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Distinct modes of molecular regulation of CCL3 induced calcium flux in monocytic cells

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Abstract

The chemokine receptor CCR5 has been shown to be targeted to cholesterol- and sphingolipid-rich membrane microdomains. Here we elucidate the effects of membrane fluidity on CCR5 signalling and expression using the monocytic THP-1 cells. MCD treatment of THP-1 cells, which removes nearly all cholesterol from the plasma membrane, leads to an increase in the signalling properties of CCR5. In contrast, the prevention of cholesterol production with lovastatin and simvastatin decreases the release of intracellular calcium and also decreases receptor cell surface expression. The loss of response in lovastatin treated cells can be rescued by MCD addition, which shows that the cholesterol content in the membrane is only one factor in determining the amount of receptor response. We show that CCR5 signalling is dependant on thapsigargin-sensitive Ca^{2+} stores and on activation of ryanodine receptors as well as InsP3 receptors or store-operated channels. Cholesterol depletion with MCD changes the thapsigargin sensitivity in THP-1 cells

1 and also alters receptor-G-protein coupling towards pertussis toxin (PTX)
2 independent G-proteins. Cholesterol removal by MCD in THP-1 cells has far
3 reaching consequences for receptor activation and signalling and emphasises the
4 need for a clearer understanding of how membrane fluidity affects receptor signalling
5 events.
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11 **Keywords**

12 chemokine receptor; caveolae; cholesterol; microdomain; statin
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17 **Abbreviations**

18 CHO cells, Chinese hamster ovary cells; FCS, foetal calf serum; FITC, fluorescein
19 isothiocyanate; HEK, human embryonic kidney; InsP3, inositol 1,4,5-trisphosphate
20 receptor, MCD, methyl- β -cyclodextrin; PBS, phosphate buffered saline; PTX,
21 Pertussis Toxin; SOCs, store-operated channels
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36 **1. Introduction**

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41 The chemokine receptor, CCR5, was originally characterised as a receptor that
42 responds functionally to the CC-chemokines CCL3, CCL4 and CCL5 [1]. We have
43 previously shown that CCR5 internalisation can be inhibited by treatment with
44 sucrose, nystatin and filipin, which inhibit clathrin-coated pit internalisation and affect
45 caveolae in the cell membrane, respectively [2]. There have been reports about a
46 number of receptors, like CCR4 [3] or CCR2 [4] where caveolae are important for
47 receptor internalisation. Furthermore it has been reported that chemokines bind
48 preferentially to receptors which are associated with cholesterol and sphingolipid-rich
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1 lipid raft microdomains in the plasma membrane [5]. It was shown that CCR5
2 requires membrane cholesterol to retain ligand binding and signalling functions [6-8].
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7 Additionally we could confirm previously that the amount of cholesterol in the plasma
8 membrane is important for CCR5 induced signalling in stably transfected CHO and
9 HEK cells. Both filipin and methyl- β -cyclodextrin (MCD) affect cholesterol in the
10 membrane, however in different ways [9, 10]. We showed that MCD treatment lead
11 to failure of CCR5 to inhibit forskolin-stimulated accumulation of cAMP and it also
12 changed the potency of CCL3 for intracellular calcium release, however cholesterol
13 depletion had no effect on receptor internalisation. Furthermore, depletion of
14 cholesterol changed the coupling of CCR5 from G α i G-protein to a PTX independent
15 G-protein, as shown by the release of intracellular calcium after PTX treatment [11].
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31 Other studies also highlighted the importance of cholesterol for receptor
32 conformation and signalling [6, 12, 13]. Recently it was revealed that cholesterol is
33 important for chemotaxis of human neutrophils, but has no influence on early
34 signalling by chemokine receptors on these cells [14], highlighting the need to
35 investigate the function of membrane cholesterol further.
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46 In this study we characterise the signalling properties of CCR5, especially the ability
47 of CCR5 to release intracellular Ca²⁺ ions and also investigate how membrane
48 fluidity influences receptor activation. Chemokine receptor induced calcium signalling
49 is not completely understood. Several inhibitors, like omega-conotoxin GVIA (an N-
50 type calcium channel blocker), thapsigargin (a sarcoendoplasmic reticulum Ca²⁺-
51 ATPase inhibitor) as well as phospholipase c and protein kinase c inhibitors seem to
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1 affect CCL2 and CXCL1 induced calcium response [15], giving some indication
2 about how chemokine receptor activation is coupled to calcium signalling. However
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4 for CCR5, no characterisation of the mechanisms for the intracellular calcium release
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6 after receptor activation has been shown so far. Here we analyse whether the
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8 endoplasmic reticulum Ca^{2+} -ATPases, inositol 1,4,5-trisphosphate (InsP3) receptors
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10 and ryanodine receptors are involved in CCR5 instigated signalling events. We also
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12 characterize the effect of membrane fluidity by either cholesterol depletion or the
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14 blockade of cholesterol production on receptor activation and expression in
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16 monocytic cells.
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24 **2. Materials and Methods**

29 **2.1 Cells and materials**

31 CHO cells expressing CCR5 were obtained as described in [2], HEK.CCR5 cells
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33 have been described previously [16] and the monocytic cell line THP-1 was obtained
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35 from the ATCC (Teddington, UK). The chemokine used for CCR5 activation was
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37 CCL3 (D26A), which was generously donated by Lloyd Czaplewski of British Biotech
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39 (UK). We have referred to the isoform used in this study in a previous publication as
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41 CCL3 (2–70) (D26A) in comparison with the full gene sequence for CCL3 [17] and it
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43 is referred to in the text as CCL3. The anti-CCR5 antibody, HEK/1/85a/7a has been
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45 described previously [2, 17] and it has been shown that the antibody binds
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47 independently of chemokines. Secondary antibodies were obtained from Sigma
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49 Aldrich (Poole, UK) and Santa Cruz (Heidelberg, Germany). Nystatin, filipin, sucrose,
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51 cholesterol, Pertussis toxin (PTX), simvastatin and methyl- β -cyclodextrin (MCD)
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53 were purchased from Sigma Aldrich. Cells were incubated for 1 hour at 37°C with
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filipin (5 $\mu\text{g}/\text{mL}$), sucrose (0.4 M), nystatin (50 $\mu\text{g}/\text{mL}$), MCD (10 mM) or cholesterol (2 mM), respectively, before an assay was performed. Lovastatin was obtained from Tocris (Bristol, UK) and activated before use as described by the manufacturer. 2-ABP, thapsigargin and ryanodine were also purchased from Tocris and cells were treated with 20 μM 2-ABP, 1 μM for thapsigargin or 10 μM for ryanodine unless stated otherwise for 1 hour before induction of calcium flux. For PTX treatment, cells were incubated 18 hours with PTX (1 $\mu\text{g}/\text{mL}$) before an assay was completed. All other chemical were purchased from Fisher Scientific (Loughborough, UK).

2.2 Analysis of intracellular calcium ion concentration

Cells were harvested with 2 mM EDTA/PBS and washed twice in buffer (148 mM NaCl, 5 mM KCl, 2.5 mM CaCl_2 , 10 mM Hepes, 1 mM glucose, pH 7.4) and incubated with 4 μM Fura-2 (Invitrogen, Paisley, UK) at 37°C for 1 hour. For assays in the absence of extracellular calcium, cells were washed twice in buffer containing no CaCl_2 and all steps of the experiment were performed in buffers without CaCl_2 . Inhibitors were present during the incubation period. After washing cells with buffer, cells were resuspended at 2×10^6 cells/mL of buffer. Chemokine-induced intracellular calcium mobilisation was determined by BMGlabtech Fluostar OPTIMA fluorometer (BMGlabtech, Offenburg, Germany). In all experiments cells were challenged with different concentrations of CCL3 after 15 or 20 sec incubation in the fluorometer and measurements were taken over a further 60 seconds. The peak values of intracellular calcium ion concentration following the chemokine challenge were determined as described by Grynkiewicz [18].

