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Establishment of a scalable microfluidic assay for characterization of population-based neutrophil chemotaxis

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Abstract

Background: Regulation of neutrophil chemotaxis and activation plays crucial roles in immunity, and dysregulated neutrophil responses can lead to pathology as seen in neutrophilic asthma. Neutrophil recruitment is key for initiating immune defense and inflammation, and its modulation is a promising therapeutic target. Microfluidic technology is an attractive tool for characterization of neutrophil migration. Compared to transwell assays, microfluidic approaches could offer several advantages, including precise control of defined chemokine gradients in space and time, automated quantitative analysis of chemotaxis, and high throughput.

Methods: We established a microfluidic device for fully automated, quantitative assessment of neutrophil chemotaxis. Freshly isolated mouse neutrophils from bone marrow or human neutrophils from peripheral blood were assessed in real time using an epifluorescence microscope for their migration toward the potent chemoattractants C-X-C-motif ligand 2 (CXCL2) and CXCL8, without or with interleukin-4 (IL-4) pre-incubation.

Results: Our microfluidic device allowed the precise and reproducible determination of the optimal CXCL2 and CXCL8 concentrations for mouse and human neutrophil chemotaxis, respectively. Furthermore, our microfluidic assay was able to measure the equilibrium and real-time dynamic effects of specific modulators of neutrophil chemotaxis. We demonstrated this concept by showing that IL-4 receptor signaling in mouse and human neutrophils inhibited their migration toward CXCL2 and CXCL8, respectively, and this inhibition was time-dependent.

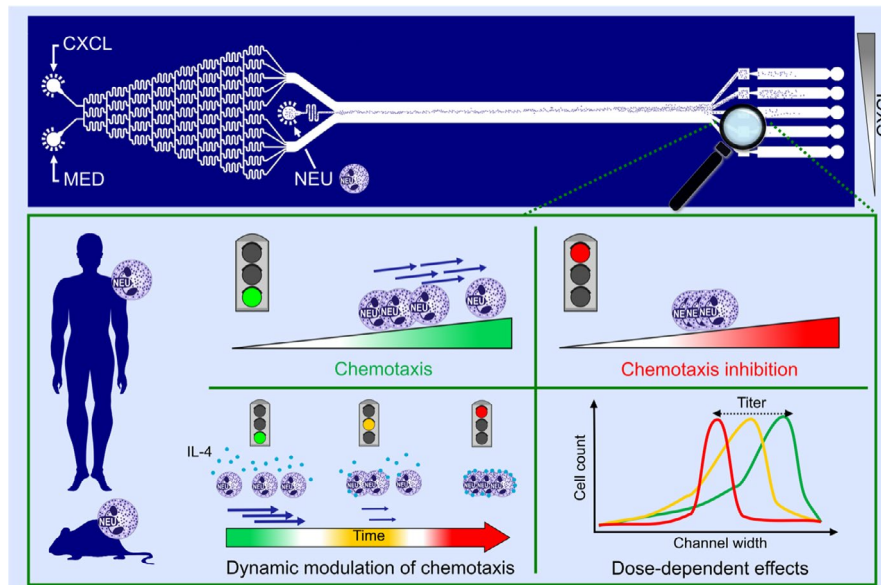
Conclusion: Collectively, our microfluidic device shows several advantages over traditional transwell migration assays and its design is amenable to future integration into multiplexed high-throughput platforms for screening of molecules that modulate the chemotaxis of different immune cells.

KEYWORDS

basic immunology, chemokines, inflammation, innate immunity

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GRAPHICAL ABSTRACT

We established a microfluidic device for fully automated, quantitative assessment of mouse and human neutrophil chemotaxis. We determined the dose and time dependency of neutrophil chemotaxis toward inflammatory chemokines. We modulated dynamic and temporal aspects of neutrophil chemotaxis by stimulation with interleukin-4, the prototypic cytokine of type 2 immunity, and allergic inflammation.

1 | INTRODUCTION

Upon infection or inflammation, numerous signaling cascades are initiated and various immune cells, both circulating and resident in tissues, become activated. Neutrophils are usually the first nonresident leukocytes recruited to the affected site.^{1,2} The majority of neutrophils is stored in the bone marrow, and the egress from and residence in the bone marrow are primarily regulated by signaling via two opposing chemokine receptors, namely C-X-C chemokine receptor 2 (CXCR2) and CXCR4, which promote neutrophil release and retention, respectively.³ An infectious or inflammatory stimulus results in release of several immune-stimulatory cytokines, which also modulate chemokine receptor expression on neutrophils. As a result, neutrophils migrate toward higher CXCR2 ligand concentrations and consequently leave the bone marrow.

After entering the blood stream, neutrophils circulate and patrol the body until they encounter activated endothelium, which acts as a signal for them to slow down and transmigrate into the surrounding tissue. This initial migration is coordinated by intermediary chemokine-chemokine receptor signals, such as CXCR2 and its ligands. These signals are subsequently overwritten by gradients of end-target chemoattractants that secure arrival to an inflamed or affected area.⁴ There neutrophils exhibit several effector functions, which all serve to contain and kill invading pathogens. All these migrational steps of neutrophils rely on chemokine-chemokine receptors binding,^{5,6} and on the subsequent directional motion, known as chemotaxis, which is guided by spatio-temporal gradients of chemokine concentrations.^{7,8}

Notably, there also exist negative regulators of neutrophil chemotaxis. It has recently been observed that interleukin-4 (IL-4) receptor (IL-4R) signaling in neutrophils exerts a potent inhibitory effect on both mouse and human neutrophil chemotaxis.⁹⁻¹² This finding might be of importance for allergic diseases and susceptibility to infections during type 2 immune responses, as IL-4 is crucial in determining type 2 immunity and allergic inflammation. Thus, IL-4 plays a central role in orchestrating type 2 immune responses involving eosinophils, IgE-producing B cells, mast cells, and T helper 2 cells.¹³

Chemotaxis serves an important role in a variety of both physiological¹⁴⁻¹⁶ and pathological^{17,18} processes. Thus, the ability of quantitatively measuring and controlling cellular chemotaxis is crucial for fundamental studies of biochemical and biophysical mechanisms involved in chemotaxis and for the design of effective therapeutic strategies. These needs have motivated the development of *in vitro* assays to study chemotaxis. Transwell migration assays, pioneered by Boyden¹⁹ and later optimized by others,²⁰⁻²² represent the current standard method to investigate chemotaxis. However, these assays suffer from important limitations, including generation of unstable chemokine gradients, limited control and characterization of the established chemotactic environment, and low flexibility and throughput.²³

In the past two decades, microfluidics has emerged as a powerful and flexible tool to quantitatively analyze cellular chemotactic motion with superior performance compared to traditional chemotaxis chambers.²⁴⁻³⁶ Key advantages of microfluidics include the establishment of highly accurate and controllable chemokine gradients, the possibility to perform real-time measurements down

to single-cell resolution, and the rigorous quantification of physical parameters describing cellular motion.³⁰⁻³³ The current challenge, however, lies in the development of microfluidic devices that operate at high throughput and remain sufficiently simple to be routinely performed in many laboratories, even by personnel who are not trained in microfluidics.

In the present work, we established a scalable and robust microfluidic device and experimental protocol for the detailed analysis of migration of thousands of cells, in the presence of well-characterized and stable chemokine gradients. The protocol described here for studying cell migration represents a reproducible and scalable tool for the high-throughput screening of extended libraries of both natural and engineered chemokines and other modulatory molecules.

2 | MATERIALS AND METHODS

2.1 | Animals

C57BL/6 mice were purchased from Charles River Laboratories. BALB/c mice, as well as *Cxcr2*^{-/-}³⁷ and *Il4ra*^{-/-}³⁸ mice (both on a BALB/c background), were purchased from The Jackson Laboratory. Experiments were approved by the Cantonal Veterinary Office of Zurich and carried out in accordance with Swiss law.

2.2 | Human subjects

Written informed consent was obtained from all volunteers donating blood for this study. All experiments with human samples were performed in accordance with the Cantonal Ethical Committee of Zurich (BASEC no. 2016-01440).

2.3 | Neutrophil isolation and sample preparation

2.3.1 | Mouse neutrophils

Mouse neutrophils were isolated from the bone marrow of fibula and tibia and enriched by negative magnetic selection with EasySep Mouse Neutrophil Enrichment Kit (STEMCELL Technologies) according to manufacturer's protocol. After isolation, neutrophils were labeled using fluorochrome-conjugated monoclonal antibodies (mAb) directed against Ly6G (1A8; BioLegend). Purified, labeled neutrophils were resuspended in RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS; Thermo Fisher) and thereafter used in experiments.

2.3.2 | Human neutrophils

Venous blood samples were collected in ethylenediaminetetraacetic acid tubes (BD Vacutainer). Neutrophils were purified with HetaSep

(STEMCELL Technologies), followed by negative magnetic selection with EasySep Direct Human Neutrophil Isolation Kit (STEMCELL Technologies). Neutrophils were then labeled with fluorochrome-conjugated mAb against human CD16 (3G8; BioLegend). Finally, neutrophils were resuspended in RPMI 1640 medium supplemented with 1% heat-inactivated FBS (Thermo Fisher) and used in experiments.

2.4 | Stimulation of neutrophils

For IL-4 prestimulation, unless otherwise indicated, neutrophils were pre-incubated for 30 minutes in an incubator at 37°C, 85%-95% humidity, and 5% CO₂, with medium alone or in combination with either mouse recombinant IL-4 (eBioscience) or human recombinant IL-4 (PeproTech). For blocking CXCR2, an anti-CXCR2 mAb was used (242216; R&D Systems) and compared to an isotype control (eBR2a; eBioscience).

2.5 | Device fabrication and gradient generation

The microfluidic device consisted of a gradient-generating serpentine network and of a migration chamber branching into five observation chambers (Figure 1, Data S1). A detailed description and protocols for the design and fabrication of the microfluidic chemotactic device and the description of the chemokine gradient generation and characterization are provided in Data S1 and Figure S1.

2.6 | Chemotactic migration in microfluidic device and analysis of cell migration

A detailed description of the microfluidic chemotaxis experiments is provided in Data S1, together with the protocol for quantitative analysis of cell migration as well as the definitions of the average neutrophil speed (v_m) and index of chemotaxis directionality (ICD).

2.7 | Transwell migration assays

Isolated neutrophils were pretreated for 30 minutes with medium alone or medium containing either mouse recombinant IL-4 (for mouse neutrophils) or human recombinant IL-4 (for human neutrophils) prior to seeding in the upper chamber of a 5- μ m transwell (mouse neutrophils) or 3- μ m transwell (human neutrophils) (Corning Costar). Cells were allowed to migrate for 2 hours toward titrated concentrations of mouse recombinant CXCL2 (PeproTech) or human recombinant CXCL8 (PeproTech) in the lower chamber. Migrated neutrophils were collected from the lower chamber and counted.

