicate. Following upper chamber attachment, background signal was measured by adding 35 μl pre-warmed RPMI medium supplemented with L-glutamine, Pen Strep, and 10% low-endotoxin, heat-inactivated FBS. Then, 100 μl neutrophil suspension was placed in the upper chambers. Unless stated otherwise, cell density was optimized to 1.2×10^6 cells per well, which corresponds to a final concentration of 4×10^6 cells/mL. When testing chemokinesis, IL-8 was added to both the upper and lower chambers, whereas IL-8 was only added to the upper chambers when testing fugetaxis. The CI was measured with an xCELLigence RTCA-DP instrument. Frequency and duration of data collection are indicated for each experiment. A more detailed version of the working protocol may be found in the Supplementary Material.

After data acquisition, slope analysis to identify the point where the maximal CI value was reached was performed using the xCELLigence RTCA software (ACEA Biosciences).

Membrane coating

Fibronectin (Sigma-Aldrich) and poly-L-lysine (Sigma-Aldrich) were tested as coating agents for the underside of the CIM-plate membrane, as these agents have been reported to induce adhesion of neutrophils (33–35). Coating agents were diluted to the desired concentrations in sterile 1× PBS: fibronectin to 50 μg/mL and poly-L-lysine to 0.01%. Fifty microliters of the diluted fibronectin solution was used to coat each well on the underside of the membrane; plates were incubated overnight at 4°C and then washed 3 times with cold sterile PBS just before the assay. Similarly, 50 μl of the diluted poly-L-lysine solution was also used to coat the

membrane, but plates were then incubated 5 min at room temperature. The membranes were then washed 3 times with cold PBS, and plates were kept in PBS overnight at 4°C before the assay. As fibronectin gave the best results, increasing concentrations of this coating agent ranging from 50 μg/mL to 400 μg/mL were also evaluated.

Results and discussion

Assay optimization

The aim of this work was to optimize an assay to assess chemotactic movements of neutrophils. Several modifications of previously reported protocols were required to assess neutrophil chemotaxis. First, coating of the underside of the CIM-plate membranes was optimized to detect neutrophil adherence resulting from chemotactic migration. Without coating, CI values remained low (Figure 1, gray and black lines), thus confirming a low natural adherence of neutrophils to the electrodes. In extravascular tissues, leukocytes migrate along chemotactic gradients by binding to proteins in the ECM such as fibronectin, which has been used as a coating agent in several neutrophil migration assays and

imaging protocols (33–35). Poly-L-lysine, a synthetic amino acid polymer, has also been used as an attachment factor to study neutrophil adhesion (33,34). In our experiments, poly-L-lysine coating (Figure 1, red line) did not allow the detection of neutrophil migration. In contrast, fibronectin coating enabled the detection of neutrophil migration in response to IL-8 (Figure 1, dark green line). These results are in agreement with previous studies that reported better suitability of fibronectin for adhesive inter-actions with neutrophils than other ECM proteins (reviewed by Lindbom and Werr in Reference 36). There was some cell adherence that could be measured with fibronectin in the absence of IL-8 (Figure 1, light green line). Preliminary results using microscopy show neutrophils passively cross the membrane in the absence of IL-8 and cells sediment in the lower chamber after 15 min. Sedimentation is not detected by the xCELLigence system since only activated cells express surface receptors that allow their adherence to be detected; therefore, this IL-8 independent signal might reflect basal activation levels of neutrophils possibly caused by the isolation procedure or the presence of FBS in the media. These

