CHEMOKINES AND THEIR RECEPTORS IN OVARIAN CANCER

Christopher John Scotton

A thesis submitted for the degree of Doctor of Philosophy

at University College London

October 2001

ICRF Translational Oncology Laboratory

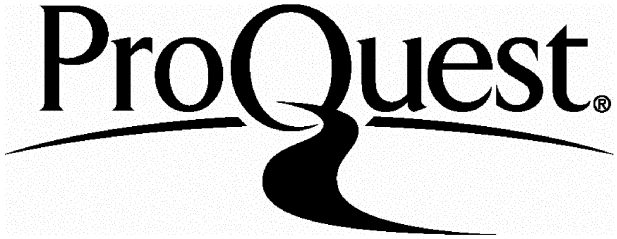
Bart's and The London

Queen Mary's School of Medicine and Dentistry

3rd Floor, John Vane Science Centre

Charterhouse Square

London, EC1M 6BQ.



Abstract

The chemokine/chemokine receptor network was investigated in solid human ovarian cancer and cancer ascites. In solid tumours, CC chemokine receptor mRNA was generally absent, the exception being CCR1 which was detected in 75 % of patients. CCR1 mRNA localised to macrophages and lymphocytes and there was a correlation between the number of $CD8^+$ and CCR1 expressing cells. mRNA for the CC chemokines CCL2, 3, 4, 5, 8 and 22 was expressed in a majority of biopsies. In a monocytic cell line *in vitro*, CCR1 mRNA expression was increased five-fold by hypoxia, which is a common feature of the tumour microenvironment. In ascites, there were variable numbers of tumour cells, macrophages and $CD3^+$ T cells (predominantly $CD4^+$) and mRNA was found for all the CC chemokines and receptors investigated. A direct correlation was found between the CCL5 protein concentration and the extent of the $CD3^+$ T cell infiltrate in ascites. The predominance of $CD4^+$ T cells in ascites may be associated with their chemokine receptor expression profile.

The possibility that chemokine gradients could influence migration of human ovarian epithelial tumour cells was also examined. Of 14 chemokine receptors investigated, only CXCR4 was expressed on ovarian cancer cells. CXCR4 mRNA localised to a sub-population of tumour cells in solid ovarian tumour biopsies. Ovarian cancer cell lines and tumour cells isolated from ascites expressed CXCR4 protein. The CXCR4 ligand, CXCL12, was found in ascites from 63 patients. Cell surface CXCR4 protein was functional on the ovarian cancer cell lines: stimulation with CXCL12 elicited an intracellular calcium flux; directed migration and invasion; and caused changes in integrin expression. CXCL12 also stimulated proliferation and/or survival of the ovarian cancer cell lines. Signalling downstream of CXCR4 involved activation of Akt/PKB and ERK1/2. CXCR4 may influence cell migration and survival in the peritoneum, a major route for ovarian cancer spread.

Chemokine receptor antagonists may be of therapeutic benefit in ovarian cancer. CCR1 receptor antagonists could affect the leukocyte infiltrate, while CXCR4 receptor antagonists could inhibit metastasis.

Acknowledgements

I'm sure there are hundreds of people I should thank for helping me either directly or indirectly during my PhD, but since I can't name you all, I'll just mention those that are foremost in my mind!

First I would like to thank my supervisor, Fran Balkwill, for giving me the opportunity to do a PhD in the first place, for lots of support and advice throughout, and for being so quick to cover drafts of each chapter in red pen.

I would like to thank everyone (past and present) in the Translational Oncology Lab for giving me advice, teaching me experimental techniques, joining me for beers, for friendship and for continually plaguing me with computer questions. By name (and alphabetically, so no-one feels slighted): Caroline Arnott, Caroline Barton, Dirk Brinkmann, Frances Burke, Maureen Cobbing, Sergio Dias, Nick East, Matt Grimshaw, Sue Hoare, Lala Khalique, David Milliken, Rob Moore, Rupert Negus, Stephen Robinson, Andy Ryan, Kate Scott, Peter Szlosarek, Parames Thavasu, Philippe Van Trappen, Lynn Turner, George Wilbanks and Julia Wilson. Extra special thanks go to Julia for being an absolute star and for reading and correcting lots of this thesis, and to David and Julia for collaborating on some of the work presented here, particularly in Chapters 4 and 6. Cheers to Kate for our little chats and for being a good friend (I still owe you a pint of vodka!), to Rob for lending me lots of funky CDs and thanks to Sue and Andy for squash sessions. I would also like to thank Gordon Stamp for help with histopathology, George Elia for help with immunohistochemistry and section cutting, Mike Bradburn for statistical advice, and Dave Lawrence for help with printing this thesis.

I must thank all my other friends who have kept me amused (and sane) over the last four years, particularly James, Phil, Jo, Steve #1, Jeremy, Rachel, James, Amanda, Steve #2, Dave, Cath, Martha, Katie, Sarah, Liz, Imogen, Clare, Riccardo, Catherine, Ribena, Mirm, Russ and Lizzie Bee, to name but a few. Thanks also to the staff at Charing Cross hospital for diagnosing me with glandular fever, two days before I was planning to submit...cheers guys!

My Mum, Andrea, Pete and the rest of the family know how much I appreciate their support, particularly over the last couple of years. We've been through some tough times, but we're getting there. Good luck to 'Dre' and Pete in their new house too!

Finally, a huge thank you goes to Caroline. Thank you for agreeing to marry me, for putting up with me while writing this thesis and for lots of TLC during the glandular fever! Milan here we come...

This thesis is dedicated to my Dad

Contents

Figures

Tables

Abbreviations

Ab	antibody
mAb	monoclonal antibody
bp	base pair
BSA	bovine serum albumin
CCL	CC chemokine ligand
CXCL	CXC chemokine ligand
XCL	C chemokine ligand
CX3CL	CX_3C chemokine ligand
CCR	CC chemokine receptor
CXCR	CXC chemokine receptor
XCR	C chemokine receptor
CX3CR	CX_3C chemokine receptor
DAB	diaminobenzidine
DEPC	diethylpyrocarbonate
DFO	desferrioxamine
DNA	deoxyribonucleic acid
cDNA	complementary DNA
DC	dendritic cell
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EST	expressed sequence tag
FBS	foetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF	hypoxia inducible factor
HPC	haematopoietic progenitor cell
HPF	high power field

IFN	interferon
Ig	immunoglobulin
IL	interleukin
kb	kilobase
kDa	kilodalton
LPS	lipopolysaccharide
MAPK	mitogen activated protein kinase
MHC	major histocompatibility complex
MIF	migration inhibitory factor
MMP	matrix metalloproteinase
NF-κB	nuclear factor kappa B
NK	natural killer
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
PKB/C	protein kinase B/C
RNA	ribonucleic acid
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
RT-PCR	reverse transcription polymerase chain reaction
SSC	sodium chloride, sodium citrate buffer
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
TAM	tumour associated macrophage
TGF	transforming growth factor
TIL	tumour infiltrating lymphocyte

TIMP	tissue inhibitor of matrix metalloproteinases
TNF	tumour necrosis factor
VEGF	vascular endothelial growth factor

Chapter 1. Introduction

1.1. Chemokines

1.1.1. Introduction

In this thesis I have investigated the expression of chemokines and their receptors in human ovarian cancer. The presence of a leukocyte infiltrate in this cancer may be determined by the chemokine network within the tumour and the response to these chemokines is likely to be controlled at the level of chemokine receptor expression. Moreover, epithelial tumour cells may be able to respond to chemokines as part of the multi-step process of metastatic spread.

The first chemokines to be discovered were Platelet Factor 4 (now called CXCL4) and β-thromboglobulin in the late 1970s (1, 2). However, not until the discovery of Interleukin-8 (CXCL8) in 1987 as an 'anionic' neutrophil-activating factor and chemoattractant (3, 4), were the chemokines recognised as a distinct subfamily of *chemo*tactic cyto*kines*. These early chemokines were identified on the basis of their biological activities in culture supernatants and were purified biochemically before being sequenced and cloned. Recently, with the advent of technologies such as EST (expressed sequence tag) databases and cDNA libraries, the identification of homologous novel genes has been rapid. At present, there are approximately 40 chemokines identified in humans, around 30 in mice [see (5, 6) for review] and the widespread use of genomics and bioinformatics means it is likely that most of the chemokines have now been discovered.

1.1.2. Chemokine classification

Chemokines are all small proteins (~8-14 kDa) with the exception of CX3CL1 (38kDa) and they are frequently glycosylated. They were originally defined according to the presence of four cysteine residues in highly conserved positions. There are now four

chemokine families: CXC, CC, C and CX3C. In the CXC family, the two amino terminal cysteine residues are separated by a single amino acid, while in the CC family they are adjacent. The C family lacks the first and third cysteine residues of the typical chemokine structure (7), while the CX3C family has three amino acids separating the amino-terminal cysteines. To date, there is only one member of the CX_3C family, called CX3CL1 or fractalkine (8). This chemokine is peculiar in that it has a multi-domain structure: the N-terminal chemokine domain is fused to a mucin-like stalk, which leads to a transmembrane domain and a C-terminal cytoplasmic tail. Thus, CX3CL1 can function as an adhesion molecule or induce migration as either a tethered or shed ligand (9). Recently, a CXC chemokine has been identified, termed CXCL16, which also has a transmembrane domain. This chemokine can also be expressed on the cell surface, or shed (10). Chemokines without a transmembrane domain can still be tethered to the cell membrane via glycosaminoglycans. Chemokines have heparin-binding capacity at their C-terminal end, such that they can bind to glycosaminoglycans and matrix glycoproteins, thereby enabling endothelial cells to present chemokines to circulating leukocytes (11, 12).

There are also differences in the genomic organisation of the chemokine families. Many of the genes for the CXC chemokines which act on neutrophils are located on chromosome 4, while a majority of the genes for the CC chemokines which act on monocytes are clustered on chromosome 17 (5). These genes may have arisen by duplication and divergence from primordial chemokine genes but remained in clusters, supporting the idea that their functions are to some extent related (Table 1.1.). The remaining chemokines are in new chromosomal locations away from these two main clusters. These genes are more conserved between species and have highly specific functions, suggesting that they are older in evolutionary terms.

Classically, the chemokines have been named according to their expression patterns or functions: for example, **M**onocyte **C**hemoattractant **P**rotein (MCP)-1 or **R**egulated upon **A**ctivation **N**ormal **T**-cell **E**xpressed and **S**ecreted (RANTES). However, the rapid discovery of new chemokines has created the problem of several laboratories identifying the same molecule but under different names. In 2000, Zlotnik and Yoshie (13) proposed a new classification system for chemokines - this systematic nomenclature follows the pattern already used for naming chemokine receptors. At present, the receptors are named CCR, CXCR, XCR and CX3CR. The new chemokine nomenclature replaces the R (for receptor) with L (for ligand); thus, MCP-1 becomes CCL2, RANTES becomes CCL5 and so on (Table 1.1.). This new nomenclature will be used throughout this thesis.

The CXC chemokines can be further subdivided by structure into ELR^+ and ELR^- CXC chemokines, depending on the presence (or absence) of a tri-peptide motif (glutamate – leucine – arginine) between the N-terminus and the first cysteine residue. ELR^+ CXC chemokines tend to be specific for neutrophils (15) and include CXCL1, 2, 3, 5, 6, 7, and 8. However, ELR^- CXC chemokines have no clear specificity and disparate activities. For example CXCL4 has no ELR motif, and is a very weak neutrophil chemoattractant, but does inhibit angiogenesis – possibly via direct inhibition of endothelial cell proliferation (16). The role of CXC chemokines in angiogenesis will be discussed in more detail later in Section 1.3.3.ii.

Systematic name	Human chromosome	Human ligand	Mouse ligand
CXC chemokines			
CXCL1	4q21.1.	GROα/MGSA-α	GRO1/KC/MIP-2
CXCL2	4q21.1	GROβ/MGSA-β	GRO1/KC/MIP-2
CXCL3	4q21.1	GROγ/MGSA-γ	GRO1/KC/MIP-2
CXCL4	4q21.1	PF4	PF4
CXCL5	4q21.1	ENA-78	LIX
CXCL6	4q21.1	GCP-2	CKα-3
CXCL7	4q21.1	NAP-2	Unknown
CXCL8	4q21.1	IL-8	Unknown
CXCL9	4q21.1	Mig	Mig
CXCL10	4q21.1	IP-10	IP-10
CXCL11	4q21.1	I-TAC	I-TAC
CXCL12	10q11.21	SDF-1α/β	SDF-1
CXCL13	4q21.1	BLC/BCA-1	BLC/BCA-1
CXCL14	5q31.1	BRAK/bolekine	BRAK
(CXCL15)	Unknown	Unknown	Lungkine
CXCL16	17p13	SR-PSOX	SR-PSOX
C Chemokines			
XCL1	1q24.2	Lymphotactin/SCM-1α	Lymphotactin
XCL2	1q24.2	SCM-1β	Unknown
CX_3C chemokines			
CX3CL1	16q13	Fractalkine	Neurotactin
CC chemokines			
CCL1	17q11.2	I-309	TCA-3
CCL2	17q11.2	MCP-1/MCAF/TDCF	JE
CCL3	17q12	MIP-1α/LD78α	MIP-1α
CCL4	17q12	MIP-1β	MIP-1β
CCL5	17q12	RANTES	RANTES
(CCL6)	Unknown	Unknown	MRP-1,C10
CCL7	17q11.2	MCP-3	MARC/MCP-3
CCL8	17q11.2	MCP-2	MCP-2
(CCL9/CCL10)		Unknown	MRP-2
CCL11	17q11.2	Eotaxin	Eotaxin
(CCL12)		Unknown	MCP-5
CCL13	17q11.2	MCP-4	Unknown
CCL14	17q12	HCC-1	Unknown
CCL15	17q12	HCC-2/MIP-1δ	Unknown
CCL16	17q12	HCC-4/LEC	LCC-1
CCL17	16q13	TARC	TARC
CCL18	17q12	DC-CK1/PARC	Unknown
CCL19	9p13.3	MIP-3β/ELC/Exodus-3	MIP-3β/ELC
CCL20	2q36.3	MIP-3α/LARC/Exodus-1	MIP-3α/LARC
CCL21	9p13.3	6CKine/SLC/Exodus-2	6CKine/SLC
CCL22	16q13	MDC	ABCD-1
CCL23	17q12	MPIF-1	Unknown
CCL24	7q11.23	MPIF-2/Eotaxin-2	MPIF-2/CKβ6/Eotaxin-2
CCL25	19p13.3	TECK	TECK
CCL26	7q11.23	Eotaxin-3	Unknown
CCL27	9p13.3	CTACK	CTACK
CCL28	5p12	CCK1/MEC	CCK1/MEC

Table 1.1. The systematic nomenclature for chemokines. The most common names for the human/mouse ligands are shown, but there may be omissions. A systematic name in brackets indicates that the human homologue has not yet been discovered. Adapted from (14).

1.1.3. Chemokine structure

The three-dimensional structures of a large number of chemokines have been determined, including CXCL4, CXCL8, CXCL12, CCL2, CCL4, CCL5, CCL11 and CX3CL1 (17-24). The amino acid sequences across the chemokine family show 20-

80% identity, yet their monomeric tertiary structures are very similar – all chemokines have a relatively disordered N-terminus, followed by three antiparallel β-sheets in a Greek key configuration, and a C-terminal α-helix that extends over the top of the β-sheets (Figure 1.1.).

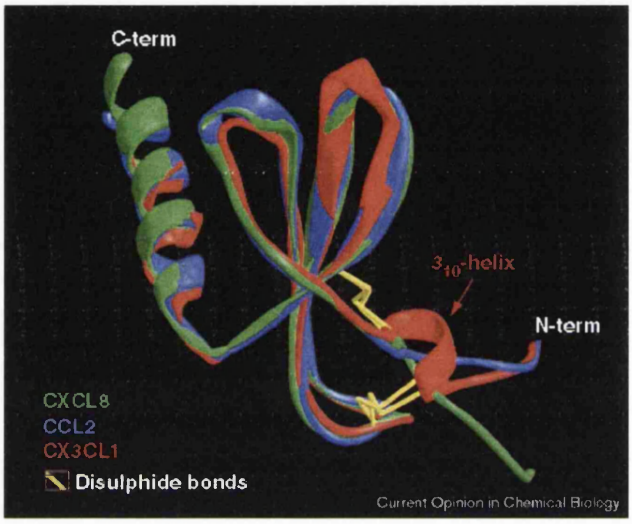


Figure 1.1. 3-dimensional structure of chemokines. Overlay of the molecular models for CXCL8 (green), CCL2 (blue) and CX3CL1 (red). Disulphide bonds are shown in yellow. These three chemokines have less than 20% sequence identity, yet their tertiary structures are extremely similar. The 3_{10}-helix in CX3CL1 is required to insert the three amino acids between the N-terminal cysteines without causing structural distortion. Adapted from (25).

However, under the conditions required for performing NMR or X-ray crystallography, the majority of the chemokines studied so far have been multimers – either dimers, or in the case of CXCL4, tetramers (26). The CXC chemokines and CC chemokines form profoundly different dimer structures: CXC chemokines dimerise via hydrophobic interactions between residues in the first β sheet (18) whereas in CC chemokines dimerisation occurs through interactions between residues near the N-termini of the monomers (21) (Figure 1.2).

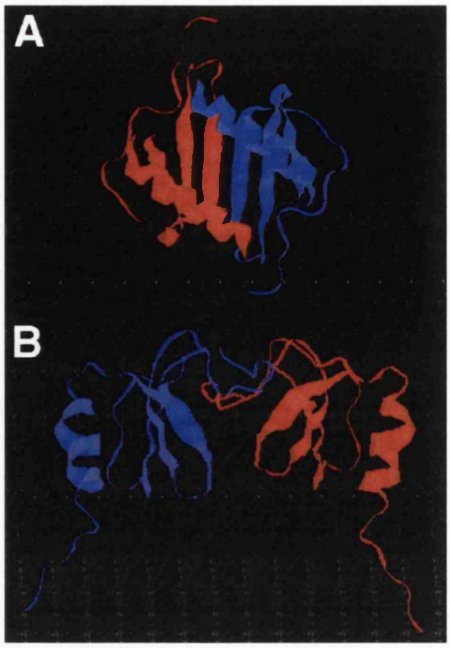


Figure 1.2. Quaternary structures of CXC and CC chemokines. The CXCL8 dimer forms through solvent exclusion along the first β-sheet (**A**). The CCL2 dimerisation interface involves N-terminal residues, including a short β-sheet (**B**). Adapted from (18, 21).

It is still unclear whether chemokines function as monomers or dimers. A number of chemokines have been shown to be monomeric over a wide range of concentrations (27, 28) and at physiologically relevant concentrations chemokines are likely to be monomeric. Mutated variants of CXCL1, CXCL7 and CXCL8 which are incapable of forming dimers are still fully functional on neutrophils (29, 30). Similarly, CCL2 and CCL4 have been shown to bind and activate their receptors as monomers (31, 32). However, other evidence suggests that chemokines do function as dimers. For example, N-terminal deletion mutants of CCL2 and CXCL8 can act as receptor antagonists (33, 34). In the case of CCL2, this mutant does not bind efficiently to its cognate receptor, instead it forms heterodimers with wild-type CCL2 – suggesting that it acts as a dominant negative inhibitor (33). The chemokine CXCL12 has been shown to exist in a monomer-dimer equilibrium at physiological concentrations, raising the possibility that both forms are functional (35). In addition, since most chemokines can bind to

glycosaminoglycans, it is possible that local chemokines concentrations could be raised sufficiently to promote the formation of dimers (36).

Given the similarities in three-dimensional structure despite having sequence identity of as little as 20 %, the potential for forming dimers and the ability to change chemokine specificity/function by making very few amino acid changes, it is virtually impossible to predict anything about the function of new chemokines from sequence alone.

1.2. Chemokine receptors

1.2.1. Introduction

Chemokines mediate their effects by binding to chemokine receptors, which are members of the seven transmembrane domain G protein-coupled receptor family. In the late 1980s, the effects of CXCL8 on neutrophils were shown to be blocked by pertussis toxin, demonstrating a role for $G\alpha_I$ in chemokine responses (37). Ligand displacement studies and SDS-PAGE analysis of chemically cross-linked ^{125}I-CXCL8 indicated that there might be two CXCL8 receptors, one which bound IL-8 alone, and a second which could also bind CXCL1, CXCL5 and CXCL7 (38, 39). Then in 1991, two unique cDNAs were discovered that encode highly homologous CXCL8 receptors (40, 41), which are now called CXCR1 and CXCR2. To date, around 19 functional chemokine receptors have been identified, along with a number of orphan receptors (with no known ligand) and viral receptors [see (6) for review].

1.2.2. Chemokine receptor nomenclature

Most chemokine receptors can bind more than one chemokine, but this is almost always of the same subclass. Therefore, chemokine receptors are named according to their specificity for a particular chemokine subclass. The receptors for the CC and CXC chemokines are identified as CCR and CXCR respectively. The receptors for the C and CX_3C chemokines are labelled XCR and CX3CR respectively (5). Splice variants are

identified by lowercase letters, for example CCR2a and CCR2b which result from alternative splicing at the C-terminus (42).

Figure 1.3. summarises the known chemokine receptors and their ligands. Some chemokines can bind to more than one receptor, and some receptors can bind multiple chemokines. A majority of the receptors fall into the 'shared' category, because they bind more than one ligand – these tend to be proinflammatory chemokine receptors. There are also several 'specific' receptors which only bind one chemokine - these receptors tend to be involved with normal leukocyte trafficking.

The Duffy antigen receptor for chemokines (Darc) can bind multiple chemokines, both CC and CXC, with high affinity, but without any apparent functional consequences (43) - this chemokine receptor is classified as 'promiscuous'. Darc is expressed on red blood cells [it is identical to the Duffy blood group antigen (44, 45)] and on postcapillary endothelium (46). At present, the consensus view is that Darc functions as a chemokine 'sink', or as a way to present chemokines to circulating or extravasating leukocytes. There is also a CC chemokine-specific promiscuous receptor called D6. This receptor can bind multiple CC chemokines, including CCL2, 3, 5 and 7, but like Darc, no signalling has been observed in response to these chemokines (47).

Chemokine receptors have also been identified which have no known ligand. These are classified as 'orphan' receptors, and include CC-chemokine receptor like 1 (CCRL1) which is expressed predominantly in the heart (48) and HCR which is expressed in lymphoid tissues such as the spleen, lymph nodes and bone marrow (49).
Functional chemokine receptor genes have also been found in viruses, for example ECFR3 in *Herpesvirus saimiri* (50), which has structural similarities to CXCR1 and CXCR2, and US28 in human CMV (51) which can bind multiple CC chemokines with

high affinity. Some viruses might selectively mimic chemokines or chemokine receptors in order to subvert or inactivate the host defence.

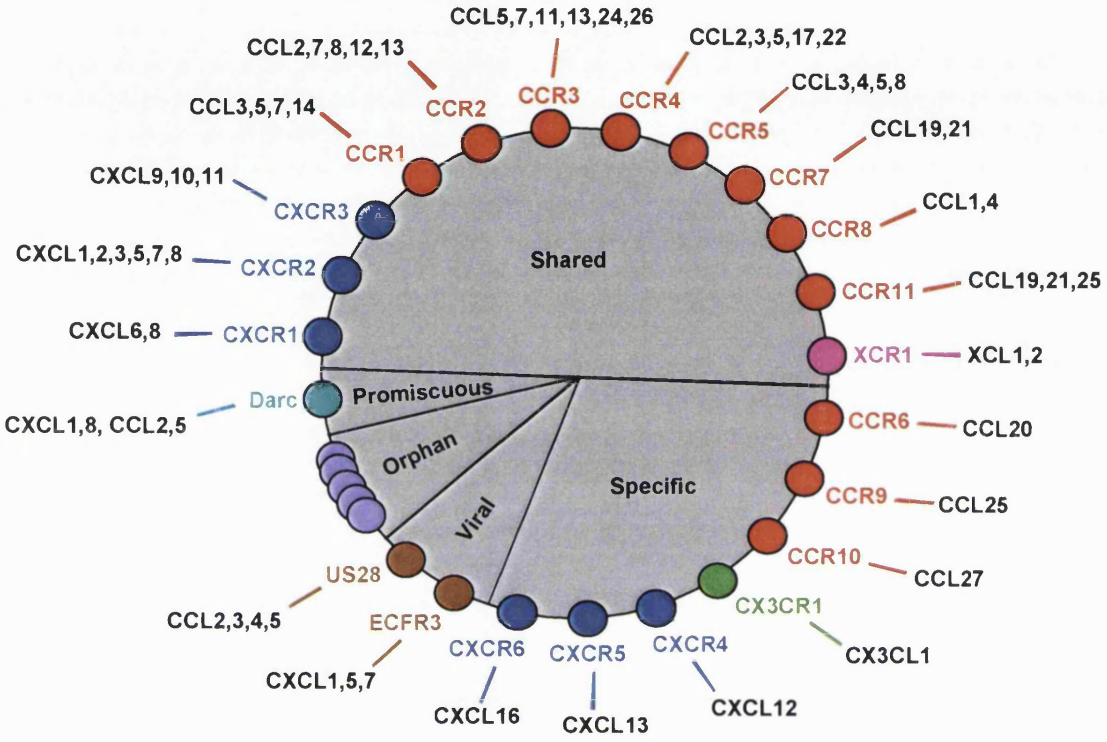


Figure 1.3. Classification of chemokine receptors and their ligands. The four chemokine receptor families are grouped as 'shared' if they bind more than one ligand, 'specific' if they only bind one ligand, and 'promiscuous' if they bind ligands of different chemokine subclasses. Adapted from (25).

1.2.3. Chemokine receptor structure and ligand binding

1.2.3.i. Three-dimensional structure

Most chemokine receptor genes have an open reading frame on a single exon of around 1000 bp (52, 53). This yields a protein of approximately 400 amino acids with a molecular mass of around 40 kDa. Chemokine receptors share structural homology with receptors for other leukocyte chemoattractants, such as fMLP (54), receptors for other signalling molecules, such as adrenaline, retinoic acid and angiotensin II, rhodopsin and herpesvirus receptor homologues (55). The structure of rhodopsin has

been recently been solved (56) and shows that the intracellular loops are spread along the inner surface of the plasma membrane, providing a large surface for interaction with intracellular signalling molecules (Figure 1.4.). This may explain how chemokine receptors, which are relatively small, can interact with a variety of downstream effectors. Chemokine receptors also have some unique structural features, such as the amino acid sequence DRYLAIV in the second intracellular loop domain (57) which is important as a docking site for heterotrimeric G proteins (58). Mutation of this motif in CCR5 prevented the release of intracellular calcium in response to CCL4 (59).

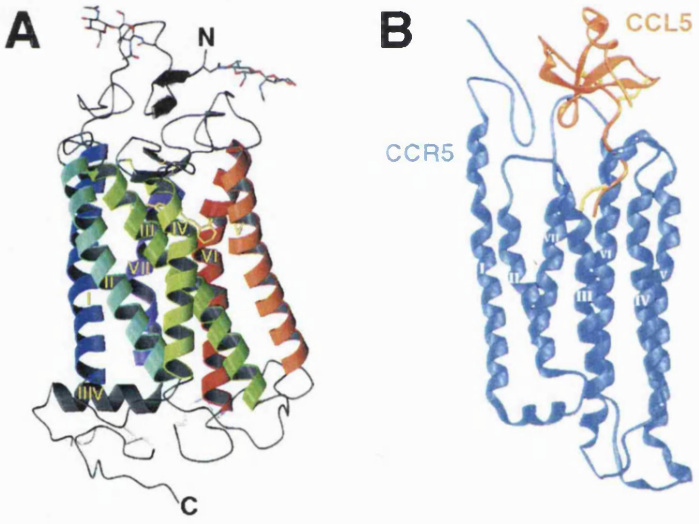


Figure 1.4. Three-dimensional structures of chemokine receptors. The crystal structure of rhodopsin has recently been solved by Palczewski *et al* (56). The C-terminal intracellular loops are spread along the inner surface of the plasma membrane, providing a large surface for interaction with intracellular signalling molecules (**A**). Chemokine receptors are likely to adopt a similar tertiary structure. A model for the interaction of CCL5 with its receptor, CCR5, is also shown (**B**). Adapted from (25).

Numerous serines and threonines are present in the C-terminal tail of chemokine receptors which, upon phosphorylation after receptor activation, are involved with receptor desensitisation and signalling (60, 61).

1.2.3.ii. Ligand selectivity

The N-terminal region of chemokine receptors tends to be composed of the most divergent amino acid sequences found between chemokine receptors, and it is thought that this region is important for chemokine selectivity. Chimaeric receptors, mutagenesis and extracellular loop-specific monoclonal antibodies have been used to evaluate chemokine selectivity (62-64). Scanning alanine mutagenesis of CXCL1 showed that various charged residues and cysteines are important for CXCL8 binding (65). CCR1/CCR3 chimaeras showed that the N-terminal segment is required for CCL3 and CCL11 binding (66), while CCR1/CCR2b chimaeras showed that the N-terminal segment is required for CCL2 binding, but not CCL3 binding (67). Monoclonal antibodies which neutralise CXCL8 binding to CXCR1 also map to the N-terminal region of the receptor (68). For CCR5 and CXCR4, the first two transmembrane domains and associated extracellular loops are dispensable for normal receptor expression, ligand binding and function (69). More work is required to narrow down the molecular determinants required for chemokine selectivity, and this information will be useful for developing specific receptor antagonists.

1.2.3.iii. Receptor dimerisation and post-translational modifications

To further complicate the chemokine/chemokine receptor system, both CC and CXC chemokine receptors can form homo- or heterodimers (70, 71). CCR2, CCR5 and CXCR4 can undergo ligand-triggered receptor dimerisation, which enables activation of the JAK signalling pathway (72). In addition, simultaneous stimulation with CCL2 and CCL5 can induce the formation of CCR2-CCR5 heterodimers in transfected HEK-293 cells; this increases the sensitivity of the chemokine response by 10 to 100-fold. Chemokine receptors can also be post-translationally modified, for example CCR2b has a sulphated tyrosine residue near its N-terminus and is also N-glycosylated. These post-translational modifications may have significant biological functions, including effects on ligand binding (73).

1.2.3.iv. Chemokine receptors are expressed on a variety of cell types

Chemokine receptors have been found predominantly on leukocytes, enabling them to respond to inflammatory chemokines and homeostatic (tissue homing) chemokines. However, various other cell types can also express chemokine receptors (Table 1.2). Vascular smooth muscle cells have been shown to express, for example, CCR1 and CCR2 (74) and CCR5 (75), which may be important in atherosclerosis. Endothelial cells can express CXCR1, 2, 3 and 4 (76, 77), which would allow these cells to respond to both pro- and anti-angiogenic chemokines such as CXCL8 and CXCL10 respectively. Epithelial cells from a number of tissues can express chemokine receptors: CXCR4 can be expressed by colonic epithelium (78); CCR3 is expressed by human airway epithelium (79) and CCR6 expression has been detected on some pancreatic cancer cell lines (80).

Chemokine Receptor	Receptor-expressing cell type
CCR1	monocytes, dendritic cells (immature), T cells, neutrophils, eosinophils, mesangial cells, platelets, smooth muscle cells
CCR2	monocytes, dendritic cells (immature), basophils, T cells, NK cells, endothelial cells, fibroblasts, smooth muscle cells
CCR3	eosinophils, basophils, T cells (Th2), dendritic cells, platelets, epithelial cells
CCR4	dendritic cells (immature and mature), basophils, T cells (Th2), platelets
CCR5	T cells (Th1), dendritic cells (immature), monocytes, NK cells, thymocytes, microglial cells, smooth muscle cells
CCR6	dendritic cells (immature), T cells, B cells, epithelial cells
CCR7	dendritic cells (mature), T cells, B cells, epithelial cells
CCR8	monocytes, T cells, B cells, thymocytes
CCR9	T cells, thymocytes
CCR10	T cells, melanocytes, dermal endothelium, dermal fibroblasts, langerhans cells
CCR11	astrocytes
CXCR1	neutrophils, monocytes, astrocytes, endothelium, epithelial cells
CXCR2	neutrophils, monocytes, eosinophils, endothelium, epithelial cells
CXCR3	T cells (Th1), B cells, mesangial cells, smooth muscle cells
CXCR4	T cells, dendritic cells (immature and mature), monocytes, B cells, neutrophils, platelets, astrocytes, endothelium, epithelial cells
CXCR5	T cells, B cells, astrocytes
CXCR6	T cells (Th1 and Tc1)
XCR1	T cells
CX3CR1	T cells, NK cells, astrocytes
Duffy	erythrocytes, endothelium
D6	B cells

Table 1.2. Cell types that express chemokine receptors. A range of different cell types, including leukocytes, endothelial cells, smooth muscle cells and epithelial cells can express various chemokine receptors. Adapted from (6).

1.2.3.v. Chemokine receptor signalling

All chemokine receptors are coupled with heterotrimeric G proteins. Binding of chemokines to their receptors can activate a variety of signal transduction pathways, but until recently, the association of receptors with some of these signal transduction pathways was poorly understood. Figure 1.5. gives an overview of signalling via chemokine receptors. The signal transduction mechanisms activated by chemokines

such as CCL2 and CCL5 have been extensively investigated (81, 82). A given chemokine may be able to activate different responses depending on the chemokine receptor or cell type to which it binds. CXCL8 can bind to CXCR1 and CXCR2, both of which have the same preference for G protein family members (83); CXCR1 activates phospholipase D (PLD) and causes the formation of superoxide, but CXCR2 does not (84). The mechanism for receptor-specific signal transduction remains to be elucidated.

It seems that most chemokines are able to elicit an increase in intracellular calcium, although the functional requirement for this is unclear, since chemotaxis can occur in situations where calcium flux is undetectable (85). During chemotaxis, the essential step upon ligation of a chemokine receptor is the release of the $G\beta\gamma$ subunit from the $G\alpha_i$ subunit (86). Experiments in *Dictyostelium discoideum* have shown that $\beta\gamma$ subunits accumulate at the leading edge of migrating amoebae, supporting their role in chemotaxis (87).

The $G\beta\gamma$ subunit can then activate PLC-β_2 and PLC-β_3; these isoenzymes are involved in chemokine signal transduction in immune cells (89) and lead to inositol-1,4,5-trisphosphate formation and a transient rise in the intracellular free calcium concentration. There is no calcium flux in neutrophils from PLC-β_2 and PLC-β_3 knockout mice, but these cells are still able to migrate normally. However, PLC can also generate diacylglycerol (DAG) and this can lead to the activation of protein kinase C (PKC). This enzyme plays a role in chemokine receptor phosphorylation and hence desensitisation (61). It can also activate the respiratory burst in neutrophils (89).

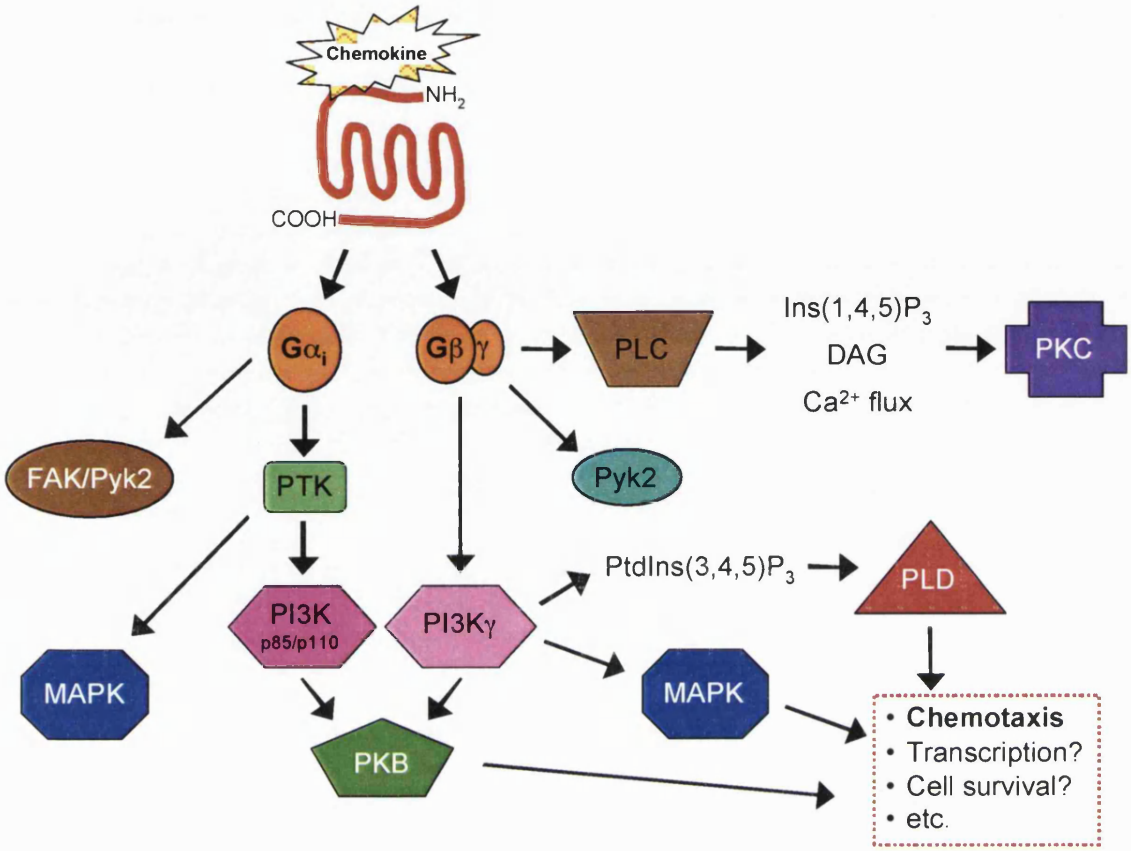


Figure 1.5. Signalling through chemokine receptors. This diagram illustrates some of the potential downstream signalling pathways following chemokine binding to a chemokine receptor. The βγ subunits can activate Phospholipase C (PLC) and PI3Kγ, leading to activation of Protein Kinase C (PKC), Phosholipase D (PLD), Protein Kinase B (PKB) and the MAPK cascade - with numerous downstream effects. PKC may be important for receptor desensitisation. The $G\alpha_i$ subunit can activate Protein Tyrosine Kinases (PTK) such as Src, which may lead to activation of FAK and Pyk2 (which are involved with cytoskeletal arrangements), the MAPK cascade and also PI3K p85/p110 which may be involved in the prolonged signalling seen with CXCR4. Adapted from (88).

Perhaps most importantly, chemokine stimulation leads to the activation of PI3Kγ (90). Mice lacking this gene have impaired signal transduction in response to chemokines, demonstrating an important role for this molecule in signalling pathways downstream of chemokine receptors (91, 92). The $G\alpha_i$ subunit can also activate signal transduction

pathways which lead to the activation of p85/p110 PI3K. Both forms of PI3K can activate protein kinase B (also known as Akt) (93). PKB can have numerous downstream effects, including promotion of cell survival [see (94) for review]. In prostate cancer, increased Akt/PKB activity has also been shown to contribute to tumour progression, by accelerating tumour growth (95). PKB is also involved in chemotaxis and is recruited to the leading edge of cells undergoing migration (96, 97).

Chemokines (including CXCL8, CXCL12, CCL2 and CCL20) have also been shown to activate the MAPK cascade (98-101). The Gβγ subunit leads to activation of PI3Kγ, and this in turn can activate the MAPK cascade, possibly through its intrinsic protein kinase activity, rather than its ability to phosphorylate lipids (102, 103). MAPK activation may or may not be required for chemotaxis. Inhibition of ERK activation using inhibitors such as PD98059 abrogates actin polymerisation and/or migration of eosinophils and T cells in response to CXCL12, CCL11 and CCL20 (101, 104, 105). Yet PD98059 does not block the migration of neutrophils to IL-8 (106).

Phospholipase D (PLD) is also activated in response to chemokines including CXCL8 and CCL5 (107, 108) and may be involved with rearrangement of the actin cytoskeleton (109). A variety of other signalling pathways may be activated in response to chemokines: Stimulation of CCR2b and CXCR4 can lead to the activation of the Jak/Stat pathway (110, 111); ligation of CXCR4 can also activate Pyk2 and NFκB (112) and CCL5 can activate focal adhesion kinase (FAK) (113).

Transient signalling is a common characteristic of most chemokine receptors, but one which requires rapid inactivation. This is achieved through receptor phosphorylation, desensitisation and internalisation. CCR2b, CCR5, CXCR1, CXCR2 and CXCR4 are all rapidly internalised after ligand-binding (114-117). Chemokine receptors are phosphorylated at serine or threonine residues in their C-termini, which may alter their

three-dimensional conformation, impairing the interaction with G proteins. Desensitisation of G protein-coupled receptors may be performed by four families of regulatory molecule: PKC, cAMP-dependent PK, arrestins and GPCR-coupled kinases (GRKs) (118). GRKs are recruited to the cell membrane by Gβγ, where they can phosphorylate the chemokine receptor; arrestins can then bind to the receptor, sterically inhibit the binding of G proteins and cause internalisation (119, 120).

CXCR4 may be unusual in this regard, since it has been shown to stimulate prolonged signalling of Akt/PKB and ERK2 in T cells, despite receptor internalisation (121). The studies by Tilton *et al* have raised various possibilities to explain this phenomenon, the most likely being that CXCR4 remaining at the surface is not desensitised (as is usual with chemokine receptors) but can continue to transduce a signal in response to CXCL12. Receptor recycling could also contribute to this effect.

Signal transduction downstream from chemokine receptors is much more complicated than the overview given here. Signalling pathways activated by a given receptor will be influenced by those from other receptor systems, and can also cross-regulate one another. The challenge now will be to understand how chemokine-induced signals are fine-tuned and integrated with other signals.

1.3. Chemokine/Chemokine receptor functions

It is becoming apparent that aside from their role as chemoattractants, chemokines and chemokine receptors have functions in a variety of other processes, including angiogenesis, development, T cell differentiation, haematopoiesis and inflammation. This section will outline some of these functions. The particular roles of CXCR4 and CXCL12 will also be described, since Chapters 5 and 6 of this thesis concentrate on this chemokine/receptor pair in the context of epithelial cells in ovarian cancer.

There is apparent redundancy in the chemokine/chemokine receptor system: chemokines can often bind to more than one receptor, each receptor can often bind more than one chemokine, and multiple receptors can be expressed on a given cell type. Despite this, the importance of individual chemokines and receptors is shown in transgenic and knockout mice (122-126). For example, CCR1 knockout mice have an imbalance in Th1/Th2 cytokines, reduced lung eosinophil recruitment in a murine asthma model, resistance to experimental autoimmune encephalomyelitis (EAE) development and prolonged allograft survival in a transplantation model (127, 128). CCR7 knockout mice have impaired B and T cell migration and defective primary T cell responses in delayed-type hypersensitivity models (129), and anti-CCL2 treatment can significantly reduce the severity of relapsing EAE in mice (130).

Chemokine/receptor functions are often classified as either 'inflammatory' or 'homeostatic'. Inflammatory chemokines are usually expressed in most tissues in response to inflammatory stimuli such as cytokines (e.g. IL-1, TNF-α or IFN-γ) and bacterial toxins (e.g. LPS). In contrast, homeostatic chemokines are constitutively expressed within discrete areas of lymphoid tissues, in the absence of inflammatory stimuli. They may control the maturation and homing of lymphocytes and dendritic cells. Aspects of these different functions will be discussed briefly below.

1.3.1. Role in normal physiology

1.3.1.i. Chemotaxis

Chemotaxis is defined as directed migration towards a gradient of chemoattractant; if the chemoattractant is immobilised (e.g. bound to glycosaminoglycans on the surface of endothelium) then this directed migration is termed 'haptotaxis'. Chemokines are so named because they are chemotactic cytokines that can stimulate the directed migration (through both chemotaxis and haptotaxis) of distinct subsets of leukocytes and other cell types. Chemokines cause cell polarisation in leukocytes, with the formation of a

'leading edge' and a uropod. A variety of molecules are concentrated at the leading edge, including chemokine receptors, integrins and intracellular signalling molecules [see (131) for review]. Cytoskeletal rearrangements then provide the locomotive force for migration: acto-myosin-based contraction of the actin cytoskeletal network pulls the cell forward. The chemotactic function of chemokines is fundamental for their role in both normal and pathophysiology.

1.3.1.ii. Leukocyte trafficking and immune responses

The trafficking of lymphocytes and dendritic cells from/to secondary lymphoid organs is a vital component of the adaptive immune system. Dendritic cells (DC) can take up antigen in peripheral tissues and migrate to draining lymph nodes where they present antigen to naïve T cells, triggering the T cells to proliferate and differentiate. These activated T cells can then migrate to the inflamed tissues to perform their effector functions. The movement of T cells and DC between lymph nodes and the periphery may be controlled by differential chemokine receptor expression (132).

Circulating monocytes and immature DC can express receptors for chemoattractants such as fMLP (representative of bacterial proteins) and C5a (generated in the complement cascade) as well as chemokine receptors including CXCR4, CCR1, 2, 3, 4 and 5 (133-135). Using these receptors, immature DC can migrate towards sites where there is a high concentration of inflammatory chemokines such as CCL2, CCL3 and CCL5. Immature DC can also respond to constitutively expressed chemokines such as CXCL12, and this may be important for localising DC in tissues under normal conditions.

At sites of inflammation, immature DC are activated by inflammatory cytokines such as TNF-α and IL-1 (136, 137), which results in DC maturation. This causes a switch in their chemokine receptor expression. Maturing DC lose their responsiveness to CCL3,

4, 5, 7 and 20 (138-141), but concomitantly upregulate CCR7 and gain responsiveness to CCL19 and CCL21 which are expressed T cell rich areas of secondary lymphoid organs including lymph nodes, spleen and tonsils (140, 142). CCR7 knockout mice have defective homing of DC (143).

Naïve T cells can express CXCR4 and CCR7, which may account for their localisation in secondary lymphoid organs. Upon interaction with antigen-presenting DC, naïve T cells are activated and alter their chemokine receptor profile. Activated T cells can express CXCR3, CXCR4, CCR1, 2, 3, 4, 5, 6, 7 and 8 (144-146). Activated T cells can polarise to T helper 1 (Th1) and T helper 2 (Th2) subsets, which differ in their cytokine production and function during an immune response. Th1 cells express IFN-γ and IL-12, while Th2 cells express IL-4, and they also express different chemokine receptors. CCR5 and CXCR3 are preferentially expressed on Th1 cells, while Th2 cells preferentially express CCR3, CCR4 and CCR8 (144, 145). However, this differential chemokine receptor expression is not clear cut – Th2 cells can also express CXCR3, albeit at ten-times lower levels and with a different dose response curve (144) and CCR4 can be expressed by skin-homing T cells (mainly Th1) (147) and central memory T cells (T_{CM}) (148). Despite this, the chemokine receptors expressed by polarised T cells may help to explain their localisation during immune responses. Other subsets of T cells, including effector memory T cells (T_{EM}) and T_{CM} may also express distinct chemokine receptor profiles [see (132) for review].

Finally, naïve B cells can express CXCR5 and CCL7 (149, 150) which directs them to the follicles of secondary lymphoid organs, where the ligands CXCL13, CCL19 and CCL21 are expressed. Upon maturation to plasma cells, B cells downregulate CXCR5 (151) and CCR7 (152), but have increased sensitivity to CXCL12 (through CXCR4) which regulates plasma cell positioning in the spleen and lodgement in the bone marrow (152).

Therefore, chemokines and chemokine receptors play a vital role in the trafficking of leukocytes to and from secondary lymphoid organs, particularly during an immune response.

1.3.1.iii. Haematopoiesis

T lymphopoiesis occurs mainly in the thymus, while B lymphopoiesis occurs predominantly in the bone marrow, leading to the development of mature naïve lymphocytes. T and B lymphocytes are thought to develop from a common haematopoietic progenitor cell (HPC) found in the bone marrow. Stem cells and progenitor cells in the bone marrow are subjected to the influence of a variety of different cytokines, resulting in either stimulation or inhibition of proliferation [see (153) for review]. Members of the chemokine family, including CXC, CC and C chemokines, have been shown to suppress haematopoiesis (154). Work by Reid *et al* (155) and Haneline *et al* (156) implicated CCL3 and CCR2 in the induction of HPC apoptosis. In contrast, various chemokines promote haematopoiesis: for example, CXCL12 has been shown to enhance HPC survival (153).

