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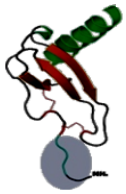
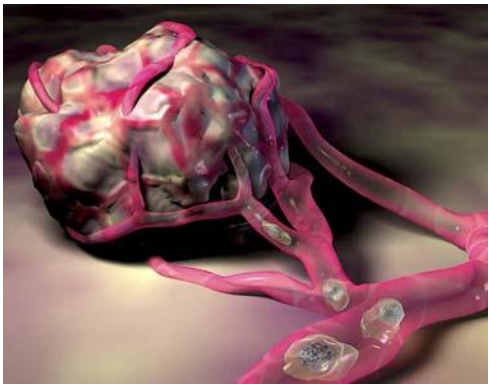


Chemokines regulate colon cancer metastasis

Master thesis

AMR A. AL-HAIDARI



Thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science in protein science

Autumn 2011

بِسْمِ اللَّهِ الرَّحْمَٰنِ الرَّحِيمِ

In the name of Allah, the most gracious, the most merciful

قال تعالى: " وَمَا تَوْفِيقِي إِلَّا بِاللَّه " سورة هود, آية 88

God says; "And my guidance cannot come except from Allāh". 88-sûrah Hûd

To my wonderful family; parents, sisters, and my brother

To the glance of my life; my wife

To SWEDEN; the country of love and peace

Chemokines regulate colon cancer metastasis

By

Amr A. Al-Haidari

Thesis submitted in partial fulfillment of the requirements for the degree of
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Thesis supervisor: Professor MD, PhD Henrik Thorlacius
Examiner: Docent, PhD Sofi K. C. Elmroth

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Kemokiner reglerar metastaseringen av koloncancer

Populärvetenskaplig sammanfattning

Nyligen gjorda upptäckter inom cancer forskningen har visat ett starkt samband mellan inflammation och cancer. Hur koloncancer sprids till andra organ orsakar fortfarande debatt och är inte helt klarlagt. Men vad som nyligen dokumenterats är att vissa proteiner som är involverade i regleringen av immunsystemet kan ha stor betydelse vid spridningen av cancer. En grupp av proteiner, så kallade kemokiner, aktiverar förflyttningen av vita blodkroppar vid den inflammatoriska processen till platsen för inflammationen som en del av immunförsvaret. Intressant nog har det visat sig att cancerceller också kan svara på den typ av aktivering på liknande sätt som vita blodkroppar gör. Cancerceller kan selektivt föredra platser till vart de ska spridas, huvudsakligen beroende på vilken mikromiljö som finns vid dessa ställen. Alla frön kan inte odlas i samma jord, en del frön, cancerceller, kräver en särskild typ av jord, mikromiljön. I denna studie försöker vi förstå de grundläggande mekanismerna för hur cancerceller skulle kunna lämna sin närmiljö och migrera till andra ställen och hur de skulle kunna utnyttja den omgivande mikromiljöns faktorer för att överleva. Här visade våra experimentella molekylära studier att vissa specifika kemokiner, särskilt CCL17 och CXCL12, är funktionella och framkallar förmågan hos cancerceller att migrera till avlägsna organ i en komplex process som metastasering är, vilket återspeglar det faktum att dessa kemokiner kan vara inblandade i regleringen av metastaseringen vid koloncancer.

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Chemokines regulate colon cancer metastasis

Biochemistry department, Center of Molecular Protein Science & Structural biology, Lund
Department of Surgery, Clinical Research Center, Skåne University Hospital, Malmö, Sweden

Abstract

Background: Chemokines are small molecular weight proteins, which mainly function as a chemoattractant cytokines for leukocyte migration. Emerging data indicate that chemokines may also play a role in cancer biology. Colon cancer is one of the most leading causes of death especially in the industrial world. The most common causes of mortality are due to the metastasis of colon cancer.

Aim: The aim of the study was to understand how chemokines may contribute to colon cancer metastasis.

Materials and Method: Chemokine receptors CXCR (*CXCR4, CXCR3, CXCR7*) and CCR receptors (*CCR4, CCR6, CCR7, CCR8*) mRNA expression were evaluated by Real Time – Polymerase Chain Reaction (RT-PCR) in HT29 and 19/97* colon cancer cell lines. Following gene expression, flowcytometry was used to assess chemokine receptors protein expression. Finally, functional receptors were verified in both cell lines using a migration assay with their corresponding ligands.

Results: Both cell lines expressed all chemokine receptors mRNA except CXCR7 with the highest expression being detected for CXCR4, CXCR3 and CCR4. Flowcytometry for the expressed receptors also revealed protein surface expression. The chemokine ligands TARC/CCL17 and SDF-1/CXCL12 induced a significant increase in colon cancer cell migration in a dose- and time- dependent manner.

Conclusions: Our data demonstrate increased expression of functional CCR4, CXCR3, and CXCR4 in colon cancer cells, suggesting that their chemokine ligands might be involved in the regulation of colon cancer metastasis.

Keywords: Chemokines; Chemokine receptors; Colon cancer; Metastasis

1. Introduction

Cancer is an abnormal cell growth that results from activation of oncoproteins or inactivation of tumor suppressor genes leading to either gaining or losing of cell function [1]. Hanahan and Weinberg [2] characterized the six phenotypic gene modification hallmarks of cancer that contribute to its initiation and progression. They can be summarized by the ability of selective growth, resistance to apoptosis, insensitivity to growth – inhibitory signals, potential uncontrolled replication, sustained angiogenesis, tissue invasion and metastasis.

Colon cancer is a major health problem and represents one of the most common causes of cancer – related deaths in men and women mainly in the industrial world. 90% of colon cancer mortality cases are due to the spread of primary tumor to other distant organs in a complex process called metastasis leading to failure of organs function. If metastasis occurred, the 5 year survival rate after surgical intervention falls from 95% to less than 10% [3, 4]. The metastatic potential of colon cancer is identified by the ability of cancer cells to interact and communicate with the tumor microenvironment [5]. In metastasis, malignant cells acquire specific characteristics that make them capable to metastasize. Such characteristics include enhanced cell adhesion to endothelial cells, increase cell migration in response to chemotactic signals released by the target organs, and higher response to some growth signals in the target organs [6]. Within tumor microenvironment, chemokines and their receptors are key players in tumorgenesis and metastasis [7-10]. Various types of cancer express different chemokine/chemokine receptors and the pattern that shapes this expression could provide some clues on the metastatic behavior of cancer cells [11]. Since long time ago and the puzzle of why different types of cancer show distinct tropism for metastatic sites was a matter of debate till recently it has been widely accepted that chemokines and chemokine receptors involved not only in overall metastasis but also in site – specific metastasis. Emerging data indicate that chemokines have a potential role in directing organ-specific metastasis. Understanding the molecular aspects behind could open up the way in front of identifying new cancer therapeutic targets.

Table (1) Important chemokine receptors expressed by different types of cancer [11].

Cancer	Receptor expressed
Breast	**CXCR3, CXCR4, CCR7, CXCR7, CCR5**
Ovarian	**CXCR4**
Prostate	**CXCR4, CXCR5, CCR9, CCR5, CX3CR1**
Pancreas	**CXCR4, CXCR1/2, CCR6**
Skin	**CXCR3, CXCR4, CXCR5, CXCR6, CCR4, CCR10, CCR7, CCR9, CXCR1/2**
Esophageal	**CXCR4, CCR7**
Lung	**CXCR4, CCR7, CXCR7**
Head and neck	**CXCR4, CCR7,CXCR5, CXCR1/2**
Bladder	**CXCR4**
Colorectal	**CXCR3, CXCR4, CCR6, CCR7, CXCR1/2**
Small intestine	**CCR9**
Osteosarcoma	**CXCR4**
Neuroblastoma	**CXCR3, CXCR4**
Acute Lymphoblastic leukemia	**CXCR4, CXCR3, CCR4**
Chronic myelogenous leukemia	**CXCR4, CXCR5, CXCR3**
Non-Hodgkin Lymphoma	**CXCR4, CXCR5**
Thyroid	**CXCR4**
Renal	**CXCR4, CCR3**
Oro-gastric	**CCR7**
Multiple myeloma	**CXCR3, CXCR4, CCR1, CCR2**
Stomach	**CCR7**

1. Chemokine Art

Chemokines are group of small molecular weight (8 – 12 kDa) chemoattractant cytokines which play a major role in leukocyte migration [12]. More than 40 chemokines and 20 functionally signaling chemokine receptors have been identified up to date [13]. Structurally, chemokines classified on the basis of conserved N- terminus cysteine residues into four main groups, two major groups; CXC, and CC, also called alpha and beta chemokines respectively and two minor groups; C, and CX3C. Chemokines usually exhibit 25 – 70% sequence identity and exist as monomers in their active state [14, 15]. Chemokines can also be classified according to their function into two main groups. First of all, inflammatory, which are involved in regulation of immune system for example

trafficking immune cells to the site of inflammation and secondly, homeostatic, which control leukocyte homing and lymphocyte recirculation under physiological conditions [16].

