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Time course of chemotaxis and chemokinesis of neutrophils following stimulation with IL-8 or FMLP

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Abstract

Polymorphonuclear cells (PMNs) attend to inflammatory sites by chemotactic movement, caused by chemoattractants (CAs) like n-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) and interleukin-8 (IL-8). However, distinct but applicable assays for investigations of PMNs' migration limit in vitro examination. We integrated CD15-bead-based isolation of PMNs with analysing their chemotaxis in a novel 3D-µ-Slide migration chamber. The PMNs were exposed to different concentrations of FMLP and IL-8 (1, 10 and 100 nM) and observed for 180 min in cell-physiological environment conditions. Moving PMNs' percentage (median and interquartile range) decreased from 62% (27%) to 36% (31%) without CA, from 88% (30%) to 22% (26%) for 1 nM IL-8, from 70% (22%) to 28% (13%) for 100 nM IL-8, from 30% (23%) to 18% (46%) for 1 nM FMLP and from 76% (20%) to 28% (13%) for 100 nM FMLP. Centres of cell movement turned towards the CAs (negative values) within a single 30-min observation period: 5.37 µm (16.82 µm) without CA, −181.37 µm (132.18 µm) with 10 nM and −239.34 µm (152.19 µm) with 100 nM IL-8; −116.2 µm (69.07 µm) with 10 nM and −71.59 µm (98.58 µm) with 100 nM FMLP. FMLP and IL-8 ensure chemotaxis without increase of chemokinesis. 3D-µ-Slide chemotaxis chambers facilitate time course analyses of PMNs' migration in stable conditions over a long time with concise distinction of chemotaxis and chemokinesis.

Keywords

Chemotaxis, FMLP, IL-8, neutrophils, PMNs

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Introduction

The rapid accumulation of polymorphonuclear cells (PMNs) at a specific site of inflammation is an essential part of host defence. PMNs are stimulated by several chemoattractants (CAs) to leave the bloodstream and migrate along a gradient towards the site of inflammation or injury. Among the CAs are the bacterial peptide n-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) and the chemokine interleukin-8 (IL-8). The secretion of these CAs at the site of inflammation ensures a chemotactic gradient, causing an aligned migration of PMNs, which is distinguishable from random chemokinesis without a chemotactic gradient.[1,2]

Several assays have been applied to investigate chemokinetic movement of PMNs. While the Boyden chamber was the first system to measure chemotaxis in vitro, a number of other systems including the Zigmond chamber, the Dunn chamber,

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Table 1. Ratio of x- to y-Centre of Mass.

Time (min)	CA (nM)				
	Without	*FMLP*		*IL-8*	
		10	*P*	*10*	*P*
0–30	0.90	8.16*	0.043	16.92*	0.043
30–60	1.60	11.50	0.177	10.87†	0.080
60–90	1.10	9.05	0.105	17.61*	0.043
90–120	0.79	11.43	0.080	12.21*	0.043
120–150	0.70	8.56	0.053	13.26*	0.043
150–180	1.65	4.06	0.066	8.04*	0.043
Average	0.94	8.57	–	13.01	–

CA: chemoattractant; FMLP: n-formyl-l-methionyl-l-leucyl-l-phenylalanine; IL-8: interleukin-8.
Ratios are displayed for CA-free cases and for FMLP and IL-8 in 10 nM concentration, because here the greatest effects were seen. *P* refers to the comparison of FMLP or IL-8 to CA-free cases.
*Value is significantly ($P < 0.05$) different from corresponding value in cases without CA.
†A greater effect was seen with 100 nM IL-8: 18.77, $P = 0.08$.

micropipette- or microfluidic assays have been used.[3] Although these assays are widespread, they are often inadequate in providing a reproducible, controllable and stable gradient or are limited by their complex handling.[4–6] To closely mimic the in vivo condition, an efficient chemotaxis system demands two steps:

1. Reliable isolation of PMNs in adequate quantities and viability;
2. Chemotaxis assays being sensitive, reproducible and easy to use.

In the present investigation, we describe a technique, which integrates the two steps of isolating PMNs by using anti-CD15 microbeads and analysing their FMLP- and IL-8-induced chemotaxis on a 3D-µ-Slide migration chamber. Whereas the microbead method enables isolation of purified PMNs in sufficient quantity, viability and without pre-activation, the 3D-µ-slide facilitates high-quality microscopy, linear concentration gradients and support of long-term assays.[7–9]

Materials and methods

The ethic committee (Ethikkommission, Faculty of Medicine, University Regensburg) approved the study (ethic vote 15–101–0043).