HPC can express CXCR4 and CCR7 and have been shown to migrate towards CXCL12, CCL19 and CCL21 (157, 158). Chemokine receptor expression by HPC may regulate the homing of these cells within the bone marrow during differentiation and maturation, and their mobilisation into the circulation. CXCR4 may be important for the retention of HPC in the bone marrow, particularly during B lymphopoiesis; retaining B cell precursors in the bone marrow would enable their regulated differentiation into mature B cells (159, 160).

1.3.1.iv. T cell differentiation

Chemokines can differentially attract naïve and activated T cells, but they may also have roles in regulating T cell differentiation. In many situations, it is still not clear

how Th1 or Th2 responses develop, yet this decision is critical to the outcome of the immune response. Chemokines, including CCL2 and CCL3, have now been shown to influence this Th1/Th2 polarisation. CCL2 can suppress Th1 responses and cause an increase in IL-4 (Th2 cytokine) production by activated and memory T cells *in vitro* (161, 162). CCL2 addition to macrophages *in vitro* can also decrease IL-12 (Th1 cytokine) expression (163). CCL2 may therefore promote Th2 polarisation both directly and indirectly by increasing IL-4 and decreasing IL-12 production, respectively. In contrast, addition of CCL3 to *in vitro* cultures of activated T cells promoted the development of IFN-γ-producing cells (161) and hence Th1 differentiation. Similarly, CCL3, 4 and 5 production by monocyte-derived DC can promote the development of IFN-γ-producing cells (164). Experiments in mice deficient in the chemokines CCL2 and CCL3 and the chemokine receptors CCR1 and CCR2 have been less conclusive, with a preference for either Th1 or Th2 polarisation depending on the experimental protocols used (126, 127, 165-167). For example, CCL2-deficient mice have increased *Leishmania* resistance, indicating a shift from Th2 to Th1 (165), while CCR2-deficient mice have diminished IFN-γ production, increased Th2-type cytokines and higher IgE, suggesting a shift from Th1 to Th2 (166, 168).

More work is required to further elucidate the requirements for chemokines in the differentiation of T cells, and also the contribution of chemokines produced by T cells themselves. This may have implications for the use of chemokine receptor antagonists in the treatment of inflammatory disease.

1.3.2. Role of chemokines in infection and inflammatory disease

The vital role of chemokines in directing leukocyte traffic during immune responses necessarily involves chemokines/receptors in infection, as well as inflammation and various other disease states. Thus, viral infection, autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, allergy, transplantation, atherosclerosis and

cancer are all affected by chemokine and chemokine receptor expression and function. Some of the roles of chemokines and their receptors in inflammatory responses, asthma, multiple sclerosis, HIV infection and cancer will be described here.

1.3.2.i. Tissue recruitment of leukocytes

During an inflammatory response, allergic reaction or in a disease state such as cancer, leukocytes are recruited into tissues by chemokines. This is similar to the normal physiological leukocyte trafficking in response to homeostatic chemokines in lymphoid organs. Inflammatory cytokines such as TNF-α or IL-1α and other stimuli (e.g. bacterial LPS) promote the production of inflammatory chemokines by stromal cells in the tissue and immigrating leukocytes. These chemokines form a gradient from the tissue to the circulation where they are presented to circulating leukocytes in the context of glycosaminoglycans (Figure 1.6.). Thus, chemokines can form a haptotactic, immobilised gradient of chemoattractant. But to do so, tissue-derived chemokines must traverse the endothelium of blood vessels. Originally this was thought to be achieved by chemokine diffusion through intercellular gaps [see (169) for review], but work by Middleton *et al* (170) suggests that chemokines, in particular CXCL8, are transcytosed across the endothelium. Immunoelectron microscopy and electron microscopic autoradiography were used to follow the localisation of radio-labelled CXCL8 injected into rabbits. The timecourse of localisation of CXCL8 was consistent with transcytosis of CXCL8 from the abluminal endothelial cell surface, via caveolae, to the luminal surface, where it was presented on the surface of microvilli. Transcytosis of the chemokine CCL19 has also been demonstrated in high endothelial venules (171).

During the multi-step process of transendothelial migration (Figure 1.6.), leukocytes first undergo a process of 'rolling' along the endothelium. This is mediated through weak adhesive interactions between L-, P- and E-selectins on the endothelium and selectin ligands on the leukocytes [see (172) for review]. Then the increased avidity of

leukocyte integrins leads to leukocyte arrest and firm adhesion (173-175). These integrins include VLA-4, which captures lymphocytes under shear flow conditions but only in response to immobilised chemokines, not soluble chemokines (176), and β_2 integrins on monocytes and neutrophils (177, 178).

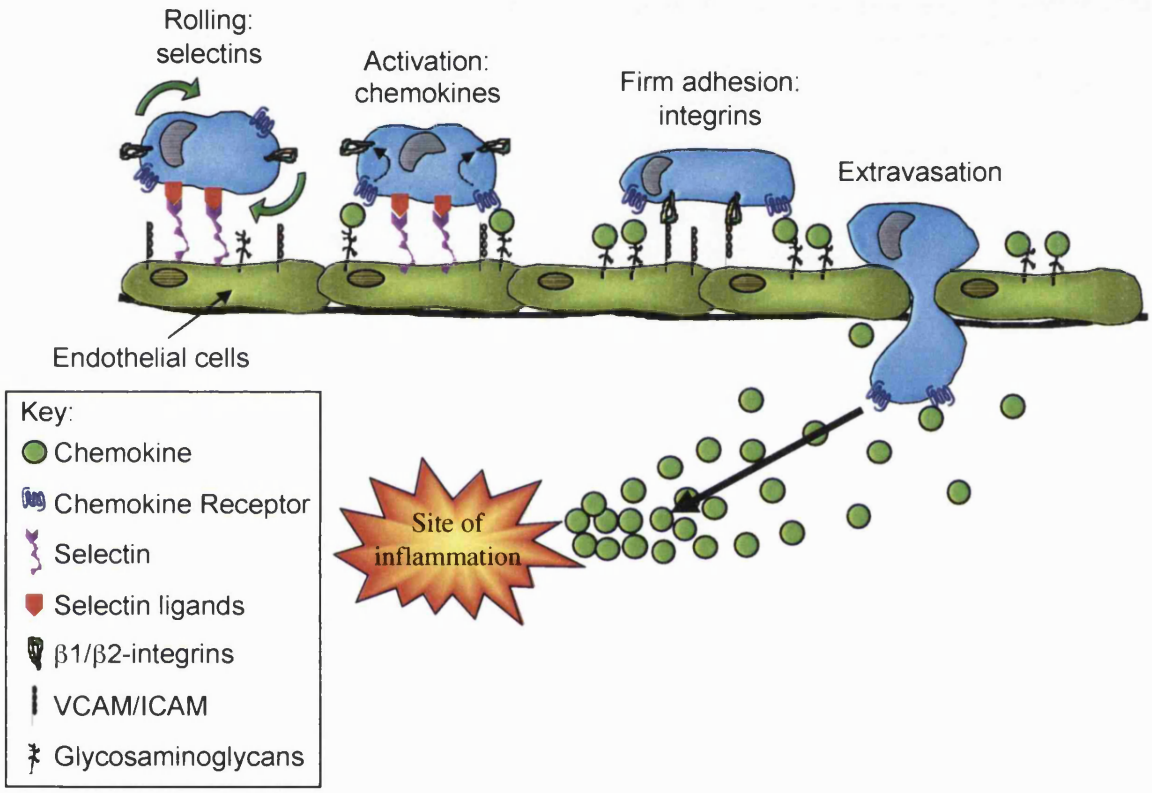


Figure 1.6. Chemokine-induced leukocyte transmigration during an inflammatory response. Circulating leukocytes loosely adhere to the endothelium through interaction with selectins and roll along the vessel wall. Activation by chemokines (possibly bound to glycosaminoglycans) causes upregulation of leukocyte integrins, leading to firm adhesion to the endothelium. This is followed by extravasation through the vessel wall, and migration into the tissue towards the chemokine source.

Subsequently, leukocytes undergo transendothelial migration, which may only initiate and progress under conditions of shear flow (176), and chemotaxis through the tissue towards the site of chemokine production. Once leukocytes reach the site of

inflammation, modulation of chemokine receptor expression by pro-inflammatory signals such as LPS and TNF-α may serve to retain leukocytes in the tissue (179-181).

1.3.2.ii. Asthma

This is a chronic disease of the small airways where chronic inflammation leads to reversible airway obstruction and bronchopulmonary hyper-responsiveness as well as mucous gland hyperplasia and subepithelial fibrosis. The late response in this disease, occurring 4 to 12 hours after allergen exposure, is characterised by a leukocyte infiltrate consisting of eosinophils, mononuclear cells (particularly Th2 cells), basophils and mast cells (182). Various chemokine receptors are expressed on the leukocytes associated with asthma, including CCR1, 2, 3, 4, 7 and 8 (182). Of these, CCR3 may be particularly important since it is the receptor for CCL11 (eotaxin); this chemokine was first described due to its ability to attract eosinophils (183) which are closely correlated with lung dysfunction clinically (184). Protein and mRNA expression of CCR3 are elevated in the bronchial mucosa and skin of patients with asthma (185); CCR3 is expressed on eosinophils, basophils, mast cells and airway epithelial cells (79, 186, 187) and can bind CCL5, 7, 11, 13, 24 and 26 (see Figure 1.3.). Airway epithelial cells are a major source of CC and CXC chemokines in asthma (188), and CCR3 expression by these cells could modulate several aspects of epithelial function, including production of more chemokines, activation, proliferation and apoptosis.

The functional importance of CC-chemokines in rodent asthma models has been demonstrated in studies using neutralising antibodies to CC chemokines, amino-terminal modified CC-chemokines that act as receptor antagonists and a pan-CC chemokine antagonist protein from pox virus (189-191). Chemokine receptor antagonists may therefore be useful for blocking the inflammatory infiltrate seen in this disease.

1.3.2.iii. Multiple sclerosis (MS)

MS is a demyelinating autoimmune disease mediated by T cells, Th1 cells in particular. These $CD4^+$ T cells are specific for one or more autoantigens in the central nervous system (CNS), and subsequently produce a variety of destructive inflammatory mediators. Antigenic re-stimulation by microglial cells in the CNS may allow these auto-reactive T cells to persist (192), and the inflammatory gene expression which follows recruits a second wave of leukocytes. Thus, MS lesions contain a variety of cell types including the original auto-reactive T cells, monocytes/macrophages and T cells which are subsequently recruited, activated microglia and activated cerebrovascular endothelium (193-195).

A number of studies have investigated the role of chemokines and their receptors in the pathogenesis of MS [for example (196-199)]. During active MS attacks there are significantly increased levels of CXCL9, CXCL10, and CCL5 in cerebrospinal fluid (CSF) (199) and CCL2, CCL7 and CCL8 have been found immunohistochemically in MS lesions (196). Various chemokine receptors are also expressed in MS lesions: CCR2 and CCR5 are found on macrophages, microglia and T cells, and CCR3 is also found on reactive astrocytes (197). MS patients heterozygous or homozygous for the CCR5 Δ32 allele (which encodes a non-functional form of CCR5) have delayed disease onset of approximately 3 years compared with affected siblings (200), suggesting that CCR5 may be a target for therapy. CXCR3 is expressed by more than 90 % of $CD3^+$ T cells in CSF, and >99 % of T cells in perivascular accumulations in active lesions (199). The presence of CXCL9 and CXCL10 in MS lesions may account for the recruitment of CXCR3 positive T cells; CXCR3 may also be a target for therapeutic intervention in MS (194).

Rodent models of experimental autoimmune encephalomyelitis (EAE), which have similar characteristics to chronic relapsing MS, have also suggested a role for

chemokines in this disease. EAE can be induced by immunising mice with antigens derived from myelin, such as myelin oligodendrocyte glycoprotein (MOG). Inflammatory lesions have mRNA and protein for CCL2, CCL3, CCL4, CCL5 and CXCL10 (130, 201-204). CCR2-deficient mice are almost completely protected from MOG-induced EAE (205, 206); CCL2-deficient mice are also resistant, suggesting that CCR2 and CCL2 may be important as therapeutic targets in MS (207). Treatment of mice with an anti-CXCR3 antibody after induction of EAE protects them from subsequent development of the disease compared with control mice, also supporting the use of CXCR3 antagonists in MS (208).

1.3.2.iv. HIV

Human immunodeficiency virus (HIV) is a retrovirus surrounded by a viral envelope which consists of virus-encoded glycoproteins embedded in a host cell-derived lipid bilayer (209). The primary cell surface receptor for HIV entry is CD4; however, in 1996, viral strain-specific co-receptors were discovered that are also required for HIV entry into cells (210-214). These co-receptors were the chemokine receptors CXCR4 and CCR5, which along with CD4 (present on T cells and macrophages) allow viral envelope fusion and entry (215). A range of chemokine receptors have now been shown to have co-receptor activity *in vitro*, including CCR1, 2, 3, 4, 5, 8, 9, CXCR2, 4, 5, 6 and CX3CR1 [see (216) for review], but so far only CXCR4 and CCR5 have been shown to act as co-receptors *in vivo* [hence X4 tropic and R5 tropic viruses (217)]. The co-receptor usage also determines the cellular tropism: R5 virus tends to be macrophage tropic (218) while X4 virus tends to be T cell tropic, despite the fact that macrophages can express CXCR4 (219). Virus entry into cells involves a process whereby the gp120 protein in the viral envelope interacts with CD4 on the cell surface. This results in a conformational change in gp120 that allows a secondary interaction with the chemokine co-receptor. This results in further conformational changes in the viral envelope protein gp41, resulting in membrane fusion and virus entry (220-222).

Since chemokine receptors act as co-receptors for HIV entry, endogenous chemokine production can regulate HIV replication. In 1995, Cocchi *et al* published that CCL3, CCL4 and CCL5 have $CD8^+$ T-cell-derived HIV inhibitory activity (223) and individuals with high levels of these chemokines have reduced HIV infectability (224). Also, individuals homozygous for the Δ32 allele of CCR5, who are deficient in cell surface CCR5 expression, can remain uninfected despite exposure to HIV (225). These observations suggest that chemokines and their receptors which are involved with HIV infection are potential targets for the development of new drugs to treat HIV. For example, small molecule antagonists of CCR5 and CXCR4, which can block HIV entry, are entering clinical trials (226, 227).

1.3.3.Role in malignancy

1.3.3.i. Leukocyte infiltrate

In 1863, Virchow noted that neoplastic tissues contain a "lymphoreticular infiltrate", suggesting a connection between cancer and inflammation. Many tumours of epithelial origin contain a leukocyte infiltrate consisting predominantly of macrophages and T lymphocytes (228). Analysis of carcinomas of the colon, lung, breast and stomach showed that the infiltrating mononuclear cells were predominantly in the stroma (229). In ovarian cancer, leukocytes can be found both within the supporting stroma and the tumour areas (230).

Leukocytes can infiltrate tumours in response to tumour-derived chemokines, including CCL2 (230, 231). These chemokines are produced by tumour cells, stromal cells and the infiltrate (230, 232, 233).

It is unclear what function is performed by the leukocyte infiltrate. Mantovani *et al* introduced the concept of a 'macrophage balance', where tumour-associated macrophages (TAM) may promote or inhibit tumour growth depending on their state of

activation (228). In recent work by Nesbit *et al*, high levels of CCL2 over-expression in melanoma cells led to tumour destruction in nude mice, due to a massive monocyte/macrophage infiltrate (234). However, low levels of CCL2 over-expression led to an angiogenic effect mediated through TAM, which resulted in increased tumour growth. In work by Lin *et al*, PyMT mice (which are susceptible to mammary carcinoma) that were deficient in CSF-1 developed primary mammary tumours at the same rate as wild-type mice. However in the CSF-1 deficient mice, the progression to malignancy and metastasis was delayed due to a paucity of TAM (235). In breast cancer, macrophage infiltration has been correlated with vascularity, suggesting that leukocytes may promote tumour angiogenesis and hence survival (236).

It is also possible that the infiltrate represents the host's attempt at mounting an ineffective immune response. Enhancing anti-tumour immune responses may lead to tumour regression. A number of chemokines have been over-expressed in murine tumour models: engineered over-expression of CCL1, 2, 5, 20, CXCL10 and XCL1 have all led to an enhanced immune response and hence tumour rejection (237-242). Vicari *et al* transduced a colon carcinoma cell line with CCL21; these cells showed a reduction in tumourigenicity in both immunocompetent and nude mice, possibly due to angiostatic mechanisms and anti-tumour immunity (243). The CCL21-transduced tumours had an increased infiltrate, which included immature dendritic cells and $CD8^+$ T cells; the data suggested that these cells participated in anti-tumour immunity. In a study of oesophageal carcinoma by Schumacher *et al*, the intratumoural $CD8^+$ T cell infiltrate showed proliferative activity and IFN-γ secretion and this infiltrate (rather than the peritumoural T cell infiltrate) was correlated with a good prognosis in both squamous cell and adenocarcinomas (244).

Tumour-derived chemokines may therefore contribute to tumour growth and spread; TAM, for example, can produce growth and angiogenic factors, and proteases which

may favour invasion and metastasis (245). In addition, reactive oxygen species released by TAM can damage DNA, causing mutation and hence tumour progression (246). Conversely, tumour-derived chemokines can be manipulated to encourage massive leukocyte infiltration and tumour destruction.

1.3.3.ii. Angiogenesis

Angiogenesis is the development of new blood vessels from pre-existing vasculature, and it depends on a balance between pro- and anti-angiogenic factors. These factors are normally precisely regulated and only allow neovascularisation when appropriate, for instance during wound repair (247). Tumour growth is also dependent on angiogenesis (248); once a tumour reaches a few millimetres in diameter, further tumour expansion requires neovascularisation. Once this is achieved, tumour growth is usually rapid and allows the potential for metastasis (249).

A variety of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), are produced by the tumour and the leukocyte infiltrate (250, 251). TAM in particular have been correlated with tumour vascularity and prognosis (252). Chemokines may also have a role in tumour angiogenesis (253).

CXC chemokines can be both pro- and anti-angiogenic, depending on the presence of a tripeptide motif (glutamate-leucine-arginine: the ELR motif) near the N-terminus of the protein (254). ELR^+ CXC chemokines are potent pro-angiogenic factors, while ELR^- CXC chemokines are angiostatic (255). Therefore, the balance between the expression of ELR^+ and ELR^- chemokines in tumours has important implications for the regulation of angiogenesis.

ELR^+ chemokines such as CXCL8, CXCL5 and CXCL6 directly promote chemotaxis and proliferation of endothelial cells *in vitro* and *in vivo* (255, 256). These effects may

be mediated through CXCR1 and/or CXCR2; CXCR1 can only bind CXCL6 and CXCL8, while CXCR2 can bind all ELR$^+$ chemokines. Work by Addison *et al* (257) with neutralising antibodies to CXCR2 and CXCR2-deficient mice suggests that this receptor is the putative receptor for chemokine-induced angiogenic activity.

Many of the angiostatic ELR$^-$ CXC chemokines are inducible by IFNs, including CXCL9, 10 and 11 (258-260). All three of these IFN-inducible chemokines bind to CXCR3, and this receptor may mediate their angiostatic functions by inhibiting the proliferation of endothelial cells (261). CXCL12 is also an ELR$^-$ CXC chemokine, but it is unclear whether this chemokine is pro- or anti-angiogenic; CXCL12 is chemotactic *in vitro* for endothelial cells (262) and can induce neovascularisation *in vivo* (263), yet it can antagonise the pro-angiogenic activity of ELR$^+$ chemokines and VEGF (264).

Recently the CC chemokine, CCL2, has also been shown to have a direct role in angiogenesis. Endothelial cells can express CCR2, the receptor for CCL2, and can migrate towards CCL2. Moreover, CCL2 can promote blood vessel formation in angiogenic assays *in vivo* (265).

CXC chemokines can have important roles in angiogenesis in tumours. Transfection of melanocytes with the ELR$^-$ chemokines CXCL1, 2 and 3 enables them to form tumours in nude mice, and these tumours are highly vascularised (266, 267). CXCL8 contributes to angiogenesis in models of non-small cell lung cancer (NSCLC) *in vivo* (268), while CXCL5 correlates with the degree of vascularity in human NSCLC biopsies (269). Work by Yoneda *et al* (270) showed a role for CXCL8 in the angiogenesis and hence progression of human ovarian cancer xenografts in nude mice. Studies in various other tumours and tumour models have indicated a role for ELR$^+$ chemokines in promoting angiogenesis and tumour progression, including prostate cancer (271), gastric cancer (272) and pancreatic cancer (273).

Angiostatic ELR$^-$ chemokines may have therapeutic benefit in cancer. The expression of CXCL9 and 10 in Burkitt's lymphoma cell lines in nude mice was higher in tumours that spontaneously regressed and was correlated with impaired angiogenesis (274). Production of CXCL10 from adenocarcinoma or squamous cell carcinoma cell lines inoculated in SCID mice was inversely correlated with tumour growth (275). Thus the balance between pro- and anti-angiogenic chemokines can regulate angiogenesis, indirectly affecting tumour cell growth; interfering with this balance may have therapeutic benefits.

1.3.3.iii. Growth-promoting effects in malignancy

Chemokines can also have direct effects on the growth of tumour cells. In 1985, an autostimulatory melanoma mitogen was discovered, termed melanoma growth-stimulatory activity (MGSA), which is now known to comprise the CXCL1/2/3 chemokines (276, 277). MGSA can stimulate the proliferation of melanoma cell lines and also pancreatic cell lines (278). CXCL8 can act as an autocrine growth factor for melanoma cell lines; inhibiting its production *in vitro* prevented cell proliferation and colony formation in soft agar (279). This chemokine can also stimulate the proliferation of some ovarian cancer cell lines (280), pancreatic cell lines (278) and colon carcinoma cell lines (281). In B-cell chronic lymphocytic leukaemia, CXCL8 does not stimulate the proliferation of leukaemic cells, instead it acts as a survival factor, protecting cells from apoptosis and causing their accumulation (282). The effects of CXCL1/2/3 and CXCL8 may be mediated through either CXCR1 or CXCR2. Both of these receptors can be expressed by tumour cells in head and neck squamous cell carcinoma (283). Therefore chemokines may be responsible for direct effects on cancer growth, as well as indirect effects through the stimulation of angiogenesis and promotion of a leukocyte infiltrate which can provide growth and survival factors.

1.3.3.iv. Invasion and metastasis

Tumour metastases probably develop due to interactions between selected tumour cells and a supportive microenvironment: the concept of 'seed' and 'soil' coined by Paget in 1889 (284). Thus, tumour cells may need enhanced adherence to the microvascular endothelium of the target organ and increased response to local mitogens, while the target site requires an appropriate microvascular endothelium, stroma and matrix, and the expression of mitogens (285). It is also apparent that metastasis is not a random process and that different tumours have preferential metastatic sites (285). Breast cancer frequently spreads to regional lymph nodes, bone marrow, lung and liver (286); malignant melanoma is similar, but also has frequent skin metastases (287). These patterns of metastasis are reminiscent of leukocyte trafficking, so it seems possible that chemokines and their receptors could have a role in tumour metastasis. It is also possible that monocytes infiltrating a tumour may provide tumour cells with a ready-made path for invasion: the 'countercurrent invasion theory' (288).

A number of different tumour cells have been shown to migrate towards chemokines, or to express chemokine receptors. The human breast cancer cell line MCF-7 migrated towards CC chemokines, including CCL3, 4 and 5 and to a lesser extent CCL2, CXCL1 and CXCL8 (289); binding studies indicated that MCF-7 possess binding sites for these chemokines, indicating the presence of multiple chemokine receptors. The prostate cancer cell line PC3 expresses CXCR2 and can migrate and invade in response to CXCL1 and CXCL8 (290). The ovarian cancer cell line SKOV-3 can express CXCR1 and CXCR2, which has implications for chemotaxis (291); however, we have never seen expression of these receptors on SKOV-3 in our laboratory. Functional CXCR4 expression has been demonstrated on a variety of leukaemic cells, including acute myelomonocytic and lymphoblastic leukaemia (292), enabling these cells to migrate towards CXCL12. CXCR4 can also be expressed by a colonic epithelial cell line (78)

and by tumour cells in pancreatic cancer (293). These results suggest that chemokine receptors may play a role in tumour metastasis.

1.3.4. CXCR4 and CXCL12

1.3.4.i. Introduction

CXCR4 is expressed on a variety of cell types, including leukocytes, haematopoietic progenitor cells, endothelial cells, epithelial cells and cells of the central nervous system (Table 1.2.). CXCR4 and its sole ligand, CXCL12, are currently the best candidates for the primordial chemokine/receptor pair because they have many functions besides those in immunity. CXCR4 and CXCL12 are now known to have important roles in leukocyte trafficking, haematopoiesis, organogenesis, vascularisation and embryogenesis [see (294), for review].

CXCL12 is a homeostatic chemokine – it is expressed constitutively in a range of tissues and does not appear to be regulated by pro-inflammatory cytokines (295). Shirozu *et al* investigated CXCL12 mRNA expression in human organs (296). Almost all organs were tested positive, with abundant mRNA expression in pancreas, spleen, ovary and small intestine. This ubiquitous expression may be due to the presence of a GC-rich sequence in the 5'-flanking region of the CXCL12 gene, a feature which is associated with 'housekeeping' genes. The human and murine CXCL12 proteins are over 92 % identical, while the CXCR4 proteins are approximately 91 % identical, therefore this chemokine/receptor pair is highly conserved between species.

1.3.4.ii. CXCR4- and CXCL12-deficient mice

CXCR4- and CXCL12-deficient mice have been generated (159, 297-299), and they have almost identical phenotypes. CXCL12-deficient mice die *in utero* and have severely reduced numbers of B cell progenitors in the foetal liver and bone marrow. Myeloid progenitor cells were also reduced in number in the bone marrow, but not in

the liver, suggesting that CXCL12 is responsible for B cell lymphopoiesis and bone marrow myelopoiesis. CXCL12-deficient mice also had severe heart defects, including defective cardiac ventricular septum formation (297), and a disorganised cerebellum (299).

CXCR4-deficient mice also die *in utero* or, in the rare instances when homozygous mutant mice are born alive, die within a few hours. As with CXCL12-deficient mice, they have profound defects in their haematopoietic systems, including severely reduced B lymphopoiesis, reduced myelopoiesis in the foetal liver, and almost absent myelopoiesis in the foetal bone marrow; T lymphopoiesis is unaffected in these mice. CXCR4-deficient mice also displayed the same defects in cerebellum formation, with abnormal neuron migration (299). Other work has shown that CXCR4/CXCL12 are also essential for vascularisation of the gastrointestinal (GI) tract; mice deficient in either CXCR4 or CXCL12 have defective formation of the large blood vessels supplying the GI tract, possibly due to defective regulation of vascular branching and/or remodelling processes in endothelial cells (298). The near identical phenotypes of the CXCR4- and CXCL12-deficient mice suggests a monogamous relationship where CXCL12 only acts through CXCR4.

1.3.4.iii. Role of CXCR4/CXCL12 in development

The phenotypes of the CXCR4- and CXCL12-deficient mice demonstrate that this chemokine/receptor pair is important in development. Elevated levels of CXCR4 mRNA have also been found in the thymus and brain of developing rat embryos (300). McGrath *et al* (301) have detected chemokine receptor mRNA (including CCR1, 2, 4, 5 and CXCR2) in developing mouse embryos at embryonic day 12.5 (E12.5). This is consistent with a role in definitive (adult-like) haematopoiesis. However, CXCR4 mRNA was the predominant receptor expressed at earlier times (E7.5 and E8.5). CXCR4 and CXCL12 had dynamic and corresponding expression patterns in the

developing neuronal, cardiac, vascular, haematopoietic and craniofacial systems, suggesting a role for CXCR4 and CXCL12 in multiple embryogenic events.

Taken together, these results point to CXCR4/CXCL12 as an important chemokine signalling system during embryogenesis and development, with broad roles in controlling the movement of cells in a variety of organs. This may explain why CXCL12 diverged from other chemokines early in evolution and remained highly conserved – due to its primordial role in development.

1.3.4.iv. Leukocyte trafficking and haematopoiesis

The role of CXCR4/CXCL12 in leukocyte trafficking and haematopoiesis was briefly discussed in Sections 1.3.1.i, 1.3.1.ii and 1.3.1.iii. CXCL12 is a potent chemoattractant for T cells and monocytes, and plays a major role in the homing of T cells to secondary lymphoid organs and inflammatory sites. CXCR4 may also be involved with the development of the thymus and positive selection of T cells: CXCR4 mRNA levels increase in the foetal thymus in parallel with T cell maturation, up to the double positive ($CD4^+$ $CD8^+$) stage of T cell development - at which point CXCR4 expression is downregulated (302).

The phenotypes of the CXCR4- and CXCL12-deficient mice clearly indicate a role for this chemokine/receptor pair in haematopoiesis. Haematopoietic progenitor cells express CXCR4 and can migrate in response to CXCL12 (158, 303). CXCR4 may also help to maintain the contact between pro/pre-B cells and bone marrow stromal cells by supporting the retention of progenitor B cells within microenvironments that promote B lymphopoiesis (158, 299). Peled *et al* have also shown a requirement for CXCR4/CXCL12 in murine bone marrow engraftment by human SCID-repopulating stem cells ($CD34^+$ cells derived from cord blood). Treatment of these cells with anti-CXCR4 antibodies prevented engraftment in the bone marrow of SCID mice. In

addition, treatment of $CD34^+$ cells with IL-6 and Stem Cell Factor (SCF) upregulated CXCR4 expression and enhanced engraftment (304). This may have clinical implications with regard to therapeutic stem cell transplantation.

In conclusion, CXCR4 and CXCL12 have the usual functions attributed to a homeostatic chemokine/receptor pair, including effects on leukocyte trafficking and homing. But the unique expression of CXCR4 and CXCL12 compared with other chemokines indicates roles in other physiological processes including organogenesis, vascularisation, haematopoiesis and embryogenesis. Pathological roles for CXCR4 include its function as an HIV co-receptor, and as will be described in this thesis, a potential role in tumour metastasis.

1.4. Ovarian Cancer

1.4.1. Epidemiology and risk factors

1.4.1.i. Incidence and mortality

Ovarian cancer is among the five leading types for incidence and mortality in women in developed areas of the world. The world-wide number of cases has been estimated at 165,500 representing 4 % of all female cancers (estimates for 1990) (305). The highest incidence areas are in Europe (particularly Nordic countries and the UK) and North America. Japan has one of the lowest incidence rates and mortality (306). Incidence and mortality rates in the United States, Canada, Scandinavia and the UK have declined over the last two decades, but the rates for Japan, India and Singapore have risen (306-308). In the UK, ovarian cancer is the 4^{th} most common cancer in women and in 1995 there were 6,262 new cases (Office of National Statistics). Over 60 % of patients diagnosed with ovarian cancer will be expected to die of the disease within five years; ovarian cancer is the gynaecologic cancer most likely to result in death among women (309). The disproportionate number of deaths seen with ovarian cancer is largely due to the fact that these tumours tend to spread silently throughout the peritoneal cavity and

only present at an advanced stage (Stage III or IV). This skew towards late presentation results in the high death rate seen with ovarian cancer.

1.4.1.ii. Risk factors for ovarian cancer

The most obvious risk factor for ovarian cancer is age. The risk of developing a tumour increases with age. Most ovarian tumours occur in post-menopausal women, and half occur in women over the age of 65.

Ovulation appears to be a major risk factor for ovarian cancer. Women with lifelong irregular menstrual cycles have a reduced risk of ovarian cancer, while women who start menstruating at an early age or go through menopause at a late age have an increased risk (310, 311). Nulliparity or low parity are associated with an increased risk, although it is sometimes unclear whether difficulty in conceiving or infertility are the actual factors involved. Ovarian hyperstimulation can also increase the risk (312). Most studies suggest that having children and breast feeding decrease the risk of developing ovarian cancer (313-315).

Familial clustering of ovarian cancer has been observed (316), suggesting that it can be a familial disease. Inherited mutations in the BRCA1 and BRCA2 genes are linked with both breast and ovarian cancer (317-319), although these cases probably account for less than 5 % of all ovarian cancers.

A number of other risk factors have been implicated in ovarian cancer, including socio-economic status (320), childhood infections such as mumps (321), obesity (322) and diet (311, 323).

Evidence suggests that factors causing epithelial inflammation promote ovarian carcinogenesis, including pelvic inflammatory disease, endometriosis and exposure to

talc or asbestos (324-326). Inflammation produces oxidants which can damage DNA (327) and a variety of cytokines, growth factors and prostaglandins which may play a role in tumourigenesis (328, 329). The process of ovulation itself may be mutagenic, due to disruption of the normal ovarian surface epithelium and the inflammation that follows. This may explain the increased risk seen with ovarian hyperstimulation, nulliparity and early menstruation/late menopause.

Oral contraceptives protect against the development of ovarian cancer, with up to a 50 % decrease in the risk of ovarian cancer for women who have used oral contraceptives for longer than five years (315). This decreased risk persists for 10-15 years after oral contraceptive use has ceased.

1.4.2. Clinical features of ovarian cancer

Ovarian cancers often spread asymptomatically throughout the peritoneal cavity, and only present at an advanced stage. Symptoms include a general feeling of malaise, abdominal pain, digestive problems (due to involvement of the GI tract), weight loss, frequent urination (due to involvement of the urinary tract) and abnormal vaginal bleeding. These symptoms are characteristic of the pattern of spread of ovarian tumours. Early growth of epithelial ovarian cancer is usually confined to the ovaries whereas in advanced stages of ovarian cancer, the peritoneal wall, diaphragm and omental structures are seeded with micro and macro-metastases of tumour cells (transcoelomic spread). Lymphatic dissemination to the pelvic and para-aortic lymph nodes is also common, especially in advanced disease (330). The most important physical sign is the presence of a solid, irregular, fixed pelvic mass. If an upper abdominal mass or ascites is present, then the diagnosis is almost certainly ovarian cancer (331). Ascites is associated with the growth of ovarian cancer stages Ic-IV (332). Ascitic fluid arises as a plasma exudate; formation results from an imbalance between the influx and efflux of fluid from the peritoneal compartment (333). This is

due to a combination of increased microvascular permeability mediated by the angiogenic development of immature blood vessels (334), the production of vascular permeability agents including VEGF (335-337) and the blockade of lymphatic drainage by cells present in the ascites (338). Ascites consists of a proteinaceous fluid with variable numbers of suspended cells and debris.

Transvaginal ultrasound, CT scans and MRI scans can be used to screen for ovarian masses in combination with blood tests for tumour markers (339). Complex ovarian cysts with wall abnormalities or solid areas, as detected by ultrasound, are associated with a significant risk for malignancy. The tumour marker CA125 is a glycoprotein that is elevated in the serum of approximately 80 % of women with epithelial ovarian cancer, but only in 50 % of Stage I ovarian tumours (340). Unfortunately, it is also elevated in a number of other conditions including fibroids, endometriosis, inflammatory conditions involving the pleura or peritoneum, and cirrhosis, which can lead to false-positive results (341). Screening programmes using CA125 and transvaginal ultrasound are of growing interest however, since early diagnosis of ovarian cancer has a better prognosis (339). Definitive diagnosis usually requires an exploratory laparotomy.

1.4.3. Pathological classification of ovarian tumours

Histopathologically and immunocytochemically, ovarian tumours are among the most complex of human malignancies. Epithelial ovarian carcinomas account for about 90 % of ovarian malignancies, with the remainder arising from granulosa cells, stromal cells or germ cells (342). Epithelial ovarian tumours arise from the ovarian surface epithelium and are subdivided into six categories: serous (fallopian tube-like); mucinous (endocervical-like); endometrioid (endometrium-like); clear cell (mesonephros-like); Brenner (urothelium-like) and unclassifiable. A simplified version of the WHO histological classification of ovarian tumours is shown in Table 1.3.

Serous tumours account for more than 50 % of all ovarian cancers. They consist of cuboid epithelial cells, lining cysts filled with serous fluid; they may also form papillae and glandular structures. Endometrioid tumours account for 5-10 % of ovarian tumours and have oval, columnar cells forming tubular gland-like structures similar to those seen in endometrial cancer. Mucinous tumours also account for around 5-10 % of ovarian tumours and they are characterised by tall columnar cells which secrete mucus and tend to have large multiloculated cystic structures filled with thick, viscid fluid. Some mucinous adenocarcinomas may be metastases from GI malignancies because the mucus in these lesions resembles that of the GI tract rather than the endocervix (343). Clear cell tumours account for around 3-5 % of primary ovarian tumours and histologically may be papillary, acinar or solid, with cuboidal cells containing clear cytoplasm. Brenner tumours are much less common (1-2 % of ovarian cancers) and consist of nests of urothelial-like epithelial cells within a dense, collagenous stroma (331, 344).

Source of tumour	Classification
Epithelial tumours	Serous tumours
	serous cystadenoma
	borderline serous tumour
	serous cystadenocarcinoma
	adenofibroma and cystadenofibroma
	Mucinous tumours
	mucinous cystadenoma
	borderline mucinous tumour
	mucinous cystadenocarcinoma
	Endometrioid carcinoma
	Clear cell carcinoma
	Brenner tumour
	Undifferentiated carcinoma
Germ cell tumours	Teratoma
	benign cystic teratoma
	benign solid teratoma
	malignant
	monodermal or specialised
	Dysgerminoma
	Endodermal sinus tumour
	Choriocarcinoma
	Others
Sex cord-stromal tumours	Granulosa-theca cell tumours
	granulosa cell tumour
	thecoma
	fibroma
	Sertoli-Leydig cell tumour
	Gonadoblastoma
Unclassified tumours	
Metastatic tumours	

Table 1.3. A simplified version of the WHO classification for ovarian tumours. Adapted from (345).

1.4.4. Genetic changes in ovarian cancer

Changes in a large number of genes associated with normal regulation of growth and proliferation have been identified in ovarian cancer. Amplification, mutation and otherwise altered expression of tumour suppressor genes and oncogenes are important

in the development of ovarian tumours. The situation is complex, so only some of these will be described in this section [for further information, see (346)].

A variety of oncogenes are overexpressed or amplified in ovarian cancer. c-myc overexpression was detected in 37 % of ovarian cancers (or 63 % of serous adenocarcinomas), particularly those of Stage III or above (347). K-ras mutations (but not H- or N-ras mutations) have been seen in 27 % of all epithelial ovarian cancers, although 75 % of mucinous carcinomas had K-ras mutations (348). HER2/neu (or c-erbB2, which encodes a cell surface receptor similar to the epidermal growth factor receptor) is overexpressed in 15-40 % of breast cancers and its overexpression is correlated with a poor prognosis. Overexpression of HER2/neu has also been seen in ovarian cancer: immunohistochemically, 68 % of ovarian cancer biopsies had normal HER2/neu staining, while 32 % had high HER2/neu expression (349). The latter were associated with significantly worse survival, and were less likely to respond well to primary therapy. The gene for PI3KCA (which encodes the p110 catalytic subunit of PI3K) is found on region 3q26 which has an increased copy number (amplification) in approximately 40 % of ovarian cancers (350). This amplification results in increased expression of PI3KCA and hence PI3K activity, which has a role in proliferation, apoptosis and oncogenic transformation.

Changes in tumour suppressor genes have also been seen in ovarian cancer. p53 is a cell cycle regulator, controlling the entry of cells into S-phase. It is also involved with the pathways leading to apoptosis. p53 is mutated in approximately 50 % of ovarian cancers (351) and this may also be related to immunohistochemical overexpression (352). p53 is not mutated or overexpressed in borderline or benign disease (353). Loss of heterozygosity (LOH) of the NOEY2 gene has been detected in 41 % of ovarian cancers. NOEY2 is related to ras and rap and is associated with growth suppression through downregulation of cyclin D1 and induction of p21 (WAF1/CIP1). The NOEY2

gene is expressed monoallelically and is imprinted maternally – LOH usually leads to deletion of the functional allele (354). As mentioned in Section 1.4.1.ii., the tumour suppressor genes BRCA1 and BRCA2 (which are involved with DNA repair) can also be mutated in ovarian cancer.

1.4.5. Management of ovarian cancer

The primary treatment for ovarian cancer is cytoreductive surgery. At surgery, the extent of the disease is staged – this is extremely important for determining the subsequent treatment. The International Federation of Gynaecology and Obstetrics (FIGO) staging system is given in Table 1.4.

It is possible to treat some Stage I or II tumours with cytoreductive surgery alone (355), but the majority of cases require chemotherapy or radiotherapy as well. Chemotherapy can be either single agent or multi-agent (331). The use of single alkylating agents such as melphalan was common until 1978, when a randomised trial demonstrated an increased response rate with combination chemotherapy (hexamethylmelamine, cyclophosphamide, methotrexate and 5-fluorouracil) (356). In 1986, clinical trials demonstrated that the inclusion of cisplatin improved the response rate further (20 months median survival compared with 16 months) (357). Since then, various other drugs have been developed including doxorubicin, paclitaxel, and carboplatin (a platinum-based compound with less toxicity than cisplatin). The various recent randomised trials indicate that platinum (either cisplatin or carboplatin) should be included in the chemotherapy of ovarian cancer, in combination with paclitaxel [see (358) for review]. Radiotherapy has limited use in ovarian cancer treatment (359).

Stage I	Growth limited to the ovaries	
	Ia	Growth limited to one ovary; no ascites containing malignant cells. No tumour on the external surface; capsule intact.
	Ib	Growth limited to both ovaries; no ascites containing malignant cells. No tumour on the external surfaces; capsules intact.
	Ic	Tumour either Stage Ia or Ib but with tumour on the surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension.	
	IIa	Extension and/or metastases to the uterus and/or tubes.
	IIb	Extension to other pelvic tissues.
	IIc	Tumour either Stage IIa or IIb but with tumour on the surface of one or both ovaries; or with capsules(s) ruptured; or with ascites present containing malignant cells or with positive peritoneal washings.
Stage III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals Stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to the small bowel or omentum.	
	IIIa	Tumour grossly limited to the true pelvis with negative nodes but with histol gically confirmed microscopic seeding of abdominal peritoneal surfaces.
	IIIb	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative.
	IIIc	Abdominal implants >2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastasis. If pleural effusion is present, there must be positive cytologic test results to allot a case to Stage IV. Parenchymal liver metastasis equals Stage IV.	

Table 1.4. FIGO staging for primary ovarian cancer.

1.4.6. The tumour microenvironment

1.4.6.i. Introduction

The tumour microenvironment consists of tumour cells supported by a stroma, plus a leukocyte infiltrate. In ovarian cancer, tumour parenchyma accounts for an average of 43 % of the total tumour volume (range 14-90 %), while the stroma occupies an average of 37 % (range 8-82 %) (230). Areas of necrosis are also common (mean 4 %; range 0-37 %). The stroma consists of connective tissue, fibroblasts, blood vessels and infiltrating leukocytes; it provides a scaffold on which cells can grow and also serves to carry blood to the tumour. The potential role of the infiltrate has already been described in Section 1.3.3.i. The areas of necrosis contain necrotic cells, apoptotic bodies, red cell fragments and eosinophilic hyaline; these regions may arise due to inadequacies in the supply of oxygen. The tumour microenvironment is also rich in cytokines and growth factors.

1.4.6.ii. Cytokines and growth factors in the tumour microenvironment

Ovarian cancer has a complex cytokine network. This network may be involved in various aspects of tumour growth and spread, including cell proliferation, motility and survival, cell:cell and cell:matrix adhesion, neovascularisation, extracellular matrix remodelling, leukocyte infiltration and nutritional balance (360).

The cytokines that have been detected in ovarian cancer can be classified as pro-inflammatory cytokines (such as TNF-α, IL-1 and IL-6), growth factors (such as TGF-α, TGF-β, PDGF, IGF-1 and EGF), macrophage regulatory cytokines (such as IL-10, M-CSF and MIF) and angiogenic factors (such as platelet-derived endothelial cell growth factor [PD-ECGF], and VEGF).

Burke *et al* investigated the mRNA expression of a range of cytokines in non-malignant and malignant ovarian tissues (361). The pro-inflammatory cytokines IL-1α, IL-6,

TNF-α and lymphotoxin were expressed in more than 80 % of tumours. The growth factors IGF-1, PDGF-A, PDGF-B, TGF-α, TGF-β and M-CSF were also expressed in more than 80 % of tumours. Interestingly, lymphocyte-associated cytokines such as IL-2 and IL-4 were expressed in less than 20 % of biopsies. IFN-γ mRNA was never detected.

Growth factors such as IGF-1, PDGF and TGF-α may contribute to tumour growth. The receptor for IGF-1 was expressed in all tumour biopsies (361), suggesting that autocrine or paracrine IGF-1 loops may contribute to tumour cell survival and proliferation (362). EGFR can also be expressed by ovarian tumour cells and allows TGF-α to act as an autocrine growth factor (363).

The presence of angiogenic factors such as PD-ECGF, CXCL8 and VEGF in ovarian cancer (361, 364) may be important for neovascularisation of tumours. Immunohistochemical detection of VEGF is associated with a poor prognosis (365) and PD-ECGF is expressed at significantly higher levels in malignant disease compared with benign tumours (366). CXCL8 can act as both an autocrine growth factor in ovarian cancer, and an angiogenic factor (280); it is also upregulated by hypoxia (367).

Pro-inflammatory cytokines such as TNF-α and IL-6 may also contribute to tumour growth. TNF-α mRNA is found in epithelial tumour cell islands and its expression correlates with tumour grade (368). The p55 TNF receptor can be expressed on both tumour cells and stromal cells, while the p75 TNF receptor is found on the leukocyte infiltrate, suggesting that both autocrine and paracrine TNF-α loops exist (368). TNF-α deficient mice are resistant to skin carcinogenesis, suggesting a role for TNF-α in malignancy (369). In ovarian cancer xenograft models, treatment of ascitic xenografts with TNF-α can promote adhesion of free-floating tumour cells to the peritoneum and formation of solid tumours (370). IL-6 can also be produced by ovarian tumour cells

(371) and may indirectly promote tumour growth through effects on tumour cell attachment and migration (372).

IL-2 and IFN-γ are Th1 cytokines that can facilitate cell-mediated immunity, while IL-4 and IL-10 are Th2 cytokines that promote humoral immunity. The absence of IL-2, IL-4 and IFN-γ mRNA in ovarian cancer, despite the presence of tumour-infiltrating lymphocytes suggests that there has been a failed attempt at mounting an immune response to the tumour. In addition, the lack of immunogenicity of the tumour in the absence of co-stimulatory molecules (such as B7 and CD28) may contribute to a state of T cell anergy (373).

1.4.6.iii. Hypoxia in the tumour microenvironment

Hypoxia results when there is an inadequate supply of oxygen to a tissue. Tumour hypoxia is generally considered to be a potential therapeutic problem because it renders them more resistant to radiotherapy (374). Hypoxia may also affect the malignant progression of tumours (375).

In solid tumours, regions of hypoxia are common due to the chaotic and intermittent blood supply, and the high metabolic rate of tumour cells (376). Acute or chronic areas of hypoxia exist with very low or zero oxygen partial pressures (pO_2), and these are distributed heterogeneously within the tumour mass. Necrotic regions are usually hypoxic and indicate the loss of vital cellular functions (374). Macrophages accumulate in regions of necrosis (377) and hypoxia can affect the migration of monocytes, but not lymphocytes (378).

Tumour hypoxia can be measured using a variety of invasive and non-invasive techniques, including microsensor techniques for direct pO_2 measurements (379), immunohistochemistry with hypoxic markers such as nitroimidazole-theophylline (380)

and nuclear magnetic resonance spectroscopy (381). Such techniques have demonstrated that most tumours have lower median pO_2 than their tissue of origin and that recurring tumours have a poorer oxygenation status than the original primary tumour (374).

Hypoxia can have profound effects on gene expression, causing up- or downregulation of genes and post-transcriptional and post-translational effects. These changes can lead to the arrest of tumour cell growth, apoptosis and necrosis (382, 383), which may explain the retardation in growth of large tumour masses with inadequate oxygenation (384).

However, hypoxia can also promote tumour propagation, by stimulating production of glycolytic enzymes, angiogenic factors [e.g. VEGF and CXCL8 (367, 385)], survival and growth factors [e.g. PDGF and IGF-2 (157, 386)], and proteins involved with tumour invasion [e.g. urokinase-type plasminogen activator (387)]. Many of these hypoxia-inducible genes are regulated by the transcription factor HIF-1 (388) or NFκB (389). Hypoxia can also promote tumour progression by increasing genomic instability and heterogeneity in tumour cells, such as point mutations, gene amplification and chromosomal rearrangements (390, 391). This acts as a selective pressure, leading to clonal selection of tumour cells with apoptotic insensitivity and increased angiogenic potential (374).