2.3 Flow cytometry

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2 Cells were treated with MCD, lovastatin or vehicle (control) cells as described,
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4 washed with PBS and stained with saturating amounts of HEK/1/85a/7a antibody or
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6 isotype control antibody for 1 hour at 4°C to prevent internalisation. To remove
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8 excess amounts of antibody, cells were washed three times with ice-cold PBS,
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10 incubated with corresponding fluorescent-labelled secondary antibody for 1 hour at
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12 4°C, washed three times with ice-cold PBS and fixed with 4% paraformaldehyde.
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14 Receptor expression levels were determined using a Coulter Elite FACS.
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2.4 Cholesterol Depletion

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23 For cholesterol depletion of cells we used two different strategies. De novo
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25 cholesterol synthesis was inhibited by lovastatin, an inhibitor of HMG-CoA reductase.
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27 Plasma membrane cholesterol was extracted by methyl- β -cyclodextrin. Methyl- β -
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29 cyclodextrin is a well established tool that selectively and quickly extracts cholesterol
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31 from plasma membranes in preference to other lipids [19]. These treatments reduce
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33 intracellular cholesterol without affecting cell viability within the time window selected
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35 for the experiments. Control cells were left untreated.
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2.5 Cholesterol Determination

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45 Cholesterol levels were determined using the Amplex Red cholesterol assay
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47 (Invitrogen) in accordance to the manufacturers protocol. Cells, which were adjusted
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49 same cell amount, were pre-incubated with cholesterol oxidase and horseradish
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51 peroxidase and Amplex red (all obtained from Invitrogen in amounts described by
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53 the manufacturer) for 20 minutes in the absence of light at 37°C. Fluorescence was
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1 measured using an excitation wavelength of 530 ± 10 nm and emission wavelength
2 of 590 ± 10 nm.
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7 **2.6 Analysis of data**

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9 Data were analysed using GraphPad Prism (GraphPad Software, LaJolla, Ca, USA).
10 Concentration/response curves for CCL3 in calcium flux assays were fitted well by
11 models assuming a Hill coefficient of 1. Statistical analyses were performed using
12 one-way ANOVA with Bonferroni's multiple comparison as a post-test were
13 appropriate. Otherwise statistical analysis were performed using Student's *t*-test
14 with a *P* value <0.05 . Data represent the mean \pm s.e.m. of at least three independent
15 experiments.
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29 **3. Results**

30 **MCD depletion of cholesterol increases signalling in THP-1 cells**

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34 We previously showed that depleting the plasma membrane of cholesterol by using
35 methyl- β -cyclodextrin (MCD) inhibits CCR5 induced calcium release in stably
36 transfected CHO and HEK cell lines, whereas complexing cholesterol with filipin or
37 nystatin does not affect signalling by the receptor [11]. Here we investigate in more
38 detail how cholesterol and resulting changes in membrane fluidity affect receptor
39 signalling in a monocytic cell line. Treatment of THP-1 cells with MCD and
40 subsequent activation of CCR5 with CCL3 leads to a substantial increase in the
41 release of intracellular calcium (Figure 1A) compared to untreated control cells
42 (Figure 1A, B). We therefore investigated whether this multiplication of the signal is
43 dependant on the dosage of MCD used. Indeed there is a perfect concentration-
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1 signal relationship between the amount of MCD used to treat THP-1 cells and the
2 signal that is induced by CCL3 (Figure 1C, D). This result is a contradiction of our
3 previous data, where MCD treatment significantly blocks the release of calcium [11].
4 To determine whether this increase can be observed with cholesterol complexing
5 agents, we also used nystatin and filipin to complex cholesterol in the membrane and
6 observed that these compounds lead to a slight increase in the release of calcium,
7 however this increase is not significant (Figure 1E, F). This is in congruence with
8 data from CHO and HEK cells [11].
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22 To understand why MCD increases the signal and does not reduce it, we analysed
23 the CCR5 receptor expression on different cells after treatment with MCD or
24 lovastatin, which prevents cholesterol production in cells. MCD treatment has no
25 significant diminishing effect on receptor expression on either CHO.CCR5 (data not
26 shown) or HEK.CCR5 cells as analysed by immunofluorescence (data not shown)
27 and flow cytometry (Figure 2 A, B) and a loss of receptor expression is therefore not
28 the reason why the signal in those cells is reduced. On the other hand in THP-1
29 cells, the receptor expression is slightly increased after MCD treatment (Figure 2 A,
30 B). This is in stark contrast to lovastatin, which blocks cholesterol production.
31 Lovastatin diminishes the number of receptors on the cell surface significantly
32 (Figure 2A), as has been described by other groups for CCR2 receptors [20]. The
33 THP-1 cells used in this study do not consist of a uniform population as can be seen
34 in Figure 2B, some cells express consistently higher levels of CCR5, this might be a
35 reflection of a different maturation state of the cells.
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1 The increase in receptor expression is not the sole reason why the signal in THP-1
2 cells increases, the loss of cholesterol and therefore changes in the fluidity of the
3 membrane are also important. Re-introducing cholesterol into the membrane after
4 depleting it first with MCD diminishes the signal to its basal level (Figure 2 C, D).
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10 The loss of cholesterol not only alters the signal, it also changes the coupling of
11 receptor to G-protein. This is similar to the results we observed earlier in CHO.CCR5
12 and HEK.CCR5 cells [11]. Treatment of THP-1 cells with PTX abolishes the
13 signalling properties of CCR5 via PTX dependent G-proteins. Treatment of cells with
14 PTX and MCD results in the release of intracellular calcium, which is due to CCR5
15 signalling via PTX independent G-proteins in the absence of cholesterol (Figure 2 E,
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27 F).

31 **Statin treatment of THP-1 cells leads to different calcium response than MCD**

32 This increase of signal in THP-1 cells is totally unexpected and therefore the
33 question whether cholesterol levels are the determining factor for the efficacy of
34 receptor signalling or whether MCD itself changes the signalling properties of the
35 receptor remain. Hence statins, which block the cholesterol production in cells were
36 used to investigate this further (Figure 3). In THP-1 cells, lovastatin as well as
37 simvastatin treatment over three days decreased the CCL3 induced release of Ca^{2+}
38 and treatment of cells with MCD after statins treatment rescued receptor signalling
39 (Figure 3A, B). Similarly in HEK.CCR5 and CHO.CCR5 cells, lovastatin inhibited the
40 CCR5 induced calcium release to similar extent as MCD and in HEK.CCR5 cells the
41 treatment of cells with lovastatin and MCD had an accumulative effect (Figure 4 A,
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58 B). Since lovastatin decreases receptor expression to some extent in THP-1 cells
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1 (Figure 2A), we can not distinguish whether the loss of signalling is due to the loss of
2 cholesterol or the decrease of receptor, although there is a population of CCR5 left
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4 on the THP-1 cells which should be sufficient to transduce a signal.
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9 **MCD as well as statins lower the amount of cholesterol in the membrane**

10 We already showed previously that different agents that affect cholesterol in cells
11 have different effect on signalling by receptors [11]. We therefore were interested in
12 understanding how these different compounds affect the overall levels of cholesterol
13 in the cells. We determined the amount of cholesterol in untreated control cells,
14 MCD, lovastatin, simvastatin and filipin treated cells (Figure 5). Increasing
15 concentrations of the statins, e.g. lovastatin and simvastatin, significantly lower the
16 amount of cholesterol that can be detected in cells, with only 50-75% of cholesterol
17 as compared to control cells present. MCD also decreased the amount of cholesterol
18 significantly with less the 50% of control cell cholesterol present in MCD treated cells
19 (Figure 5 C, D). MCD reduces the amount of cholesterol, whereas filipin complexes
20 the cholesterol in the membrane without reducing the net amount of it.
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41 Overall there is no marked difference between the three different cell lines tested.
42 These results show that the amount of cholesterol levels alone can not be
43 responsible for the difference in signalling that is observed in different cells, on the
44 contrary, it seems as if MCD has a second effect on THP-1 cells, apart from lowering
45 the cholesterol level, which predicts and enhances chemokine receptor signalling.
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Ca²⁺ responses require a thapsigargin-sensitive Ca²⁺ store and activation of InsP3 receptors or SOCs

We wanted to clarify in more detail where the calcium released by CCR5 activation originates and therefore cells were treated with the endoplasmic reticulum Ca²⁺-ATPase inhibitor thapsigargin before stimulation with CCL3. Thapsigargin abolished the Ca²⁺ responses in THP-1, HEK.CCR5 cells (Figure 6) and CHO.CCR5 cells (data not shown), indicating that the released calcium originates from the endoplasmic reticulum. Interestingly, in MCD treated THP-1 cells, thapsigargin inhibition of calcium release is overridden by the MCD treatment and an increased signal can be observed (Figure 6 A). This was confirmed in experiments where THP-1 cells were incubated with calcium free medium for the duration of the experiment (Figure 6 C), therefore indicating that MCD alters the endoplasmic reticulum membrane and calcium release originates from the endoplasmic reticulum and not from the medium via transport through the plasma membrane. Incidentally, thapsigargin treatment had no effect on the viability of the cells treated (data not shown).