Isolation of PMNs

Six test persons participated in the investigation. After confirming their written informed consent, 5 mL of blood was drawn from each healthy test person. To isolate the PMNs at high purity, the method of pluriSelect Life Science (Leipzig, Germany) was chosen, targeting $CD15^+$ cells (Table 1 in Zengel et al.[10]) with anti-human CD15 antibodies located on the surface of spherical beads (S-Beads). Following the instruction manual, the heparinized blood was incubated with CD15S-Beads at room temperature. After incubation, the sample was repeatedly washed to separate the S-Beads from non-binding cells. At the end of the isolation process, the connection between PMNs and S-Beads was resolved by adding a detachment buffer (w/o calcium) and a highly purified isolate of PMNs was retrieved. After final centrifugation, all extracted cells were suspended in RPMI 1640 (PAN-Biotech GmbH, Aidenbach, Germany) with 10% foetal calf serum (FCS; Sigma-Aldrich Chemie GmbH, Steinheim, Germany).

In three representative samples, the cells were further examined for purity and cellular death by flow cytometry. To detect any effect of the CAs on the viability of the cells, the three samples were split and separately either suspended in medium (RPMI 1640 with 10% FCS) or exposed to FMLP (Sigma) or IL-8 (Abcam Plc, Cambridge, UK) in medium in 1, 10 and 100 nM concentrations for up to 3 h. No effect of the CAs on the cells' viability was detectable in the fluorescence-activated cell sorting (FACS) analyses, and the isolation method proofed to be highly selective on PMNs (95.1%, interquartile range (IQR) 1.6%). The median cells' viability was 93.91% (IQR 7.15%).

Preparation of the migration chamber

For the chemotaxis experiments, a 3D-µ-Slide migration chamber (Ibidi GmbH, Planegg, Germany) was used, which primarily consists of one channel for the cells and two reservoirs for the CAs or respectively the CA-free media on either side of the channel.

For our observations, we applied to the instructions given by Ibidi in the application notes 17, 22, 23 and 26 (Ibidi GmbH). In accordance with these instructions, we produced a collagen–cell gel at a concentration of 0.5 mg/mL collagen and 3×10^6 cells/mL and filled it into the channels. During the gelation process of the gel, the filled migration chamber was incubated in a humid Petri dish at 37°C and 5% CO_2 for 35 min. To prevent the cells from sedimentation through the gelling liquid and getting attached on the bottom of the

channel, the Petri dish was turned upside down after 15 min and again turned upside after another 10 min.

As CAs IL-8 and FMLP in 1, 10 and 100 nM concentrations in a medium of RPMI 1640 with 10% FCS were applied, a solution of RPMI 1640 with 10% FCS was placed as a negative control.[11] After the end of the gelation process, the reservoirs of the µ-Slide were filled with either one of the CAs in medium or CA-free medium.

For experiments with CAs, the reservoir sited left to the channel was filled with the chemoattractive solution, and the opposite reservoir on the right side of the channel was filled with the inert negative control. In case of control experiments, we filled both reservoirs with the inert solution. Once a reservoir contains a chemoattractive solution, a gradient of the CA towards the migration channel is established instantly. As a result, the cells within the gel are exposed to the stimulating effects of the specific CA.[10]

Microscopy

After the preparation of the chemotaxis chamber, cell movement was observed under a BZ-8100E microscope (Keyence Deutschland GmbH, Neu-Isenburg, Germany). During observation the µ-slide chamber was heated at 37°C and fumigated with 5% CO_2 using the climate box INUA EF-F1 (Tokai Hit Co. Ltd., Fujinomiya-shi Shizuoka-ken, Japan). Observation was performed at a 10× magnification in a bright field modification at a lens wide of 0.17 with a Plan Fluor objective (20×/0.45, Keyence). At our setting, 1 pixel is consistent with 1.31 µm.

