Analysis of transient migration behavior of natural killer cells imaged \textit{in situ} and \textit{in vitro}†

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We present a simple method for rapid and automatic characterization of lymphocyte migration from time-lapse fluorescence microscopy data. Time-lapse imaging of natural killer (NK) cells \textit{in vitro} and \textit{in situ}, both showed that individual cells transiently alter their migration behavior. Typically, NK cells showed periods of high motility, interrupted by transient periods of slow migration or almost complete arrests. Analysis of \textit{in vitro} data showed that these periods frequently coincided with contacts with target cells, sometimes leading to target cell lysis. However, NK cells were also commonly observed to stop independently of contact with other cells. In order to objectively characterize the migration of NK cells, we implemented a simple method to discriminate when NK cells stop or have low motilities, have periods of directed migration or undergo random movement. This was achieved using a sliding window approach and evaluating the mean squared displacement (MSD) to assess the migration coefficient and MSD curvature along trajectories from individual NK cells over time. The method presented here can be used to quickly and quantitatively assess the dynamics of individual cells as well as heterogeneity within ensembles. Furthermore, it may also be used as a tool to automatically detect transient stops due to the formation of immune synapses, cell division or cell death. We show that this could be particularly useful for analysis of \textit{in situ} time-lapse fluorescence imaging data where most cells, as well as the extracellular matrix, are usually unlabelled and thus invisible.

Introduction

NK cells can clear the body of virus-infected, foreign, or tumor cells. NK cell recognition and killing is based on a balance between activating and inhibitory signals generated by a repertoire of receptors and ligands.\textsuperscript{1} A central question in NK cell recognition is how inhibitory and activating signals are balanced to determine the outcome of an interaction with a target cell.\textsuperscript{2} NK cells survey other cells by forming tight intercellular contacts allowing interactions between receptors and ligands expressed by the respective cells. This interaction is often accompanied by segregation of proteins into micrometre-sized

Insight box

Through recent advances in multiphoton microscopy it is now possible to study the behavior of individual lymphocytes inside, e.g. tumors or lymph nodes. Although studies have largely focused on how these cells respond to different conditions, such as foreign cells or antigen, they have also revealed variations in migration between individual cells and that individual cells alter their migration behavior over time. Here, we introduce an \textit{in vitro} microchip-based imaging method making it possible to follow small cell populations over long times and a mathematical model allowing quantification of transient migration behavior. These tools could easily be implemented in any studies of cell migration \textit{in vitro} and to objectively assess migration behavior both \textit{in situ} and \textit{in vitro}.
domains called supramolecular activation clusters (SMACs), an arrangement that has been called the immune synapse (IS). The NK cell IS has been intensely studied in recent years leading to an increased knowledge about its function as a platform for signaling and directed secretion. Thus, it is recognized that NK cell mediated immunity is regulated by the expression level of receptors and ligands, their spatial distribution, and their interactions across the tight intercellular contact at the IS. Another important regulating factor could be migration dynamics. For example, it is possible that the number of previous encounters with target cells or the speed of migration or polarization may also influence how efficiently NK cells find and survey new target cells. To assess if such factors are important it is necessary to image populations of NK cells as they interact with target cells over extended periods of time. Data analysis will be accelerated and facilitated by implementation of methods for automatic and quantitative image analysis.6,7

Migration of NK cells to sites where target cells are located is critical for their function. Two-photon imaging has recently been used to visualize and elucidate NK cell dynamics in situ.8 NK cell migration has been studied inside lymph nodes using adoptively transferred NK cells under steady-state and activating conditions9,10 in a bacterial model,11 in a viral model system in situ,12 and in tumors.13 The 3D steady state motility of NK cells has been found to be in the order of 10 μm min⁻¹, which is intermediate between that reported for B and T cells.12,14 Although it is possible to use multiple fluorescence probes to visualize several cell populations inside lymph nodes or in tumors, it remains difficult to obtain a complete picture of the interactions a particular cell makes with other cells as the majority of cells in the tissue are often unlabeled.