Consequently, hypoxia is found to be an adverse prognostic indicator in a variety of cancers. In soft tissue sarcomas, the more hypoxic tumours had statistically worse overall survival at 5 years (392). In cervical cancer, patients with hypoxic tumours or with immunohistochemically strong expression of HIF-1 also had significantly shorter survival time (393, 394). HIF-1 protein expression alone does not have an impact on

patient survival in epithelial ovarian cancer. However, patients with strong HIF-1 expression plus p53 overexpression do have significantly shorter overall survival (395).

1.5. Aims of the thesis

The initial aim of this thesis was to assess the CC chemokine and CC chemokine receptor expression in human ovarian cancer (both solid tumours and ascites). CC chemokines may be responsible for the leukocyte infiltrate seen in these tumours (230), but it is unknown whether the infiltrating cells express receptors that would allow them to respond to these chemokines. Manipulation of the leukocyte infiltrate could have possible therapeutic implications.

The second aim was to investigate the potential involvement of chemokine receptors in ovarian tumour growth and spread. Again, manipulation/prevention of tumour cell migration and growth could have important therapeutic implications in this disease.

Chapter 2. Materials and Methods

2.1. Patient material and cell lines

2.1.1. Solid tumour biopsies

25 biopsies from human ovarian tumours were collected at the time of cytoreductive surgery. Half of each sample was fixed in formol saline for 24 hours, then embedded in paraffin prior to cutting 4 μm sections for immunohistochemistry. The other half was frozen in isopentane which had been cooled to near freezing point on liquid nitrogen. Isopentane penetrates tissues better than liquid nitrogen, thereby improving tissue preservation; frozen samples were stored at –70 °C.

The tumour biopsies were classified as serous adenocarcinoma (19), clear cell carcinoma (2), mucinous adenocarcinoma (1), anaplastic carcinoma (1), signet ring carcinoma (1), and endometrioid carcinoma (1).

2.1.2. Ascites

Samples of ascitic fluid were collected from patients with ovarian carcinoma at the time of cytoreductive surgery or by paracentesis for palliative/diagnostic purposes. Volumes of between 1 L and 1.5 L were taken. All 66 ascites samples were from patients with advanced epithelial ovarian cancer (FIGO Stage III or IV). The pathology of the 20 isolates used for FACS analysis was: 16 papillary serous adenocarcinomas; 2 adenocarcinomas; 1 mucinous adenocarcinoma; 1 unknown gynaecological malignancy. Each sample was spun down and the ascitic fluid removed for ELISA analysis. The cell isolate was treated with ACK lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 100 mM Na_2EDTA) for 2-3 minutes to remove red blood cells, prior to RNA extraction or flow cytometry. In some patients, a peripheral blood sample was also collected, plasma was removed and stored at –70 °C and peripheral blood mononuclear cells (PBMC) were isolated (described in Section 2.1.3.).

2.1.3. Peripheral blood mononuclear cells (PBMC) and macrophages

2.1.3.i. Purification of PBMC

PBMC were purified by Ficoll-Hypaque density centrifugation. Briefly, heparinised venous blood from healthy donors, or buffy coat from the Blood Transfusion Service (London, UK) was diluted 1:1 with sterile PBS (without Ca^{2+} or Mg^{2+}; Gibco BRL, Paisley, UK) and centrifuged on Lymphoprep™ (Nycomed, Birmingham, UK) for 30 minutes at 500 *g*. The mononuclear cell layer was removed using a sterile pastette, and washed thoroughly by resuspension in sterile PBS (Gibco BRL). The PBMC were then used for RNA extraction or monocyte isolation.

2.1.3.ii. Isolation of monocytes

Isolation of monocytes from PBMC was performed by negative selection using a MACS monocyte isolation kit (Miltenyl Biotech, Bisley, UK) according to the manufacturer's instructions. Briefly, PBMC were incubated with FcR blocking reagent (human Ig), followed by hapten-antibody cocktail (a cocktail of monoclonal hapten-conjugated anti-CD3, CD7, CD19, CD45RA, CD56 and anti-IgE antibodies) and then MACS microbeads conjugated to a monoclonal anti-hapten antibody. The magnetically-labelled cell suspension was washed, then passed through a magnetic field. Unlabelled cells were the negative fraction (the enriched monocyte population), which was then evaluated by flow cytometry using anti-CD14 conjugated to FITC (see Section 2.7). Purity of the monocyte population was always >95 %.

2.1.3.iii. Generation of monocyte-derived macrophages and dendritic cells

MACS-purified monocytes were cultured in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 10 % human AB serum (Sigma, Poole, UK) for 7 days in Teflon-coated bags (Sud-Laborbedarf, Gauting, Germany) or Teflon-coated pots (Tuf Tainer, Perbio Science UK Ltd, UK). The resulting macrophages were washed and resuspended in RPMI 1640 supplemented with 10 % foetal bovine serum (FBS; Gibco

BRL) prior to use in the three-dimensional tumour cell model (spheroids). The viability of the cell population was assessed by Trypan Blue staining.

Dendritic cells were generated from MACS-purified monocytes by culture in RPMI 1640 supplemented with 10 % FBS, 800 U/ml recombinant human (rh) IL-4 (R&D Systems, Abingdon, UK) and 50 ng/ml rhGM-CSF (R&D Systems) for 7 days in teflon-coated bags or teflon-coated pots.

2.1.4. Cell lines and culture

The ovarian cancer cell lines PEO1 and PEO14 (from S. Langdon, ICRF Oncology Unit, Edinburgh, UK), OVCAR-3 (purchased from the American Type Culture Collection [ATCC], Rockville, MD, USA) and IGROV (from J. Bénard, Institut Gustave Roussy, Villejuif, France) were grown in RPMI 1640 (Gibco BRL, Paisley, UK) supplemented with 10 % FBS and, for PEO1 and PEO14, 10 μg/ml insulin (Sigma, Poole, UK); SKOV-3 and CAOV-3 (purchased from ATCC) were grown in DMEM (Gibco BRL) supplemented with 10 % FBS. The monocytic cell line, THP-1 (purchased from ATCC) was grown in RPMI 1640 supplemented with 10 % heat inactivated FBS (RPMI/HIBS) and 50 μM β-mercaptoethanol (Sigma). All cell lines were cultured in pyrogen-free conditions in a humidified atmosphere at 37 °C (5 % CO_2). The adherent ovarian cancer cell lines were passaged twice weekly or when confluent – the cells were trypsinised and seeded at an appropriate density in fresh flasks.

2.1.4.i. Post-trypsinisation recovery of ovarian cancer cell lines

Prior to their use in invasion assays, cell signalling experiments or flow cytometry, the ovarian cancer cell lines were trypsinised. However, to allow recovery of cell surface receptors (including chemokine receptors) on these cells after trypsinisation, cells were

cultured as single cell suspensions in teflon-coated pots (Tuf Tainer, Perbio Science UK Limited) at 0.5-1 x 10^6 cells/ml for a minimum of 5 hours.

2.1.4.ii. Hypoxic culture of THP-1 cells

2 x 10^6 THP-1 cells were cultured in 2 ml serum-free RPMI 1640 supplemented with 1 % bovine serum albumin (BSA; Sigma, Poole, UK) and 50 μM β-mercaptoethanol, in each well of a 6-well plate. The cells were then incubated for up to 24 h at 37 °C under normoxia (5 % CO_2, in air) or hypoxia (gassed with 5 % CO_2, balanced N_2 until <0.1 % O_2) in a modular incubation chamber (made in-house). Gases were purchased from BOC (Manchester, UK).

Chemical mimics of hypoxia were also used; Cobalt chloride ($CoCl_2$) and desferrioxamine (DFO) were purchased from Sigma. Medium supplemented with 25 μM, 50 μM or 100 μM $CoCl_2$ or 50 μM, 100 μM or 200 μM DFO was added to THP-1 cells for 24 hours, then RNA was extracted for northern analysis (see Section 2.4.).

2.1.4.iii. Growth of tumour spheroids

96-well plates were coated with a solution of 2 % agarose, 1 x DMEM supplemented with 10 % FBS (all Gibco BRL, Paisley, UK). 2 x 10^3 SKOV-3 cells were added to each well in 100 μl of DMEM supplemented with 10 % FBS. The cells were unable to adhere to the agarose, and instead adhered to each other, forming spherical cell aggregates (spheroids). Spheroids were grown for up to two weeks; half the culture medium was replaced with fresh medium every three days.

To obtain spheroid sections for histological examination, spheroids were transferred from the 96-well plate into a microfuge tube. The spheroids were allowed to settle, the medium was aspirated and the spheroids were fixed in formol saline for 24 hours before

being embedded in paraffin. Sections were cut and stained with haematoxylin and eosin, or used for immunohistochemistry (see Section 2.5.).

2.2. Chemotaxis and invasion assays

Chemotaxis was assayed using Falcon Transwells (24-well format, 8 μm pore; Becton Dickinson, Oxford, UK). Invasion was assayed using Biocoat growth factor-reduced matrigel™ invasion chambers (24-well format, 8 μm pores; Becton Dickinson). Cells were allowed to recover from trypsinisation according to Section 2.1.4.i. 0.5 ml medium (RPMI 1640 or DMEM plus 1 % BSA) containing 5 x 10^5 cells was added to the upper chamber and 0.5 ml of medium alone or medium supplemented with 100 ng/ml CXCL12 (Peprotech) was added to the lower chamber. After overnight incubation at 37 °C and 5 % CO_2, cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained using DiffQuik (Dade Behring, Düdingen, Switzerland). For each transwell/invasion chamber, the number of migrated cells in 10 medium power fields (x 20) was counted. Three transwells/invasion chambers were used per condition. The results of the migration assays were evaluated using the Welch's approximate t-test (which is a more stringent analysis than the student t-test).

2.3. Proliferation assays

0.5 ml of medium (RPMI 1640 or DMEM supplemented with 10 % FBS) containing 2 x 10^5 cells was added to each well of a 24-well plate. The cells were allowed to adhere and recover overnight. The medium was aspirated, the cells were washed twice with sterile PBS, and fresh medium (RPMI 1640 or DMEM plus 1 % BSA) was added. Cells were treated with either medium alone, medium supplemented with 100 ng/ml CXCL12 (Peprotech), medium supplemented with 10 μg/ml anti-CXCR4 neutralising antibody (R&D Systems, Abingdon, UK), or medium supplemented with both. After 3 days, fresh medium (containing the appropriate supplements) was added. At 2, 4 and 6

days of culture, cells were trypsinised using 100 µl of trypsin/versene mix (Gibco BRL, Paisley, UK) and counted using a haemocytometer. 8 wells were counted per condition at each time-point. The results of the proliferation assays were evaluated using the Welch's approximate t-test (which is a more stringent analysis than the student t-test).

2.4. Methods for analysing RNA expression

2.4.1. Preparation of RNA from tissue samples and cell lines

Total RNA was prepared from all samples using Tri Reagent™ (Sigma, Poole, UK) according to the manufacturer's instructions. Solid tumour biopsies were homogenised in Tri Reagent™ using an Ultra-turrax T25 tissue homogeniser (Janke & Kunkel, Staufen, Germany).

2.4.1.i. DNase-treatment of RNA and phenol/chloroform extraction

For RT-PCR and RNase protection analysis, total RNA was DNase-treated to remove contaminating genomic DNA, using RNase-free DNase I (Pharmacia Biotech, St. Albans, UK). The following reagents were added to an RNase-free 1.5 ml microfuge tube, followed by incubation at 37 °C for 1 hour:

(a) 10 µl Transcription optimized 5x buffer (Promega, Southampton, UK)

(b) 1 µl RNasin® ribonuclease inhibitor (Promega)

(c) x µg total RNA (where x was between 1 and 20 µg of total RNA)

(d) 2 µl DNaseI (Pharmacia Biotech)

(e) DEPC-treated water to a final volume of 50 µl

The contents were then phenol/chloroform extracted as follows: 50 µl of DEPC-treated water was added to the tube, followed by 100 µl of citrate-buffered phenol (Sigma). The contents were vortexed thoroughly, then centrifuged at 10,000 *g*/13,000 rpm for 5

min at 4 °C. The upper aqueous phase was transferred to a fresh tube and 100 µl of chloroform/isoamyl alcohol (24:1 ratio; Merck/BDH, Lutterworth, UK) was added, followed by vortexing and centrifugation at 10,000 *g*/13,000 rpm for 5 min at 4 °C. The upper phase was again transferred to a fresh tube. 0.5 µl of carrier tRNA (Sigma; 10 mg/ml stock made up in DEPC-treated water), 1/5 volume (i.e. 20 µl) of 10 M ammonium acetate (Sigma) and 2.5 volumes (i.e. 250 µl) of absolute ethanol (Merck/BDH) were added. The tube was put on dry ice for 30 min, then centrifuged at 10,000 *g*/13,000 rpm for 15 min at 4 °C. The supernatant was removed and the pellet was washed by adding 0.5 ml 75 % ethanol (made with DEPC-treated water) and vortexing. The tube was centrifuged at 10,000 *g*/13,000 rpm for 10 min at 4 °C. The supernatant was removed and the pellet was air dried. Finally, the pellet was resuspended in an appropriate volume of DEPC-treated water (usually at a concentration of 1 µg/µl).

2.4.2. RT-PCR

2.4.2.i. cDNA synthesis

cDNA was synthesised from 5 µg of DNase-treated total RNA using the Ready-to-Go™ T-primed First Strand kit (Pharmacia Biotech, St. Albans, UK) according to the manufacturer's instructions. Briefly, the volume of 5 µg of DNase-treated total RNA was adjusted to 33 µl with DEPC-treated water, and placed in a microfuge tube. The RNA was incubated at 65 °C for 5 min, then at 37 °C for 5 min. At the same time, a First-Strand Reaction Mix tube was incubated at 37 °C for 5 min. The RNA sample (33 µl) was transferred to the Reaction Strand Mix tube and incubated at 37 °C for 5 min. The contents of the tube were then mixed by pipetting up and down several times. The tube was then incubated at 37 °C for 1 hour, to allow cDNA synthesis to proceed. The volume of the sample was adjusted to 50 µl by adding 17 µl of distilled water; the cDNA was then stored at –20 °C. 2 µl (equivalent to 200 ng of total RNA) were used

for PCR.

2.4.2.ii. Primers

The primers for CCL2, CCL22, CCR1 and GAPDH were designed from sequences submitted to Genbank, using Primer 3.0 (Steve Rozen, Helen J. Skaletsky, 1998). Primer sequences for CCL3 and CCL4 were from (396), and CCL5 and CCL8 primer sequences were from (397) and (398) respectively. The primers for matrix metalloproteinases (MMP), membrane-type MMPs (MT-MMP), tissue inhibitors of MMPs (TIMP), urokinase plasminogen activator (uPA), uPA receptor (uPAR) and plasminogen activator inhibitor (PAI) were designed by Thomas Leber, Stephen Robinson and Kate Scott in our laboratory. The primer sequences and product sizes are shown in Table 2.1.

The 'housekeeping' gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used in all PCR reactions to control for reverse transcription of the total RNA.

Target	Forward Primer Reverse Primer	Product size (bp)
CCL2	CAAACTGAAGCTCGCACTCTCGCC ATTCTTGGGTTGTGGAGTGAGTGTTCA	354
CCL3	CCTTGCTGTCCTCCTCTGCA CACTCAGCTCTAGGTCGCTG	254
CCL4	TGTCTCTCCTCATGCTAGTA GTACTCCTGGACCCAGGAT	233
CCL5	CCTCTCCACAGGTACCAT TCAGGTTCAAGGACTC	314
CCL8	ATGCTGAAGCTCACACCCTTGCCC CAGATGCTTCATGGAATCCCTGACC	300
CCL22	CCCTACCTCCCTGCCATTAT CAGGGAGCTAGAACCCAACA	338
CCR1	AAAGCCTACGAGAGTGGAAGC AGAGGAAGGGGAGCCATTTA	426
MMP-1	AATTACACGCCAGATTTGCC CTGGTTGAAAAGCATGAGCA	205
MMP-2	TCCTTTCACAACCTTCTGTGG GGGAACCATCACTATGTGGG	316
MMP-3	GTACCTCATTTCCTCTGATGGC TGCTTTGTCCTTTGATGCTG	230
MMP-7	GAGCTACAGTGGGAACAGGC ATGCAGGGGGATCTCTTTG	345
MMP-9	ACCGCTATGGTTACACTCGG GCAGGCAGAGTAGGAGCG	584
MMP-10	GGCTCTTTCACTCAGCCAAC TCAGATCCCGAAGGAACAGA	181
MMP-11	TGACTTCTTTGGCTGTGCC GTTGTCATGGTGGTTGTACCC	199
MMP-13	CTGGCTGCCTTCCTCTTCTT ATGTCAGCAATGCCATCGTG	473
MT1-MMP	CACTGCCTACGAGAGGAAGG TGAATGACCCTCTGGGAGAC	296
MT2-MMP	CGTGTCCTGCTTTACTGCAA CTCCAACTGGGCAAAGAGAG	430
MT3-MMP	CAGGGTGATGGATGGATACC CCTTGAGGATGGATCTTGGA	470
TIMP-1	CCAAGTTCGTGGGGACAC TGCAGTTTTCCAGCAATGAG	208
TIMP-2	AGAAGAACATCAACGGGCAC CTTGGAGGCTTTTTTTGCAG	172
TIMP-3	CCTGCTACTACCTGCCTTGC TCAGGGGTCTGTGGCATT	191
uPA	GTGGCCAAAAGACTCTGAGG ATTTTCAGCTGCTCCGGATA	499
uPAR	CGGTGCATGCAGTGTAAGAC CAGGAAGTGGAAGGTGTCGT	501
PAI	ATCGAGGTGAACGAGAGTGG ATGGCAATGTGACTGGAACA	596
GAPDH	TGAAGGTCGGAGTCAACGGATTTGG ACGACGTACTCAGCGCCAGCATCGC	277

Table 2.1. Primer sequences used for RT-PCR. The table shows the forward/reverse primer sequences and the PCR product sizes.

2.4.2.iii. Polymerase chain reaction (PCR)

PCR was performed using a GeneAmp® PCR System 9700 thermal cycler (Perkin Elmer, Beaconsfield, UK). For each primer pair, a master mix was prepared containing

all reagents except for the cDNA. The final volume of each PCR reaction was 25 µl, containing 200 ng cDNA, 1 U AmpliTaq DNA polymerase, GeneAmp PCR buffer, GeneAmp dNTPs (all from Perkin Elmer) and 4 µM each primer. The following protocol was used for the PCR reaction: 94 °C (5 min); 35 cycles 94 °C (30 s), 60 °C (30 s), 72 °C (30 s); 72 °C (7 min).

15 µl of each PCR reaction was added to 5 µl of loading buffer (40 % w/v sucrose; 0.25 % w/v bromophenol blue; 0.25 % xylene cyanol [all from Sigma, Poole, UK]; made up in distilled water) and electrophoresed through a 1.2 % agarose gel containing 0.5 µg/ml ethidium bromide (Sigma). Bands were visualised by UV transillumination and their sizes were estimated using a co-migrated DNA size marker (123 bp marker; Gibco BRL, Paisley, UK). PCR products were gel extracted (QiaQuick Gel Extraction kit, Qiagen, UK) and sequenced to confirm their identity.

2.4.3. RNase protection assay (RPA)

The theory behind RPA is as follows: a radiolabelled antisense RNA probe is hybridised to target mRNA in the sample, forming double-stranded RNA, which is resistant to digestion by single-strand specific ribonucleases such as RNase A. After purification, the protected fragments are sized on a sequencing gel and can be quantitated by autoradiography and image analysis or phospho-imaging. A radiolabelled antisense GAPDH or L32 probe added to each sample makes a good loading control, allowing for semi-quantitation of each mRNA species in the original sample.

All RPAs were performed using the Riboquant® RPA system from Pharmingen (Becton Dickinson, Oxford, UK). The hCR5 template set from Pharmingen contained DNA templates for CCR1, CCR2, CCR2a, CCR2b, CCR3, CCR4, CCR5, CCR8, GAPDH and L32. The hCR6 template set contained DNA templates for CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CCR7 and CX3CR1. These template sets were

used to generate radiolabelled antisense riboprobes by *in vitro* transcription. RPA was carried out strictly according to the manufacturer's instructions. However, [α^{35}S]UTP (Amersham International plc, Aylesbury, UK) was used for the probe labelling instead of [α^{32}P]UTP; it was much easier and safer to work with, but required longer exposure times during autoradiography. The RNase-protected fragments were run out on an acrylamide-urea sequencing gel (BioRad Laboratories Ltd, Hemel Hempstead, UK), which was then adsorbed to filter paper and dried under vacuum. Autoradiography was subsequently carried out using Kodak Biomax MS film with a Transcreen LE intensifying screen (Sigma, Poole, UK). Densitometry was performed using NIH Image 1.61.

2.4.4. Northern blotting

2.4.4.i. cDNA probes

cDNAs for CCR1, CCR2b and CXCR4 were a kind gift from Dr Antonio Sica (Istituto di Ricerche Farmacologiche 'Mario Negri', Milan, Italy). The β-actin cDNA was obtained from Dr L Kedes (Stanford University, Stanford, CA, USA). Appropriate restriction enzymes were used to cut out each cDNA insert; each digest reaction was added to 5 µl of loading buffer (40 % w/v sucrose; 0.25 % w/v bromophenol blue; 0.25 % xylene cyanol [all from Sigma, Poole, UK]; made up in distilled water) and electrophoresed through a 1 % agarose gel containing 0.5 µg/ml ethidium bromide. Insert cDNA bands were visualised by UV transillumination and gel extracted (QiaQuick Gel Extraction kit, Qiagen, UK). The purified cDNA insert was subsequently used for probe labelling (Section 2.4.4.iii).

2.4.4.ii. Northern blotting

15 µg of total RNA (Section 2.4.1.) was mixed with 5 µl of loading buffer (48 % deionised formamide [Gibco BRL, Paisley, UK], 6 % formaldehyde [Merck/BDH, Lutterworth, UK], 5 % glycerol, 20 mM MOPS [3-*N*-morpholinopropanesulphonic

acid], 5 mM sodium acetate and 1 mM EDTA pH 8.0 [all from Sigma, Poole, UK] made up in DEPC-treated water). Each RNA sample was heated to 65 °C for 5 minutes, then placed on ice prior to loading. The RNA was subjected to electrophoresis through a 1 % agarose-formaldehyde gel (1 % agarose [Gibco], 6 % formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA pH 8.0, 0.5 μg/ml ethidium bromide) then blotted by capillary transfer onto nylon membrane (Hybond N^+, Amersham, UK). After transfer, the membrane was UV crosslinked (1200 J) in a Stratalinker (Stratagene, La Jolla, CA, USA).

2.4.4.iii. Probe labelling and hybridisation

The membrane was placed in a suitable hybridisation tube and pre-hybridised for 1-2 hours at 42 °C with 20 ml of hybridisation buffer (0.2 M sodium phosphate buffer pH 7.2, 1 mM EDTA pH 8.0, 1 % BSA, 7 % SDS [Merck/BDH, Lutterworth, UK], 45 % formamide, made up in distilled water).

cDNA probes were labelled by random priming using the Stratagene Prime-It II kit (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Unincorporated [α^{32}P]CTP nucleotide was removed by passing the radiolabelled probe through a Clontech Chromaspin TE 100 spin column (Clontech, Basingstoke, UK), following the manufacturer's instructions. Prior to use, the radiolabelled probe was heated to 100 °C for 5 minutes, then quenched on ice for up to 30 min. Probe was then added to 20 ml of hybridisation buffer (approximately 1 x 10^6 cpm/ml of buffer), which was poured on to the membrane in place of the pre-hybridisation buffer. Hybridisation was performed overnight at 42 °C.

Following hybridisation, membranes were washed twice with 2 x SSC, 0.1 % SDS for 5 min at room temperature, twice with 0.1 x SSC, 0.1 % SDS for 15 min at 68 °C and finally once with 2 x SSC for 10 min at room temperature. The membrane was then

wrapped in Saran wrap and exposed overnight to Kodak Biomax MS film with an intensifying screen, at –70 °C. Densitometry was performed using NIH Image 1.61.

Radiolabelled probe was stripped from the membrane by washing the membrane in boiling 0.1 % SDS. The membrane was left on a shaker until the solution cooled down; it could then be re-probed.

2.4.5. *In situ* hybridisation (ISH)

The method described by Naylor *et al* was followed (399). For tumour biopsies, 5 μm cryostat sections were cut onto baked slides coated with 3-aminopropyltriethoxysilane (TESPA; Sigma, Poole, UK). For non-adherent cells used as positive controls (THP-1 and PBMC), cytospins were prepared using a Shandon Cytospin 3. 5 x 10^5 cells/ml were loaded per chamber and spun onto TESPA-coated baked slides at 500 rpm for 5 min.

2.4.5.i. Pre-hybridisation

All incubations were carried out at room temperature in DEPC-treated buffers and baked slide dishes (400 ml dishes) on a rotary shaker. Cryostat sections were brought to room temperature and air dried for one hour prior to pre-hybridisation. Cytospin slides were treated the same as cryostat sections, except that treatment with Proteinase K was omitted. All reagents were from Sigma, Poole, UK. The following steps were carried out:

1.	4 % paraformaldehyde (PFA)	20 min
2.	PBS	5 min
3.	DEPC-treated water	5 min
4.	0.2 M HCl	20 min
5.	PBS	5 min

6.	4 % PFA	15 min
7.	PBS	2 x 5 min
8.	5 μg/ml Proteinase K made up in appropriate buffer (50 mM Tris, 5 mM EDTA pH 7.5)	7.5 min
9.	PBS	5 min
10.	4 % PFA	5 min
11.	DEPC-treated water dip	
12.	0.1 M triethanolamine + 0.25 % acetic anhydride	2 x 10 min
13.	PBS	5 min
14.	Dehydrate through graded alcohols (30 %, 50 %, 70 %, 95 %, 100 %)	2 min in each
15.	Air-dry in a dust-free environment	

Pre-hybridisation optimises tissue penetration by the radiolabelled riboprobe, while preserving tissue morphology.

2.4.5.ii. Probe labelling

cDNA probes for CCR1, CCR2b and CXCR4 were in vectors containing promoters for *in vitro* transcription by either T3, T7 or Sp6 RNA polymerases. The vector was linearised by digestion with a single restriction enzyme at either the 5' or 3' end of the cDNA insert, and purified by standard phenol/chloroform extraction (see Section 2.4.1.i). The linearised template was then suitable for *in vitro* transcription of radiolabelled riboprobes. All reagents were RNase-free.

The following components were added to a sterile ribonuclease-free 1.5 ml microcentrifuge tube and incubated at 37 °C for 1 hour:

(a)	5 x transcription buffer (Promega, Southampton, UK)	4 µl
(b)	100 mM DTT (Promega)	2 µl
(c)	RNasin® ribonuclease inhibitor (Promega)	0.8 µl
(d)	ATP, CTP, GTP mix (Promega)	4 µl
	(from 10 mM stocks in a ratio of 1:1:1:1 with DEPC-treated water)	
(e)	Linearised transcription vector template	1 µl (= 1 µg)
(f)	[^{35}S]UTP (> 1000 µCi/nmol) (Amersham, UK)	10 µl
(g)	Relevant polymerase (T3, T7 or Sp6 from Promega)	1 µl

Two units (2 µl) of RQ1 DNase (Promega) were then added to the tube, followed by incubation at 37 °C for a further 30 min. The reaction volume was made up to 100 µl with DEPC-treated water, then phenol-extracted and precipitated (see Section 2.4.1.i). After air drying, the pellet was resuspended in 100 µl of alkaline digestion buffer (40 mM $NaHCO_3$, 60 mM Na_2CO_3, 10 mM DTT, pH 10.2) and incubated at 60 °C. The incubation time was determined according to the formula:

$$t = \frac{(L_o - L_f)}{kL_oL_f}$$

where t = time in min; L_o = initial probe length in kb; L_f = desired probe length in kb; k = 0.11, from (400). The desired probe length was approximately 200 bp because short probe fragments generally yield higher signals.

The alkaline digestion reaction was stopped with 10 µl of 1M sodium acetate (Sigma), 10 µl of 5 % acetic acid (Merck/BDH) and 2 µl of 10 mg/ml carrier tRNA (Sigma). The probe was then phenol-extracted twice (see Section 2.4.1.i with the chloroform step omitted), precipitated and resuspended in 40 µl of 10 mM DTT (Sigma). 1 µl of

radiolabelled probe was added to 10 ml of scintillation fluid and counted in a Beckman LS 6000IC scintillation counter.

2.4.5.iii. Hybridisation

All reagents and equipment were RNase-free. Chemicals were from Sigma, Poole, UK, unless otherwise stated. The following reagents were mixed in a microfuge tube, to make approximately 1 ml of hybridisation mix:

Reagent	Amount (µl)	Final conc.
(a) 1 M DTT	10	10 mM
(b) Deionized formamide (Gibco BRL)	600	60 %
(c) 100 x Denhardts	10	1 x
(d) 1 M Tris pH 8	10	10 mM
(e) 5 M NaCl	60	0.3 mM
(f) 0.5 M EDTA	10	1 mM
(g) 10 mg/ml Poly A	30	300 µg/µl
(h) 10 mg/ml carrier tRNA	30	300 µg/µl
(i) 20 mM cold S-UTP (optional)	22	500 µM
(j) 50 % dextran sulphate	200	10 %
Total volume (µl)	982	

Radiolabelled probe was added to the hybridisation mix to a final concentration of 5 x 10^4 cpm/µl. The hybridisation mix was then heated to 80 °C for 2 min; 10-15 µl were applied to each section, which were then covered with a baked coverslip. The slides were incubated overnight at 50 °C in a sealed box humidified with a tissue soaked in 50 % formamide, 5 x SSC.

2.4.5.iv. Post-hybridisation washes

Slides were then transferred to racks and incubated in the following buffers in 400 ml slide dishes equilibrated in 37 °C, 50 °C, and 65 °C waterbaths as directed.

Buffer	Temp.	Time
(a) 5 x SSC, 0.1 % β-mercaptoethanol (2-ME)	50 °C	3 x 20 min
(b) 50 % formamide, 2 x SSC, 0.1 % 2-ME	65 °C	30 min
(c) Ribonuclease buffer	37 °C	2 x 10 min
(0.5 M NaCl, 10 mM Tris, 5 mM EDTA, pH 8.0)		
(d) Ribonuclease A, 20 μg/ml in the above	37 °C	30 min
(e) Ribonuclease buffer	37 °C	15 min
(f) 50 % formamide, 2 x SSC, 0.1 % 2-ME	65 °C	30 min
(g) 2 x SSC	room temp.	15 min
(h) 0.1 x SSC	room temp.	15 min

The slides were then dehydrated through graded alcohols (30 %, 50 %, 70 %, 95 % and 100 %; 2 min in each) and air dried in a dust-free environment.

2.4.5.v. Autoradiography

The slides were dipped in 0.1 % gelatin (made up in distilled water and filtered; Sigma) and air dried. In a dark room, 10 ml of 0.1 % gelatin was pre-warmed to 50 °C in a waterbath; Ilford K5 emulsion (Ilford Imaging UK Ltd., Knutsford, UK) was then added to a final volume of 20 ml. The emulsion was poured into a slide mailing box; the slides were dipped in emulsion, then air dried for 2 hours at room temperature in a light-proof box. The slides were then transferred to a rack and placed in a box containing silica gel. The box was wrapped in foil and stored at 4 °C for 7-10 days.

2.4.5.vi. Development and counterstaining

In a dark room, the slides were incubated in the following solutions at room temperature:

(a) Kodak D-19 developer (Sigma) or equivalent for 2.5 min.

(b) 1 % acetic acid for 0.5 min

(c) 30 % sodium thiosulphate (freshly made) for 5 min.

The slides were then rinsed in running distilled water for 1 hour and counterstained in toluidine blue. Briefly, slides were stained for 2 minutes in 0.01 % filtered toluidine blue solution, followed by a rinse in distilled water, incubation in 100 % ethanol (to remove excess stain) then incubation in xylene before mounting with DPX mountant (Merck/BDH, Lutterworth, UK). Sections were examined using a Nikon Labophot II microscope (Nikon, Kingston, UK). Image capture was with Image Grabber PCI (Neotech Ltd, London, UK).

2.4.5.vii. Cell counting and statistics

The CCR1-expressing cells were counted in 15 High Power Fields (HPF) selected at random. 15 HPF corresponded to a total tumour area of 1.095 mm^2, so the counts were expressed as cells/mm^2. As the data were not normally distributed, the nonparametric Spearman's rank correlation was used to calculate P values (401).

2.5. Immunohistochemistry

2.5.1. Antibodies

In order to stain for $CD8^+$ T cells and $CD68^+$ macrophages in cryostat and paraffin sections, the following antibodies were used: anti-CD8 mouse monoclonal DK25 and anti-CD68 mouse monoclonal PG-M1 (both from DAKO, Ely, UK). To stain for CXCL12 in paraffin sections, anti-SDF-1α rabbit polyclonal antibody (clone 500-P87A,

Peprotech, UK) was used. Biotinylated rabbit anti-mouse antibody and biotinylated swine anti-rabbit antibody were from DAKO.

2.5.2. Streptavidin-peroxidase method for immunostaining

2.5.2.i. Crytostat sections

Cryostat sections were removed from the freezer, and air dried at room temperature for 1 hour in a dust-free environment. The following steps were then carried out at room temperature (using slide dishes for washes):

a) Sections were fixed in 400 ml of 4 % paraformaldehyde (Sigma, Poole, UK) in a slide dish for 20 min.

b) Sections were washed twice in 400 ml PBS for 3 min.

c) The slides were laid out in a humidified box and endogenous peroxidase was blocked by adding ~100 µl of 0.3 % H_2O_2 (in PBS) to each section, for 10 min.

e) Sections were washed in 400 ml PBS for 3 min.

f) The slides were laid out in a humidified box and excess PBS was blotted from around each section using a tissue.

g) ~100 µl of 5 % normal rabbit serum (DAKO) was added to each section for 20 min, then drained off.

h) To each section, enough primary antibody (at appropriate dilution) was added to cover the section. The slides were incubated for 1 hour in a humidified box at room temperature.

i) Sections were washed twice in 400 ml PBS for 3 min.

j) The slides were laid out in a humidified box and excess PBS was blotted from around each section using a tissue.

k) To each section, enough secondary antibody (biotinylated rabbit anti-mouse [DAKO], diluted 1:300 with PBS) was added to cover the section. The slides were incubated for 30 min in a humidified box at room temperature.

l) Sections were washed twice in 400 ml PBS for 3 min.

m) The slides were laid out in a humidified box and excess PBS was blotted from around each section using a tissue.

n) Streptavidin-peroxidase (DAKO, diluted 1:500) was added to each section and incubated for 30 min in a humidified box at room temperature.

o) Sections were washed twice in 400 ml PBS for 3 min. Then the slides were laid out in a humidified box and excess PBS was blotted from around each section using a tissue.

p) The peroxidase substrate 3,5-diaminobenzidine (DAB) was prepared: 5 mg DAB (Sigma), 10 ml PBS, 20 µl 30 % H_2O_2.

q) DAB solution was added to each section and incubated for less than 5 min (until colour developed).

r) Sections were washed in 400 ml distilled water.

s) Sections were counterstained for 2 minutes in 0.01 % filtered toluidine blue solution.

t) The slides were dipped in distilled water.

u) Sections were dehydrated through graded alcohols (50 %, 70 %, 95 %, 100 %; 1 min in each), then incubated in xylene until being mounted with DPX mountant (Merck/BDH, Lutterworth, UK).

2.5.2.ii. Paraffin sections

Paraffin sections were deparaffinised in xylene (2 x 10 min) and taken down through graded alcohols (50 %, 70 %, 95 %, 100%; 1 min in each), using a Bayer DRS 601 automatic slide stainer. Endogenous peroxidase was blocked by adding ~100 µl of 0.3 % H_2O_2 (in PBS) to each section, for 10 min. Sections were then microwaved in boiling 0.01 M sodium citrate pH 6.0. for 10 min to retrieve antigen, and rinsed in 400 ml PBS. Steps (f) to (u) were then carried out (Section 2.5.2.i above), except that sections were blocked with normal swine serum in step (g) and incubated with biotinylated swine anti-rabbit antibody in step (k).

2.5.3. Cell counting and statistics

The CD8 and CD68-expressing cells were counted using the method described in Section 2.4.5.vii.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Concentrations of the chemokines CCL2, CCL3, CCL4, CCL5, CXCL12 and the cytokine TNF-α were measured in samples of ascitic fluid and patient plasma samples using Quantikine® ELISA kits (R&D Systems, Abingdon, UK) according to the manufacturer's protocol. The sensitivity of the assays was as follows: CCL2: 5 pg/ml, CCL3: 7 pg/ml, CCL4: 4 pg/ml, CCL5: 8 pg/ml, CXCL12: 18 pg/ml and TNF-α: 4 pg/ml.

To determine correlation coefficients for the ascitic fluid chemokine concentrations the non-parametric Spearman's rank correlation was used to calculate P values, as the data were not normally distributed.

2.7. Flow cytometry

2.7.1. Monoclonal antibodies

FITC- and PE-labelled monoclonal antibodies (mAb) against CD3 (UCHT1), CD4 (MT310), CD8 (DK25) (all from DAKO, Ely, UK), CD14 (UCHM-1) (Sigma, Poole, UK), CD19 (SJ25C1), CD56 (MY31), HER2/neu (neu 24.7) (all from Becton Dickinson, Oxford, UK), CD83 (Immunotech, Marseilles, France), CCR1 (a kind gift from Shixin Qin, Millennium Pharmaceuticals, Cambridge, MA, USA), CCR2 (48607.211), CCR5 (45531.111) and CXCR4 (12G5) (all from R&D Systems, Abingdon, UK) and isotype-matched labelled controls were obtained and used to characterise cell surface phenotypes by flow cytometry. Unconjugated antibodies against β_1 integrin, β_4 integrin, α_2 integrin, α_v integrin, α_6 integrin, CD54 (ICAM) and E-cadherin were a gift from Fiona Watt (ICRF, London, UK); a PE-conjugated

polyclonal secondary antibody (BD Pharmingen, Oxford, UK) was used to detect these antibodies.

2.7.2. Immunofluorescent staining protocol

For staining, cells were washed in PBS supplemented with 1 % BSA and 0.01 % NaN_3 (FACS buffer) Approximately 5 x 10^5 cells were resuspended in 200 µl of FACS buffer in a microfuge tube, then 10 µg of human IgG (Sigma, Poole, UK; from 4 mg/ml stock made up in PBS) was added to block Fc receptors. After 15 min incubation at room temperature, primary antibody was added to a final concentration of between 2-20 µg/ml. Cells were incubated with the primary antibody for 30 min on ice. The cells were then washed twice by addition of 1 ml of cold FACS buffer, centrifugation at 13,000 rpm for 10 seconds in a microfuge at 4 °C, and resuspension in cold FACS buffer. Cells stained with FITC- or PE-conjugated antibodies were then ready for flow cytometric analysis. For unconjugated antibodies, cells were resuspended in 200 µl of FACS buffer and PE-conjugated secondary antibody was added (at an appropriate dilution). Following a further 30 minute incubation on ice, the cells were washed twice as before, then analysed by flow cytometry.

For two-colour flow cytometry, cells were incubated with the first antibody, washed, then incubated with the second antibody, washed, and analysed. Cells were analysed on a FACScan® flow cytometer using Cellquest software (Beckton Dickinson, Oxford, UK).

2.8. Calcium Flux

1 x 10^7 cells were resuspended in 2 ml of Flux buffer (1 x HBSS [Gibco BRL, Paisley, UK], plus 0.5 % BSA, 1mM Ca^{2+}, 1mM Mg^{2+} and 10 µM Zn^{2+}). Fluo-3 (Molecular Probes, Cambridge Biosciences, Cambridge, UK; 1 mM stock in DMSO) was added to a final concentration of 5 µM, and the cells were incubated for 30 minutes at 37 °C on a

roller, in the dark. Cells were then washed by adding 10 ml of flux buffer and centrifuging at 500 *g* for 5 minutes. After resuspension at 2 x 10^6 cells/ml, 2 ml of cells were placed in a quartz cuvette (Sigma, Poole, UK) with a cuvette stirbar (Sigma). Fluorescence was measured in a PTI fluorimeter, excitation wavelength 485 nm, emission wavelength 530 nm, in response to addition of various concentrations of CXCL12 (Peprotech, UK).

2.9. Western blotting

2.9.1. Antibodies

Antibodies against Akt (#9272), Phospho-Akt (#9271), p44/42 MAPK (#9102) and Phospho-p44/42 MAPK (#9101S) were obtained from New England Biolabs (NEB, Hitchin, UK). MAPK control proteins (positive and negative controls for western blotting) were also obtained from NEB. Anti-β-actin mouse monoclonal antibody (clone AC-15) was from Sigma, Poole, UK. HRP-conjugated donkey anti-rabbit secondary antibody and HRP-conjugated rabbit anti-mouse secondary antibody were from Amersham, UK. Chemiluminescence was performed using Renaissance® Western Blot Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Cambridge, UK).

2.9.2. Cell lysates

2.9.2.i. Cell culture

IGROV and CAOV-3 cell lines were cultured overnight in teflon-coated pots (see Section 2.1.4.i.) at 5 x 10^5 cells/ml, 5 ml per pot. At various time-points after stimulation with 100 ng/ml CXCL12 (Peprotech, UK), 1 ml aliquots were removed onto ice and centrifuged at 13,000 rpm for 10 seconds in a microfuge. The cell pellets were washed twice with 1 ml of ice-cold PBS containing 20 mM NaF (Sigma; 0.5M stock), 1 mM Na_3VO_4 (Sigma; 0.2M stock) and β-glycerophosphate (Sigma; 0.5M stock). The cells were then lysed in lysis buffer (see below).

2.9.2.ii. Preparation of total cell lysates

Lysis buffer was prepared on ice, containing: 50 mM Tris-HCl pH 8.0, 1 % Triton X100, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM Na_3VO_4, 20 mM NaF, 1 mM β-glycerophosphate, 25 μg/ml aprotinin, 25 μg/ml pepstatin A and 50 μg/ml leupeptin (all from Sigma). Then 100 μl of ice-cold lysis buffer was added to each aliquot of 5 x 10^5 cells. Cells were lysed by pipetting up and down, then genomic DNA was sheared by repeatedly passing the lysate through a 25 g needle connected to a 1 ml syringe.

The protein concentration of each lysate was determined using a standard BCA assay. Briefly, 200 μl of BCA reagent (Sigma) was added to 10 μl of lysate in one well of a 96-well plate. The 96-well plate was incubated at 37 °C for 30 min then cooled to room temperature. The absorbance at 562 nm was measured in a plate reader (Opsys MR, Dynex Technologies), and the protein concentration determined by reference to a BSA standard curve. Lysates were adjusted to 1 μg/μl and stored at –70 °C.

2.9.3. Western immunoblotting protocol

2.9.3.i. SDS-PAGE and electroblotting

2.5 μl of 5x loading buffer (312.5 mM Tris-HCl pH 6.8, 10 % SDS, 20 % glycerol, 10 % β-mercaptoethanol, 1 % w/v bromophenol blue, made up in distilled water) were added to 10 μl (equivalent to 10 μg) of each lysate. The samples were boiled at 100 °C for 5 minutes, then cooled on ice prior to loading onto a 10 % SDS-acrylamide gel (made with buffers from National Diagnostics, Hessle, UK, in a Mini-PROTEAN 3 gel electrophoresis system from Bio-Rad, Hemel Hempstead, UK). Seeblue pre-stained protein standards (Invitrogen, Paisley, UK) were used to estimate molecular weight. Following electrophoresis, protein was transferred from the gel to Hybond ECL nitrocellulose membrane (Amersham, UK) using the Mini trans-blot electrophoretic transfer cell (Bio-Rad), according to the manufacturer's instructions.

2.9.3.ii. Protein detection

The membrane was incubated in 25 ml of blocking buffer (1x PBS, 0.1 % Tween-20, 5 % non-fat dry milk) for 1 hour at room temperature, in a small tray. The blocking buffer was removed, and 10 ml of primary antibody (diluted in 1x PBS, 0.1 % Tween-20, 5 % BSA, according to the manufacturer's instructions) was added to the membrane. The membrane was incubated overnight at 4 °C with gentle shaking, then washed with 15 ml of 1x PBS, 0.1 % Tween-20 (3 x 5 minutes). The HRP-conjugated donkey anti-rabbit secondary antibody (diluted 1:2000 in 10 ml of blocking buffer) was then added to the membrane, and incubated with gentle shaking for 1 hour at room temperature. The membrane was washed again (3 x 5 minutes in 1x PBS, 0.1 % Tween-20), then 4 ml of Renaissance® Western Blot Chemiluminescence Reagent Plus mix was added to the membrane for 1 min. Excess reagent was drained, then the membrane was wrapped in saran wrap and exposed to x-ray film (Hyperfilm ECL, Amersham, UK). Densitometry was performed using NIH Image 1.61.

2.9.3.iii. Membrane stripping

Membranes were stripped by incubation at 50 °C for 30 minutes in 20 ml of stripping buffer (62.5 mM Tris-HCl pH 6.8, 0.7 % β-mercaptoethanol, 2 % SDS), followed by 2 washes in 1x PBS, 0.1 % Tween-20. The membrane could then be reprobed with other antibodies.

Chapter 3. CC Chemokine and CC Chemokine Receptor Expression in Solid Ovarian Tumours

3.1. Introduction

Several studies have examined the leukocyte infiltrate in ovarian cancer; the majority showed that T cells and macrophages were the dominant component of the leukocyte infiltrate, with very few B cells or NK cells. Work by Haskill *et al* (402) and Dietl *et al* (403) concluded that the T cells were $CD8^+$, while work by Kabawat *et al* (404) found that they were $CD4^+$ T cells. However, $CD4^+$ T cells can be difficult to assess due to expression of CD4 by other cell types, including macrophages. An extensive characterisation of the leukocyte infiltrate in solid human ovarian tumours was subsequently carried out by Rupert Negus in our laboratory (230). He concluded that $CD68^+$ macrophages and $CD3^+/CD8^+/CD45RO^+$ T cells accounted for the majority of the leukocyte infiltrate. Macrophages were found throughout the tumour stroma and parenchyma, with an accumulation in areas of necrosis (231). $CD8^+$ T cells were distributed more evenly, and were not associated with regions of apoptosis or necrosis. B cells, NK cells and mast cells were only present in low numbers. The presence of macrophages and lymphocytes in this tumour microenvironment may be related to local production of chemokines (232, 405). Further work by Negus *et al* assessed the expression of the CC chemokines CCL2 (MCP-1), CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES), and correlated this with the leukocyte infiltrate. CCL3, 4 and 5 localised to infiltrating leukocytes, while CCL2 localised to infiltrating leukocytes and also to the tumour cells themselves. He found a relationship between mRNA expression of CCL2 and the lymphocyte ($P<0.005$) and macrophage ($P=0.026$) counts in ovarian tumours (230). He also found a correlation between the expression of CCL5 and the $CD8^+$ lymphocyte infiltrate ($P<0.005$). This work suggested that a chemokine network exists in ovarian cancer. However, it was not clear how this network was controlled. Changes in the profile of chemokine receptors expressed by individual cells

can inhibit cell migration or change their path. Thus, to understand the chemokine network in a tissue, both chemokine and chemokine receptor expression must be studied.

3.2. Aims of the chapter

The initial aims of this chapter were to examine CC chemokine expression by ovarian cancer cells lines and leukocytic cell lines. The subsequent aim was to compare CC chemokine receptor mRNA expression and regulation in solid tumours with the expression of CC chemokine mRNA, and to relate this to the leukocyte infiltrate.

3.3. Results

3.3.1. Chemokine expression in solid tumours

3.3.1.i. RT-PCR for chemokines in cell lines

Due to its speed and sensitivity, RT-PCR was used to examine the expression of six CC chemokines, CCL2, CCL3, CCL4, CCL5, CCL8 and CCL22 in four ovarian cancer cell lines, five leukocytic cell lines and a fibroblastic cell line. These chemokines were chosen because they are representative ligands for the chemokine receptors CCR1, CCR2b and CCR4 which are investigated in Section 3.3.2.; the cell lines were chosen to represent the potential cellular sources of chemokines in solid ovarian tumours. The results are summarised in Table 3.1. The ovarian cancer cell lines were SKOV-3, OVCAR-3, PEO1 and PEO14. The leukocytic cell lines were THP-1 and MonoMac6 (monocytic cell lines), Jurkat and Hut-78 (T cell lines); PBMC and human foreskin fibroblasts (HFF) were also used. Total RNA from MonoMac6, Jurkat, Hut-78 and HFF was a gift from Rupert Negus and Frances Burke in our laboratory.

