Immature dendritic cells navigate microscopic mazes to find tumor cells†

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Dendritic cells (DCs) are potent antigen-presenting cells with high sentinel ability to scan their neighborhood and to initiate an adaptive immune response. Whereas chemotactic migration of mature DCs (mDCs) towards lymph nodes is relatively well documented, the migratory behavior of immature DCs (imDCs) in tumor microenvironments is still poorly understood. Here, microfluidic systems of various geometries, including mazes, are used to investigate how the physical and chemical microenvironment influences the migration pattern of imDCs. Under proper degree of confinement, the imDCs are preferentially recruited towards cancer vs. normal cells, accompanied by increased cell speed and persistence. Furthermore, a systematic screen of cytokines, reveals that Gas6 is a major chemokine responsible for the chemotactic preference. These results and the accompanying theoretical model suggest that imDC migration in complex tissue environments is tuned by a proper balance between the strength of the chemical gradients and the degree of spatial confinement.

Introduction

Dendritic cells (DCs) are surveillance agents and messengers of the immune system. When these cells migrate within an organism, their trajectories and degree of recruitment to specific organs or lymph nodes provide clues about the immunological response and the organism’s health status. 1, 2 Although the chemotactic migration of mature DCs (mDCs) towards lymphatic vessels is well studied both in vivo and in vitro, 3–5 much less is known about the migratory patterns and taxis of immature DCs (imDCs), 6 which are involved in the initial immunological reaction and in response to nearby tumors. In this context, tumor-associated imDCs are known to have contradictory effects on tumor cells (pro- or anti-tumor). 7–10 They usually do not uptake antigens from these cells, and do not undergo maturation to mediate adaptive immune response, unless subjected to inflammatory reactions. 11, 12 Still, many details of how imDCs navigate complex tumor environments – containing multiple types of chemokines secreted by tumor cells as well as other nearby immune and stromal cells – remain elusive, and the more complete understanding is important for the development of successful immunotherapies. One approach to gain more insight is via the studies of cell motility in systems of microfluidic channels. While this approach has several limitations, microchannel systems can mimic, at least to some extent, the effects of confinement the cells experience during migration through tight spaces of interstitial tissues or three-dimensional (3D) matrices. 13–15 In such studies, the dimensionality of the system is known to matter, and differences in the cellular motility in 1D microchannels, 16–19 3D cellular (or gel) environments, 20–22 or confined 2D substrates 23 from conventional 2D platforms, such as Petri dishes, are well documented.

In particular, DC movements have been previously investigated in 1D microchannel systems, wherein they exhibit highly persistent movements with regular alterations between fast and slow motility that may reflect an effective antigen search mechanism. 6, 24–26 Enhanced recruitment of mature or interferon-conditioned DCs in response to cancer cells have also been demonstrated in artificial 3D or 1D platforms. 27–29 On the other hand, migration behaviors of immature DCs in different microenvironments and under different degrees of confinement have not been studied in detail.

Here, we use microfluidic systems of geometries ranging from straight channels to complex mazes to study migratory behaviors of imDCs in response to various cues (especially the presence of cancerous vs. non-cancerous cells) and under different degrees of imposed confinement. We show that for intermediate degrees of confinement, the ability of imDCs to

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“solve” microfluidic mazes, as well as the speeds and persistence of these cells are enhanced when cancerous “bait” cells are placed at the channels’/maze’s outlet. This response scales with the concentration of the baits and can thus be ascribed to the presence of soluble gradients of chemotacticants emitted by the cancerous cells. We support this conjecture by systematic screens of cytokines that are emitted from the cancerous cells and known to affect DC motility. Of these, Gas6 shows the most pronounced effects in attracting imDCs. The structure of cellular pathways we observe experimentally agree with those predicted theoretically based on a model of an overdamped active Brownian particle. Overall, this work is unprecedented in distinguishing migration patterns of imDCs towards different neighboring normal vs. tumorigenic cells originated from the same parental cell line. These results provide new insights into cancer-immune cell interactions and stress the role of the proper “balance” between the strengths of chemical gradients mediating these interactions and the degree of imposed confinement. Extrapolating to the tissue environments, one could then expect that the geometry and/or dimensions of the cell migration environment can either reveal or conceal the complex chemotactic effects.