To test whether Ca²⁺ stores gated by activation of ryanodine receptors are involved in the release of intracellular Ca²⁺ in response to activation of CCR5 receptor, cells were incubated for 30 min in the presence of 10 or 100 μ M ryanodine, respectively before addition of CCL3 (Figure 7). Ryanodine treatment resulted in a slight increase of the CCL3 induced response. In CHO.CCR5 cells, after treatment with 10 μ M ryanodine, this increase was significant, however at 100 μ M ryanodine, which should inhibit activation of ryanodine receptor, the observed changes were not considerable (Figure 7 B). These results show that 100 μ M ryanodine does not block ryanodine receptors or if it does, this effect is independent of InsP3 receptors. Interestingly, like

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in CHO.CCR5 cells, when THP-1 cells were treated with ryanodine, a slight increase in calcium release could be observed (Figure 7 A) and ryanodine increased even further the MCD induced calcium signalling.

We used 2-APB, an inositol 1,4,5-trisphosphate (InsP3) receptor antagonist and blocker of store-operated channels (SOCs) [21] to analyse the involvement of InsP3 in release of intracellular calcium in THP-1 cells (Figure 7C). 2-APB brings the enhanced MCD-signal back to basal levels, which suggest that MCD treatment of THP-1 cells results in an increased activation of either InsP3 or SOCs. These results indicate that MCD changes the endoplasmic reticulum membrane and increases the open probability of all ryanodine and inositol 1,4,5-trisphosphate (InsP3) receptors.

4. Discussion

The detailed mechanisms of how chemokine receptors induce the release of intracellular calcium are still not completely identified. Previous reports by us and others highlighted the importance of cholesterol in the plasma membrane for effective CCR5 signalling [6, 8, 11, 22]. Altogether there is a certain problematic with comparing the different literature values for CCR5. Most of the experiments were performed in different cell lines and there is no conclusive study so far, whether the release of calcium ions in cell lines like CHO cells is actually similar to monocytic cells like THP-1 cells. Cell type specificity has been shown for some chemokine receptor signalling events. For example chemokine receptors can be crosslinked to purinergic receptors like P2Y, when pre-treatment of cells with ATP increases the signal via the chemokine receptor [23]. This crosslinking seems to be cell type

1 specific, since it can be observed in CHO.CCR5 cells, but not in THP-1 cells [24].
2 Therefore we analysed in this study the release of intracellular calcium in different
3 cell lines with an emphasis on understanding the receptor activation pattern in the
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10 Changing the cholesterol concentration in the plasma membrane of cells using MCD
11 or similar agents generally decrease the signalling properties of the receptor [6, 8,
12 11], but it can also disrupt lipid rafts and modulate the activity of multiple signalling
13 pathways, like activating the Ras-Erk pathways in T-lymphocytes [25]. Unlike other
14 cell types, treatment of THP-1 cells with MCD leads to a vast increase of the CCL3
15 induced signal. This actually contradicts the results obtained by Nguyen et al. in
16 CEM-NKR-CCR5 cells and shows that cholesterol extraction has distinct effects in
17 chemokine receptors signalling depending on the cell type. The increase in calcium
18 release observed here is not due to the lack of cholesterol, since lovastatin and
19 simvastatin treatment, which both decrease the amount of cholesterol in the
20 membrane, have the opposite effect as MCD. In fact lovastatin and simvastatin
21 significantly decrease CCL3 induced signal in THP-1 cells, similarly to the results
22 observed in HEK.CCR5 and CHO.CCR5 cells. However unlike MCD, lovastatin also
23 decreases the amount of receptor on the cell surface, which could account for
24 inhibitory effect of statins when compared to MCD.
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51 The question remains how MCD treatment increases the signal. There is a
52 suggestion that MCD treatment leads to a loss of $G\alpha_i$ expression in THP-1 cells [26],
53 this might then lead to preferential coupling of CCR5 to $G\alpha_q$ and therefore to an
54 enhanced release of calcium. We already showed for CHO.CCR5 and HEK.CCR5
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1 cells that MCD treatment changes coupling of CCR5 from $G_{\alpha i}$ to PTX independent
2 G-proteins [11] and there is the possibility that in THP-1 cells this change in coupling
3 is more distinct and effective than in other cells. PTX treatment in THP-1 cells does
4 not completely block CCL3 induced calcium flux and in the presence of MCD there is
5 no effect of PTX on receptor induced Ca^{2+} release.
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14 Inhibition of endoplasmic reticulum Ca^{2+} -ATPases with thapsigargin completely
15 blocks calcium release which is a clear indicator that in all cell lines tested the
16 intracellular calcium stores used are situated in the endoplasmic reticulum. However
17 MCD completely reversed thapsigargin effect in THP-1 cells, suggesting that a more
18 fluid membrane in these cells might change the ability of this compound to block
19 Ca^{2+} -ATPases. The involvement of ryanodine receptor on the calcium release is less
20 clear however and it is distinct in the different cell systems. Ryanodine locks the
21 Ryanodine receptors (RyRs) at half-open state at nanomolar concentrations, yet fully
22 closes them at micromolar concentration [27]. Importantly, ryanodine at 10 μ M
23 significantly enhances calcium release in THP-1 cells and this increase is even more
24 pronounced in MCD-treated THP-1 cells. This implicates that MCD changes RyR
25 function. Whereas in CHO and HEK cells ryanodine at low concentrations open up
26 the calcium release channel and therefore increase the amount of Ca^{2+} ions that are
27 entering the cytoplasm in response to CCR5 activation, high concentrations of
28 ryanodine, which should close the calcium channels, do not seem to affect CCR5
29 induced calcium release significantly. These findings suggest that although RyR
30 activation can potentiate calcium release, its open state does not seem essential for
31 chemokine induced signal transduction. This leaves a role for inositol 1,4,5-
32 trisphosphate (InsP3) receptors in transducing CCR5 induced calcium release. InsP3
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diffuses in the cytoplasm and binds to a receptor, the InsP3R, a polytopic integral membrane protein in the endoplasmic reticulum (ER), activating it as a Ca^{2+} channel to liberate stored Ca^{2+} from the ER lumen into the cytoplasm [28, 29]. Some studies have shown that MCD treatment of cells resulted in phosphorylation and activation of PLC [30]. This increase in PLC activity could directly enhance the amount of calcium released due to an increase of InsP3 formation. 2-APB, an InsP3 antagonist and SOCs inhibitor reduces the calcium released in MCD treated THP-1 cells, which shows that at least in MCD treated cells part of the calcium released after activation is due to action of either the InsP3 receptor or SOCs.

Altogether this study shows the release of intracellular calcium is regulated in a distinct manner in different cells and highlights the importance of cholesterol depletion for optimal signal transduction in THP-1 cells and the involvement of ryanodine and InsP3 receptors in chemokine receptor responses. This work therefore offers more insight into key molecular mechanisms of chemokine receptor signalling

5. Acknowledgments

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6. Figure legends

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5 **Figure 1: Changes in intracellular Ca²⁺ in THP-1 cells in the presence of**
6 **inhibitors.** A) shows single traces in real time in the presence or absence of 10mM
7 MCD, B) shows control THP-1 cells challenged with different amounts of CCL3, C)
8 shows cells pre-treated with different concentration of MCD and then challenged with
9 CCL3 as indicated, D) shows concentration response curve for cells pre-treated with
10 different concentrations of MCD, E) shows cells pre-treated with filipin, nystatin or
11 vehicle (control) as described in materials and methods and then challenged with
12 CCL3, F) shows single traces of THP-1 cells pre-treated with filipin, nystatin or
13 vehicle (control) and then challenged with 100nM CCL3. Data are expressed as
14 either changes in fluorescence ratio [340nm/380nm] where the basal before addition
15 of chemokine is subtracted from peak fluorescence after addition of chemokine or as
16 percentage of stimulation over basal where the basal level is normalised to 100
17 percent. Data represent Mean \pm s.e.m. from at least three independent experiments
18 for the bar charts and a representative tracer for the calcium flux.
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41 **Figure 2: Changes in receptor expression and intracellular Ca²⁺ in THP-1 cells.**

