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8 Dynamamin function is important for CC-chemokine receptor induced cell migration
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Abstract

The HIV viral entry co-receptors CCR5 and CXCR4 function physiologically as typical chemokine receptors. Activation leads to cytosolic signal transduction that results in a variety of cellular responses such as cytoskeletal rearrangement and chemotaxis (CTX). Our aim was to investigate the signalling pathways involved in CC- and CXC-receptor mediated cell migration. Inhibition of dynamin I and II GTPase with dynasore completely inhibited CCL3 stimulated chemotaxis in THP-1 cells, whereas the dynasore analogue dyngo-4a, which is a more potent inhibitor, showed reduced ability to inhibit CC-chemokine induced CTX. In contrast, dynasore was not able to block cell migration via CXCR4. The same activation/inhibition pattern was verified in activated-T-lymphocytes for different CC- and CXC-chemokines. Cell migration induced by CC- and CXC-receptors is not relying on active internalisation processes driven by dynamin since the blockade of internalisation does not affect migration, but they might rely on dynamin interaction with the cytoskeleton. We identify here a functional difference in how CC- and CXC-receptor migration is controlled, suggesting that specific signalling networks are being employed for different receptor classes and potentially specific therapeutic targets to prevent receptor migration can be identified.

Keywords

Chemokine receptor, chemotaxis, dynamin, signalling, internalisation

Introduction

Cellular migration can be activated by chemokine receptors, which are part of the G protein coupled receptors family (GPCRs) [1]. In different disease settings and different cancer types it has been shown that chemokine receptors play a crucial role in promoting cell migration and even cancer growth [2, 3]. Several chemokines (CCL5, CCL8) act as agonists for CCR1, CCR3 and CCR5, whereas a few chemokines, like CCL2, only activate CCR2 and CCR4, but not CCR5 [4, 5]. It has been shown that chemokine receptor activation leads to activation of heterotrimeric G proteins and phosphorylation of the receptor via G protein coupled receptor kinases (GRKs), which in turn leads to binding of β -arrestins to the receptor and is followed by receptor internalisation [4]. Activation is also followed by actin polymerisation but the signalling networks become which activated to allow this to happen have yet to be fully defined.

In recent years it has become clear that GPCRs do not signal solely via G proteins [6]. The so called receptosome of these receptors, which includes β -arrestins and other associating proteins makes signalling of GPCRs comparatively complex. β -arrestins can associate directly with a range of proteins including ERK1/2, cofilin, filamin and Jnk3 and therefore activate a variety of cellular responses without the involvement of G proteins [7]. Ligand biased signalling is important for chemokine receptors and it allows different ligands to activate different signalling cascades by encouraging specific ligand-receptor conformations. Receptors adopting such ligand dependent conformations then display specificity or bias towards certain signalling pathways dependent on which ligand is binding to the receptor [8] and which

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3 receptor class is involved. There is also a marked difference in the regulation of
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5 CXC-receptors and CC-receptors expression on the cell surfaces. Whereas CCR5,
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7 CCR2 and CCR4 internalise via clathrin-coated-pits and caveolae, CXCR3 and
8
9 CXCR4 only use clathrin-coated pits [9-13]. Similarly, CCR5 recycles back to the cell
10
11 surface, whereas CXCR3 and CXCR4 are targeted to the late endosomes and
12
13 lysosomes [10, 12, 13].
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15
16 Traditionally it is thought that $\beta\gamma$ -subunits of the G proteins induce migration via
17
18 activation of PI3K [14], however we have recently shown that this seems not to be
19
20 the case for CCL3 induced migration in THP-1 cells [15]. For CXCR4 it has been
21
22 shown that migration under certain circumstances is dependent on β -arrestins as
23
24 well as filamin-A, a protein, which can bind actin and interacts with β -arrestins [16,
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26 17]. This raises the possibility that β -arrestin and actin interacting proteins are
27
28 activated downstream of different types of chemokine receptors. One of these actin
29
30 interacting proteins is dynamin. Several groups have shown that dynamin, an
31
32 enzyme which has traditionally been linked to internalisation of receptors via clathrin-
33
34 coated pits, is important for the integral structure of actin polymers [18-20].
35
36 Dynamins are large multi-domain proteins (~100 kDa) that constitute an N-terminal
37
38 GTPase domain, a middle domain, a PH domain, a GTPase effector domain (GED),
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40 and a C-terminal proline-rich domain (PRD), which interacts with proteins that
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42 contain SH3 domains [21] and there are several types of the protein: dynamin I is
43
44 primarily found in neurones where it is involved in synaptic vesicle endocytosis [22,
45
46 23] and it has been linked with several neurological processes such as long-term
47
48 memory formation [24]. Dynamin II is ubiquitously expressed and is found in all cell
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50 types, dynamin III is primarily found in the testis. Dynamin II interacts with numerous
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52 GPCRs as well as non-GPCR receptors, including the chemokine receptor CCR5 [25]
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1
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3 and various cytokine receptors [26] and is, therefore, an interesting target protein to
4
5 investigate chemokine receptor triggered migration.
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8 Here we analysed different small molecule inhibitors for their effects on chemokine
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10 receptor-induced migration and release of intracellular calcium. We investigated
11
12 whether dynamin plays a role for both CC-receptor- as well as CXC-receptor-
13
14 induced migration or whether distinct signalling pathways are activated by different
15
16 subsets of receptors.
17

