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Research paper

Analysis of CCR7 mediated T cell transfectant migration using a microfluidic gradient generator

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ABSTRACT

T lymphocyte migration is crucial for adaptive immunity. Manipulation of signaling molecules controlling cell migration combined with *in-vitro* cell migration analysis provides a powerful research approach. Microfluidic devices, which can precisely configure chemoattractant gradients and allow quantitative single cell analysis, have been increasingly applied to cell migration and chemotaxis studies. However, there are a very limited number of published studies involving microfluidic migration analysis of genetically manipulated immune cells. In this study, we describe a simple microfluidic method for quantitative analysis of T cells expressing transfected chemokine receptors and other cell migration signaling probes. Using this method, we demonstrated chemotaxis of Jurkat transfectants expressing wild-type or C-terminus mutated CCR7 within a gradient of chemokine CCL19, and characterized the difference in transfectant migration mediated by wild-type and mutant CCR7. The EGFP-tagged CCR7 allows identification of CCR7-expressing transfectants in cell migration analysis and microscopy assessment of CCR7 dynamics. Collectively, our study demonstrated the effective use of the microfluidic method for studying CCR7 mediated T cell transfectant migration. We envision this developed method will provide a useful platform to functionally test various signaling mechanisms at the cell migration level.

1. Introduction

Chemoattractant concentration gradient directed immune cell migration (chemotaxis) critically orchestrates the trafficking and homing of various immune cell types in tissues (Cahalan and Parker, 2008; Jin et al., 2008; Gambardella and Vermeren, 2013; Heuzé et al., 2013). T lymphocytes are the key players in adaptive immunity (Garside et al., 1998; Miller et al., 2002; Bromley et al., 2008; John et al., 2009). These cells have highly regulated tissue-specific migratory properties (Campbell and Butcher, 2000; Moser and Loetscher, 2001; Kunkel and Butcher, 2002). In particular, chemokine receptor CCR7 is required for naïve and central memory T cell migration to lymph nodes (LN), which is important to initiate adaptive immune response and immune surveillance (Forster et al., 1999, 2008; Gunn et al., 1999). Chemokine CCL21, a ligand for CCR7, is highly expressed in LN and is responsible for T cell recruitment to LN (Ngo et al., 1998; Luther et al., 2002). Chemokine CCL19, another ligand for CCR7, is a potent chemoattractant for CCR7 expressing T cells *in vitro*. However, CCL19 is only produced in LN at low level and seems not to participate in T cell recruitment to LN (Luther et al., 2002; Link et al., 2007). CCL19 but not CCL21 triggers robust CCR7 desensitization and internalization (Bardi et al., 2001; Kohout et al., 2004). Interestingly, deletion of the C-terminus of CCR7 does not completely inhibit the CCL19-induced internalization (Otero et al., 2008). The mechanisms underlying these unique features of CCR7 and their roles in mediating T cell

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migration are not clear. Further studies to better understand CCR7-mediated T cell migration will benefit from an advanced experimental platform, which is capable of quantitative single cell migration analysis and imaging studies of cell migration signaling at the molecular level in well-defined chemical gradients.

Most *in-vitro* studies of T cell migration employed the conventional transwell assay. Its capacity is limited by poor gradient control and inability to visualize migrating cells (Keenan and Folch, 2008). Microfluidic devices, which can precisely configure gradient conditions, offer useful tools for cell migration and chemotaxis studies (Li and Lin, 2011; Wu et al., 2013). Previously, we used a microfluidic gradient-generating device to study the migration of activated human peripheral blood T cells (ahPBT) (Lin and Butcher, 2006; Nandagopal et al., 2011). Microfluidic devices were also used to study the migration of other subsets of human peripheral blood T cells such as memory T cells (Lin et al., 2008). However, there have been few reports for studying signaling mechanisms in chemotaxing immune cells by combining genetic manipulations and controlled chemoattractant gradient profiles generated by microfluidic devices, especially in lymphocytes (Long et al., 2004; Sai et al., 2006; Cavnar et al., 2011). Among them, several studies employed microfluidic devices to study chemotactic signaling for neutrophil chemotaxis using neutrophil-like cell lines expressing transfected mutants of specific signaling molecules such as CXCR2 (Sai et al., 2006) and Hax1 (Cavnar et al., 2011). We previously employed a microfluidic gradient generator to quantitatively evaluate the role of tandem PH domain-containing protein 2 (TAPP2) for regulating malignant B lymphocyte migration (Li et al., 2013). However, the effective use of microfluidic device for studying T cell transfectants chemotaxis targeting specific signaling mechanisms has not been demonstrated.