Experimental

Bone marrow-derived DCs (BMDCs)

BMDCs were generated based on a modified protocol from literature. Briefly, tibias and femurs from BALB/c mice (8–12 weeks of age, females) were flushed and red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer (Gibco). Bone marrow cells were plated on 24-well plates (1 × 10^6 cells per mL) in complete medium (RPMI 1640, supplemented with 5% fetal-bovine serum (FBS), 1% antibiotic–antimycotic solution, 1% HEPES buffer, 0.1% 2-mercaptoethanol, all from Gibco) containing 20 ng mL⁻¹ recombinant mouse GM-CSF (granulocyte-macrophage colony-stimulating factor) (Peprotech). The medium was replaced by complete fresh medium with GM-CSF on day 2 and 4. On day 6, non-adherent and loosely adherent cells were harvested by gentle pipetting and transferred to Petri dishes. After 1 day of culture, immature BMDCs, which appeared as floating cells, were collected. To generate mature BMDCs, immature BMDCs were stimulated overnight with 100 ng mL⁻¹ LPS (lipopolysaccharide, Sigma-Aldrich). ImDCs and mDCs were characterized for their specific surface markers (Fig. S1†). All animal experiments were conducted under protocols approved by the Institutional Animal Care and Use Committee of Ulsan National Institute of Science and Technology (UNISTIACUC-16-13).

Migration of imDC in microchannels and its analysis

As baits cells, we selected a mouse mammary epithelial cell line EpH4-Ev to represent normal cells, and β-MEKDD 116 as cancer cells, the latter generated by transfecting parental EpH4 cell line. We compared the migration patterns of imDCs towards these cell lines in different geometries of microchannels and under different degrees of confinements, using microchannels of different widths. The microchannels were incubated with 20 μg mL⁻¹ fibronectin for 1 h and washed with PBS before loading the cells. The inlet was seeded with imDCs at plating density of 4.2 × 10^3 cells per mm² and the outlet was filled with either media (control) or cell lines (EpH4-Ev or β-MEKDD 116) in the same conditioned media, at the ratio of imDCs: cell lines to be 6:1, 3:1, or 3:2. The loading density was chosen to limit the number of DCs simultaneously entering the channel to minimize the effects due to cell-cell interactions. We checked on the microscope that the cells are evenly distributed in the inlet and the outlet after loading.

The devices were mounted in a custom-built live-cell chamber with temperature of 37 °C and 5% CO₂. Six devices could be imaged using an EMCCD camera (Andor iXon 897; Till Photonics GmbH) on the motorized stage of an inverted microscope (IX81-ZDC; Olympus Corp). Time-lapse images of the cells were taken in DIC (differential interference contrast) every 1 min using a 10× objective, for the total duration of 18 h. The observation window for each device was 425 μm × 425 μm. For cell tracking, ImageJ manual tracking plug-in was utilized. Only imDCs within the 380 μm length of the microchannels were tracked from the time of entrance until they left the track. Directionality ratios over time from the cell trajectories were calculated using the program developed by Gorelik et al. More information on culture of breast cell lines, preparation of microchannels, Transwell migration assay, gene expression of imDCs, cytokine antibody array studies of the cell lines, and method of simulation analysis, is given in (ESI†).

Method of trajectory simulation as Brownian particles

We simulated imDC movement in confinement using the overdamped Brownian particle model adopted from Camley et al. In this model, the particle aligns its polarity with velocity. The dimensionless model equations for a cell’s position \( r = (x, y) \), velocity \( v = (v_x, v_y) \), and polarity \( p = (\cos \theta, \sin \theta) \) can be expressed as follows:

\[
\frac{\partial x}{\partial t} = v_x = v_s \cos \theta + \lambda \\
\frac{\partial y}{\partial t} = v_y = v_s \sin \theta
\]

\[
\frac{\partial \theta}{\partial t} = \frac{1}{\tau} \sin^{-1} \left[ \frac{v_x}{|v|} \cos \theta - \frac{v_y}{|v|} \sin \theta \right] + \zeta(t)
\]