2.8 | Flow cytometry

Isolated neutrophils were stained for analysis by flow cytometry using fluorochrome-conjugated mAbs directed against the following mouse antigens: CD3 (145-2C11; BD Biosciences), CD11b (M1/70; BioLegend), and Ly6G (1A8; BioLegend). Cell viability was analyzed with Annexin V Apoptosis Detection Kit (eBioscience). Cells were acquired on a BD LSR Fortessa flow cytometer and analyzed using FlowJo software (Tristar).

2.9 | Statistical analysis

For each set of experimental conditions, three or more independent replicates were performed. Variations in the percentage chamber occupancies were calculated with respect to the corresponding control experiments, performed in the absence of chemokine gradients. The statistical significance of the observed variations was assessed by means of two-sample Welch's *t* test for data sets with unequal variances.

3 | RESULTS

3.1 | Neutrophil chemotaxis is driven by the spatial derivative of chemokine gradients

To validate our device (Figure 1A-C), we first compared the chemotactic behavior of C57BL/6 wild-type (WT) mouse neutrophils in the absence (Figure 1D) or presence (Figure 1E) of a CXCL2 chemokine gradient. In the absence of a chemokine gradient, the resulting cell migration distribution featured a symmetric Gaussian-like curve, centered on the longitudinal axis of the migration chamber (Figure 1D). The average migrational speed of neutrophils (v_m) was 0.3 ± 3.2 $\mu\text{m}/\text{min}$, while the overall migrational directionality (ICD) was 0.02 ± 0.10 . We next generated a 100 ng/mL CXCL2 gradient, which increased from Chamber 1 toward Chamber 5 (termed regular direction from here on). In the presence of this gradient, the chemotactic migration distribution featured a right-skewed shape (Figure 1E-F): The associated measured average migrational speed increased to 11.9 ± 8.0 $\mu\text{m}/\text{min}$, and the migrational directionality changed to 0.84 ± 0.14 .

Physical models of chemotaxis predict that cellular chemotactic motion is driven by relative variations in the spatial chemokine distribution, rather than by the absolute values of the chemokine concentration.³⁹⁻⁴¹ Such relative variations are mathematically represented by the spatial derivative of the chemokine concentration profile. As a consequence, the driving force for cellular chemotaxis is expected to be strongest where the spatial derivative of a chemokine profile is maximum. Consistently, we observed the largest cell displacement toward Chamber 4 (Figure 1E) where the spatial derivative of the established chemokine gradient was maximal according to our computations (Figure 1C).

To assess potential biases of our microfluidic assay, we tested the chemotactic migration of WT mouse neutrophils toward 100 ng/mL CXCL2 gradients that were symmetrically mirrored to each other. In contrast to the right-skewed migration of neutrophils observed for the regular direction gradient (Figure 2A), the migration was symmetrically left-skewed when the direction of the CXCL2 gradient was reversed (Figure 2B). With respect to the positive parameters of the control experiments (8.4 ± 6.6 $\mu\text{m}/\text{min}$ (v_m), and 0.66 ± 0.11 (ICD)), in the presence of a reversed gradient, we observed a negative average migrational speed (-6.3 ± 4.1 $\mu\text{m}/\text{min}$) and migrational directionality (-0.52 ± 0.12).

To further validate that neutrophil chemotaxis observed in our microfluidic setup was driven exclusively by chemokine-mediated migration, we took advantage of neutrophils lacking CXCR2 (*Cxcr2*^{-/-}), the receptor of CXCL2. The migration of *Cxcr2*^{-/-} neutrophils in a 100 ng/mL CXCL2 gradient of regular direction exhibited a symmetric Gaussian-like distribution (Figure 2C), similar to the one observed for C57BL/6 and BALB/c WT mouse neutrophils in the absence of a CXCL2 gradient (Figure 1D; Figure S1, Table S1). A similar behavior was observed for the migration of C57BL/6 WT neutrophils pre-incubated with an anti-CXCR2 mAb. Thus, while pre-incubation with an isotype-matched mAb did not impair the cells' migrational ability (Figure S2A), the anti-CXCR2 mAb effectively suppressed the neutrophils' chemotaxis toward a regular 100 ng/mL CXCL2 gradient (Figure S2B, Table S1).

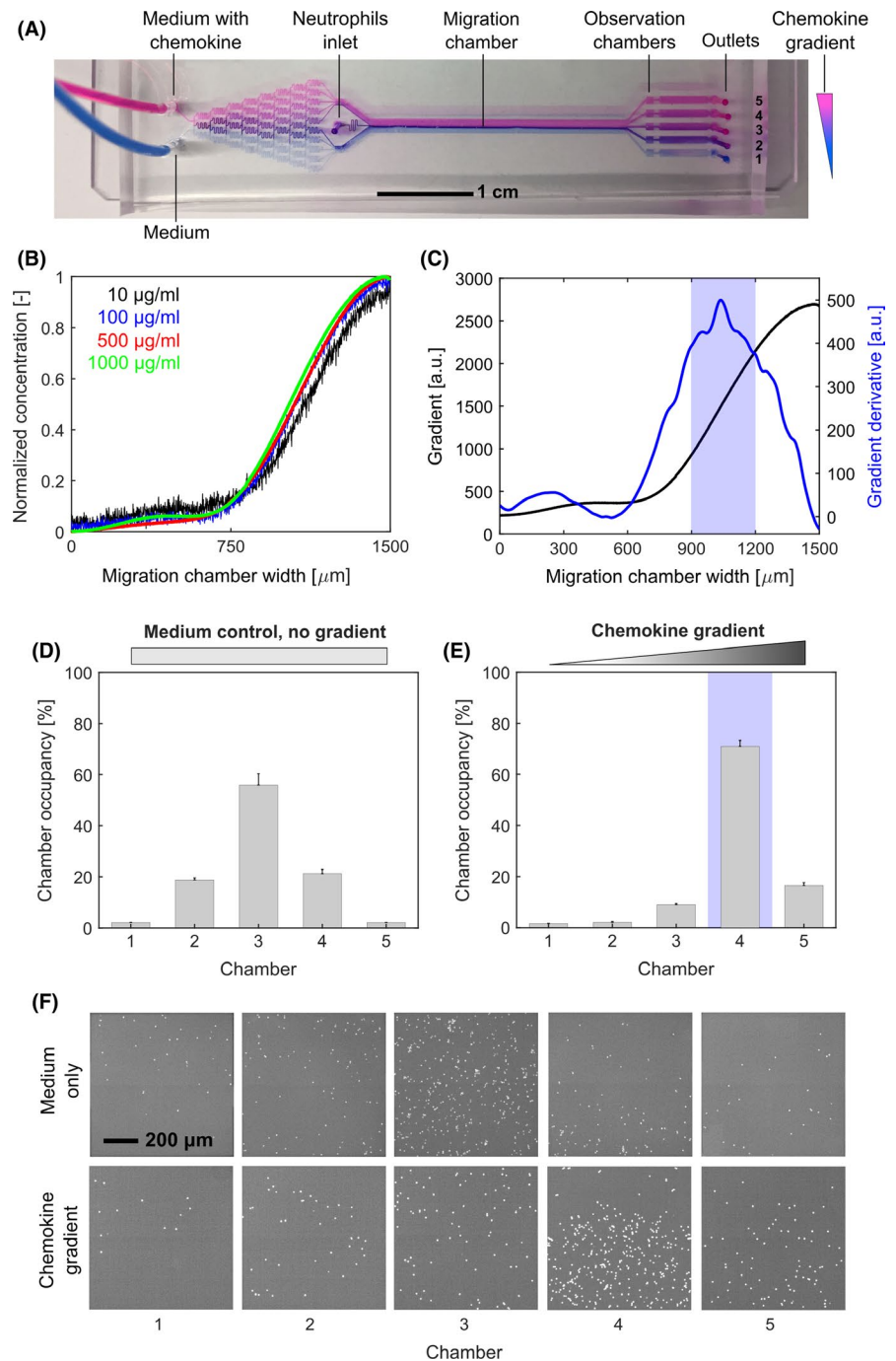
Overall, these results indicate that our microfluidic assay represents a reliable and robust platform for analyzing neutrophil chemotaxis under well-defined experimental conditions. Moreover, the observed chemotactic migrations are driven by chemokine-receptor interactions and by the spatial derivative of the established chemokine gradients.

3.2 | Effects of CXCL2 concentration on mouse neutrophil migration

Our microfluidic setup allows the accurate control of the generated chemokine gradients, robust reproducibility, and precise quantification of neutrophil chemotactic migration. These features open the possibility to study in detail chemokine dose-dependent effects in chemotaxis.

In the presence of a 1 ng/mL CXCL2 gradient, we did not observe any differences in the observation chambers compared to medium alone (Figure 3A,B, Table S1). Increasing the CXCL2 concentration to 10 ng/mL reduced the cell occupancy of Chamber 2 and increased the occupancies of Chambers 4 and 5 (Figure 3C, Table S1): These results were associated with an estimated net positive average chemotactic speed of 7.1 ± 4.6 $\mu\text{m}/\text{min}$ and migrational directionality of 0.49 ± 0.08 (Figure 3C). The migration became even more pronounced with a 100 ng/mL CXCL2 gradient (Figure 3D). The reduced cell occupancy observed for Chamber 2 and the increase in cell occupancies observed for Chambers 4 and 5 (Figure 3D, Table S1) were reflected by the higher positive migrational parameters of 9.4 ± 6.0 $\mu\text{m}/\text{min}$

FIGURE 1 Characterization of the microfluidic device. (A) Picture of the microfluidic chemotactic device. The channels are filled with Rhodamine B and Coomassie Blue solutions for visualization. (B) Experimentally measured gradients of fluorescein (FITC)-labeled dextrans at different concentration: 10 $\mu\text{g/mL}$ (black), 100 $\mu\text{g/mL}$ (blue), 500 $\mu\text{g/mL}$ (red), and 1000 $\mu\text{g/mL}$ (green). (C) Representative gradient generated within the microfluidic channel (black) and associated spatial derivative (blue). The region of the gradient derivative's maximum is shown as light-blue-shaded area. (D-E) Distribution of mouse neutrophils in the observation chambers in the absence (D) and presence (E) of a gradient with 100 ng/mL CXCL2. The region of the gradient derivative's maximum is shown as light-blue-shaded area. (F) Neutrophils were stained with a FITC-conjugated anti-Ly6G monoclonal antibody and monitored online by epifluorescence microscopy, as shown by the representative images of medium (top) and CXCL2 chemokine gradient (bottom). Results of one representative experiment are shown as mean and standard error of the mean (SEM)



(v_m) and 0.63 ± 0.24 (ICD). In the 250 ng/mL and 1000 ng/mL CXCL2 gradients (Figure 3E,F, Table S1), the cell distribution became more similar to that of medium (Figure 3A), as confirmed by calculation of the parameters v_m and ICD (Table S1). In a side-by-side comparison, it became clear that 100 ng/mL of CXCL2 elicited the strongest chemotaxis among the tested chemokine concentrations (Figure 3G), and the additional cells that migrated to Chamber 4 came primarily from reduced migration to Chambers 2 and 3 (Figure 3H).