background levels did not interfere with CI measurement in the presence of IL-8 (Figure 1, dark green line), especially when the signal was further optimized (Figures 2, 3, and 4). However, while detecting neutrophil chemotaxis, CI values were below the lower value recommended by the manufacturer (0.2), likely resulting from insufficient cell density or an inappropriate fibronectin concentration. Increasing cell density from 6×10^5 cells/mL to 1×10^6 cells/mL was sufficient to obtain CI values above 0.2 (Figure 2A). Slope analysis, area under the curve, and maximal CI values were compared for data interpre-tation. Although all methods led to similar conclusions (data not shown), we selected slope analysis for follow-up experiments as it reflects not only maximal CI values, but also migratory speed. Slope analysis was performed on CI curves at points where the maximum value was reached. Iqbal and colleagues previously validated this analysis method when they examined chemotaxis of different leukocyte populations with the xCELLigence instrument and a scanning electron microscope at different time points (28). Optimal results in terms of maximal slope values and sensitivity to increasing
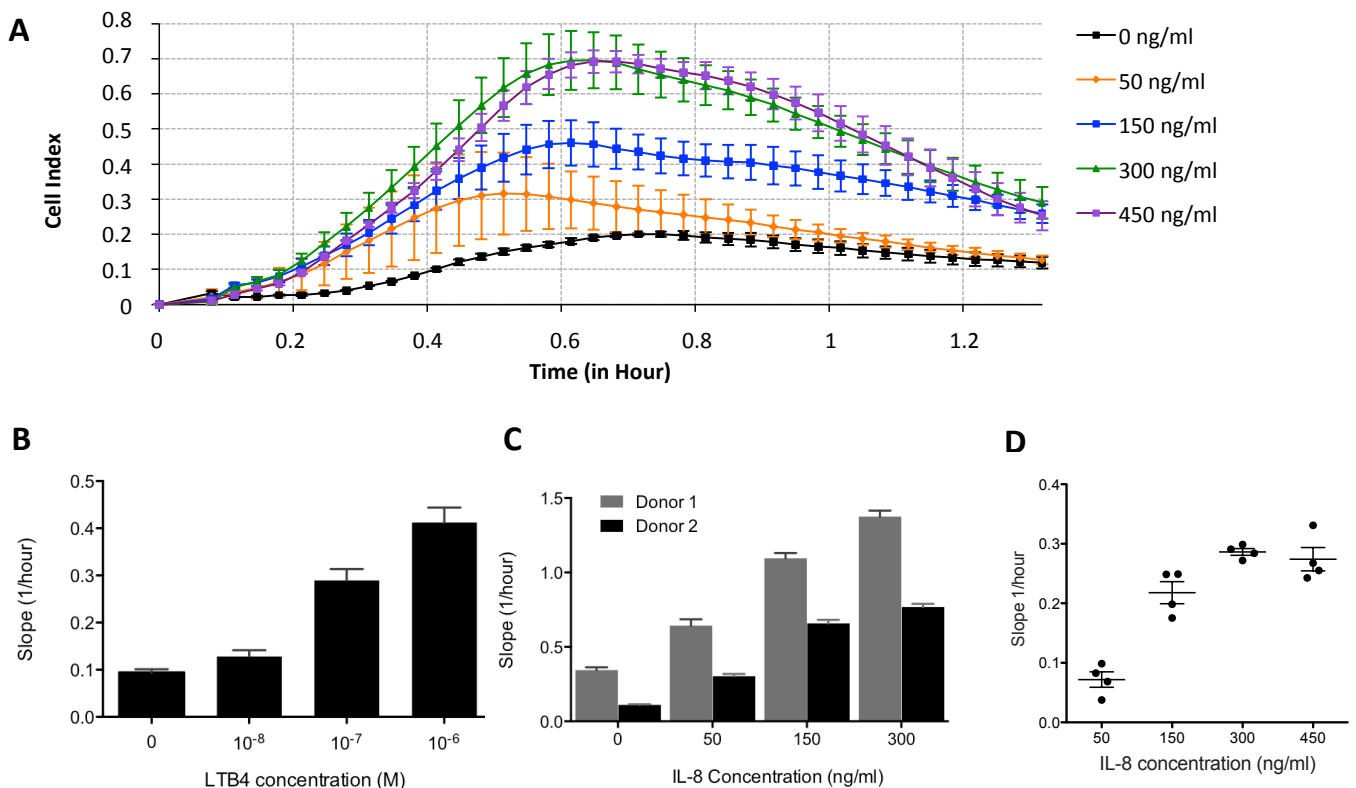
Figure 3. Dose-dependent chemotactic effect of interleukin 8 (IL-8) and leukotriene B4 (LTB4) on neutrophils. The membrane was coated with fibronectin (50 μg/mL), neutrophils (4×10^6 cells/mL) were added to the upper wells, and increasing concentrations of IL-8 (A) or LTB4 (B) were placed in the lower wells. The cell index (CI) was measured as cell electrical impedance every 2 min for 1 h, 20 min. (C) Slope analysis was performed with RTCA software for two independent experiments with IL-8. The results are shown as the mean of two technical replicates ± SD. (D) A reproducibility assay was performed using four technical replicates of the same assay at each of four IL-8 concentrations. The results are shown as the mean ± SD.