18 19 20 21 **Methods**

22 23 24 *Cells and materials*

25
26 Culture conditions for THP-1 cells have previously been described [9]. Jurkat cells
27
28 were obtained from ATCC and grown in RPMI containing 10% FCS and 2 mM L-
29
30 glutamine. Blood was sampled from healthy normal subjects according to a protocol
31
32 approved by a local ethics committee (reference number 2008042). Peripheral blood
33
34 mononuclear cells (PBMCs) were subsequently isolated as previously described by
35
36 Sabroe et al. [27]. Lymphocytes were separated from monocytes by allowing the
37
38 latter to adhere to a tissue culture flask for 2 hours at 37 °C and 5% CO₂ and were
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40 activated by culture in the presence of IL-2 (200 mg/mL) and concanavalin A (30
41
42 mg/mL) for at least 10 days. The chemokine used for CCR5/CCR1 activation was
43
44 human CCL3 (D26A) and has been described before [9, 28]. CXCL11 and CXCL12
45
46 were from Peprotech (UK). Dynamin inhibitors dynasore, dyngo-4a, MiTMAB,
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48 OcTMAB, dynole-34-2, dynole-31-2 (negative control), iminodyn-22 and iminodyn-17
49
50 (negative control), pyrimidin-7 were purchased from Abcam (for an overview of
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52 dynamin inhibitors see Table 1). Clathrin-mediated endocytosis inhibitor pitstop 2
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3 and the corresponding negative control were from Abcam. All other chemicals were
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5 from Fisher Scientific.
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8 9 *Chemotaxis Assays*

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11 Cells were harvested and washed twice with pre-warmed, sterile PBS, then
12
13 resuspended in serum-free RPMI 1640 which contained 0.1% BSA. The
14
15 concentration of cells was adjusted to 6.25×10^7 cells/mL. Chemoattractants were
16
17 loaded in a final volume of 31 μ L at indicated concentrations in the lower
18
19 compartment and 20 μ L of resuspended cells were loaded onto the upper
20
21 compartment of a microchemotaxis chamber (Receptor Technologies, Adderbury,
22
23 UK). The two compartments were separated by a polyvinylpyrrolidone-free
24
25 polycarbonate filter with 5 μ m pores. For inhibitor treatment, cells were incubated for
26
27 30 minutes with the inhibitor or with vehicle control before loading onto the upper
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29 compartment of the chamber. Chambers were incubated at 37°C and 5% CO₂ for 4
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31 hours before cells were counted. Data was analysed as previously described [15].
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38 *Analysis of data*

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40 Data were analysed using GraphPad Prism 5 (GraphPad Software). Statistical
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42 analyses were performed using a one-way ANOVA with a Bonferroni multiple
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44 comparison test as post-hoc test with a p value <0.05 deemed significant. In all
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46 figures, data represent the mean \pm S.E.M. of at least three independent experiments.
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52 **Results**