To critically assess the performance of our microfluidic setup compared to traditional transwell assays, we performed side-by-side dose titration experiments using our microfluidic device and a standard transwell setup. Measurements of neutrophil chemotaxis

performed in traditional transwell migration assays (Figure 3I) supported our findings, although the variations in the transwell migration assays were larger than those of our microfluidic assays. In particular, both setups could identify the optimal chemokine concentration (100 ng/mL CXCL2, Figure 3G,I) able to elicit the strongest chemoattraction on mouse neutrophils.

3.3 | Migration of human neutrophils toward CXCL8

We next investigated the effects of different CXCL8 concentrations on the chemotaxis of human neutrophils from healthy

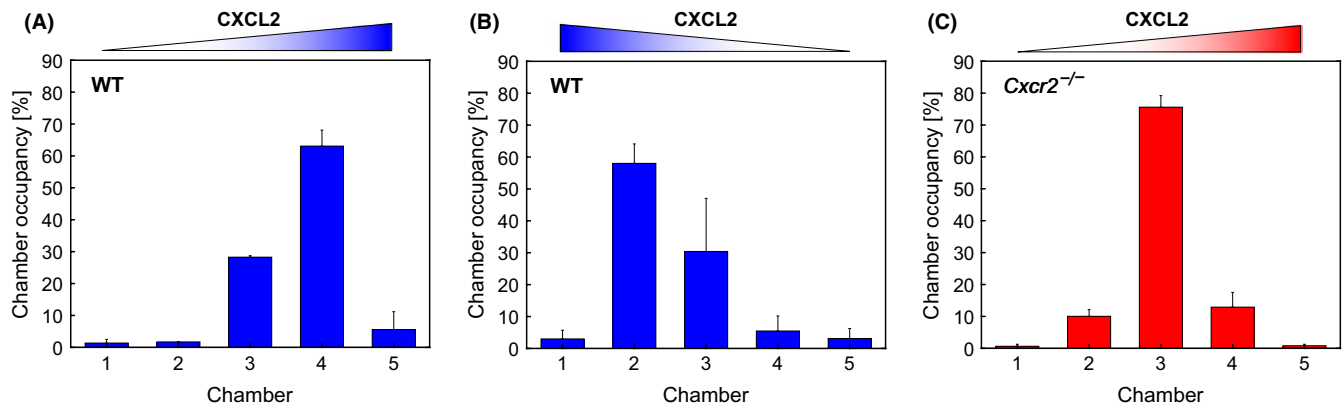


FIGURE 2 Specificity of chemotactic migration of neutrophils. (A) Migration of wild-type (WT) mouse neutrophils in the presence of a gradient with 100 ng/mL CXCL2, with the highest absolute chemokine concentration applied at the side of Chamber 5. (B) Migration assay in the presence of an inverted 100 ng/mL CXCL2 gradient, in which the highest absolute chemokine concentration was applied at the side of chamber 1. (C) Migration of *Cxcr2*^{-/-} neutrophils in the presence of a gradient with 100 ng/mL CXCL2, with the highest absolute concentration at the side of chamber 5. Data are shown as mean and SEM of at least 3 independent replicates

donors, following the same strategy as for mouse neutrophils. The chemokine CXCL8 is a ligand for the human CXCR2 and thus analogous to mouse CXCL2.

For human neutrophils, also the chemokine concentration of 100 ng/mL resulted in the most right-skewed cell migration distribution (Figure 4). Lower and higher tested chemokine concentrations were not as potent (Figure 4A-F, Table S1), in analogy to what we observed for mouse neutrophils. The migration parameters obtained using 100 ng/mL CXCL8 ($6.4 \pm 5.4 \mu\text{m}/\text{min}$ (v_m) and 0.51 ± 0.23 (ICD)) were congruent with the observed reduction in cell counts in Chamber 2 and the corresponding increase in Chamber 4 (Table S1). Moreover, for the migration of neutrophils in a 100 ng/mL chemokine gradient, the changes in chamber occupancy were remarkably similar: For both mouse and human neutrophils, we observed a net reduction in Chambers 1 to 3 and a corresponding net increase in Chambers 4 and 5 (Figures 3H and 4G, Table S1).

Finally, the dose-response obtained using our microfluidic device was again consistent with the data from traditional transwell migration assay measurements (Figure 4H).

3.4 | Detailed quantification of IL-4-mediated inhibition of mouse and human neutrophil chemotaxis

The microfluidic setup presented here can be conveniently used also to thoroughly characterize the modulatory effects of specific proteins or small molecules on neutrophil chemotaxis. To test this feature of our microfluidic setup, we characterized the recently described inhibitory effect of IL-4 on neutrophil migration.

For both mouse and human neutrophils prestimulated with medium only prior to injection in the microfluidic device, the resulting migrational distributions exhibited the expected right skewness toward regular gradients of chemokines (Figure 5A,B) and positive parameters of migration toward 100 ng/mL chemokine (Table S1). In contrast, neutrophils that had been prestimulated with IL-4 prior

to injection in the microfluidic device and exposure to chemokines failed to migrate sideways, as indicated by migration distributions with a central symmetry, both for mouse and for human neutrophils (Figure 5C,D). The chamber occupancy variations of IL-4-prestimulated neutrophils (Figure 5E,F, Table S1) mirrored those observed for both mouse and human neutrophils in the absence of a chemokine gradient (Figures 3H and 4G), confirming that IL-4 potently inhibited their chemotaxis. We further confirmed that this was a direct action of IL-4 on neutrophils by performing experiments with neutrophils lacking IL-4R α (*Il4ra*^{-/-}), a crucial receptor subunit of the IL-4R. As expected, stimulation of *Il4ra*^{-/-} neutrophils with IL-4 prior to their injection in the microfluidic device did not result in any appreciable impairment of chemotaxis (Figure S1, Table S1). Furthermore, the percentage variations observed for the cell occupancies of Chamber 4 (Figure 5E,F) were in a similar range (40%–60%) as the percentage of IL-4-induced inhibition of chemotaxis as measured by transwell migration assays (50%; Figure 5G,H).

3.5 | Temporal dynamics of the inhibitory effect of IL-4 on neutrophil migration

Another advantage of our microfluidic setup is the ability to measure the dynamics of inhibition or stimulation over time. To illustrate this feature, we monitored the migrational distributions of mouse neutrophils that had been incubated with IL-4 at room temperature (RT, $24.6 \pm 0.4^\circ\text{C}$). We found that IL-4-induced inhibition was dependent on the duration of incubation (Figure 6A-C). Only after 3 hours of stimulation, the inhibitory action of IL-4 reached a maximum and plateau, as evidenced by a perfectly symmetric, Gaussian-like migrational distribution (Figure 6C), and migrational parameters close to 0 (Table S1).

To rule out the possibility that the observed progressive decrease in chemotaxis was due to cell death of the neutrophils over the incubation time, we performed control experiments. We checked neutrophils incubated at RT in medium for 4 hours and observed that their

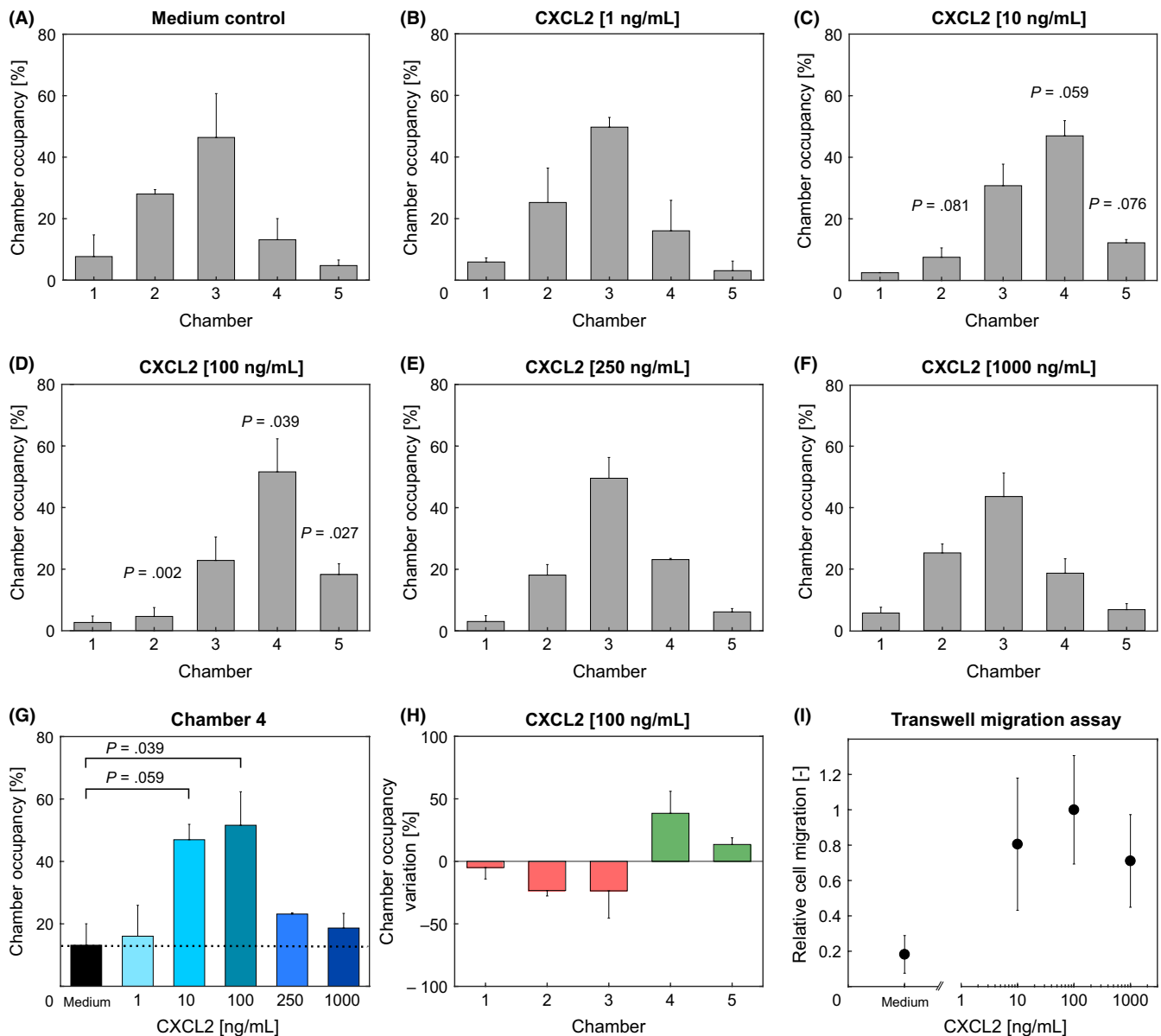


FIGURE 3 Migration of mouse neutrophils toward titrated concentrations of chemokine. (A–F) CXCL2 concentrations were 0 ng/mL (A), 1 ng/mL (B), 10 ng/mL (C), 100 ng/mL (D), 250 ng/mL (E), and 1000 ng/mL (F). Results were quantified as percentage chamber occupancy and are shown as mean and SEM of 3 or more replicates. (G) Overview of neutrophil chemotaxis observed in Chamber 4 over the complete titration range of CXCL2. (H) Percentage variations in the chamber occupancies observed at 100 ng/mL CXCL2. Variations, calculated with respect to the 0 ng/mL control migration experiments, are shown as mean and SEM of 3 or more replicates. (I) Results of CXCL2-induced chemotactic migration of mouse neutrophils over 2 h in a traditional transwell migration assay. Results, calculated with respect to the maximal migration, are shown as mean and SEM of 3 independent replicates

migratory ability remained fully intact (Figure 6D). Moreover, we also evaluated the viability of neutrophils by Annexin V and 7-AAD, both right after isolation (Figure 6E, left) and after 4 hours of incubation in either medium (Figure 6E, middle) or IL-4-containing medium (Figure 6E, right). Under all tested conditions, more than 96% of neutrophils remained negative for both Annexin V and 7-AAD, showing that the neutrophils remained perfectly viable.