42 A) flow cytometry analysis of HEK.CCR5 and THP-1 cells after MCD and lovastatin
43 treatment, cells were treated with lovastatin for 3 days and MCD for 1 hour,
44 respectively, stained with an anti-CCR5 antibody and the corresponding FITC-
45 conjugated secondary antibody and analysed using flow cytometry, data represents
46 percentage of Geo Mean fluorescence compared to vehicle treated (control) cells, B)
47 flow cytometry analysis of CCR5 expression on control THP-1 cells and cells after
48 MCD treatment, respectively. MCD treatment increases the expression level of
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CCR5. C) THP-1 cells were treated with 10mM MCD as described and when indicated 10mM cholesterol was fed back to the cells before activation of receptor with 100nM CCL3. D) shows single calcium trace of THP-1 cells either pre-treated with 10 mM MCD, 10mM cholesterol or 10mM MCD plus 10mM cholesterol before receptor activation with 100nM CCL3 when indicated. E) cells were treated with PTX or vehicle overnight and MCD was added 1 hour before calcium flux was induced with 100nM CCL3, F) shows single calcium trace of THP-1 cells treated with PTX overnight and/or MCD for 1 hour before addition of 100nM CCL3. Significant changes towards control cells are indicated by stars (*= $p < 0.05$, **= $p < 0.01$). Data are expressed as either changes in fluorescence ratio [340nm/380nm], where the basal line before addition of chemokine is subtracted from the peak fluorescence after addition of chemokine or as percentage of stimulation over basal, where the basal level is normalised to 100 percent. Data represent Mean \pm s.e.m. from at least three independent experiments for the bar charts and a representative tracer for the calcium flux.

Figure 3: Effect of statins on release of intracellular calcium in THP-1 cells.

Cells were incubated with different statins for 3 days before cells were stimulated with 100nM CCL3. A) shows THP-1 cells pre-treated with lovastatin for 3 days, MCD for 1 hour or vehicle (control) before receptor was activated with 100nM CCL3, and single trace of THP-1 cells pre-treated with vehicle (control), MCD, lovastatin or MCD and lovastatin before the addition of 100nM CCL3 as indicated, B) shows the change of intracellular calcium in THP-1 cells, pre-treated with different concentrations of simvastatin for 3 days or vehicle (control) and challenged with 100nM CCL3 and single trace of THP-1 cells pre-treated with vehicle (control) or simvastatin before the

1 addition of 100nM CCL3 when indicated. Significant changes to control data are
2 shown by stars (**=p<0.01), Data are expressed as either changes in fluorescence
3 ratio [340nm/380nm], where the basal line before addition of chemokine is
4 subtracted from the peak fluorescence after addition of chemokine or as percentage
5 of stimulation over basal, where the basal level is normalised to 100 percent. Data
6 represent Mean \pm s.e.m. from at least three independent experiments for the bar
7 charts and a representative tracer for the calcium flux.
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19 **Figure 4: Effect of statins on release of intracellular calcium in HEK.CCR5 and**
20 **CHO.CCR5 cells.**
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22 A) shows HEK.CCR5 cells pre-treated with vehicle (control), lovastatin for 3 days,
23 MCD for 1 hour, or both before the receptor is activated with 100nM CCL3.
24 Significant changes to control data are shown by stars (*=p<0.05, **=p<0.01), B)
25 shows CHO.CCR5 cells pre-treated with vehicle (control), lovastatin for 3 days, MCD
26 for 1 hour, or both before the receptor is activated with 100nM CCL3. Data are
27 expressed as either changes in fluorescence ratio [340nm/380nm], where the basal
28 line before addition of chemokine is subtracted from the peak fluorescence after
29 addition of chemokine or as percentage of stimulation over basal, where the basal
30 level is normalised to 100 percent. Data represent Mean \pm s.e.m. from at least three
31 independent experiments for the bar charts and a representative tracer for the
32 calcium flux.
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Figure 5: Quantification of cholesterol levels in the plasma membrane of different cells.

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5 A) THP-1 cells were incubated with different concentrations of lovastatin for 3 days
6 and cholesterol levels in the cells were determined using the Amplex Red cholesterol
7 assay as described. Increasing concentrations of lovastatin decrease the amount of
8 cholesterol present in the cells. B) THP-1 cells were treated with different
9 concentrations of simvastatin for 3 days, C) THP-1 cells were treated with MCD and
10 filipin for 1 hour, D) HEK.CCR5 cells were treated with either MCD or filipin for 1 hour
11 or lovastatin for 3 days. Data are represented as percentage of cholesterol content
12 compared to vehicle treated (control) cells. Significant changes towards control are
13 shown by stars (*= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.001$). Data represent Mean \pm s.e.m. of
14 at least three independent experiments.
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Figure 6: Release of intracellular calcium in THP-1, HEK.CCR5 and CHO.CCR5 cells is dependent on sarcoendoplasmic reticulum Ca^{2+} -ATPase.

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36 A) THP-1 cells and B) HEK.CCR5 were treated with vehicle (control), MCD,
37 thapsigargin or thapsigargin plus MCD for 1 hour. Thapsigargin significantly inhibits
38 the release of calcium in all cell lines compared to vehicle treated control cells. MCD
39 treatment in THP-1 cells reverses thapsigargin inhibition. C) shows THP-1 cells
40 treated as in (A) in a calcium free buffer. Significant changes towards control are
41 shown by stars (**= $p < 0.01$, ***= $p < 0.001$). Data are expressed as either changes in
42 fluorescence ratio [340nm/380nm], where the basal line before addition of
43 chemokine is subtracted from the peak fluorescence after addition of chemokine or
44 as percentage of stimulation over basal, where the basal level is normalised to 100
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1 percent. Data represent Mean \pm s.e.m. from at least three independent experiments
2 for the bar charts and a representative tracer for the calcium flux.
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7 **Figure 7: Differential regulated release of intracellular calcium in THP-1,**
8 **HEK.CCR5 and CHO.CCR5 cells.**
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11 A) THP-1 cells and B) CHO.CCR5 cells were treated with vehicle (control),
12 ryanodine or ryanodine plus MCD for 1 hour before receptor activation with 100nM
13 CCL3. Ryanodine slightly increases the release of intracellular calcium in these cells.
14 Significant changes towards control are indicated by stars (*= $p < 0.05$, **= $p < 0.01$). C)
15 THP-1 cells were pre-treated with vehicle (control), MCD or 2APB plus MCD for 1
16 hour before addition of 100nM CCL3. 2-APB reduces the release of intracellular
17 calcium in the presence of MCD. Data are expressed as either changes in
18 fluorescence ratio [340nm/380nm], where the basal line before addition of
19 chemokine is subtracted from the peak fluorescence after addition of chemokine or
20 as percentage of stimulation over basal, where the basal level is normalised to 100
21 percent. Data represent Mean \pm s.e.m. from at least three independent experiments
22 for the bar charts and a representative tracer for the calcium flux.
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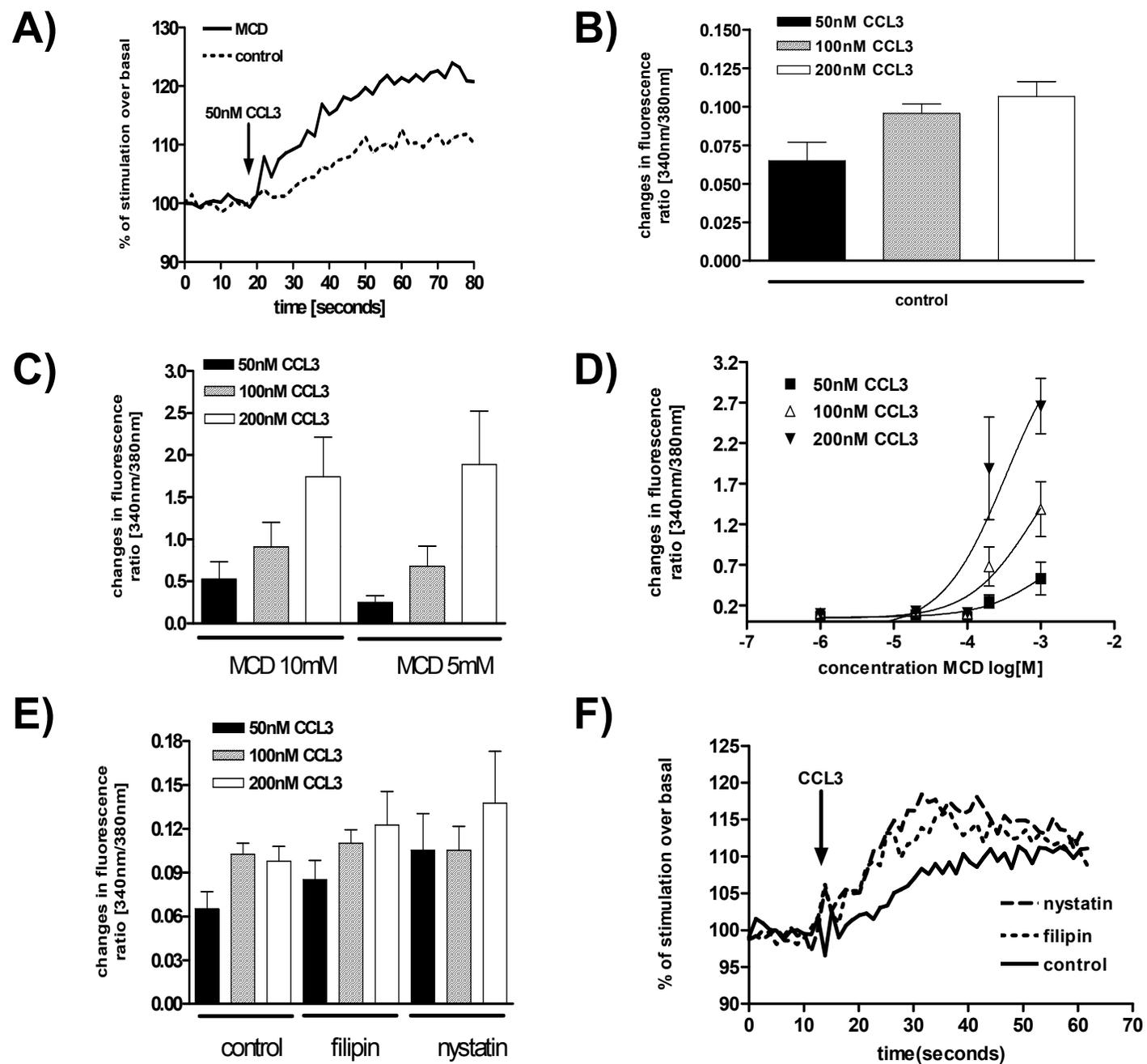