	Chemokine					
Cell line	CCL2	CCL3	CCL4	CCL5	CCL8	CCL22
SKOV-3	-	-	-	-	-	-
OVCAR-3	+	nd	nd	nd	-	+
PEO1	+	-	-	+	-	nd
PEO14	+	-	-	-	+	+
THP-1	+	+	+	+	-	+
MonoMac6	+	+	-	+	-	nd
Jurkat	-	nd	nd	nd	-	nd
Hut-78	-	-	-	+	-	nd
PBMC	+	+	+	+	+	nd
HFF	+	-	-	+	+	-

Table 3.1. Expression of CC chemokines by ovarian cancer cell lines, leukocytic cell lines, and fibroblasts. + = expression detected; - = no expression; nd = not done.

The ovarian cancer cell lines had restricted chemokine expression – only CCL2 and CCL22 were expressed by the majority of the cell lines. CCL3, 4, 5 and 8 were only expressed by 1/4 ovarian cancer cell lines. THP-1 and PBMC were able to express a majority of the chemokines examined.

3.3.1.ii. RT-PCR for chemokines in solid tumour biopsies

The expression of six CC chemokines, CCL2, CCL3, CCL4, CCL5, CCL8 and CCL22 was analysed by RT-PCR in 25 solid ovarian tumour biopsies. These biopsies were classified as serous adenocarcinoma (19), clear cell carcinoma (2), mucinous adenocarcinoma (1), anaplastic carcinoma (1), signet ring carcinoma (1), and endometrioid carcinoma (1). CCL2, CCL3, CCL4, CCL5 and CCL8, were detected in more than 80 % of the solid tumour samples, while CCL22 was detected in 6/25 samples (Figure 3.1.). This agrees with previous work from our laboratory where CCL2, 3, 4 and 5 were detected by *in situ* hybridisation (ISH) (230). Thus, a range of chemokine mRNAs are expressed in solid ovarian tumours.

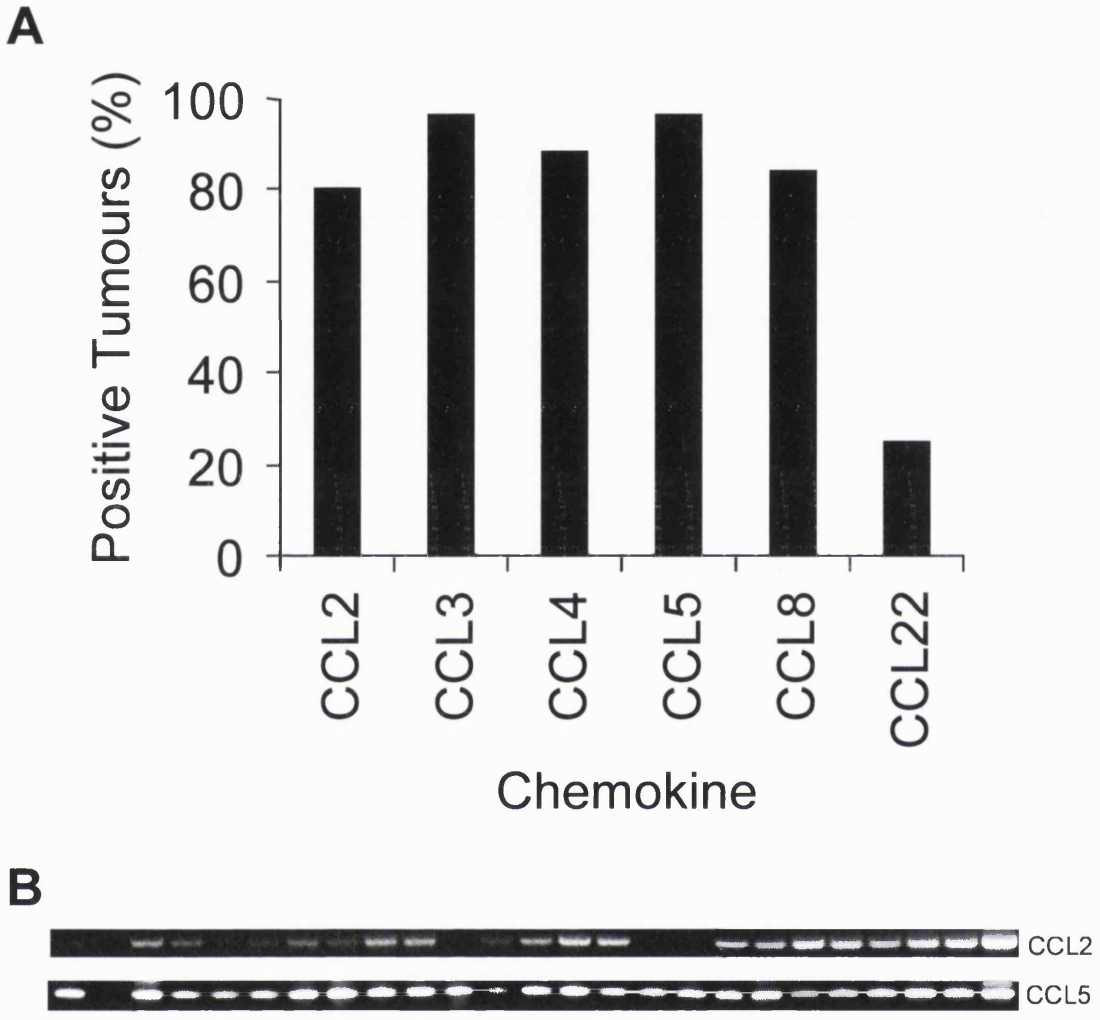


Figure 3.1. The mRNA expression of six CC chemokines was assessed by RT-PCR in 25 solid human ovarian tumour biopsies. (**A**) The percentage of samples positive for each chemokine is shown. (**B**) Representative gels for CCL2 and CCL5 RT-PCR.

3.3.2. Chemokine receptor expression in solid tumours

3.3.2.i. RNase protection assay (RPA) and RT-PCR for chemokine receptors in solid tumours

RPA was used to analyse CC chemokine receptor mRNA expression in the twenty-five solid tumour biopsies described in section 3.3.1.ii. RPA was performed with 5 μg of total RNA and a template set containing probes for CCR1, 2, 2a, 2b, 3, 4, 5, 8, and the

'housekeeping' genes GAPDH and L32. All the CC chemokine receptor mRNAs were expressed by a control PBMC preparation (Figure 3.2.). In contrast, CC chemokine receptor mRNA was weakly expressed in the solid tumour biopsies. CCR1 was the only CC chemokine receptor present in the majority of the samples, with 75 % of the biopsies positive for this receptor mRNA. Less than 15 % of the biopsies were positive for the remaining CC chemokine receptor mRNAs (Figure 3.2. and 3.3. A).

Leukocytes are likely to be the main source of chemokine receptor mRNA in the tumours and their mRNA would have been diluted by other cells in the tumour microenvironment. To confirm the RPA results, therefore, the more sensitive technique of RT-PCR was used on RNA from the same biopsies. No mRNA was detected for CCR2, 2a, 2b, 3 or 5 in samples that were negative by RPA. CCR1 was detected in those samples positive by RPA (Figure 3.3. B). 72 % of the samples also gave a positive signal for CCR4 by RT-PCR, although these were negative by RPA (Figure 3.3. B).

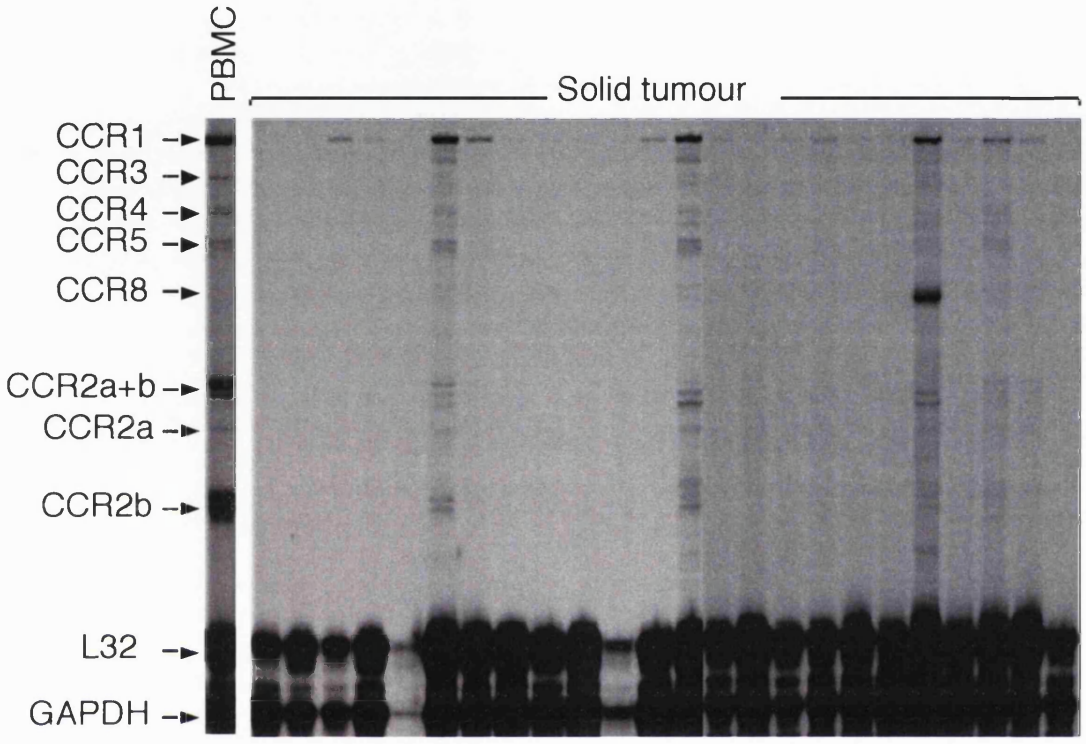


Figure 3.2. RNase protection assay of CC chemokine receptor expression in solid human ovarian tumour biopsies. RNA from normal PBMC was used as a positive control.

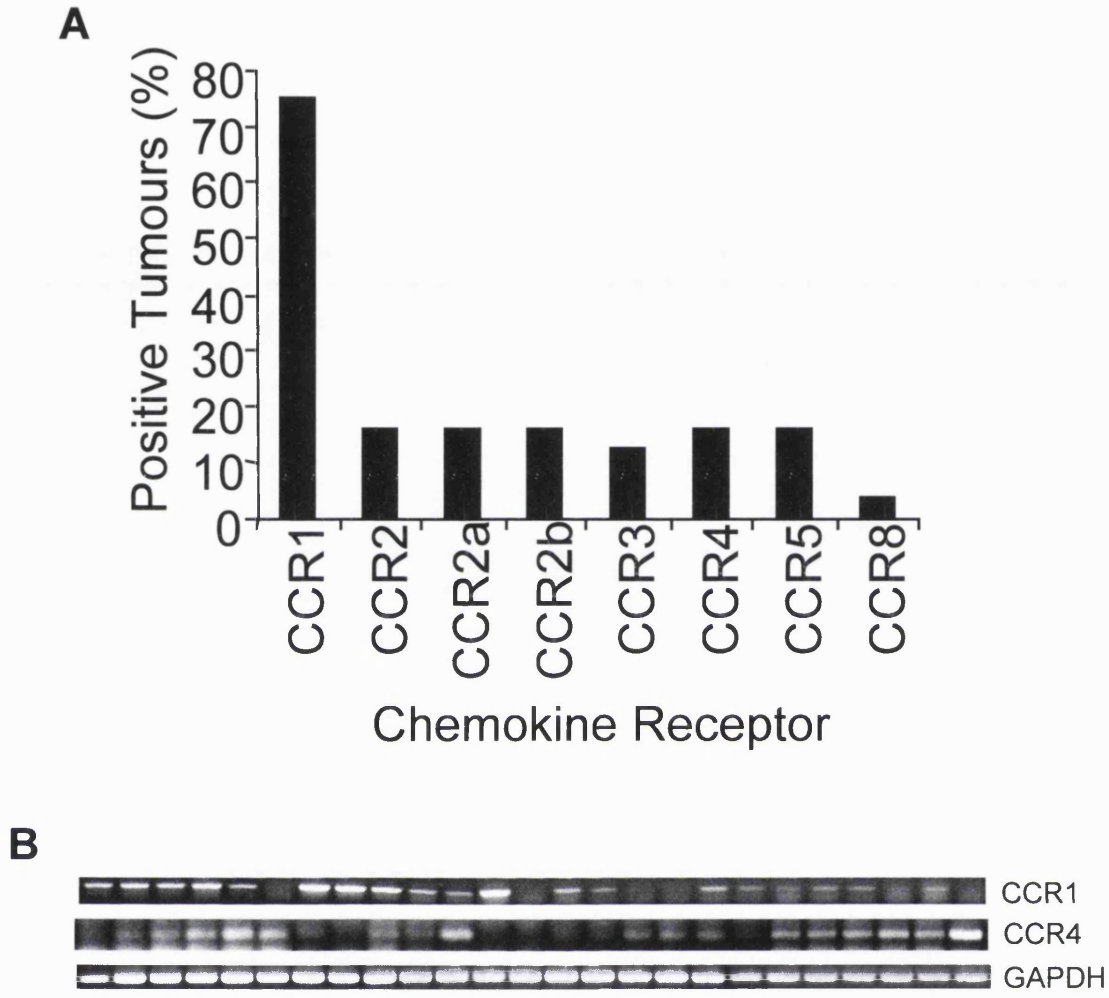


Figure 3.3. Chemokine receptor mRNA expression in solid human ovarian tumours. (**A**) Percentage of samples expressing CC chemokine receptor mRNA in solid human ovarian tumours, using the data derived from the RPA shown in Figure 3.2. Only CCR1 is expressed by the majority of solid tumours. (**B**) The presence of CCR1 and CCR4 mRNA in the solid tumours was demonstrated by RT-PCR. Amplification of GAPDH is shown as a loading control.

3.3.2.ii. In situ *hybridisation (ISH) for chemokine receptors in solid tumours*

ISH to mRNA was performed on frozen sections from eleven solid tumour biopsies to localise CCR1 and CCR4 expression. Positive and negative controls were used: β-actin always gave a positive signal, demonstrating RNA integrity; sense riboprobes for CCR1 and CCR4 were always negative with minimal background scatter (Figure 3.4.).

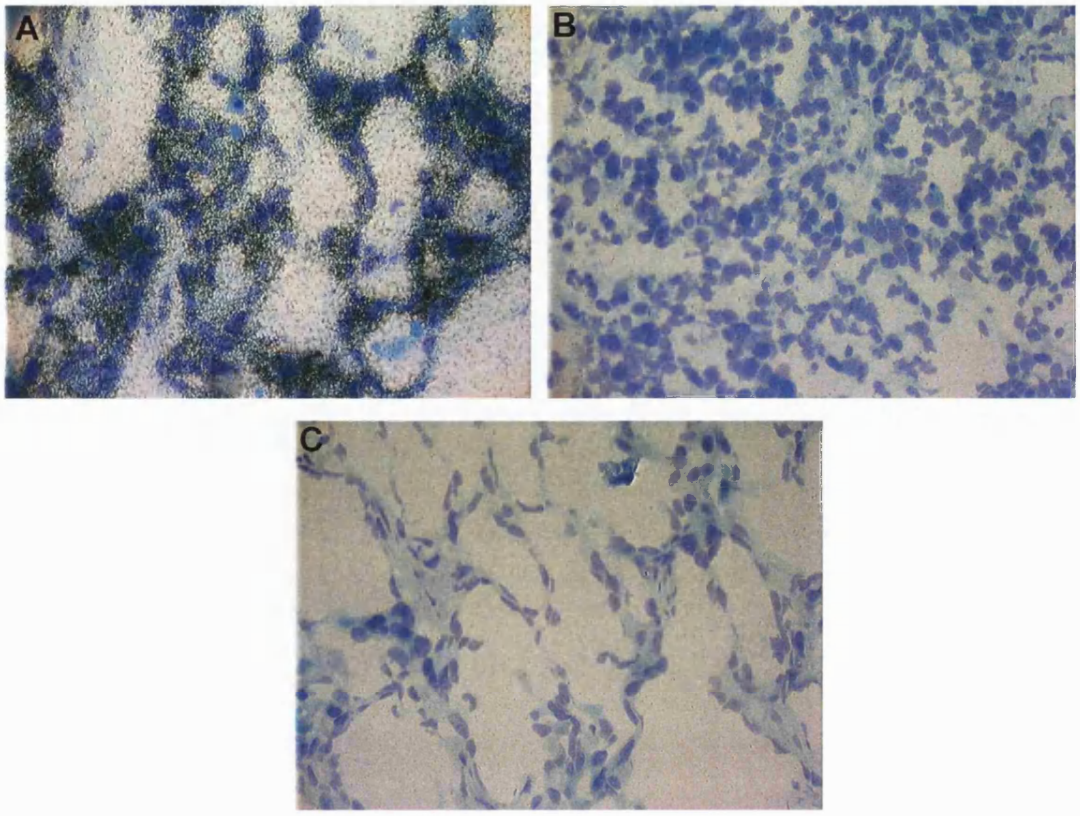


Figure 3.4. Positive and negative controls for *in situ* hybridisation. As a control for RNA integrity, tumour sections were probed for the 'housekeeping' gene β-actin, x 400 (**A**). As a negative control, tumour sections were probed with sense riboprobes for CCR1, x 400 (**B**) and CCR4, x 400 (**C**).

Nine of the eleven biopsies were positive for CCR1 by RPA and CCR1 mRNA could be detected in cells in these biopsies by ISH. The number of cells expressing CCR1 were counted in 15 high power fields (x 40 objective, x 10 eyepiece) and the results were expressed as cells/mm^2. Numbers of CCR1 expressing cells ranged from 13.7-83.1 cells/mm^2, with a median 41.4 cells/mm^2. Two of the biopsies used for ISH were negative by RPA, yet very occasional cells (mean 2.7 cells/mm^2) could be found that were positive for CCR1 mRNA, demonstrating the increased sensitivity of ISH compared with RPA. CCR1 mRNA localised mainly to clusters of cells in the stromal areas of the ovarian tumour biopsies and the distribution was consistent with expression

by infiltrating cells (Figures 3.5. and 3.6.). Epithelial tumour cells did not appear to express CCR1 mRNA.

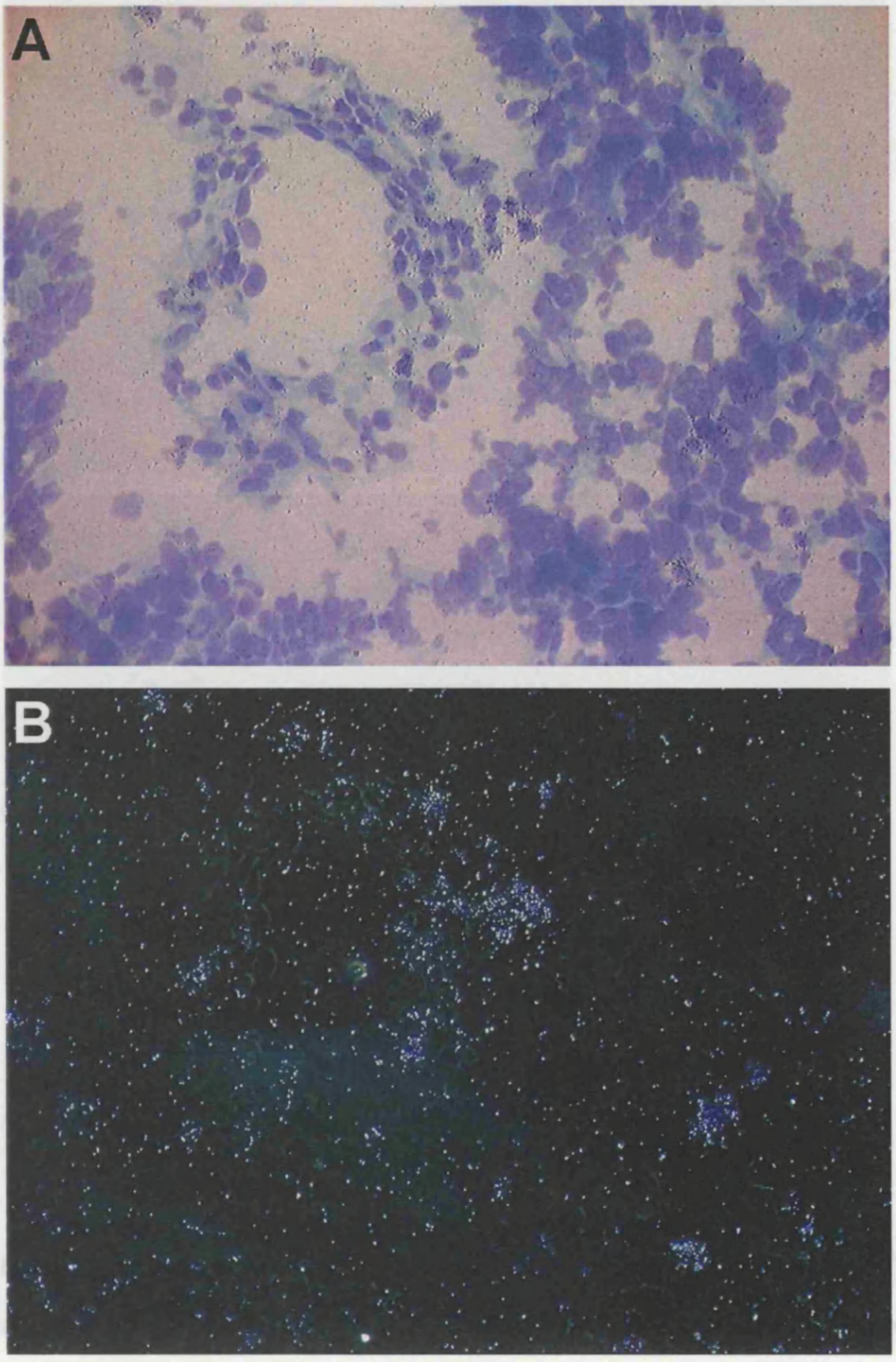


Figure 3.5. ***In situ*** **hybridisation to CCR1 in solid ovarian cancer.** The figure shows expression of CCR1 on stromal cells adjacent to a blood vessel under brightfield (**A**) and darkfield (**B**) illumination, x 400.

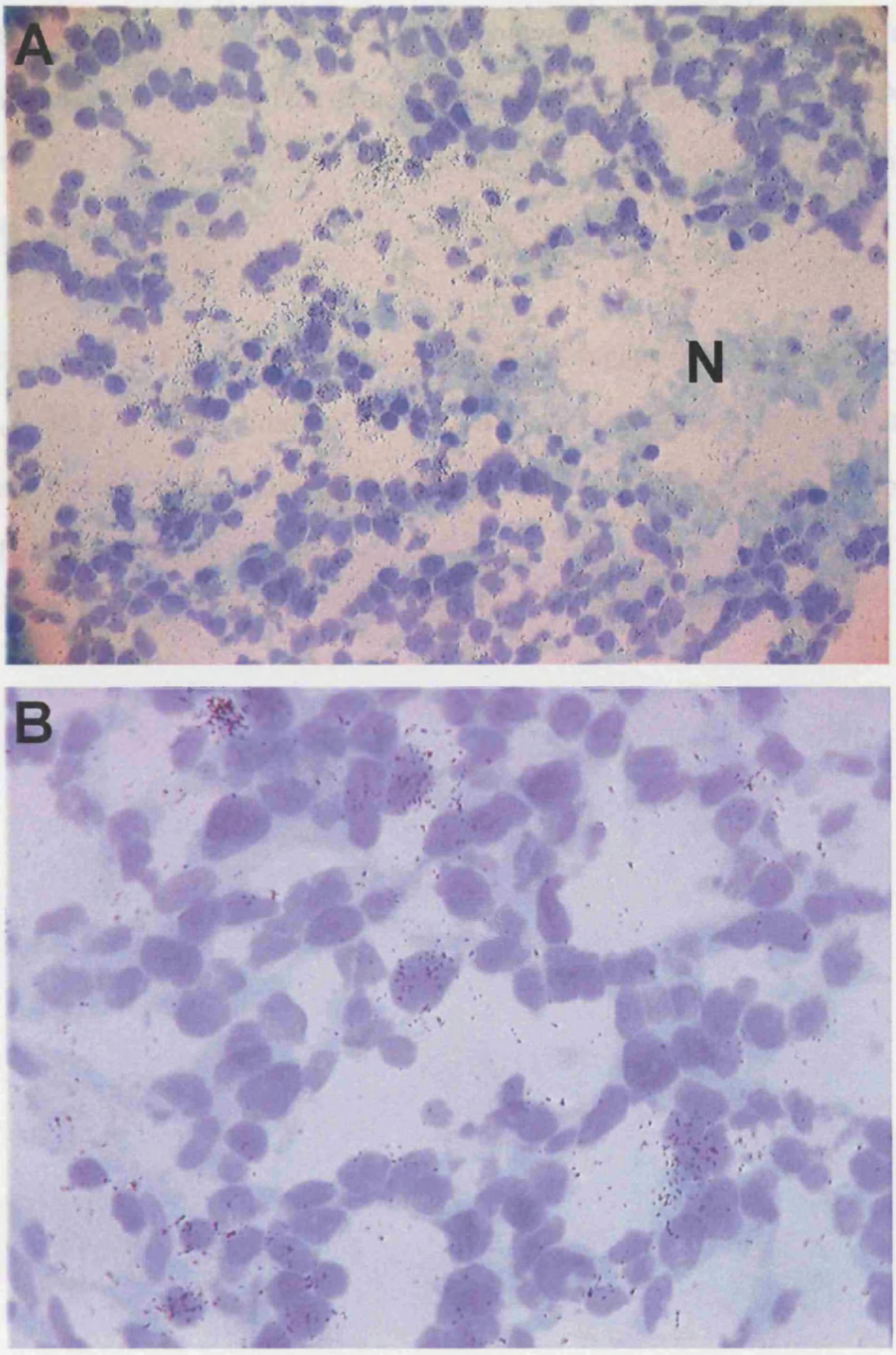


Figure 3.6. ***In situ*** **hybridisation to CCR1 in solid ovarian cancer.** (**A**) Expression of CCR1 adjacent to an area of necrosis (N), x 400. (**B**) Expression of CCR1 on infiltrating cells within a tumour area, x 1000.

As described in Section 3.3.2.i, CCR4 mRNA was detected by RT-PCR but not by RPA in RNA from solid tumours. As might be expected, individual cells expressing CCR4

were rare (Figure 3.7.). They were seen in 4/11 biopsies, with only 2-3 positive cells detected in the entire section. This demonstrates the extreme sensitivity of RT-PCR, but suggests that RPA and ISH give more meaningful data on chemokine receptor mRNA expression in tissue samples.

Thus a combination of techniques for measuring RNA suggests that expression of CCR2, 3, 5 and 8 in the solid tumour microenvironment is extremely low both in terms of RNA and number of expressing cells, although cells that might be expected to express these receptors are present.

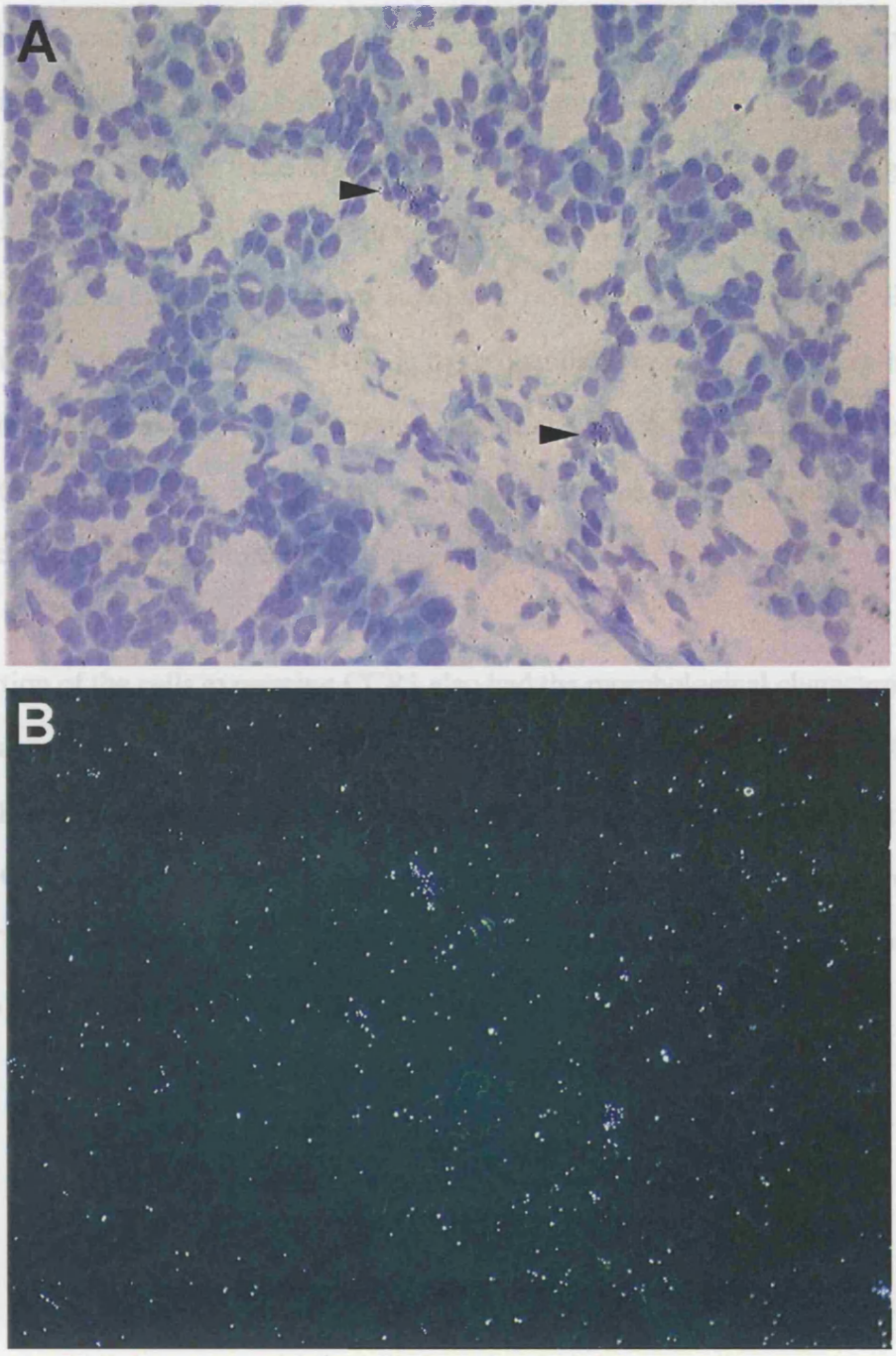


Figure 3.7. ***In situ*** **hybridisation to CCR4 in solid ovarian cancer.** Expression of CCR4 on two individual cells, marked by arrowheads, under brightfield (**A**) and darkfield (**B**) illumination, x 400.

3.3.2.iii. Immunohistochemistry for CD8⁺ T cells and CD68⁺ macrophages in solid tumours and correlations with CCR1 expression

Immunohistochemistry for CD8 and CD68, and ISH to CCR1 mRNA were performed on sequential sections from 10 solid human ovarian tumour biopsies. CCR1 expression was often seen at the same location as $CD8^+$ T cells in the sequential section (Figure 3.8. and 3.9.). The number of cells expressing CCR1 was counted in 15 HPF, corresponding to a total tumour area of 1.095 mm^2. Similarly, the number of cells expressing CD8 was counted in 15 HPF in the sequential section; only those cells with obvious nuclei and good cytoplasmic staining were scored. A possible correlation was found between the number of cells expressing CCR1 and the number of infiltrating $CD8^+$ T-cells in individual tumour sections ($r_s = 0.682$; $P = 0.031$).

A proportion of the cells expressing CCR1 also had the morphological characteristics of macrophages. Immunohistochemistry to CD68 was not of sufficient quality to obtain statistical correlation with the ISH results but there were examples of concordance between CD68 positivity and CCR1 expression on the sequential sections (Figure 3.10.). Tumour associated macrophages from ovarian cancer have previously been shown to be positive for CCR1 (180).

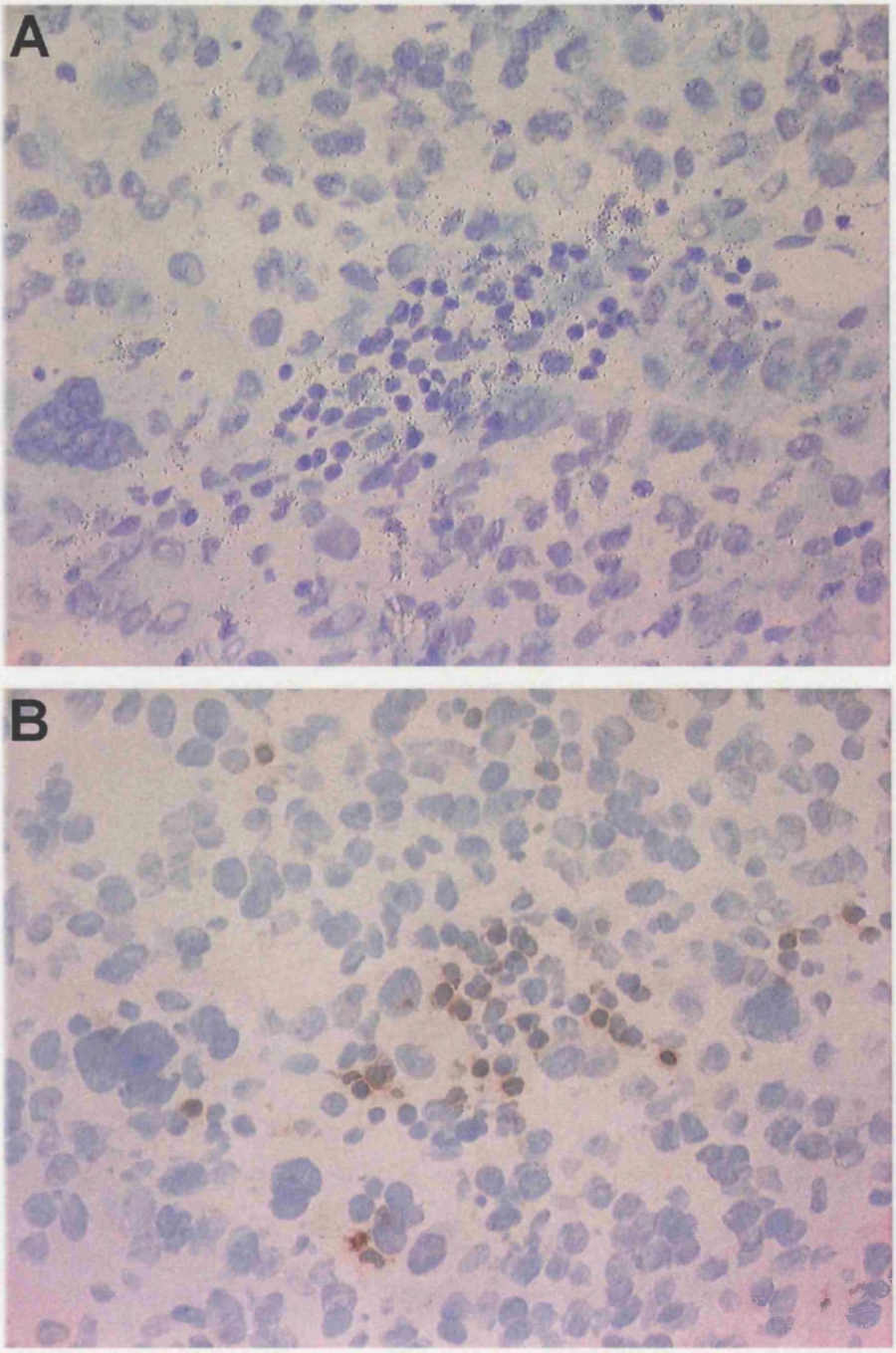


Figure 3.8. Co-localisation of CCR1 expression with $CD8^+$ T cells. *In situ* hybridisation for CCR1, x 400 (**A**). Immunohistochemistry for CD8 on a sequential section, x 400 (**B**).

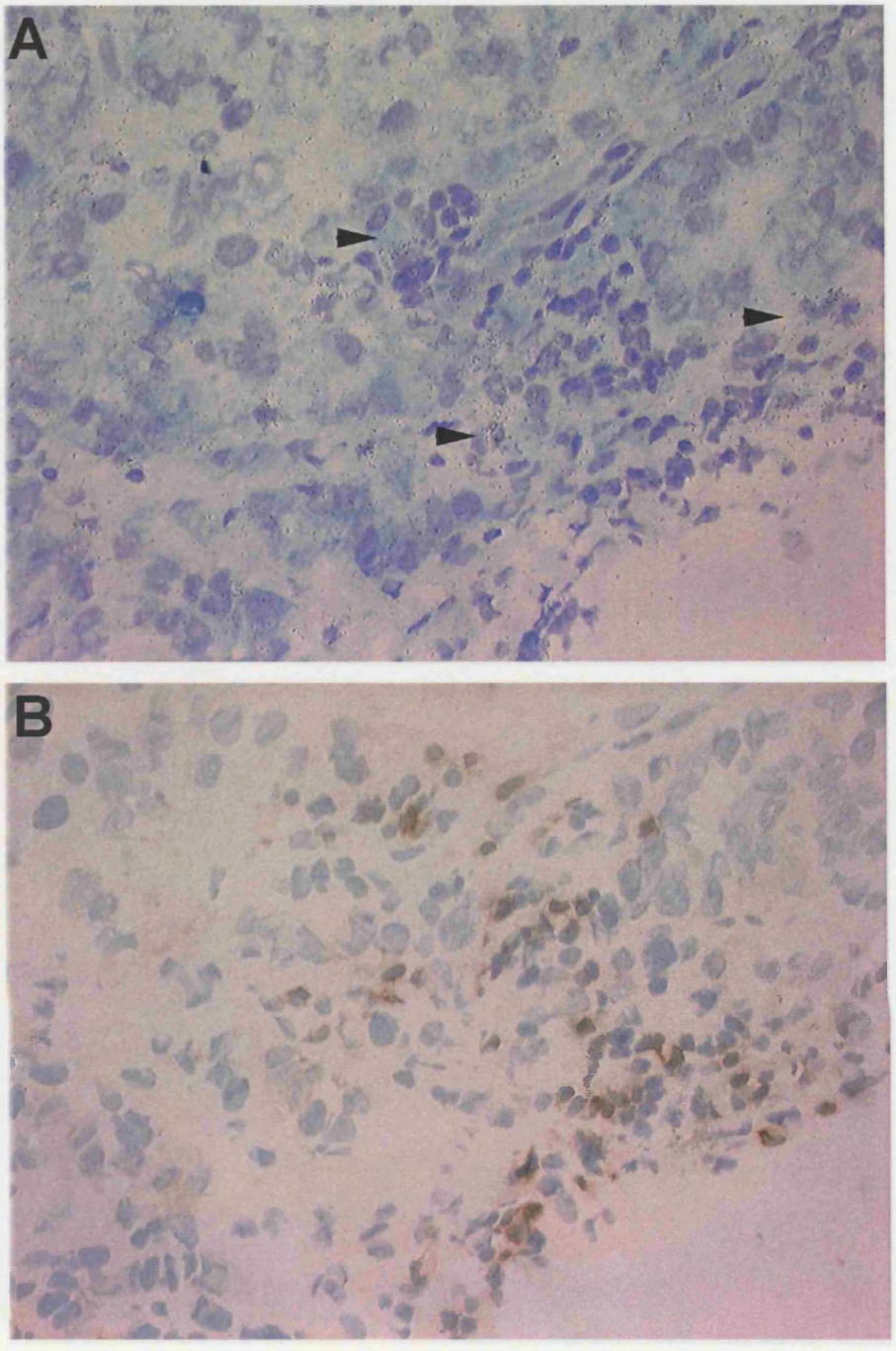


Figure 3.9. Co-localisation of CCR1 expression with CD8⁺ T cells. *In situ* hybridisation for CCR1, marked by arrowheads, x 400 (**A**). Immunohistochemistry for CD8 on a sequential section, x 400 (**B**).

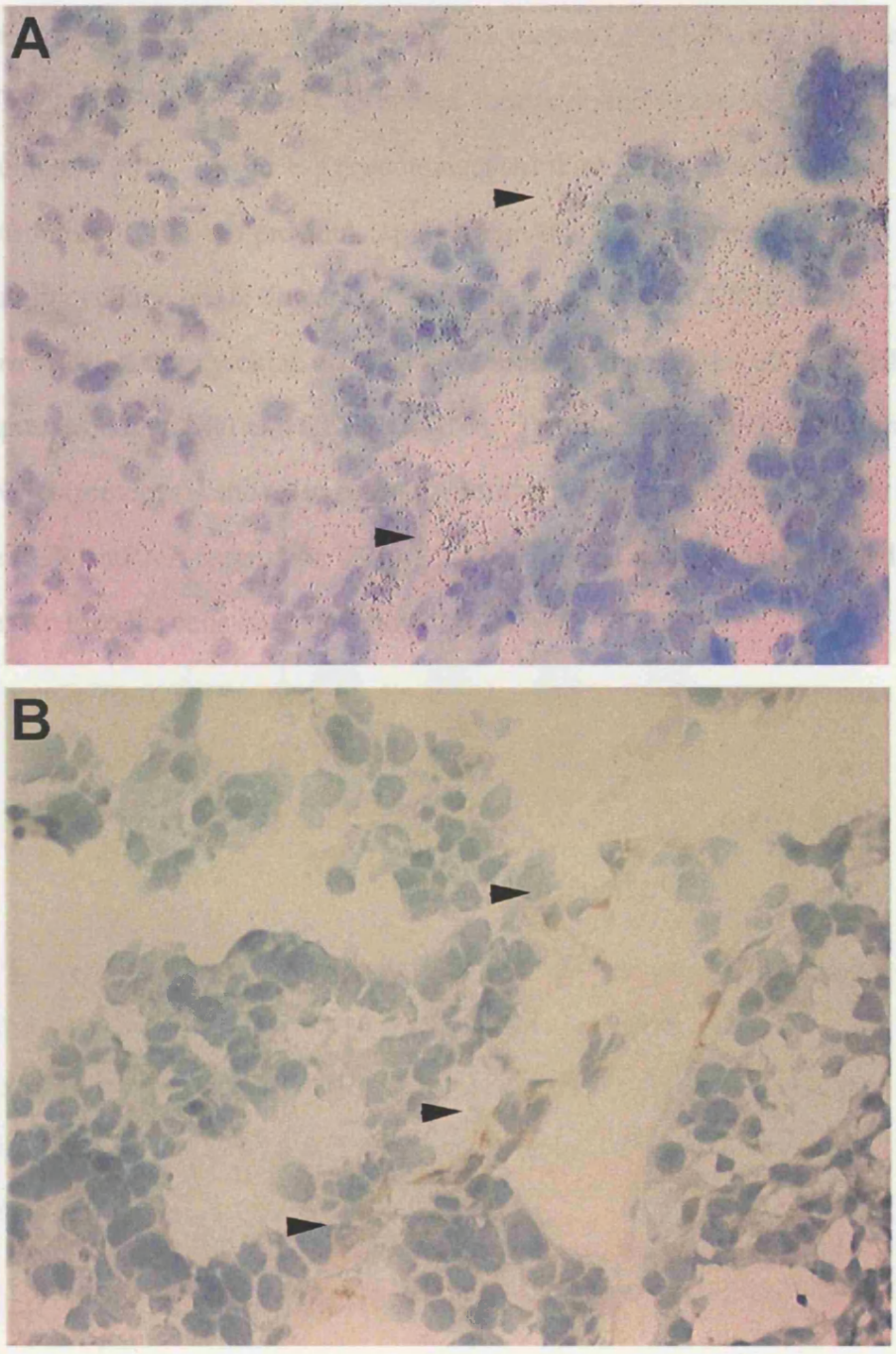


Figure 3.10. Co-localisation of CCR1 expression and CD68$^+$ macrophages. *In situ* hybridisation for CCR1, marked by arrowheads, x 400 (**A**). Immunohistochemistry for CD68 on a sequential section, marked by arrowheads, x 400 (**B**).

3.3.3. Regulation of CCR1

3.3.3.i. TNF-α

A number of agents are known to regulate CCR1 such as LPS (179), and the cytokines IL-12, IFN-γ and IFN-α (406-408). However, these cytokines have not been reported in ovarian tumours (361). TNF-α is a predominant cytokine in the ovarian tumour microenvironment (368) and previous reports suggested that the presence of this pro-inflammatory cytokine might downregulate the CCL2 receptor, CCR2b (180). To study the *in vitro* effect of this cytokine on CCR1, we used the monocytic cell line THP-1 which expresses both CCR1 and CCR2b mRNA. Treatment of THP-1 cells with 10 ng/ml TNF-α (previously shown to be optimal for stimulation of these cells) did not influence CCR1 mRNA expression (Figure 3.11.); 1 ng/ml was also inactive and 100 ng/ml was toxic to the cells (data not shown).

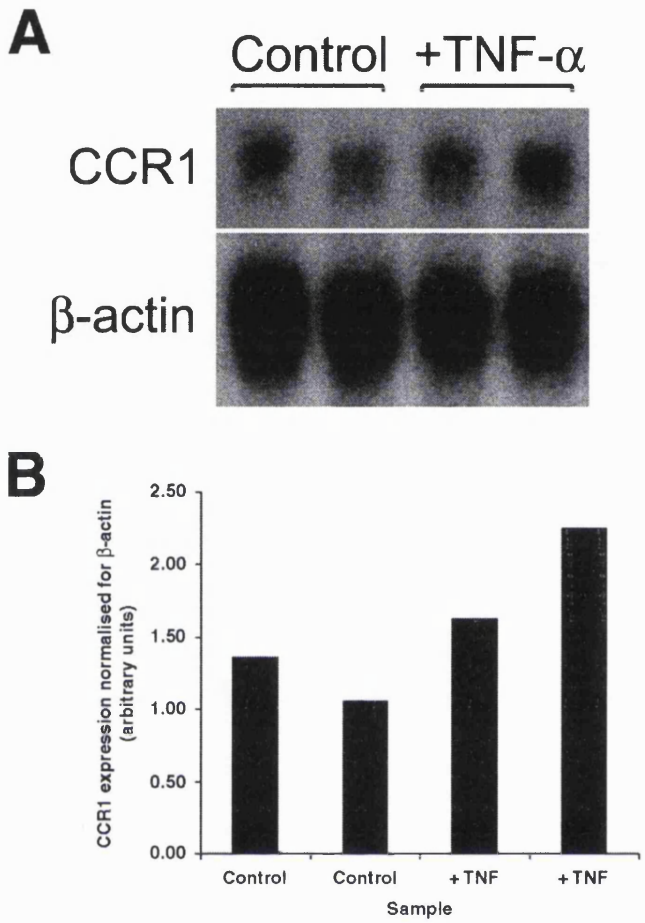


Figure 3.11. Effect of TNF-α stimulation on CCR1 mRNA expression in THP-1 cells.
THP-1 cells were stimulated with 10 ng/ml TNF-α for 24 hours. CCR1 mRNA expression was analysed by northern analysis, with β-actin as a loading control (**A**). Densitometric analysis showing the relative expression of CCR1 mRNA by control and TNF-stimulated cells, normalised for β-actin (**B**).

3.3.3.ii. Hypoxia

Another feature of the ovarian tumour microenvironment that is likely to influence cell behaviour is intratumoural oxygen tension. The effect of hypoxia on CCR1 and CCR2b expression was therefore studied. Regions of hypoxia are common in solid tumours due to the chaotic and intermittent blood supply, and the high metabolic rate of tumour cells (376). Macrophages accumulate in regions of necrosis, which are usually hypoxic (377)

and hypoxia can affect the migration of THP-1 cells and monocytes, but not lymphocytes (378). Thus, THP-1 cells were cultured under hypoxic conditions for 24 hours, and CCR1 mRNA expression was assayed by northern blot analysis (Figure 3.12.). After 24 hours hypoxia, the expression of CCR1 mRNA (normalised for the housekeeping gene, β-actin) was approximately 5-fold higher compared with cells cultured under normoxia.