concentrations of IL-8 were obtained with 4×10^6 cells/mL (Figure 2B). This cell density is similar to that used in other migration assays with human neutrophils (6×10^6 cells/mL) (21) and equine neutrophils (3×10^6 cells/mL, 2×10^6 cells/mL) (31,37), and in other impedance-based assays with monocytes and macrophages (8×10^6 cells/mL) (28). Interestingly, increasing concentrations of fibronectin were not associated with higher slope values (i.e., increased neutrophil chemotaxis) (Figure 2C). Fifty micrograms per milliliter fibronectin allowed maximal detection of neutrophil migration and also the best sensitivity to increasing concentrations of IL-8. Coating the upper side of the membrane did not allow the detection of neutrophil chemotaxis as no signal could be observed when coating was performed on both sides of the membrane or on the upper side only (data not shown). These experiments highlight the specificity of the assay in measuring only adherence of the cells to the underside of the membrane resulting from chemotactic migration. Although optimized for equine neutrophils, our method should also be suitable for murine and human cells given the high
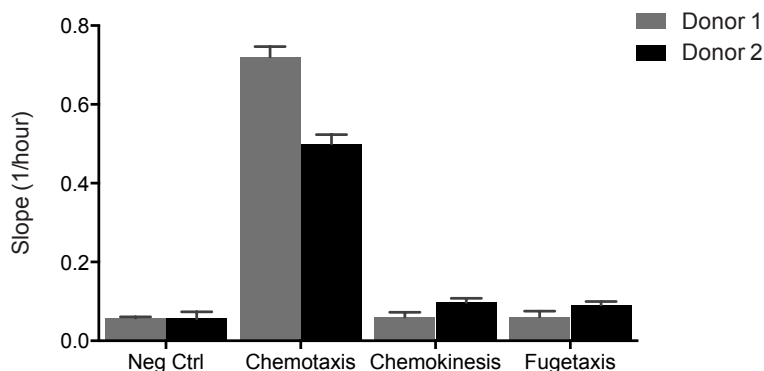
functional homology among mammalian neutrophils, especially regarding the insulating properties of cellular plasma membranes (29). However, it is important to note that minor adjustments in terms of chemoattractant concentration, migration time, and coating agent may be necessary. If magnetic beads are used for neutrophil isolation, the use of a negative control is essential as this technique may induce minor cellular activation prior to the chemotaxis assay.

Dose-dependent chemotactic effect of IL-8 and LTB4

To test the sensitivity of our assay, neutrophil chemotaxis was measured in response to increasing concentrations of IL-8 and LTB4 (Figure 3). A dose-dependent response with concentrations ranging from 0 ng/mL to 450 ng/mL was observed in the presence of IL-8 (Figure 3A). At 450 ng/mL, no further increase in neutrophil migration was detected, suggesting that chemotactic properties of neutrophils may have been saturated at concentrations >300 ng/mL. Another important chemoattractant of neutrophils is the lipid mediator

LTB4 (38). When tested with the xCELLigence instrument, LTB4 induced neutrophil chemotaxis in a dose-dependent manner with concentrations raging from 1×10^{-8} M to 1×10^{-6} M (Figure 3B).