Here we apply a simple method for analysis of transient behavior of NK cells. This analysis allows, for example, the determination of when and where NK cells undergo transient migration arrest periods (TMAPs), i.e. periods when the NK cells stop or display low motilities, caused for example by contacts with surrounding cells. We use the analysis method to study NK cell migration both in situ and in vitro. For the in situ experiments, NK cells were imaged under two distinct conditions: at steady state and in an inflammatory situation. For the in vitro data, we used a novel microchip-based assay where small populations of NK cells were tracked as they interacted with adherent target cells for extended periods of time (>12 h). In this setup, human NK and target cells are spatially confined in miniature, deep wells etched in silicon, allowing observation of the same population of cells for the duration of the experiment.15

Materials and methods

Cell culture and reagents

Human embryonic kidney (HEK) 293T cells were cultured in RPMI-1640 media supplemented with 10% FCS, 2 mM l-glutamine, 1 × non-essential amino acids, 1 mM sodium pyruvate, 50 U ml⁻¹ penicillin-streptomycin, 50 μM β-mercapto-ethanol at 37 °C, 5% CO₂. Human polyclonal NK cells were isolated from peripheral blood lymphocytes by negative selection according to manufacturers’ instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). NK cell purity was >95% CD3 CD56⁻. Freshly isolated NK cells were cultured in IMDM supplemented with 10% human AB serum, 2 mM l-glutamine, 1 × non-essential amino acids, 1 mM sodium pyruvate, 50 U ml⁻¹ penicillin-streptomycin, 50 μM β-mercapto-ethanol and 100 U ml⁻¹ IL-2 (Peprotech, Rocky Hill, USA). Polyclonal NK cells used were cytotoxic against 293T in standard chromium release assays (data not shown). Reagents were purchased from Sigma Aldrich (St Louis, USA) unless otherwise stated.

Mice

C57BL/6 (B6), BALB/c, B6.129S7-RAG<sup>tm1Mom/J</sup> (B6.Rag<sup>−/−</sup>) mice were purchased from the Jackson Laboratory. CD11c-EYFP reporter mice were a kind gift from M. Nussenzweig16 and were backcrossed to B6 for 10 generations. Mice were housed in a pathogen-free animal facility and all procedures were performed in accordance with protocols approved by the animal care and use committee of the University of California, Irvine.

Cell labeling

293T cells (10<sup>6</sup> cells ml⁻¹) were loaded with 1 μM ml⁻¹ calcein-AM and 2 μM ml⁻¹ Far Red DDAO-SE at 37 °C, 5% CO₂ for 10 min. Polyclonal NK cells (0.5 × 10⁶) were loaded with 0.4 μM ml⁻¹ Calcein Red-Orange 37 °C, 5% CO₂ for 10 min. After staining, cells were washed twice in RPMI-1640 culture medium. Fluorescent probes were purchased from Invitrogen (Carlsbad, USA).

Imaging

In vitro. A silicon-glass microchip containing 672 wells, each well being 500 μm deep with a bottom area of 650 × 650 μm², was used for the in vitro assay.17 Dye-labeled 293T cells were seeded onto the dry microchip at 20 × 10⁶ cells ml⁻¹ (Fig. 1A). Cells were left to sediment and attach to the glass surface for 30 min at 37 °C, 5% CO₂. Dye-labeled polyclonal NK cells were subsequently seeded at 10⁶ cells ml⁻¹ for 5 min. The chip was submerged in NK cell culture medium and imaged at 10× magnification (Fig. 1B) using a LSM 5 Pascal confocal microscope (Carl Zeiss, Oberkochen, Germany) equipped with an environmental chamber kept at 37 °C, 5% CO₂. The pinhole was opened to capture maximum fluorescence and images were acquired every 2 min for approximately 12 h. For reference experiments, NK cell migration was imaged in the absence of target cells. The data presented is cumulated from 3 in vitro experiments with target cells (n = 175) and 2 in vitro experiments without target cells (n = 98).