Table (2) Chemokine families

Family	Systematic name	Alternate name(s)
CXC	CXCL1	Gro-a, MGSA-a, MIP-2, KC
	CXCL2	Gro-b, MGSA-b, MIP-2a
	CXCL3	Gro-g, MGSA-g, MIP-2b
	CXCL4	Platelet factor 4
	CXCL5	ENA-78
	CXCL6	GCP-2
	CXCL7	Platelet basic protein and its products (b-TG, CTAP-III, NAP-2) (human only)
	CXCL8	IL8 (human only)
	CXCL9	Mig
	CXCL10	IP-10
	CXCL11	I-TAC
	CXCL12	SDF-1
	CXCL13	BLC, BCA-1
	CXCL14	MIP-2g, BRAK, bolekine
	CXCL15	Lungkine (mouse only)
	CXCL16	
CC	CCL1	I-309 (mTCA3)
	CCL2	MCP-1
	CCL3	MIP-1a, LD78
	CCL4	MIP-1b, Act-2
	CCL5	RANTES
	CCL6	c10 (mouse only)
	CCL7	MCP-3, FIC, MARC
	CCL8	MCP-2
	CCL9/CCL10	MIP-1g, MRP-2 (mouse only)
	CCL11	Eotaxin
	CCL12	mMCP-5 (mouse only)
	CCL13	MCP-4, CKb10 (human only)
	CCL14	HCC-1, CKb1 (human only)
	CCL15	HCC-2, MIP-5, MIP-1d (human only)
	CCL16	HCC-4, CKb12 (human only)
	CCL17	TARC
	CCL18	DC-CK1, PARC, MIP-4, CKb7 (human only)
	CCL19	MIP-3b, ELC, exodus-3, CKb11
	CCL20	MIP-3a, LARC, exodus-1, CKb4
	CCL21	SLC, 6Ckine, exodus-2, TCA4
	CCL22	MDC
	CCL23	MPIF-1, CKb8, MIP-3 (human only)
	CCL24	MPIF-2, CKb6, eotaxin-2
	CCL25	TECK, CKb15
	CCL26	Eotaxin-3, MIP-4a (human only)
	CCL27	CTAK, ESkine, skinkine
	CCL28	MEC (human only)
C	CL1	Lymphotactin, SCM-1a
	CL2	SCM-1b (human only)
CX3C	CX3CL1	Fractalkine

Chemokines function through signaling of 7 transmembrane G protein coupled receptors (GPCR). Some chemokine receptors bind multiple chemokines while others have exclusive chemokine receptor/ligand interactions. For example, CXCL12 has the only identified chemokine receptor CXCR4 [17].

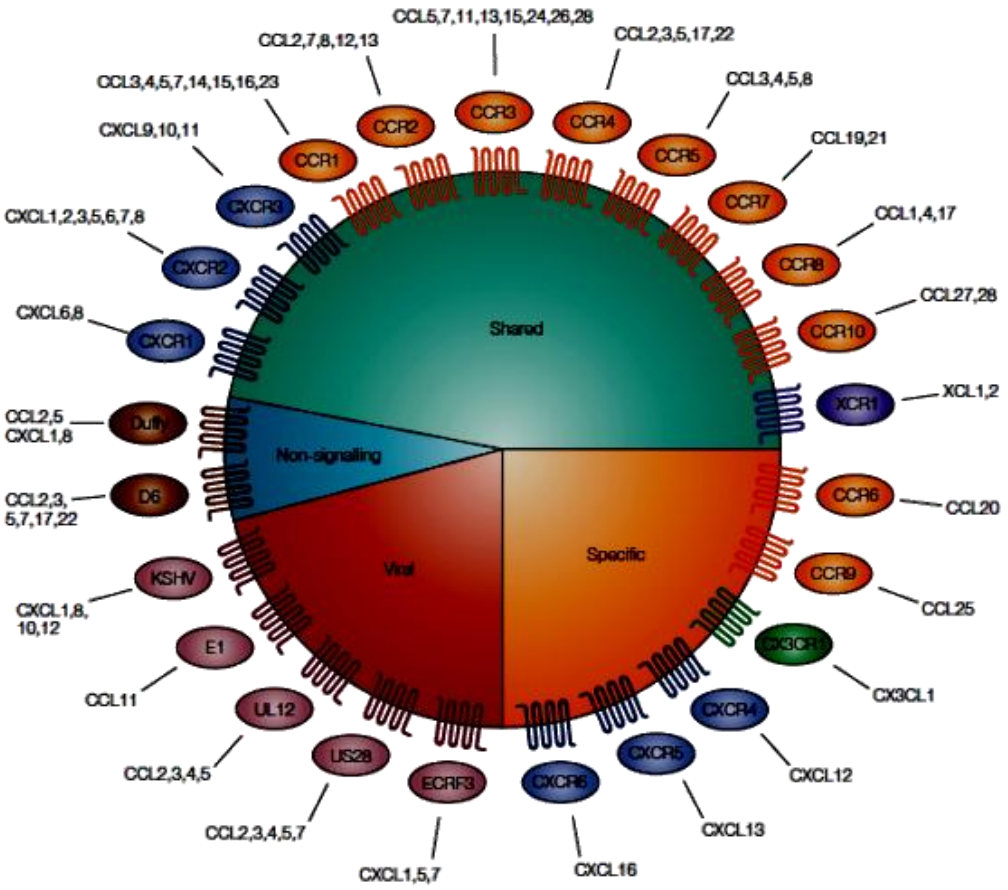

Figure (1). Chemokine wheel. This illustration explains the chemokine/chemokine receptor binding pattern. Some chemokines bind several receptors while others share single chemokine receptor. *Fran Balkwill, CANCER AND THE CHEMOKINE NETWORK, NATURE REVIEWS | CANCER, VOLUME 4 | JULY 2004 | 541*

2. Chemokine/chemokine receptors in colon cancer metastasis

Several studies have linked the connection between chemokines /chemokine receptors expression and metastasis due to the fact that cancer cells could express chemokine receptors which enable them to migrate to distant sites based on chemokine ligands gradient released by the target organs. For example, there is strong evidence showing that CXCR4/CXCL12 axis is involved in lung, bone, and lymph nodes metastasis in several lines of cancers and in abdominal lymph nodes and liver metastasis in colorectal carcinoma in particular [18]. Other studies indicated that CXCR3 expression promotes colon cancer metastasis to lymph nodes [19]. Moreover, previous reports also predicted lymph node metastasis in colorectal carcinoma by expression of chemokine receptor 7

(CCR7) [20] while CCR6 and CCL20 were shown a significant upregulation in liver metastasis of colorectal cancer [21, 22].

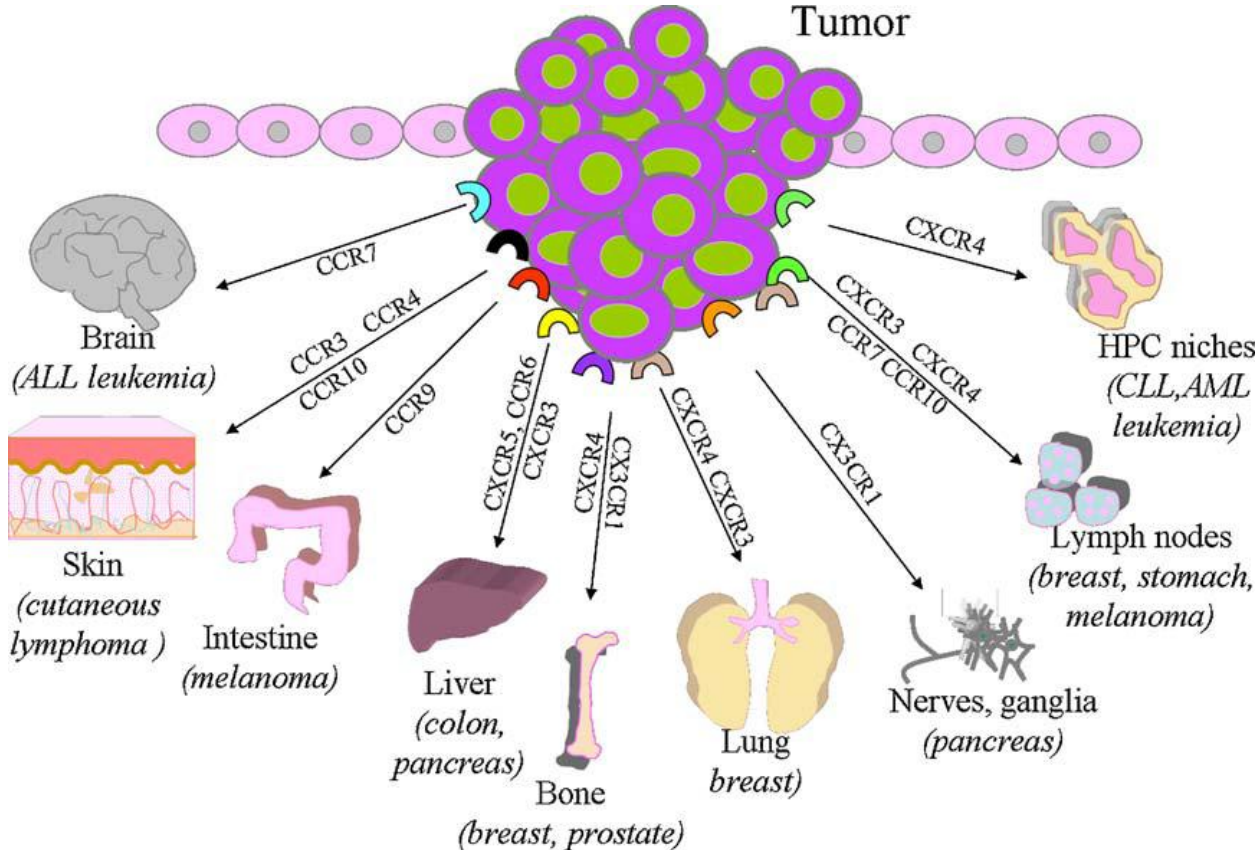

Figure (2) Chemokine receptor – mediated tumor metastasis.
Tumors express different types of chemokine receptors that are involved in the process of distant organ metastasis. Secondary localization of tumor is shown in parenthesis. HPC: hematopoietic precursor cell; CCL: chronic lymphocytic leukemia; AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia. *Alberto Mantovani, etc. al, The chemokine system in cancer biology and therapy, Cytokine & Growth Factor Reviews 21 (2010) 27–39*

3. Molecular aspects of colon cancer metastasis from chemokine approach

Up on activation, chemokine receptors stimulate downstream intracellular signaling cascades which result not just only in colon cancer cell migration but also in cell survival and proliferation [23]. Several lines of evidence indicate that chemokines and chemokine receptors have a crucial role in the survival of colon cancer cells under stressed conditions such as hypoxia and serum starvation [24]. Such unfavorable tumor microenvironment conditions, along with increase release of cytokines from stromal and tumor cells, upregulate chemokines/chemokine receptor expression and activate some transcriptional factors, resulting in increased cell migration and inhibition of apoptosis

[25]. Figure (3) summarizes most of the chemokine receptor intracellular signaling pathways involved in migration and survival/proliferation.