The equations are scaled by the characteristic length \( l_c = 10 \mu m \) and time \( t_c = 1 \text{min} \). Here, \( v_s \) is the cell speed, statistically chosen from the Maxwell distribution with the average
value of the cell speed obtained from our experiments, for every relaxation time scale $\tau$. The external chemotactic force term is given by $\lambda$. The Gaussian Langevin noise $\xi(t)$ has zero mean and variance $\sigma$. The confinement effect is accounted for by a simple reflection strategy: when a cell collides with the channel wall, it is assumed that before proceeding, the cell is reflected symmetrically about the plane perpendicular to the wall.

The parameter, $\sigma$ was tuned – according to the directionality distribution from our experiments – when there is no external chemotactic force term. Large value of $\sigma$ indicates the increase in randomness. We set the value of $\sigma$ the same for $10$ and $50 \mu m$ channels, but used a different value for $5 \mu m$ channel, since the average speed of imDC distinctly increases, with its distribution shifting to higher values in the $5 \mu m$-wide channel. We also assumed the randomness of cell motility to be different with different cell lines in the outlet; this assumption was based on the experimental observation that a ballistic jump of the cell was frequently observed in the environment with $\beta$-MEKDD 116 cell lines. Then, we set the value of $\lambda$ to simulate the effect from the chemotactic force term, also to correspond with the directionality distribution from our experiments.

Results and discussion

Navigation of imDCs through a complex maze

The first system we used was a microfluidic maze with one inlet and one outlet, and all channels having $10 \times 10 \mu m$ cross-sections (Fig. 1A and B; ESI† Movie S1). Mazes can represent complex pathways cells encounter in vivo, and we can characterize their migratory behaviors with their patterns of choosing the pathways.33,34 The key question addressed herein was whether and how the presence of bait cells at the outlet would affect the trajectories of the imDC cells introduced at the inlet.

We placed imDCs at the inlet, and either media, EpH4-Ev, or $\beta$-MEKDD 116 cells at the outlet. The outlet was separated from the maze by a $3 \mu m$-wide filter array to block the “bait” cells from entering the maze – in effect, only the imDC cells could navigate the maze from inlet to outlet. From the outlet, the bait cells will begin and continue to secrete various cytokines during the entire $18$ hours of the experiment, and the concentration gradient will be preserved throughout the shortest path, with a continuous increase in its steepness (Fig. S2A†). As represented in Fig. 1B, time-lapse trajectories of imDCs in this particular case were seen to follow the shortest path towards the outlet filled with $\beta$-MEKDD 116 cells.

The number of imDCs recruited ($n$) to enter the maze over the period of $18$ h was higher when the outlet contained EpH4-Ev ($n = 279$) or $\beta$-MEKDD 116 cells ($n = 303$), compared with the outlet containing just the medium ($n = 182$). Among the total number of cells that entered mazes, the percentages of imDCs that solved the maze were ca. 9% higher when the outlet hosted cells (normal or cancer) rather than just the medium, as quantified in Fig. 1C.

Mann–Whitney test of the percentage of DCs exiting the maze among $28$ mazes showed statistically significant differences $^* p < 0.05$, when comparing media and EpH4-Ev or $\beta$-MEKDD 116 conditions. The difference in the percentage of DCs exiting the maze was statistically non-significant when compared between EpH4-Ev and $\beta$-MEKDD 116 cases. Possible reasons that might explain the $22\%$ of DCs escaping the maze, even in the medium condition, may include a self-generated gradient of nutrients,35 hydraulic resistance at the dead end the channel,36 and cell–cell interactions from the occasional simultaneous entrance of multiple cells in the maze.37 However, among the cells that did reach the outlet, there was a pronounced difference in how they did so: when the outlet contained normal EpH4-Ev cells, $44\%$ of maze-solving imDCs took the shortest path with no detours, while $55\%$ did so when the outlet contained cancerous $\beta$-MEKDD 116 cells; when just the medium was at the outlet, only $17\%$ of imDCs took the shortest path (Fig. 1D). By comparing the number of cells taking the shortest path per
chip, the Mann–Whitney test was also applied for media vs. β-MEKDD 116 cells (***p < 0.001), media vs. EpH4-Ev (**p < 0.01), and EpH4-Ev vs. β-MEKDD 116 cells (not significant). Based on these experiments, we surmise that EpH4-Ev and β-MEKDD 116 cells secrete some soluble factors that attract the imDCs, which may follow the attractant’s concentration gradient along the shortest path as chemotaxis.\(^3\)