We observed similar results in human neutrophils in terms of the kinetic actions of IL-4-mediated inhibition on neutrophil chemotaxis (Figure S3, Table S1).

Taken together, our microfluidic assay allowed us to show a time-dependent effect of IL-4-induced inhibition of neutrophil chemotaxis.

4 | DISCUSSION

Many of the previously described microfluidic devices measuring chemotaxis focused on the analysis of single-cell chemotactic migration and relied on manual tracking of the trajectories of

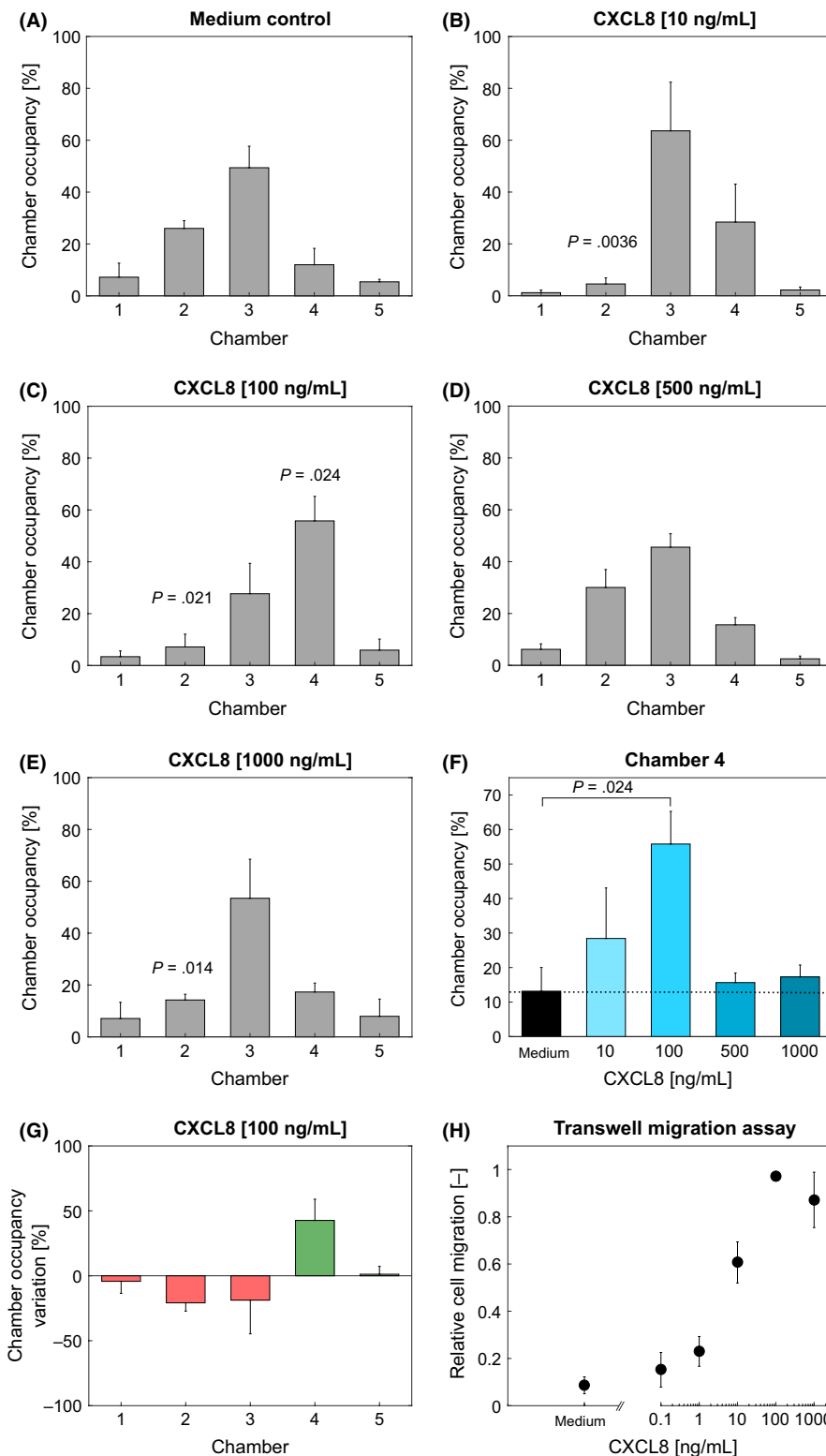


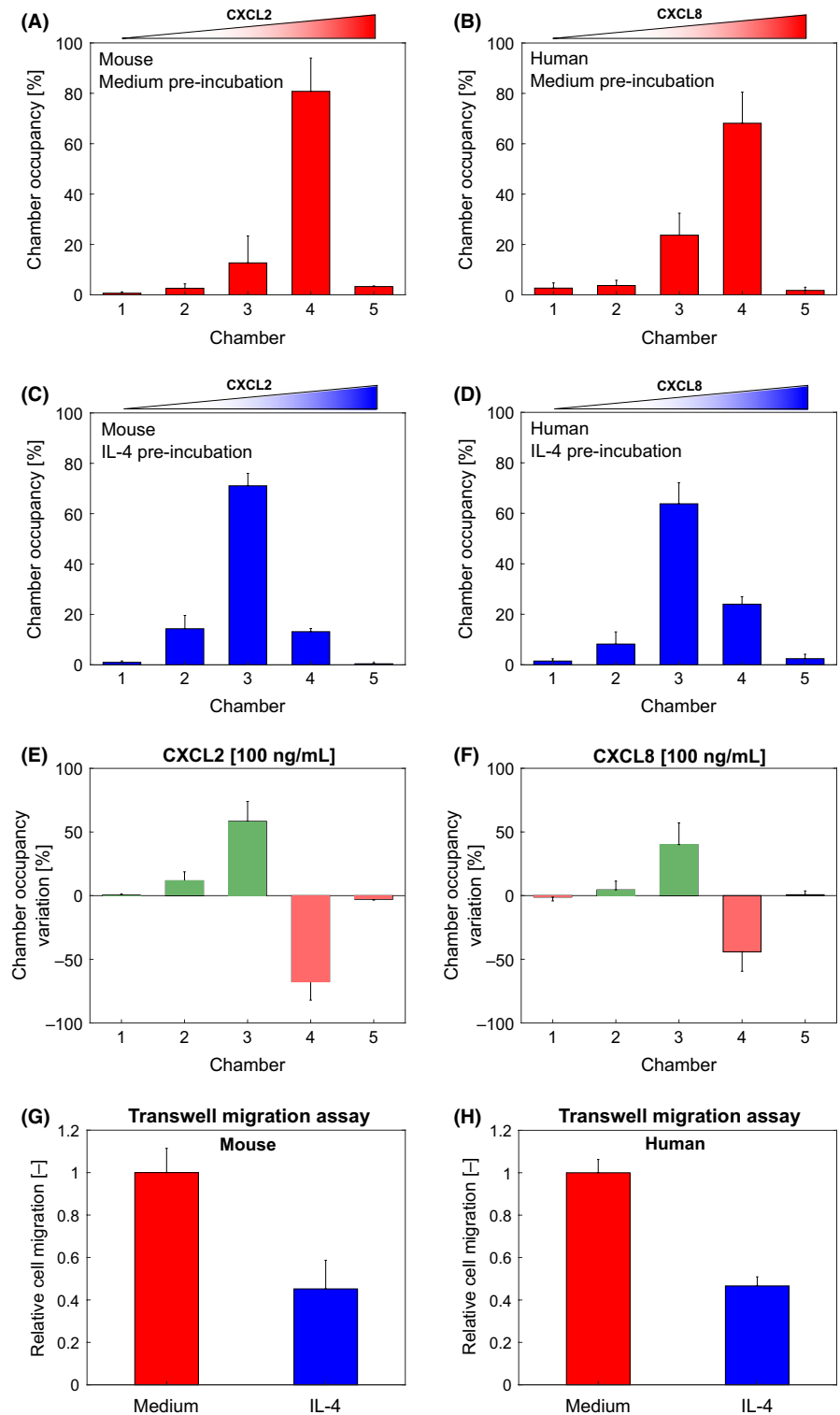
FIGURE 4 Migration of human neutrophils toward titrated concentrations of chemokine. (A-E) CXCL8 concentrations were 0 ng/mL (A), 10 ng/mL (B), 100 ng/mL (C), 500 ng/mL (D), and 1000 ng/mL (E). Results were quantified as percentage chamber occupancy and are shown as mean and SEM of 3 or more replicates. (F) Overview of neutrophil chemotaxis observed in Chamber 4 over the complete titration range of CXCL8. (G) Percentage variations in the chamber occupancies observed at 100 ng/mL CXCL8. Variations, calculated with respect to the 0 ng/mL control migration experiments, are shown as mean and SEM of 3 or more replicates. (H) Results of CXCL8-induced chemotactic migration of human neutrophils over 2 h in a traditional transwell migration assay. Results, calculated with respect to the maximal migration, are shown as mean and SEM of 5 independent replicates

10^0 - 10^2 selected cells.^{28,31,32,40,42,43} In contrast, the device presented here allows a fully automated and trajectory-free analysis of the chemotaxis of populations of $\geq 10^3$ cells, which self-sort in the observation chambers of the device according to the undergone chemotactic migration. This analysis provides information averaged over the entire population, rules out potential cell-to-cell variations within the same population,⁴⁴ and minimizes the

influence of abnormally behaving cells on the estimated biophysical parameters of migration.