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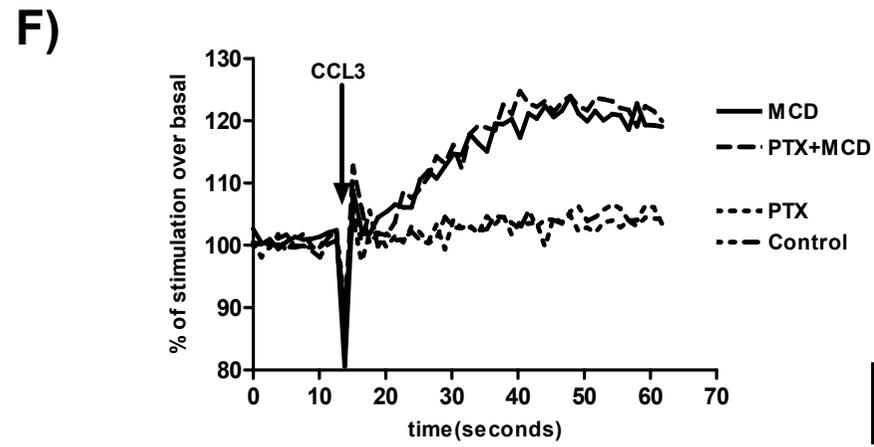
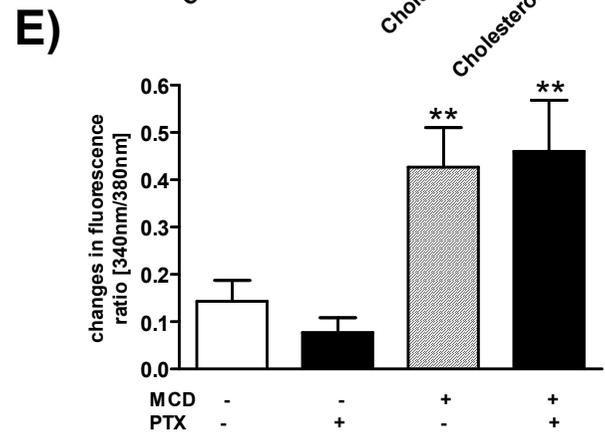
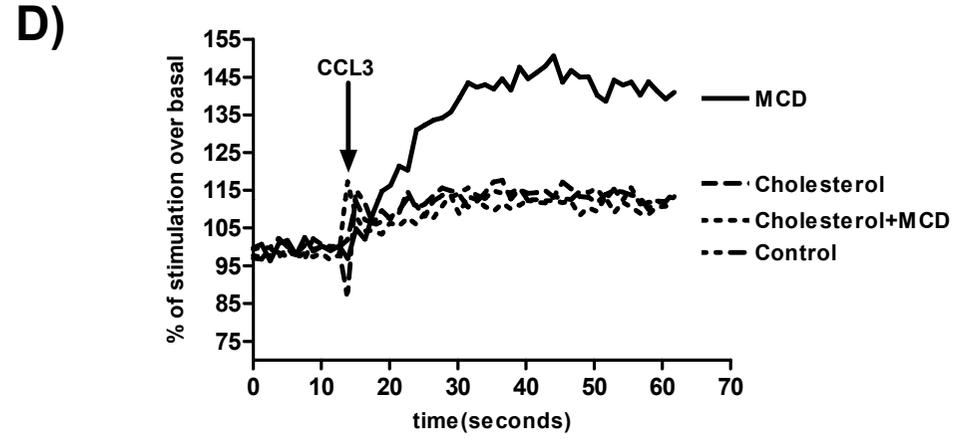
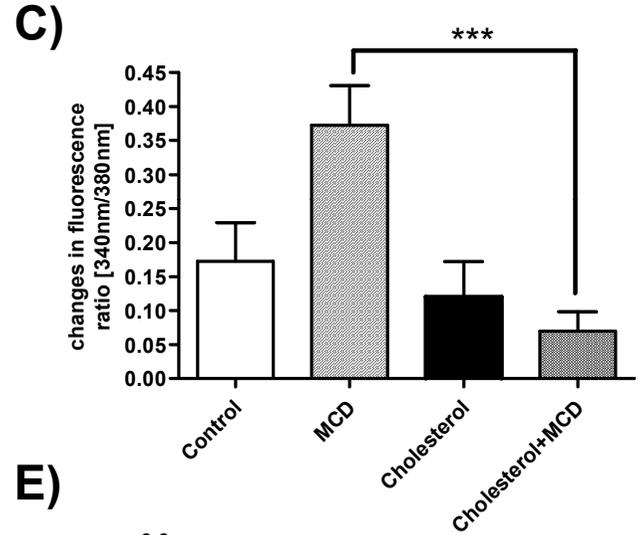
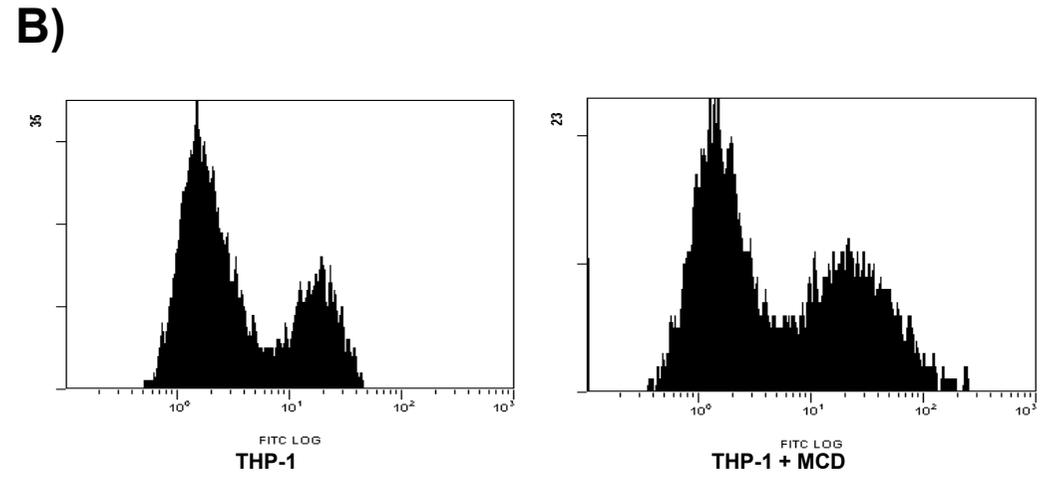
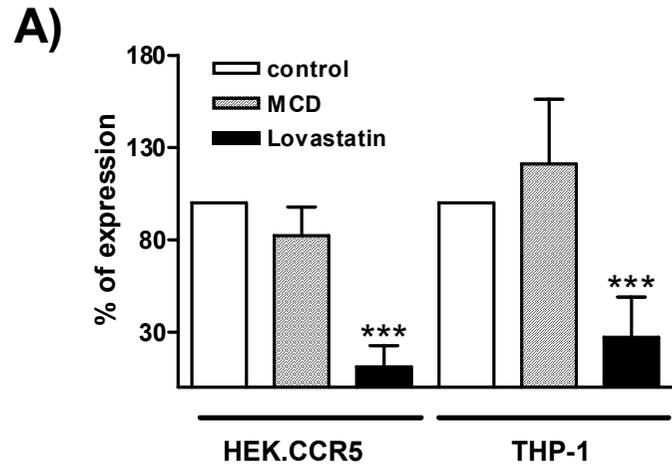