Directional preference of imDCs towards a specific cell type

The maze experiments described so far suggested that imDCs are attracted toward the outlet cells but did not directly measure the relative “strengths” of this effect for the different cell lines. To gauge whether imDCs are preferentially attracted towards cancerous vs. normal cells (Fig. 2A), we designed a system of microchannels bifurcating near the entrance but then converging – with “lateral” connections between the branches – towards two different outlets (Fig. 2B), each filled with either the medium, EpH4-Ev, or β-MEKDD 116 cells. For each of the three pairwise comparisons listed in the table in Fig. 2C, we compared the percentages of DCs selecting the “left” vs. “right” branches at the entrance, as well as at the outlet (Fig. 2C).

When one of the outlets contained the medium (cases 1 and 2), imDCs preferred to navigate towards the “right”, cell-containing reservoir. As seen from the top two panels in Fig. 2C, the attraction by the cancerous β-MEKDD 116 cells was significantly “stronger” (84% of imDCs ended up in this reservoir) in the process amplifying the initial preference for the “right” turn at the first bifurcation, than that by the normal EpH4-Ev’s cells (65%). This was further confirmed by direct comparison, in which the two cell lines were placed at the two outlets. In this case (case 3), the imDCs initially divided almost evenly at the first bifurcation (52% turned “left”, towards the EpH4-Ev outlet), but in the end, 69% of imDCs navigated towards the outlet housing β-MEKDD 116 cells. We performed statistical analysis of the number of escaped DCs using Mann–Whitney test from 14 chips, ****p < 0.0001 in case 1 of media vs. β-MEKDD 116 cells, *p < 0.05 in case 2 of media vs. EpH4-Ev, and ***p < 0.001 in case 3 of EpH4-Ev vs. β-MEKDD 116.

Examples of the DC trajectories when the outlets contained EpH4-Ev cells (left), and β-MEKDD 116 cells (right), are shown in Fig. 2D and E and in ESI† Movie S2. Some cells took the shortest path from the entrance to the outlet (Fig. 2D). However, most of the time, DCs navigated...
the branches more thoroughly, passing through the same channel multiple times before exiting (Fig. 2E). The same simulation for the presence of a concentration gradient was performed in the complex maze (Fig. S2†), and the concentration gradient of a representative protein, from the inlet to the outlet, is depicted throughout the experiment considering that the cells will keep secreting cytokines in the outlet. One possible reason why the majority of cells did not take the shortest path could be due to a complex mix of cytokines (attractants) emitted by cells of different types (see later in the text), a possible cross-talk between these gradients, and multiple cells (typically 5–7) migrating through the channel network concurrently.

**Migration of imDCs along straight channels**

To further quantify the motility characteristics of DCs more systematically – notably directionality and speed – and between different cell lines, we fabricated arrays of straight channels connecting two reservoirs, respectively housing imDCs and the medium, with Eph4-Ev, or β-MEKDD 116 cells at different concentrations; Fig. 3A and ESI† Movie S3.

The histogram in Fig. 3B indicates the numbers of imDCs reaching the outlet reservoir and mirrors the results obtained with the maze systems: Eph4-Ev and β-MEKDD 116 cells attracted significantly higher numbers of imDCs compared with the control media, and these numbers increased with increasing concentrations of the “bait” cells. Furthermore, the number of imDCs recruited to β-MEKDD 116 compared with Eph4-Ev cells was higher, primarily when the cell plating density was above $1.4 \times 10^3$ cells per mm² in the outlet (statistical analysis was performed using Mann–Whitney tests and $p$-values $<0.05$ were considered statistically significant; $p < 0.05$ when the outlet cell density was $1.4 \times 10^3$ cells per mm², and $p < 0.0001$ when it was $2.8 \times 10^3$ cells per mm²).