In situ. NK cells from B6.Rag<sup>−/−</sup> spleens were labeled with 9–10 μM 5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) for 30 min and admittively transferred into syngeneic B6 CD11c-EYFP (steady state conditions) or WT recipients. Bone marrow was harvested from long bones and plated at 5 × 10⁶/10 ml DC media (IMDM with 10% FCS (HyClone), l-glutamine, 50 μM β-ME, and 30–50 ng ml⁻¹ GM-CSF) and cultured for up to 8 days as previously described.18 Following overnight stimulation with
1 µg ml\(^{-1}\) lipopolysaccharide (LPS), 1–5 \times 10^6 2 µM carboxy-fluorescein succinimidyl ester (CFSE)-labeled allogeneic bone marrow-derived dendritic cells (BM-DCs) and 1–5 \times 10^6 20–40 µM 7-amino-4-chloromethylcoumarin (CMAC)-labeled syngeneic BM-DCs were injected subcutaneously into the footpads of recipients that had previously received NK cells (inflammatory conditions). The following day, lymph nodes were secured with cyanoacrylate adhesive onto a coverslip and placed in an imaging chamber superfused with RPMI medium bubbled with carbogen (95% O\(_2\), 5% CO\(_2\)) at 37 °C. Two-photon imaging was performed as previously described.\(^{19}\) In brief, two femtosecond lasers provided simultaneous excitation at 780 and 900 nm. 510 and 560 nm dichroic mirrors were used to split fluorescence emission into three photomultiplier detector channels. Successive imaging volumes of 50–60 µm were acquired using MetaMorph software. The data presented is cumulated from 2 and 3 individual in situ experiments for steady state (\(n = 84\)) and inflammatory conditions (\(n = 137\)), respectively. The total imaging times were 25 min 22 s and 21 min 59 s for the two steady state movies and 35 min 44 s, 56 min 24 s and 1 h 0 min 5 s for the three movies acquired under inflammatory conditions.

### Image analysis

Analysis of in situ and in vitro data was performed using Volocity software (PerkinElmer, Waltham, USA). NK cells were manually tracked (Fig. 1C). Analysis of in situ data was performed on 2D images created by projecting the 3D volumes along the optical axis (z-axis). By comparing with previously published results we estimate that flattening of in situ imaging data resulted in a reduction of the measured average speed by approximately 20–30%.\(^{14}\) It is possible that some TMAPs detected in the flattened images were caused by cells moving only in the direction of the z-axis, hence appearing immobile in the \(x\), \(y\)-plane. Conjugate formation occurring in the in vitro movies was determined by assessing the proximity between NK and target cells using both the bright field and fluorescence images.

### Characterization of transient migration behavior

To categorize transient migration behavior exhibited by NK cells, three classes of migration were defined: (1) transient migration arrest periods (TMAPs); (2) directed migration and (3) random movement. Detection of these modes of migration is outlined below. All analysis was done by home-developed routines in Matlab (The Mathworks, Natick, USA).

### Mean squared displacement

Individual trajectories were analyzed by forming the mean squared displacement (MSD). The MSD for two dimensional data is defined as:

\[
\text{MSD}(t) = \frac{1}{N-n} \sum_{i=1}^{N-n} \left( (x_{i+n} - x_i)^2 + (y_{i+n} - y_i)^2 \right) \quad (1)
\]

where the NK cell position in 2-dimensional space is written as \(x_i\) and \(y_i\) and \(N\) is total number of NK cell positions. The time lag for the MSD calculation is \(t = n\Delta t\), where \(\Delta t\) is the time between two consecutive NK cell positions (the time between image frames) and \(n\) is an integer number. For a randomly migrating cell, the MSD increases linearly with time and the slope is directly proportional to the migration coefficient (M).

**Sliding window analysis.** To evaluate transient migration behavior, the trajectories from individual NK cells were analyzed using a sliding window approach, i.e. the MSD was evaluated using only a small part of the trajectory and the center of the window was moved step-by-step along the trajectory.\(^{20}\) Thus, each trajectory was divided into sections of \(W\) consecutive points, where \(W\) is the size of the sliding window. Here the value of \(W\) was set to 25, since this value gave a satisfactory level of noise for reliable valuation of the MSD parameters (analysis not shown). Trajectories that were shorter than the length of the sliding window were excluded from the analysis.

After extraction of the MSD parameters using the sliding window, a rolling average filter with the same width as the sliding window was used to smooth the parameter profiles. Each point of the parameter profile was smoothed as

\[
P_i = \frac{1}{W} \sum_{k=1}^{\text{int} W-1} P_k
\]

where \(1 \leq i \leq N\) and the size of the smoothed parameter profile is \(N\).

To evaluate the MSD parameters at the beginning and end of each trajectory in conjunction with the rolling average filter, \(W – 1\) points were padded at the ends of the trajectory by using the value of the start and end point, respectively. Hence, the size of the trajectory increased to \(N + 2(W – 1)\) points and the final profile size was \(N + W – 1\).