The period of observation was 180 min with one frame per minute taken for each channel. The migration chamber was fixed on the microscope table and the exact same location was electronically monitored every minute. Due to technical limitations, we could observe only two of the three channels in the 3D-µ-Slide Chamber at our time interval between two pictures. For each observation field in the two channels, 10 pictures were taken at different z-levels to minimize the impact of vertical movement during the observation. The picture with the highest contrast was chosen by the picture analysing software BZ 9000 Generation II Analyzer (Version 2.2, BZ-H2A/H2AE, Keyence) for further assessment. The resulting 180 pictures – representing 180 min of observation – were divided into six episodes of 30 pictures each. This procedure guaranteed good conditions for manual analysis of the episodes by ImageJ (Version 1.47; National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/), as it also enabled us to scrutinize cell movement over the time.

Analysis of the chemotaxis parameters

All cells were tracked by Manual Tracking (Fabrice Cordelières; Institut Curie, Orsay, France), a plugin of ImageJ. In our analysis, we focused on the percentage of moving cells and on different chemotaxis parameters.

Therefore, we defined as criterion for cell movement: An observed cell must not show a velocity of 0 µm/min in more than 50% of the frameshifts of a 30-min period. As single movement of one cell from one frame to the next frame, we defined that the cell must move completely out of the courser's crosslines – corresponding to a distance of 8.8 µm. In that case, we would move the courser's crosslines to the cell's new centre, acknowledging the cell's movement. By this definition, all moving cells must at least show 15 single movements in a 30-frame episode. In the first picture of every episode, that is, at minute 0, 30, 60, 90, 120 and 150, 50 cells were numbered by chance and subsequently tested according to the moving criterion. As a result, we could determine the percentage of moving cells for each episode.

If more than 20 cells were denominated as 'moving', only the first 20 cells were taken into further chemotaxis analysis by ImageJ's Chemotaxis Tool (Version 1.01, National Institutes of Health, Bethesda, Maryland, USA, http://www.ibidi.de/applications/ap_chemo.html). If less than 20 cells could be identified as moving, only these ones were taken into further assessment. To detect effects on the PMNs' motility, we compared the fraction of moving cells in cases, when CAs were present, to the fraction of moving cells, when no CAs were present. This ratio of moving cells indicates whether the fraction of moving cells rose in the presence of a CA (ratio >1) or declined (ratio <1) compared to the situation, when no CA was present.

For chemotaxis analysis, we used the x- and y-Forward-Migration Index (FMI) and the x- or y-Centre of Mass (CoM). Because the CA was

always placed left to the channel, a migration towards the CA would result in a negative xCoM and xFMI. The CoM parameter indicates the average translocation of the cells along the x-axis (xCoM) or the y-axis (yCoM). To detect chemotaxis of PMNs, we referred the PMNs' movement in parallel direction to the CA (xCoM) to their movement in perpendicular direction (yCoM). Using only the absolute ratios of xCoM to yCoM, we could distinguish between chemokinesis (ratio ~1) and chemotactic movement in x-direction (ratio >1) or y-direction (ratio <1).

Chemotactic effects could additionally be quantified by the FMI, which describes the efficiency and direction of any movement along either the x-axis (xFMI) or the y-axis (yFMI). To distinguish between chemotaxis in x- (ratio >1) and y-direction (ratio <1) and chemokinesis (ratio ~1), we analogously determined a ratio for the FMI index, as we did before with the CoM, again using only absolute values.

For further distinction of chemokinesis and chemotaxis, the Rayleigh test was applied. By comparison of the FMI of both axes, the Rayleigh test for uniformity enables the analysis of circular distribution of cells. At a significance level of 0.05, its null hypothesis of an aligned migration was rejected.

Further on, we could quantify the effectiveness of any chemotactic movement using the Directionality. This parameter is the relation of Euclidean to accumulated distance and, thus, measures the efficiency of the cells' movement. Finally, we measured the mean velocity of the PMNs during observations.

All experiments were manually analysed by one person. Thus, we investigated the individual influence on the results. A priori not involved staff (n = 5) analysed two different, blinded frame sets from 30 min periods, and their results were compared to the ones of the main investigator. No significant difference in the results could be found between the main investigator and the controlling staff (data not shown). Five experiments for each different concentration of IL-8, FMLP and for the negative controls were performed.

All data are displayed as median with interquartile ratio (IQR). Variables were compared between the groups by applying the Kruskal–Wallis test. In case of significant findings, the Wilcoxon rank-sum test with Bonferroni correction was used. Analyses were performed using R (R Development Core Team, www.R-project.org), Excel (Microsoft, Redmond, WA, USA) and SPSS (IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered to be statistically significant.