Next, we defined a “directionality ratio” as the net, start-to-end displacement divided by the length of the whole trajectory of the cell. Representative trajectories of imDCs towards Eph4-Ev with directionality ratio 0 (cell reverting to the inlet), and β-MEKDD 116 cells with directionality ratio 0.97 (cell going almost ballistically towards the outlet), are shown in Fig. 3C and D, respectively. The average directionality ratios at the last tracking point, just before the cell exits the channel, are plotted in Fig. 3E - the values are 0.34 for the medium, 0.43 for Eph4-Ev, and 0.57 for β-MEKDD 116 cells. The average speeds of imDCs also increased significantly in the presence of β-MEKDD 116 cells (Fig. 3F). When either the medium or the Eph4-Ev cells were present at the outlet, ImDCs displayed significant speed fluctuations (defined as the standard deviation normalized by mean speed), especially for cells in the slow-speed phase, corresponding to the repeated disruption of the motor proteins such as myosin II.²¹ In sharp contrast, when β-MEKDD 116 cells were present at the outlet, speed fluctuations were significantly reduced.

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*Fig. 3 Migration of imDCs in arrays of straight channels. (A) Schematic of an array of microchannels – 27 such microchannels, each 380 μm long and 10 × 10 μm in cross-section – connecting the inlet reservoir with imDCs and the outlet reservoir housing the medium or other cell lines. The outlet reservoir is separated from the channels by a 3 μm-wide filter array (same as in the maze systems) to prevent the escape of the “bait” cells into the main channel. Microscope image of the imDCs migrating inside the microchannels. (B) The average number of DCs that exited towards the outlet, is depicted throughout the experiment considering that the cells will keep secreting cytokines in the outlet. One possible reason why the majority of cells did not take the shortest path could be due to a complex mix of cytokines (attractants) emitted by cells of different types (see later in the text), a possible cross-talk between these gradients, and multiple cells (typically 5–7) migrating through the channel network concurrently.

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(Fig. 3G). We note that the microchannel may have selected DCs with higher motility from the inlet of whole heterogeneous populations, and our analysis is based only on the DCs that entered through the channel.

Migration of imDCs along straight channels imposing different degrees of confinement

An important question that remains to be answered is whether and how the abovementioned trends depend on the degree of confinement imposed by the microfluidic channels. Accordingly, we conducted a series of experiments using microchannels of different widths – 5 μm and 50 μm, all having heights of 10 μm – and compared the results with those obtained using the 10 μm-wide channels (Fig. S3 and S4; ESI† Movie S4 and S5). In particular, we analyzed time-averaged MSD (mean square displacement) of imDCs and the distributions of MSD exponents $\alpha$ (MSD $\sim t^\alpha$, with lag time 5 min) between $\alpha = 1$ (diffusive motion) and $\alpha = 2$ (ballistic motion). As shown in Fig. 4A–C, the directional persistence of imDCs was affected by the degree of confinement. While imDCs moving along 50 μm (Fig. 4A) and 10 μm (Fig. 4B) channels were characterized by relatively broad distributions of $\alpha$, the distributions for 5 μm channels were significantly sharper and shifted towards $\alpha = 2$ (Fig. 4C).

The prominent differences in imDC trajectories under different confinements were further corroborated by the decay of directionality ratio, plotted over normalized time, $T$ (i.e., time divided by the average residence time of imDCs in a channel of a given width) (Fig. 4D). Under all confinements, the directionality ratio decreased as the cells spent more time within the channel, i.e., as $T$ increased. However, the slope of the directionality ratio vs. $T$ decreased most steeply for 50 μm-wide channels, indicating that their trajectories followed more random directions than in other confinements. On the other hand, for the imDCs migrating in 5 μm-wide channels, the directionality ratio remained above 0.6 for all $T$s, even without any chemokines present as in control, which is congruent with more directional and often almost ballistic motions observed under such high confinement.

