THE PRODUCTION AND PURIFICATION OF D6 AND DARC CHEMOKINE DECOY RECEPTOR RECOMBINANT PROTEINS AND THEIR EFFECTS ON MIGRATION AND INVASION IN MDA-MB-231 AND MCF-7 CELLS

TAN WEE YEE

UNIVERSITI SAINS MALAYSIA

2018

THE PRODUCTION AND PURIFICATION OF D6 AND DARC CHEMOKINE DECOY RECEPTOR RECOMBINANT PROTEINS AND THEIR EFFECTS ON MIGRATION AND INVASION IN MDA-MB-231 AND MCF-7 CELLS

by

TAN WEE YEE

Thesis submitted in fulfillment of the requirements

for the degree of

Doctor of Philosophy

July 2018

ACKNOWLEDGEMENT

First of all, I would like to express my sincere thanks and deepest gratitude to my main supervisor, Dr. Chew Ai Lan, for spending her times and efforts on supervising me throughout the research. Without her dedicated guidance and constructive advice, this project will not be completed smoothly. Again, hearty thanks to Dr Chew for being a great supervisor.

Not forgetting my co-supervisors, Dr Khoo Boon Yin for her valuable advice on cell culture studies and also Professor Darah Ibrahim from School of Biological Sciences. The helps provided by scientific, academic and administrative staff of INFORMM throughout the study are highly appreciated.

Special gratitude goes to National Science Fellowship awarded by Ministry of Science, Technology and Innovation (MOSTI) which covered my tuition fees and living expenses in USM. Besides, the Exploratory Research Grant Scheme (ERGS) from the Ministry of Higher Education Malaysia (grant no: 203/CIPPM/6730059) is acknowledged for funding this work.

Furthermore, I would like to convey my sincere thanks to all of my best fellow colleagues and friends in the lab and institute who never fail in lending me a hand when I was in the dark. The support, help and more importantly, the valuable friendship and also the great time we spent together, will not be forgotten.

Last but not least, I dedicate this milestone to my family, husband and daughter. Million of thanks to my family for helping me to look after my daughter while I was working in the lab. This journey will not be possible without the unfailing love, care and encouragement from my beloved husband. To my lovely daughter who has taught me to become a better parent and person, you are forever my source of motivation. Thank you!

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

ºC	Degree Celsius
%	Percent
®	Registered Trademark
<	Less Than
>	More Than
1×	1 Time
µg	Microgram (s)
µL	Microlitre (s)
A_{260}	Absorbance at 260 nm Wavelength
A_{280}	Absorbance at 280 nm Wavelength
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Base Pair (s)
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleoside Triphosphates
E. coli	*Escherichia coli*
ELISA	Enzyme-Linked Immunosorbent Assay
g	Gram (s)
H_2O	Water
HCl	Hydrogen Chloride
HRP	Horseradish Peroxidase

kb	Kilobase (s)
kDa	Kilodalton (s)
LB	Luria Broth
M	Molar (s)
mg	Milligram (s)
mg/mL	Milligram (s) per Millilitre (s)
min	Minute (s)
mL	Millilitre (s)
mM	Millimolar (s)
NCBI	National Center for Biotechnology Information
ng/µL	Nanogram (s) per Microlitre (s)
nm	Nanometer (s)
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
pH	Power of Hydrogen
rpm	Revolutions per Minute
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl Sulfate-polyacrylamide Gel Electrophoresis
UV	Ultraviolet
v/v	Volume Per Volume
w/v	Weight Per Volume

PENGHASILAN DAN PENULENAN PROTEIN REKOMBINAN PENARIK RESEPTOR KEMOKINA D6 DAN DARC SERTA KESANNYA KE ATAS MIGRASI DAN INVASI SEL MDA-MB-231 DAN MCF-7

ABSTRAK

D6 dan DARC telah dilaporkan sebagai penarik reseptor kemokina dalam kajian kanser. Penglibatan D6 dan DARC dalam kanser payudara telah dikaji dan dilaporkan berkorelasi negatif dengan perkembangan dan metastasis sel kanser payudara. Kajian ini bertujuan membina klon rekombinan D6 dan DARC, mengekspres, menganalisis dan menulenkan protein rekombinan dan kemudian menentukan kesan protein rekombinan ke atas migrasi dan invasi sel kanser payudara. Gen D6 dan DARC dalam sel MDA-MB-231 diamplifikasikan oleh RT-PCR satu-langkah dengan primer gen spesifik dan polimerase Pfu DNA pada mulanya. Selain itu, DARC juga diamplifikasikan dengan PCR menggunakan polimerase DNA Phusion. Bagi setiap klon, dua pasang primer spesifik digunakan untuk menghasilkan jujukan nukleotida penuh yang kemudian diklonkan ke dalam vektor ekspresi pPICZ dan pPICZα. Pembinaan klon TA dan analisis penjujukan DNA menunjukkan pemadanan sempurna antara TA-D6 dan TA-D6α dengan jujukan DNA rujukan. Walau bagaimanapun, penggantian bes diperhatikan pada lokasi 131 bp TA-DARC dan TA-DARCα. Mutagenesis tapak terarah telah dijalankan untuk membetulkan ketidakpadanan itu. Penjanaan vektor ekspresi yis rekombinan dilakukan dan penjujukan DNA dijalankan semula untuk mengesahkan ligasi sebingkai gen yang diingini dengan jujukan hujung N dan C vektor-vektor

ekspresi. Analisis ClustalW sekali lagi menunjukkan ketidakpadanan pada 131 bp pPICZ-DARC dan pPICZ-DARCα, selaras dengan penemuan TA-DARC dan TA-DARCα. Ini mencadangkan bahawa penggantian bes yang ditemui bukan satu ketidakpadanan tetapi mungkin merupakan polimorfisme di mana jujukan nukleotida DARC daripada sel MDA-MB-231 mempunyai satu nukleotida yang berlainan pada 131 bp dan jujukan gen tersebut telah disimpan dalam GeneBank (ID: JX081310). Selepas itu, elektrotransformasi *Pichia* GS115 telah dijalankan untuk membolehkan integrasi vektor ekspresi yis rekombinan linear ke dalam genom yis. Selepas penyaringan antibiotik dan analisis PCR untuk mengesahkan integrasi gen yang diingini dalam genom yis, klon-klon positif dikulturkan atas medium dengan peningkatan kepekatan Zeocin^{TM}. Klon-klon yang dapat merintang kepekatan Zeocin^{TM} sehingga 2 mg/ml dipilih untuk ekspresi protein rekombinasi. Profil awal ekspresi protein rekombinan menunjukkan penghasilan protein heterologus yang rendah oleh klon pPICZα. Oleh itu, hanya klon pPICZ yang menghasilkan protein rekombinan intraselular dipilih untuk kajian ekspresi selanjutnya. Pengoptimuman parameter fizikal dan kimia terpilih telah dilakukan dan ekspresi protein rekombinan intraselular terlarut didapati meningkat secara mendadak dengan peningkatan sebanyak 1011.97% untuk klon rekombinan D6, 451.75% untuk klon rekombinan DARC dan 394.72% untuk klon rekombinan DARC bermutasi. Parameter yang dioptimumkan ialah: BMMY pada pH 6.0, saiz inokulum pada OD_{600} 2.5 (untuk D6) dan 1.0 (untuk DARC dan DARC bermutasi), induksi dengan 1.0% (v/v) metanol sekali setiap 24 jam dan inkubasi pada 16°C selama 48 jam. Protein rekombinan kemudian ditulenkan dengan menggunakan kromatografi afiniti dengan resin Cobalt. Analisis SDS PAGE dan Western Blot yang telah dioptimumkan menunjukkan bahawa rekombinan D6, DARC dan DARC bermutasi hasilan yis yang