The extensive validation of our microfluidic setup and analysis protocol confirmed that the observed neutrophil migration patterns were exclusively determined by chemokine-chemokine receptor interactions. Consistent with the fundamental physics of chemotaxis⁸ and with previous findings in the literature,³⁹⁻⁴¹ we showed that

FIGURE 5 Quantification of IL-4-mediated inhibition of neutrophil chemotaxis. (A-D) Migration of neutrophils pre-incubated in medium without (A-B) or with IL-4 (C-D) toward 100 ng/mL CXCL2 (for mouse; A, C) or 100 ng/mL CXCL8 (for human; B, D). (E-F) Percentage variations in the chamber occupancies observed at 100 ng/mL CXCL2 (E) and CXCL8 (F). Variations, calculated with respect to the medium-prestimulation migration experiments, are shown as mean and SEM of 3 or more replicates. (G-H) Migration of mouse and human neutrophils pre-incubated with medium without or with IL-4 in a transwell migration assay. Results, calculated with respect to the maximal migration, are shown as mean and SEM of 3 or more replicates



chemotaxis of both mouse and human neutrophils was driven by relative variations in the spatial chemokine concentrations rather than by absolute concentration differences.

We found that both mouse and human neutrophils exhibited the strongest chemotactic migration in gradients of 100 ng/mL CXCL2 and CXCL8, respectively, in agreement with our recent data obtained from traditional transwell assays.^{9,11} Our findings demonstrated that neutrophil migration toward titrated CXCR2 ligand gradients reached

a maximum for a specific threshold chemokine concentration as indicated by bell-shaped dose-response curves. Although not further investigated here, this type of response has been ascribed to chemokine receptor desensitization and internalization, which can decrease the cellular response to the ligands.⁴⁵⁻⁴⁸ This finding was also in line with previous reports based on transwell migration assays.^{49,50}

Although the results obtained with our microfluidic device were often in agreement with those of traditional transwell

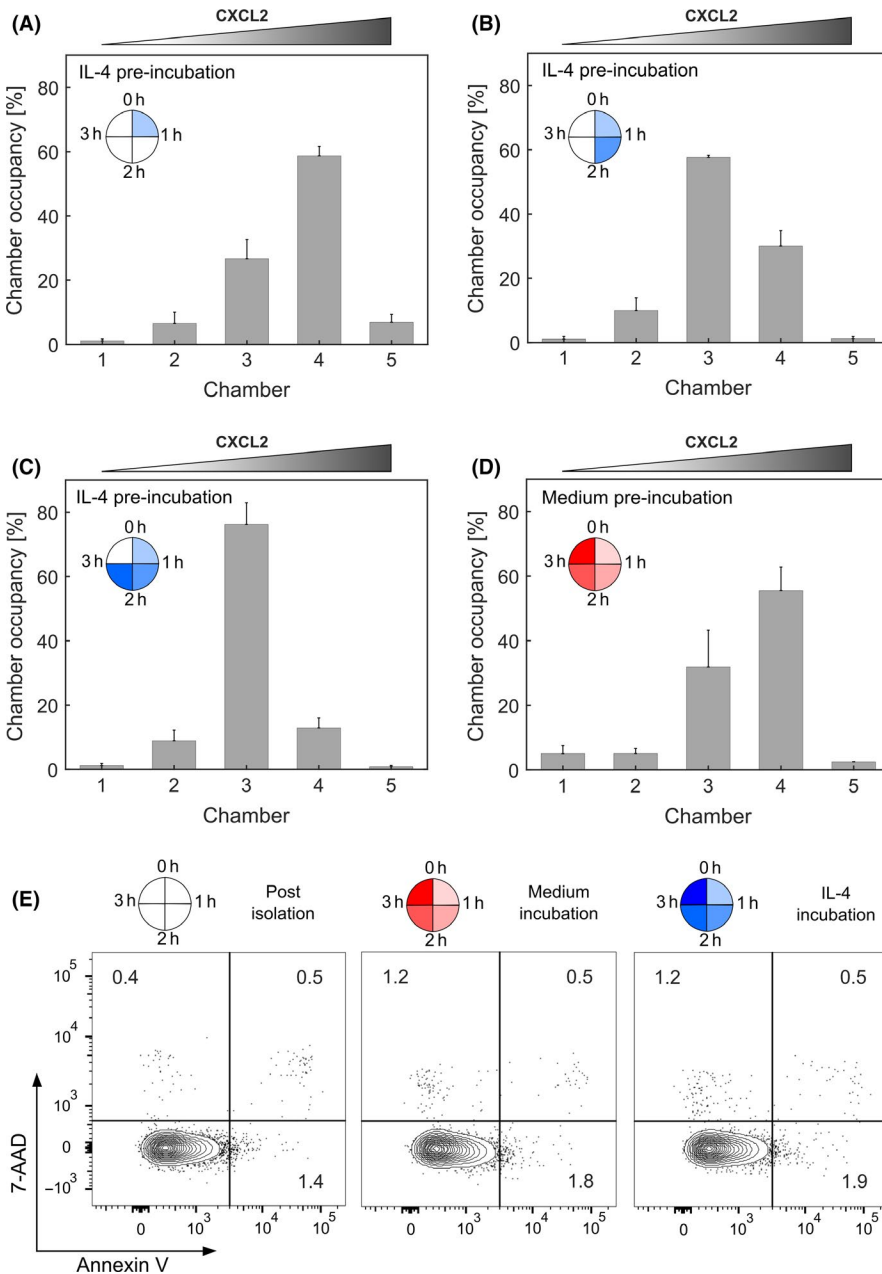


FIGURE 6 Kinetics of IL-4-mediated inhibition of neutrophil chemotaxis. (A-D) Mouse neutrophils were pre-incubated at room temperature with medium containing IL-4 (100 ng/mL) for 1 h (A), 2 h (B), and 3 h (C), or medium alone for 4 h (D), prior to assessment of chemotaxis in a 100 ng/mL CXCL2 gradient. Results were quantified as percentage chamber occupancy and are shown as mean and SEM of 3 independent replicates. (E) Flow cytometric analysis of isolated CD3⁻ CD11b⁺ Ly6G⁺ neutrophils right after isolation (left) as well as after 4 h of incubation in medium (middle) or medium containing 100 ng/mL IL-4 (right)

migration assays, it is important to highlight the significant differences between these setups. Firstly, in transwell assays, an exact characterization of the established chemokine gradient is difficult to achieve, and the spatio-temporal control of the gradient is either severely limited or impossible.²³ This is exemplified by migration in the presence of the highest chemokine concentration: The transwell assay suggested that 1000 ng/mL led to migration that was only modestly different from the optimal concentration of 100 ng/mL, while with our microfluidic setup we were able to appreciate that 1000 ng/mL actually did not induce any chemotactic response. This apparent disagreement can be explained by differences in the shape of the chemokine gradient established in the two setups. In the microfluidic device, a well-defined, temporally stable gradient is formed under controlled conditions (Figure S4). Conversely, during transwell migration, formation of

the gradient is initiated as soon as the insert containing medium and cells get into contact with the chemokine-containing medium in the lower chamber. Thereafter, the gradient gradually flattens and dynamically reduces the difference in the chemokine concentration between the upper and lower compartments. This dynamic gradient causes an initial potent migration that diminishes over time, as previously demonstrated.⁹ In contrast, the stable gradient in our microfluidic assay induces constant chemotaxis throughout an experiment (Figure S5). Secondly, both the operative and data analysis steps in the transwell assay involve a high degree of manual work. By contrast, our microfluidic setup offers tightly controlled and reproducible experimental conditions, in combination with a requirement of much smaller reagent volumes (10¹-10² µL) and a high level of automation throughout all stages of the assay. Thirdly, in standard transwell assays, the established chemokine

gradient is considerably affected by user-dependent motion. This significantly increases inter-experimental variation.

The simplicity and accessible level of expertise required to establish and operate the device discussed here make our setup an efficient and valuable tool that could be conveniently transferred to every biomedical and biological laboratory in future. The realization and functionalization of the device described in the present work involve cheap, nontoxic materials, and basic laboratory equipments. The preparation and execution of the migration assay can take place over the time span of few hours, and operators do not require special training other than that needed for standard laboratory practices.

The advantages of microfluidics over more traditional migration assays also become evident when studying the dynamic aspects of chemotaxis and its modulation. Whereas transwell assays provide only the total number of cells that migrated over the whole time course of an experiment, our microfluidic platform and experimental protocol allowed us to investigate both the equilibrium and real-time dynamic inhibitory effects of IL-4 on the migration of mouse and human neutrophils.

Modulation of neutrophil chemotaxis by means of specific molecules is emerging as an attractive therapeutic strategy of clinical relevance. Since neutrophils play a crucial role in the first lines of defense against invading pathogens, inadequate neutrophil recruitment can result in pathology.⁵¹ For instance, accumulation and over-abundance of neutrophils can cause damage to the surrounding healthy tissues due to their potent effector functions.^{52,53} Conversely, in allergic disease settings such as atopic dermatitis where neutrophils are usually absent in the skin, patients suffer from frequent bacterial infections.^{54,55} The efficient and reliable identification of therapeutic molecules capable of modulating neutrophil chemotaxis requires an accurate and unambiguous quantitative and qualitative characterization of their effects on cellular migration. The microfluidic setup and analysis protocol outlined here represents an accessible and precise tool to address these needs.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

FG and CE designed, performed, and analyzed experiments and wrote the manuscript. DI designed, performed, and analyzed experiments involving human samples. PA and OB designed and analyzed experiments, supervised the study, and wrote the manuscript, with input from all authors. FG and CE are equally contributing first authors. PA and OB are equally contributing senior and corresponding authors.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Figure S1. Comparison of migration of neutrophils from wild type (WT) and *Il4ra*^{J/J} mice (both on a BALB/c background). Migration of BALB/c WT (left) and *Il4ra*^{J/J} (right) mouse neutrophils pre-incubated in medium in the absence (A) and presence (B) of a 100 ng/ml CXCL2 gradient. (C) Migration of BALB/c WT (left) and *Il4ra*^{J/J} (right) mouse neutrophils pre-incubated in medium containing IL-4 toward a 100 ng/ml CXCL2 gradient. Results were quantified as percentage chamber occupancy, and are shown as mean and SEM of 3 independent replicates.