What is most important, however, is that for both the very narrow (5 μm) or very wide (50 μm) channels, the migrating imDCs showed no preference towards the cancerous β-MEKDD 116 cells vs. normal EpH4-Ev cells at the outlet (see Fig. S3B and S4B†). A reasonable explanation of this observation is that in narrow channels the motility is dominated by strong confinement, akin to other cell types in which confinement is known to induce faster and more directional motions caused by the change in the internal motor protein structure, such as actin and myosin,6,17,21,39,40 whereas in wide channels, random movement of the cells dominates, and the effect of gradients of chemotactants emitted by both types of target cells is less significant to elicit a clearly differentiating response. The 10 μm-wide channels seem to offer the optimal trade-off between the stochastic movements of DCs and the directional motion imposed by the confinement, in our current steepness of attractants’ gradients. We emphasize that there is no “correct” dimension to study cell migration. We should always consider that an optimal dimension or the design of the track can differ between assays, and the result can vary depending on the degree of confinement applied when exploring the cell motility. For further quantification of cell’s trajectories in each confinement, see Fig. S5†.

We note that such confinement effects are likely very subtle, and pronounced differences in the cell’s tactic behaviors can be elicited by relatively small changes in the system’s geometry and/or dimensions. In one set of experiments, we designed a system of channels $7 \times 10$ μm in cross-section (7 μm in width is still narrower than the average diameter of imDCs), with three perpendiculars, shorter branches (Fig. S6A and ESI† Movie S6). In this system, the cells were just a
bit less confined than in 5 μm-wide channels but were also given the choice of exploring the side branches. In the presence of media or EpH4-Ev cells at the outlet, the imDCs stayed longer within the channel, while exploring the side branches, compared to the straight channel without branches. However, in the presence of β-MEKDD 116 cells at the outlet, most of the imDCs did not spend time in the branches and rapidly made their way towards the outlet (for quantification, see Fig. S6B–D†).

Gas6: cytokine candidate emitted from β-MEKDD 116 cells that affects DCs migration

Irrespective of the effects of confinement being intertwined with those of chemical taxis, the above studies confirm that cancerous β-MEKDD 116 cells are more effective in attracting imDCs than either the medium or the non-cancerous EpH4-Ev’s. A logical hypothesis is then that these cells emit a chemoattractant to whose gradients the imDCs respond. As candidates for this chemoattractant, we considered multiple cytokines known to affect the motility of DCs, including CCL19 and CCL21. Specifically, we compared the expression levels of 144 mouse cytokines from 18 h cell-cultured media of EpH4-Ev and β-MEKDD 116 cells using a multiplex cytokine array (Table S1†). Among the tested cytokines that may be relevant to imDC migration, the difference between supernatants of β-MEKDD 116 vs. EpH4-Ev cells was most pronounced for Gas6 (Fig. 5A). Other cytokines well-known to induce DC migration, such as CCL2 and CCL5 for imDCs, or CCL19 and CCL21 for mDCs, were not significantly secreted from β-MEKDD 116 cells, compared with EpH4-Ev cells.

The Gas6 protein is a ligand for Axl, one of the TAM (Tyro3, Axl and Mer) receptors involved in cell migration. Activation of Axl on immune cells, upon binding with Gas6, is reported to suppress immune responses and increase migration through multiple downstream pathways.41–43 Furthermore, Gas6-induced chemotaxis of DCs has been reported from Transwell experiments for the Axl positive DCs differentiated from human peripheral blood mononuclear cells in the presence of interferon-alpha.44 The Axl expression was relatively high for imDCs used in this study but decreased upon maturation (Fig. S7†). To verify the effect of Gas6 secreted from β-MEKDD 116 on imDC migration in our experiments, we reduced the effect of Gas6 secreted from the cell lines by pre-incubating them with Gas6-antibodies before loading the cells into the reservoir (Fig. 5B). We then studied imDC migration in 10 μm wide tracks in the presence of these antibody-treated cells. When the outlet was filled with the anti-Gas6-treated β-MEKDD 116 cell line, the number of DCs exiting the track decreased significantly (Fig. 5C), along with decreased directionality ratio (Fig. 5D) and speed (Fig. 5E), and increased speed fluctuation (Fig. 5F). The reduced recruitment of imDCs was not observed in the case of EpH4-Ev treated with anti-Gas6 antibody.