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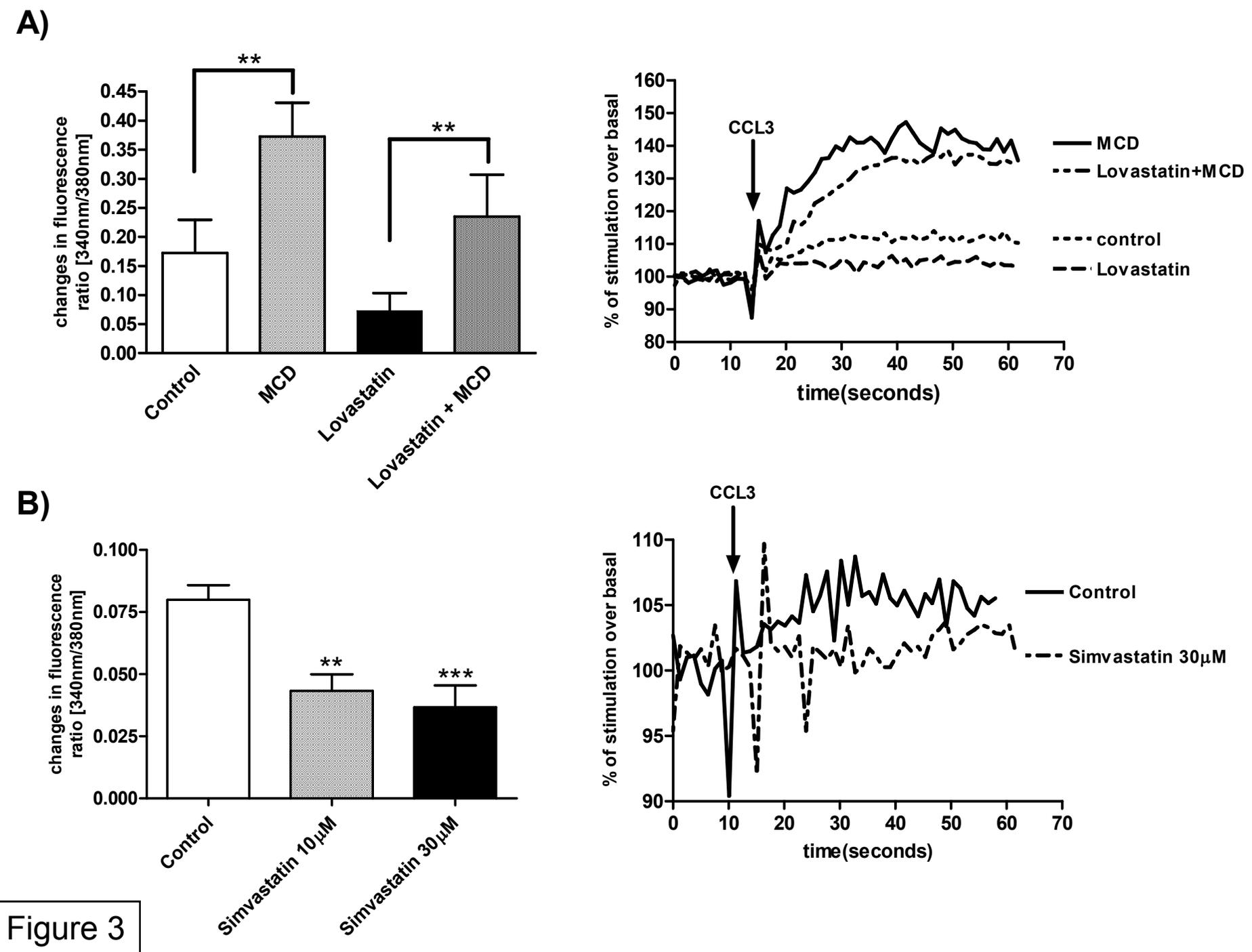


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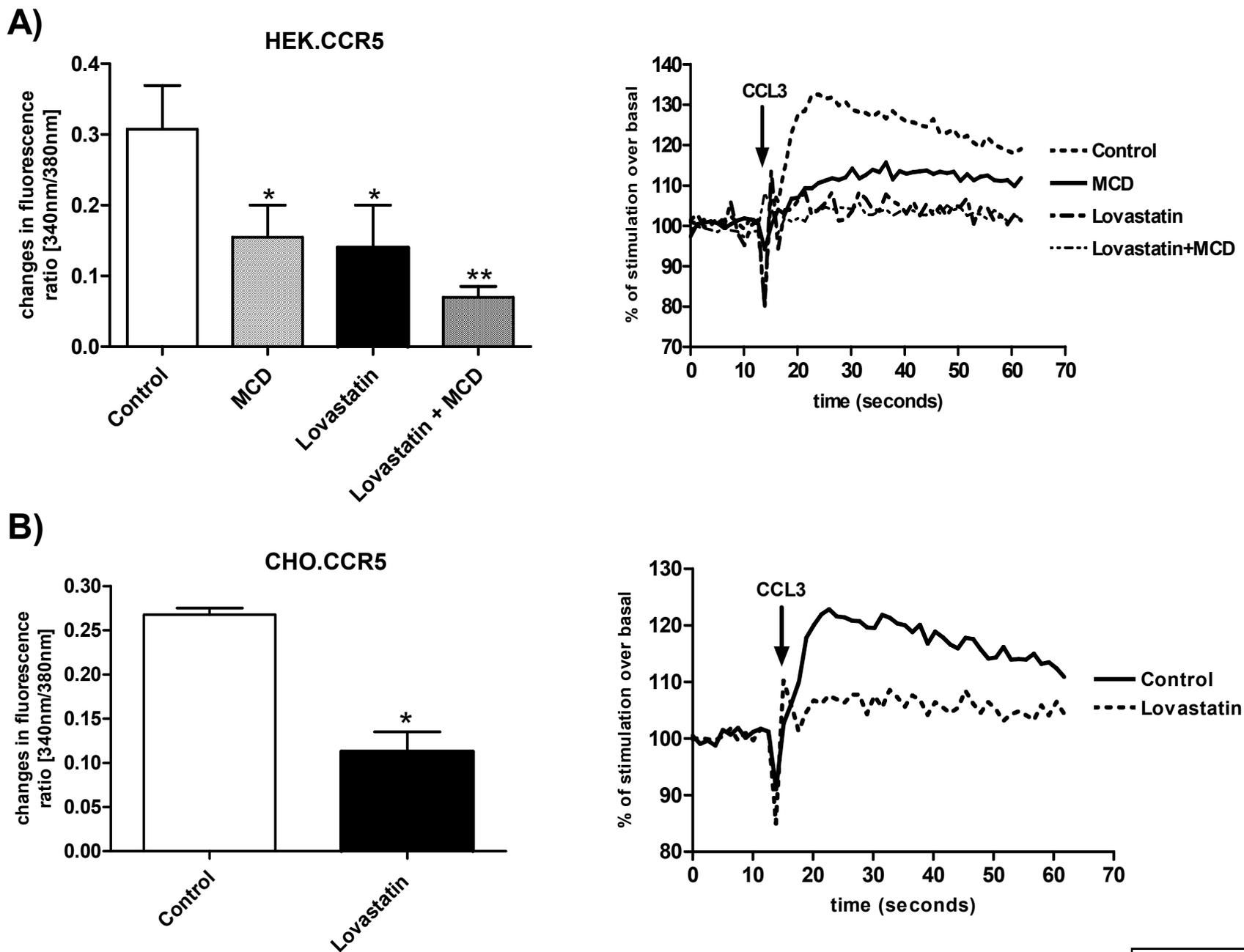