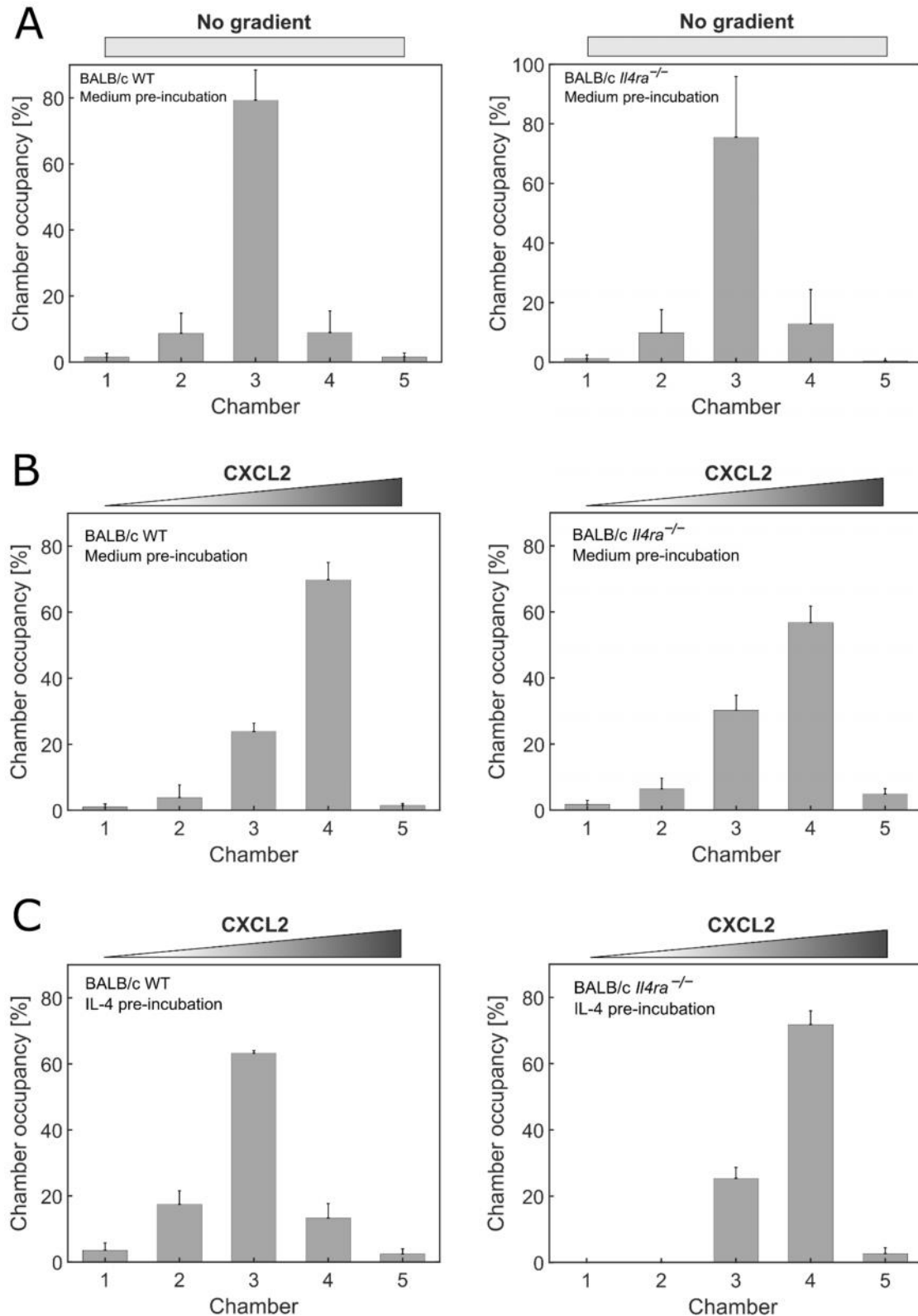


Figure S2. Blocking of CXCR2 on neutrophils migrating toward CXCL2. Migration toward a 100 ng/ml CXCL2 gradient of mouse neutrophils pre-incubated with an isotype-matched antibody (A) or with an anti-CXCR2 monoclonal antibody (clone 242216) (B). Results were quantified as percentage chamber occupancy, and are shown as mean and SEM of 3 independent replicates.

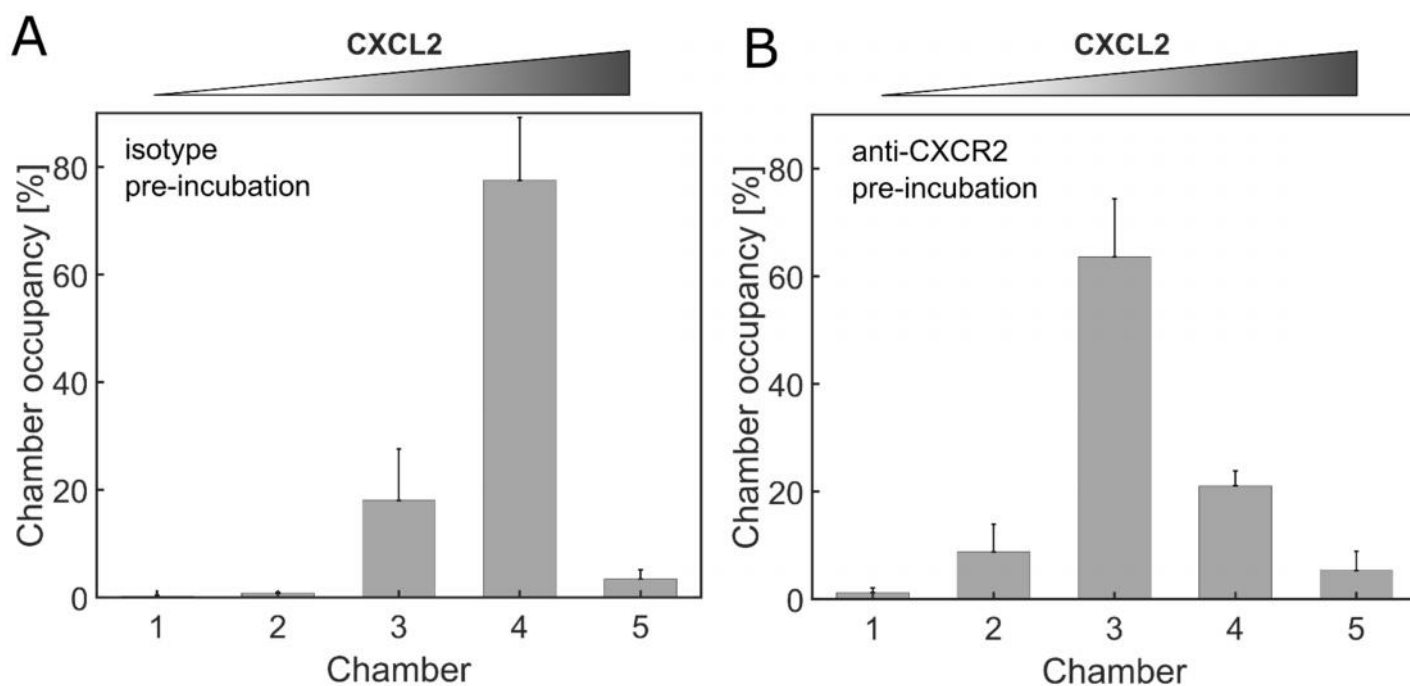


Figure S3. Kinetics of IL-4-mediated inhibition of human neutrophil chemotaxis. (A–D) Human neutrophils were pre-incubated at room temperature with medium containing IL-4 (150 ng/ml) for 1 h (A), 2 h (B), and 3 h (C), or medium alone for 4 h (D), prior to assessment of chemotaxis in a 100 ng/ml CXCL8 gradient. Results were quantified as percentage chamber occupancy and are shown as mean and SEM of 3 independent replicates. **(E)** Flow cytometric analysis of isolated human neutrophils right after isolation (left) as well as after 4 h of incubation in medium (middle) or medium containing 150 ng/ml IL-4 (right).

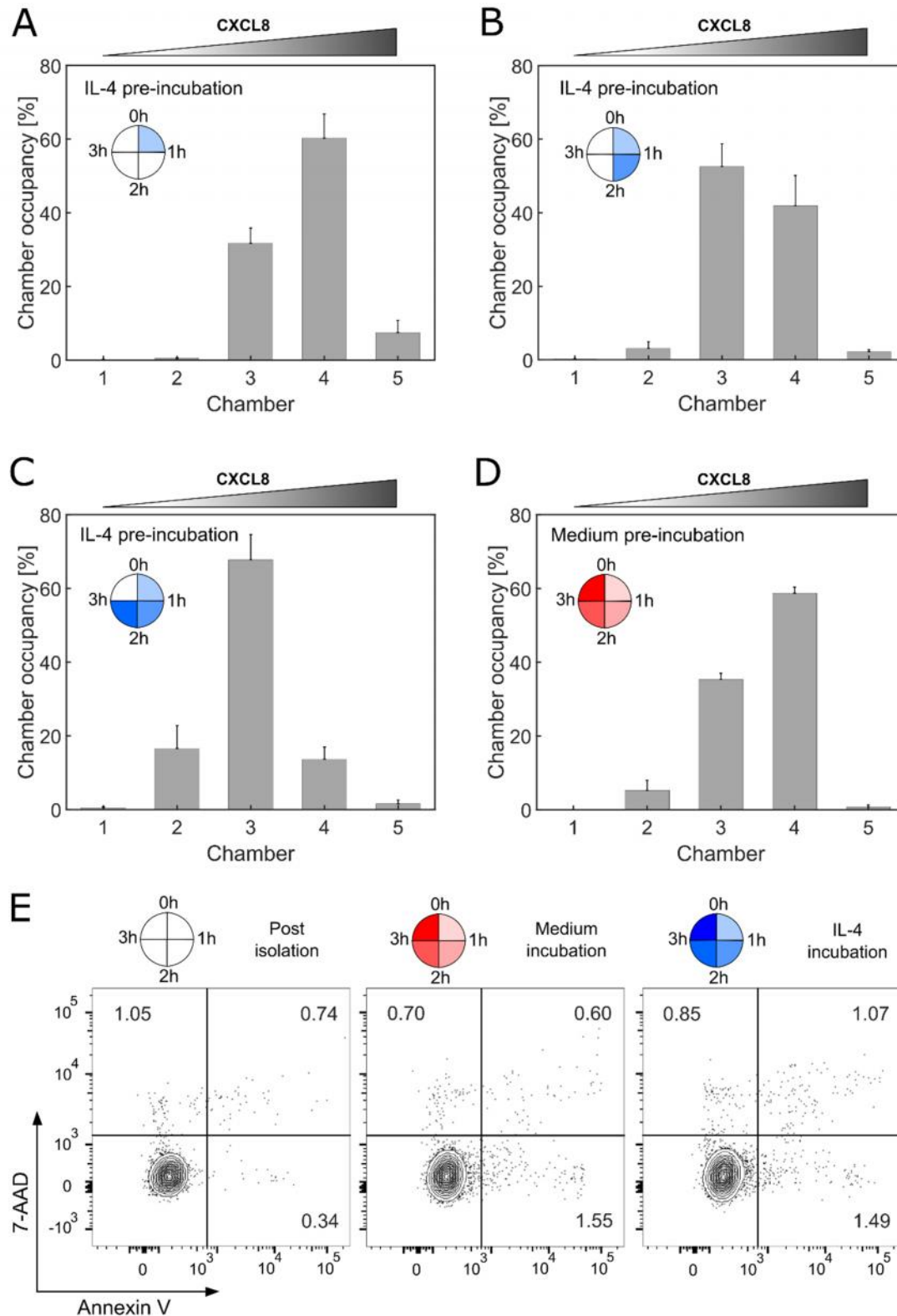


Figure S4. Characterization of shape and spatio-temporal stability of gradients in microfluidic assay. Experimental fluorescence profiles of gradients generated with FITC-labelled 10-kDa dextrans of concentrations varying from 10 $\mu\text{g/ml}$ (left) to 1000 $\mu\text{g/ml}$ (right). The profiles were acquired at the beginning (top), in the middle (middle), and at the end (bottom) of the main migration chamber, right after establishment of the gradient (black solid lines) or after 30 and 60 min (gray and light gray solid lines, respectively). The gradients were recorded via fluorescence detection on an epi-fluorescence microscope, and the distributions of fluorescence intensity were extracted from the acquired images via ImageJ (Fiji). The FITC-dextrans concentration ranged from 10 $\mu\text{g/ml}$ to 1000 $\mu\text{g/ml}$ in order to span the same difference in orders of magnitude of the chemokine concentrations tested in the migration assays. Irrespective of the different absolute values for the pixel fluorescence intensity/dextran concentration, all the generated gradients overlapped once normalized (Fig. 1B, Main Text). This proved the independence of the shape and steepness of the generated gradients on the particular tracer/chemokine concentration. In addition to the linear gradient itself, its spatial derivative was also characterized by performing numerical derivation of the experimentally acquired fluorescence profiles (see Fig. 1C). Shown are results of one representative experiment out of 3 independent replicates.