telah ditulenkan mempunyai berat molekul yang lebih tinggi berbanding dengan berat molekul kiraan teori. Selepas itu, protein rekombinan yang telah ditulenkan digunakan dalam kajian berasaskan sel untuk menguji aktiviti biologinya. Ujian viabiliti sel menunjukkan bahawa rekombinan D6, DARC dan DARC bermutasi tidak mempengaruhi viabiliti sel dengan ketara dan dengan itu mencadangkan bahawa mereka tidak terlibat dalam kematian sel kanser payudara. Ujian penyembuhan luka menunjukkan bahawa kehadiran rekombinan D6, DARC atau DARC bermutasi pada 10 μg/ml menghalang migrasi sel kanser payudara dengan optimumnya. Kajian ELISA menunjukkan hubungan songsang antara protein-protein rekombinan dengan tahap CCL2 dalam sel yang dirawat. Ujian migrasi menggunakan "Boyden chamber" memaparkan fungsi protein rekombinan dalam merencat aktiviti kemotaksis sel-sel yang dirawat. Ujian invasi menggunakan "Boyden chamber" yang dilapisi matrigel menunjukkan keupayaan protein rekombinan dalam menyekat invasi sel yang dirawat. Dalam perbandingan kesan tunggal dan kesan kombinasi protein rekombinan, gabungan D6 dan DARC pada nisbah 1:1 (10 μg/ml) didapati paling baik dalam mengurangkan tahap CCL2 dalam sel-sel yang dirawat dan seterusnya menghalang migrasi dan invasi sel-sel yang dirawat. Ini telah menunjukkan bahawa rekombinan D6, DARC dan DARC bermutasi hasilan yis yang ditulenkan bukan sahaja bertindak sebagai pengawal selia negatif bagi migrasi dan invasi sel kanser payudara malah kesan perencatannya adalah lebih tinggi apabila digunakan bersama.

THE PRODUCTION AND PURIFICATION OF D6 AND DARC CHEMOKINE DECOY RECEPTOR RECOMBINANT PROTEINS AND THEIR EFFECTS ON MIGRATION AND INVASION IN MDA-MB-231 AND MCF-7 CELLS

ABSTRACT

D6 and DARC had been reported as a decoy chemokine receptor in cancer study. The involvement of D6 and DARC in breast cancer had been investigated and it was reported to negatively correlate with the progression and metastasis of breast cancer cells. This study aimed to construct recombinant clones of D6 and DARC, express, analyze and purify the proteins and then determine the effects of the recombinant proteins on breast cancer cell migration and invasion. D6 and DARC genes in MDA-MB-231 cell line were first amplified by one-step RT-PCR with gene specific primers and Pfu DNA polymerase. Besides, DARC was also amplified by PCR using Phusion DNA polymerase. For each of the clones, two pairs of specific primers were used to generate full length nucleotide sequences which were cloned into pPICZ and pPICZα expression vectors. The construction of TA clones and DNA sequencing analysis showed perfect match of TA-D6 and TA-D6α to reference sequence. However a base substitution was observed at 131 bp of TA-DARC and TA-DARCα. Site-directed mutagenesis was carried out to correct the mismatch. Generation of recombinant yeast expression vector was performed and DNA sequencing was carried out again to confirm in frame ligation of gene of interest to the N and C-terminal sequences of expression vectors. ClustalW analysis again showed a mismatch at 131 bp of pPICZ-DARC and

pPICZ-DARCα, which was identical to the findings of TA-DARC and TA-DARCα. This suggested that the base substitution found was not a mismatch but might be a polymorphism where the nucleotide sequence of DARC from MDA-MB-231 cells posses a different nucleotide at 131 bp and the gene sequence had been deposited to GeneBank (ID: JX081310). After that, electrotransformation of *Pichia* GS115 was carried out to allow integration of linearized recombinant yeast expression vectors into yeast genome. Upon antibiotic screening and PCR analysis to confirm the integration of gene of interest in the yeast genome, the positive clones were plated at increasing Zeocin™ concentrations. Clones which were able to confer resistance to Zeocin™ concentration of up to 2 mg/ml were selected for recombinant protein expression. Initial profile of recombinant protein expression showed low yields of heterologous proteins from pPICZα clones. Hence, only pPICZ clones, which expressed recombinant protein intracellularly were selected for further expression studies. Optimizations of selected physical and chemical parameters were performed and the intracellular expression of soluble recombinant proteins were found to increase dramatically with an increase of 1011.97% for recombinant D6, 451.75% for recombinant DARC and 394.72% for recombinant mutated DARC. The optimized parameters are: BMMY at pH 6.0, inoculum size at OD_{600} of 2.5 (for D6) and 1.0 (for DARC and mutated DARC), induction with 1.0% (v/v) of methanol once every 24 hours and incubated at 16°C for 48 hours. The recombinant proteins were then purified by using affinity chromatography with Cobalt resins. SDS PAGE analysis and optimized Western Blot showed that the purified yeast expressed recombinant D6, DARC and mutated DARC were higher in apparent molecular weight compared to the theoretical calculated molecular weight. The purified recombinant proteins were then used in cell based studies to test on its

biological activity. Cell viability tests showed that recombinant D6, DARC and mutated DARC did not affect the viability of cells significantly and thus suggested that they were not involved in breast cancer cell death. Wound healing assays showed that the presence of recombinant D6, DARC or mutated DARC at 10 μg/ml inhibited the migration of breast cancer cells optimally. ELISA showed the inverse relationship between the recombinant proteins and CCL2 level in treated cells. Migration assay using Boyden chamber demonstrated the function of the recombinant proteins in inhibiting chemotaxis activity of treated cells. Invasion assay using matrigel coated Boyden chamber further showed the ability of the recombinant proteins in inhibiting the invasion property of treated cells. Comparing single and combinatorial effects of the recombinant proteins, the combination of D6 and DARC at ratio 1:1 (10 μg/ml) was found to be the best in reducing CCL2 level in treated cells and subsequently inhibit the migration and invasion of treated cells. It was shown that the purified yeast expressed recombinant D6, DARC and mutated DARC are not only negative regulators of breast cancer cell migration and invasion but the inhibition effects were greater when they were used in combination.