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3 Chemokine receptors are expressed on different cell types, and THP-1 cells express
4 naturally CCR1, CCR2 and CCR5 as well as CXCR4 and migrate towards stimuli
5 with CCL2, CCL3, CCL8, CCL23 and CXCL12 whereas Jurkat cells express CXCR4
6 and migrate towards stimuli with CXCL12, but not towards CCL3. Activated T-cells
7 have been shown to express functional CXCR3 and CCR5 and migrate towards
8 CXCL11 and CCL3 [29]. We therefore used these different cells to investigate the
9 effect of dynamin inhibitors on cell migration with the view to differentiate between
10 CC-receptor and CXC-receptor family behaviour. In our hand Jurkat cells do not
11 migrate towards CCL3 and hence we used THP-1 cells for both CCL3 and CXCL12.
12 Dynasore blocks migration towards CCL3 in THP-1 cells in a dose dependant
13 manner (Figure 1a). At a concentration where dynasore clearly blocks CCL3 induced
14 migration (40 μ M), it does not affect CXCL12 induced migration in THP-1 (Figure 1b).
15 To rule out any ambiguities, we used the higher concentration of dynasore (80 μ M) in
16 activated T-cells. Dynasore does not block migration of activated T-cells towards
17 CXCL11, whereas, there is a clear trend of inhibition towards CCL3 induced
18 migration (Figure 1 c,d), showing a distinct difference in the activation pattern of
19 CXC- and CC-receptors. Confirming the differences between CC- and CXC-
20 receptors are results with CXCL12 in Jurkat cells, where dynasore has no effect at
21 all on migration, even at 80 μ M (Figure 1 e). At the concentration used, none of the
22 dynamin inhibitors showed any cytotoxic effects in the experimental set up, as shown
23 by MTS assays (data not shown).
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51 The dynasore analogue dyngo-4a, which is more potent than dynasore (dyngo-4a
52 IC_{50} 16 \pm 1.2 μ M versus dynasore 79.3 \pm 1.3 μ M [30]) blocks migration towards CCL3
53 in THP-1 cells to a lesser degree than dynasore. Remarkably dyngo-4a blocks
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3 migration towards CXCL11 in activated T-cells and CXCL12 in THP-1 and Jurkat
4 cells (Figure 2). Dyngo-4a shows selectivity towards dynamin I versus dynamin II
5 whereas dynasore is non-selective and therefore these results might reflect a
6 different usage of the dynamin isoforms by different receptors (see Table 1). We
7 further investigated which domains of the dynamin proteins are essential for cell
8 migration and whether they are equally important for different receptor families. In
9 the first instance, we used iminodyn-22 and dynole-34-2 which are both non-
10 selective dynamin I and II inhibitors, and their negative controls which are iminodyn-
11 17 and dynole-31-2. Neither iminodyn-22 nor dynole-34-2 block migration in THP-1
12 cells towards CCL3 or in Jurkat cells towards CXCL12 (Figure 3 a-d). However there
13 is a distinct difference between CCL3- and CXCL12-induced chemotaxis for the non-
14 selective MiTMAB and OcTMAB inhibitors which bind to the dynamin PH domain [30],
15 and completely block any migration in Jurkat cells towards CXCL12 (Figure 3 f), but
16 have no significant effect on CCL3-induced migration in THP-1 cells (Figure 3 e,g)
17 but still effect CXCL12 migration in THP-1, even though with less of an effect than in
18 Jurkat cells (Figure 3 h). Again this data shows a clear difference in the reliance of
19 CC- and CXC-receptors on dynamin usage. We also used pyrimidyn-7, which
20 competitively inhibits both GTP and phospholipid binding and is the only inhibitor
21 available up to now which targets two distinct domains of dynamin. There is no effect
22 on CCL3 induced migration in THP-1 cells (Figure 4 a), but CXCL12 induced
23 migration is significantly blocked in Jurkat cells (Figure 3 b) as well as THP-1 cells
24 (data not shown).

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Dynamin is classically known as being of importance for clathrin-coated pit triggered internalisation of receptors, even though recently its importance for actin-dynamics have become more apparent [19]. We previously showed that CCR5 can use

1
2
3 clathrin-coated pits for internalisation [9, 31] and indeed dynamin inhibition via
4
5 dynasore completely abrogates internalisation on CHO.CCR5 as well as THP-1 cells
6
7 as analysed via immunofluorescence (data not shown). To investigate whether it is
8
9 the prohibition of internalisation which prevents cell migration, we used another
10
11 clathrin-coated pit endocytosis inhibitor, pitstop 2 and its negative control analogue in
12
13 THP-1 cells for CCL3 activation as well as in Jurkat cells for CXCL12 activation.
14
15 Pitstop 2 does not block cell migration (Figure 4 c,d). The concentration of pitstop 2
16
17 used for migration assays actually inhibits internalisation of CCR5 receptor in THP-1
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19 cells (data not shown). An increase of the concentration of pitstop 2 used in THP-1
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21 cells, actually increased the number of migrating cells by a small, but significant
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23 amount.
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30 Discussion