Results

Fraction of moving cells and motility ratio

During all observations, between 20% and 85% of the initially targeted cells were identified as moving cells according to the above-mentioned criterion. Without CAs, the fraction of moving cells decreased from 62% (IQR 27 %) at the first observation period to 36% (IQR 31%) after 3 h.

1 nM FMLP caused statistically insignificant lower ratios of moving cells than the CA-free cases. Among the CAs, 10 nM FMLP showed constant high-motility ratios with the highest score (1.89, IQR 0.16); 100 nM FMLP activated the cells especially in the first hour, and then, its ratio decreased insignificantly beneath the unstimulated level.

IL-8 in 1 and 100 nM concentrations caused an elevated ratio in the first hour, and then, their ratios decreased insignificantly below the ratio of unstimulated cells; 10 nM IL-8 caused high ratios of moving cells throughout the time. All CAs had no statistically significant influence on the motility ratio compared to CA-free cases – except for 10 nM FMLP in the observation interval from 30–120 min ($P = 0.043$).

Displacement of centre of mass

The CoM along the x-axis (xCoM) for non-stimulated cells lay in a range from −3.47 µm (IQR 7.53 µm) to +5.5 µm (IQR 4.36 µm).

Stimulation with FMLP achieved a nearly continuous shift of the xCoM towards the CA in all concentrations. Compared to non-stimulated cells, 10 nM FMLP had significant higher xCoM values throughout the observation. 1 and 100 nM FMLP only partially had significant higher values (Supplemental Data). Stimulation with 10 nM FMLP effected a higher absolute shift of xCoM (−116.20 µm, IQR 69.07 µm) than stimulation with 100 nM FMLP (−71.59 µm, IQR 98.58 µm).

Towards the stimulation origin, 1 nM IL-8 achieved a continuous shift of the xCoM. Stimulation with 10 or 100 nM of IL-8 resulted in a strong shift of the xCoM. However, their effects on the xCoM were declining within the beginning of the observation (100 nM) after 90 min (10 nM).

Among IL-8, only 10 nM had a significant higher xCoM than non-stimulated cells for the complete observation; 1 and 100 nM IL-8 showed partially significant differences (Supplemental Data).

The PMNs' displacement along the y-axis was low without any significant differences between cases with CAs and CA-free cases (data not shown). The ratio of xCoM to yCoM for experiments without CAs was nearly 1. In contrast, all CAs – especially 10 nM FMLP and IL-8 – induced a greater shift along the x-axis than along the y-axis (ratio >1, Table 1).

Parallel (xFMI) and perpendicular (yFMI)

Without CAs, the xFMI was constantly next to 0 (Supplemental Data). In contrast, all concentrations of FMLP caused an increase in the xFMI and a turn towards the stimulation origin (negative values).

After 30 min, 1 nM FMLP increased the xFMI, with a constant time course afterwards; 10 nM FMLP showed the highest scores of xFMI among all FMLP concentrations and a constant course, similar to 100 nM FMLP.

In the first half of the observation, 1 nM IL-8 increased the xFMI and showed a constant course afterwards. Stimulation with 10 and 100 nM IL-8 showed the highest scores of xFMI among all CAs; 10 nM IL-8 reached its maximum after 1 h, gradually declining afterwards. With 100 nM IL-8, the xFMI rose to a maximum of 0.72 (IQR 0.26) in the first 30 min, which declined over the rest of the observation.

The yFMI was low and without any statistical difference between cases with CA and the CA-free cases (data not shown). The ratio of xFMI to yFMI was high (ratio >1) for CA-experiments. In contrast, experiments without CAs showed a ratio next to 1 (Table 2). Finally, the Rayleigh test for unstimulated cells did not show any difference ($P>0.05$), whereas stimulation with IL-8 and with FMLP in all concentrations demonstrated a significant migration along the x-axis ($P<0.05$, data not shown).

Mean velocity

The PMNs' mean velocity in cases without CAs declined during the observation period from 4.7 µm/min (IQR 2.0) to 2.8 µm/min (IQR 1.48).

Stimulation with FMLP demonstrated an increase in the mean velocity with continuous reduction over the time. Stimulation with 10 nM FMLP (max. 11.60 µm/min, IQR 1.01) resulted in a higher velocity of the cells than stimulation with 100 nM FMLP (max. 10.15 µm/min, IQR 3.87). Stimulation with 10 nM FMLP resulted in a significant higher velocity throughout the time compared to CA-free cases.