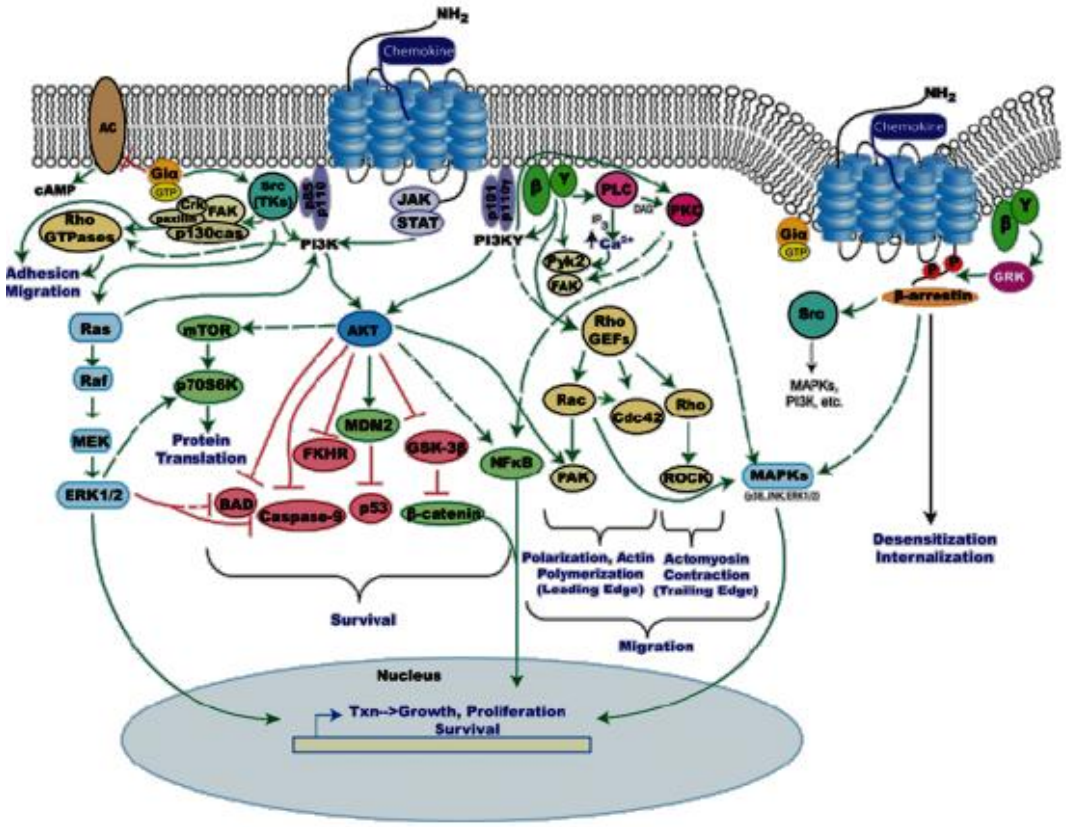

Figure (3) Intracellular downstream signaling pathways involved in migration, proliferation and cell survival. Signaling by Gi protein usually initiates through PI3K and other protein kinases which activate Akt and Rac pathways resulting in actin polymerization and reorganization. Rho kinase, MAPKinases such as Erk, and Akt all play an important role in cell migration, survival and proliferation. *Morgan O'HAYRE, Catherina L. SALANGA, Tracy M. HANDEL and Samantha J. ALLEN, Chemokines and cancer: migration, intracellular signaling and intercellular communication in the microenvironment, Biochem. J. (2008) 409, 635–649*

Not much has been reported regarding the role of chemokines and chemokine receptors in colon cancer, particularly in colon cancer metastasis. The existing mechanisms which regulate chemokine/chemokine receptor expression still poorly understood and require fundamental work. The present study, for the first time, shows a novel expression of functional chemokine receptor 4 (CCR4) by colon cancer cells that could improve our understanding of colon cancer metastasis.

Practical considerations

Materials and Methods

- **Cell lines and Cell culture**

  The human epithelial colon adenocarcinoma cell lines HT29 and 19/97* were cultured in Dulbecco's Modified Eagle Medium (DMEM); (*Sigma–Aldrich Co. catalog# D5796)*, supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mmol/L L-glutamine at 37C, 5%CO_2 as the following:

- **1st Day:**

  The vial containing cells is taken from liquid nitrogen and the lower part submerged in 37C water for 1 minute and then wiped by alcohol for sterilization. The content is pipetted to 5ml fresh DMEM and centrifuged at 800 r.p.m for 5minutes. The supernatant then discarded and the pellets resuspended in 10ml DMEM. Two culture flasks were used, T-25 and T-75 in which 15ml of fresh DMEM was added to both and 2ml of the resuspended cells was added to T-25 culture flask and the rest to the T-75 culture flask and incubated at 37C, 5% CO_2 for 24 hours.

- **2nd Day:**

  The media from the flasks is changed by pouring out and replacing 10ml DMEM to T-25 culture flask and 20ml to T-75 culture flask and incubated for further 24 hours at 37C, 5% CO_2 atmosphere.

- **3rd Day:**

  The media is poured out from the culture flasks and 2ml PBS is added for washing and poured out. Then 1ml Trypsin 0.25% PBS-EDTA is added to the T-25 culture flask and 3ml to T-75 culture flask and incubated 6 minutes. The trypsin then poured out and flasks is shacked and ticked by hands several times to ensure all cells detachment. 5ml DMEM is then added to the T-25 culture flask and 10ml to the T-75 culture flask with mixing using the pipette and 1μl is taken for cell counting in haemocytometer.

19/97*: colon adenocarcinoma cell line constructed by the department of surgery, Skåne university hospital, Malmö, Lund University

Gene expression

- **RNA isolation**

Total RNA was isolated using RNeasy columns from (Qiagen - Hilden, Germany) following the manufacturer's instructions. The Total RNA concentration of HT29 and 19/97 cell lines were measured using nanodrop spectrophotometer and were 929.5ng/µl and 854ng/µl respectively. The purity of total RNA was determined by the A260: A280 ratios and were 2.07 for HT29 and 2.09 for 19/97 cell lines. Before cDNA synthesis, the integrity of the RNA samples was confirmed by electrophoresis on 1% agarose gels demonstrating both 28S rRNA and 18S RNA.

Materials:

1- RNA isolation kit (Qiagen- Hilden, Germany)
2- Phosphate buffer saline (PBS)
3- Beta merceptophenol
4- 70% Ethanol
5- Microcentrifuge
6- Nano drop spectrophotometer
7- Agarose
8- 1X TAE buffer
9- GelRed stain
10- Microwave
11- Loading dye
12- DNA electrophoresis device
13- Ultraviolet (UV) source and Gel photographing device
14- HT29 and 19/97 colon cancer cell lines
15- 0.25% trypsin-EDTA in PBS
16- Hematocytometer for cell counting
17- Microscope

Method:

1- Trypsinize cells for 5 minutes and then wash by PBS
2- Lysis step:

Count cells to know how much lysis buffer should be added. 4×10^6 cells were counted for each cell line. And then centrifuge to pellet the cells.

3- Prepare beta merceptophenol to be added to RLT lysis buffer (1:100) by adding 10 µl of beta merceptophenol to 1000 µl RLT lysis buffer and then accordingly add 400 µl of the solution to 4×10^6 cells of each cell line. Then mix and wait for 10 minutes.
4- Pipette 400 µl of each lysate to two QIA-shredder spin columns and centrifuge at maximum speed 13000 r.p.m for 2 minutes.
5- Take 350 µl 70% ethanol to new eppendorf and add 350 µl lysate of each cell line and mix and transfer to RNeasy spin columns. Then centrifuge at 10000 r.p.m for 30 seconds and discard the flow through
6- Add 700 µl buffer, supplied with the kits, to each cell line spin columns and centrifuge 1 minute at 10000 r.p.m and carefully transfer the spin columns to new collection tubes and discard the flow through.
7- Wash by adding 500 µl washing buffer, supplied with the kits, to each spin columns and centrifuge at 10000 r.p.m for 60 seconds.
8- Repeat the washing step but at higher speed; 13000 r.p.m for 2 minutes.
9- For elution:

   Transfer the spin columns to new eppendorf tubes and add to each one 50 µl RNeasy free water, supplied with the kits, and wait 5 minutes and then centrifuge at 10000 r.p.m for 1 minute. Total RNA is now in the eppendorf tubes.
10- Measure RNA by nano drop spectrophotometer to determine the concentration and run 1% agarose to check RNA integrity.

- **Reverse Transcription PCR**

Reverse transcription was done with 2.5 µg of total RNA in a final reaction volume of 20 µL; AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent technologies catalog # 200436) according to the recommendations of the manufacturer. The reaction conditions were 42 ºC for 1 minute, 70 ºC for 15 minute, and 4 ºC holding.

- **Quantitative RT- PCR**

Real time quantitative polymerase chain reaction (qRT – PCR) was done for gene expression in a final volume of 25 µl using syber green dye for absolute gene quantification (Stratagene's Mx Real-Time PCR Platform). The RT PCR program was as follows: initial denaturation (10 minutes at 95 ºC) followed by 40cycles of denaturation (30 seconds at 95 ºC), annealing (1 minute at 55 ºC) and elongation (1 minute at 72 ºC). After the last cycle a final extension (1 minute at 95 ºC). Thereafter 12µl of each product was run on 2% agarose gel electrophoresis and stained by GelRed and analyzed under Ultraviolet (UV) light. mRNA reference sequences were used to design primers using web based primer design tools of national center of biotechnology information (NCBI), the Primers used were:

Table (3) indicates the primers sequences used in the qRT – PCR in the study. National center of bioinformatics institute (NCBI) tool was used to design the primers