The potential of advanced microfluidic platforms to study chemotaxis mechanisms in immune cells is strengthened by recent development of molecular probes and imaging technologies (Wang et al., 2008; Deng et al., 2011). Immunofluorescence staining against specific cell surface and intracellular markers is a well-established technique (Fritschy and Härtig, 2001; Robinson et al., 2010). For example, this method can be used to visualize surface distribution and endocytosis of chemoattractant receptors as well as cytoskeleton in migrating cells at the time point of interest (Ballestrem et al., 1998; Otero et al., 2006). In addition, advanced methods were developed to visualize the dynamics of signaling events in migrating cells in real-time (Servant et al., 2000; Ridley et al., 2003; Riedl et al., 2008; Arai et al., 2010). New F-actin binding probes have become available for real-time visualization of cytoskeleton dynamics in cell migration (Riedl et al., 2008). State-of-the-art imaging modules such as confocal and total internal reflection fluorescence microscopy (TIRF) have been widely used in cell migration research to enable high-resolution molecular imaging (Blow, 2007). It will provide a powerful research approach for studying T cell migration and chemotaxis by integrating molecular imaging with microfluidic devices.

The main goal of the present study is to demonstrate the feasibility of combining a microfluidic gradient generator with molecular immunology methods to quantitatively analyzing the migration of genetically modified T cell transfectants. We wanted to determine whether chemotactic signaling constructs can be effectively transfected to T cells and mediate functional migration and chemotaxis of the T cell transfectants in microfluidic devices. Furthermore, we wanted to examine the function of both wild-type (wt) CCR7 and C-terminus mutated CCR7 in mediating T cell transfectant chemotaxis in the microfluidic system.

2. Materials and methods

2.1. Plasmid construction

Lifeact-RFP plasmid was purchased from ibidi GmbH. The CCR7-WT and CCR7-WT-EGFP plasmids were described previously (Otero et al., 2008). The CCR7 C-terminus truncated sequence was generated by polymerase chain reaction (PCR) using primer design 5′-ATA GAA TTC CGT CAT GGA CCT GGG GAA AC-3′ (EcoRI) (restriction site underlined) and 5′-TGC GGC CGC GCC CAG GTC CTT GAA GAG C-3′ (NotI) (restriction site underlined) based on CCR7 truncation site. This PCR product was digested by EcoRI and NotI and ligated into pcDNA3 CCR7-WT-EGFP vector to remove CCR7-WT. After transformation into competent *Escherichia coli*, clones containing the truncated CCR7 insert were sequence verified.

2.2. Cell line and transfection

Jurkat T cells (human leukemia T cell) were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) and 1% penicillin–streptomycin (P/S). Transient transfections were carried out by the Neon Transfection System following the manufacturer's protocol. Cells were passaged one day before transfection. 2.5×10^5 cells were resuspended in the R buffer containing 1 μg plasmid in a 10 μL Neon tip for each electroporation. The cells were electroporated at 1325 V pulse voltage, 10 ms pulse width for 3 pulses.

2.3. Cell surface receptor expression

CCR7 transfected Jurkat cells were stained with anti-CCR7-Alexa647 (BD Biosciences) for 30 min on ice. After washing twice, cells were analyzed by flow cytometry using a flow cytometer (FACS Canto II, BD Biosciences). The flow data were further analyzed using Flowjo (Tree Star, OR).

2.4. Transwell assay

Transwell assays (Corning Inc, NY) were performed across bare polycarbonate membranes. Briefly, 5×10^5 cells in 100 μL of medium were added to the top well of a 6.5 mm-diameter transwell culture insert with a pore size of 5 μm. The insert was then transferred to wells containing 600 μL of medium with or without different concentrations of chemoattractants. Cells were allowed to migrate in the transwell assay for 4 h at 37 °C with 5% CO_2. Transmigrated cells into the lower well were resuspended in medium and collected for counting using a flow cytometer (FACS Canto II, BD Biosciences). The migration rate was measured as the percentage of the total input cells that migrated into the lower well. The flow data were further analyzed using Flowjo. At least triplicate was performed for the medium control group and CCL19 group.