A timecourse was also performed to examine CCR1 and CCR2b mRNA expression during hypoxia. CCR1 mRNA expression increased steadily up to 24 hours, but during the same time, CCR2b mRNA expression was relatively constant (Figure 3.13.).

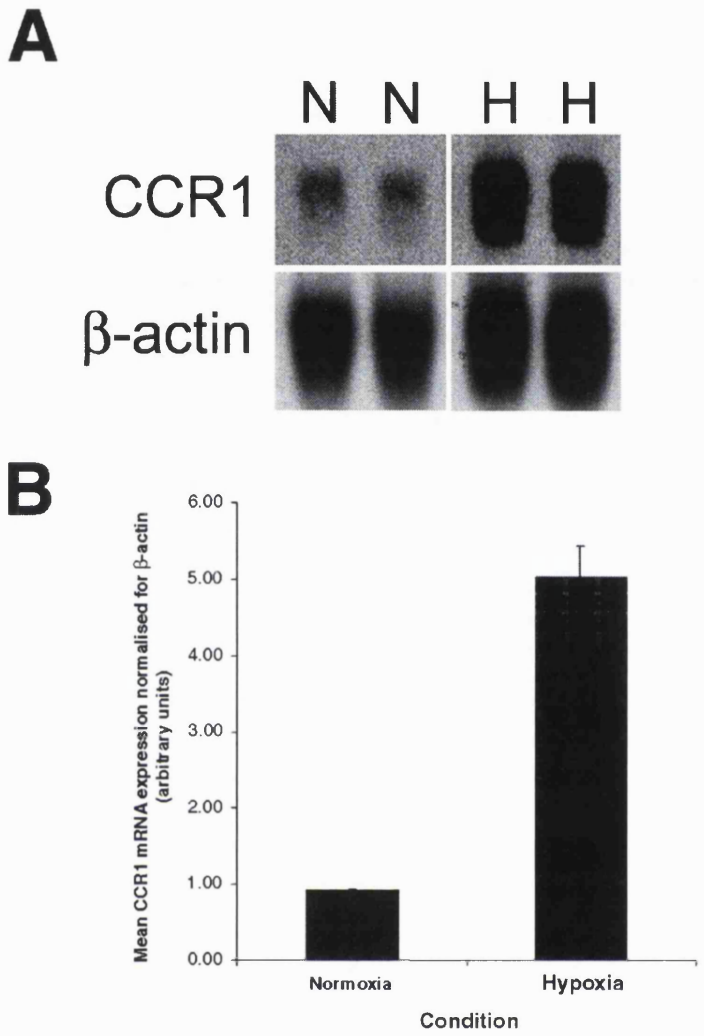


Figure 3.12. Upregulation of CCR1 mRNA expression under hypoxia. THP-1 cells were cultured for 24 hours in normoxic or hypoxic conditions. CCR1 mRNA expression was analysed by northern analysis. N = normoxic, H = hypoxic. The results are representative of 3 separate experiments (**A**). Densitometric analysis of CCR1 expression, normalised for the housekeeping gene, β-actin (**B**). Error bars represent the SEM (n = 6).

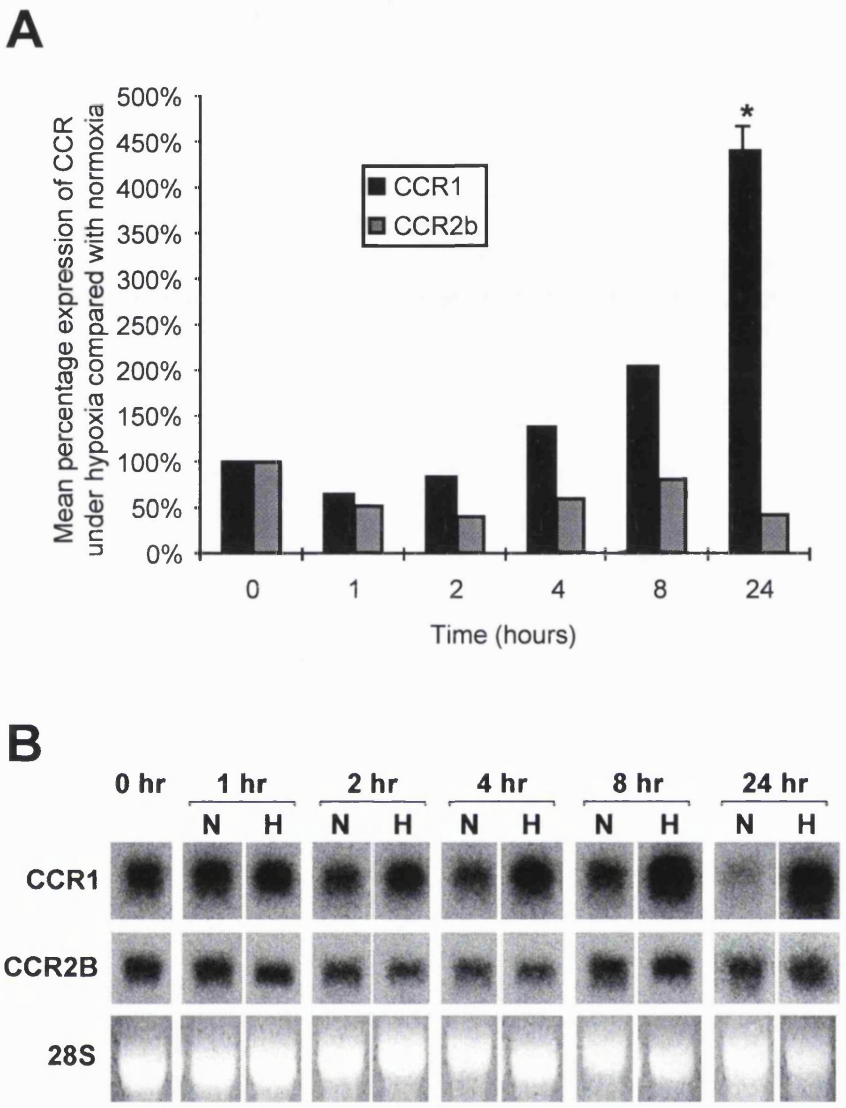


Figure 3.13. Timecourse of CCR1 and CCR2b mRNA expression under hypoxia. THP-1 cells were cultured under hypoxic conditions for 24 hours. (**A**) The expression under hypoxia is shown as a percentage of the expression under normoxia, normalised for the housekeeping gene β-actin. * 24 hr time-point includes the SEM (n = 6); all other time-points were representative of two experiments. (**B**) Representative northern blots for CCR1 and CCR2b showing mRNA expression at each time-point, with the 28S band from the ethidium bromide stained gel shown as a loading control. N = normoxia; H = hypoxia.

3.3.3.iii. Potential binding sites for the transcription factor HIF-1 in the CCR1 promoter.

The promoter region of CCR1 has been cloned by Lee *et al* (direct submission to Genbank, accession no. AF051305). Analysis of the promoter sequence using MatInspector V2.2 (409) revealed two potential binding sites (at –892 and –760 from the transcriptional start site) for the transcription factor hypoxia inducible factor (HIF)-1. This transcription factor is stabilised under hypoxia, and controls the expression of a number of target genes including VEGF and erythropoietin (410, 411) through a hypoxia responsive element (HRE). HREs always contain the sequence RCGTG (where R is a purine) which is critical for HIF-1 binding; the flanking residues are also important, but no strong consensus has been observed. The presence of potential HIF-1 binding sites suggests that CCR1 could be a target for transcriptional regulation by HIF-1, and could account for the upregulation seen in THP-1 cells under hypoxic conditions.

3.3.3.iv. Effect of cobalt chloride and desferrioxamine on CCR1 expression

The hypoxic state can be mimicked by using cobalt chloride and the iron chelator desferrioxamine (DFO). As in hypoxia, cobalt chloride and desferrioxamine activate HIF-1α (412). THP-1 cells were treated with 25 μM, 50 μM or 100 μM $CoCl_2$ or 50 μM, 100 μM or 200 μM DFO for 24 hours. Northern analysis was used to determine CCR1 mRNA expression (Figure 3.14.).

Cobalt chloride had no effect on CCR1 expression, while DFO gave only a slight increase over control levels. However, over 24 hours, both compounds displayed some toxicity and caused a decrease in cell number. Thus, any effects on CCR1 expression may have been masked, and a role for HIF-1 cannot be ruled out.

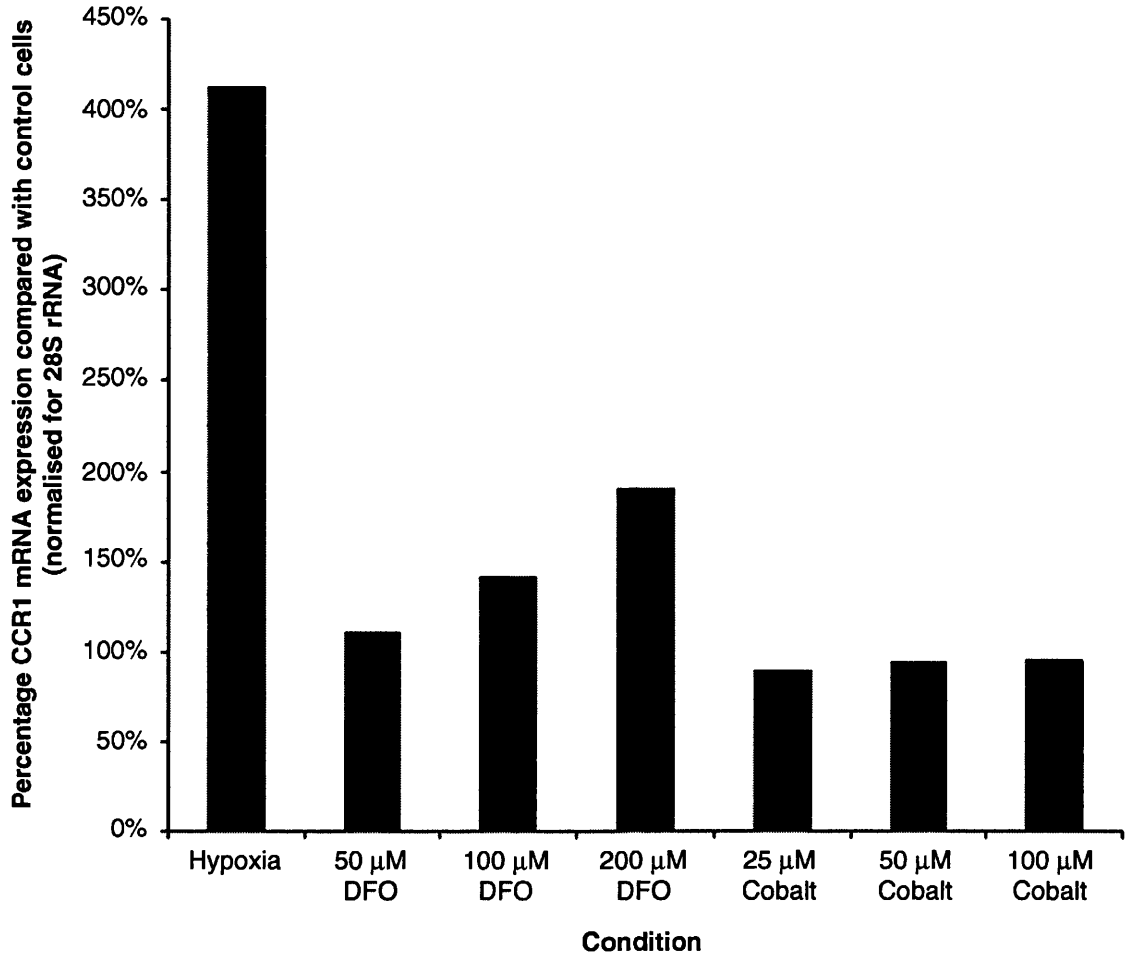


Figure 3.14. Effect of desferrioxamine (DFO) and cobalt chloride on CCR1 expression.

THP-1 cells were treated for 24 hours with either DFO or cobalt at the concentrations indicated. CCR1 mRNA expression was determined by northern analysis, and normalised for 28S rRNA. Results are representative of two experiments.

3.3.3.v. CCR1 regulation by hypoxia in primary cells

During the course of these studies, I was unable to demonstrate that hypoxia can upregulate CCR1 expression on primary monocytes, monocyte-derived macrophages or monocyte-derived dendritic cells. The methods used to isolate primary cells invariably caused some activation, leading to very high basal levels of CCR1 (Figure 3.15.). Further increases in CCR1 mRNA expression under hypoxia were not seen (data not shown).

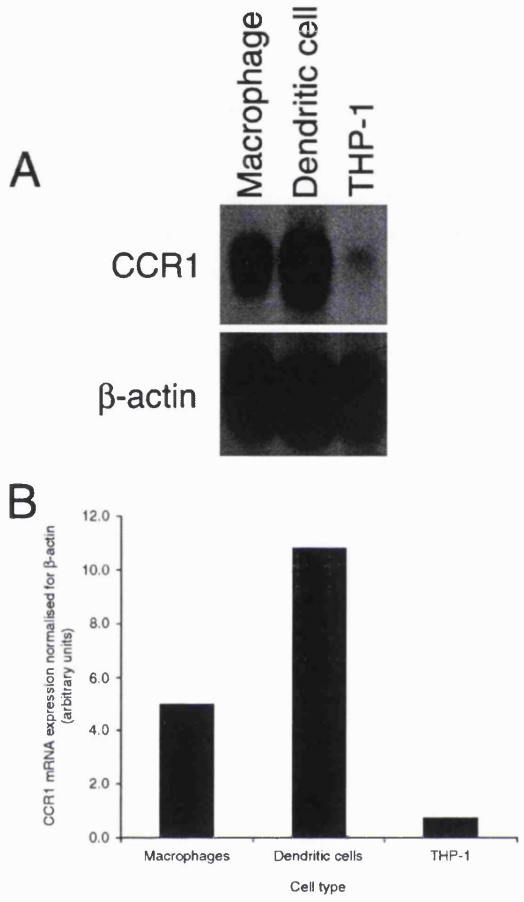


Figure 3.15. Comparison of CCR1 expression on primary cells and THP-1. Monocytes purified from peripheral blood were cultured for seven days in the presence of human AB serum, or IL-4/GM-CSF, to generate macrophages or dendritic cells respectively. CCR1 expression was then determined by northern analysis (**A**). Densitometric analysis was performed using β-actin as the housekeeping gene (**B**). The results are representative of three experiments.

3.3.4. Development of an *in vitro* three-dimensional tumour model (spheroids)

There is no suitable *in vivo* model for studying the role of chemokines and chemokine receptors in the immune cell infiltrate in ovarian cancer. Human ovarian cancer cell lines can be grown as xenografts in nude mice, but the infiltrate is therefore murine. Chemokines and their receptors are not totally conserved between mouse and human, nor do all of the chemokines cross react. Therefore, an *in vitro* model of human ovarian

cancer was developed whereby cancer cells cultured in a non-adherent 96 well plate can coalesce to form a cellular "ball", or spheroid. Leukocytes can then be added to the well, to examine their ability to infiltrate the three-dimensional tumour spheroid.

3.3.4.i. Growth of spheroids

Six ovarian cancer cell lines were tested for their ability to form spheroids *in vitro*. PEO1, PEO14, OVCAR-3, SKOV-3, CAOV-3 and IGROV were cultured in agarose-coated 96 well plates at a density of 200, 500, 1000, 2000 and 5000 cells per well. Only SKOV-3 were able to form spheroids; 2000 cells per well being the optimum seeding density.

SKOV-3 spheroids could be cultured for up to 2 weeks, with fresh medium added every 3 days. During this time, the spheroid diameter reached over 1 mm (Figure 3.16.). Once the diameter reaches approximately 400 μm, the centre of the spheroid can become hypoxic (413), and an area of necrosis develops. This mimics the situation seen in solid human ovarian tumours – areas of necrosis which are generally hypoxic, surrounded by healthy tumour parenchyma.

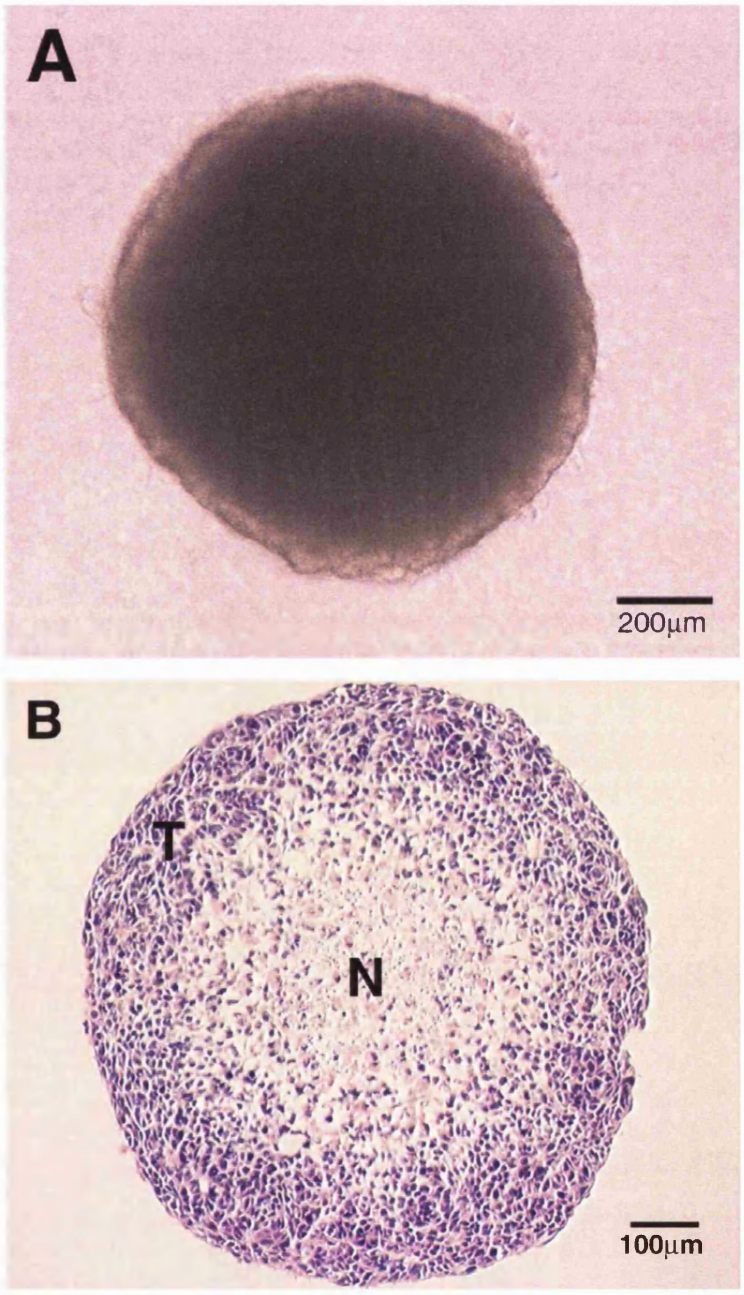


Figure 3.16. Appearance of a tumour spheroid. (**A**) External view of a SKOV-3 spheroid in one well of a 96-well plate. (**B**) Cross-section through a SKOV-3 spheroid, stained with H&E to reveal the histological detail. A necrotic core (N) is surrounded by a halo of healthy tumour cells (T), x 20.

3.3.4.ii. Infiltration of SKOV-3 spheroids by peripheral blood monocyte-derived macrophages

To generate macrophages, peripheral blood monocytes were cultured for seven days in a teflon bag in the presence of human AB serum. 1 x 10^4 macrophages were then added to each well of a 96-well plate containing 7 day old SKOV-3 spheroids (approximately 1 mm in diameter). The spheroids were incubated for 48 hours, to allow macrophages to infiltrate the spheroid, and distribute themselves accordingly. Immunohistochemistry for CD68 revealed that macrophages could infiltrate the spheroids and accumulate in the necrotic core. This mimics the situation seen in solid tumours, where macrophages tend to accumulate in areas of necrosis – a phenomenon attributed to a cessation of migration under hypoxic conditions (378, 414).

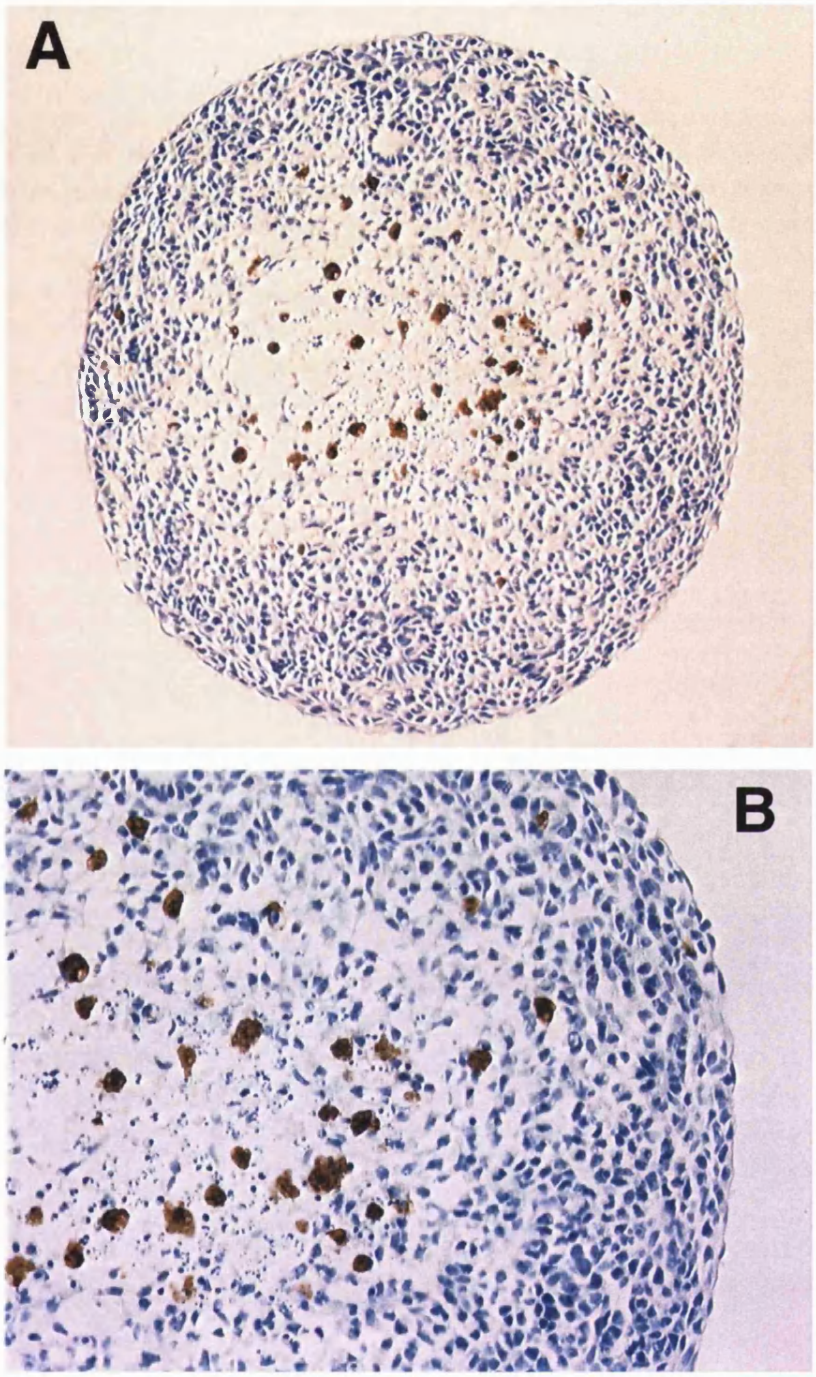


Figure 3.17. Infiltration of a tumour spheroid by macrophages. (**A**) Immunohistochemistry for CD68 in a seven day old SKOV-3 spheroid to which 1 x 10^4 macrophages have been added, x 200. (**B**) The same spheroid at x 400 magnification, showing the clustering of macrophages in the necrotic core, with very few macrophages within the healthy tumour cell layer.

Future work in the laboratory could utilise this model to examine chemokine receptor expression and regulation in an *in vitro* system which has similarities to the *in vivo* situation.

3.4. Discussion

The tumour microenvironment of human epithelial ovarian cancer comprises a mixture of normal and tumour cells (230, 361). There was considerable variability within and between individual biopsies. However, a median of 37 % of the area of each tissue section was occupied by stromal cells, 43 % by epithelial tumour areas, the rest being regions of necrosis and space (real or artefactual) (230). Within the stromal areas, the majority of infiltrating leukocytes were $CD3^+/CD8^+/CD45RO^+$ T cells and $CD68^+$ macrophages. These cells were also found amongst the epithelial tumour cells.

The cytokine context of the ovarian tumour microenvironment was generally proinflammatory. There was frequent expression of TNF-α, IL-1α, IL-6 and several growth factors, but little expression of IFN-γ, IL-2, IL-4 and IL-7, which are required for lymphocyte functions (361). CC chemokines were also present and they may control the leukocyte infiltrate (230). The CC chemokine CCL2 was expressed by both tumour cells and infiltrating cells (231). Correlations between the number of CCL2 expressing cells and the $CD8^+$ and $CD68^+$ infiltrate suggested that CCL2 was a predominant chemokine but it was not clear how the chemokine network was functioning.

In contrast to the findings with CC chemokines, receptor expression was limited in solid tumours, with CCR1 predominating. CCR1 mRNA expression correlated with the $CD8^+$ infiltrate, and CCR1 mRNA also appeared to be expressed by macrophages. CCL2 is not a ligand for this receptor and the major receptor for CCL2, CCR2b, was not detected in the solid tumours, as has previously been reported (180). CCR2 may be

downregulated by proinflammatory cytokines present in solid tumours (179). Expression of CC chemokine receptors was not seen on ovarian tumour cells, or ovarian cancer cell lines.

The present study demonstrates the importance of studying both chemokines and their receptors in a tissue and suggests that the CC chemokine network in solid tumours of ovarian cancer is controlled at the level of CC chemokine receptors. It is possible that CC chemokines such as CCL2 attract peripheral leukocytes into the tumour tissue but once there, they lose the ability to respond to this and other CC chemokines because of receptor downregulation. This traps cells in the tumour microenvironment and changes their chemokine response profile.

Control of the chemokine network by receptor expression makes sense. As a range of chemokines are expressed by the tumours there will be conflicting chemoattractant gradients that an individual cell can follow. These gradients may be difficult to regulate, with chemokines being retained by the extracellular matrix. Chemokine receptor expression, in contrast, can be rapidly modulated by LPS, chemokines, cytokines (179, 406-408, 415, 416) and, as shown in this chapter, hypoxia . Thus, microenvironmental control of chemokine receptor expression will track an individual cell along an appropriate gradient.

Hypoxia is an important factor in solid tumours; it can be an important prognostic indicator in gynaecological cancer (417) and has been shown to regulate the expression of the chemokines CCL2 (377) and CXCL8 (367) and the chemokine receptor CXCR1 (418). Hypoxia is also being exploited as a therapeutic strategy in tumours (419). Hypoxia was a strong stimulus for the upregulation of CCR1. The presence of potential HIF-1 binding sites suggests that CCR1 could be a target for transcriptional regulation by HIF-1, and could account for the upregulation seen in THP-1 cells under hypoxic

conditions. However, further work is required to determine if any of these binding sites are functional. Preliminary analysis of the CCR2 promoter sequences showed no similar HRE consensus sequence in the promoter region.

Hypoxia may have time dependent effects on cell migration because it has previously been shown that hypoxia is a rapid and potent 'stop' signal, inhibiting migration after as little as 30 minutes of exposure (377, 378, 414); this may account for the accumulation of macrophages seen in areas of necrosis in solid ovarian tumours. The mechanism for this inhibition of migration was shown to involve upregulation of a MAPK phosphatase, MKP-1. The speed of the response suggested that HIF-1 was not involved. The upregulation of CCR1 mRNA expression reported here peaked much later at 24 hours. Chronic hypoxia may enable macrophages to respond again to CCL3, 4 and 5, and thereby move away again from hypoxic regions.

Tumour-associated macrophages (TAM) may contribute to tumour growth and spread by providing growth and survival cytokines, angiogenic factors and proteases for remodelling the extracellular matrix (228, 420). Nesbit *et al* recently showed that high levels of CCL2 over-expression in melanoma cells led to tumour destruction in nude mice, due to a massive monocyte/macrophage infiltrate. However, low levels of CCL2 over-expression led to an angiogenic effect mediated through TAM, which resulted in increased tumour growth (234). Inhibiting the tumour infiltrate may inhibit growth and spread of the malignant cells and chemokine receptors are an obvious target for such intervention. A recent study of acute and chronic graft rejection models is of interest. Graft survival in mice with a targeted gene disruption of CCR1 was significantly prolonged and permanent engraftment occurred in some of these mice (421). We propose that the CCR1 receptor may also be a therapeutic target in human epithelial ovarian cancer.

Development of a three dimensional model of human ovarian cancer (tumour spheroids), where leukocytes can infiltrate in a manner akin to that *in vivo*, allows various aspects of leukocyte trafficking to be explored. Potential chemokine receptor antagonists, and other factors which may influence chemokine/chemokine receptor expression can be assessed for their ability to inhibit host cell infiltration of solid tumours.

The next chapter investigates the role of chemokines/chemokine receptors in generating the leukocyte infiltrate in ovarian cancer ascites.

Conclusions from this chapter:

- A range of CC chemokines are expressed in solid tumours
- CC chemokine receptor expression is restricted. Only CCR1 is expressed in the majority of biopsies.
- CCR1 expression correlates with the $CD8^+$ infiltrate, and is also expressed by $CD68^+$ macrophages.
- CCR1 is upregulated by hypoxia in a monocytic cell line.
- CCR1 may be a therapeutic target in human ovarian cancer.
- An *in vitro* three dimensional model of human ovarian cancer can be used as a tool to study leukocyte infiltration of solid tumours.

Chapter 4. CC chemokine and CC chemokine receptor expression in ascites

4.1. Introduction

Early growth of epithelial ovarian cancer is usually confined to the ovaries whereas in advanced stages of ovarian cancer, the peritoneal wall, diaphragm and omental structures are seeded with micro and macrometastases of tumour cells. Growth of ovarian cancer (stages Ic-IV) is also associated with the development of ascites (332). Abdominal distension caused by intractable ascites can be a major cause of discomfort and distress for patients, hence the need for palliative paracentesis.

Ascites consists of a proteinaceous fluid with variable numbers of suspended cells and debris. Ascitic fluid arises as a plasma exudate; formation results from an imbalance between the influx and efflux of fluid from the peritoneal compartment (333). This is due to a combination of increased microvascular permeability mediated by the angiogenic development of immature blood vessels (334), the production of vascular permeability agents including VEGF (335-337) and the blockade of lymphatic drainage by cells present in the ascites (338). The cellular population in ascites consists of differing proportions of tumour cells, mesothelial cells, fibroblasts, macrophages, leukocytes and red blood cells (402, 422). Changes in cell-cell and cell-extracellular matrix interactions may be responsible for the peritoneal dissemination of ovarian carcinoma (423).

The ascitic fluid is rich in cytokines and growth factors that are secreted by the tumour cells and by mesothelial cells lining the peritoneal cavity, some of which can act to directly stimulate tumour cell growth (363, 424-426).

With the aim of further understanding chemokine action in human ovarian cancer, we investigated CC chemokine receptors and their ligands in ascitic fluid samples. The cellular populations in human ovarian ascitic disease were also quantitatively assessed and related to chemokine/chemokine receptor expression.

4.2. Aim of the chapter

The aim of this chapter was to investigate the cellular composition of ovarian cancer ascites and relate this to the expression of chemokines and chemokine receptors.

This work was performed in collaboration with Dr David Milliken and Dr Julia Wilson in our laboratory: David Milliken collected the ascites samples and performed many of the ELISAs; Julia Wilson performed a majority of the FACS analysis.

4.3. Results

4.3.1. Cell populations in ascitic fluid

Flow cytometry was used to determine the cellular composition of 20 samples of ascites from patients with ovarian cancer. Tumour cell content was assessed by staining for HER2/neu. This antigen is overexpressed in around 75 % of ovarian cancer cells (427); it is possible that in this study not all tumour cells within the ascitic fluid were detected and the actual numbers of tumour cells may be greater. There was considerable variation within the cell populations (Figure 4.1.) Tumour cell content was variable (median 8 %, range 0-61 %), numbers of $CD14^+$ macrophages were also variable (median 27 %, range 0-66 %). There were usually high numbers of $CD3^+$ T lymphocytes present (median 59 %, range 1-89 %) which were predominantly $CD4^+$ T cells (median 51 %, range 1-65 %), rather than $CD8^+$ T cells (median 10 %, range 0-31 %) (Figure 4.2.). In no sample were B cells ($CD19^+$), NK cells ($CD56^+$) or mature dendritic cells ($CD83^+$) detected (data not shown). The T cell subsets in ascitic fluid differed significantly from solid ovarian tumours which contain predominantly $CD8^+$ T

cells (with few or no $CD4^+$ T cells) and macrophages (230). As T lymphocytes and macrophages typically respond to CC chemokines, we studied a range of these and their related receptors in ascites.

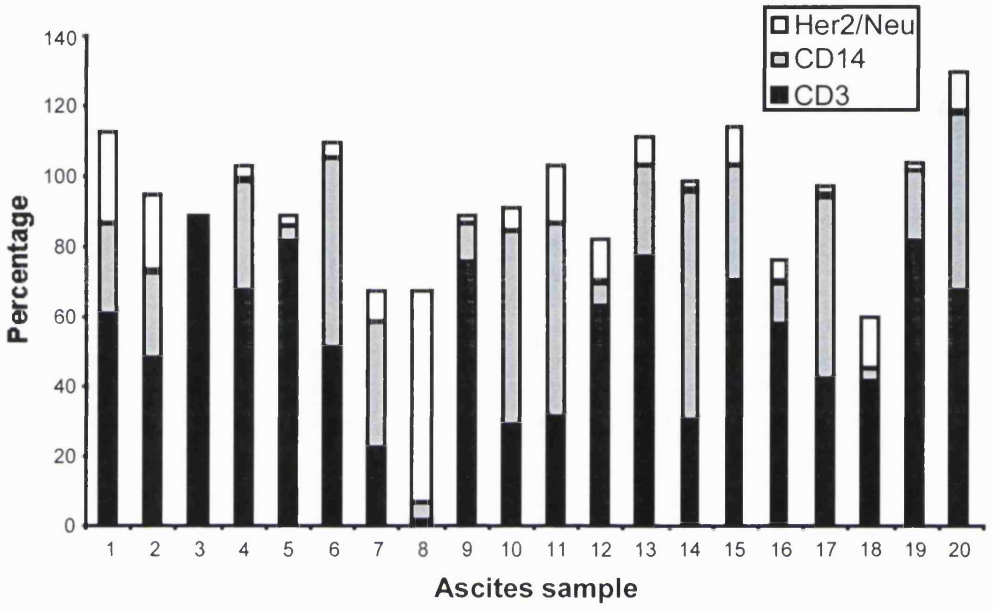


Figure 4.1. Cellular composition of ascitic fluid. Percentage of cells positive for CD3 (black), CD14 (grey) and HER2/neu (unfilled) was determined by flow cytometry in 20 ovarian ascites samples.

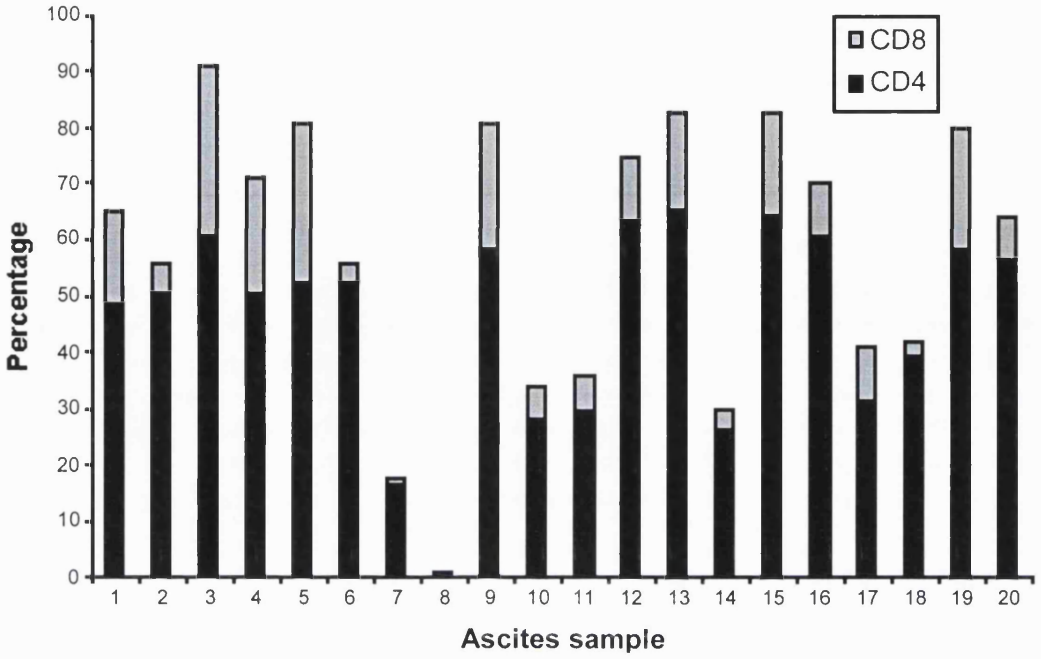


Figure 4.2. T cell component of ascitic fluid. Percentage of cells positive for CD4 (black) or CD8 (grey) within the $CD3^+$ T cell population in ascitic fluid was determined by flow cytometry in 20 ovarian ascites samples.

4.3.2. Chemokine expression in ascites

4.3.2.i. RT-PCR for chemokines in ascites cell isolates

The cellular component of ascitic fluid was isolated by centrifugation and RNA was prepared. RT-PCR was then used to screen for six CC chemokines (CCL2, CCL3, CCL4, CCL5, CCL8 and CCL22). Each of these chemokines was expressed in 85-100 % of the samples studied (Figure 4.3.).

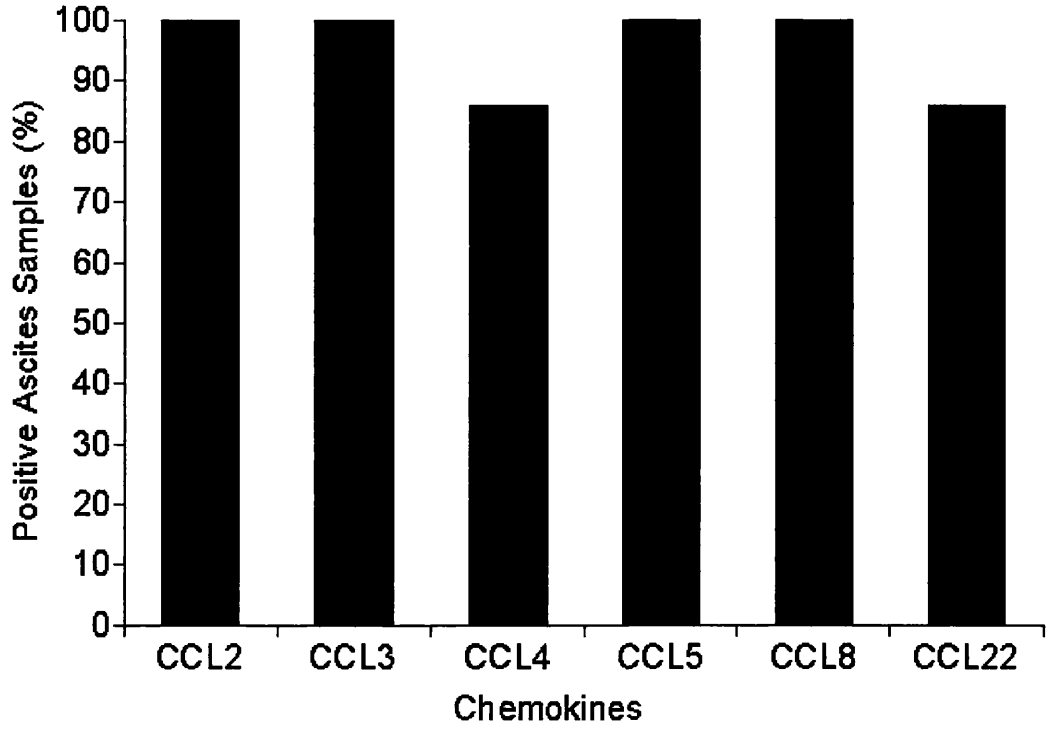


Figure 4.3. Chemokine mRNA expression in ascites. RT-PCR was used to screen for mRNA expression of six CC chemokines in cells isolated from ovarian cancer ascites.

4.3.2.ii. ELISA for chemokine protein levels in ascitic fluid

CCL2, CCL3, CCL4, and CCL5 protein levels were measured in 66 samples of ascitic fluid from ovarian cancer patients by ELISA (Figure 4.4.). The total protein concentration in ascites was reasonably constant (range = 13-75 mg/ml; median = 44 mg/ml), therefore, chemokine concentrations were expressed as pg/ml. All 66 samples contained the CC chemokine CCL2 with a range from 103-19,195 pg/ml (median 1778 pg/ml), this was the most abundant CC chemokine. The median concentrations of CCL3, CCL4 and CCL5 were approximately one log lower at 60, 182 and 44 pg/ml, respectively. CCL3 was found in 62/66 samples, range 6-4840 pg/ml. CCL4 was found in all 66 samples, range 33-11,040 pg/ml. CCL5 was found in 63/66 samples, range 3-1903 pg/ml.

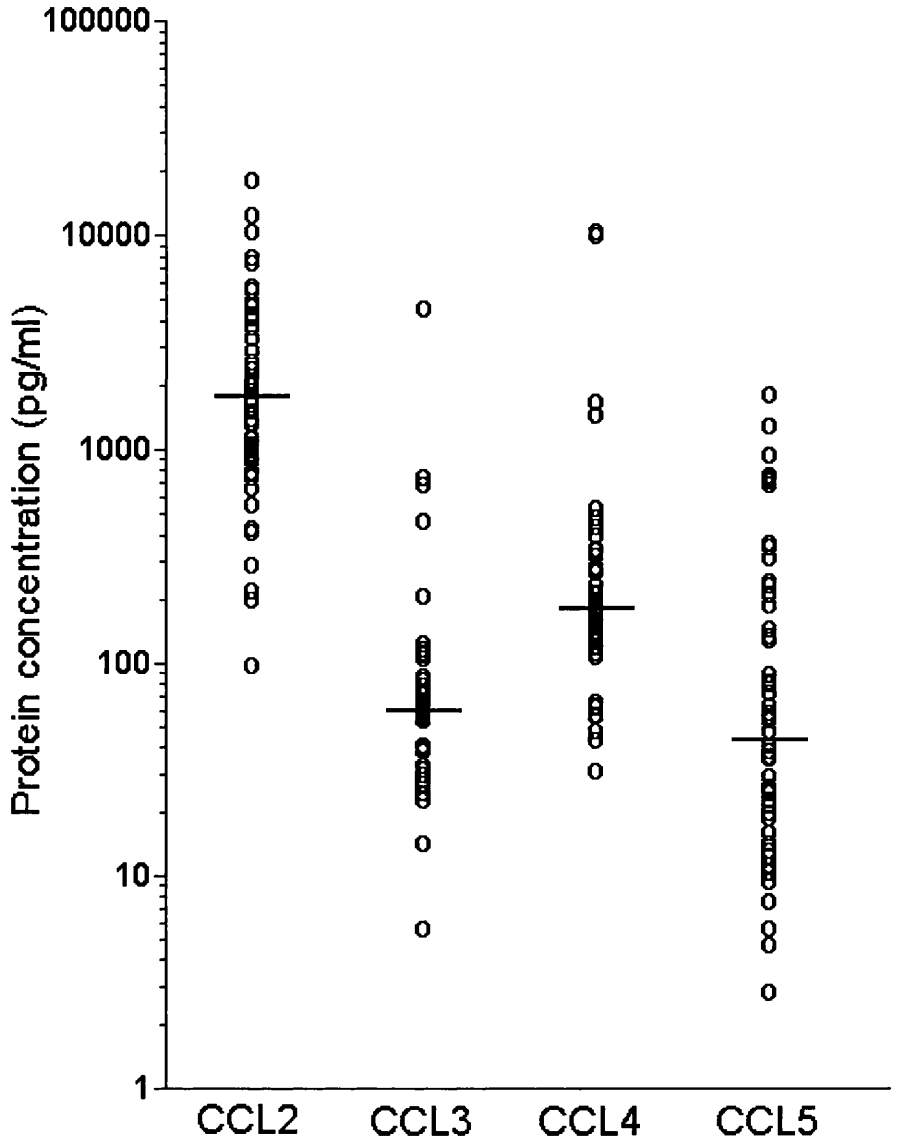


Figure 4.4. ELISA for chemokine protein in 66 samples of ascitic fluid. Bars represent median values.

4.3.2.iii. ELISA for chemokine protein levels in plasma from ovarian cancer patients

In addition to ascitic fluid, limited amounts of plasma were obtained from a small number of patients. Protein concentrations for CCL2, CCL3, CCL4 and CCL5 were determined by ELISA in these plasma samples. CCL2, CCL3 and CCL4 were present in low concentrations, with medians of 341 pg/ml, 12 pg/ml and 54 pg/ml respectively (Figure 4.5.). CCL2 was present in 12/12 samples, with range 110-1888 pg/ml; CCL3 was present in 4/10 samples, with range 3-19 pg/ml; and CCL4 was present in 12/13

samples, with range 5-120 pg/ml. CCL5 was detected at extremely high levels, with a median of 28,950 pg/ml, however this was likely due to release of stored CCL5 from platelets (data not shown) (428). These results suggest that a chemokine gradient may exist between plasma and ascites, which may partially explain the leukocyte infiltrate seen in ascites.

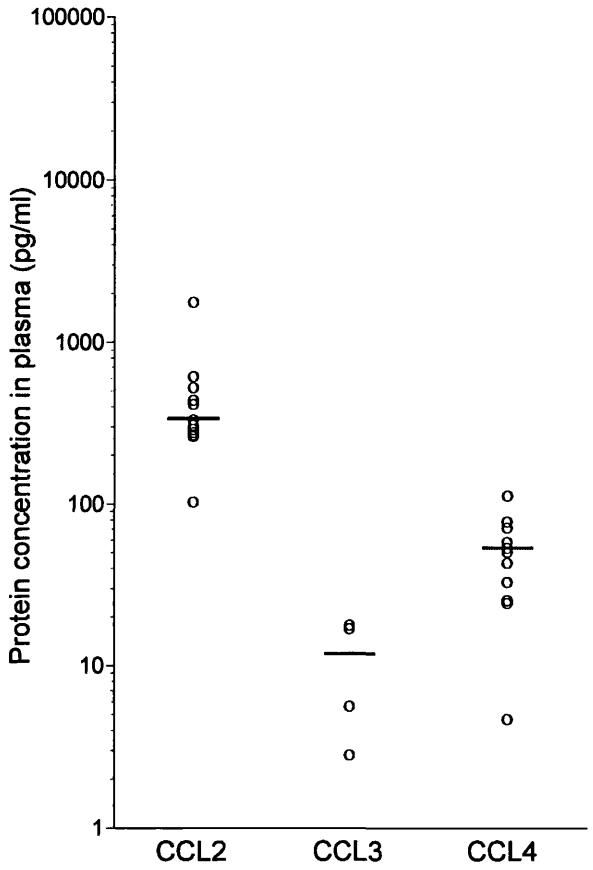


Figure 4.5. ELISA for chemokine protein in samples of patient plasma. Bars represent median values. CCL2 was present in 12/12 samples, CCL3 was present in 4/10 samples and CCL4 was present in 12/13 samples.

4.3.3. Chemokine receptor expression in ascites

4.3.3.i. RNase protection assays for chemokine receptors in ascites cell isolates

CC chemokine receptor expression was analysed by RPA in seven samples of RNA isolated from ascites. The receptor expression was similar to that of the normal PBMC

control (Figure 4.6. A and B), with a majority of samples positive for each receptor. This contrasts with the findings described in Chapter 3 for solid ovarian tumour biopsies, where CCR1 was the only CC chemokine receptor detected in a majority of cases. The level of chemokine receptor expression in five of the ascites samples was compared with that of PBMC from the same patients using densitometry. When normalised to the "housekeeping" gene, L32, the level of expression for CCR2, 3 and 4 was similar in PBMC and ascites, but CCR1 and CCR5 appeared to be more strongly expressed by the ascites cells ($P<0.05$) (Figure 4.7.).