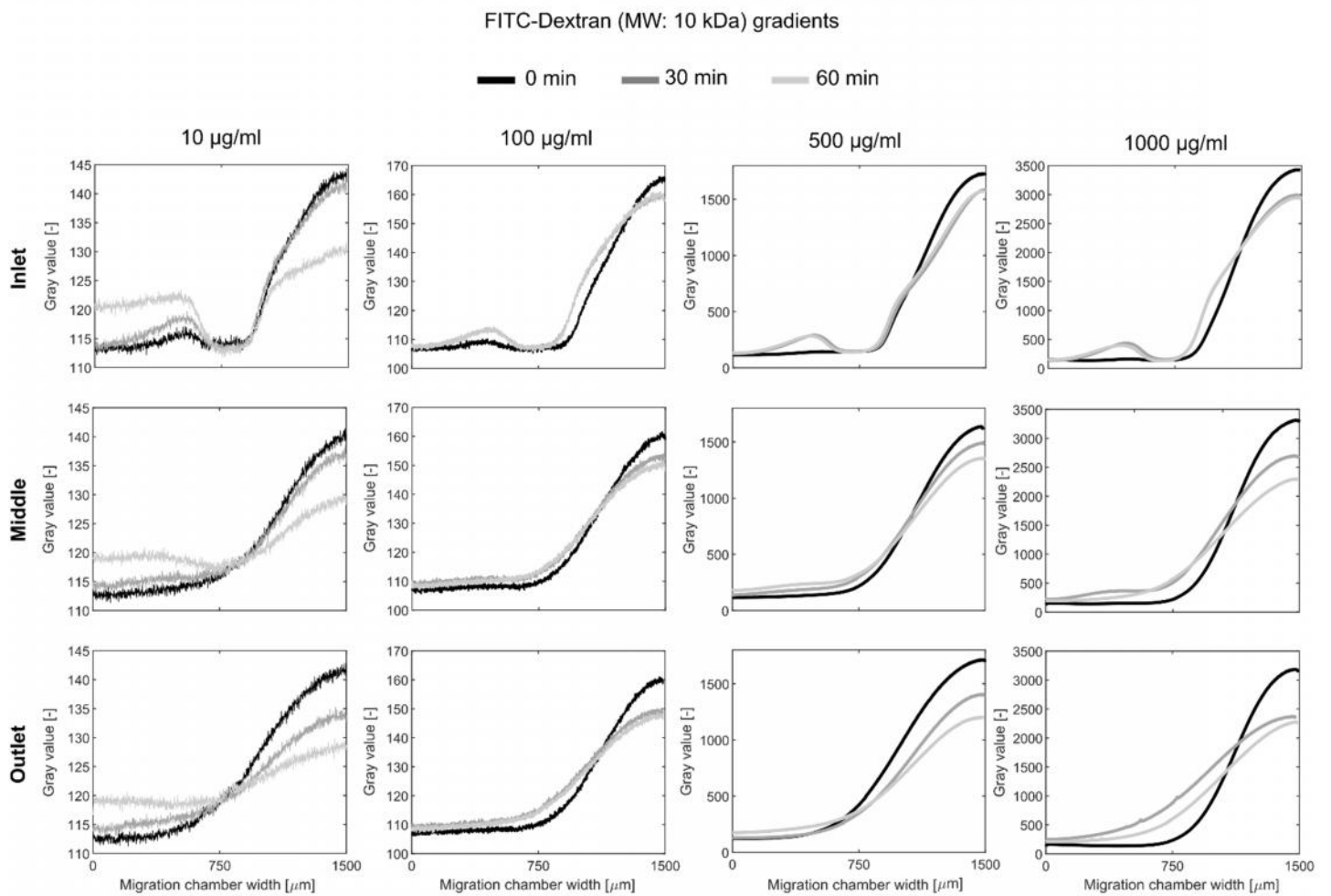
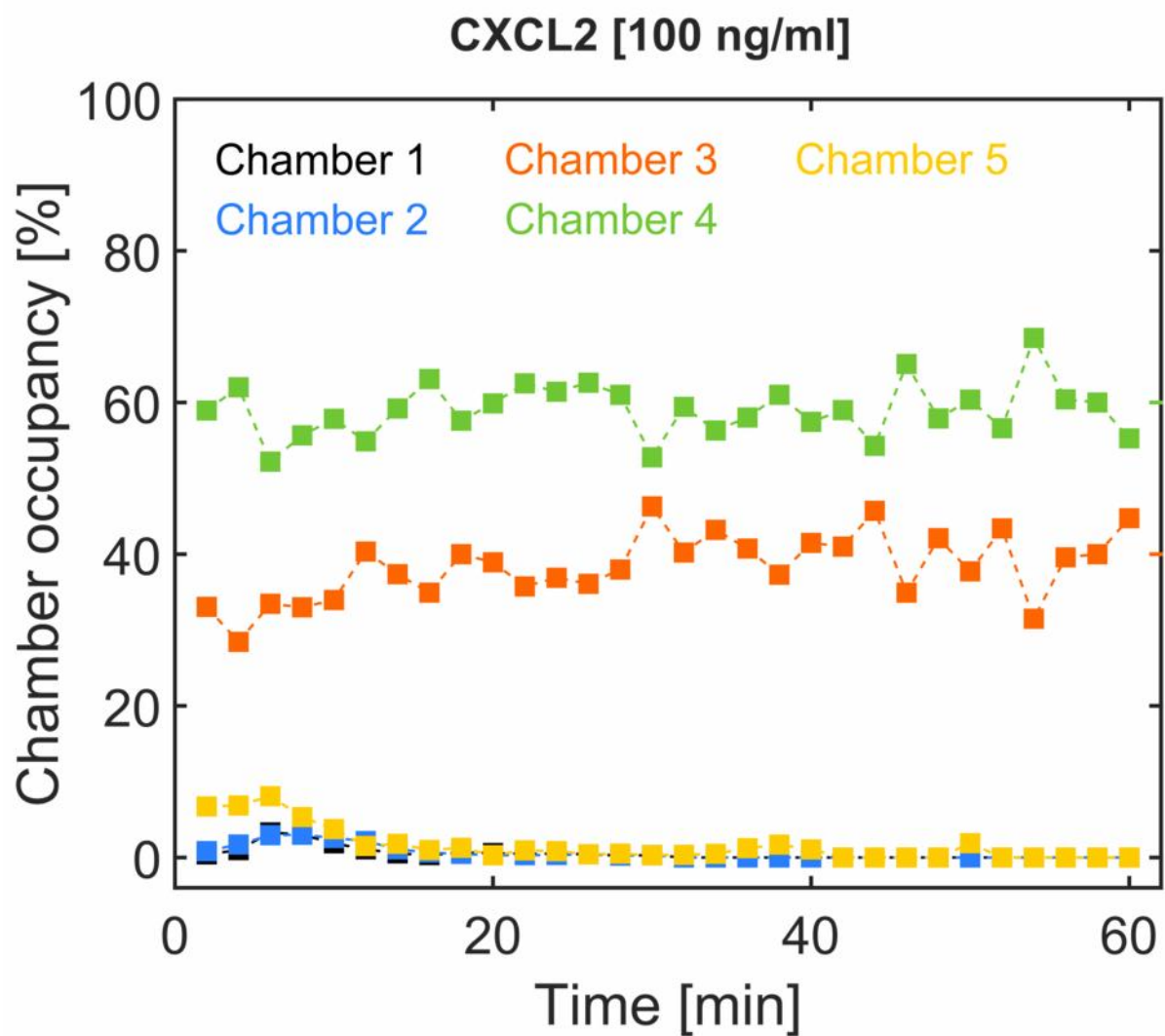


Figure S5. Migration of mouse neutrophils in a 100 ng/ml CXCL2 gradient over time. Percentages of chamber occupancy for the individual observation chambers are plotted over time, including for Chamber 1 (black), Chamber 2 (blue), Chamber 3 (orange), Chamber 4 (green), and Chamber 5 (yellow). Shown are results of one representative experiment.



Data S1: Supplementary methods

Device design and fabrication

The device consisted of a gradient-generating serpentine network (overall length: 12.75 mm, overall width: 9.6 mm, width of individual serpentine channels: 100 μm , height: 50 μm) and of a main migration chamber of width of 1500 μm (length: 2.7 cm, height: 50 μm). The main migration chamber branched at its end into five outlet channels with each a width of 300 μm , with a symmetrically layout with respect to the device's longitudinal axis (Fig. 1A), and each chamber led to a square observation chamber (side: 800 μm).

The geometry of the microfluidic device was designed using the AutoCAD software (Autodesk Inc.) and printed on a photolithographic mask (Micro Litho), which was applied in combination with UV radiation to pattern a layer of negative photoresist (SU8, Microchem) and which was previously spin-coated on a 3-inch silicon wafer (MicroChemicals GmbH). The subsequent processing steps were performed according to the specifications of the photoresist manufacturer.

The silicone elastomer Sylgard 184 (Dow Corning) was then poured on the patterned wafer. After degassing and thermal curing, the resulting cured poly(dimethylsiloxane) (PDMS) sheet was peeled off the silicon wafer. Inlets and outlets were created using a 0.75 mm-gauge puncher (WellTech® Rapid-Core, WPI Inc.). The microfluidic channels were generated by bonding the PDMS patterned matrix on a standard microscopy glass slide (Fisher Scientific) by means of plasma bonding (Zepto plasma cleaner, Diener Electronics).

After the bonding step, the device was functionalized with a 1% w/w solution of poly(vinyl-alcohol) (PVA) in MilliQ water, as described previously.¹ This hydrophilic functionalization step minimized the formation of air bubbles and interactions between the cells and walls of the device.

Gradient generation and characterization

The shape and spatio-temporal characterization of the gradients generated inside the migration channel were assessed using a solution of FITC-labelled dextrans with a molecular weight of 10 kDa (Sigma-Aldrich), which is comparable to the molecular weights of CXCL2 (7.6 kDa) and CXCL8 (11 kDa). The gradient was generated by injecting the solution of dextrans and milliQ water at the two inlets of the gradient-generating region of the device.