CHAPTER ONE

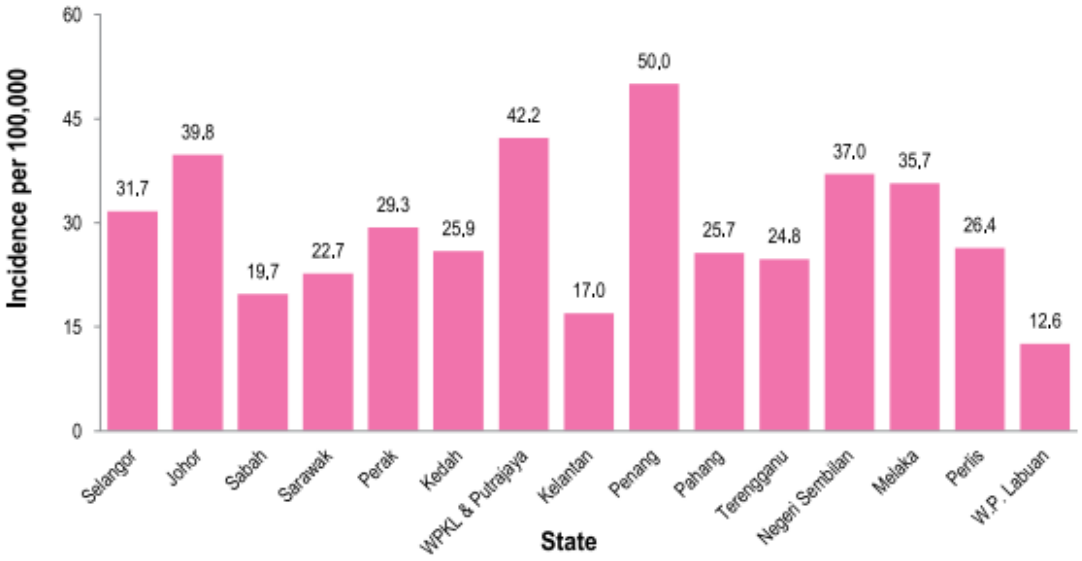
INTRODUCTION

1.1 Breast cancer

Breast cancer is the most common cancer in women in most parts of the world. Majority of the breast cancer patients were found to surrender to this disease due to cancer invasion and metastasis (Chew *et al.*, 2013). In oncology study, breast cancer research had become one of the most evolving fields (Vora *et al.*, 2009). To date, with the advanced understanding of key molecular features, breast cancer is no longer considered a single disease but a combination of different subtypes with different biological behaviours and clinical outcomes (Sandhu *et al*., 2010). Novel molecules and new diagnostic methods are being discovered and developed constantly, globally. Recently, the identification of various signaling pathways implicated in the cellular processes of breast cancer cells has drawn the attention of researchers worldwide. The involvement of growth factors or signaling molecules in breast cancer cell proliferation and invasion were reported worldwide (Adams *et al.*, 1991; Adnane *et al.*, 1991; Cabioglu *et al.*, 2009; Ahmad *et al.*, 2011). For examples, chemokines and chemokine receptors were reported to be involved in cancer growth and metastasis (Addison *et al.*, 2004; Balkwill, 2004; Ali and Lazennec, 2007; Allavena *et al.*, 2011; Tang *et al.*, 2011; Zeng *et al.*, 2011). Noteworthy attention had been placed on mediators which are known to be inflammatory, such as cytokines and chemokines (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Allavena *et al.*, 2008).

1.1.1 Breast cancer in Malaysia

Ho *et al*. (2017) reported that breast cancer is the fourth leading cause of death in Malaysia. According to the Malaysia Breast Health Information Centre, there is a marked geographical variation in the breast cancer incidence rate where the statistics are more frightening in Western countries, such as the U.S., than in developing countries. In the U.S., approximately 184,000 new breast cancer cases are detected annually. The breast cancer incidence is also rising in Malaysia. Malaysian National Cancer Registry Report 2007-2011 (Azizah *et al*., 2016) showed that breast cancer is the most common cancer among females in Malaysia during the period of 2007-2011. Colorectal, cervix uteri, ovary and lung cancer are the other 4 common cancers after breast cancer. According to the report, breast cancer constituted 32.1% of all the cancers in Malaysian females. 56.9% of the breast cancer cases were detected at stage I and stage II. Breast cancer incidence was found to be the highest in Chinese females followed by Indian and Malay. Moreover, the report also showed that the highest cumulative risk was observed in Chinese females and the lowest in Malay females. Furthermore, lifetime risk was found to be 1 in 30 as a big total. Lifetime risk in Chinese was 1 in 22, 1 in 24 for Indian and Malay was observed to be 1 in 35. The age-standardization rate (ASR) distribution according to state among female breast cancers in Malaysia from year 2007-2011 is presented in Figure 1.1.



Adopted from Azizah *et al*. (2016)

Figure 1.1 The age-standardization rate (ASR) distribution according to state in Malaysia, 2007-2011

1.1.2 Breast cancer studies in Malaysia

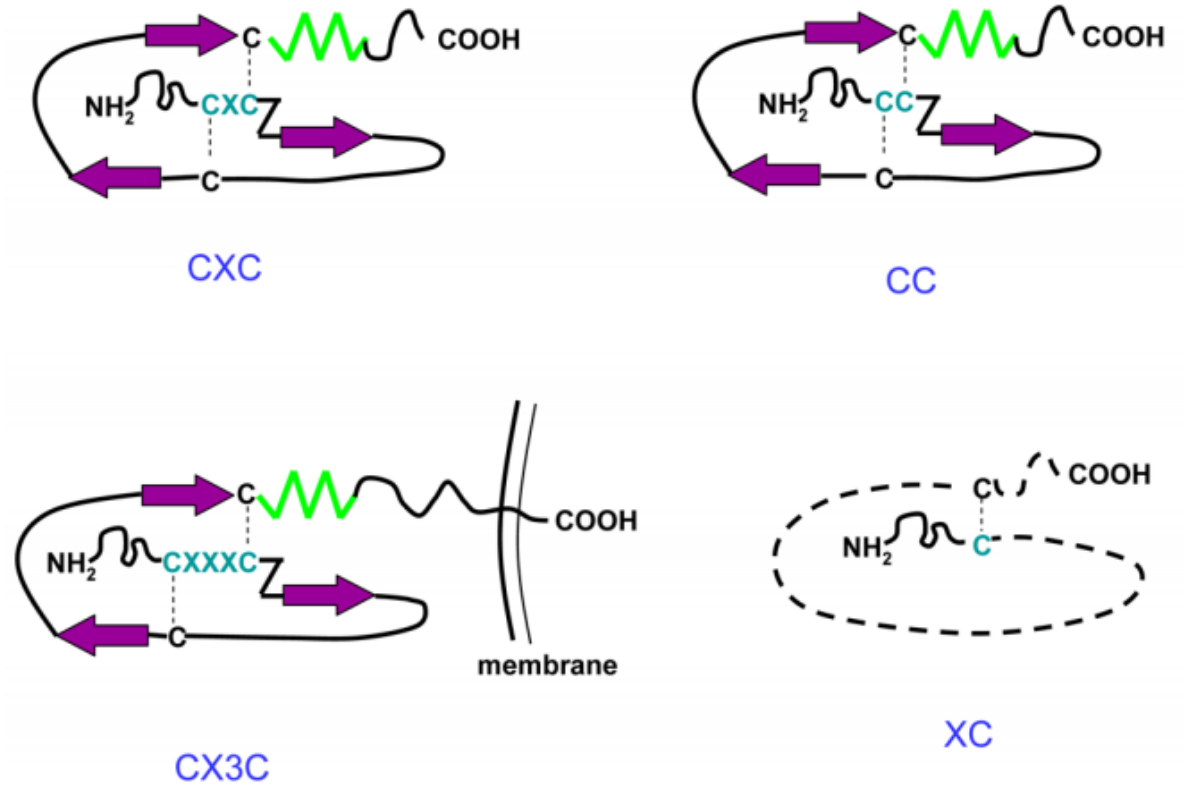
According to the Cancer Research Initiatives Foundation (CARIF), current breast cancer research in Malaysia includes:

- Determination of genes that cause breast cancer and contribute to an increased risk of breast cancer.
- Identification of biomarkers that may be used in identification and early detection of breast cancer and development of these biomarkers into clinically useful tools.
- Identification and development of methods for the rapid and robust identification of individuals who are at risk of breast cancer.
- Development of appropriate screening, preventive and therapeutic strategies for individuals who are at high risk for breast cancer.
- Discovering ways to prevent and treat breast cancer such as using drugs, plant extracts, herbal tualang honey and other traditional medicines.