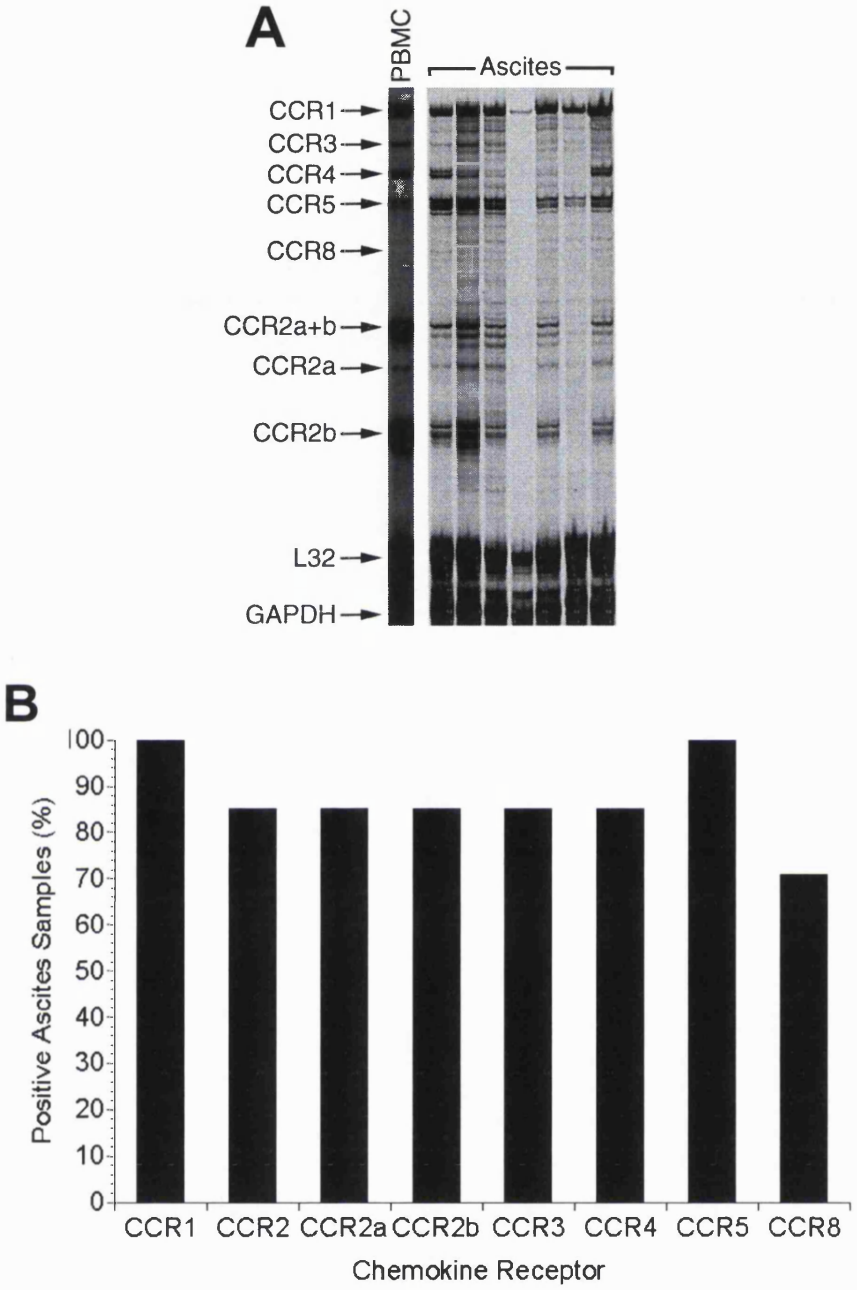


Figure 4.6. Chemokine receptor expression in ovarian cancer ascites. (**A**) RPA for CC chemokine receptor expression in normal PBMC, and cell isolates from ovarian cancer ascites. (**B**) Percentage of samples expressing CC chemokine receptor mRNA in cell isolates from ovarian cancer ascites, using the data derived from A.

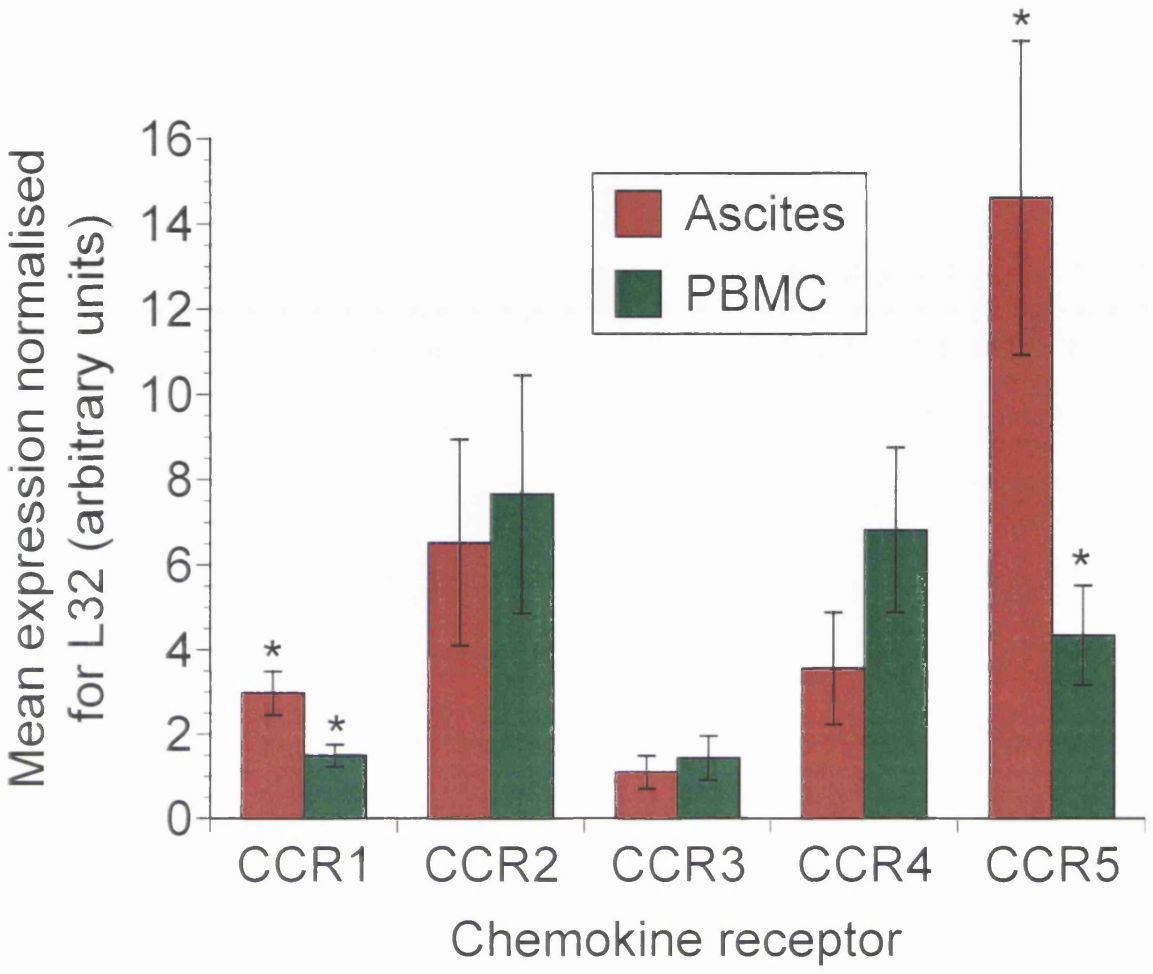


Figure 4.7. Densitometric analysis of CC chemokine receptor expression in ascites and PBMC from the same patients. * denotes statistical significance (P<0.05). Bars represent the SEM.

4.3.3.ii. Chemokine receptor protein on ascites cells

Two-colour flow cytometric analysis was used to determine the phenotype of chemokine receptor expressing cells, freshly isolated from three samples of ovarian cancer ascites (Figure 4.8. and Table 4.1.). Cell surface expression of CCR1, CCR2 and CCR5 was detected on the majority of $CD14^+$ cells. On $CD4^+$ T cells, CCR1 was expressed by the majority of cells; CCR2 expression was detected on between 25-80 % of cells, and CCR5 was detected on 23-70 % of the cells. On $CD8^+$ T cells CCR1 was expressed by the majority of cells; CCR2 and CCR5 were expressed by approximately

5-45 % of cells. Thus, more $CD4^+$ than $CD8^+$ T cells expressed CCR2 and 5, suggesting a difference in the chemokine receptor profile of these cell types. No CCR expression was detected on $HER2/neu^+$ tumour cells (data not shown).

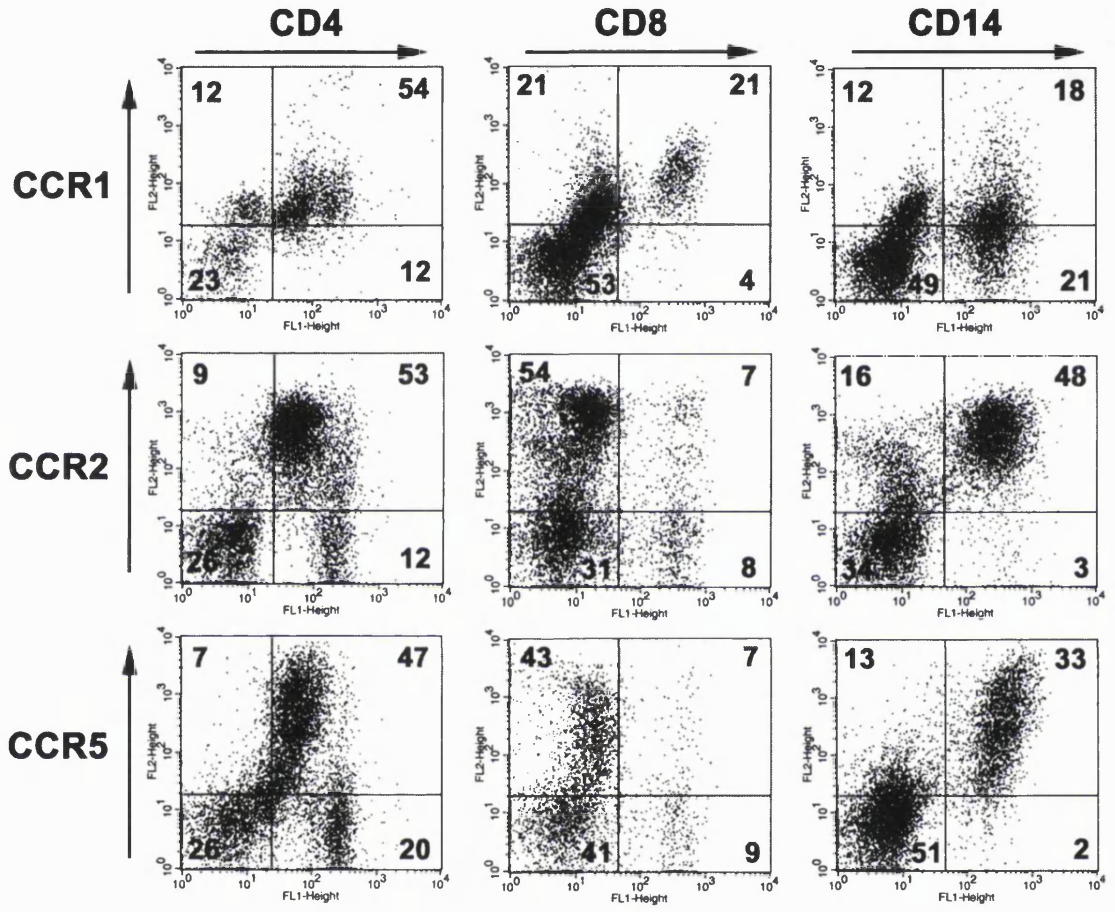


Figure 4.8. Chemokine receptor expression on cells isolated from ovarian ascites as determined by flow cytometry. CCR1, CCR2 and CCR5 expression on $CD4^+$, $CD8^+$ and $CD14^+$ cells. Numbers show the percentage of cells in each quadrant.

		Percentage of cells positive for each receptor		
Sample	Cell type	CCR1	CCR2	CCR5
A	$CD4^+$	82	82	70
	$CD8^+$	84	44	45
	$CD14^+$	45	94	93
B	$CD4^+$	88	75	42
	$CD8^+$	87	48	27
	$CD14^+$	80	96	68
C	$CD4^+$	57	26	19
	$CD8^+$	91	6	4
	$CD14^+$	57	88	60

Table 4.1. Chemokine receptor protein expression in ascites. Percentage of each leukocyte type expressing CCR1, CCR2 and CCR5, in 3 samples of ovarian cancer ascites (A, B and C).

4.3.4. Correlations between chemokines, chemokine receptors and cell types

4.3.4.i. Correlations between chemokines

The non-parametric Spearman's rank correlation was used to examine the relationship between the protein concentrations of the four CC chemokines studied. Significant correlations were found between the levels of CCL3 and CCL4 ($r_s = 0.56$; $P = 0.0001$), (Table 4.2.) and between the levels of CCL2 and CCL5 ($r_s = 0.32$, $P = 0.008$). Weaker associations were observed between CCL3 and CCL5 ($r_s = 0.29$; $P = 0.02$) and CCL4 with CCL5 ($r_s = 0.28$; $P = 0.02$). No other chemokine pair gave a significant correlation. These correlations may suggest that CCL3 and CCL4 are produced by the same cell type, or are induced by the same stimulus in ovarian cancer ascites. This could also be true for CCL2 and CCL5.

	CCL2	CCL3	CCL4	CCL5
CCL2	-			
CCL3	0.04 (0.78)	-		
CCL4	0.11 (0.11)	**0.56 (<0.0001)**	-	
CCL5	**0.32 (0.008)**	**0.29 (0.02)**	**0.28 (0.02)**	-

Table 4.2. Statistical analysis between the concentrations of chemokines present in ascites. Spearman correlation coefficients and (P-value) between the chemokines are shown. P<0.05 indicates statistical significance (bold).

4.3.4.ii. Correlations between chemokines and cell counts

Despite the high levels of chemokine protein, there were few correlations found between the total cell counts in the ascites and the concentration of any chemokine (Table 4.3.). CCL5 was expressed at higher levels when $CD3^+$ cells were prevalent (r_s = 0.54; P = 0.01), and there was a negative correlation between CCL5 and $CD14^+$ cell presence (r_s = -0.47; P = 0.03).

	CCL2	CCL3	CCL4	CCL5
CD3	-0.14 (0.57)	0.09 (0.70)	-0.12 (0.62)	**0.54 (0.01)**
CD14	0.08 (0.75)	-0.24 (0.31)	-0.26 (0.28)	**-0.47 (0.03)**
HER2	0.34 (0.14)	-0.03 (0.90)	0.11 (0.64)	-0.09 (0.71)

Table 4.3. Statistical analysis between the chemokines and cell populations within ovarian cancer ascites. Spearman correlation coefficients and (P-value) between the chemokines and cell populations within ovarian ascites are shown. P<0.05 indicates statistical significance (bold).

4.3.5. Comparisons with the solid tumour

In this chapter and the previous chapter, differences have become apparent between the solid tumour microenvironment, and the ascitic microenvironment in terms of chemokines and chemokine receptors. Chemokine receptor expression was restricted in solid tumours, and this was possibly related to the effects of cytokines and oxygen tension on chemokine receptor regulation. In ascites however, the majority of chemokine receptors were expressed, and this may reflect a substantially different microenvironment. Two potentially important factors were therefore investigated: oxygen tension and TNF-α expression. In chapter 3, low oxygen tension (hypoxia) was shown to be important for the upregulation of CCR1, while TNF-α can cause downregulation of chemokine receptors such as CCR2b.

4.3.5.i. Gas analysis of ascitic fluid

At the time of therapeutic tap, samples of ascitic fluid from four patients were subjected to gas analysis. This revealed that the $p0_2$ (range 59 - 95 mmHg) and pCO_2 (range 41 – 52 mmHg) were similar to that of arterial blood (approx. 95 mmHg and 40 mmHg respectively) and were therefore normoxic. This is in contrast to the solid tumour, where regions of hypoxia are probably common, particularly in areas of necrosis (see Section 3.3.3.ii.).

4.3.5.ii. TNF-α levels

TNF-α can regulate the expression of chemokine receptors, including CCR2b. TNF-α levels were assayed in 37 of the ascites samples by ELISA. Only 16 of the 37 samples had detectable levels of TNF-α (range 20-41 pg/ml). Thus, TNF-α levels are very low or absent in ascitic fluid. This is in contrast to the solid tumour where TNF-α mRNA is abundantly expressed and TNF-α protein levels are expected to be high (368, 399).

4.4. Discussion

This work is the first detailed study of chemokines and chemokine receptor expression in human ovarian cancer ascites. A range of cell types were found in ovarian cancer ascites. There were variable numbers of tumour cells, T cells and macrophages, but no B cells, NK cells or mature dendritic cells in the 20 different samples investigated. Ascitic fluid was rich in CC chemokines and the macrophages and T cells expressed CC chemokine receptor mRNA and protein.

Is the extent and phenotype of the leukocyte infiltrate in ovarian ascites related to chemokines and chemokine receptor expression? Gradients of chemokines usually cause tissue recruitment of leukocytes through effects on adhesion and endothelial transmigration (429). Therefore, chemokines present in ascites could form a gradient for leukocyte migration into the peritoneal cavity. However, not many associations were found between chemokine concentration and leukocyte numbers; this could be because multiple chemokines co-operate to attract a particular leukocyte.

There was a correlation between CCL5 expression in ascites and the presence of $CD3^+$ T cells suggesting that this chemokine could be important for recruiting T cells. This chemokine also correlated with the presence of $CD8^+$ T cells in solid ovarian tumours (230). The fact that the CD4:CD8 ratio differs between solid tumour and ascites may be related to CC chemokine receptor expression on the T cells. There were differing proportions of $CD4^+$ and $CD8^+$ T cells expressing CCR2 and CCR5 (the receptor for CCL5). CCR2 was expressed more by $CD4^+$ T cells than $CD8^+$ T cells and the same was true of CCR5. The lower number of $CD8^+$ T cells expressing CCR5 could account for the defective recruitment of these cells to the ascites compared with the solid tumour.

Overall, the profile of CC chemokine receptor expression in ascites was similar to that of leukocytes in peripheral blood and contrasted with the restricted expression of CC

chemokine receptors in solid ovarian tumours described in Chapter 3. This may be due to differences in the solid tumour and ascitic microenvironment. TNF-α levels in ascites are low, so this cytokine is unlikely to be responsible for the stimulation of chemokine production or downregulation of CC chemokine expression (180). Another important stimulus regulating chemokine production is hypoxia (377, 430). Solid tumours are likely to be hypoxic (376) and have high TNF-α levels (368) whereas ascites is normoxic with low TNF-α levels. These factors may contribute to the differences in CC chemokine receptor expression between the two tumour states.

There was a negative correlation between CCL5 concentration and $CD14^+$ macrophages. A majority of $CD14^+$ cells within ascites consistently expressed CCR1, CCR2 and CCR5; CCR5 and CCR1 are both receptors for CCL5. Chemokines typically induce migration *in vitro* with a bell-shaped dose response curve: for example, at 0.1–10 nmol/ml, CXCL8 induces neutrophil migration, but at higher concentrations, migration is inhibited (431). It is therefore possible that CCL5 was inhibiting macrophage migration. Alternatively, we found a correlation between the concentrations of CCL2 and CCL5, although CCL2 levels were consistently one log higher; at high CCL5 concentrations, CCL2 levels could even be sufficient to cause repulsion of macrophages, as has been shown for CXCL12 (SDF-1α) with T cells (432).

CCR1 was the predominant receptor found on leukocytes within the solid tumour (Chapter 3) and this receptor was also found on the majority of leukocytes within the ascites. The expression of CCR2 on $CD14^+$ cells within the ascites contrasts with the work of Sica *et al* who suggest CCR2 downregulation on ovarian tumour infiltrating macrophages (180). However, this may reflect the fact that the cells used in this study are freshly isolated from ascitic fluid and not purified or cultured.

In the next chapter, the expression of chemokine receptors by tumour cells was investigated. The role of chemokines/chemokine receptors in attracting leukocytes into the peritoneal cavity would be a useful mechanism to also explain the presence of tumour cells in ascites.

Conclusions from this chapter:

- There is a complex chemokine/chemokine receptor network in human ovarian cancer ascites.
- Ascites consists of variable numbers of tumour cells, T cells and macrophages.
- A range of CC chemokines are expressed by cells present in ascites.
- CC chemokine protein levels are variable, with MCP-1 being present at the highest concentration.
- CC chemokine receptor expression by cells in ascites is similar to that of PBMC.
- No CC chemokine receptors studied are expressed by tumour cells.
- Few associations exist between chemokine receptor expression, chemokine levels and cell counts - this is in contrast to the strong associations found in the solid tumour.
- High levels of chemokine protein in ascites may cause receptor desensitisation or inhibition of migration.

Chapter 5. Expression of CXCR4 on ovarian cancer cells

5.1. Introduction

The results of the previous two chapters and work by other laboratories show that there is strong evidence that chemokines are major determinants of the macrophage and lymphocyte infiltrate found in various cancers, including melanomas, carcinomas of the ovary, breast and cervix, and sarcomas and gliomas (230, 231, 433, 434). However, chemokines may play other roles in cancer. Some are potent angiogenic factors, while others can be angiostatic (255, 435). Alterations in the balance between these may contribute to the development of the tumour vasculature.

There is also the possibility that the malignant cells in common epithelial tumours may use chemokine gradients as part of the process of metastatic spread. The previous chapter suggested that chemokines/chemokine receptors are responsible for the movement of leukocytes into ascites. This is also the major route for the spread of ovarian cancer. If ovarian tumour cells expressed chemokine receptors then they could also move into the peritoneal cavity in response to chemokines. In addition, chemokines/chemokine receptors are important for directing leukocytes to secondary lymphoid organs: for example, maturing dendritic cells change their chemokine receptor profile and specifically migrate to secondary lymphoid organs (140). Ovarian cancer also metastasises to lymph nodes (330). This chapter investigates the expression of chemokine receptors by ovarian cancer cells at the level of mRNA and protein.

5.2. Aims of the chapter

This chapter will examine the expression of chemokine receptors by normal ovarian epithelium and epithelial ovarian cancer cell lines and primary cells in solid tumours and cancer ascites.

5.3. Results

5.3.1. Expression of CXCR4 mRNA by normal ovarian epithelium and ovarian cancer cells

5.3.1.i. RNase protection assay for chemokine receptors in normal ovarian epithelium

Total RNA from three samples of primary human ovarian surface epithelium was a kind gift from Dr Nelly Auersperg (Department of Obstetrics and Gynaecology, University of British Columbia, Vancouver, Canada). The samples were screened for expression of 13 chemokine receptors by Ribonuclease Protection Assay (RPA). No chemokine receptor expression was detected in any of the samples (Figure 5.1. A and B).

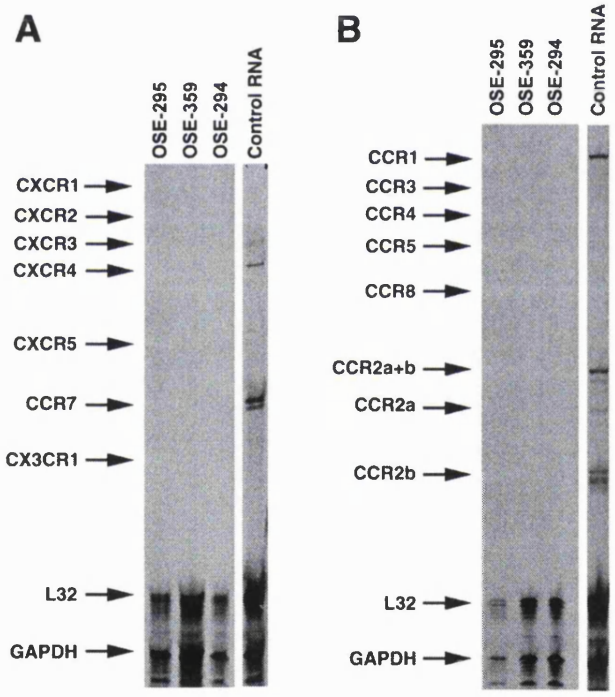


Figure 5.1. RNase protection assay for chemokine receptor expression in normal ovarian surface epithelium. (**A**) CXC chemokine receptors; (**B**) CC chemokine receptors. Control RNA (from Pharmingen) containing mRNA transcripts for some of the chemokine receptors, was included as a positive control for the assay.

5.3.1.ii. RNase protection assay for chemokine receptors in cell lines

Total RNA was extracted from six ovarian cancer cell lines (SKOV-3, IGROV, OVCAR-3, CAOV-3, PEO1, PEO14) and screened for chemokine receptor expression by RPA. The cells did not express CCR1, 2a, 2b, 3, 4, 5, 7, 8, CXCR1, 2, 3, 5, or CX_3CR1, but two of the six cell lines (IGROV, CAOV-3) strongly expressed CXCR4 mRNA (Figure 5.2. A and B). PEO1 and PEO14 also had detectable levels of CXCR4 mRNA (Figure 5.2. A).

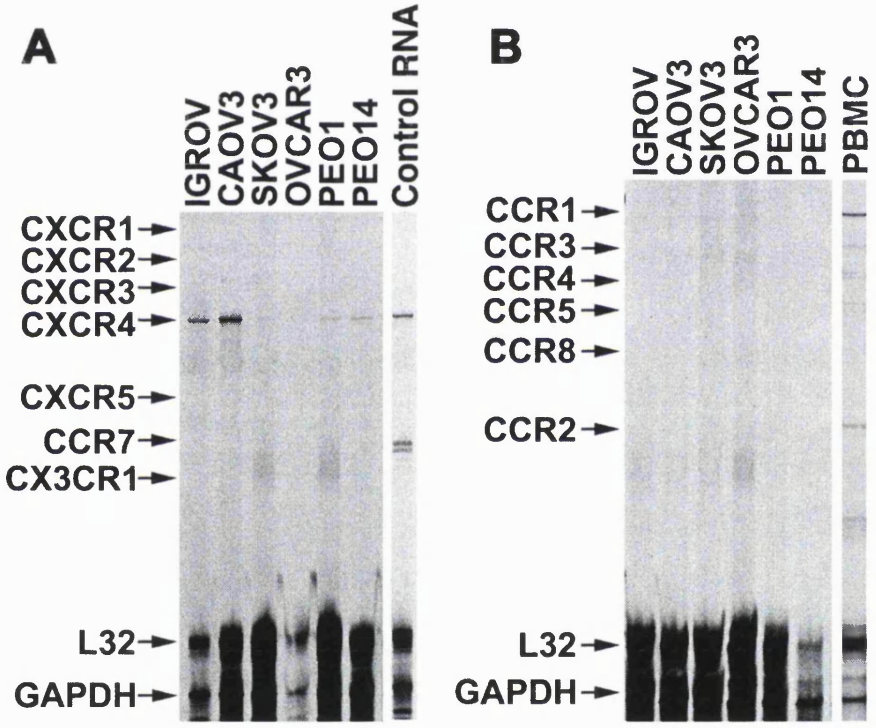


Figure 5.2. RNase protection assay for chemokine receptor expression in ovarian cancer cell lines. (**A**) CXC chemokine receptors. Control RNA (from Pharmingen) containing mRNA transcripts for some of the chemokine receptors, was included as a positive control. (**B**) CC chemokine receptors. PBMC total RNA was included as a positive control.

5.3.1.iii. RNase protection assay for CXC chemokine receptors in solid tumour biopsies

RPA was performed with 5 μg of total RNA to analyse CXC chemokine receptor mRNA expression in ten solid tumour biopsies (Figure 5.3.). CXC chemokine receptor mRNA expression was restricted in these solid tumours. CXCR4 was the only CXC chemokine receptor present in the majority of the samples, with 8/10 of the biopsies positive for this receptor mRNA. None of the other CXC chemokine receptors were detected using RPA.

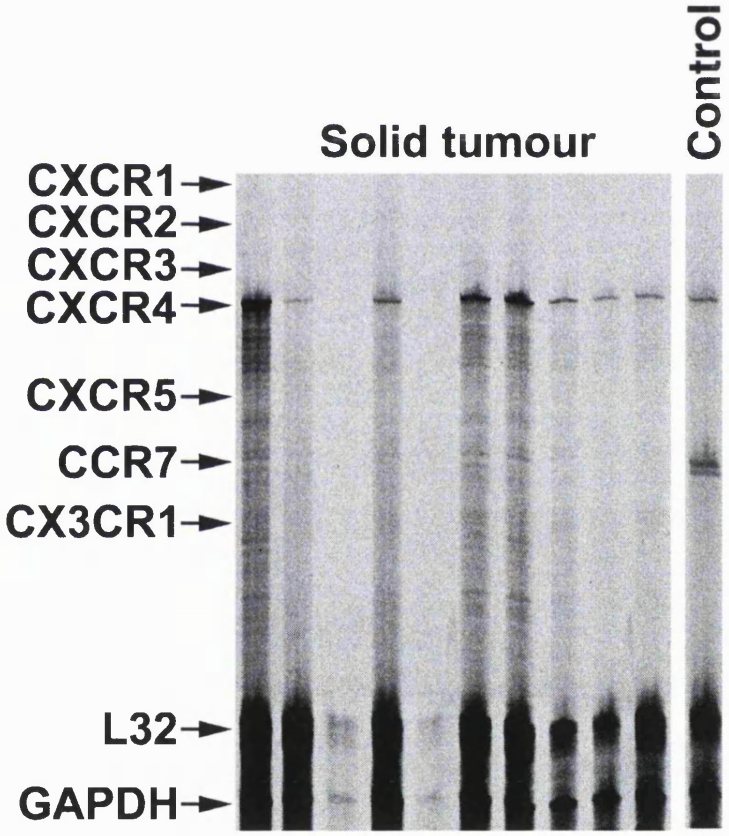


Figure 5.3. RNase protection assay for CXC chemokine receptors in solid human ovarian tumour biopsies. Control RNA (from Pharmingen) was included as a positive control.

Thus, CXC chemokine receptor mRNA expression was limited in solid tumours, analogous to the finding with CC chemokine receptors.

5.3.1.iv. RNase protection assay for CXC chemokine receptors in ascites cell isolates

RPA was used to analyse CXC chemokine receptor mRNA expression in 20 samples of ovarian cancer ascites (Figure 5.4.). CXCR4 was detected in 19/20 samples. mRNA for other receptors was also detected: 12/20 biopsies expressed CXCR3; 10/20 biopsies expressed CCR7 and 6/20 biopsies expressed CX3CR1.

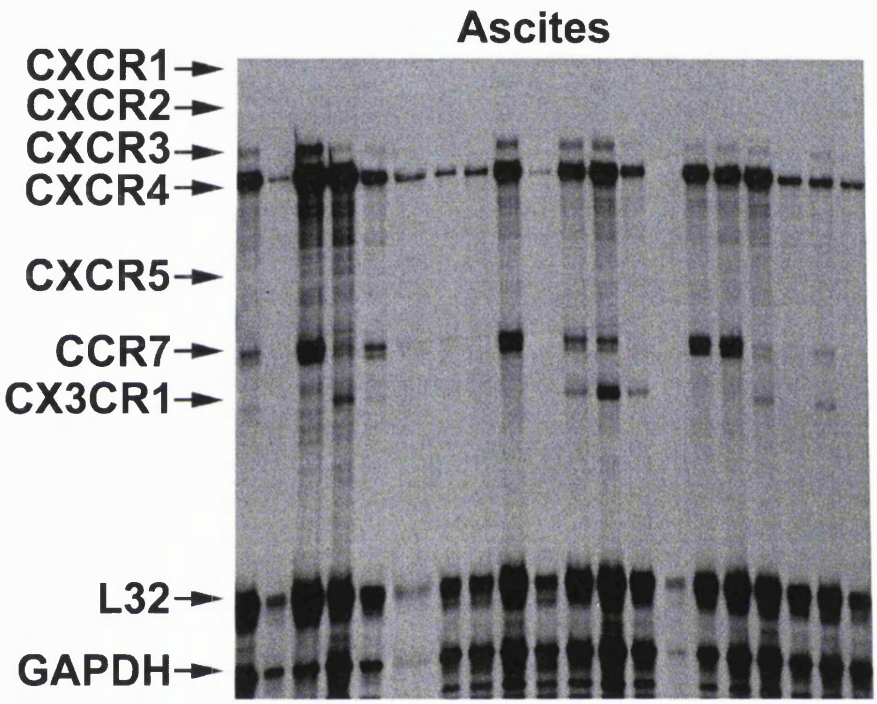


Figure 5.4. RNase protection assay for CXC chemokine receptors in ovarian cancer ascites.

5.3.2. Localisation of CXCR4 mRNA expression in solid tumour biopsies

In situ hybridisation to mRNA was performed on frozen sections from ten solid tumour biopsies to localise CXCR4 expression. Positive and negative controls were used: β-actin always gave a positive signal, demonstrating RNA integrity (data not shown); a sense riboprobe for CXCR4 was always negative with minimal background scatter (Figure 5.5.).

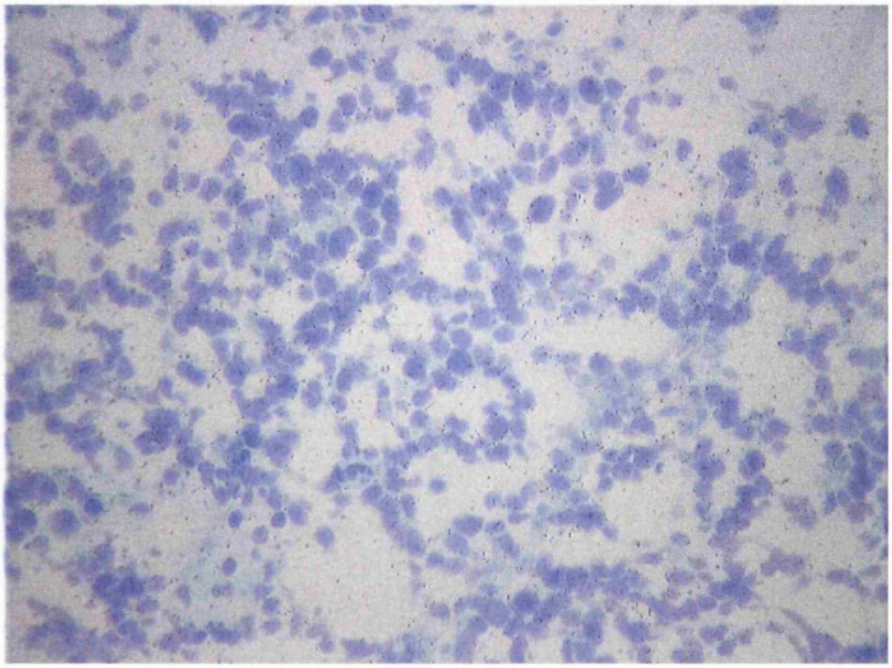


Figure 5.5. Negative control for *in situ* hybridisation. Tumour sections were probed with sense riboprobes for CXCR4, x 400.

5.3.2.i. CXCR4 mRNA expression on tumour cells

CXCR4 mRNA localised to a proportion of neoplastic cells in 10/10 biopsies (Figure 5.6., 5.7. and 5.8.). Expression of CXCR4 mRNA was not uniform throughout the tumour. In five of the biopsies, between 5 % and 20 % of tumour cells were labelled and in the other five, 1 – 5 % of tumour cells were positive.

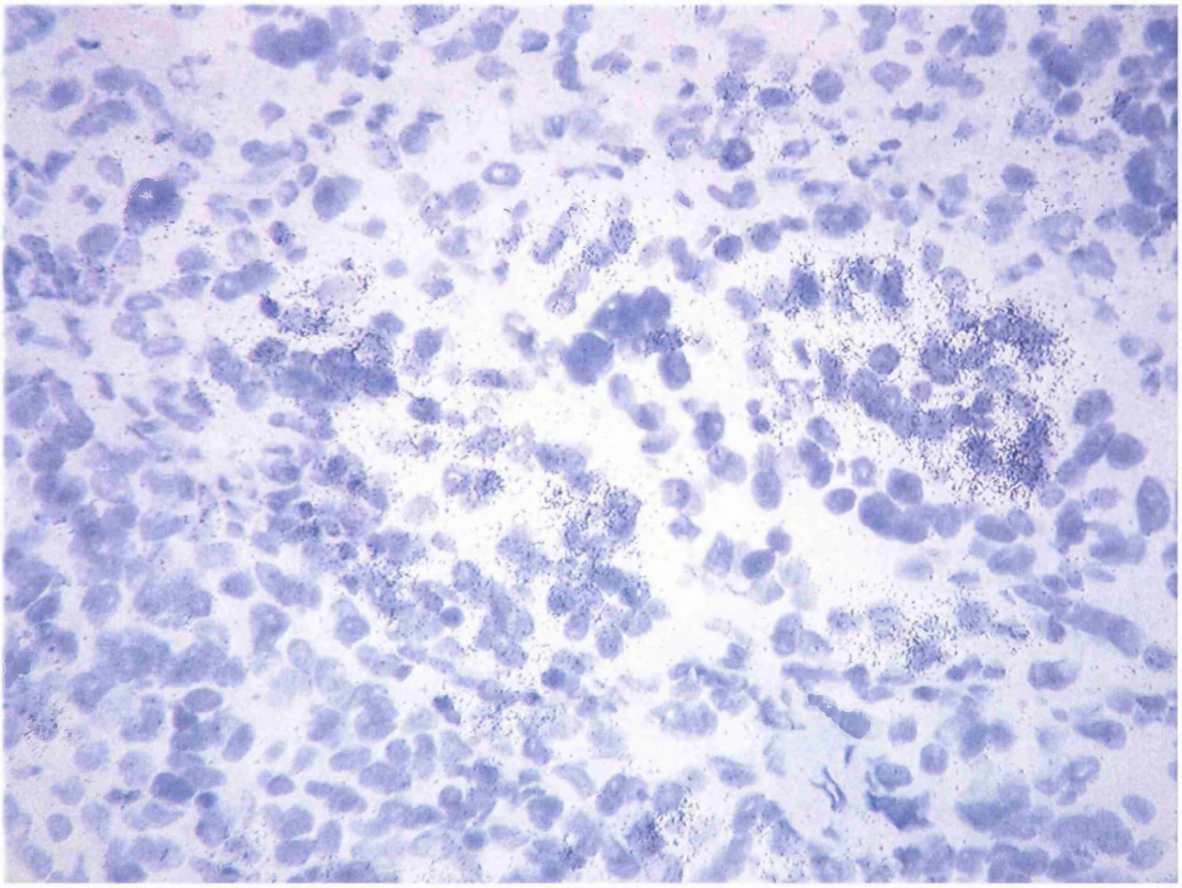


Figure 5.6. *In situ* hybridisation to CXCR4 in a solid tumour. There is a variable degree of labelling of two tumour epithelial cell groups in a serous carcinoma separated by a cellular stroma that is largely unlabelled, x 400.

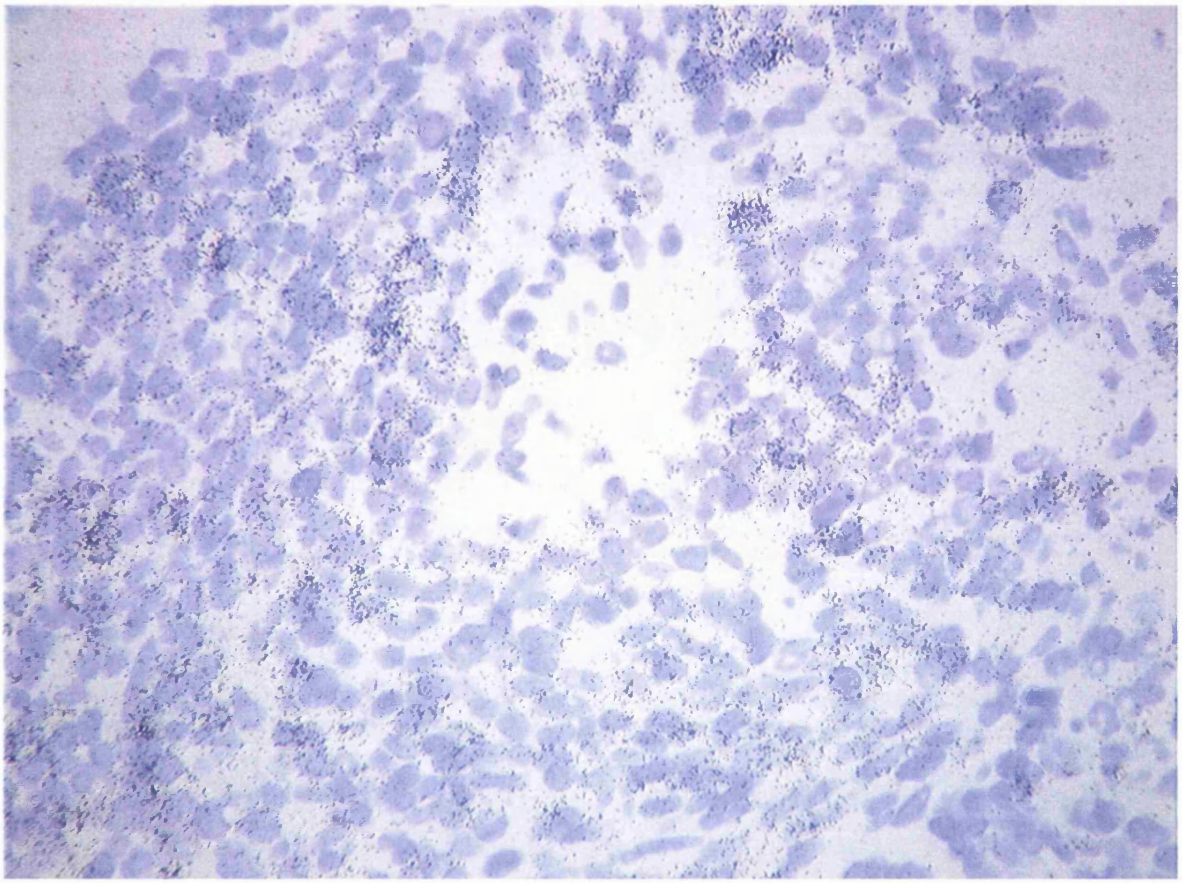


Figure 5.7. *In situ* hybridisation to CXCR4 in a solid tumour. A grade 3 serous carcinoma with heterogeneous labelling of tumour epithelial cells and focal weak labelling of some cells in the stroma, x 400.

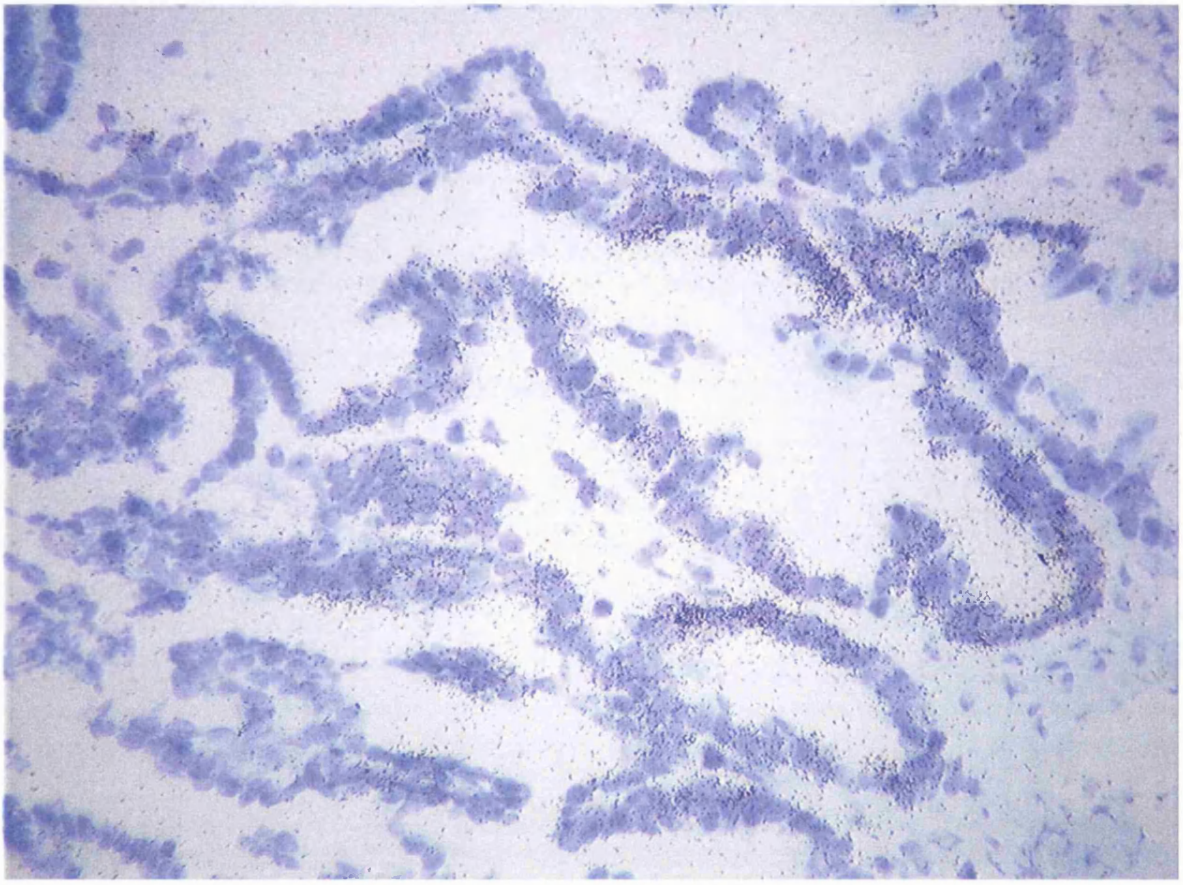


Figure 5.8. *In situ* hybridisation to CXCR4 in a solid tumour. A grade 1 serous carcinoma with neoplastic epithelial cells clearly labelled for CXCR4, x 400.

There was no correlation between CXCR4 positivity and areas of necrosis or 'hot spots' of angiogenesis.

5.3.2.ii. CXCR4 mRNA expression on stromal cells

CXCR4 mRNA was also detected in some mononuclear and endothelial cells. Tumours with a strong lymphoid infiltrate in the stroma showed highest mononuclear cell labelling for CXCR4 (Figure 5.9.). The presence and distribution of tumour infiltrating lymphocytes (TIL) was assessed by H&E staining and CD8 immunostaining, and the CXCR4 labelling pattern was clearly in excess of these populations in every sample

(Figure 5.10.). CD8 cells also showed a different distribution from CXCR4-labeled tumour cells. In addition it was possible to distinguish between neoplastic and TIL cell nuclei. Some endothelial cells within the tumour were also labelled in each biopsy (Figures 5.11. and 5.12.). CXCR4-expressing endothelial cells were detected in fibrovascular cores of papillary tumours, adjacent to the epithelial tumour cells, or within stromal invaginations (Figure 5.11.). Endothelial cells in tissue adjacent to the tumours were not positive for CXCR4.