The gradients were recorded via fluorescence detection on an epi-fluorescence microscope Ti2-U (Nikon) equipped with a LED light source (Omicron Laserage Laserprodukte GmbH) and a camera (Zyla sCMOS 4.2P-CL10, Andor). The distributions of fluorescence intensity were extracted from the acquired images via ImageJ (Fiji) (Fig. 1B, 1C, Fig. S1).

Chemotactic migration in microfluidic device

The flows in the setup were controlled using external syringe pumps (Cetoni neMESYS, Cetoni, GmbH) and Hamilton quartz gas-tight syringes. Specifically, we used a 500 μl syringe

HA-81020 (Model 1750 TLL SYR) for the medium and a 100 μ l syringe HA-81020 (Model 1710 TLL SYR) for the solutions of chemokines and neutrophils. The syringes and the device inlets were connected by means of polytetrafluoroethylene (PTFE) microtubing (Adtech Polymer EngineeringTM, Fisher Scientific). All experiments were carried out at room temperature.

The microfluidic chemotactic device was gently filled with the gradient-generating solutions at 1 ml/h before injection of the neutrophils, paying special attention to avoid the formation of air bubbles within the device. After the establishment of a stable linear gradient, the neutrophil suspension was loaded into the device at 4 μ l/h. The overall flow rate was selected to ensure an adequate residence time of the neutrophils (of approximately 25 min) to allow detectable chemotactic motion in the chemokine gradient direction, as estimated from characteristic neutrophil chemotactic speeds (10^0 - 10^1 μ m/min) reported in the literature for devices with comparable geometry.²

The chemotactic migration of neutrophils was characterized along the direction of the established chemokine gradient. The end of the main migration chamber branched into multiple separated lanes, which self-sorted neutrophils according to their chemotactic motion. Stack images of the neutrophils in each observation chamber were automatically acquired on the microscopy setup described above. The frequency of image acquisition was set to 2 minutes, and the duration of a typical migration experiment was 1 hour.

Analysis of cell migration

The observation chambers were numbered from 1 to 5, corresponding respectively to the lowest to highest absolute chemokine concentration of the established linear gradient. The central observation chamber, located along the longitudinal symmetry axis of the main migration chamber, was denoted as Chamber 3.

The acquired image stacks were processed and analyzed by a Matlab code written in house. This analysis automatically counted the number of neutrophils without the need to track single-cell trajectories over the duration of the experiments. For each experiment, the number of cells observed in a given chamber was integrated over time and normalized by the total number of counted cells to obtain the time-averaged chamber percentage occupancy. The total number of counted cells corresponded to the number of neutrophils injected into the device, calculated from the flow rate, the cell density and the experimental time, thereby confirming that no cells remained trapped within the migration chamber. Measurement errors for the time-averaged chamber percentage occupancies were quantified as standard error of the mean (SEM).

Cells occupying different observation chambers travelled different distances along the gradient direction during the same residence time. Each chamber was therefore associated with a distinct average speed of migration, calculated as the ratio between the net distance travelled by the cells and the residence time within the migration channel. To distinguish whether the cells moved downhill (negative) or uphill (positive) a chemokine gradient, we assigned a negative sign (-) to Chambers 1 and 2 and a positive sign (+) to Chambers 4 and 5,

while Chamber 3 was taken as reference value 0. Thus, we established the following rules between observation chambers and average chemotactic speeds:

Chamber 1: $v_m = (-24 \pm 6) \mu\text{m}/\text{min}$, direction of migration < 0

Chamber 2: $v_m = (-12 \pm 6) \mu\text{m}/\text{min}$, direction of migration < 0

Chamber 3: $v_m = (0 \pm 6) \mu\text{m}/\text{min}$, direction of migration $= 0$

Chamber 4: $v_m = (12 \pm 6) \mu\text{m}/\text{min}$, direction of migration > 0

Chamber 5: $v_m = (24 \pm 6) \mu\text{m}/\text{min}$, direction of migration > 0

The overall speed of migration of the injected neutrophils was calculated by weighing the characteristic migration speed of each observation chamber with the corresponding experimental percentage chamber occupancy.

Moreover, an adimensional index of chemotaxis directionality (ICD) was introduced to quantify the net direction of chemotactic migration and the skewness of the resulting distribution. The ICD was defined as the sum of the fractional occupancies of the observation chambers, each weighed by a multiplicative factor according to the position of the observation chamber with respect to the longitudinal symmetry axis of the microfluidic device (-1 for Chambers 1 and 2, 0 for Chamber 3, +1 for Chambers 3 and 4). By definition, the ICD can take any value in the $[-1, 1]$ interval. A negative (respectively, positive) ICD value denotes a left- (respectively, right-) skewed cell migration distribution, while an ICD value close to 0 corresponds to a symmetrically centered cell migration distribution.

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Table S1. List of all of experimental conditions and quantitative migration parameters associated with the migration distributions discussed in the text and shown in the figures. The overall chemotactic speed (v_m) and the index of chemotaxis directionality (ICD) values are provided as mean plus standard deviation (SD).

Neutrophils	Chemokine	Figure	v_m [$\mu\text{m}/\text{min}$]	ICD [-]	Chamber occupancy [%]				
					1	2	3	4	5
C57BL/6 WT	/	1D	0.3 ± 3.2	0.02 ± 0.10	2.0	18.9	55.9	21.2	2.0
C57BL/6 WT	100 ng/ml CXCL2	1E	11.9 ± 8.0	0.84 ± 0.14	1.5	2.0	9.0	71.0	16.5
C57BL/6 WT	100 ng/ml CXCL2	2A	8.4 ± 6.6	0.66 ± 0.11	1.3	1.7	28.3	61.3	5.6
C57BL/6 WT	100 ng/ml CXCL2	2B	-6.3 ± 4.1	-0.52 ± 0.12	3.0	58.0	30.4	5.5	3.1
BALB/c <i>Cxcr2</i> ^{3/3}	100 ng/ml CXCL2	2C	0.4 ± 1.8	0.03 ± 0.07	0.6	10.1	75.6	12.9	0.8
C57BL/6 WT	/	3A	-2.5 ± 2.6	-0.12 ± 0.21	7.6	28.0	46.4	13.2	4.8
C57BL/6 WT	1 ng/ml CXCL2	3B	-1.8 ± 3.3	-0.18 ± 0.14	5.9	25.3	49.7	16.0	3.1
C57BL/6 WT	10 ng/ml CXCL2	3C	7.1 ± 4.6	0.49 ± 0.08	2.5	7.5	30.8	47.0	12.2
C57BL/6 WT	100 ng/ml CXCL2	3D	9.4 ± 6.0	0.63 ± 0.24	2.7	4.6	22.8	51.6	18.3
C57BL/6 WT	250 ng/ml CXCL2	3E	1.4 ± 2.5	0.08 ± 0.06	3.0	18.1	49.6	23.1	6.2
C57BL/6 WT	1000 ng/ml CXCL2	3F	-0.5 ± 3.6	-0.05 ± 0.09	5.6	25.3	43.6	18.7	6.8
Human	/	4A	-2.1 ± 2.4	-0.16 ± 0.20	7.2	26.0	49.4	12.0	5.4
Human	10 ng/ml CXCL8	4B	3.1 ± 3.9	0.25 ± 0.26	1.2	4.6	63.6	28.4	2.2
Human	100 ng/ml CXCL8	4C	6.4 ± 5.4	0.51 ± 0.23	3.4	7.2	27.7	55.8	5.9
Human	500 ng/ml CXCL8	4D	-2.6 ± 2.4	-0.18 ± 0.11	6.2	30.1	45.6	15.6	2.5
Human	1000 ng/ml CXCL8	4E	0.6 ± 3.2	0.04 ± 0.17	7.1	14.2	53.5	17.3	7.9
C57BL/6 WT	100 ng/ml CXCL2	5A	10.0 ± 7.1	0.81 ± 0.19	0.7	2.6	12.6	80.8	3.3
Human	100 ng/ml CXCL8	5B	7.5 ± 6.4	0.64 ± 0.22	2.7	3.7	23.7	68.1	1.8
C57BL/6 WT	100 ng/ml CXCL2	5C	-0.28 ± 0.98	-0.01 ± 0.08	1.0	14.3	71.1	13.2	0.4
Human	100 ng/ml CXCL8	5D	2.1 ± 2.1	0.17 ± 0.10	1.5	8.2	63.8	24.0	2.5
C57BL/6 WT	100 ng/ml CXCL2	6A	7.7 ± 4.7	0.58 ± 0.05	1.1	6.6	26.6	58.7	7.0
C57BL/6 WT	100 ng/ml CXCL2	6B	2.5 ± 2.6	0.20 ± 0.06	1.1	10.0	57.6	30.1	1.2

C57BL/6 WT	100 ng/ml CXCL2	6C	0.4 ± 1.4	0.04 ± 0.05	1.2	8.9	76.2	12.9	0.8
C57BL/6 WT	100 ng/ml CXCL2	6D	5.5 ± 4.2	0.48 ± 0.08	5.0	5.1	31.9	55.5	2.5
BALB/c WT	/	S1A, left	0.04 ± 1.31	0.003 ± 0.091	1.6	8.7	79.3	8.9	1.5
BALB/c WT	100 ng/ml CXCL2	S1B, left	8.1 ± 4.6	0.67 ± 0.07	1.0	3.8	23.9	69.8	1.5
BALB/c WT	100 ng/ml CXCL2	S1C, left	-0.8 ± 2.1	-0.05 ± 0.07	3.6	17.5	63.2	13.3	2.4
BALB/c <i>Il4ra</i> ^{-/-}	/	S1A, right	0.2 ± 1.6	0.02 ± 0.14	1.2	9.9	75.5	12.9	0.5
BALB/c <i>Il4ra</i> ^{-/-}	100 ng/ml CXCL2	S1B, right	6.8 ± 4.5	0.54 ± 0.06	1.7	6.4	30.2	56.8	4.9
BALB/c <i>Il4ra</i> ^{-/-}	100 ng/ml CXCL2	S1C, right	9.2 ± 5.4	0.74 ± 0.04	0.0	0.1	25.4	71.8	2.7
C57BL/6 WT	100 ng/ml CXCL2	S2A	10.0 ± 6.7	0.80 ± 0.12	0.2	0.9	18.0	77.5	3.4
C57BL/6 WT	100 ng/ml CXCL2	S2B	2.5 ± 2.5	0.16 ± 0.07	1.2	8.8	63.6	21.1	5.3
Human	100 ng/ml CXCL8	S3A	8.9 ± 5.6	0.67 ± 0.07	0.1	0.4	31.7	60.3	7.5
Human	100 ng/ml CXCL8	S3B	5.2 ± 3.7	0.41 ± 0.08	0.2	3.1	52.6	41.9	2.2
Human	100 ng/ml CXCL8	S3C	-0.1 ± 1.8	-0.02 ± 0.07	0.5	16.6	67.7	13.6	1.6
Human	100 ng/ml CXCL8	S3D	6.6 ± 3.9	0.54 ± 0.03	0.1	5.2	35.3	58.7	0.7