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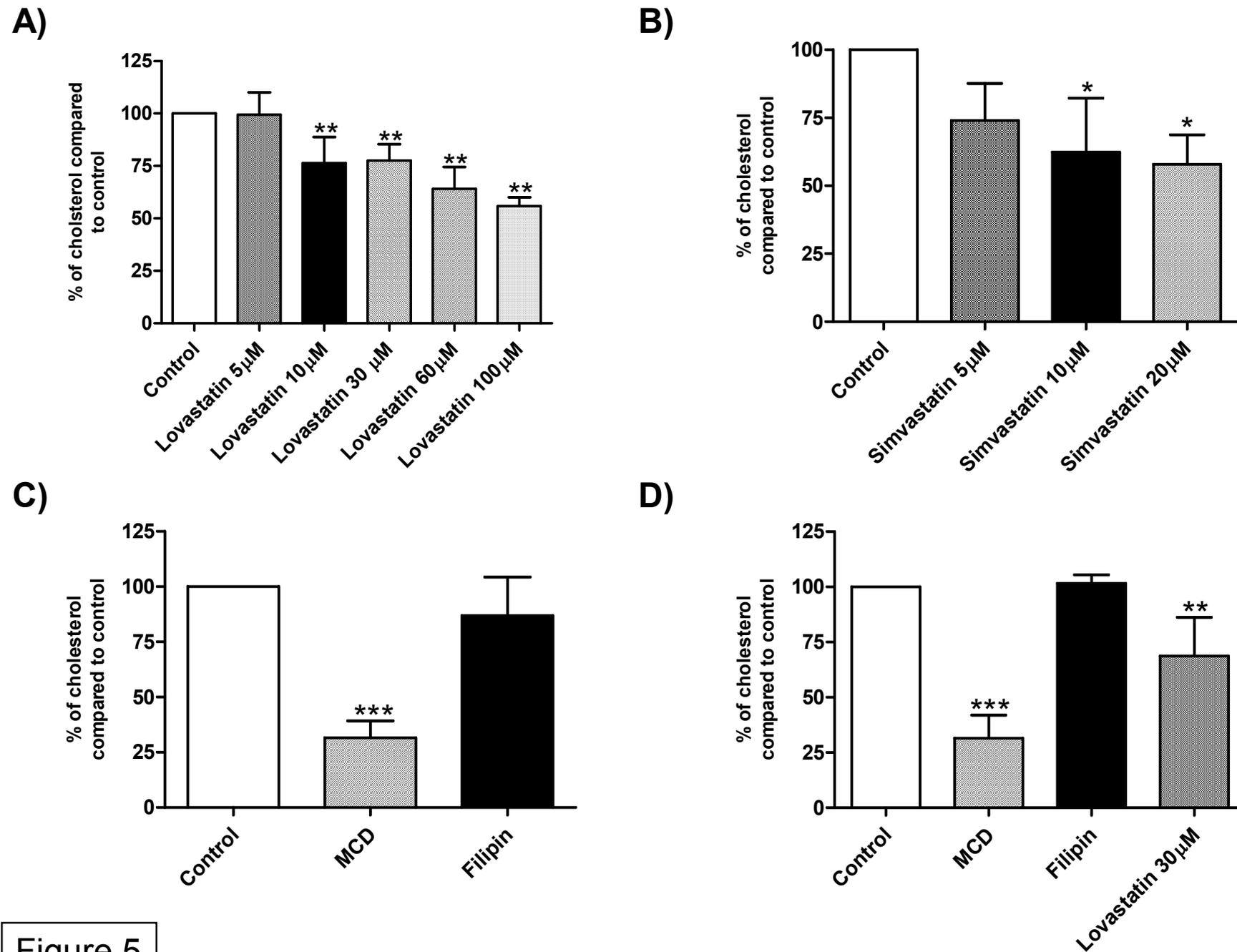