![Fig. 5](https://example.com/fig5.png)  
**Fig. 5** Effects of Gas6 and other cytokines on imDC migration. (A) Relative ratios of signal intensities (β-MEKDD 116/EpH4-Ev) from selective cytokines; Gas6 stands out as a dominant cytokine released from β-MEKDD 116 vs. EpH4-Ev cells. (B) ELISA results of soluble Gas6 concentration from the supernatants of EpH4-Ev and β-MEKDD 116 cells cultured in normal media, and media treated with anti-IgG or anti-Gas6 antibodies. (C–F) imDC migration assay in 10 μm-wide microchannels showing (C) reduced number of imDCs migrated, (D) reduced directionality ratio at the last point, (E) reduced average speed, and (F) increased speed fluctuation, when the outlet contains anti-Gas6 treated β-MEKDD 116 cells, compared with anti-IgG treated β-MEKDD 116 cells.
Maturation status of migrated DC

It is well known that mature DCs can migrate due to chemotactic response to chemokines such as CCL19 and CCL21. However, from our cytokine array data (Table S1†), CCL19 was only slightly secreted from either EpH4-Ev and β-MEKDD 116, and CCL21 was secreted in similar amounts from both cell lines. Therefore, we deduced that the observed preferential migration of DCs towards β-MEKDD 116 is not primarily due to CCL19/CCL21-dependent chemotaxis, and the migrated DCs remained to be in an immature state.

We further compared the surface expression of the activation markers, CD86, CD80, and CD40, between the migrated DCs and the stationary DCs in Transwell, filled with normal medium as well as EpH4-Ev or β-MEKDD 116-conditioned media (Fig. S8†). ImDCs that had migrated across the Transwell membrane in the medium case exhibited higher expression levels of CD86, CD80, and CD40, compared to the stationary cells. On the other hand, DCs that migrated in EpH4-Ev and β-MEKDD 116-conditioned media did not show any significant increase in these activation markers.

We also collected, after 18 h of incubation, the migrated imDCs in the region of 10 μm channels and near the 3 μm-wide filter array at the chip’s outlet, in order to study differences in their gene expressions depending on the outlet conditions (Fig. S9†). Higher expression levels of markers for DCs maturation, namely CD83 and CCR7, along with CD86, CD40, and CD80 are in good agreement with the above mentioned Transwell experiment in migrated imDCs, for the case when the outlet was filled with control media, as compared to the ones filled with EpH4-Ev and β-MEKDD 116 cells. We also examined the expression of some toll-like receptors (TLRs), which are known to trigger innate immune pathways. It has been reported that TLR expression (TLR2 to TLR5) is increased in these activation markers.

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Comparison of DC movement in confinement with Brownian particles

The motions of imDCs in the presence of normal or cancer bait cells are virtually random and unaffected by shallow chemokine gradients. Motility differences due to chemoattractants emitted by either of the baits are not significant for very wide, 50 μm-wide, channels. On the other hand, for imDCs migrating along 5 μm channels, strong physical confinement (5 μm) seems to be the dominant factor driving the cells to move faster and with higher directional consistency – though, once more, nullifying any differences due to chemoattractants emitted by different bait cells. It is only when the effects of confinement and attractant gradients find the optimal balance that the differences in terms of imDCs migration towards normal vs. cancer cells can be appreciated. Whether or not such a balance exists in vivo cannot be established based on these results but should definitely be studied in the future.

Contemplating further the interplay between physical confinement and chemotactic effects, we compared the experimentally recorded imDC movements with those predicted by a minimal model of an overdamped active Brownian particle, aligning its polarity with velocity as described by Camley et al. Interaction between particles or non-diffusive motions such as Lévy walks were not considered except for the constant attracting force in the x-direction (λ), representing chemotaxis. The different chemotactic sources in the outlet were represented as different values of λ, which were tuned according to the distribution of directionality ratio from the experimental results (Fig. 6A). The confinement effect was prescribed by the mirror-like reflecting angles of particles colliding with channel’s walls.