Human Chemokine Receptor	Sequences of primers	Genbank accession Number	Product length (bp)
CCR7			
	Forward 5`-TCCTTCTCATCAGCAAGCTGTC-3`	NM_001838	529
	Reverse 5`-GAGGCAGCCCAGGTCCTTGAA-3`		
CCR4			
	Forward 5`- AAGCAAGCTGCTTCTGGT-3`	NM_005508	282
	Reverse 5`- TCAGTCATGGACCTGAGC-3`		
CCR6			
	Forward 5`-CTAGCGGAGTTCCAGCAAAC-3`	NM_004367	223
	Reverse 5`-TCAGCCACACATTGCTCTTC-3`		
CCR8			
	Forward 5`-GTGGCCTCTGAAGATGGTGT-3`	NM_005201	188
	Reverse 5`-GCCTTGGTCTTGTTGTGGTT-3`		
CXCR4			
	Forward 5`-TGGGCAGTTGATGCCGTGGC-3`	NM_001008540	136
	Reverse 5`-CGATGGCCAGGTAGCGGTCC-3`		
CXCR3			
	Forward 5`-TCAGCTTTGACCGCTACCTGAACA-3`	NM_001504	191
	Reverse 5`-TGGGAAGTTGTATTGGCAGTGGGT-3`		
CXCR7			
	Forward 5`-TCCAGGCCAAGACCACAGGCT-3`	NM_020311	125
	Reverse 5`-GGGCCACTGGTTGTGCTGCA-3`		
Human β-actin			
	Forward 5`-AGAGCCTCGCCTTTGCCGATCC-3`	NM_001101	103
	Reverse 5`-CACATGCCGGAGCCGTTGTCG-3`		

Protein expression

Flowcytometry

Surface expression of chemokine receptors were assessed using flow cytometry. A confluent of 80% HT29 and 19/97 cell lines were detached with 0.25% trypsin-EDTA in Phosphate Buffer Saline (PBS) and suspension of 5×10^6 cells/ml is prepared. Cells were then Fc blocked using 1 μg of human IgG, *eBioscience Catalog Number: 14-9161*, and incubated 15 minutes at room temperature. Then without washing, 10μg of PE, FITC, APC fluorescent conjugated antibodies, *catalog Numbers: FAB170P, FAB160A, FAB1567F, FAB195F, FAB197A, FAB1429P* (R&Dsystem) were added and incubated for 90 minutes at room temperature. Cells were then washed twice by FACS buffer and resuspended in 400 μl final volume FACS Buffer and analyzed in duplicates at least three times using BD *FACSCalibur* (*Becton Dickinson*). For intracellular staining, cells were fixed by 0.25% paraformaldehyde and incubated for 1 hour at 4 ºC and then centrifuged for 5 minutes at 300 r.p.m and all the supernatant is removed and the samples stored at 4 ºC overnight without PBS. Next day, FACS buffer was added and samples were centrifuged at 1000 r.p.m for 5 minutes and permeabilized by resuspending the pellets in 1ml room temperature 0.2% Tween 20 and incubated at 37 ºC for 15 minutes. Cells then washed by FACS buffer twice and Fc blocked using 1μg of human IgG, *eBioscience Catalog Number: 14-9161*, and incubated 15 minutes at Room Temperature. Then without washing, cells were stained using the mentioned conjugated antibodies and incubated on ice protected from light for 45 minutes. Cells then washed twice and resuspended in 400 μl final volume FACS Buffer and analyzed in duplicates at least three times using BD *FACSCalibur*. Unstained cells were used as negative control. Histograms were made using *CellQuest* software with assessment of 20000 events per sample.

Materials:

1- HT29 and 19/97 colon adenocarcinoma cell lines
2- Dublecco's modified eagle media (DMEM) supplemented by 10% FBS with 100x penicillin and streptomycin
3- Flowcytometry facility equipped with 488 nm, 405 nm, and 633 nm lasers
4- 5 ml BD falcon tubes
5- FACS Buffer containing 0.1% Sodium azide

6- Hematocytometer and cell counter
7- Ice box
8- Water bath
9- The following flourochrome conjugated antibodies (R&Dsystems):

A- Mouse Anti-human CXCR3-Allophycocyanin (APC), Catalog Number: FAB160A, Clone #: 49801

B- Mouse Anti-human CXCR-4-Phycoerythrin (PE),Catalog Number: FAB170P, Clone #: 12G5

C- Mouse Anti-human/rat CCR4-Fluorescein (FITC), Catalog Number: FAB1567F, Clone #: 205410

D- Mouse Anti-human CCR-6-Fluorescein (FITC),Catalog Number: FAB195F, Clone #: 53103

E- Mouse Anti-human CCR7-Allophycocyanin (APC),Catalog Number: FAB197A,Clone #: 150503

F- Anti-human/rat CCR8-Phycoerythrin (PE), Catalog Number: FAB1429P, Clone #: 191704

Method:

Cell surface staining:

1- Trypsinize cells using 0.25% trypsin-EDTA in PBS and incubate 7 minute with checking under microscope till cells detach.
2- Wash cells in 3ml media and centrifuge at 800 r.p.m for 5 minutes then resuspend in 3ml FACS buffer for 25cm culture flask and 5ml for 75cm culture flask and prepare 5×10^6 cells/ml.
3- Incubate 20 minutes prior to staining at room temperature.
4- Prepare your BD FACS 5ml tubes and add to each 100µL cells
5- Add 20µL human Fc block, eBioscience Catalog Number: 14-9161 and incubate 15 minutes at room temperature.
6- Then without washing add directly 10µL of 10µg of PE,FITC,APC conjugated antibodies (R&Dsystem) and incubate at room temperature for 90 minutes.
7- Add 1ml FACS buffer and centrifuge 10 minutes at 1400 r.p.m for 10 minutes
8- Repeat washing step
9- Resuspend pellets in 400 µL FACS buffer and vortex briefly then analyze using BD FACSCalibur.

Note: Negative control was run and treated the same way as above except there was no antibody staining step.

Intracellular staining:

1- Trypsinize cells using 0.25% trypsin-EDTA in PBS and incubate 7 minute with checking under microscope till cells detach.
2- Wash cells in 3ml media and centrifuge at 800 r.p.m for 5 minutes then resuspend in 3ml FACS buffer for 25 cm culture flask and 5ml for 75 cm culture flask and prepare 5×10^6 cells/ml.
3- Prepare your BD FACS 5ml tubes and add to each 100 µL cells
4- Fix cells by 0.25% paraformaldehyde as following:
   A- Prepare 2% paraformaldehyde (mix equal volumes of 4% paraformaldehyde and PBS ex. 1ml PFA and 1ml PBS)
   B- Take from the prepared solution 125 µl and add to the 100 µl cells and add 875 µl 1X PBS, mix gently and vortex immediately and incubate 1 hour at 4 ºC.
   C- Centrifuge for 5 minutes at 300 r.p.m and remove all the supernatant and store at 4 ºC overnight without PBS.
   D- Next day add 1ml FACS buffer and vortex then centrifuge at 1000 r.p.m for 5 minutes.
   E- Permeabilize cells by resuspending the pellets in 1ml room temperature 0.2%Tween 20 and incubate at 37 ºC for 15 minute.
   F- Add 1ml FACS buffer and centrifuge for 5minute at 350 r.p.m for washing and repeat this step.
   G- Remove the supernatant and shake, and then add 90 µl and vortex briefly.
   H- Add 20µL human Fc block, eBioscience Catalog Number: 14-9161 and incubate 15 minutes at room temperature.
   I- Then without washing add directly 10 µl of 10 µg of PE,FITC,APC conjugated antibodies (R&Dsystem) and incubate on ice protected from light for 45 minutes.
   J- Add 1ml FACS buffer and centrifuge 10 minutes at 1400 r.p.m for 10 minutes
   K- Repeat washing step
   L- Resuspend pellets in 400 µl FACS buffer and vortex briefly then analyze using BD FACSCalibur.

Note: Negative control was run and treated the same way as above except there was no antibody staining step.

Cell migration assay

Chemotactic responses of colon cancer cells were evaluated by using 24-well cell migration chambers with 8μm pore size inserts *(Corning Coster Corporation)* for HT29 and 19/97 cell lines. The colon cancer cells were suspended in serum free DMEM with 0.5% BSA at cell density of 8×10^5 cells/ml and 0.3 ml of the suspension was added to the inserts. DMEM 10% FBS either with/without CXCL12/SDF-1 and CCL17/TARC, *PeproTECh* catalog # 300-28A, and 300-30 respectively, was added to the lower chambers and incubated for 12, 24, and 48 hours at 37C, 5% CO_2. The cells on the lower surface of the insert membrane were stained with giemsa stain and non migrated cells were removed by cotton swabs. Migrated cells then counted microscopically using 10× high power fields for 5 different fields. Migration index was then calculated as the ratio of the number of migrated cells on wells – containing potential chemoattractant divided by the number of cells in the control wells.

Materials:

1- HT29, 19/97 cell lines.
2- DMEM with/without serum
3- Phosphate buffer saline (PBS)
4- Trypsin 0.25% PBS- 1mM EDTA
5- Coaster transmigration well culture plate 24 wells, 0.8μm pore size (*Corning Coster Corporation Catalog# 3422*)
6- SDF-1 (10μg/ml PBS or media containing 0.1% BSA), PeproTECh (catalog # 300-28A), TARC, PeproTECh (catalog # 300-30) 20μg/ml PBS or media containing 0.1% BSA.
7- Cotton swabs
8- Culture pate 24 wells
9- Giemsa stain
10- Inverted light microscope
11- Sterile forceps to handle the inserted chambers
12- 3.7% paraformaldehyde in PBS
13- 100% methanol
14- CO_2 incubator

Method:

1- Overnight cell starvation in serum free DMEM containing 0.5% BSA.
2- Next day trypsinize the cells and prepare suspension containing 8×10^5 cells/ml in free serum DMEM.
3- Add 700 µl of DMEM containing serum with /without SDF-1 or TARC (diluted in PBS or media with 0.5% BSA) to the lower chambers.
4- Add 300 µl serum free DMEM containing the cells to the upper chambers.
5- Put the upper chambers in the lower chambers and incubate at 37 ºC, 5% CO_2 for 12, 24, and 48 hours.
6- Remove the media from the upper chambers and wash them twice by PBS and place them in a new culture plate 24 wells.
7- Add 700 µl paraformaldehyde (3.7% in PBS) to the lower chambers and 300 µl to the upper chambers to fix the cells at room temp for 2 minutes.
8- Remove the paraformaldehyde and wash twice by PBS.
9- Permeabilize cells by adding 700 µl 100% methanol to the lower chambers and 300µl to the upper chambers and incubate at room temp for 20 minutes.
10- Remove methanol and wash twice by PBS.
11- Stain with giemsa stain (1:20) by adding 700 µl giemsa stain to the lower chambers and 300 µl stain to the upper chambers and incubate at Room temp for 15minutes.
12- Remove giemsa stain by washing twice with PBS.
13- Scrap off non migrated cells from the edges all around with cotton swab.
14- Count migrated cells under light inverted microscope/10HPF (at least 5 fields required).