However, investigation of the invasiveness of breast cancer cells by targeting the invasion-associated molecules, such as CCL2 remains rare.

1.2 Chemokines and chemokine receptors in cancer

Chemokines are small proteins range from approximately 8 to 17 kDa and belong to a family of chemoattractant cytokines, which can be induced by cytokines, growth factors and pathogenic stimuli. Chemokines mostly involved in chemoattraction to regulate the migration of cells, particularly leukocyte, to inflammation sites (Luster, 1998). Charo and Ransohoff (2006) reported that chemokines were also involved in other cytokine-like activitives, such as proliferation, apoptosis susceptibility, angiogenesis and fibrosis. Chemokine structure comprises an N‑terminal loop region, three-strand antiparallel β-sheets forming the typical core fold of the chemokines and a C‑terminal α helix which overlays the β-sheet. The production of chemokines can be either constitutive or induced by environmental stimuli. Thus, chemokines can be subdivided into two major categories, namely homeostatic and inflammatory chemokines. Constitutive chemokines always regulate homeostatic trafficking of leukocytes and lymphocyte recirculation under normal or steady state condition; whereas inflammatory chemokines are generated in response to inflammatory and immune stimuli which subsequently direct leukocytes to inflammed peripheral tissues (Chew *et al.*, 2013). Chemokines are classified into four subfamilies, C, CC, CXC and CX3C (where X is any amino acid), based on the number and spacing of the first two cysteines in the amino terminus (Slettenaar and Wilson, 2006). The schematic representation of these four different groups of chemokines is shown in Figure 1.2.



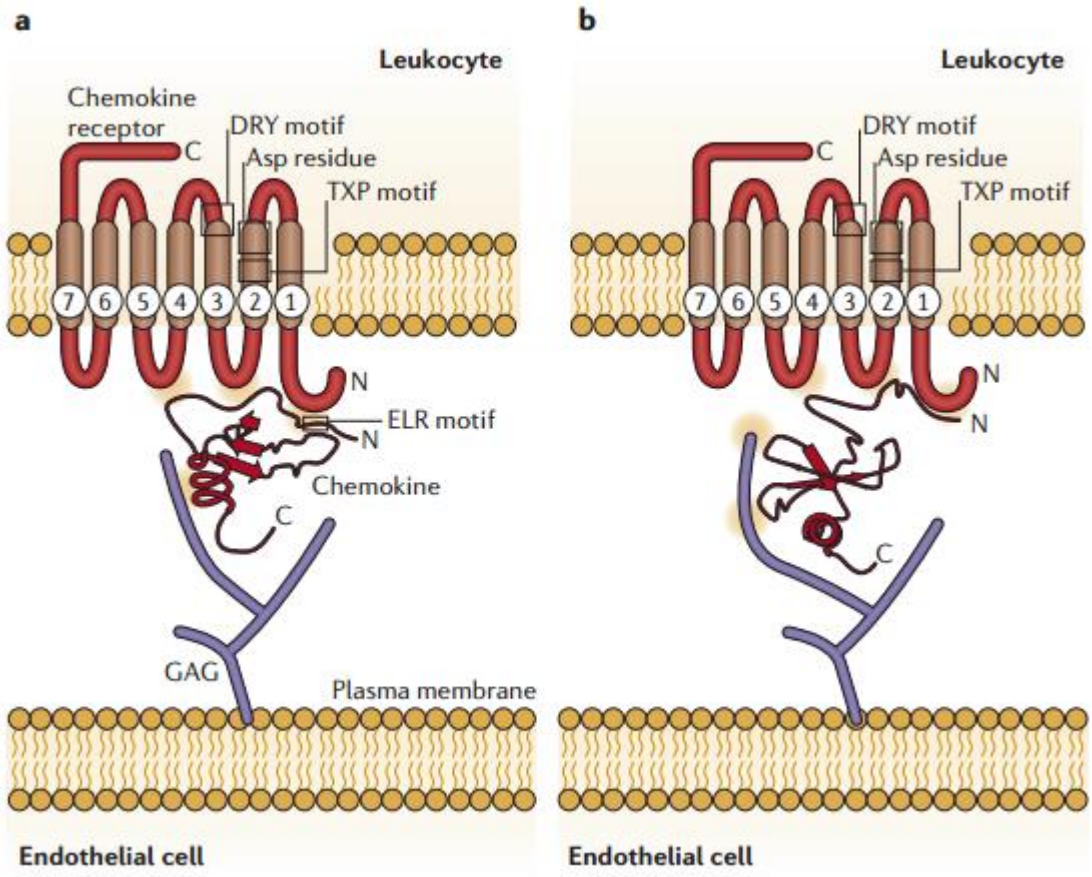
Adopted from Ali and Lazennec (2007)

Figure 1.2 Chemokines are classified into four major groups, namely CXC, CC, CX3C and XC (where X is any amino acid)

Besides leukocyte chemoattraction, Ali and Lazennec (2007) reviewed that chemokines were the first members of the cytokine family to show interaction with G-protein-coupled receptors (GPRC). These chemokine receptors are known to be embedded in the lipid bilayer of the cell surface and also to possess seven transmembrane domains. These members of G-protein-coupled receptor (GPCR) superfamily have single polypeptide chains that consist of three extracellular loops and also three intracellular loops. There is also a serine/threonine-rich intracellular carboxyl-terminal domain and an acidic amino-terminal extracellular domain which is involved in the binding of ligand. Besides, there are important conserved motifs in chemokine receptors, such as Thr-X-Pro; where X refers to any amino acid, Asp-Arg-Tyr (DRY) and Glu-Leu-Arg (ELR) (Figure 1.3). These conserved motifs play a role in signaling.

Figure 1.3 depicted the interaction of chemokine receptors with endothelial cells. Chemokines interact with endothelial cells via glycosaminoglycans (GAGs). Some chemokines bind GAGs through C-terminal α-helix amino acids, whereas other chemokines bind GAGs via residues in the loop that links N terminus with the first β-strand and residues in the loop which links to second and third β-strands (Mantovani *et al.*, 2006).

.