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34 In this study we investigated the role of dynamin in the signalling events that occur
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36 after the activation of CC- and CXC-receptors. Dynamin involvement in cell migration
37
38 is related to its role as a focal adhesion regulator and it has been shown that
39
40 inhibition of dynamin 2 inhibits focal adhesion disassembly and impairs cell migration.
41
42 We, therefore, used different dynamin inhibitors, which either have a higher potency
43
44 for dynamin I over II (dyngo-4a) or are non-selective dynamin I and II inhibitors
45
46 (dynasore, dynole-34-2, MiTMAB, OcTMAB, iminodyn-22, pyrimidyn-7) [30, 32, 33].
47
48 Dynasore has been shown previously to block endocytosis via clathrin-coated pits
49
50 [34-36] and indeed it blocks CCL3-induced endocytosis of CCR5 in CHO.CCR5 cells.
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52 Dynasore blocks CCL3 induced migration in THP-1 cells and activated T-
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54 lymphocytes, but it has no effect on either CXCL12-induced migration of THP-1 or
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3 Jurkat cells or CXCL11-induced migration of activated T-lymphocytes. These results
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5 point towards a significant difference between CC- and CXC-receptor activated
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7 signalling networks. Dyngo-4a, a close analogue of dynasore, which is more potent
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9 than dynasore and has a higher potency for dynamin I over dynamin II [30], is less
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11 effective in blocking CCL3-induced migration in THP-1 cells, however it blocks
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13 CXCL11- and CXCL12-induced migration in activated T-lymphocytes and Jurkat
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15 cells, respectively. Similarly, dynasore can significantly reduce CCL2 induced
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17 migration in THP-1 cells, whereas dyngo-4a shows a trend to inhibit migration, but
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19 does not reach significance. The functional differences between dynasore and
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21 dyngo-4a have not been fully analysed yet, but with the knowledge available today,
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23 our data points towards either a different usage of dynamin isoforms by chemokine
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25 receptor subtypes or to the usage of a varying set of dynamin interacting protein by
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27 different receptor subfamilies. This difference between CC- and CXC-receptors was
28
29 further highlighted by the use of dynole-34-2, MiTMAB, OcTMAB, iminodyn-22 and
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31 pyrimidyn-7. None of those blocked CCL3-induced migration in THP-1 cells, but
32
33 pyrimidyn-7, MiTMAB and OcTMAB block CXCL12-induced migration in THP-1 and
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35 Jurkat cells. Unlike MiTMAB and OcTMAB, which block dynamin recruitment to the
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37 membranes, dynole-34-2, dyngo-4a and dynasore block dynamin function after its
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39 recruitment [30]. MiTMAB, OcTMAB also bind to the PH-domain of the dynamin
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41 molecule, unlike the other inhibitors, which bind to the G domain. An obvious reason
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43 for the prevention of migration after the use of dynamin inhibitors is the potential
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45 importance of internalisation for receptor activation and ultimately signal transduction.
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47 We, therefore, employed a different clathrin-coated pits endocytosis inhibitor, pitstop
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49 2 and its negative control compound to analyse whether internalisation is a pre-
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51 requisite for migration. In both THP-1 cells and Jurkat cells pitstop 2 did not prevent
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3 CCL3- and CXCL12-induced migration, respectively, which is evidence that receptor
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5 internalisation is not necessary to activate cell migration as had been described
6
7 already for the CCR2b receptor [37].
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10 In our study we detect distinct differences between the CC- and CXC-receptors.
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12 Traditionally it has been shown that CXCR4 activation leads to chemotaxis in a β -
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14 arrestin 2, ERK 1/2, G β γ dependent manner and is PI3K dependent [38, 39].
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16 Therefore the implication of the dynamin PH domain in cell migration for CXCL12 is
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18 in line with the already published signalling networks, whereas the PH domain is not
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20 necessary for CCL3-induced migration, since this migration is independent of PI3K
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22 activation [15]. Overall our study showed that there are distinct differences in
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24 signalling networks used by CC-receptors compared to CXC-receptors which will
25
26 yield novel therapeutic targets to prevent cell migration triggered by specific
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28 receptors.
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37
38 support for SCM from the Novartis studentship fund.
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10 11 12 13 14 15 **Figures and legends**