**TMAP detection.** The detection of TMAPs was based on finding periods where M was below a threshold defined by the diffusion coefficient estimated for a spherical particle of a comparable size of a typical NK cell. This threshold can be computed as:

\[
M_{\text{min}} = \frac{k T}{3 \pi \eta d}
\]

where \(k\) is the Boltzmann’s constant, \(\eta\) is the viscosity of the medium (approximated to \(\eta\) of water), \(d\) is the mean diameter of the particle (the mean diameter of human NK cells was determined to be \(d = 8.3\) µm) and \(T\) is the absolute temperature of the medium (\(T = 310\) K). Thus, this value
of M (4.2 μm² min⁻¹) is comparable to a situation when the NK cell’s migration machinery is completely turned off or engaged in other activities than migration.

For 2D data, M is proportional to the slope of the MSD curve. In agreement with previous work measuring the diffusion coefficients of single vesicles,²¹,²² we considered only the initial portion (the first 6 points) of the MSD curve for assessing the slope. Then, M was calculated along NK cell trajectories using the sliding window to detect TMAPs, i.e. periods when the value of M was below the defined threshold (Fig. 2A).

**Detection of directed migration.** Directed migration of NK cells was detected by assessing the MSD curvature. The MSD can be written as MSD ∝ t², where z is quantifying the MSD curvature. For a symmetric random walk, z = 1, whereas z > 1 indicates directed movement.²³,²⁴ To simulate Brownian motion we generated one thousand 2D trajectories for each condition in silico. Briefly, for migration in vitro the starting points of the simulated tracks were randomly picked to be somewhere in the microwell (0 ≤ x ≤ 650 μm; 0 ≤ y ≤ 650 μm). Then a step was generated every two minutes, assuming a Gaussian distribution of step lengths symmetric around zero, with an average absolute step length corresponding to the speed measured for NK cells in vitro with or without target cells. The direction of each step was picked randomly and if the endpoint of a step was outside the boundaries defined by the wells a new step was generated. The total time-length of the trajectories was 12 h. To simulate random walk in situ, a similar approach was used but assuming no physical boundaries and a time interval between steps of 30 s and step lengths corresponding to the speeds measured under steady state and inflammatory conditions, respectively. The total trajectory time was 1 h.

For assessing the MSD curvature as a function of time the exponent z was calculated from the initial slope, using 6 points of a linear fit of log MSD versus log t, using the sliding window analysis. To limit the detection of false directed movement a threshold was introduced, so that directed movement was only detected when z > 1.5 for at least 10 consecutive points, corresponding to <2.2% of the time points in the simulated tracks. The MSD curvature was assessed in the same way for the experimental data after the time points that had been classified as TMAPs were excluded from each trajectory (Fig. 2B). Thus, all periods where z > 1.5 for at least 10 consecutive points were classified as directed migration.

**Random movement.** The parts of NK cell trajectories which were not classified as either TMAPs or directed migration were defined as random movement. In this way each NK cell trajectory could be divided into TMAPs, periods of directed migration or random movement (Fig. 2C).

**Statistical analysis**

Statistical significance was determined by using either student’s t-test or Mann-Whitney U test. p < 0.05 was considered significant. Statistical correlation between two parameters was assessed by Spearman’s rank correlation coefficient. Data is presented as mean ± standard error of the mean (SEM).

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**Results**

Small populations of human peripheral blood NK cells (50–100 cells) were studied as they migrated and interacted with adherent 293T tumor target cells, which are highly susceptible to NK cell-mediated lysis. As a reference, we also analyzed NK cells migrating in the absence of target cells. By confining the cells in small wells made in silicon¹⁷ we were able to monitor the same population of cells for extended periods of time. Fluorescently-labeled NK cells were imaged every 2 min for 12 h and individual NK cells were tracked (Fig. 1).