The reproducibility of our assay was ascertained using four replicates of the same assay (Figure 3D), with slopes being similar in replicates for each condition. When comparing the dose-dependent chemotactic effect of IL-8 between two independent donors, differences in maximal CI value and migratory speed were observed, as depicted by slope differences (Figure 3C). These variations most likely reflect individual differences in basal activation of neutrophils and biological specificities between donors. Similar observations have been reported with human monocytes when using the xCELLigence instrument (28). These results emphasize the importance of real-time assays, without which early or delayed signals could be missed. Also, compared with other leukocytes such as macrophages (28), neutrophil chemotaxis occurred rapidly, highlighting the importance of these cells as a first line

IL-8 300 ng/ml		Neg Ctrl	Chemotaxis	Chemokinesis	Fugetaxis
	Upper well	-	-	+	+
	Lower well	-	+	+	-

Assay chemotaxis specificity

In addition to chemotaxis, neutrophils also have the ability to migrate randomly (chemokinesis) or to migrate away from a chemotactic agent (fugetaxis), depending on the concentration of the chemoat-tractant and the intensity of the gradient (10,11). Therefore, the specificity of our assay for measurement of chemotaxis was evaluated with a concentration of IL-8 known to induce only this type of movement in equine neutrophils (37). Detection of artifactual signals was tested with IL-8 placement (*i*) only in the lower wells, thus simulating chemotaxis, as a positive control; (*ii*) at similar concentrations in the upper and the lower wells for chemokinesis; and (*iii*) only in the upper wells to test fugetaxis. As shown in Figure 4 for two different donors, only chemo-tactic movements could be detected, thus confirming the specificity of the assay for measurement of neutrophil chemotaxis. However, the present protocol is not limited to the study of chemotaxis and could be adapted to neutrophil chemokinesis and fugetaxis by using lower concentrations of IL-8 for chemokinesis (10) and higher concentration for fugetaxis (11).

Figure 4. Detection of other cellular migratory movements. The membrane was coated with fibronectin (50 μg/mL) and neutrophils (4×10^6 cells/mL) were added to the upper wells as described previously. In this experiment, 300 ng/mL interleukin 8 (IL-8) was added only to the lower wells in order to measure chemotaxis, in both the upper and lower wells to mea-sure chemokinesis, or only to the upper wells to measure fugetaxis. No IL-8 was added in the control condition (Neg Ctrl). The cell index (CI) was measured as cell electrical imped-ance every 2 min for 2.5 h. Slope analysis was performed with RTCA software for two inde-pendent experiments. The results are shown as the mean of two technical replicates ± SD.

of defense. Given the speed at which neutrophil chemotaxis takes place, the 30 min incubation time recommended by the manufacturer for cell sedimentation prior to each experiment for other cell types was removed from our protocol. Otherwise, chemotactic movements were already achieved, and the increase in neutrophil migration in the presence of IL-8 could not be detected.

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Here we have described an impedance-based protocol to monitor the chemotaxis of label-free neutrophils in real time. Unlike previous methods, our assay has the advantage of being reproducible, fast, user friendly, and easy to analyze and interpret. This technique could facilitate the selection of drug candidates for regulating the influx of neutrophils, aiding the development of therapies against chronic inflammatory diseases. Our results also suggest that this type of assay could be used to study other migratory movements of neutrophils, as well as migration of other non-adherent cells such as lymphocytes.

Author contributions

P.M.C. contributed to the execution and analysis of the data as well as in the writing and editing of the manuscript. A.V. was involved in the conception and execution of the experiments as well as in the writing and editing of the manuscript. J.P.L. supervised the conception, execution, and analysis of the experiments as well as the writing and editing of the manuscript.

Acknowledgments

Competing interests

The authors declare no competing interests. However, ACEA Biosciences provided the CIM-plates required for the study but had no further involvement in the experimental design or data analysis.

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