Consistent with the results regarding FMLP, stimulation with 10 nM IL-8 in average effectuated a higher velocity (max. 11.85 µm/min, IQR 3.47) than stimulation with 100 nM IL-8 (max. 11.89 µm/min, IQR 3.72) – except for the observation time 30–60 min. Both concentrations increased the velocity of PMNs significantly for the complete observation compared to CA-free cases.

Table 2. Ratio of x- to y-Forward-Migration Index.

Time (min)	CA (nM)				
	Without	*FMLP*		*IL-8*	
		10	*P*	*10*	*P*
0–30	0.83	9.75*	0.043	16.25*	0.043
30–60	1.67	13.67	0.199	9.75	0.080
60–90	1.00	5.50	0.088	21.50*	0.043
90–120	1.00	9.60	0.224	11.75	0.080
120–150	0.62	5.75*†	0.043	30.00*	0.043
150–180	1.25	5.40‡	0.059	7.00*	0.043
Average	1.00	8.00	–	13.5	–

CA: chemoattractant; FMLP: n-formyl-l-methionyl-l-leucyl-l-phenylalanine; IL-8: interleukin-8.
Ratios are displayed for CA-free cases and for FMLP and IL-8 in 10 nM concentration, because here the greatest effects were seen. *P* refers to the comparison of FMLP or IL-8 to CA-free cases.
*Value is significantly ($P<0.05$) different from corresponding value in cases without CA.
†A greater ratio was reached by 100 nM FMLP: 6.40, $P=0.043$.
‡A greater effect was monitored with 1 nM FMLP: 7.00, $P=0.225$.

Directionality

The directionality of unstimulated PMNs was low, but steady (Supplemental Data). In contrast, all concentrations of FMLP demonstrated high and steady values for the directionality parameter. Stimulation with 1 and 10 nM FMLP showed a greater directionality compared to 100 nM concentration.

Stimulation with IL-8 in all concentrations also demonstrated an increase in the directionality, but their values were more declining over the observation time. IL-8 in 10 and 100 nM concentrations showed significant higher values for the directionality than non-stimulated PMNs for the complete observation.