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19 **Figure 1:** Cell migration towards CCL3 but not towards CXCL11 or CXCL12 is
20 blocked by dynasore. a) THP-1 cells were treated with 16, 40 or 80 μ M of dynasore.
21 b) THP-1 cells were treated with 40 μ M of dynasore. Migration was induced with 1
22 nM CXCL12. c) Activated T-lymphocytes were treated with 80 μ M of dynasore and
23 migration was induced with 20 nM CCL3. d) Activated T-lymphocytes were treated
24 with 80 μ M of dynasore and migration was induced with 1 nM CXCL11. e) Jurkat
25 cells were treated with 80 μ M of dynasore and migration was induced with 1 nM
26 CXCL12. Base level of migration was determined in the absence of chemokines.
27 Statistical analysis were performed using a one-way ANOVA with a Bonferroni
28 Multiple comparison test as post-test ** showing a p value <0.01 and *** <0.001 .
29 Data represent the mean \pm S.E.M. of at least three independent experiments.
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46 **Figure 2:** Cell migration towards CCL3, CXCL11 and CXCL12 is blocked by dyngo-
47 4a. a) THP-1 cells were treated with 80 μ M of dyngo-4a. Migration was induced with
48 1 nM CCL3. b) Activated T-lymphocytes were treated with 80 μ M of dyngo-4a and
49 migration was induced with 20 nM CXCL11. c) THP-1 cells were treated with 80 μ M
50 of dyngo-4a and migration was induced with 1 nM CXCL12. d) Jurkat cells were
51 treated with 80 μ M of dyngo-4a and migration was induced with 1 nM CXCL12.
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3 Statistical analysis were performed using a one-way ANOVA with a Bonferroni
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5 Multiple comparison test as post-test with * showing a p value <0.05, ** showing a p
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7 value <0.01 and *** showing a p value < 0.001. Data represent the mean \pm S.E.M. of
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9 at least three independent experiments.
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14 **Figure 3:** Effect of different dynamin inhibitors on migration towards CCL3 and
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16 CXCL12. a) THP-1 cells were treated with 1 μ M of iminodn-17 and 1 μ M of
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18 iminodn-22. Migration was induced with 1 nM CCL3. b) Jurkat cells were treated
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20 with 1 μ M of iminodn-17 and 1 μ M of iminodn-22. Migration was induced with 1 nM
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22 CXCL12. c) THP-1 cells were treated with 15 μ M dynole-31-2 and 15 μ M dynole-34-
23
24 2 and migration was induced with 1 nM CCL3 d) Jurkat cells were treated with 15 μ M