Proliferation assay

Cell proliferation was evaluated in triplicates using Cell Counting Kit-8 assay (CCK-8; Sigma-Aldrich, Catalog # 96992). Briefly, Cells were seeded in 96 – microplate at 1×10^5 cells/well in media either with/without CCL17/TARC (100ng/ml) for 24, 48, and 72 hours. To assess proliferation, 10µl CCK-8 was added per well, and the cells were incubated for an additional 4 hours and absorbance at 450 nm was recorded using 96-well plate ELISA reader.

Materials:

- HT29 and 19.97 colon adenocarcinoma cell lines
- DMEM, 10% FBS
- Serum free DMEM
- Microplate reader 450 nm – 490 nm filter
- 96 well microplate
- Multipipette 8 – 12 channels for 10 -100 µl
- Cell counter and hematocytometer
- CO_2 incubator

Method:

- Trypsinize cells and wash them twice by media
- Prepare cell suspension of starved cells 5×10^4 - 10^5 cells/ml
- Add to the first column only media without cells. This will serve as blank
- Add 100 µl of starved cell suspension to the wells required for testing in two sets, one for control and one for test without adding the ligand.
- Incubate all for 24hrs at 37 ºC, 5% CO_2 and the next day add 10µl potential ligand and incubate for 24, 48, and 72hrs.(prepare the ligand with normal media containing 10% FBS)
- Next day add 10 µl of CCK-8 kit to the control and test wells and incubate 4hrs at 37 ºC, 5% CO_2
- Repeat the previous step for each incubation period you set above.
- Read O.D at 450 nm.

Plates were read with an Enzyme – linked Immunosorbent assay plate reader and data expressed in optical density (O.D) units. Experiments were conducted in triplicate (N=3).

Statistical Analysis

All statistical analyses were performed using SigmaPlot 10 software. Statistical comparisons between datasets were made with Mann–Whitney rank-sum test. And P value < 0.05 was considered as significant.

Results

1. Gene Expression profile

❖ **Expression of chemokine receptors in colon cancer cell lines**

QRT-PCR was performed to investigate the mRNA expression levels of two human colon cancer cell lines. All beta chemokine receptors (CCR4, CCR6, CCR7, CCR8) and alpha chemokine receptors (CXCR3, CXCR4) expressed mRNA for these colon cancer cell lines, HT29 and 19/97, except CXCR7 with higher expression being detected for CXCR4, CXCR3, and CCR4. The PCR products were further confirmed by gel electrophoresis in which single bands were detected to the corresponding product length of each chemokine receptor. Expression pattern was almost similar in both HT29 and 19/97 cell lines, (Figure 4).

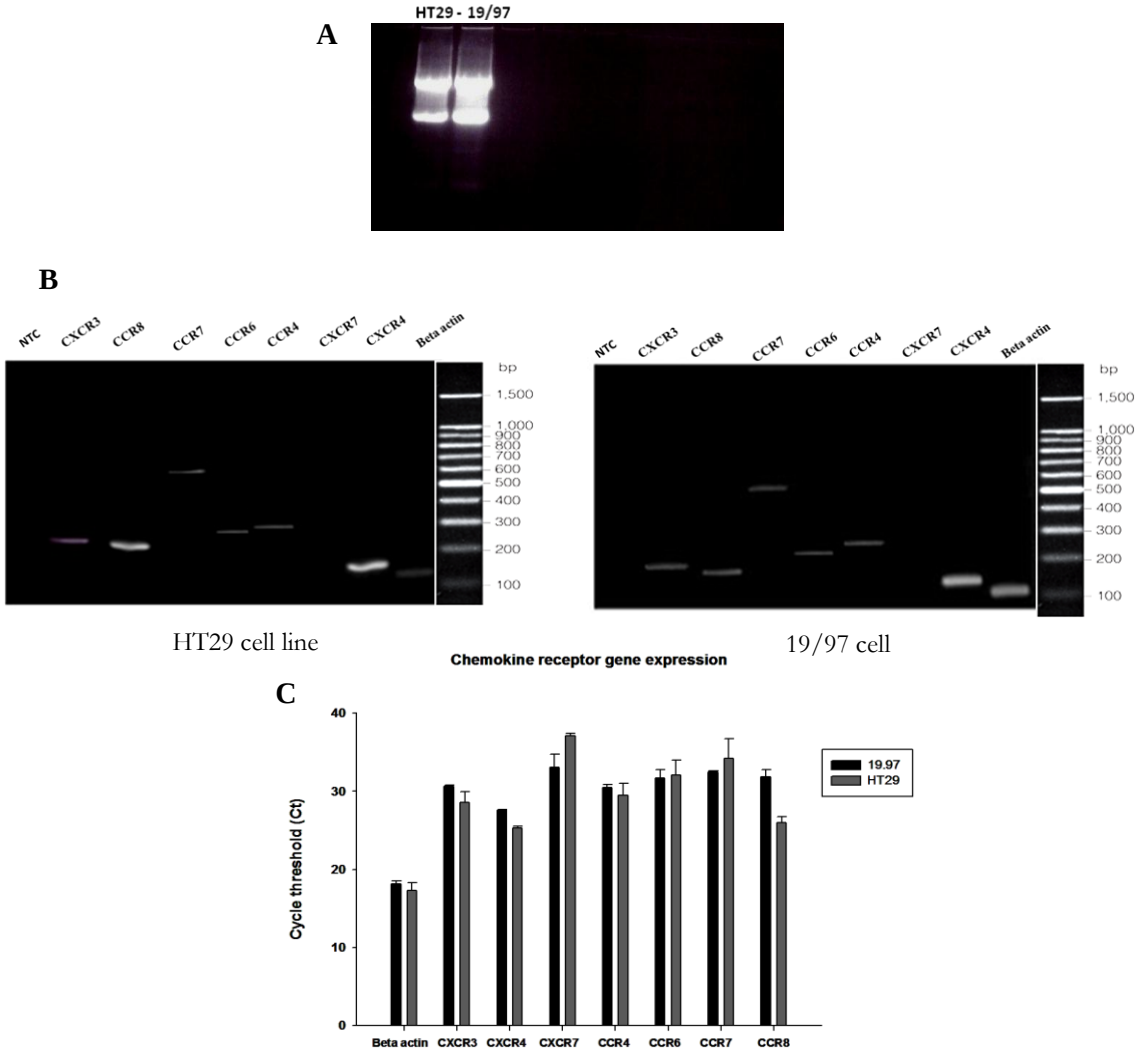

Figure (4) 1% agarose electrophoresis for HT29, and 19/97 cell lines show RNA integrity in which both 28 rRNA and 18 sRNA are appeared, **A**. Gel electrophoresis of QRT-PCR products indicate that all chemokine receptors are expressed except CXCR7 in both cell lines, **B**. gene expression profile histogram indicates cycle threshold of each receptor in both cell lines where beta actin used as housekeeping gene. The lower cycle threshold the higher gene expressed, **C**. Data shown represents a typical one of three independent experiments.

2. Protein expression profile

❖ **Chemokine receptors are expressed on the cell surface of colon cancer cells**

Further studies were performed using flowcytometry to analyze the highest receptors expressed on the cell surface. As shown in Figure 5, unstained cells used as negative control in which negative cells fit within the negative region (left lower quadrant) of each channel filters of flowcytometry. Single color staining process was done for each receptor separately to exclude unnecessary overlapping of cell staining. On the other hand, HT29 and 19/97 cell lines expressed all beta chemokines receptors (CCR4, CCR6, CCR7, CCR8) and alpha chemokine receptors (CXCR3, and CXCR4) on the cell surface with the highest expression being detected for both CXCR3, and CCR4 in both cell lines (Figure 6). Moreover, intracellular chemokine receptor expression also revealed results similar to those demonstrated on the cell surface, (Figure 7).