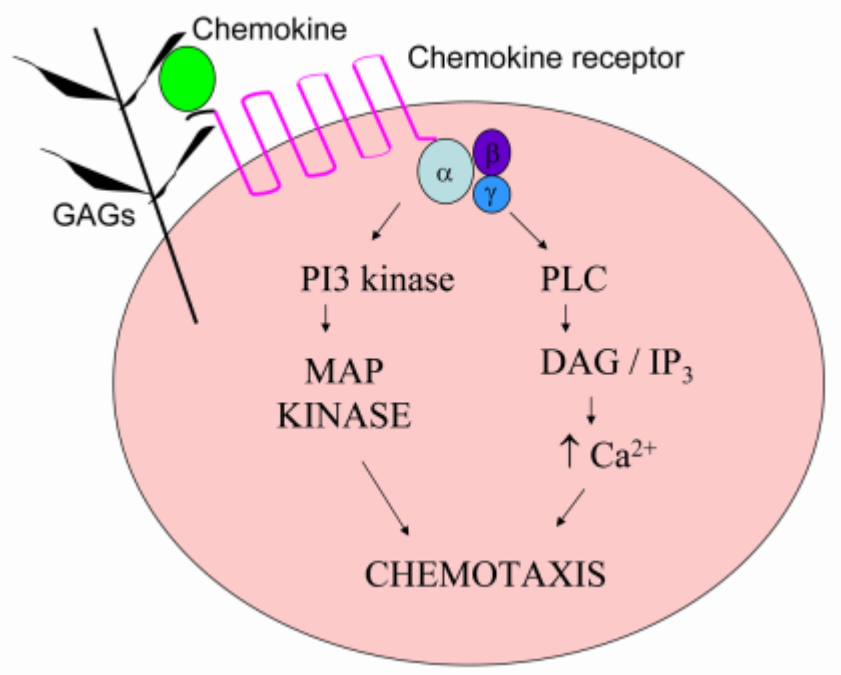
Adopted from Mantovani *et al.* (2006)

Figure 1.3 The interaction of chemokine receptors with endothelial cells

(a) Some chemokines bind GAGs through C-terminal α-helix amino acids

(b) Some chemokines bind GAGs via residues in the loop that links N terminus with the first β-strand and residues in the loop which links to second and third β-strands.

Chemokines bind to the seven transmembrane spanning G-protein-coupled receptors (GPCRs) to exert their actions. Typical receptor then specifically binds to its ligand leading to typical signaling pathways. Upon binding of chemokine receptor to its ligand, the βγ subunits of heterotrimeric G-protein were released. The detached βγ subunits activate phosphoinositide-specific phospholipase C (PLC) isoenzymes directly. This leads to the formation of inositol-1, 4, 5-triphosphate. The rise of intracellular calcium concentration subsequently leads to chemotaxis (Ali and Lazennec, 2007; Yadav *et al.*, 2010). However, PI3K pathway is another alternative. Activation of MAP Kinase pathway following PI3K will ultimately leads to chemotaxis too (Roussos *et al.*, 2011) (Figure 1.4). The short and transient signals mediated by the chemokine receptor to induce chemotaxis can be terminated rapidly by phosphorylation at multiple sites of the cytoplasmic C-terminus, homologous and heterologous desensitization and internalization eventually (Yadav *et al.*, 2010).



Adopted from Ali and Lazennec (2007)

Figure 1.4 The binding of chemokine receptor to respective ligand elicits a cascade of signaling pathways

Allavena *et al*. (2011) reported that serving as key-player in cancer-related inflammations, chemokines was found affecting a variety of tumor progression pathways. Those pathways include cancer cell survival and proliferation, cell migration and cell invasion. Chemokine ligands and its receptors are plentifully expressed in cancer cells of chronic inflammatory conditions.

In the recent years, chemokines and chemokine receptors have been widely reported on their roles in the process of malignant progression (Muller *et al.*, 2001; Ben-Baruch, 2006; Rollins, 2006; Ali and Lazennec, 2007; Ben-Baruch, 2008). Cancer cells were observed to produce chemokines and chemokine receptors which were able to response specifically to these chemokines, thus forming a complex chemokine network which is involved in influencing tumuor cell survival, spreading and growth (Balkwill, 2004). Typical receptor binds specifically to its ligand and leading to typical signaling pathway. However, a few "silent" receptors are included in chemokine system as they bind to respective ligands with high affinity but do not bring out signal transduction. The details on these silent receptors are discussed in Section 1.4.

Raman *et al.* (2011) stated that chemokines play a vital role in physiology, homeostasis and also pathogenesis of tumors and their metastasis. The applications of chemokines and chemokine receptors in pre-clinical and clinical settings suggested chemokine system as an important target for the improvement of current therapeutic strategies (Allavena, 2011).