**Transient migration behavior of NK cells in vitro**

The in vitro mean speed of individual NK cells was found to vary greatly, with an average value of 1.6 ± 0.1 μm min⁻¹ (Fig. 3A), which was slightly lower than the mean speed for NK cells migrating in the absence of target cells (2.4 ± 0.1 μm min⁻¹, see Table S1, ESI for a summary of measured mean values ± SEM†). A closer look at the raw data revealed that there were transient variations within individual trajectories. NK cells often moved intermittently, continuously starting and stopping.
This stop-and-go behavior has previously been observed both for NK and T cells in situ.\textsuperscript{10,19}

To characterize transient variation in the migration of NK cells, we analyzed small sections of the NK trajectory using a sliding window to distinguish TMAPs, periods of directed migration and periods of random motion (see Fig. 2, Materials and Methods). From this analysis it was evident that NK cells continuously shifted between these different modes of migration. Importantly, these shifts in migration were irregular and differed from cell to cell. This is evident, for example, by plotting the fraction of time spent in TMAPs (Fig. 3B) or the fraction of time spent in directed migration for individual cells (Fig. 3C). Furthermore, the mean NK cell speed varied through these distinct modes of migration; low mean speeds for TMAPs which increased for directed migration and random motion (Fig. S1, ESI\textsuperscript{†}).

Thus, alternating between different modes of migration can influence the overall mean NK cell speed. The mean NK cell speed decreased as the fraction of time NK cells spent in TMAPs increased (Fig. 3D) while the mean speed increased for the fraction of time spent in directed movement (Fig. 3E). These data indicate that the heterogeneity observed in the mean speed for individual NK cells is largely due to variations in the fraction of time spent in different modes of migration.

\textbf{Properties of TMAPs in vitro}

In the presence of target cells, 97\% of NK cells were found to enter a TMAP at some point during their migration, some cells often moving in and out of several TMAPs (Fig. 4A). The size (Fig. 4B) and duration (Fig. 4C) of individual TMAPs varied substantially. A large proportion of TMAPs lasted less than one hour although several longer TMAPs, sometimes spanning the whole experiment, were also observed. Interestingly, the majority of TMAPs had 10–20 \( \mu \)m radii which correlates with the size of the target cells used,\textsuperscript{15} suggesting that TMAPs could be associated with formation of conjugates with target cells. However, 74\% of NK cells migrating in the absence of target cells entered a TMAP at some point during their migration and their size was comparable to when target cells were present but their average duration was shorter without target cells (Fig. S2, Table S1, ESI\textsuperscript{†}). Importantly, the mean fraction of time individual NK cells spent in TMAPs approximately doubled (from 35\% to 70\%) when target cells were present, indicating that formation of contacts with target cells could be responsible for TMAPs.

\textbf{Correlation between TMAPs, conjugate formation and target cell lysis}

As both NK and target cells were fluorescently labeled in the \textit{in vitro} experiments it was possible to directly quantify when and for how long individual NK cells were conjugated to target cells. NK cells spent on average 55\% of their time in conjugates with target cells. To investigate whether actual periods of conjugation overlapped with TMAP formation we correlated conjugation periods (CPs) with the time and location that NK cells were in TMAPs. This was achieved by comparing the overlap between the two parameters, \textit{i.e.} finding coordinates for when and where the cells were in both TMAPs and CPs simultaneously. The ratio between the overlap with either the time spent in TMAPs (Fig. 5A) or the time spent in CPs (Fig. 5B) was plotted. When looking at the conjugation overlap with TMAPs (Fig. 5A), two distinct populations were observed. One population had little or no overlap, representing TMAPs that form independently of target cell conjugation, whereas the other population showed almost 100\% overlap. On average, 61\% of the time NK cells spent in TMAPs was correlated with formation of conjugates. Thus, TMAPs were frequently formed upon conjugation but could also form spontaneously. Interestingly, when looking at TMAP overlap with conjugation, a large population was observed with high overlap, showing that conjugate formation most often leads to TMAPs (Fig. 5B). On average 74\% of the time NK cells spent in conjugates was accompanied by TMAPs. A few representative trajectories were plotted to demonstrate NK cell behavior when there was a high overlap between TMAP and CP (Fig. 5C–D), no overlap between TMAPs and CP (Fig. 5E) and conjugates that did not lead to a TMAP (Fig. 5F).