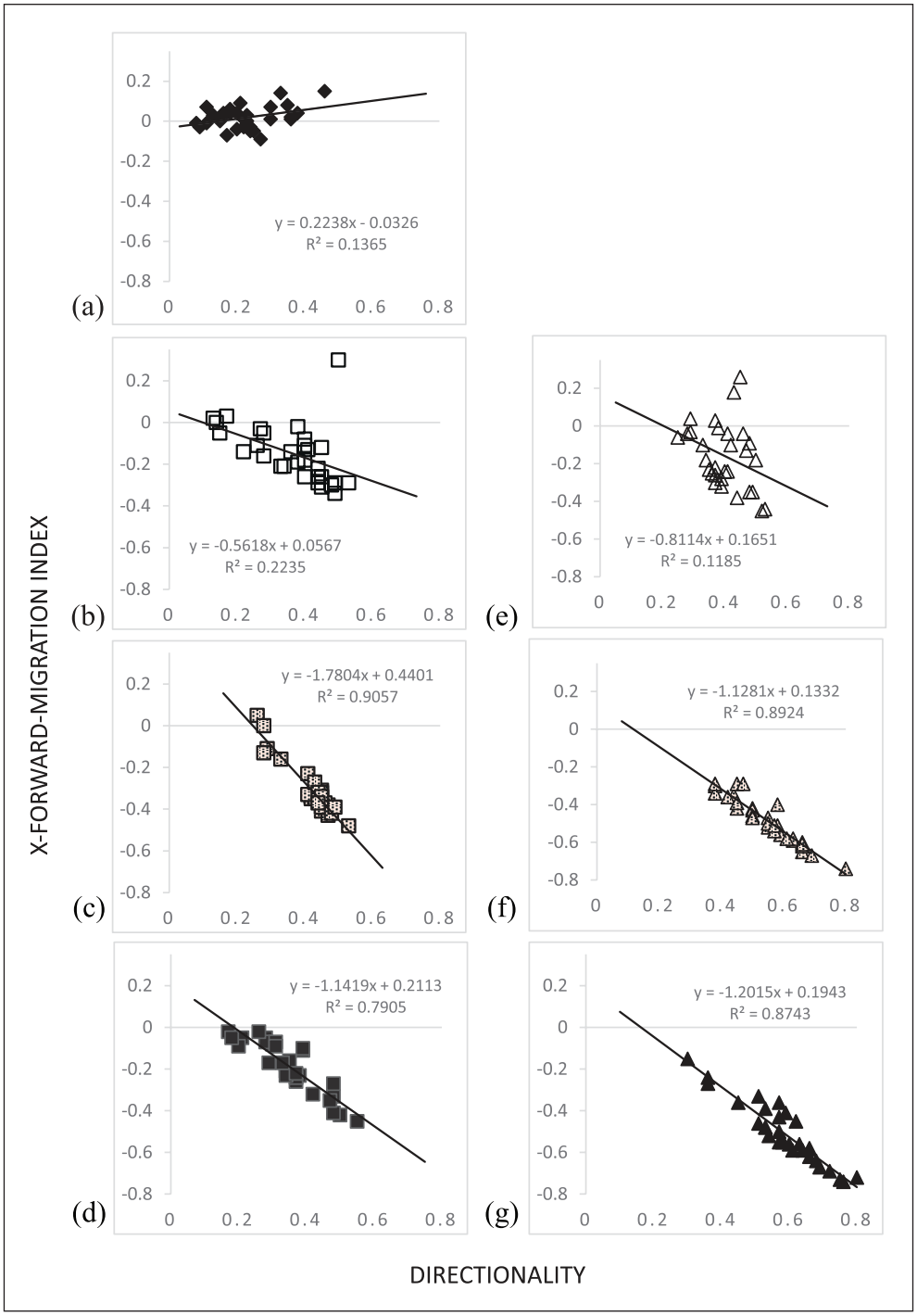

Figure 1. Correlation between the x-Forward-Migration Index and Directionality. ((b)–(d)) FMLP was used in 1 (), 10 () and 100 () nM concentrations, and likewise, ((e)–(g)) IL-8 in 1 (), 10 (), 100 () nM concentrations. Cases without CAs (, (a)) were introduced as controls. Straight line migration correlates with high xFMI (displayed on y-axis) and Directionality (displayed on x-axis) values. In our settings, the CA was placed into the left reservoir, so negative high xFMI values indicate straight-line migration towards the CA.

Correlation

Directionality and xFMI were compared using Spearman's rank correlation analysis. Whereas xFMI and directionality without CA did not show any correlation, for cells exposed to FMLP or IL-8, the correlations were high (Figure 1).

Discussion

The key advantages of the presented migration assay include the opportunity to distinguish and measure chemokinesis and chemotaxis independently from each other and computed parameters can precisely describe multiple cell responses. Also, the system facilitates a persistent gradient of CAs over a long time in a three-dimensional collagen–cell environment.[9,10]

Our results show that FMLP and IL-8 – in concentrations seen in clinical settings – increase the percentage of moving cells compared to unstimulated cells. Notably, the fraction of moving PMNs showed a time-dependent decrease independent of the CA. This could be interpreted as either a physiological reduction of moving PMNs following a continuous stimulation or a physiological aging of PMNs' motility as a response to activation bearing a subpopulation of resting PMNs.[2] A lack of energy, however, could be excluded in prior studies.[9,10] FMLP and IL-8 both ensure chemotaxis without an increase in chemokinesis. Consistent with other studies, our results support the hypothesis of different possible modulations of PMNs' migration as part of the host defence.[1,2] While FMLP in optimal concentration shows continuous cell responses, the responses to IL-8 are limited. This might concur with the understanding of IL-8 as a chemokine recruiting PMNs and attracting them to the site of inflammation, whereas FMLP indicates the actual location, where host defence needs to take place. A disadvantage of our migration assay is that generated data require a time-consuming manual cell tracking, because semi-automated tracking algorithms cannot thoroughly follow fast moving cells at our observational time shift. However, a greater interval between two pictures would omit cellular movement, which is crucial for the analysis of chemokinesis and chemotaxis.

In the long term, the presented migration assay could facilitate monitoring further effects on PMNs, for example, by in vitro exposure to drugs or in fluorescing studies.

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M.H., S.-M.P. and S.M. performed the experiments; M.H., M.G. and B.T. analysed the data and made the figures; and M.H., M.G., K.L. and B.T. wrote the manuscript.

Declaration of conflicting interests

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Supplemental material

Supplemental material for this article is available online.

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