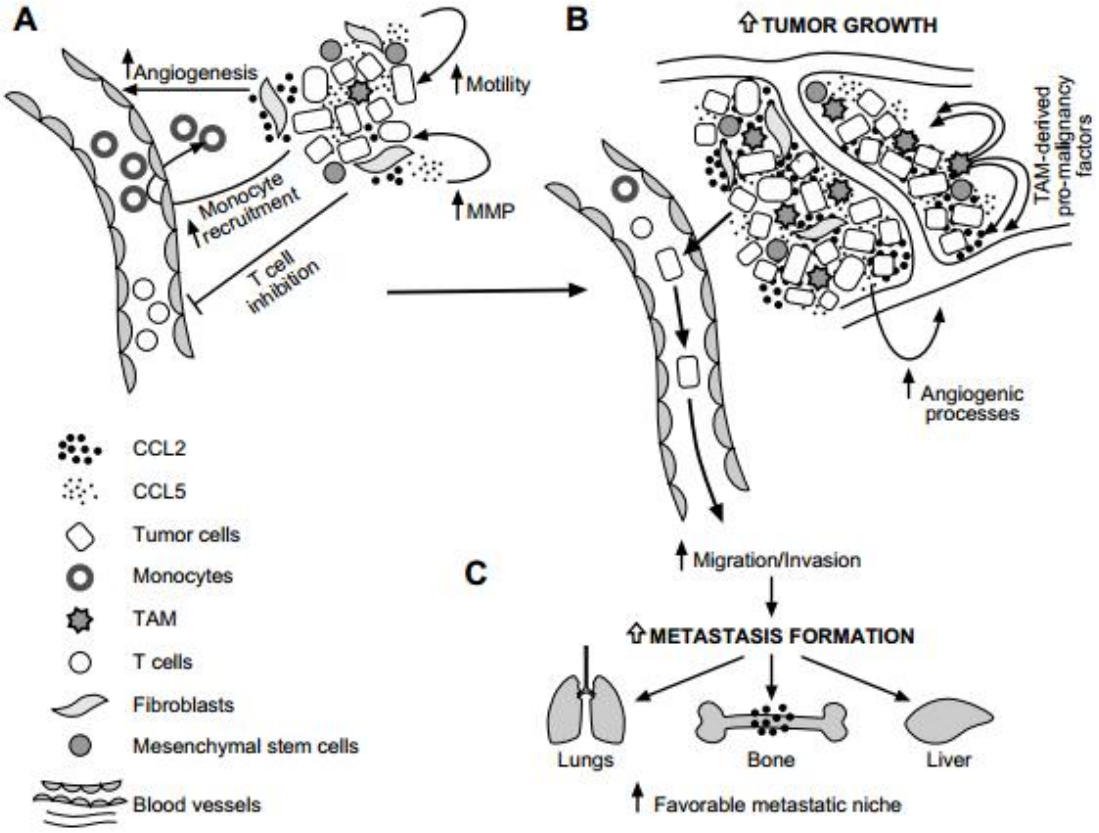
1.2.1 CCL2 and breast cancer

Chemokine (C-C motif) ligand 2 (CCL2), also referred to as monocyte chemotactic protein-1 (MCP-1), is a member of the C-C chemokine family and is primarily secreted by monocytes, macrophages and dendritic cells (Conti and Rollins, 2004). It is known as a potent chemoattractant for monocytes, memory T-lymphocytes and also natural killer cells (NK cells). It is one of the soluble growth factors, detected in high level in serum and not presented on cell surface (Jiang *et al.*, 1990; Jiang *et al.*, 1991; Proost *et al.*, 2006). An elevated serum level of CCL2 has been found to be significantly associated with breast cancer invasion and metastasis and higher tumorigenicity phenotype of breast cancer cells (Neumark *et al.*, 1999; Wang *et al.*, 2006). CCL2 expression in breast carcinomas was reported to correlate with the lack of estrogen receptor (ER) and expression of progesterone receptor (Chavey *et al*., 2007).

The tumor associated macrophages (TAM) are myeloid monocytic cells which are employed to the tumor cells via CCL2. TAM was correlated to invasive phenotype and poor diagnosis (Mantovani *et al.*, 2006b; Sozzani *et al.*, 2007; Lewis and Pollard, 2006). Besides serving the role as pro-maglinancy in breast cancer, CCL2 expressed by TAM and/or tumor cells was observed to significantly correlate with microvessel density and vessel invasion of tumor cells (Saji *et al.*, 2001; Balkwill, 2004; Wang *et al.*, 2006; Wu *et al*., 2008; Galzi *et al.*, 2010; Mantovani *et al*., 2010; Ueno *et al.*, 2000). TAM-expressed CCL2 was also found to be significantly related to the expression of membrane type 1-matrix metalloproteinase (MT1-MMP), tumor necrosis factor α (TNFα), thymidine phosphorylase (TP) and other angiogenic factors.

Figure 1.5 showed the suggested mechanisms interceding roles of CCL2 and breast cancer. Different from normal breast cells, breast cancer cells express CCL2 in high level. Besides, CCL2 was also released by different cells types at the tumor microenvironment. Figure 1.5A showed that CCL2 promotes angiogenesis and stimulates the tumor cells to release MMP, subsequently induces the motility of tumor cells. This explains the roles of CCL2 in breast cancer cell migration and invasion. Activities of CCL2 further aggravate the effects of TAM-derived pro-maglinancy factors at the tumor sites (Figure 1.5B). This lead to the growth and establishment of tumor cells at primary tumor sites. Tumor cell migration and dissemination also take place. Ultimately, the tumor-promoting activities of CCL2 lead to metastatic spread of tumor cells to preferred metastatic sites such as lungs, bones and liver (Figure 1.5C)

In summary, CCL2 plays a causative role in the maglinancy of breast cancer (Soria and Ben-Baruch, 2008). Nam *et al.* (2006) used MDA-MB-231, the metastatic human breast cancer cells to study the direct effect of CCL2 in breast cancer maglinancy. CCL2 knocked down by shRNA in the study was observed to result in approximately 3-fold decrease of metastatic lung nodules in mice. Salcedo *et al.* (2000) reported significant inhibition of lung metastasis and increment of mice survival rate with CCL2 blocking by neutralizing antibodies in MDA-MB-231 cells.



Adopted from Soria and Ben-Baruch (2008)

Figure 1.5 Roles of CCL2 on the maglinancy of breast cancer

1.3 Role of chemokines and chemokine receptors in cancer cell migration and invasion

Expression of chemokine receptors has been found to be restricted and specific in many cancer cells. Beside aiding in cell growth and survival, chemokine receptors were also found to be facilitating the characteristic patterns of metastasis (Slettenaar and Wilson, 2006).

To date, CXCR4 is the most overexpressed and best characterized chemokine receptor in cancer cells that demonstrated the involvement of CXCR4 in cancer cells metastasis. CXCR4 was found up regulated in more than 20 different types of tumor histotypes (Mantovani *et al.*, 2010). In hematopoietic stem cells, CXCR4 was used to reach and settle down in bone marrow niches. The expression of CXCR4 in primary tumor has shown positive co-relationship with the degree of lymph node metastasis, poor patient overall survival and also tumor grade (Ali and Lazennec, 2007). Besides, the expression of CXCR4 had also been linked to the metastasis ability of breast cancer cells to the lung (Helbig *et al.*, 2003).

Other CXC chemokine receptors had also been reported in the malignancy of different types of hematological neoplasia either alone or in combination. For examples, CXCR and CXCR2 in malignant melanoma (Varney *et al.*, 2006), CCR5, CCR9 and CX3CR1 in prostate cancer (Murphy *et al.*, 2005). Besides, CXCR3 was found to be associated with the development of lymph node metastasis in murine melanoma

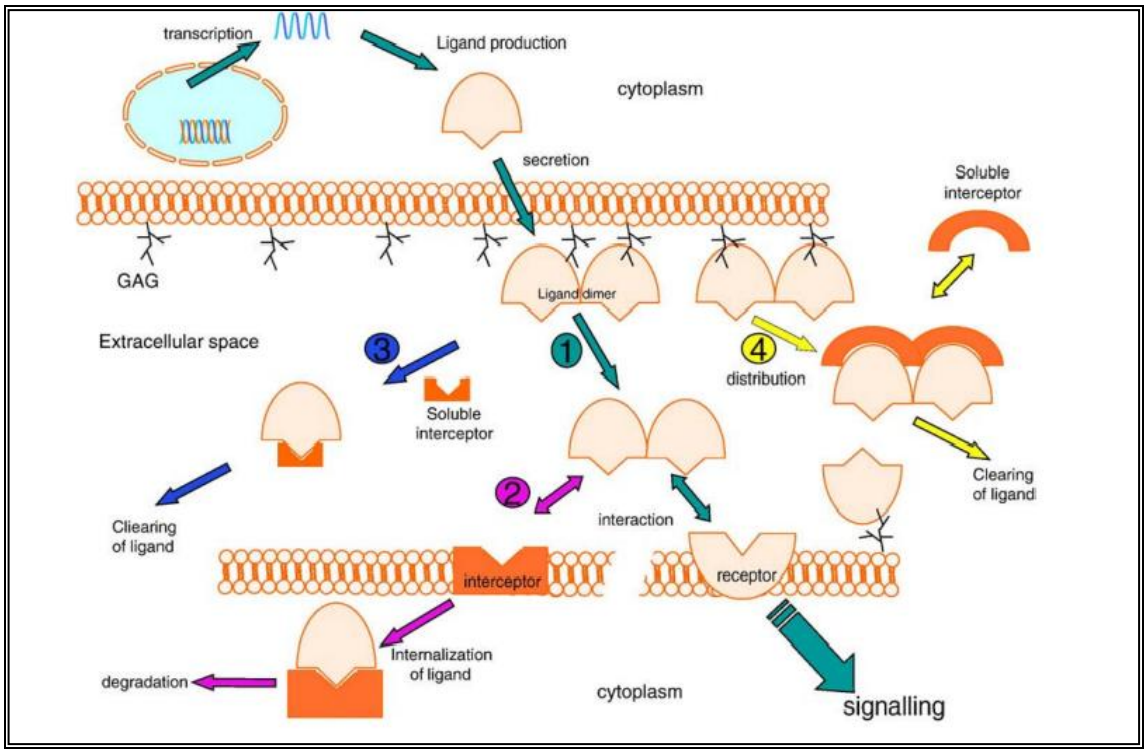
(Kawada *et al.*, 2004) and colon cancer (Kawada *et al.*, 2007) and lung metastasis in murine mammary cancer models (Walser *et al.*, 2006; Ma *et al.*, 2009). Apart from that, CXCR5 had been observed to promote liver metastasis of colorectal carcinoma (Meijer *et al.*, 2006) whereas CXCR6 was found to be associated with inhibition of tumor growth in breast and renal cancer (Meijer *et al.*, 2008; Gutwein *et al.*, 2009) but up regulated in advanced stage of prostate cancer (Darash-Yahana *et al.*, 2009).