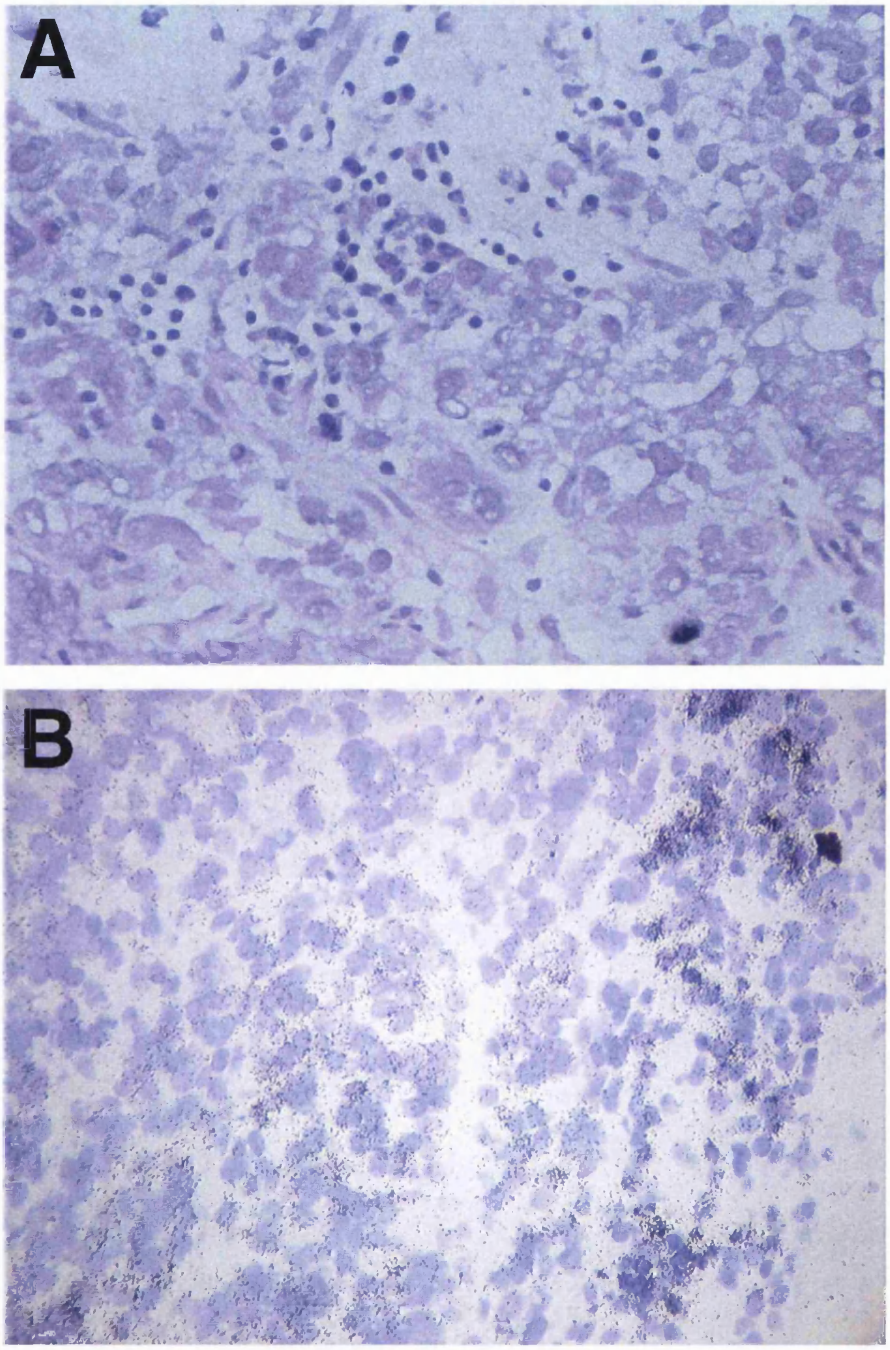


Figure 5.9. *In situ* hybridisation to CXCR4 mRNA in a solid tumour. (**A**) H&E section of Grade 3 serous carcinoma with stromal lymphoid infiltrate. (**B**) Serous carcinoma as in (A) with CXCR4 labelling of tumour epithelial cells (left of field). The lymphoid cells within the stroma (right of field) show a denser pattern of label, x 400.

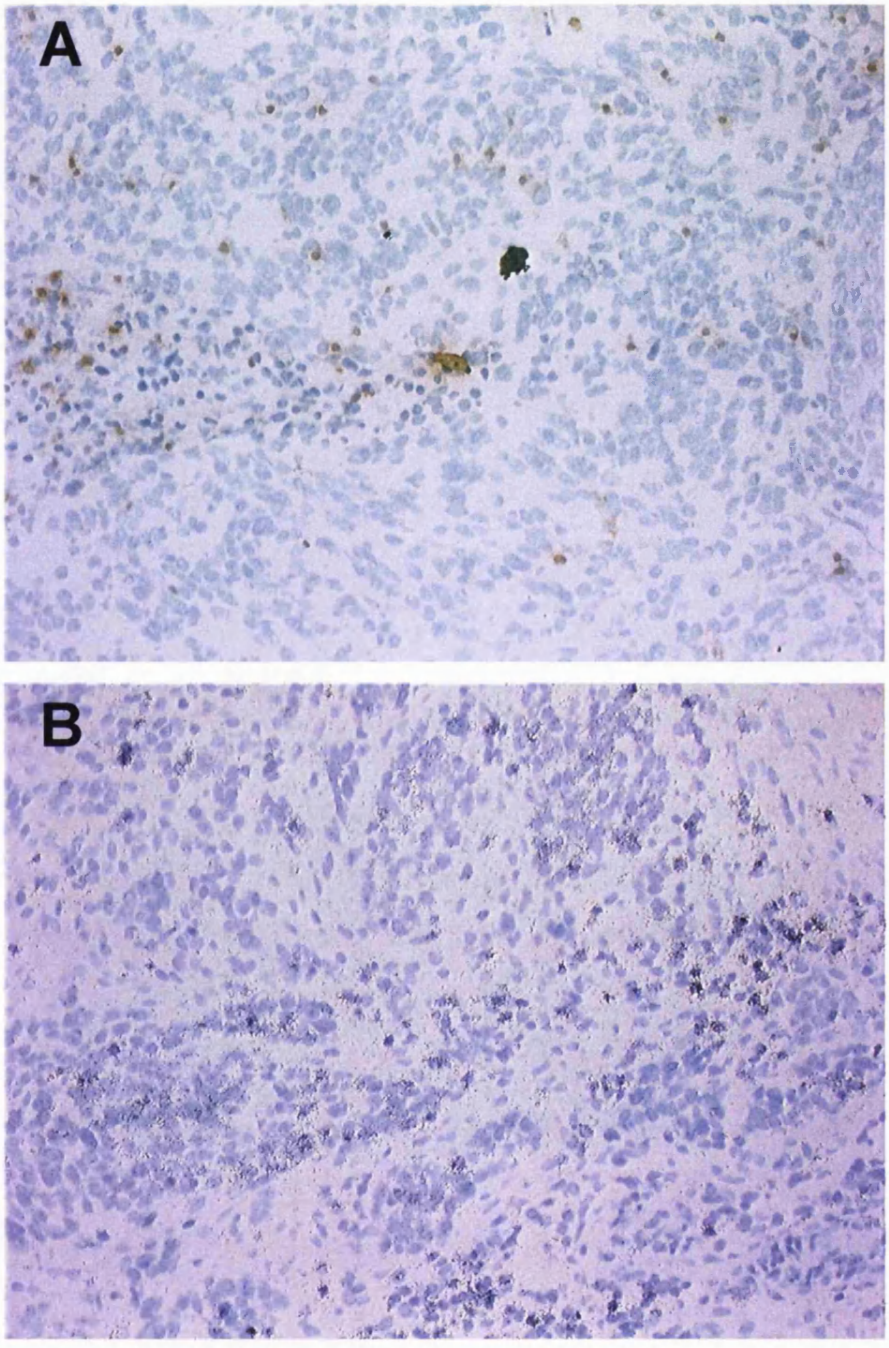


Figure 5.10. CXCR4 expression by $CD8^+$ T cells. (**A**) Immunohistochemistry showing $CD8^+$ T cells in a Grade 3 serous carcinoma, x 200 (**B**) ISH showing CXCR4 expression co-localising with $CD8^+$ expression, x 200.

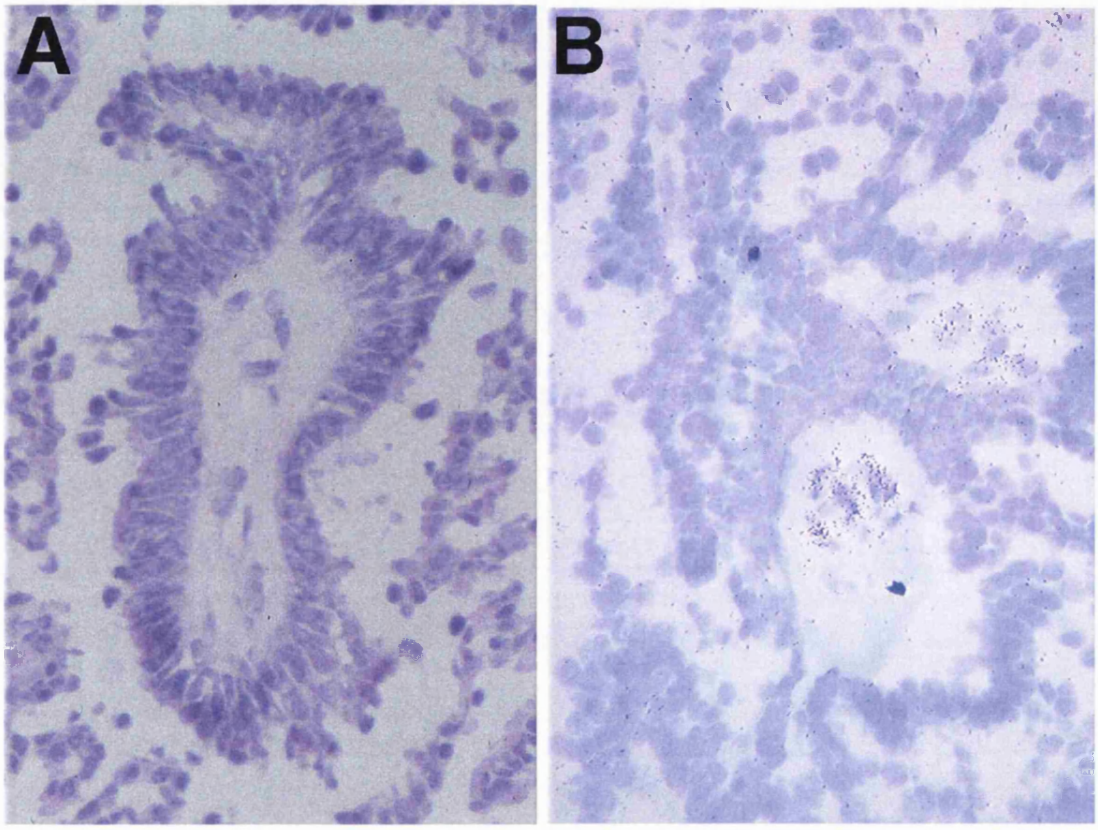


Figure 5.11. *In situ* hybridisation to CXCR4 mRNA in a solid tumour. Endothelial cells in the papillary core of a Grade 1 serous carcinoma (H&E stain, **A**) are clearly labelled for CXCR4 (**B**), x 400.

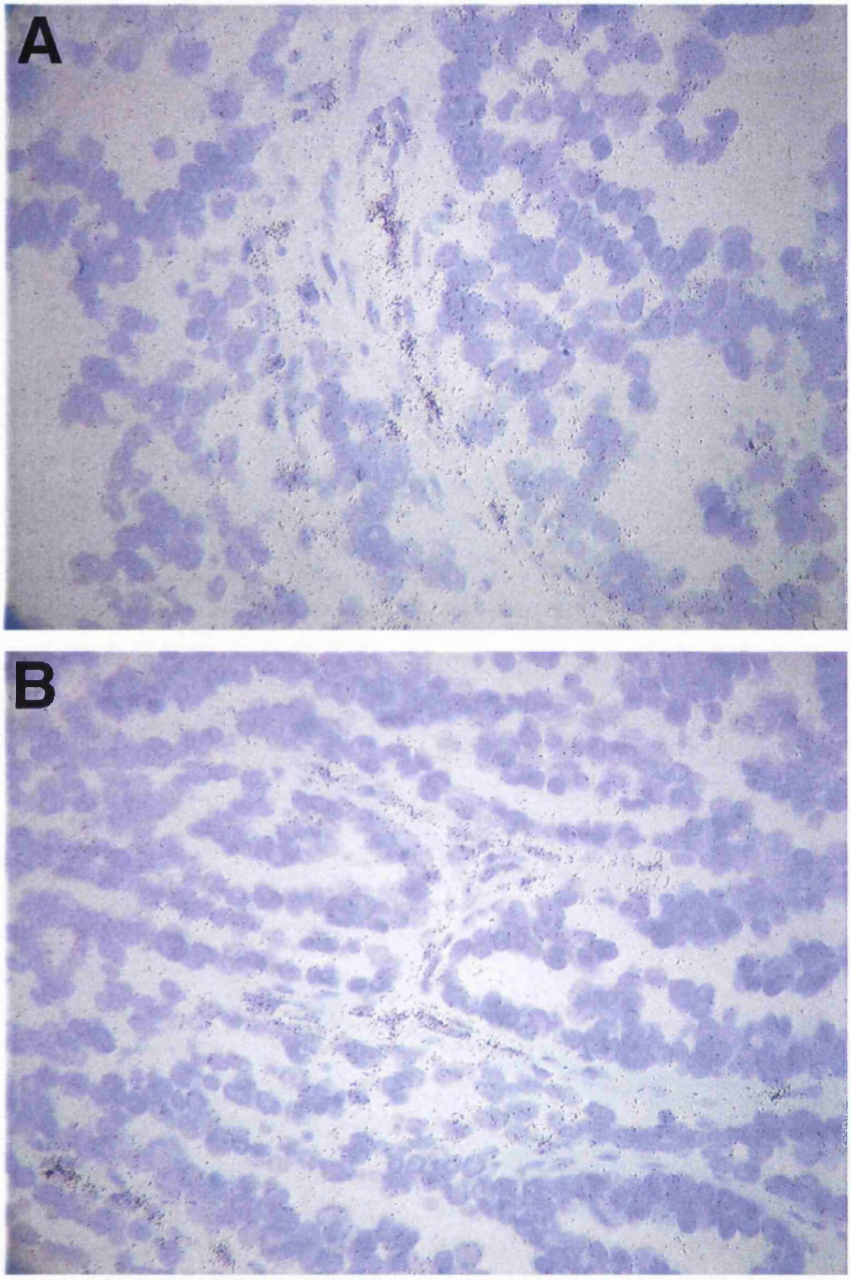


Figure 5.12. *In situ* hybridisation to CXCR4 in a solid tumour. (**A**) Stromal endothelial cell labelling in a Grade 2 serous carcinoma with equivocal epithelial cell labelling. (**B**) Tumour epithelial cells show a low index of CXCR4 labelling together with a linear pattern of CXCR4 labelled endothelial cells in the adjacent stroma, x 400.

5.3.3. CXCR4 protein expression on ovarian cancer cells

5.3.3.i. Detection of CXCR4 protein on IGROV and CAOV-3 cell lines

Flow cytometry was used to assess CXCR4 protein expression by IGROV and CAOV-3 cells, which were shown to express CXCR4 mRNA (Section 5.3.1.ii). Both these cell lines had detectable cell surface CXCR4 protein, although IGROV had greater expression than CAOV-3 (Figure 5.13.). Interestingly, CXCR4 protein expression diminished with increasing passage number, until becoming almost undetectable at around passage ten (data not shown).

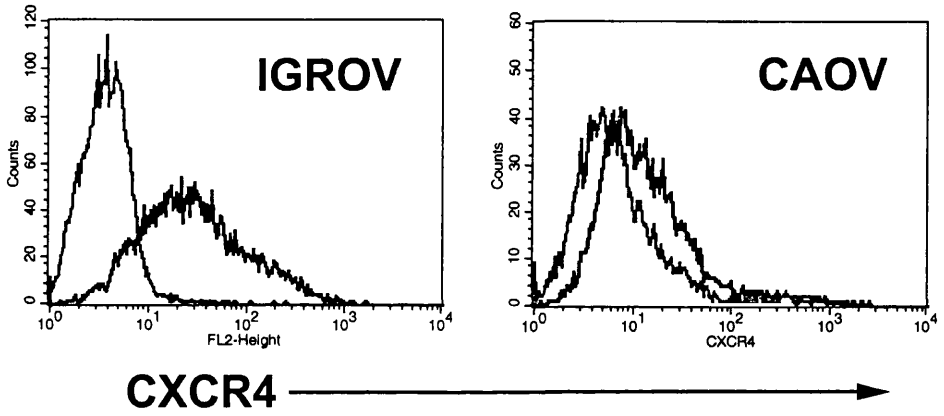


Figure 5.13. Flow cytometry histograms for cell surface expression of CXCR4 on the ovarian cancer cell lines IGROV and CAOV-3. Cells were incubated with IgG2a isotype control mAb (grey line) or anti-CXCR4 mAb (black line). IGROV had higher expression of CXCR4 protein than CAOV-3.

5.3.3.ii. Detection of CXCR4 protein on HER2/neu positive cells from ascites

Two colour flow cytometric analysis was used to investigate CXCR4 protein expression in cells isolated from five samples of ovarian cancer ascites. Cells positive for HER2/neu were positive for CXCR4 (Figure 5.14.). In the sample shown in Figure 5.14., around 50 % of the cells present in ascites were positive for CXCR4. HER2/neu

positive tumour cells accounted for half of these. Thus, the majority, if not all, of the tumour cells within the ascitic fluid were positive for CXCR4.

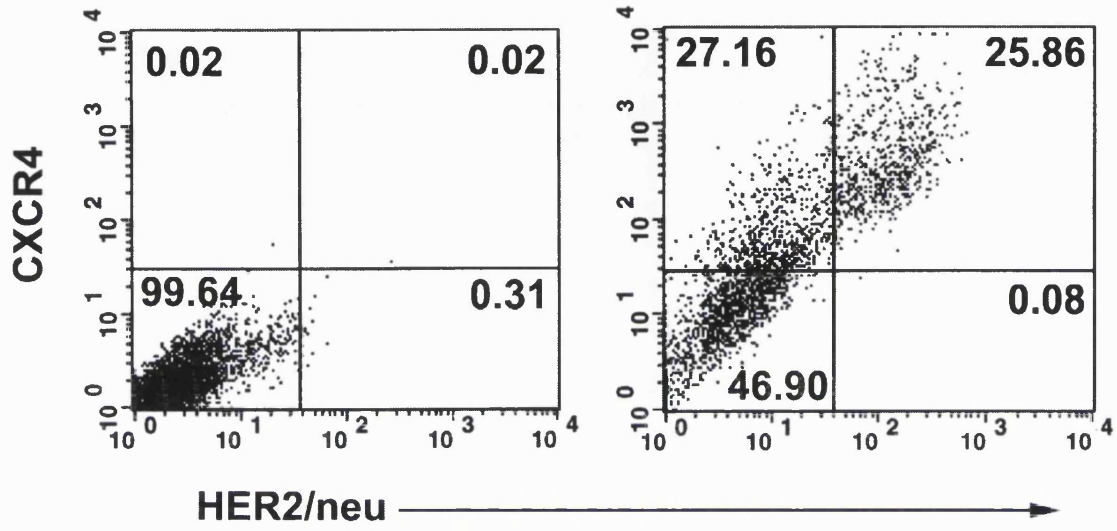


Figure 5.14. Two colour flow cytometric analysis of CXCR4 versus HER2/neu expression on cells from ovarian cancer ascites. Representative plots are shown with isotype control mAbs (left panel) and percentages of HER2/neu positive, CXCR4 positive cells (right panel). Cells from five different patients gave similar results.

5.3.4. Expression of CXCL12 in normal ovary, solid tumour biopsies and ascites

5.3.4.i. CXCL12 protein expression in normal ovary

Immunohistochemistry was used to detect CXCL12 expression in three biopsies of normal ovary. No CXCL12 protein was detected on normal ovarian epithelium (Figure 5.15.).

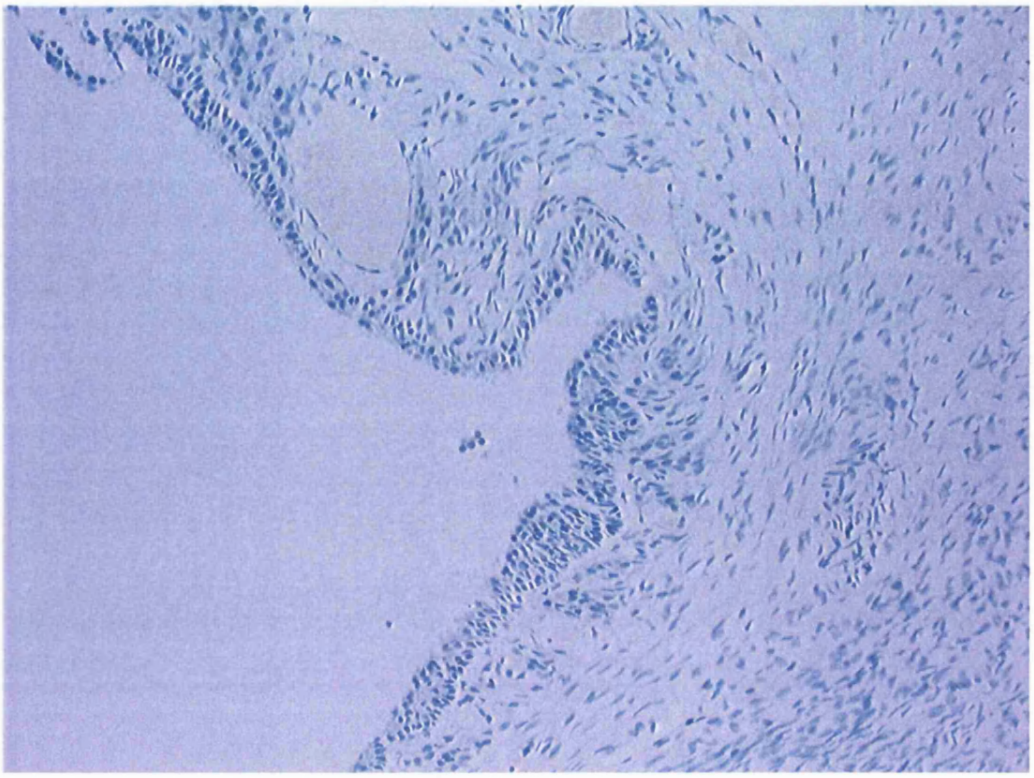


Figure 5.15. CXCL12 protein expression in normal ovarian surface epithelium.
Immunohistochemistry did not detect any CXCL12 protein expression in biopsies of normal ovary, x 200.

5.3.4.ii. CXCL12 protein expression in solid tumour biopsies

Preliminary results from immunohistochemistry for CXCL12 in solid tumour biopsies demonstrated CXCL12 protein expression in 6/8 samples. CXCL12 protein was mainly found within the tumour parenchyma, with a widespread distribution (Figure 5.16.). CXCL12-positive tumours also had tumour areas that were negative for CXCL12 (Figure 5.17.).

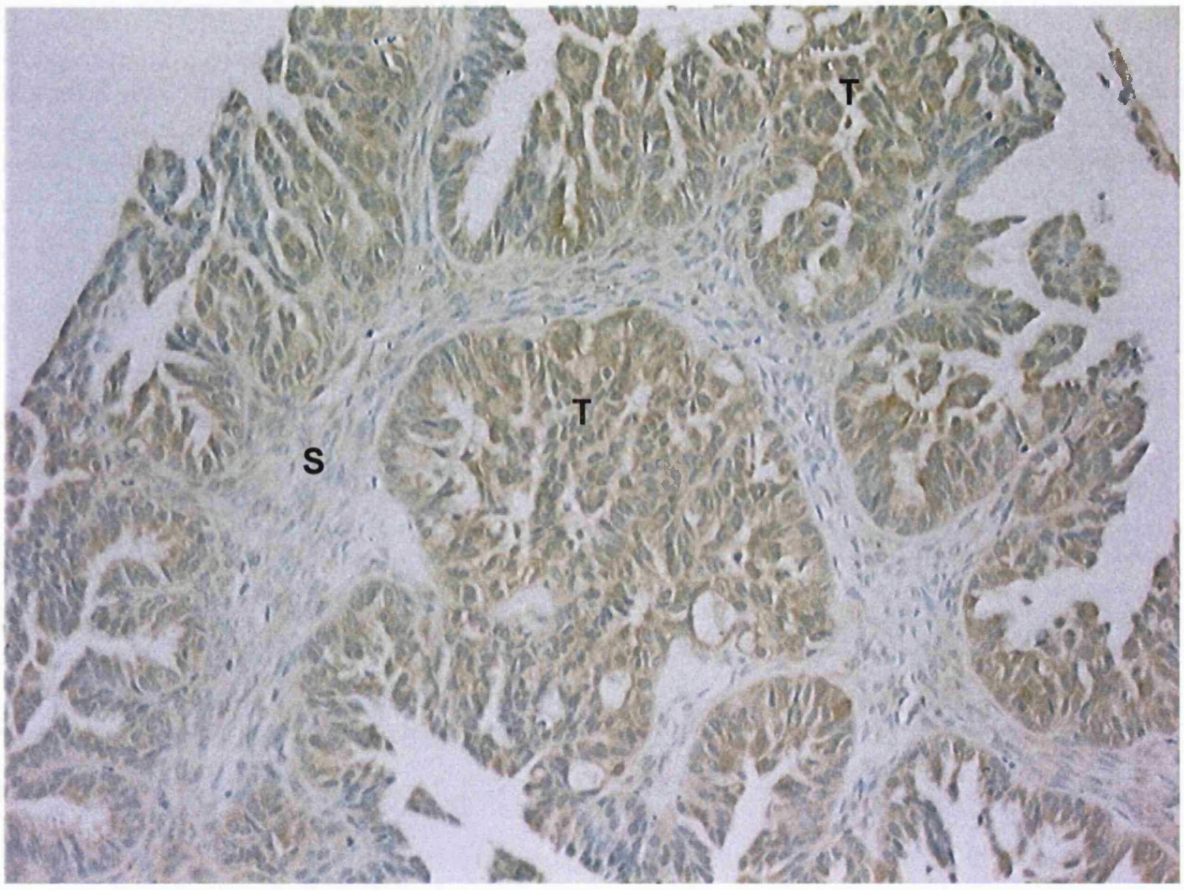


Figure 5.16. Immunohistochemistry for CXCL12 in a solid tumour biopsy. CXCL12 protein was distributed throughout the tumour parenchyma (**T**). Less CXCL12 protein was associated with the stroma (**S**), x 200.

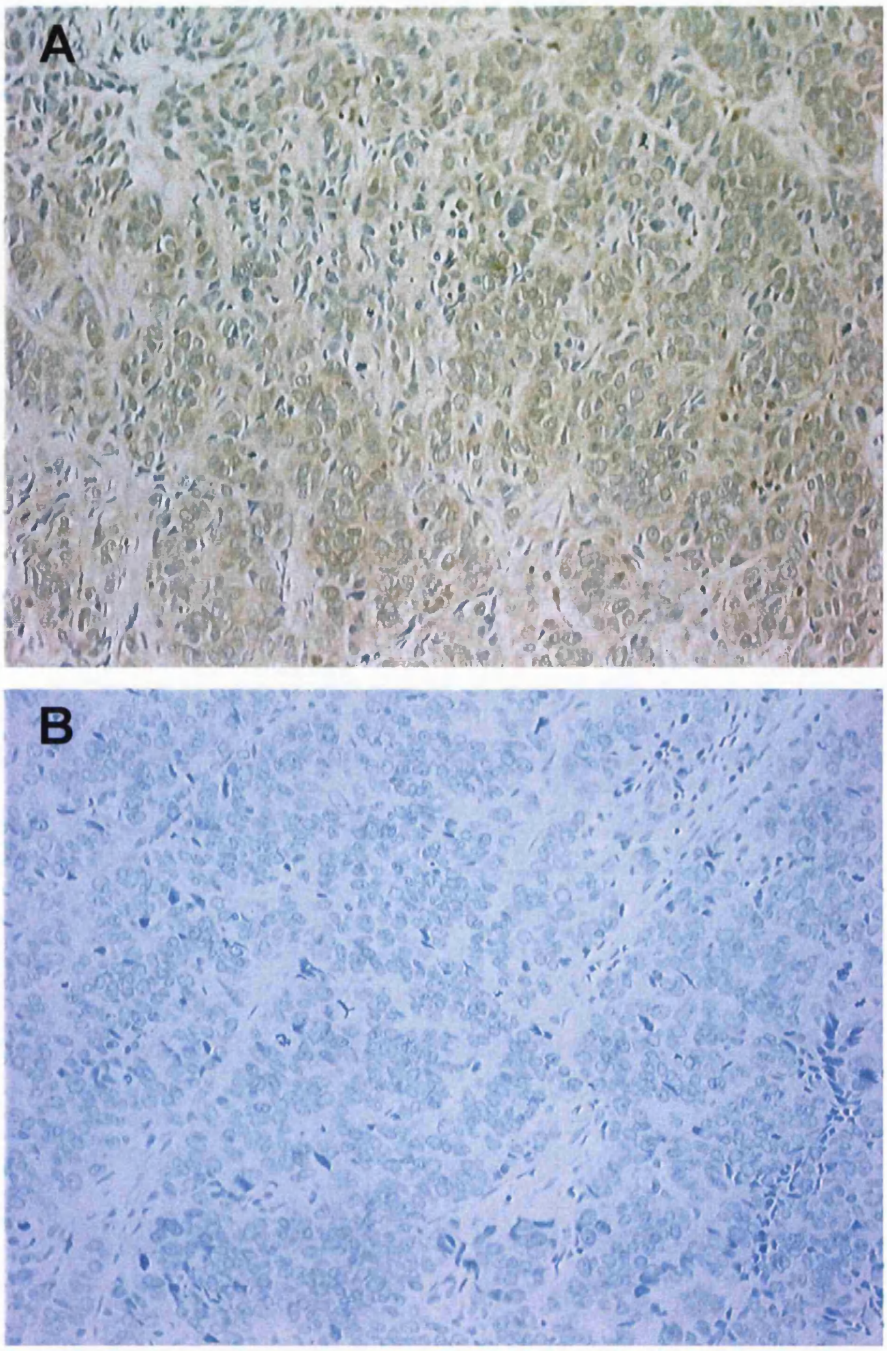


Figure 5.17. Immunohistochemistry for CXCL12 in a solid tumour biopsy. Widespread distribution of CXCL12 protein was seen within tumour parenchyma (**A**), x 200. Adjacent tumour areas had little or no detectable CXCL12 protein (**B**), x 200.

5.3.4.iii. Lack of CXCL12 expression by IGROV and CAOV-3 cell lines

ELISA was used to determine whether CXCL12 protein was present in conditioned medium collected from IGROV and CAOV-3 cells grown for 48 hours. No CXCL12 was detected, suggesting that these cells do not produce this chemokine (data not shown).

5.3.4.iv. CXCL12 protein expression in ascites

CXCL12 levels were assayed by ELISA in 63 samples of ascitic fluid from patients with ovarian cancer (Figure 5.18.). High concentrations of this chemokine (range 613-9333 pg/ml; median 6021 pg/ml) were detected in all samples, making this the predominant chemokine so far detected in human ovarian cancer ascites (c.f. Section 4.3.2.ii).

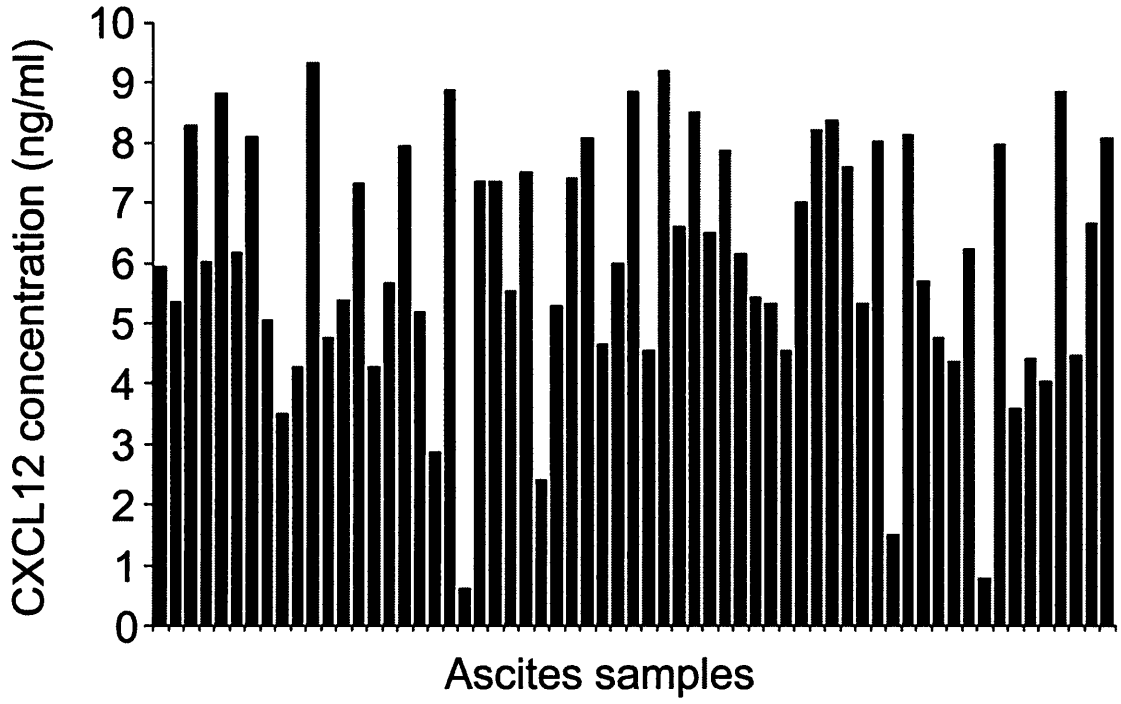


Figure 5.18. ELISA for CXCL12 in 63 samples of ascitic fluid. CXCL12 was detected at high levels (range 613-9333 pg/ml; median 6021 pg/ml) in all samples.

5.4. Discussion

CXCR4 and its ligand CXCL12 are not classical pro-inflammatory chemokines. CXCL12 is expressed in a wide range of tissues and cell types in the absence of infectious stimuli, allergic or autoimmune diseases (295, 296). Other functions of CXCL12 include roles in B cell lymphopoiesis (297, 436), bone marrow myelopoiesis, cardiac ventricular septum formation (297), architecture of the cerebellum (159) and megakaryocyte development (437).

CXCR4 is expressed on haematopoietic cells, including T cells (438), B cells and B precursors, neutrophils, monocytes, dendritic cells (5), macrophages and Langerhans cells (439), some epithelial cells (78, 440, 441) and endothelial cells (262). In the brain CXCR4 has been detected on microglia, neurons and astrocytes (442).

This chapter demonstrates that normal ovarian surface epithelium does not express mRNA for the chemokine receptor CXCR4. However, tumour cells in both solid ovarian tumours and in ovarian cancer ascites can express CXCR4. Only a proportion (between 1 and 20 %) of the tumour cells in the solid ovarian cancer biopsies were positive for this receptor. Receptor levels could be regulated by cytokines such as TGF-β, or other factors in the tumour microenvironment; this could explain why a proportion of the tumour cells was positive at any one time. Alternatively, some cells may have acquired constitutive CXCR4 expression during malignant progression. Peritoneal dissemination is the major route for spread of ovarian cancer and all of the HER2/neu positive tumour cells that had spread into ascites were positive for CXCR4 protein.

Analysis of mRNA by RPA showed expression of other chemokine receptors in the tumour and ascites samples, which are a mixture of tumour cells, leukocytes and connective tissue cells. CC chemokine receptor expression in solid tumours was restricted to expression of the CC chemokine receptor CCR1, but this localised to

infiltrating leukocytes (see Chapter 3). More chemokine receptor mRNA was expressed in cells from ascites but, apart from CXCR4, these were also detected on leukocyte but not tumour cell populations (see Chapter 4).

Thus, the only chemokine receptor we have found on ovarian cancer cells is CXCR4. Chemokine gradients may well influence routes of tumour cell migration within the body and response to these gradients may be one component of the multi-step process of metastatic spread. Of particular interest is the spread of tumour cells to lymphatic vessels where the tumour cells seem to follow the routes used by immune cells responding to chemokine gradients in the lymph nodes. The migration of mature dendritic cells from the tissues to draining lymph nodes is associated with changes in chemokine receptor expression and hence response to lymph node chemokines (443). Ovarian cancer cells can be detected in draining lymph nodes (330); it is tempting to speculate that they migrate there specifically.

The presence of high CXCL12 concentrations in ascitic fluid could provide a gradient for the movement of CXCR4-expressing tumour cells from the solid tumour into ascites; ovarian cancer primarily forms metastases via dissemination throughout the peritoneal cavity. The preliminary finding that CXCL12 protein is present in solid tumour biopsies suggests that CXCL12 may also have autocrine or paracrine effects. These effects could include promotion of angiogenesis and tumour cell proliferation.

Two recent papers described tumour and endothelial cell CXCR4 expression in human pancreatic cancer and glioblastoma (293, 444). Koshiba *et al* observed CXCR4 protein expression on pancreatic cancer cells and infiltrating leukocytes, but not on normal acinar or ductal epithelial cells. There was no statistically significant correlation with clinicopathological variables such as tumour stage or metastasis. In contrast to our work, CXCR4 expression was observed on endothelium in large vessels adjacent to the

tumour area, but not on microvessels within the tumour parenchyma. CXCL12 mRNA was detected in the pancreatic cancer biopsies. Koshiba *et al* suggest that CXCR4/CXCL12 may play a role in tumour progression and angiogenesis (293). Rempel *et al* identified CXCL12 and CXCR4 expression on glioblastoma multiforme tumour cells in regions adjacent to areas of necrosis, and CXCR4 was frequently seen on endothelial cells in areas of neovascularisation. Their data suggest an important role for CXCR4/CXCL12 in the promotion of angiogenesis in glioblastoma (444). Neither of these studies assessed whether tumour cells expressed chemokine receptors other than CXCR4.

Most persuasively, Muller *et al* recently reported high expression of CXCR4 and CCR7 on human breast cancer cells (286). They used real-time quantitative PCR to investigate the expression of 17 different chemokine receptors in breast cancer cells lines and RNA derived from primary tumours. Immunohistochemistry localised CXCR4 protein to cancer cells, but not normal cells, in invasive ductal carcinoma. Axillary lymph node metastases and distant metastases to lung and liver also expressed CXCR4 protein. mRNA levels of the respective ligands CXCL12 and CCL21, were highest at sites of breast cancer metastasis; these chemokines induced migration of tumour cells and furthermore, neutralising antibodies to CXCR4 reduced experimental metastasis in a xenograft model of breast cancer. Thus, it seems that malignant cells from common tumours express restricted and specific patterns of chemokine receptors. CXCR4 may be important for tumour progression, metastasis and angiogenesis in a variety of different cancers.

Endothelial cells in the solid tumour biopsies also expressed CXCR4. Only endothelial cells within the tumour area were positive for CXCR4; endothelial cells in well-formed blood vessels or in tissue adjacent to the tumour did not express CXCR4 mRNA. This suggests that CXCR4 may be involved with angiogenesis in ovarian cancer.

Alternatively, these cells may not be endothelial cells at all. Recent work by Sood *et al* on ovarian cancer plasticity showed that ovarian cancer cells can employ molecular vasculogenic mimicry to form networks and channels when cultured in a three-dimensional matrix *in vitro* (445). These cells expressed vascular-associated markers such as CD31 and FLT-1 (VEGFR-1). Moreover, tumour cell-lined channels containing red blood cells were seen in solid tumour biopsies. More work is required to determine whether CXCR4 is involved with angiogenesis in ovarian cancer.

The work in Chapter 6 will investigate whether CXCR4 expression by ovarian cancer cells is functional and whether binding of CXCL12 to CXCR4 can induce actions that would permit a role in tumour cell migration, metastasis and survival.

Conclusions from this chapter:

- Of 14 chemokine receptors investigated, only CXCR4 was expressed by ovarian cancer cell lines. Normal ovarian surface epithelium did not express any chemokine receptors.
- 4/6 ovarian cancer cell lines expressed CXCR4 mRNA.
- 2/6 ovarian cancer cell lines expressed CXCR4 protein.
- CXCR4 mRNA was detected in 8/10 solid tumour biopsies and 19/20 ascites samples, by RNase protection assay.
- CXCR4 mRNA localised to a proportion of tumour cells in 10/10 solid tumour biopsies, by *in situ* hybridisation. Expression was also seen on the mononuclear cell infiltrate and some endothelial cells.
- HER2/neu positive tumour cells in five ascites samples were positive for CXCR4.
- CXCL12 protein, the ligand for CXCR4, was detected in solid tumour biopsies but not in normal ovarian epithelium.
- Ascitic fluid contained high concentrations of CXCL12 protein.

Chapter 6. The function of CXCR4 expression in ovarian cancer cell lines and its potential role in metastasis

6.1. Introduction

The previous chapter illustrated that tumour cells in solid ovarian tumour biopsies and ascitic fluid expressed CXCR4. Cell lines derived from human ovarian cancer also expressed CXCR4 mRNA and protein.

Functional CXCR4 has been reported in primary cultures of type II alveolar epithelial cells, and A549, RT-4 and MCF-7 cell lines (441). CXCR1 and 2 expression was detected on SKOV-3 ovarian cancer cells (291), although neither of these receptors was detected in the SKOV-3 cells grown in our laboratory (see Chapter 5). CXCL8 and CXCL1 induced both proliferation and migration of melanoma cells in tissue culture (446). Sub-lines of MCF-7 breast cancer cells migrated towards CCL3, CCL4 and CCL5, although chemokine receptor expression was not investigated on these cells (447).

For CXCR4 to have a role in the metastasis of human ovarian cancer, it must be functional. If CXCL12, the ligand for CXCR4, can stimulate ovarian cancer cells to migrate for example, then it would imply a possible role for CXCR4 in metastasis. Ovarian cancer spreads primarily via dissemination throughout the peritoneal cavity, with subsequent formation of micro- and macrometastases. The presence of CXCL12 in ascitic fluid could have effects on proliferation and adhesiveness of CXCR4-expressing tumour cells. CXCL12 protein in solid tumours could also have autocrine or paracrine effects on tumour cells.

Invasion of malignant tumours and formation of metastases at remote sites usually requires the proteolytic degradation of components of the extracellular matrix and basement membranes; this requires specific proteases. Matrix metalloproteinases (MMPs), including the membrane-type MMPs (MT-MMPs) are a family of zinc-dependent endopeptidases that can degrade most components of the basement membrane, including collagen [see (448) for review]. An important regulatory mechanism for MMP activity involves tissue inhibitors of MMPs (TIMPs). The plasminogen activator system, including urokinase plasminogen activator (uPA), its receptor (uPAR) and plasminogen activator inhibitor (PAI) can also be important for cell invasion and migration [see (449) for review].

6.2. Aims of the chapter

This chapter will investigate the function of CXCR4 on ovarian cancer cell lines. The effect of ligation of CXCR4 with its ligand, CXCL12, will be studied in terms of migration, modulation of matrix-degrading enzymes and their inhibitors, proliferation and signalling. Regulation of CXCR4 on ovarian cancer cells will also be examined.

Some of this work was performed in collaboration with Dr Julia Wilson and Dr Kate Scott in our laboratory: Julia Wilson helped with proliferation assays and flow cytometry; Kate Scott performed the RT-PCR for MMPs.

6.3. Results

6.3.1. Chemotaxis

Migration of IGROV and CAOV-3 cells in response to CXCL12 was investigated using the transwell migration assay (Figure 6.1.). During overnight incubation, CXCL12 induced significant migration of IGROV and CAOV-3 cells at concentrations of 100 ng/ml (IGROV, P=0.0001; CAOV-3, P=0.0002) and 300 ng/ml (IGROV, P=0.0001; CAOV-3, P=0.0003).

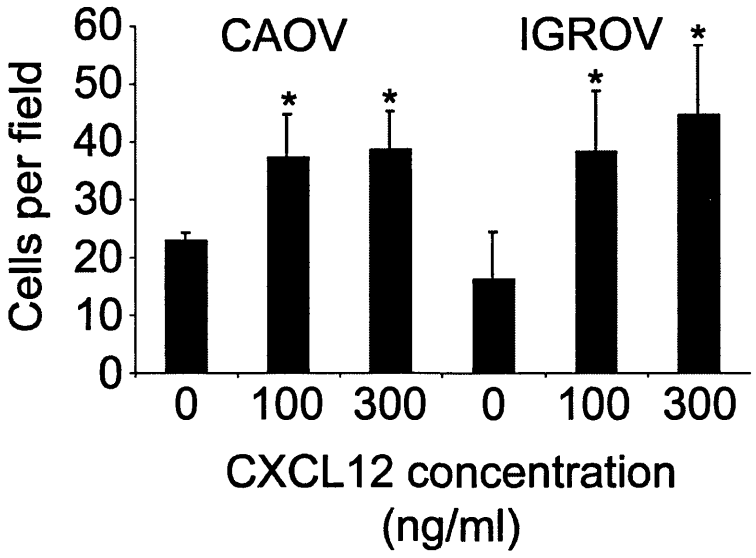


Figure 6.1. Migration of ovarian cancer cells in response to CXCL12. IGROV and CAOV-3 cell lines showed significant migration towards 100 ng/ml and 300 ng/ml CXCL12. Values are the mean ± SD of 10 determinations from two experiments. * denotes statistically significant results, P<0.0005.

6.3.2. Invasion

6.3.2.i. Invasion through matrigel™

IGROV and CAOV-3 cells were also tested for their ability to invade through growth factor-reduced matrigel™ invasion chambers. Both cell lines showed an ability to invade through matrigel™ towards 100 ng/ml CXCL12 (Figure 6.2.). This suggests that CXCL12 can stimulate production/release of matrix-degrading enzymes, in addition to causing chemotaxis.

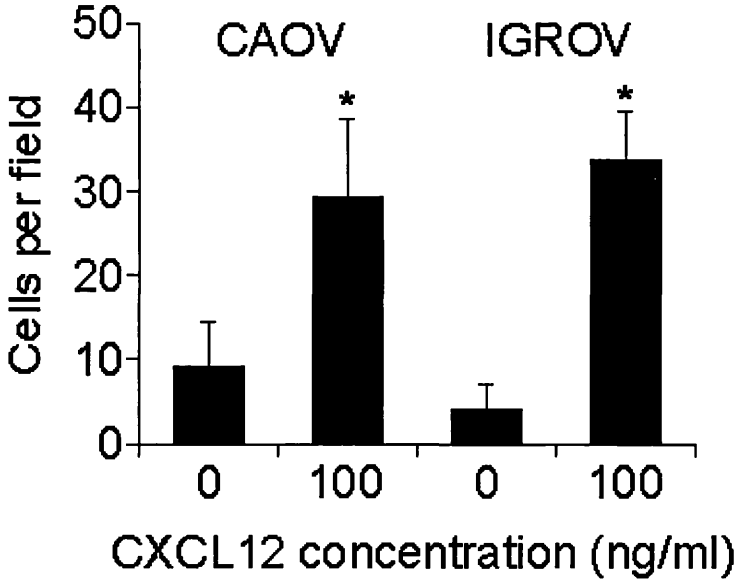


Figure 6.2. Invasion of ovarian cancer cells in response to CXCL12. IGROV and CAOV-3 cell lines showed significant invasion through matrigel™, towards 100 ng/ml CXCL12. Values are the mean ± SD of 15 determinations and the results are representative of two experiments. * denotes statistically significant results, P<0.0001.

6.3.2.ii. Modulation of MMP production by CXCL12

IGROV and CAOV-3 cells were cultured for 24 hours with or without 100 ng/ml CXCL12. Total RNA was prepared and RT-PCR was performed to assess the expression of matrix metalloproteinases (MMP), membrane-type MMPs (MT-MMP), tissue inhibitors of MMPs (TIMP), urokinase plasminogen activator (uPA), uPA receptor (uPAR) and plasminogen activator inhibitor (PAI).

There was no detectable expression of MMP-1, 2, 3, 9, 10, 11, MT1-MMP, MT2-MMP, TIMP-1, uPA or PAI (data not shown). IGROV expressed mRNA for MMP-7, MMP-13, MT3-MMP, TIMP-2, TIMP-3 and uPAR (Figure 6.3.). CAOV-3 expressed MMP-13, TIMP-2, TIMP-3 and uPAR. CXCL12 stimulation did not appear to switch on expression of any of these genes. It is possible that CXCL12 caused upregulation of existing mRNA expression; this could be investigated using real-time quantitative PCR

or northern analysis. Expression of uPAR could allow ovarian cancer cells to bind uPA, and hence cause localised proteolysis.