Next we set out to investigate to what extent TMAPs that overlapped with conjugation led to target cell lysis. NK cell mediated lysis of target cells was followed in real time by analyzing the target cell morphology as well as the calcein fluorescence intensity which rapidly declines due to leakage of the dye from dying cells.\textsuperscript{15} The initial drop in calcein fluorescence was scored as the time point of cell death. Our analysis showed that of the TMAPs that overlapped with conjugate formation, 74 resulted in NK cell mediated killing of target cells and 113 resulted in no killing (Fig. 6A). Frequently the NK cell interacted with several target cells within the same TMAP, and if lysis of at least one target cell was detected the TMAP was scored as a lytic TMAP. Conjugates between individual...
NK cells in TMAPs and individual target cells was also commonly observed to result in killing (Fig. 6B and C, Movie S1, ESI†) or no killing (Fig. 6D and E, Fig. S3, ESI†). A closer investigation of NK TMAPs that formed without target cell contact showed that some cells were stationary for the duration of the movie, while other cells stopped spontaneously, remaining stationary for some time before continuing migration (Fig. 6F and G, Fig. S3, ESI†). TMAPs occurring without conjugate formation was observed for 35% of the NK cells and sometimes coincided with NK cell death or proliferation (Fig. 6A). Amongst conjugates where no TMAPs were detected, we found brief contacts or contacts where the NK cells picked up target cells and dragged them along their path as they migrated, a behavior which has also been observed before.25 In summary, for NK cells migrating in vitro, TMAPs were frequently formed due to transient contacts with target cells but could also form spontaneously, due to NK cell death or division.

Detection of transient migration behavior of NK cells imaged in situ

Next we tested if our analysis method could detect a similar migration behavior for NK cells imaged in situ and whether distinct differences could be observed under steady state and inflammatory conditions. To represent a steady state condition, we analyzed the migration pattern of NK cells that had been adoptively transferred into a syngeneic recipient and rested overnight (see movie S2, ESI†). To mimic inflammatory conditions, we analyzed the migration pattern of NK cells that had been adoptively transferred into a syngeneic recipient, rested overnight and then injected with both syngeneic and allogeneic LPS matured BM-DCs. Notably, the distribution of the mean NK cell speed was higher under inflammatory conditions (average mean 9.3 ± 0.2 μm min⁻¹) compared to steady state conditions (average mean 6.0 ± 0.3 μm min⁻¹) (Fig. 7A). Similar to what was observed in vitro, the mean speeds of NK cells were significantly decreased in TMAPs compared to the periods of directed movement and random movement (ESI Fig. S3†).

The fraction of time that NK cells spent in TMAPs (Fig. 7B) as well as the number of TMAPs/cell (Fig. 8A and C) was drastically reduced under inflammatory conditions. The mean percentage of time in TMAPs decreased from 41% to 5% and the average number of TMAPs/cell decreased from 0.7 ± 0.1 to 0.15 ± 0.1 in the presence of allogeneic LPS-activated DCs. These values depend on the total imaging times, which on average were longer for the inflammatory conditions (see Materials and Methods), meaning that directly comparing these values underestimates the difference observed between the two conditions. A summary of each individual movie analyzed separately can be found in the ESI (Fig. S5†). Interestingly, NK cells also spent more time migrating in a directed fashion
under inflammatory conditions compared to the steady state conditions (mean values 56% vs. 24%, Fig. 7C). Thus, under inflammatory conditions NK cells were found to stop less and migrate in a much more directed fashion. Similar to what was found in vitro, the shift between different modes of migration can explain the significant difference observed in the average mean speed measured under steady state and inflammatory conditions.

Despite the distinct NK cell behavior between the two conditions, the mean durations of TMAPs were similar at steady state (10.4 ± 1.1 min) and inflammatory conditions (8.4 ± 3.4 min). However, the distribution of TMAPs over time differed; under steady state conditions TMAPs of various lengths were observed, whereas under inflammatory conditions the TMAPs were either short (<10 min) or long (>30 min) (Fig. 8B, D and Fig. S5†). Although the presence of long TMAPs under steady state conditions could not be excluded due to the shorter imaging time, the few long TMAPs observed under inflammatory conditions could come from stable contacts formed between allogeneic cells and NK cells, which have been observed previously under similar conditions.9

Discussion

NK cells imaged in situ or in vitro were observed to move intermittently, transiently stopping, e.g. to form contacts with surrounding cells, to divide or to die. Common for lymphocyte migration is the significant heterogeneity observed between individual cells. This emphasizes the importance of implementing methods for rapid, objective and quantitative data analysis that can help to detect and classify subpopulations of cells. Various approaches have previously been used to characterize transient behavior in vesicular movement20 or for the detection of molecules trapped in transient confinement zones.26,27 Here, we applied a simple method to characterize the migration behavior of NK cells imaged both in vitro and in situ. This method categorizes migration into three different classes: TMAPs, directed migration and random movement. This analysis allowed the immediate extraction of several parameters important for the characterization of cell migration, e.g. the size, location and duration of TMAPs, the mean speed of cells and fraction of time that they spent in different modes of migration.