CCR7 had been used as a potential marker in prediction of breast and colorectal cancer (Gunther *et al.*, 2005). Lower survival rates were observed from patients with CCR7 positive carcinomas than those with CCR7 negative cancer cells. Besides, CCR7 was also found to be associated with lymph node metastasization, lymphatic invasion and also stage of tumor (Takanami, 2003). Other CC chemokine receptor such as CCR6 was observed to play significant roles in organ selective liver metastasis of colorectal cancer (Ghadjar *et al.*, 2006). CCR9 was reported to be related to intestinal melanoma metastasis (Letsch *et al.*, 2004) while CCR5-positive cancer cells were observed to enhance the growth and metastatic ability of tumor cells upon interacting with CCL5 (Karnoub *et al.*, 2007). Besides, the expression of CX3CR1 by prostate cancer cells was observed to mediate metastasis to bone (Shulby *et al*., 2004). It was also reported to be expressed by pancreatic adenocarcinoma and was implicated in perineural invasion and tumor recurrence (Marchesi *et al*., 2008).

1.4 Decoy chemokine receptor proteins

Many studies have recently reported findings on the atypical action of chemokine receptor proteins (Figure 1.6). These receptor proteins were termed “decoy proteins” or “scavenger proteins” due to the fact that the binding of these proteins to the respective ligands does not lead to typical signaling pathway, but intercept the respective pathway and neutralize the action of chemokines (Wang *et al.*, 1998; Mantovani *et al.*, 2006; Wang *et al.*, 2006; Wu *et al.*, 2008; Galzi *et al.*, 2010). Hence they are also well known to be “intercepting receptors” as the decoy chemokine receptors confiscate chemokines with no activation of respective signaling pathway (Galzi *et al.*, 2010; Hansell *et al.*, 2006). The binding of chemokines without triggering G protein signaling pathway is a way of regulating chemokine activity and may function as a tumor suppressor (Mantovani *et al.*, 2006; Graham, 2009). It has emerged as a general strategy in recent years to tune the actions of cytokines and growth factors.

The decoy chemokine receptors that had been reported are Duffy antigen for chemokines (DARC), D6 (which is also known as CCBP2) and also CCX-CKR (or CCRL1). Different from antibodies and small molecule receptor antagonists, chemokine decoy receptors generally have broad specificity of ligands that are recognized by different receptors (Mantovani *et al.*, 2006a). In this study, DARC and D6 will be expressed and hence details of DARC and D6 are described as follows.



Adopted from Galzi *et al.* (2010)

Figure 1.6 A global illustration of the routes taken by chemokines with different types of receptors

Route 1: Chemokine binding with a typical specific receptor leads to signalling in the cell that expresses the receptor.

Route 2: Chemokine binding with an interceptor intracellularly leads to either chemokine degradation or transcytosis.

Route 3: Chemokine binding with a soluble interceptor leads to ligand sequestering.

Route 4: Soluble interceptor binding prevents chemokine binding to glycoaminoglycans, which results in a collapse of the chemotactic gradient.

1.4.1 D6

D6 was first cloned from haematopoietic stem cells (Nibbs *et al.*, 1997) and placenta (Bonini *et al.*, 1997) in the 1990s. It is also known as CCBP2 (Locati *et al.*, 2005) or ACKR (atypical chemokine receptor) 2 (Wilson *et al.*, 2017). It was highly expressed by endothelial cells of skin, gut and lung and also lymphatic endothelium, trophoblast, leukocyt, maglinant vascular tumuor, T-cell large granular lymphocyte leukemia cells, choriocarcinoma (Nibbs *et al.*, 2001; Martinez de la Torre *et al.*, 2007; Zeng *et al.*, 2011) and human breast cancer cells (Wu *et al.*, 2008).

D6 was observed to bind almost all pro-inflammatory CC chemokines. However, it does not recognize and bind to homeostatic CC-chemokines or other families such as CXC-chemokines (Mantovani *et al.*, 2006). The binding of D6 to its ligand happened in such a way that D6 takes part in ligand-independent constitutive internalization. D6 enters cell through endosomal compartments rapidly upon binding with chemokine. Then, it detaches from the ligand and this made the internalized chemokines remain trapped in the cell. The trapped chemokines are then targeted for degradation by cellular organelle (Xu *et al.*, 2007). At the same time, D6 recycles back to cell surface to bind new chemokine and rapidly enter the cell through endosomal compartments again (Chew *et al.*, 2013). The repeated rounds of chemokine internalization leads to reduction of free extracellular pro-inflammatory chemokines and subsequently down regulate respective chemokine related biological activities (Figure 1.7). The binding mechanism of D6 to its ligands had been reported widely. Researches found that D6 took part in

chemokine degradation and at the same time reduced the inflammatory activity of CC chemokines (Savino *et al.*, 2009). This agrees with the findings from Fra *et al.* (2003), Bonecchi *et al.* (2004) and Weber *et al.* (2004) that D6 can speedily internalize and degrade its ligands. Mantovani *et al.* (2006) also reported similar findings that D6 mediates chemokine degradation but does not mediate chemokine transfer through cell monolayer.