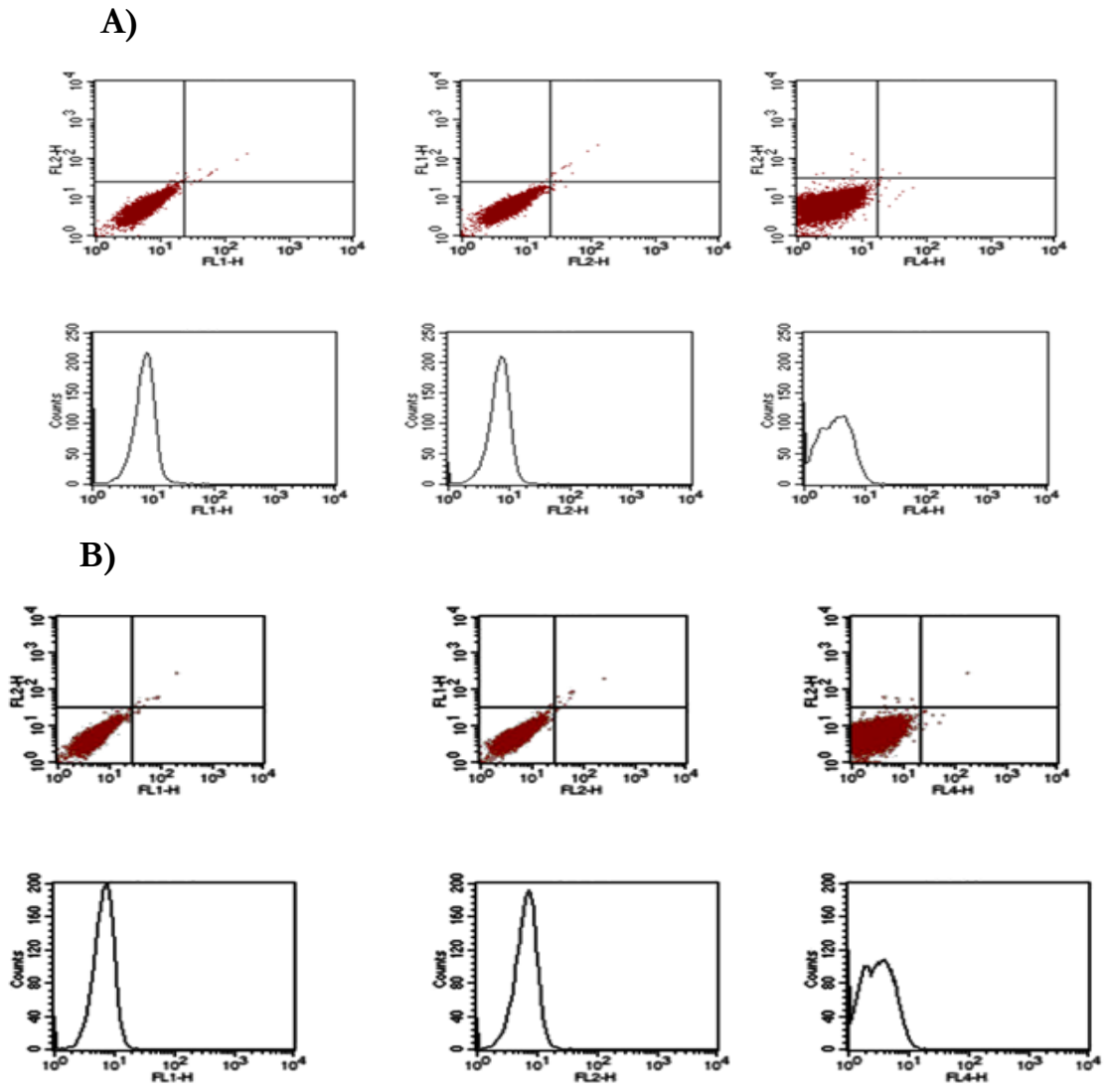

Figure (5) Unstained cells used as negative control, **A)** 19/97 cell line , and **B)** HT29 cell line dot plots and histograms show negative cells. Single color analysis was performed for each conjugated flourochrome antibody in which FL1 channel filter used for FITC, FL2 for PE, and FL4 for APC.

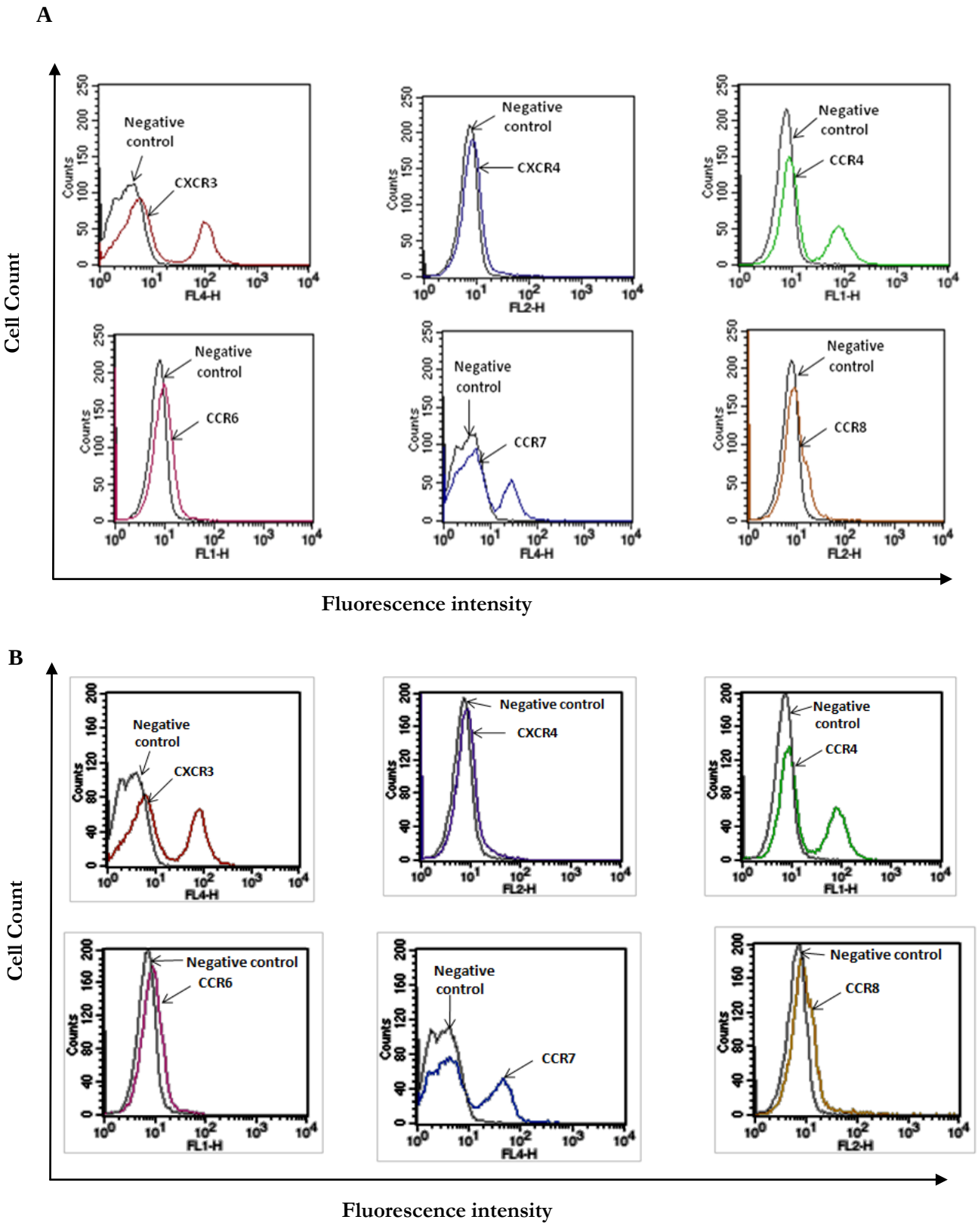

Figure (6) Cell surface chemokine receptors expression histograms **A)** 19/97 cell line, and **B)** HT29 cell line show that CXCR3, and CCR4 are the highest receptors expressed on the cell surface of both cell lines. Data were analyzed in duplicate at least three times.

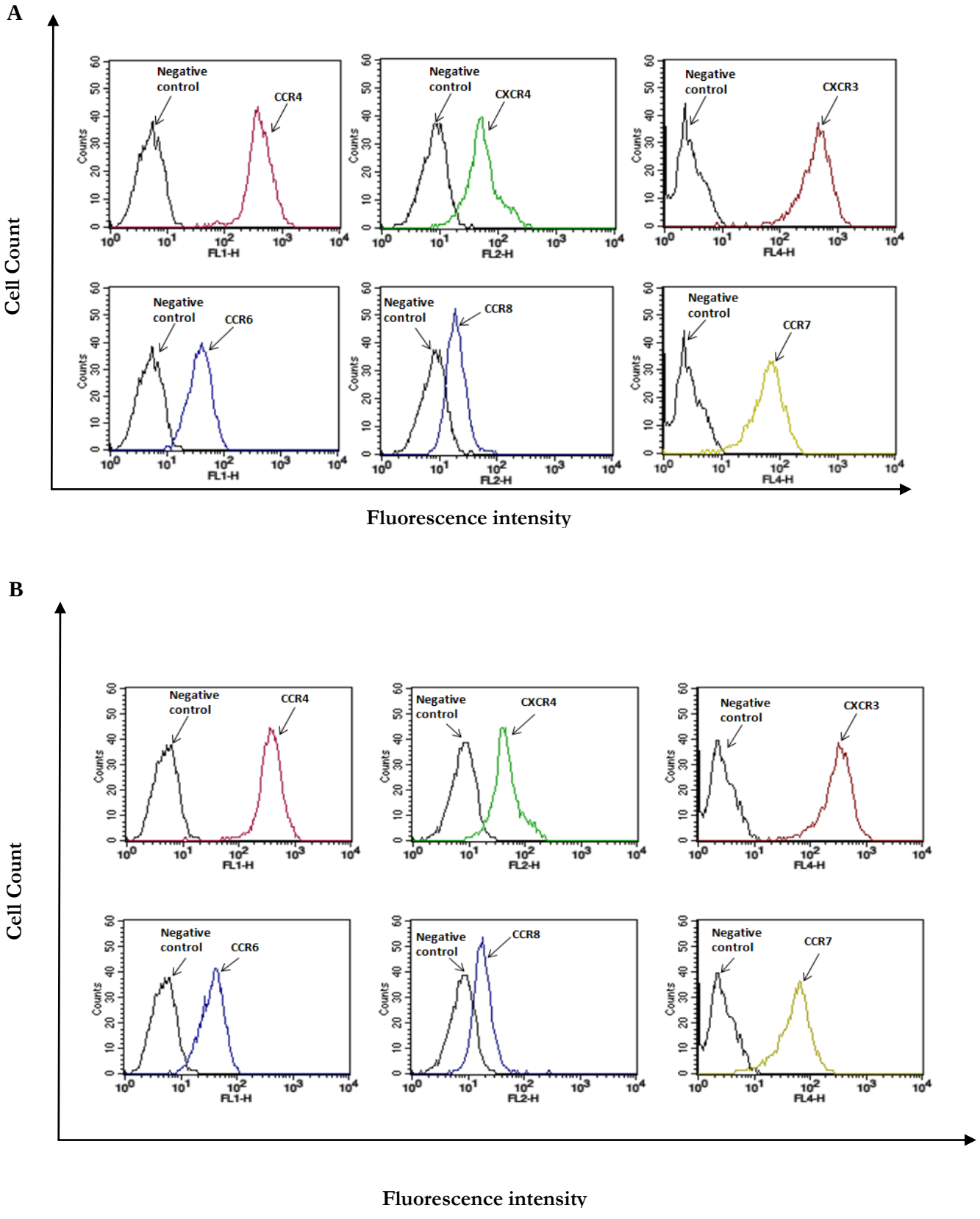

Figure (7) Intracellular staining histograms indicate intracellular expression of chemokine receptors with the highest expression being detected for CXCR3, and CCR4 in **A)** HT29 cell line, and **B)** 19/97 cell line.

3. Migration assay results

❖ **Expression of CCR4 and CXCR4 on the surface of colon cancer cells are functional and increase chemotaxis**

Migration assays were performed to investigate whether CCR4 and CXCR4 chemokine receptors induce migration of colon cancer cell lines or not since these receptors have shown the highest expression pattern in protein and gene levels respectively. CCR4 revealed significant increase of HT29 and 19/97 colon cancer cell migration rate when stimulated by its ligand TARC/CCL17 comparing with those cells with no potential ligand (Figures 8 and 9).