Despite the simplicity of the model, the simulation reproduced our experimental trends of directionality-ratio distributions, and the change of the slope in MSD curves depending on the different degrees of confinement (Fig. 6B and C). Both in experiments and in simulations, more than half of the migrating cells exhibited directionality ratio, below 0.2, even in the presence of bait cells in 50 μm-tracks. As the confinement increased from 10 to 5 μm-tracks, the populations of the migrating cells with directionality ratio above 0.8 increased significantly. The simulation revealed the effect of confinement playing a major role in determining the overall shape of the distribution of directionality of DCs, whereas the effect from the chemotactic force term (λ), was negligible, as shown in both experiments and simulations.

We are aware that the simple Brownian particle model we implemented omits many biophysical details in the internal and external characteristics of the imDCs, such as shape changes, actual behaviors near the walls, or directional memory, and disturbance of the concentration gradient profile due to the cell migration in the channel. The average diameter of imDCs resting on a fibronectin-coated surface is 9.6 (±1.3) μm, suggesting that they must elongate...
their shapes to move through the channels narrower than ∼10 μm. With such pronounced, confinement-induced changes in cells shape, internal organization of motor proteins (myosin II) must be significantly changed,23 likely explaining the observed high directionality and high speed in DCs (Fig. S3†). Moreover, the speeds of the cells applied in the simulation are taken to follow Maxwell distribution with the average values obtained in our experiments and therefore do not capture the existence of very rapid speeds of imDCs (>40 μm s⁻¹) migrating towards β-MEKDD 116 cells. Such almost ballistic movements of some cells or pronounced increase in the speeds of imDCs under strong confinement (as in the 5 μm-wide channels) could not be fully explained by the model. Still, the simplified theoretical treatment is adequate for reproducing the key trends in directional persistence depending on different degrees of confinement – as such, the assumption that the DCs movement is similar to random Brownian motion can serve as a baseline model for cell movements in simple confined geometries.

**Conclusions**

In sum, this work addressed the unexplored question of how recruitment and migration of imDCs can be affected by different physical environments and neighboring cells. We demonstrated (i) that imDCs are more prone to migrate towards cancerous than same-cell-line normal cells; and (ii) that the degree of confinement modulates the directional persistence of migrating cells, which can either reveal or conceal subtle chemotactic force from the surroundings. Of course, this work calls for further generalization, especially in terms of the effects imparted on the imDC by cancer cells of different origins.

Our results emphasize that in the study of imDC–cancer interactions, deeper understanding of the geometry and dimensions of the cell-migration domain is as essential as the chemical microenvironment. In fact, DCs are found in diverse parts of the body, migrating through 1–3 μm pores from the tissue interstitium towards draining lymph nodes. Typically, the lymphatic vessels are comprised of capillaries ranging between 10 and 80 μm in diameter.50 Understanding
DCs movement in different confinement conditions will enhance the knowledge of DCs recruitment to cancer in relation to the locations they are found. Moreover, the difference in cancer cell migration strategies that depend on the matrix confinement has been explored in vivo, and techniques based on multiphoton microscopies now seem to offer an adequate resolution for such studies. Indeed these new techniques have recently been applied to differentiate the motility profiles of DCs can also be important for the development of standardized assays, which will be useful to screen effective immune cells for clinical applications. The recruitment of potent immune cells to the tumor site is a critical issue in immunotherapy, and screening of heterogeneous populations based on their migration abilities might provide insight toward this direction.

Author contributions

E. U., C. S., B. A. G., and Y.-K. C. designed the research; E. U., J. P., T.-E. K., Y. C., C. S., and D. K. performed the experiments; E. U., J. M. O., T. S, and J.-H. J. performed simulation and statistical analysis; all authors wrote the manuscript.

Conflicts of interest

There are no conflicts to declare.

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