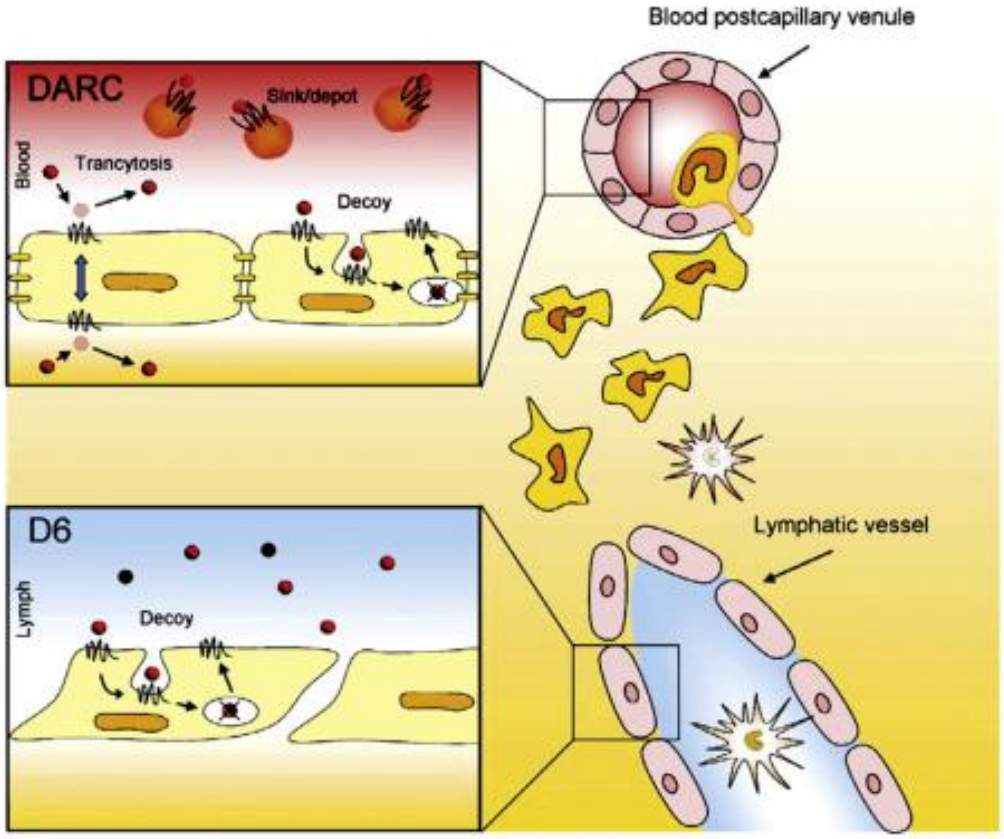


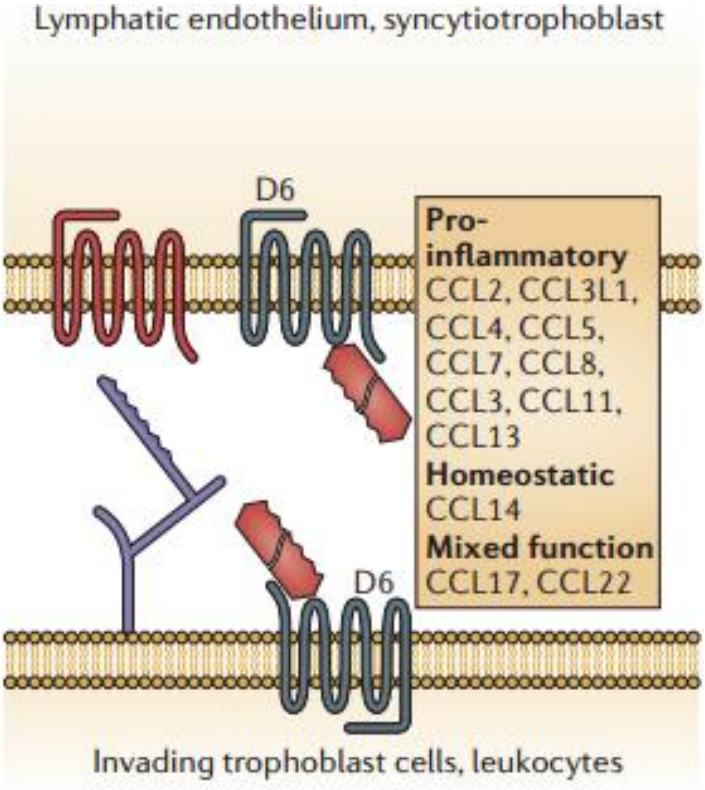
Figure 1.7 The coordinated actions of DARC and D6 in peripheral tissues

Adapated from Bonecchi *et al.* (2008)

- Expression of DARC takes place on blood vessels and erythrocytes. Besides acting as chemokines' depot on erythrocytes, it serves as decoy chemokine receptors on endothelial cells and transport chemokines through cell barriers by trancytosis.

- D6 is expressed at lymphatic endothelium and leokocytes at lower levels. It acts as scavenger to degrade pro-inflammatory chemokines, preventig them from transfering to lymph nodes.

D6 was a decoy chemokine receptor as it was found lacking of sequence motif used for G-protein coupling and also signaling functions of respective chemokine receptors (Mantovani *et al.*, 2001). D6 is structurally typical of chemokine receptor family and thus it has the similar gene structure with signaling CC chemokine receptors (Nibbs *et al.*, 2003). However, alterations were observed in D6 conserved motif. Instead of DRYLAIV motif on the second intracellular loop of GPCRs, it was observed being altered to DKYLEIV in D6 (Nibbs *et al.*, 2003). DRYLAIV motif is essential for ligand-induced signaling, having the conserved motif altered, making D6 a silent chemokine receptor. Figure 1.8 showed the presence of D6 in chemokine system to compete with signaling chemokine receptors to bind with its ligand. The binding of D6 to its ligand will not elicit signal transduction so does the cascade of signaling pathways. Wu *et al*. (2008) reported that over expression of D6 reduced chemokines such as CCL2 and CCL5, subsequently inhibited proliferation of breast cancer cells *in vitro* and *in vivo*. The tumorigenesis of lung metastasis *in vivo* was observed to be inhibited too. Coexpression of D6 in invasive breast cancer cells was observed to be negatively correlated with lymph node status and tumor stage (Zeng *et al.*, 2011).



Adopted from Mantovani *et al.* (2006)

Figure 1.8 D6, the decoy chemokine receptor proteins compete with signaling chemokine receptor for ligands

1.4.2 DARC

DARC or Duffy Antigen was originally reported by Horuk *et al.* (1993) as the receptor on erythrocyte to bind chemokines in infection by malaria parasites, the *Plasmodium vivax* and *P. knowlesi*. It was then well known and intensively studied in malaria research as its role is remarkable in this aspect. Pogo and Chaudhuri (2000) reported that human red blood cells which are Duffy-negative are resistant to malaria parasite infection. Similar results were reported by Miller *et al.* (1975) that the infection of malaria parasite in Duffy-positive individuals happened occasionally but Duffy-negative individuals were not prone to the infection. All the four extracellular domains in DARC were shown to be involved in the interaction between DARC and chemokines but the first extracellular domain was found vital for the interactions of erythrocyte-binding proteins from malarial parasites (Choe *et al*., 2005; Tournamille *et al*., 2003; Chitnis *et al*., 1996).

DARC was found to be expressed by vascular endothelium, red blood cells and also several tumors (Wang *et al.*, 2006; Galzi *et al.*, 2010). The expression of DARC on endothelial cells suggested its role in vascular biology (Peiper *et al.*, 1995). Rot (2005) showed that DARC interacts with many inflammatory chemokines on red blood cells and may act as a reservoir besides involving in transendothelial chemokine transport process (Figure 1.7). It was found to interact with 11 pro-inflammatory CXC and CC chemokines but not the homeostatic one (Gardner *et al*., 2004; Mantovani *et al*., 2001; Wang *et al*., 2006; Wu *et al.*, 2008; Galzi *et al*., 2010). Being promiscuous chemokine