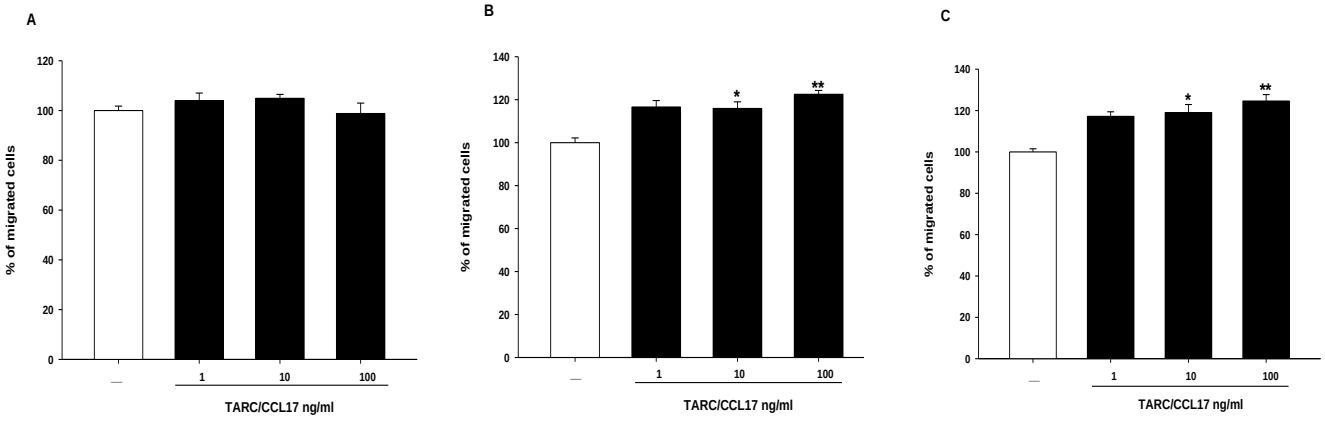

Figure (8) Cell migration in response to CCL17/ TARC stimulation indicates HT29 cell migration rate with or without 1, 10, and 100 ng/ml CCL17/TARC in **A)** 12hrs, **B)** 24hrs, **C)** 48hrs. CCL17/TARC induces significant cell migration in a dose and time dependent manner ******P= 0.008 vs control cells (without CCL17/TARC stimulation), *****P=0.016 vs control cells. Data shown represent %(mean ± SEM) of at least two independent experiments. Migration index was calculated as the ratio of the number of migrated cells on wells – containing potential chemoattractant divided by the number of cells in the control wells.

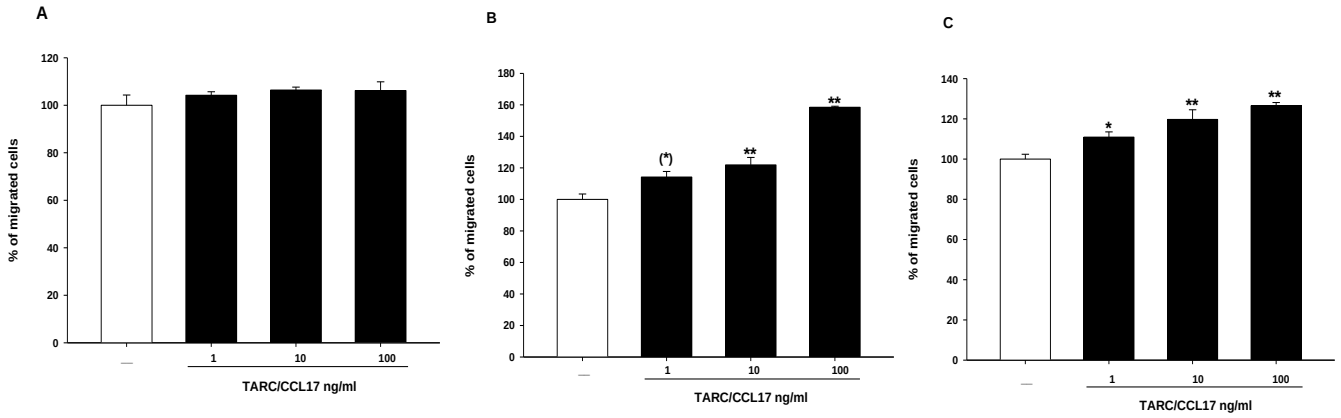

Figure (9) indicates HT29 cell migration rate with or without 1, 10, and 100ng/ml CCL17/TARC in **A)** 12hrs, **B)** 24hrs, **C)** 48hrs. CCL17/TARC induces significant cell migration in a dose and time dependent manner ******P= 0.008 vs control cells (without CCL17/TARC stimulation), **(*)**P= 0.032 vs control cells, and *P=0.016 vs control cells. Data shown represent %(mean ± SEM) at least of two independent experiments. Migration index was calculated as the ratio of the number of migrated cells on wells – containing potential chemoattractant divided by the number of cells in the control wells.

Migration assays also indicated functional CXCR4 which induce HT29 and 19/97 cell migration upon activation by different doses of its corresponding ligand SDF-1/CXCL12 in different sets of time points, (Figures 10 and 11) which indicates that colon cancer cell lines bearing these receptors are more prone to migrate toward high chemokine ligand gradients.

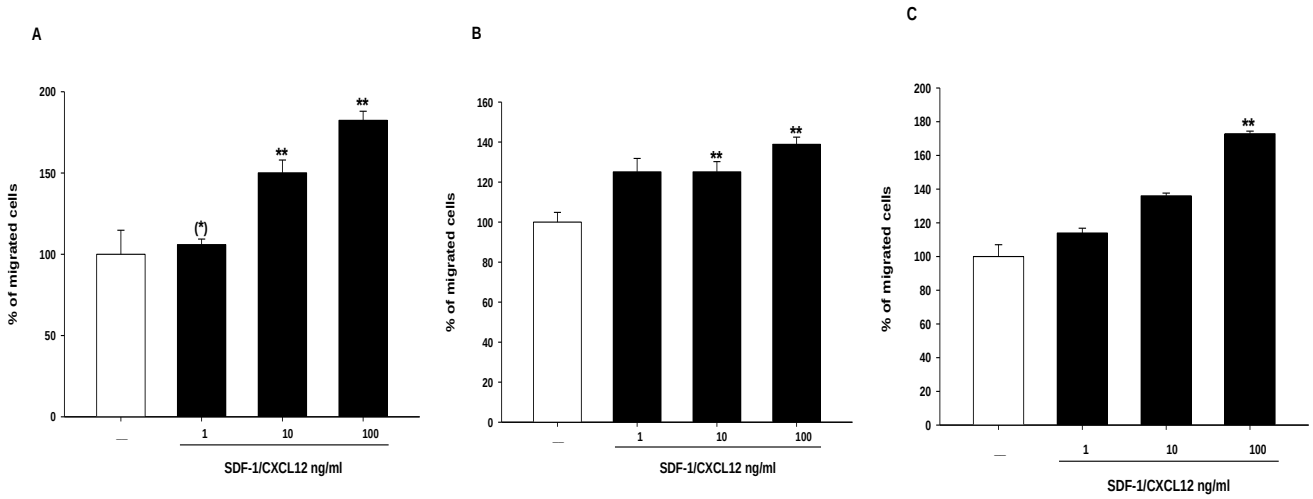

Figure (10) Cell migration in response to CXCL12/SDF-1 stimulation indicates HT29 cell migration rate with or without 1, 10, and 100ng/ml CXCL12/SDF-1 in **A)** 12hrs, **B)** 24hrs, **C)** 48hrs. CXCL12/SDF-1 induces significant cell migration in a dose and time dependent manner**. ****P= 0.008 vs control cells (without CXCL12/SDF-1 stimulation), **(*)**P= 0.032 vs control cells, Results shown as %(mean ± SEM) of at least 2 independent experiments. Migration index was calculated as the ratio of the number of migrated cells on wells – containing potential chemoattractant divided by the number of cells in the control wells.

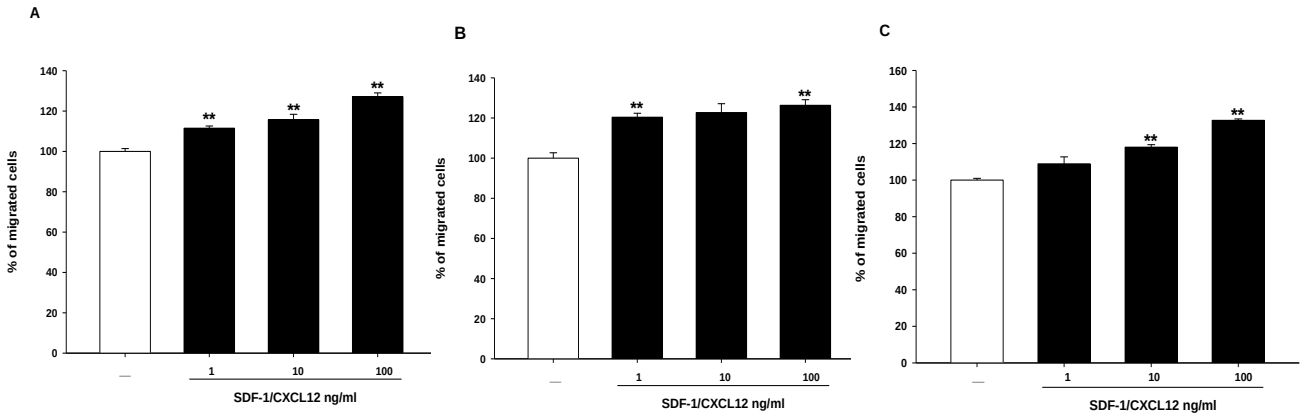

Figure (11) Represents 19/97 cell migration rate with or without 1, 10, and 100ng/ml CXCL12/SDF-1 in **A)** 12hrs, **B)** 24hrs, **C)** 48hrs. CXCL12/SDF-1 induces significant cell migration in a dose and time dependent manner. ******P= 0.008 vs control cells (without CXCL12/SDF-1 stimulation), Results shown as % (mean ± SEM) of at least 2 independent experiments. Migration index was calculated as the ratio of the number of migrated cells on wells – containing potential chemoattractant divided by the number of cells in the control wells.