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26 dynole-31-2 and 15 μ M dynole-34-2 and migration was induced with 1 nM CXCL12.
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28 e) THP-1 cells were treated with 10 μ M of MiTMAB and migration was induced with 1
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30 nM CCL3. f) Jurkat cells were treated with 10 μ M of MiTMAB and 5 μ M of OcTMAB.
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32 Migration was induced with 1 nM CXCL12. g) THP-1 cells were treated with 5 μ M of
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34 OcTMAB. Migration was induced with 1 nM CCL3. h) THP-1 cells were treated with
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36 10 μ M of MiTMAB and 5 μ M of OcTMAB. Migration was induced with 1 nM CXCL12.
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38 Statistical analysis were performed using a one-way ANOVA with a Bonferroni
39
40 Multiple comparison test as post-test with *** showing a p value < 0.001. Data
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42 represent the mean \pm S.E.M. of at least three independent experiments.
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50 **Figure 4:** Effect of endocytosis inhibitors on migration towards CCL3 and CXCL12. a)
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52 THP-1 cells were treated with 10 μ M of pyrimidyn-7. Migration was induced with 1
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54 nM CCL3. b) THP-1 cells were treated with 10 μ M of pyrimidyn-7. Migration was
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56 induced with 1 nM CXCL12 and migrated cells were counted after 4 hours. c) THP-1
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3 cells were treated with 1.25 μM of pitstop 2 and pitstop 2 negative control compound
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5 and 30 μM of pitstop 2 and pitstop 2 negative control compound, respectively.
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7 Migration was induced with 1 nM CCL3. d) Jurkat cells were treated with 30 μM of
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9 pitstop 2 and pitstop 2 negative control compound, respectively and migration was
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11 induced with 1 nM CXCL12. Statistical analysis were performed using a one-way
12
13 ANOVA with a Bonferroni Multiple comparison test as post-test with ** showing a p
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15 value < 0.01 and *** showing a p value < 0.001. Data represent the mean \pm S.E.M.
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17 of at least three independent experiments.
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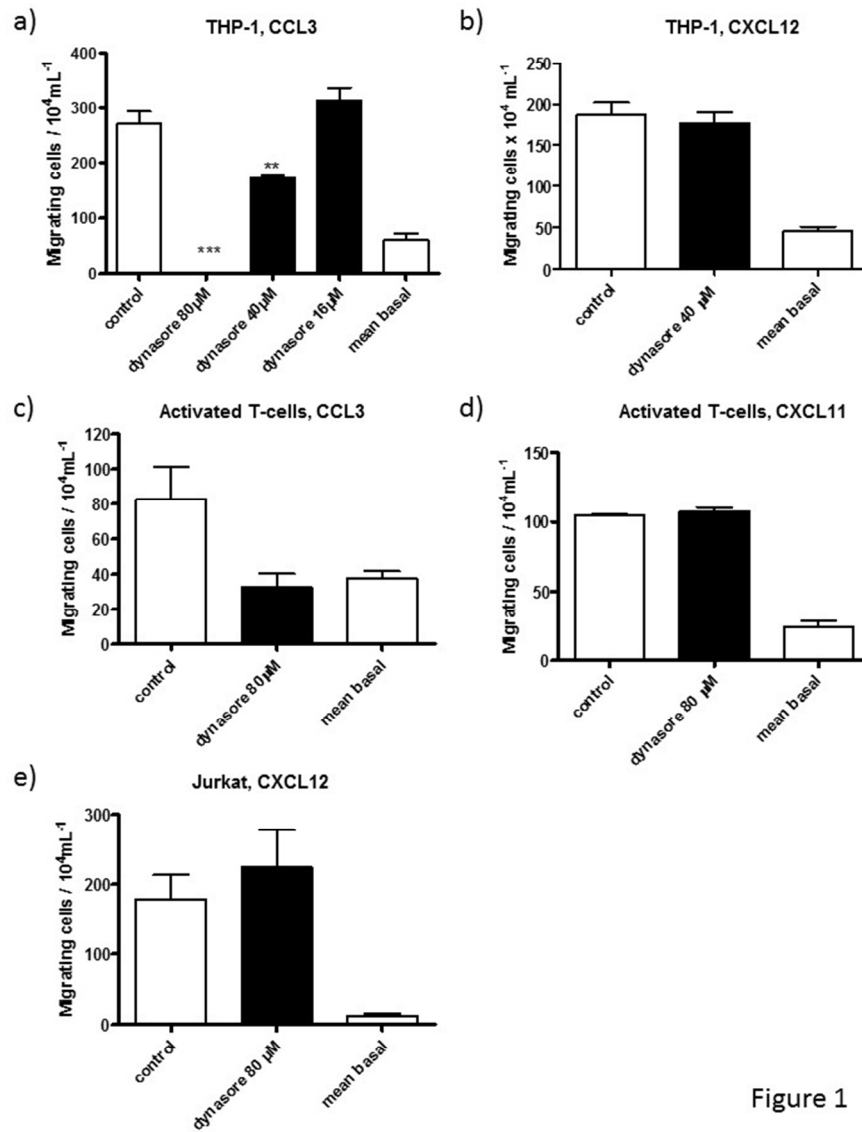