Figure 5

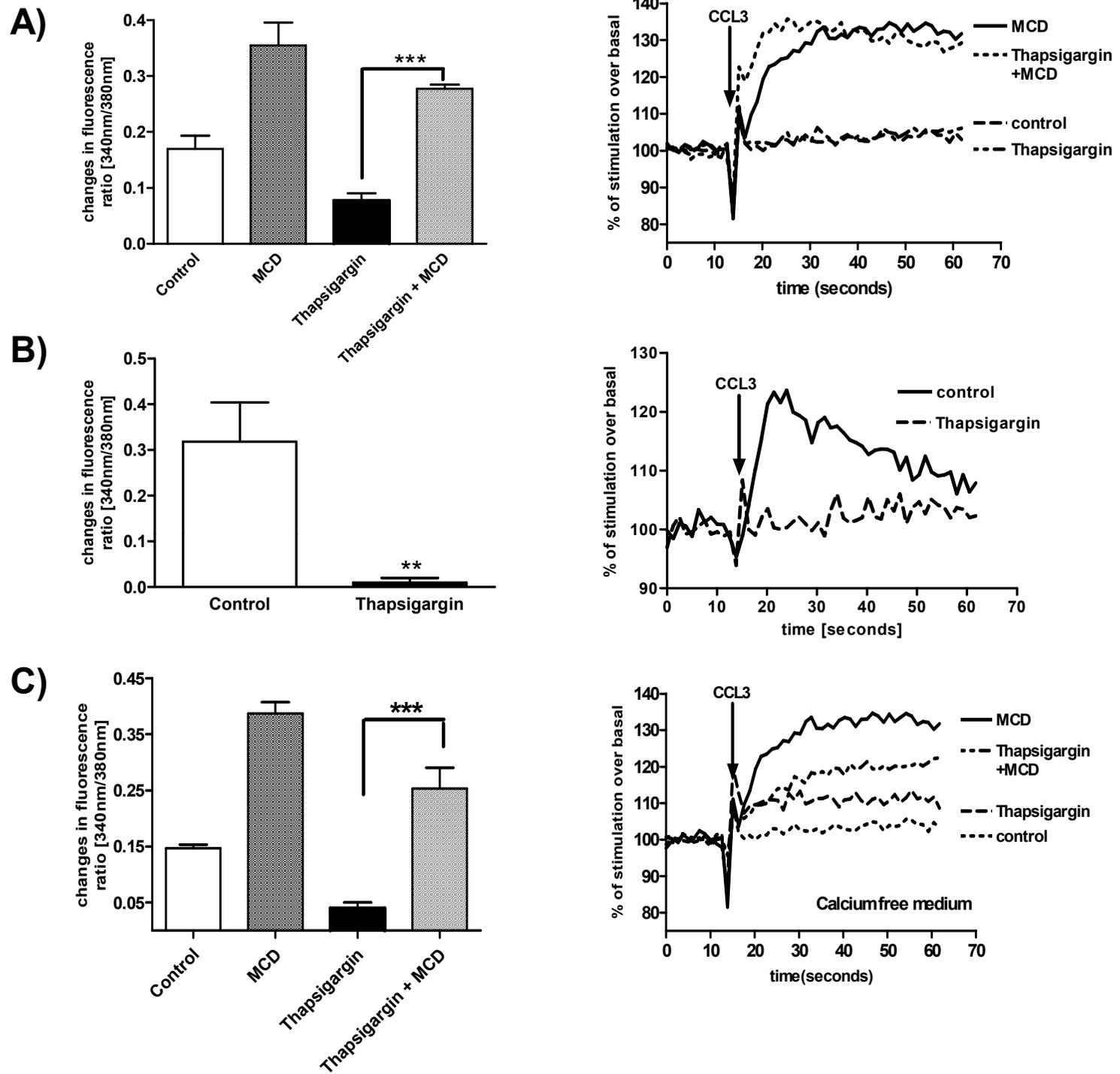


Figure 6

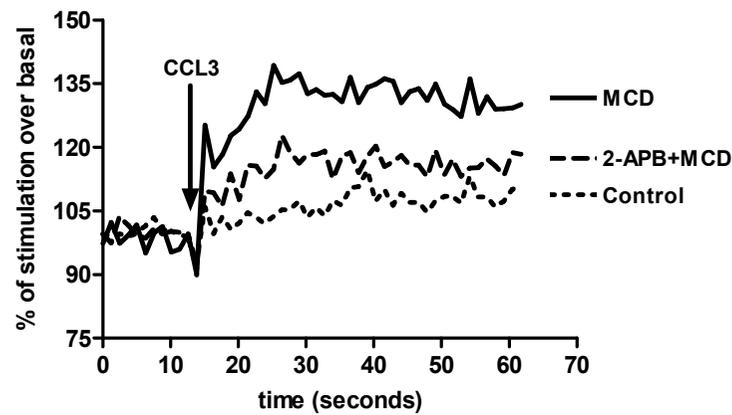
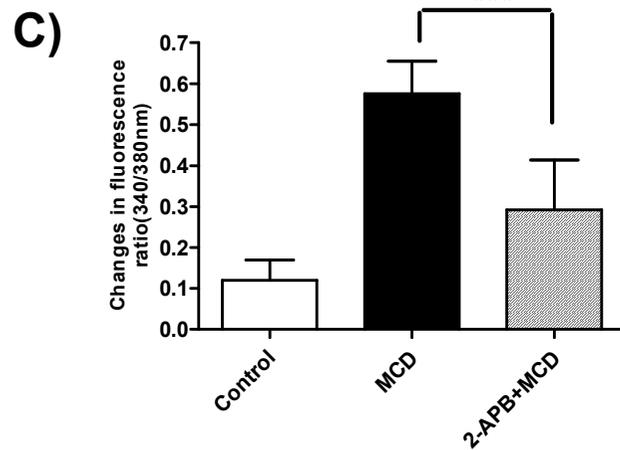
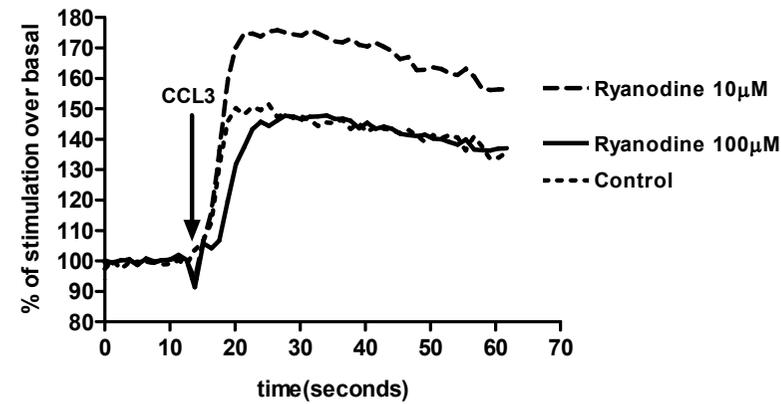
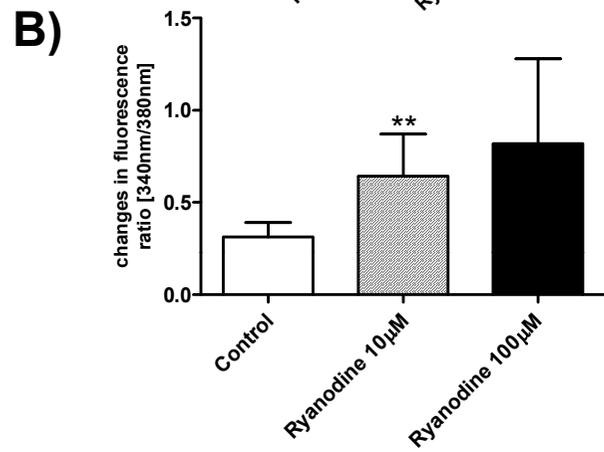
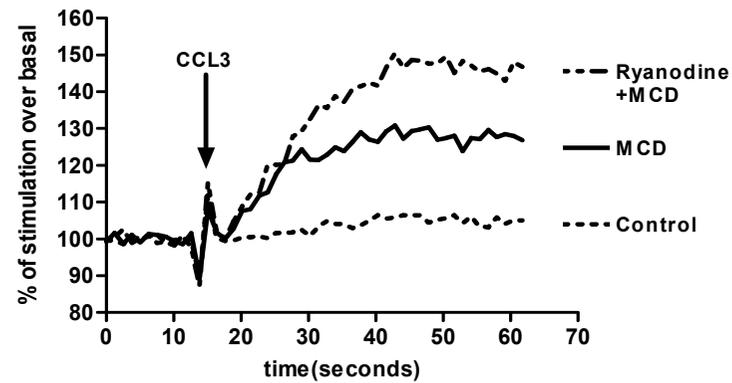
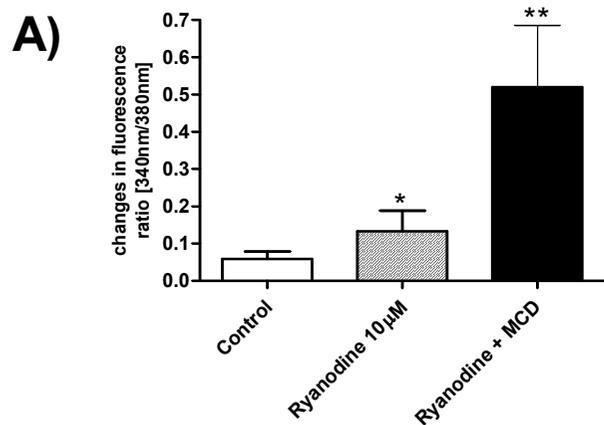
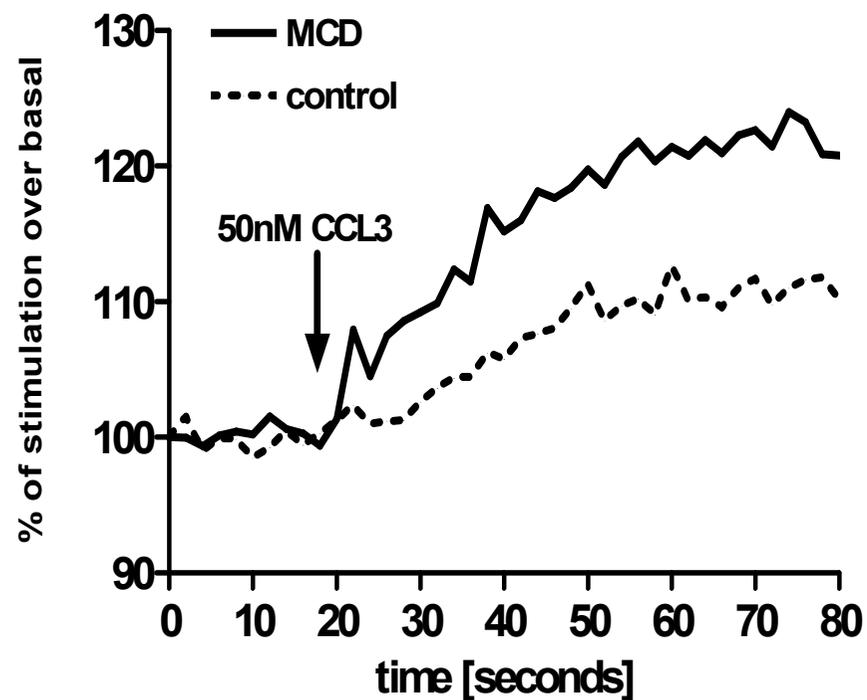


Figure 7



MCD treatment of monocytic THP-1 cells leads to an increase in the signalling properties of CCR5.