Fig. 6 NK cell TMAPs can form during lytic and non-lytic interactions with target cells as well as spontaneously. The number of TMAPs overlapping with lytic and non-lytic interactions with target cells as well as the number of TMAPs formed due to NK cell death, NK cell division or spontaneous arrest with (black bars) or without (white bars) target cells (A). Snapshots from time-lapse movies showing bright field (B, D, F) and fluorescence (C, E, G) of NK cells (arrow-heads) in TMAPs correlated with lysis of the target cell (B, C), no lysis of the target cell (D, E) or spontaneous arrest (F, G). The white lines in (B, D, F) show the trajectories immediately outside of the TMAP while the trajectories of the TMAPs are shown by red lines. Target cell death occurring in (C) is seen as decreased calcine fluorescence intensity. Movie S1 and Fig. S3, ESI†, show time sequences of the cells in (B–G). Scale bars, 20 μm.

Fig. 7 Transient behavior of NK cells migrating in situ. A range of different NK cell migration speeds was observed under steady state and inflammatory conditions (A). The fraction of time that NK cells spent in TMAPs (B) or directed migration (C) plotted for steady state and inflammatory condition. The bars have been normalized to the total number of NK cells to facilitate comparison between the two conditions.
Several results are noteworthy. Analysis of *in vitro* data showed that NK cells spent significantly more time in TMAPs when target cells were present, and on average more than half of the time (61%) spent in TMAPs overlapped with formation of conjugates. Thus, interaction with target cells was a common reason for TMAPs to occur. NK cell mediated killing was observed in 40% (74/187) of the TMAPs that coincided with NK cell conjugation to target cells. While this number could appear low it is important to stress that little is currently known about the dynamics of how polyclonal populations of NK cells eliminate tumor cells. Our preliminary data indicate a significant heterogeneity within the NK populations so that some NK cells can kill several targets in a consecutive fashion (serial killers\textsuperscript{28}), while other NK cells kill more stochastically (8.4 ± 3.4 min for inflammatory conditions, 3.4 min for steady state conditions, respectively). Importantly, the difference in speeds observed under inflammatory and steady state conditions could partly be explained by a relative shift in time that the cells spend in the different migration classes.

Based on the duration of TMAPs, most stops were relatively short under both *in situ* conditions. The mean duration of TMAPs did not change significantly between inflammatory (8.4 ± 3.4 min) and steady state conditions (10.4 ± 1.1 min). Interestingly, these times are fairly similar to the average conjugation times measured between NK cells and syngeneic DCs (5 min) and non-lytic interactions between NK cells and allogeneic DCs (6 min) under similar conditions.\textsuperscript{10,12} Notably, the TMAP analysis does not distinguish why a particular TMAP is formed unless it is correlated with other imaging data. However, the similarity observed between TMAP analysis and previously reported conjugation data indicate that occurrence of TMAPs *in situ* could be related to interactions with other cells. Previous studies have shown that T cells\textsuperscript{29} as well as NK cells\textsuperscript{10,25,30} can receive a stop signal when encountering activating ligands, *e.g.*, expressed by a susceptible target cell. Consistent with that, Garrod *et al.* recently reported that a low fraction (<5%) of conjugates between NK cells and allogeneic DCs resulted in lysis of the DCs and that these conjugates lasted for extended periods of time (20 min).\textsuperscript{9} Interestingly, under inflammatory conditions, we observed a few long lasting TMAPs but were unable to determine if these originated from allogeneic DC-NK cell conjugates. Thus, although some long-lasting TMAPs were seen under inflammatory conditions, our analysis indicates that most NK cells adopt a more rapid scanning behavior with less transient stops under inflammatory conditions *in situ*.

Although the background to why cells enter TMAPs is not completely straightforward, this automatic analysis gives a rapid fingerprint of the properties of the individual cells within the studied cell population. This, together with our *in vitro* microchip assay, could be useful for high-throughput applications for rapid screening, *e.g.*, how drugs or genotype affect cell migration. As we have shown, it could also be useful for *in situ* imaging studies where the majority of the surrounding cells are usually unlabeled and therefore invisible by fluorescence imaging.

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