Figure 1

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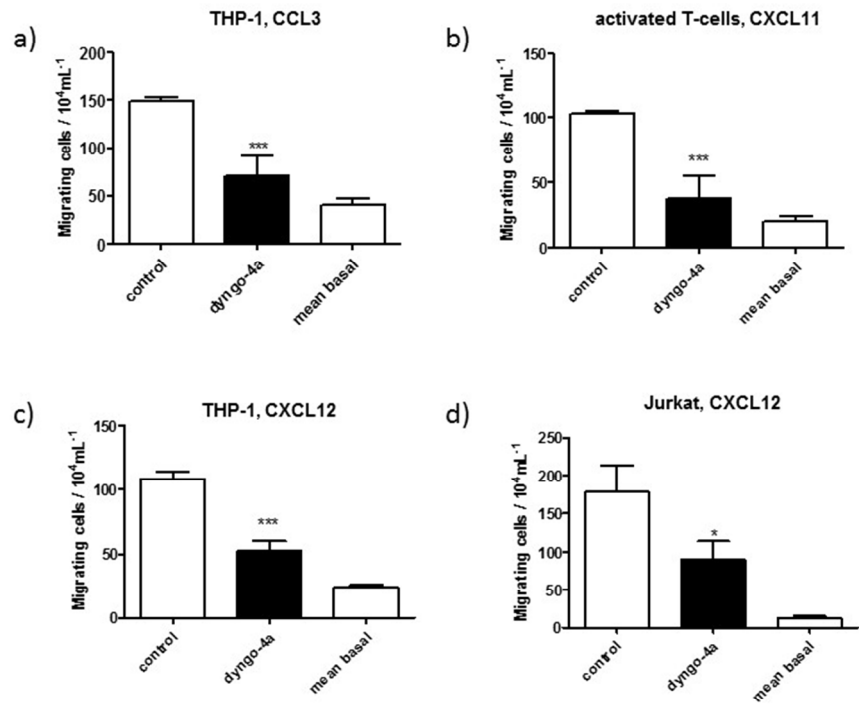


Figure 2

190x254mm (96 x 96 DPI)