4. Proliferation assay results

❖ CCR4 is not involved in colon cancer growth in vitro

To examine the role of CCR4 in colon cancer growth, CCR4 was tested for its ability to induce colon cancer cell proliferation in vitro. As (Figure 12) shows, HT29 and 19/97 cell line proliferation was not increased when cells stimulated with high TARC/CCL17 concentration (100 ng/ml) in 3 days. There was no significant difference between control and stimulated cells in proliferation curves.

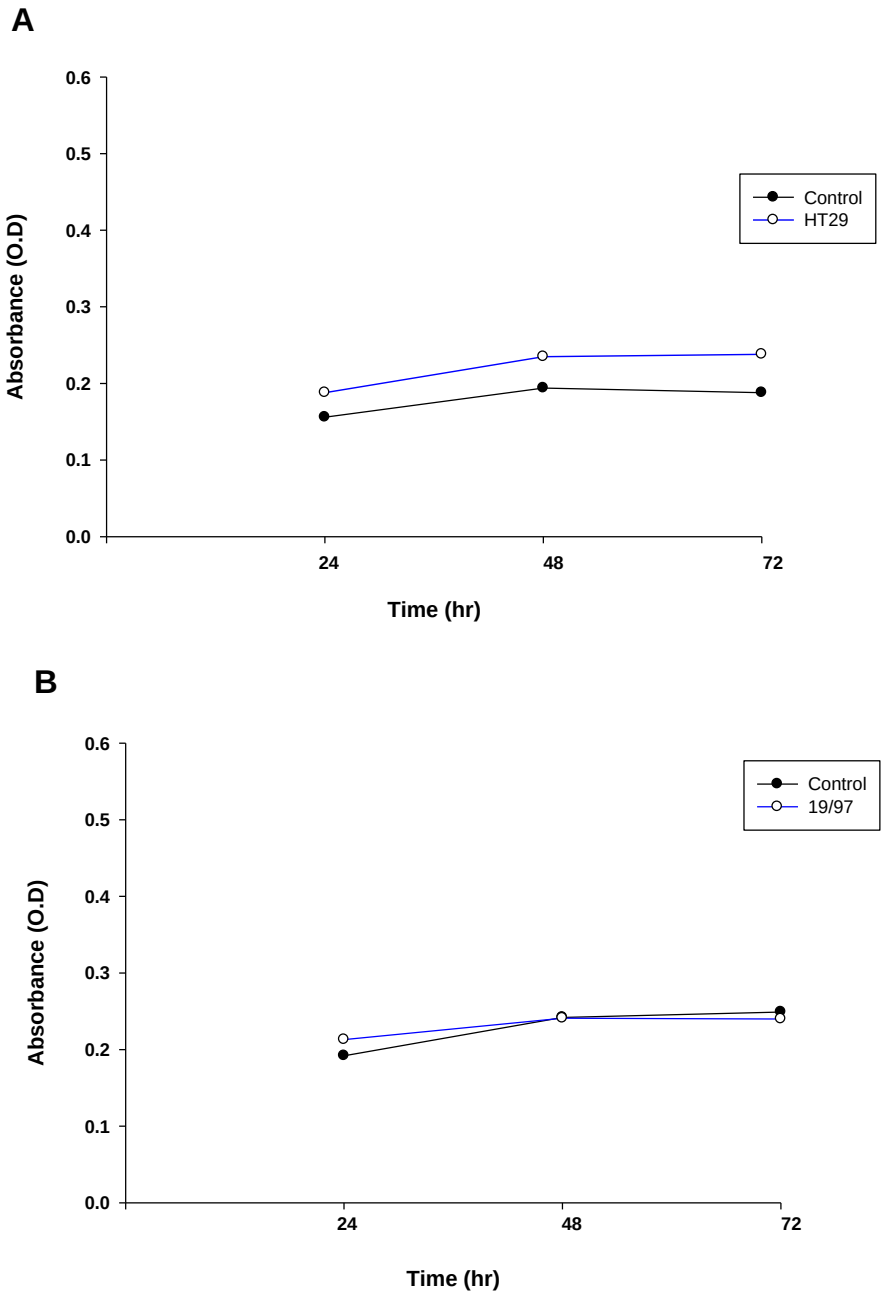

Figure (12) Proliferation curves. CCL17 100ng/ml does not induce significant cell proliferation (P>0.05) in 24, 48, and 72 hrs in **A)** HT29 and **B)** 19/97 cell lines.

Discussion

An increasing number of strong evidences highlight the importance of chemokines and their receptors in the metastasis of wide varieties of cancer including colon cancer [26-28]. Our data based on experimental molecular studies demonstrated that colon cancer cell lines express functional CXCR4, chemokine receptor for the chemokine CXCL12/SDF-1 and CCR4, chemokine receptor for the chemokine CCL17/TARC. In this study we have investigated a set of chemokine receptors that some of them are known to have a significant role in colon cancer metastasis [19, 21, 29], while others exist for the first time to be novel receptors that might be key players in such complex process. Two primary colon adenocarcinoma cell lines were used in the study, HT29 and a new constructed cell line called 19/97 which has a metastatic behavior characteristics. Gene expression was made using real time polymerase chain reaction (RT-PCR) for seven chemokine receptors, Alpha CXC chemokine receptors; CXCR3, CXCR4, CXCR7 and Beta CC chemokine receptors; CCR4, CCR6, CCR7, CCR8. All chemokine receptors expressed mRNAs except CXCR7 in both cell lines. CXCR4, CXCR3, and CCR4 were the highest receptors expressed mRNA levels. The high mRNA expression pattern reflects the fact that these cells could be a target for chemokine stimulation. We have also assessed cell surface protein expression which reveals a consistently expression of CCR4 and CXCR3 on both colon cancer cell lines. Further investigations were made on CCR4 to test its functionality since it was the most abundantly expressed receptor on the cell surface. On the other hand, we found that CXCR4 has a low cell surface expression; however, it induces a significant colon cancer cell migration in a dose - and time - dependent manner when stimulated by its ligand CXCL12/SDF-1. The high mRNA expression of CXCR4 and its low surface expression can be explained by either receptor internalization or protein posttranslational modification [30, 31]. Moreover, receptor shedding due to structural changes induced by the receptor when binding to the flourochrome - conjugated antibody could be a technical explanation.

Interestingly, we observed intracellular store of the chemokine receptors in a profile that is similar to the cell surface expression which reflect the fact that these intracellular store could be recycled to the cell surface in response to proinflammatory stimuli that upregulate their cell surface expression or they might be involved in cellular functions by binding to intracellular stores of their ligands [32]. The reason behind why we have selected CXCR4 along with CCR4 for further investigations although it has a low cell surface expression that CXCR4 is the most receptor being studied and proved to play a key role in the metastasis of different types of cancers including colon cancer [33, 34]. Besides, we thought that it would be good idea to compare and evaluate results that have been achieved by our new findings to those already existed for CXCR4 for better understanding how these receptors might contribute to colon cancer metastasis.

There is some evidence showing that cancer bearing CXCR4 cells upon stimulation by CXCL12/SDF-1 influence cell migration and proliferation [35, 36]. Several studies have indicated that alteration in intracellular signaling pathways may be responsible for cell survival and cell migration [37]. The mechanism behind was reported by recent findings which shed the light on Rho kinase and MAP kinases as a pivotal intracellular signaling steps that mediate cell migration and cell proliferation when cells expressing CXCR4 stimulated by the chemokine CXCL12/SDF-1 [18, 38, 39]. Furthermore, blocking CXCR4 on the cell surface results in a major reduction in the metastasis of colon cancer to the liver and lung [40].

Here we demonstrate, for the first time, CCR4 expression induce a significant colon cancer cell migration. In previous studies CCR4 was described to be involved in the metastasis of some tumors either with solid or hematopoietic origin but not in colon cancer [41-44]. In another contest, Michael et. al [32] were unable to express CCR4 in both gene and protein levels when using HT29 colon adenocarcinoma cell line while in our study we were successful in obtaining a unique expression pattern of CCR4 mainly on the cell surface which lead us to address an important question whether this receptor is functional or not or has any

specific role in colon cancer metastasis. The migration assays revealed functional CCR4 when stimulated with its ligand CCL17/TARC and induce cell migration in both colon cancer cell lines. The exact mechanism how CCR4 regulate cell migration is not yet identified and this question required fundamental work to be answered.

Cell proliferation is an essential step by which tumor starts growing and increase its size. We tested the proliferation ability of both colon adenocarcinoma cell lines, HT29 and 19.97, under the stimulation of CCL17/TARC to understand how CCR4 can regulate tumor growth. No significant proliferation rate was observed when cells treated by 100 ng/ml TARC for 3 days. Our findings were consistent with Lee et al.'s findings [43] in gastric cancer and Ji-Yu et al's in breast cancer [41]. In murine models of breast cancer however, CCR4 increases tumor growth in vivo but not in vitro due to factors that might have a role in vivo rather than in vitro [41].

Table (4). Summary of Chemokine Receptor expression on mRNA, Intracellular Protein, and Cell Surface Protein by HT29 and 19/97 colon cancer cell lines

Chemokine Receptor	mRNA		Intracellular protein		Cell surface protein	
	HT29	19/97	HT29	19/97	HT29	19/97
CXCR3	+	+/-	+	+	+	+
CXCR4	+	+	+	+	+/-	+/-
CCR4	+/-	+/-	+	+	+	+
CCR6	+/-	+/-	+/-	+/-	+/-	+/-
CCR7	+/-	+/-	+	+	+	+
CCR8	+	+/-	+/-	+/-	+/-	+/-

−, not detected; +/−, detected; +, strong expression

In summary, we demonstrate that chemokine receptor CXCR4 and CCR4, for the first time, are expressed by colon cancer cells both in mRNA and protein levels. The cell surface receptors indicate functional receptors upon stimulation with their ligands CXCL12/SDF-1 and CCL17/TARC respectively. Further studies required on CCR4 to elucidate how cell migration activation is regulated through CCR4 which could open new horizons in the molecular target therapy of colon cancer.

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