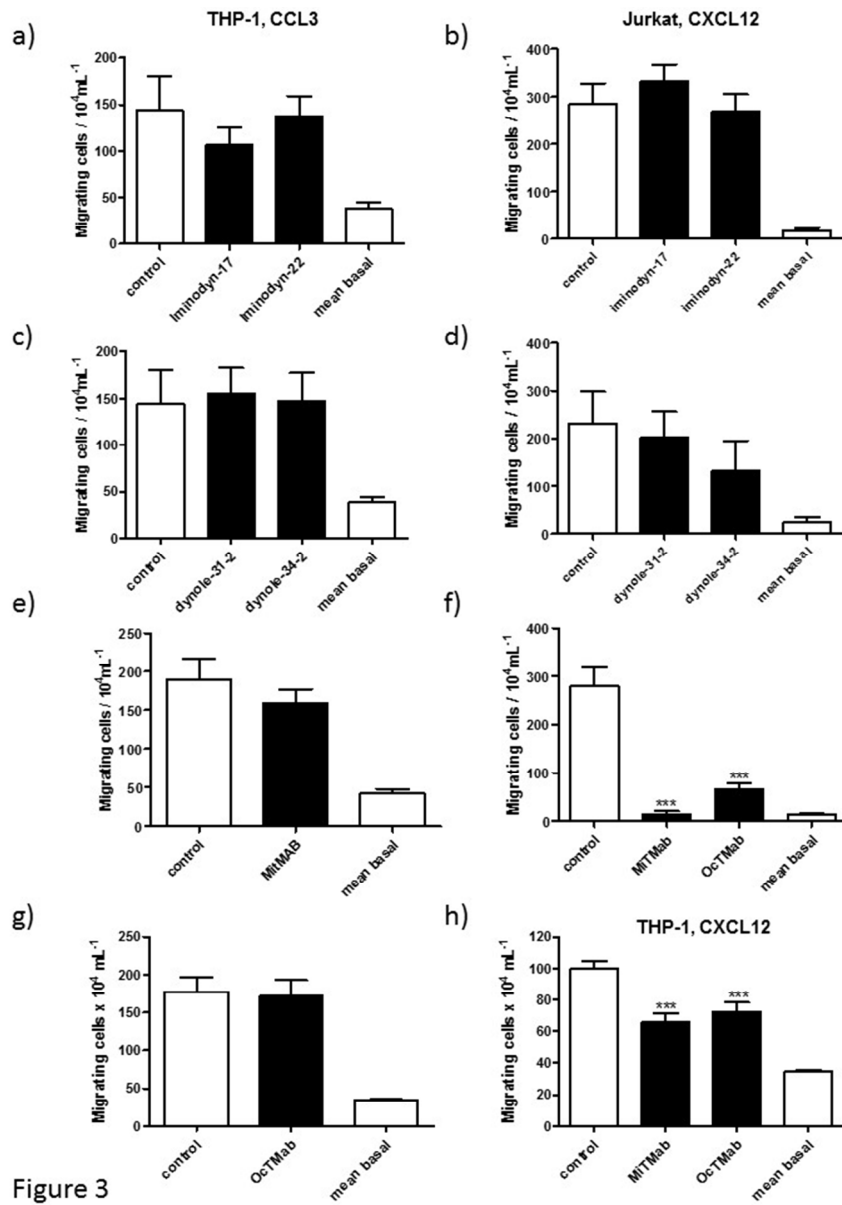


Figure 3

190x254mm (96 x 96 DPI)

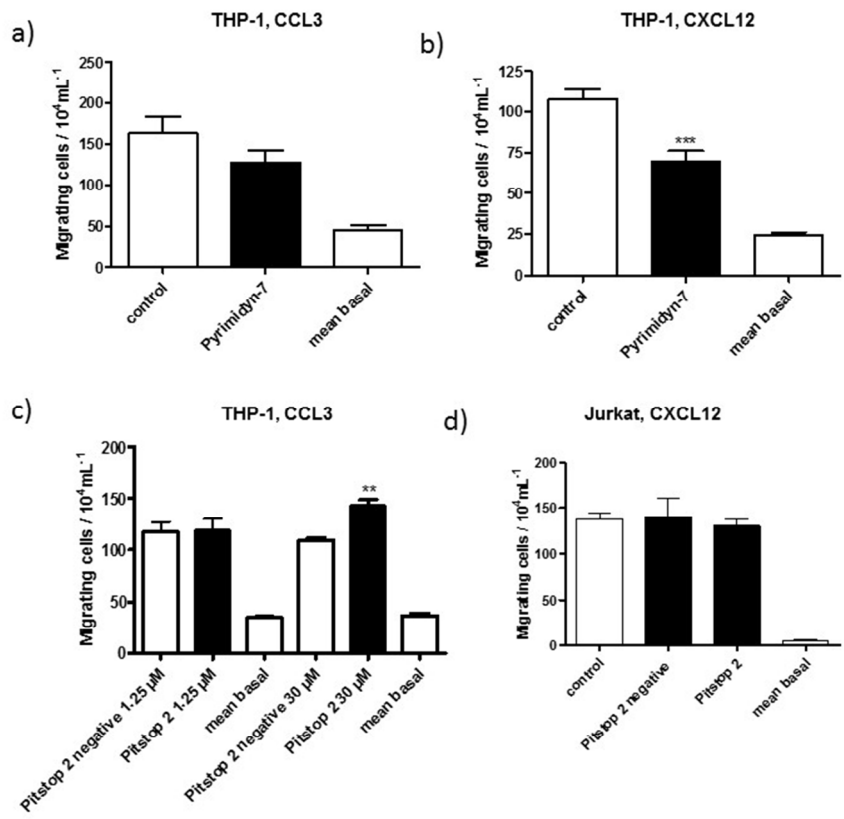


Figure 4

190x254mm (96 x 96 DPI)

Table 1: Overview of dynamin inhibitors

Inhibitor	Mode of action	IC _{50(SVE)} ^a (μM)	Specificity Dynamin I versus II (μM)	Refs
Dyngo-4a TM	G domain: allosteric site	16±1.2	Dynamin I selective 0.38±0.05 vs 2.6±0.12	[30, 35]
Dynasore	Unknown: non-competitive inhibition	79.3±1.3	Non-selective	[35]
Dynole-34-2 TM	G domain: uncompetitive with GTP	105	Non-selective	[36]
Iminodyn 22 TM	G domain: uncompetitive with GTP	99.5±1.7	Non-selective	[33]
Pyrimidin-7 TM	Competitively inhibits both GTP and phospholipid binding	Not reported	Non-selective 1.1vs1.8	[32]
MiTMAB TM	PH domain: competitive with lipid and non-competitive with GTP	105	Non-selective	[36]
OcTMAB TM	PH domain: competitive with lipid and non-competitive with GTP	Not reported	Non-selective	[36]

^a FM4-64 uptake in synaptosomes