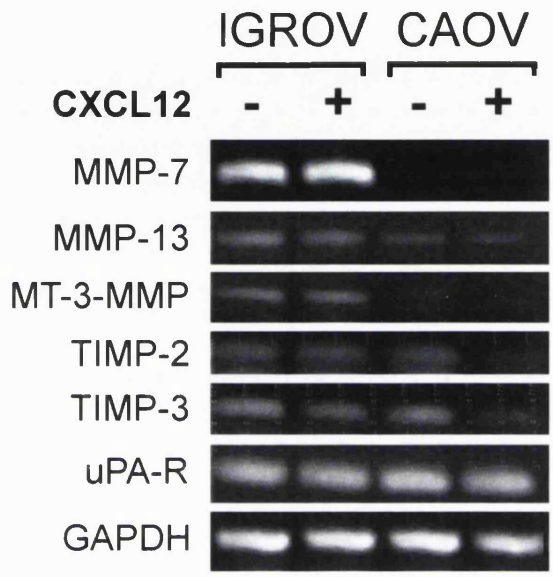


Figure 6.3. Expression of MMPs and TIMPs by ovarian cancer cell lines. RT-PCR was used to screen for expression of a variety of matrix-degrading enzymes and their inhibitors in IGROV and CAOV-3 cells after 24 hours culture with (+) or without (-) 100 ng/ml CXCL12. The results are representative of two experiments.

6.3.3. Modulation of integrin expression after CXCL12 stimulation

For tumour cells in ascitic fluid to form metastatic deposits on the peritoneal wall, it may be anticipated that CXCL12 stimulation could increase the expression of adhesion molecules. The cell surface expression of various adhesion molecules was assessed by flow cytometry after 24 hours stimulation with 100 ng/ml CXCL12. The levels of α_2, α_v, α_6 and β_4 integrins, CD54 (ICAM) and E-cadherin remained unchanged after CXCL12 stimulation. However, β_1 integrin levels were increased (Figure 6.4.). β_1 forms dimers with a wide range of α subunits (172), which could effect an increase in adhesion.

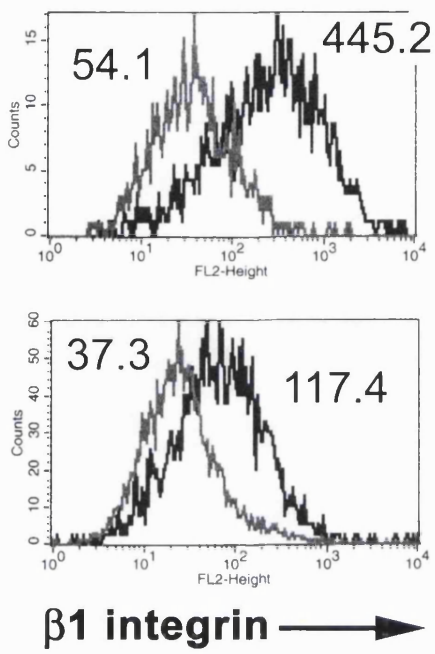


Figure 6.4. Upregulation of β_1 integrin after CXCL12 stimulation. Flow cytometry was used to analyse β_1 integrin expression on IGROV (top panel) and CAOV-3 (bottom panel) after 24 hours stimulation with 100 ng/ml CXCL12 (unstimulated control, grey line; CXCL12-stimulated cells, black line). The results are representative of three experiments. Numbers show the mean fluorescence intensity for each histogram.

6.3.4. Proliferation in response to CXCL12 stimulation

6.3.4.i. Increased proliferation/survival in response to CXCL12

The growth kinetics of IGROV and CAOV-3 were investigated after stimulation with CXCL12 (Figure 6.5.). Cells were seeded at 2 x 10^4 cells per well of a 24-well plate, with 8 wells per condition, in serum-free medium + 0.5 % BSA ± 100 ng/ml CXCL12. At 2, 4 and 6 days after seeding, the cells were trypsinized and counted using a haemocytometer. Fresh medium ± 100 ng/ml CXCL12 was added after 3 days.

Both IGROV and CAOV-3 showed a significant increase in cell number following stimulation with CXCL12, suggesting a role for CXCL12 in the proliferation and/or survival of ovarian cancer cell lines expressing CXCR4.

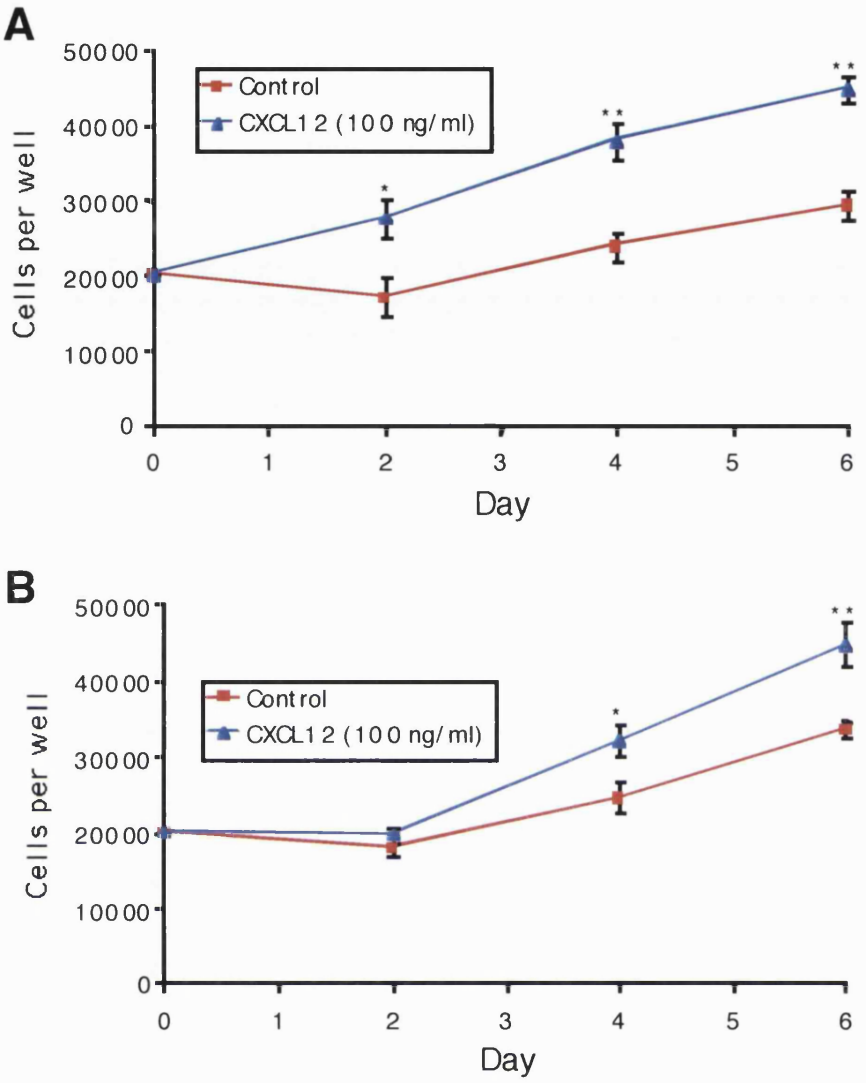


Figure 6.5. Growth of ovarian cancer cell lines after stimulation with CXCL12. IGROV and CAOV-3 cells were grown in serum-free medium ± 100 ng/ml CXCL12. The number of cells was counted after 2, 4 and 6 days of culture. CXCL12 stimulation resulted in a significant increase in cell number of IGROV (**A**) and CAOV-3 (**B**). Values are the mean ± SEM of 8 determinations and the results are representative of three experiments. * denotes $P<0.05$; ** denotes $P<0.01$.

6.3.4.ii. Effect of CXCR4-neutralising antibodies

To confirm the necessity for CXCR4 expression on the cell lines, proliferation experiments were repeated with the addition of CXCR4-neutralising antibodies. Cells were treated with serum-free medium ± 100 ng/ml CXCL12 ± 10 μg/ml anti-CXCR4

mAb for 4 days (Figure 6.6.). Stimulation with 100 ng/ml CXCL12 resulted in a significant increase in cell number in both IGROV and CAOV-3, compared with the untreated control. Addition of anti-CXCR4 neutralising antibodies inhibited this CXCL12-dependent increase in cell number; no significant difference was seen between cells treated with CXCL12/anti-CXCR4 mAb and the untreated control. These results show that CXCR4 is necessary for the CXCL12-dependent increase in cell number.

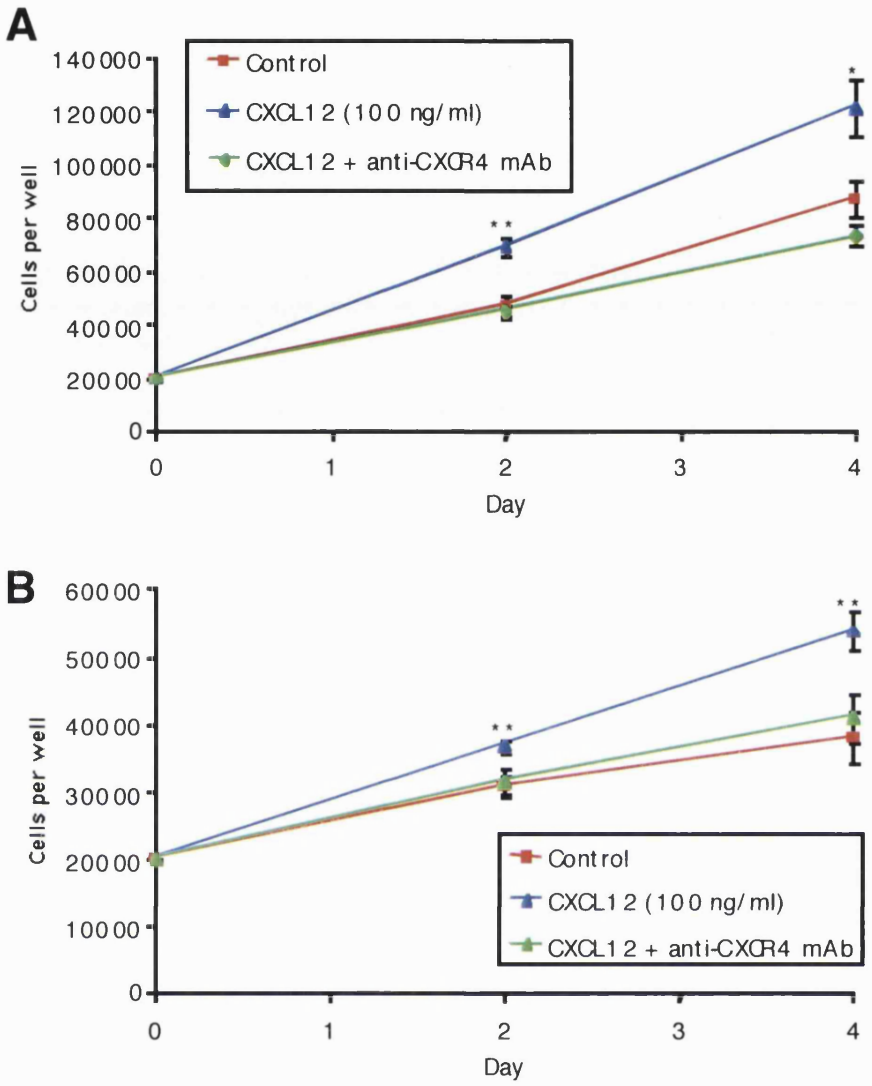


Figure 6.6. Growth of ovarian cancer cell lines after stimulation with CXCL12 and anti-CXCR4 neutralising antibodies. IGROV and CAOV-3 cells were grown in serum-free medium ± 100 ng/ml CXCL12 ± 10 μg/ml anti-CXCR4 mAb. The number of cells was counted after 2 and 4 days of culture. CXCL12 stimulation resulted in a significant increase in cell number of IGROV (**A**) and CAOV-3 (**B**), but this increase was inhibited by the anti-CXCR4 neutralising mAb. Values are the mean ± SEM of 8 determinations and the results are representative of two experiments. * denotes $P<0.05$; ** denotes $P<0.01$.

6.3.5. Signalling downstream of CXCR4

Chemokine receptors signal via heterotrimeric G_i proteins. Depending on the receptor, this can lead to the subsequent activation of a variety of downstream signalling

pathways, including activation of PLC and PI3Kγ, activation of Src-like kinases, and stimulation of the MAPK cascade [see (88, 450) for review].

6.3.5.i. Calcium flux in response to CXCL12

Intracellular calcium flux was measured in the ovarian cancer cell lines IGROV and CAOV-3 in response to CXCL12. Stimulation with 100 ng/ml CXCL12 elicited a detectable intracellular calcium flux in both IGROV and CAOV-3 (Figure 6.7.). No release of intracellular calcium was detectable when cells were stimulated with 10 ng/ml CXCL12 (data not shown). These results show that ligation of CXCR4 with CXCL12 activates downstream signalling events, leading to the release of intracellular calcium.

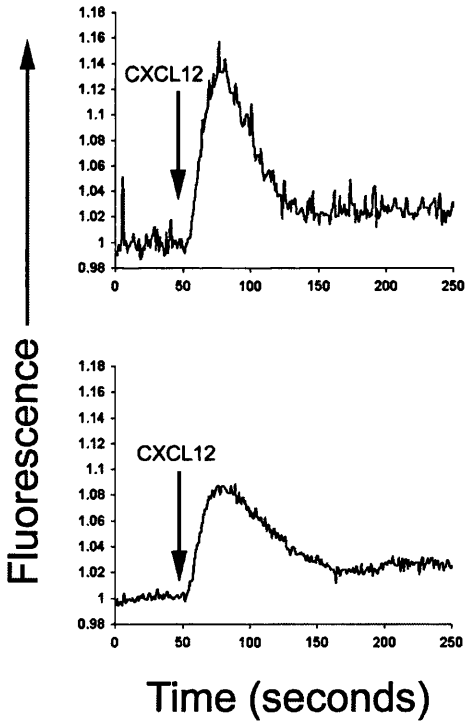


Figure 6.7. CXCL12-stimulated calcium flux. Intracellular calcium flux was seen in response to 100 ng/ml CXCL12 in the ovarian cancer cell lines IGROV (top panel) and CAOV-3 (bottom panel). Arrows indicate the point at which chemokine was added. The results are representative of three experiments.

6.3.5.ii. CXCR4 internalisation following CXCL12 stimulation

Binding of CXCL12 to its receptor caused internalisation of CXCR4 on IGROV cells after approximately 15 minutes, as determined by flow cytometry. The receptor was recycled to the cell surface within 30 minutes. On CAOV-3 cells, there was considerable internalisation of CXCR4 after 5 minutes, but restoration of CXCR4 cell surface expression was slower (Figure 6.8.).

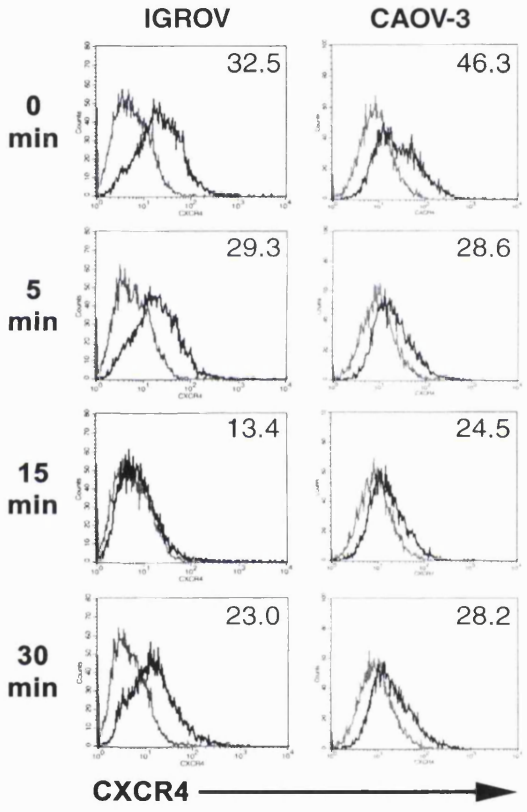


Figure 6.8. CXCL12-induced CXCR4 internalisation. The expression/internalisation of CXCR4 by IGROV and CAOV-3 cells was determined by flow cytometry at various timepoints following stimulation with CXCL12 (isotype control, grey line; anti-CXCR4 mAb, black line). The numbers on the figures represent the mean fluorescence intensity of CXCR4. Results are representative of two experiments.

6.3.5.iii. Activation of Akt/PKB

PKB is a downstream effector of PI3K, and has been implicated in several signal transduction pathways, many of which promote cell survival (94). It is also important in the chemotactic response (96).

IGROV and CAOV-3 cells were stimulated with 100 ng/ml CXCL12, and total cell lysates were prepared at various timepoints. Western blots were performed using 10 μg of total protein, and these were probed for phospho-Akt/PKB, Akt/PKB and β-actin. Activation of Akt/PKB was seen in IGROV cells, with a three to four-fold increase in phospho-Akt (relative to Akt) within 10 minutes. This induction of phospho-Akt was still maintained at 2 hours (Figures 6.9. and 6.10.). No activation of Akt was seen in CAOV-3 cells; the ratio of phospho-Akt to Akt remained the same in response to CXCL12 treatment (Figure 6.11.).

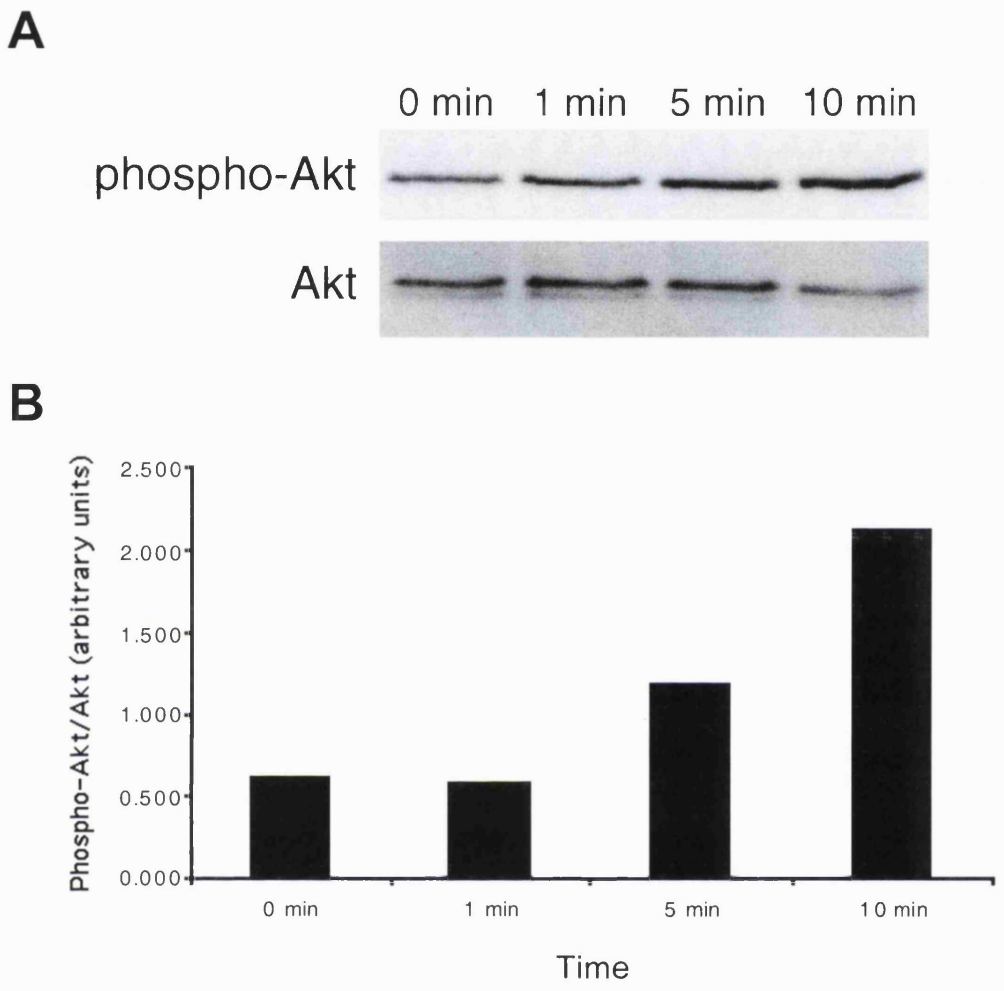


Figure 6.9. Western blots for phospho-Akt and Akt in IGROV cells following CXCL12 stimulation. Total cell lysates were prepared at various timepoints following stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein and probed for Akt and its active form, phospho-Akt (**A**). Densitometry was performed on the blots from (**A**) and the ratio of phospho-Akt to Akt was calculated (**B**). Results are representative of two experiments.

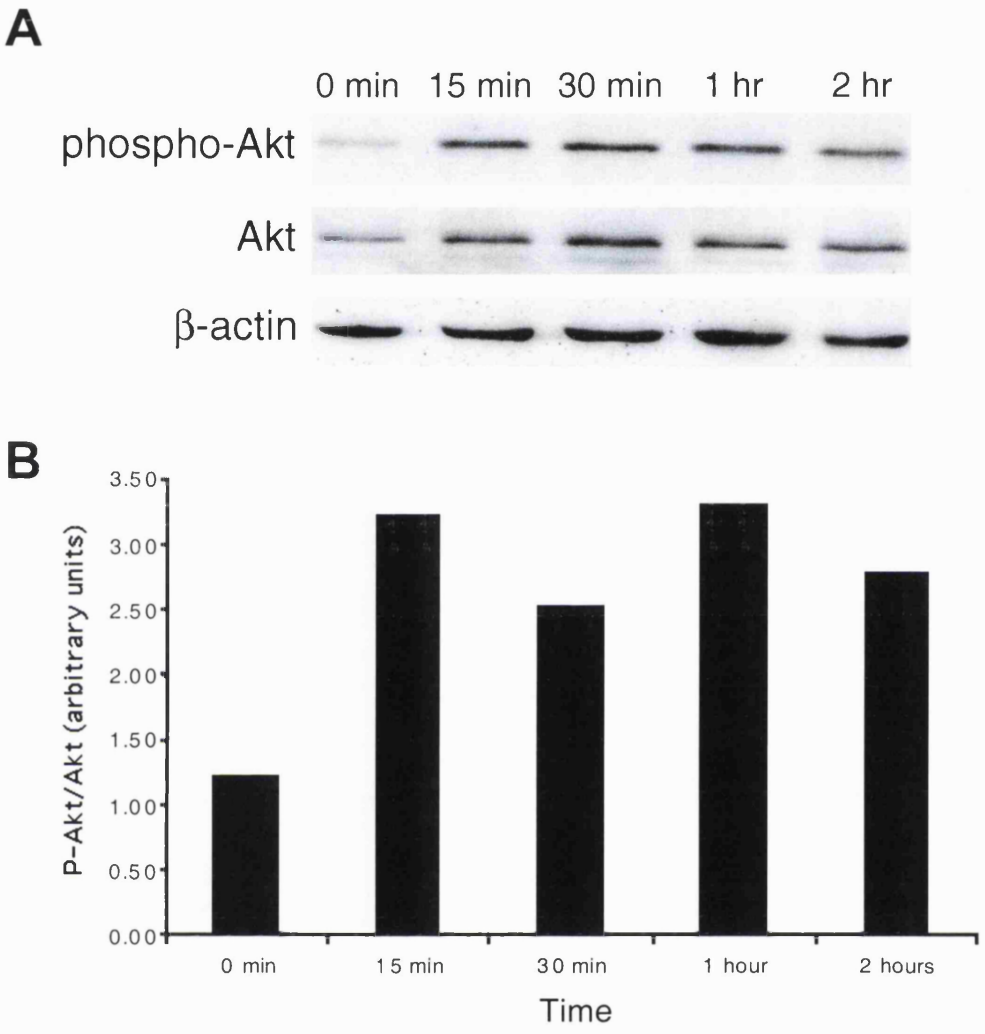


Figure 6.10. Western blots for phospho-Akt and Akt in IGROV cells following CXCL12 stimulation. Total cell lysates were prepared at various timepoints following stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein and probed for Akt and its active form, phospho-Akt (**A**). Densitometry was performed on the blots from (**A**) and the ratio of phospho-Akt to Akt was calculated (**B**). Results are representative of two experiments.

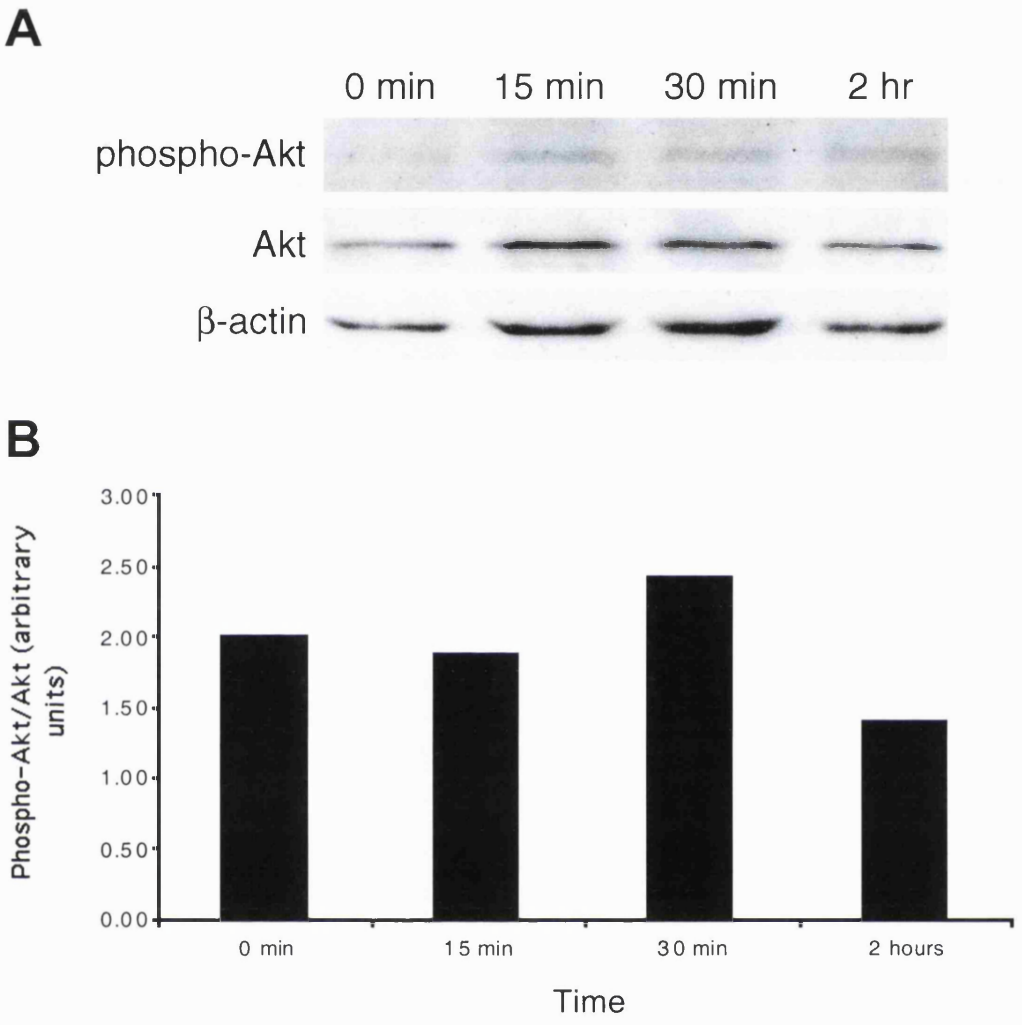


Figure 6.11. Western blots for phospho-Akt and Akt in CAOV-3 cells following CXCL12 stimulation. Total cell lysates were prepared at various timepoints following stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein and probed for Akt and its active form, phospho-Akt (**A**). Densitometry was performed on the blots from (**A**) and the ratio of phospho-Akt to Akt was calculated (**B**). Results are representative of two experiments.

6.3.5.iv. Activation of ERK1/ERK2

The MAPK pathway is thought to be activated via PI3Kγ (100) and can lead to a variety of cell-type specific effects and gene induction. p44 and p42 MAPK (ERK1 and ERK2 respectively) are important components of this MAPK cascade.

IGROV and CAOV-3 cells were stimulated with 100 ng/ml CXCL12, and total cell lysates were prepared at various timepoints. Western blots were performed using 10 μg of total protein, and these were probed for phospho-p44/42 MAPK, non-phosphorylated p44/42 MAPK and β-actin. Phosphorylation of p44/42 MAPK was seen in both IGROV cells and CAOV-3 cells with slightly different kinetics. In IGROV cells, there was approximately a three-fold increase in the ratio of phopsho-p44/42 MAPK to p44/42 MAPK after 15 minutes (Figure 6.12.). The ratio then decreased back to control levels after 30 minutes, before increasing to four/five-fold of control levels after 2 hours. In CAOV-3 cells, there was a four-fold increase in the ratio of phopsho-p44/42 MAPK to p44/42 MAPK after 15 minutes and this was still sustained after 2 hours (Figure 6.13.).

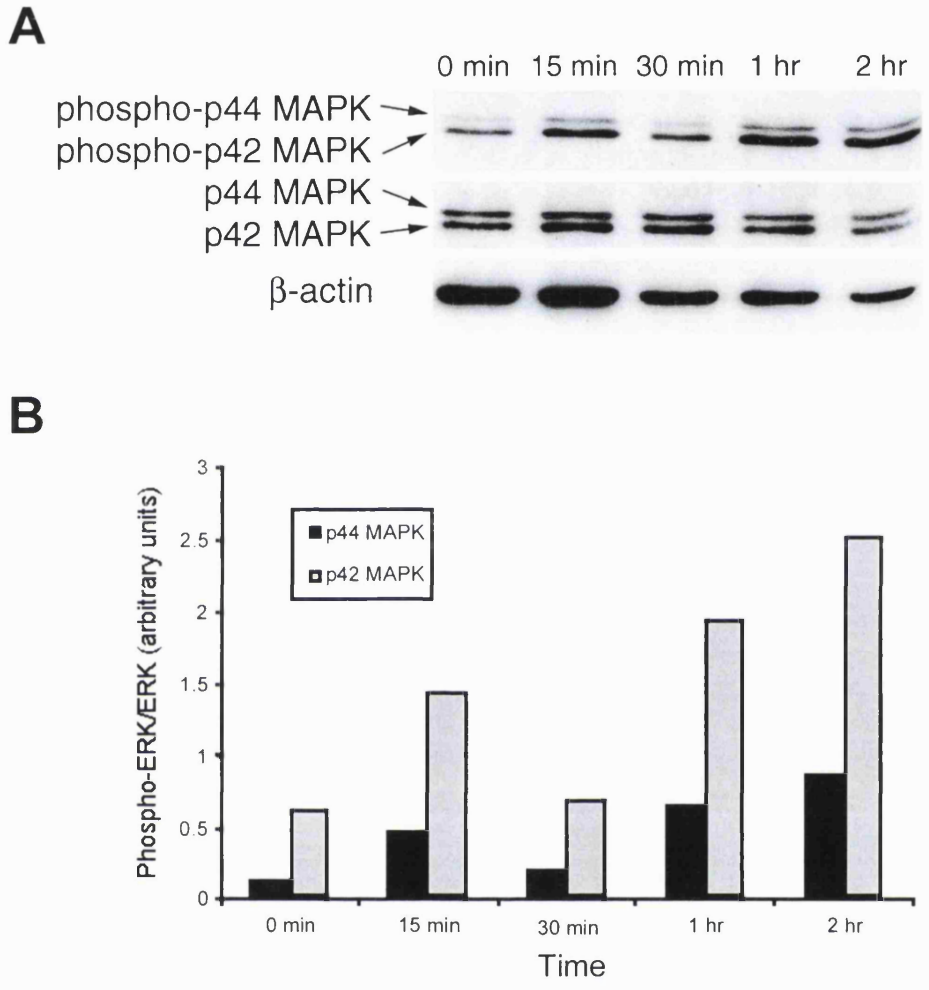


Figure 6.12. Western blots for phospho-p44/42 MAPK and p44/42 MAPK in IGROV cells following CXCL12 stimulation. Total cell lysates were prepared at various timepoints following stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein and probed for p44/42 MAPK and the active form, phospho-p44/42 MAPK (**A**). Densitometry was performed on the blots from (**A**) and the ratio of phospho-p42 MAPK or phospho-p44 MAPK to the non-phosphorylated form was calculated (**B**). Results are representative of two experiments.

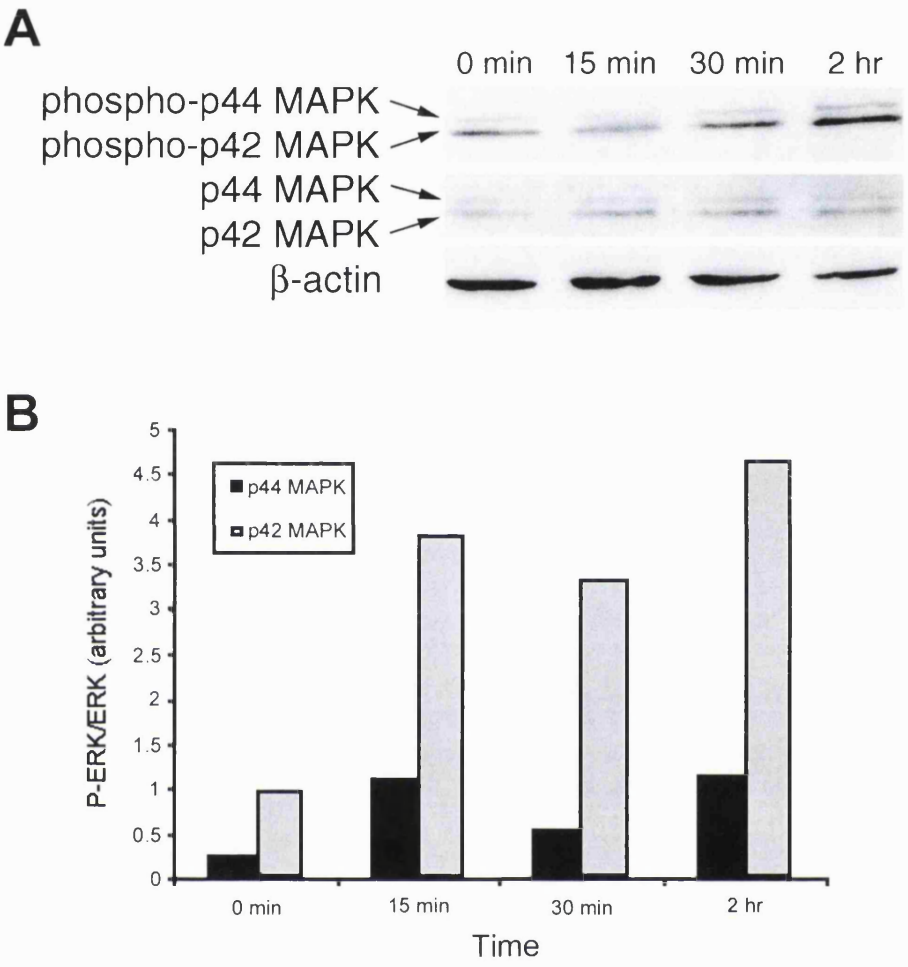


Figure 6.13. Western blots for phospho-p44/42 MAPK and p44/42 MAPK in CAOV-3 cells following CXCL12 stimulation. Total cell lysates were prepared at various timepoints following stimulation with 100 ng/ml CXCL12. Western blots were performed with 10 μg of total protein and probed for p44/42 MAPK and the active form, phospho-p44/42 MAPK (**A**). Densitometry was performed on the blots from (**A**) and the ratio of phospho-p42 MAPK or phospho-p44 MAPK to the non-phosphorylated form was calculated (**B**). Results are representative of two experiments.

6.3.6. Regulation of CXCR4 by cytokines

The effect on CXCR4 expression after stimulation with a range of cytokines and bacterial lipopolysaccharide was investigated in IGROV and CAOV-3 cell lines. Both protein and mRNA expression of CXCR4 were studied.

6.3.6.i. Flow cytometric analysis of protein

Surface expression of CXCR4 protein on IGROV was relatively unchanged after stimulation with TNF-α (10 ng/ml), IFN-α (1000 U/ml), IFN-γ (1000 U/ml), TGF-β (10 ng/ml) and LPS (100 ng/ml), with intensity of fluorescence varying by no more than 10 %. However, in CAOV-3 cells, CXCR4 protein expression was reproducibly upregulated by TGF-β (mean fluorescence intensity of 29.1 compared with control, 21.2), and downregulated by IFN-γ (mean fluorescence intensity of 13.5 compared with control, 21.2), although none of the other cytokines nor LPS had any effect (Figure 6.14.).

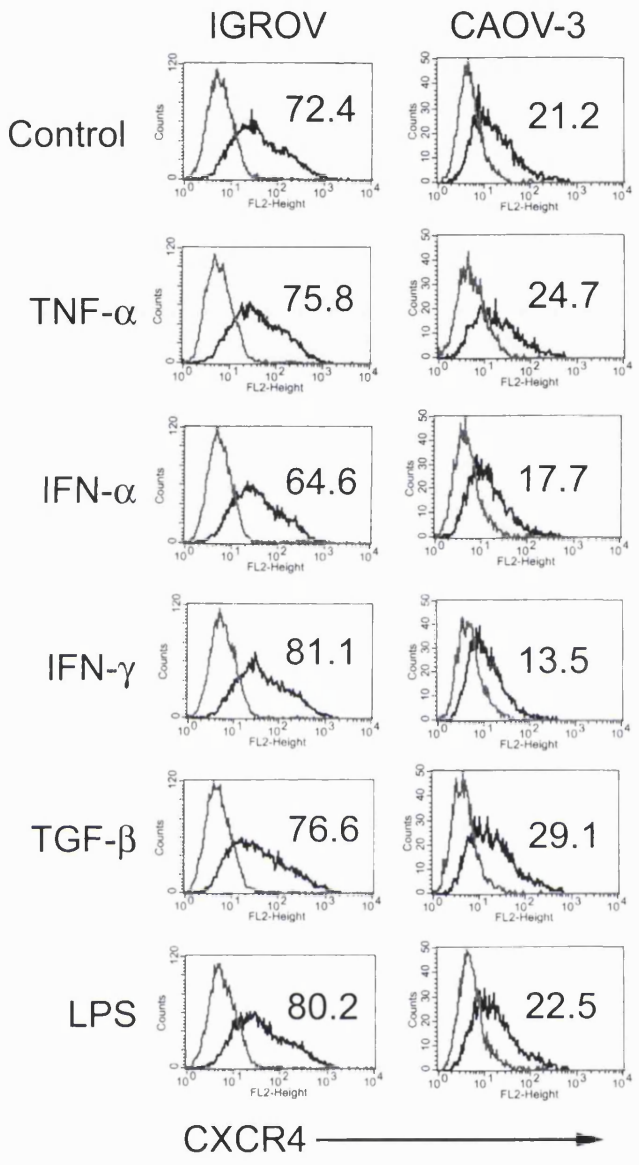


Figure 6.14. Modulation of CXCR4 cell surface expression on IGROV and CAOV-3 cells. A range of cytokines were tested for their ability to modulate CXCR4 expression on IGROV and CAOV-3 cell lines as detected by flow cytometry; CXCR4 mAb (black line), isotype control Ab (grey line). Numbers represent the mean fluorescence intensity of CXCR4 expression. Results are representative of two separate experiments.

6.3.6.ii. Northern analysis of mRNA

CXCR4 mRNA expression was also examined in the IGROV and CAOV-3 cells used in Section 6.3.6.i. Northern analysis showed that CXCR4 mRNA levels were relatively

unchanged after cytokine stimulation of IGROV cells (Figure 6.15.); only TGF-β gave a slight increase in CXCR4 mRNA levels, which was not reflected at the protein level (see Section 6.3.6.i.). In CAOV-3 cells, IFN-γ caused a significant decrease in CXCR4 mRNA levels, which concurred with the decrease in cell surface protein expression (Figure 6.16.). However, TGF-β did not significantly increase CXCR4 mRNA expression, suggesting that the increased protein expression seen after stimulation with this cytokine is due to post-transcriptional events.

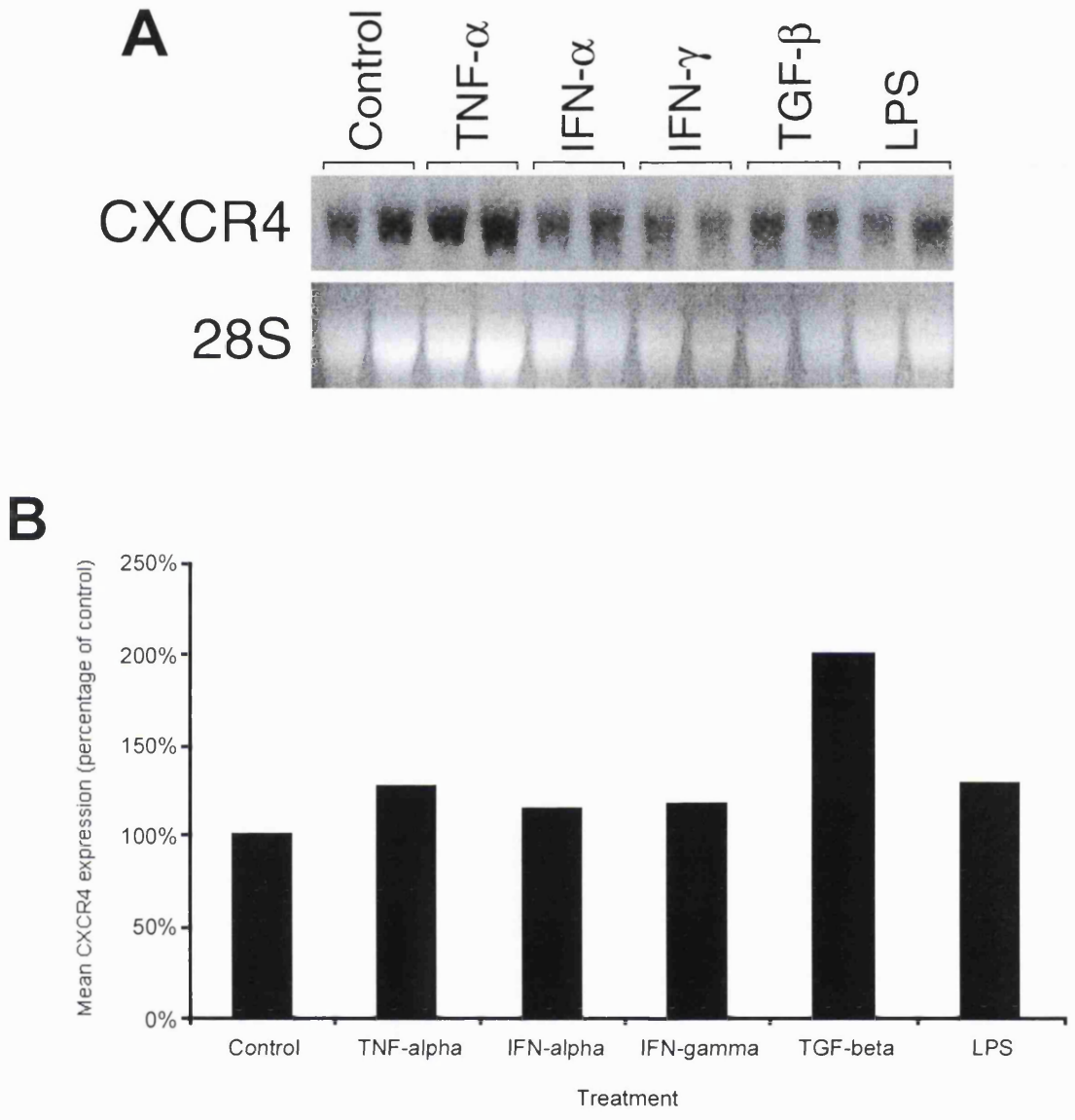


Figure 6.15. Modulation of CXCR4 mRNA expression in IGROV cells. A range of cytokines were tested for their ability to modulate CXCR4 expression in IGROV cells as detected by northern analysis (**A**). 28S rRNA is shown as a loading control. Densitometric analysis was performed on the blots from **A**; the results show CXCR4 mRNA expression as a percentage of the control levels (**B**). Results are representative of two separate experiments.

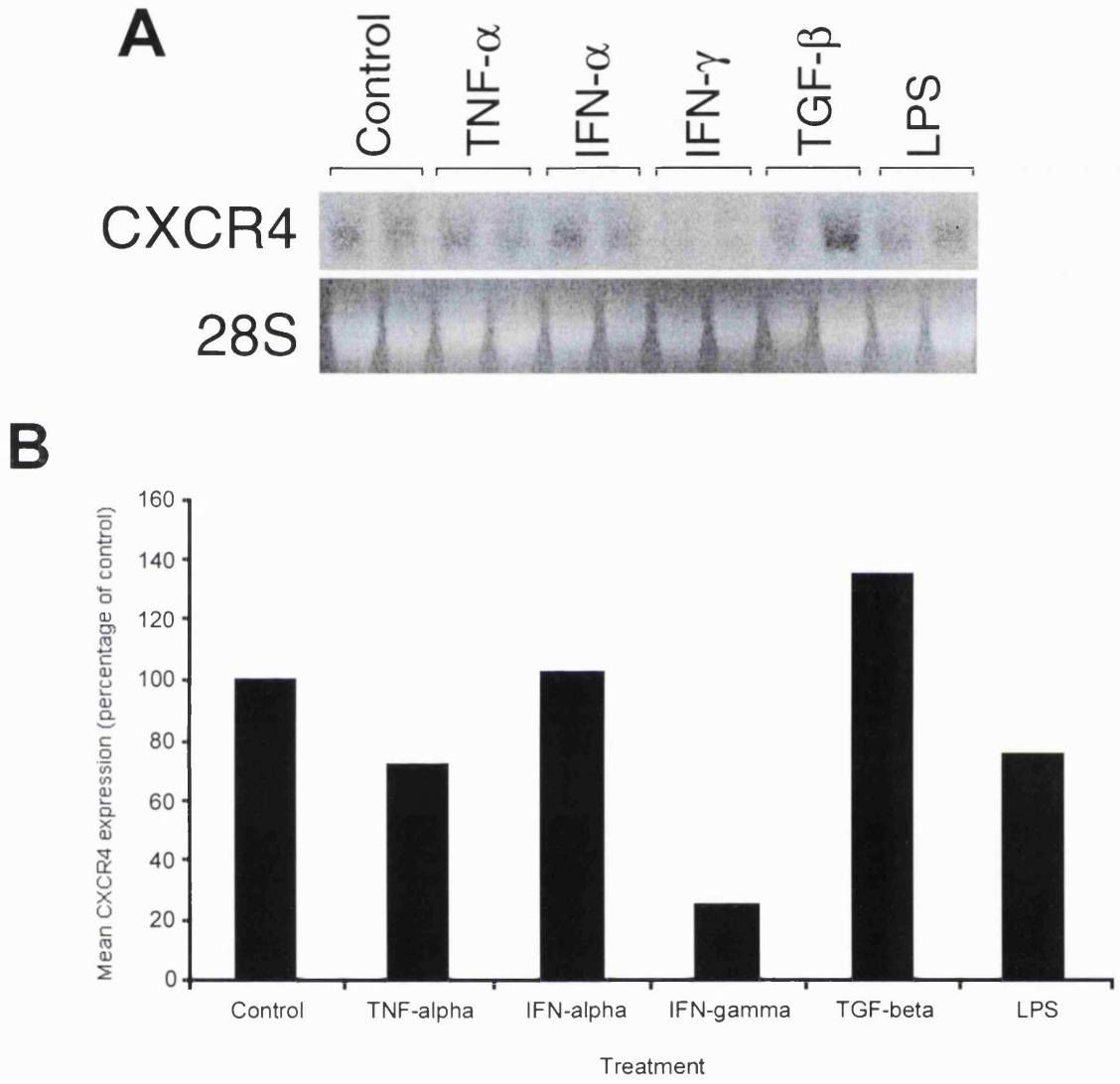


Figure 6.16. Modulation of CXCR4 mRNA expression in CAOV-3 cells. A range of cytokines were tested for their ability to modulate CXCR4 expression in CAOV-3 cells as detected by northern analysis (**A**). 28S rRNA is shown as a loading control. Densitometric analysis was performed on the blots from **A**; the results show CXCR4 mRNA expression as a percentage of the control levels (**B**). Results are representative of two separate experiments.

6.4. Discussion

CXCR4 expression by the ovarian cancer cell lines IGROV and CAOV-3 is functional. This has implications for CXCR4 expression by tumour cells in solid human ovarian cancer and ovarian cancer ascites.

Importantly, both IGROV and CAOV-3 cells were able to migrate in response to CXCL12. 100 ng/ml and 300 ng/ml CXCL12 induced significant migration in a transwell assay. Moreover, both IGROV and CAOV-3 cells were able to invade through matrigel™ along a gradient of CXCL12. The ability to migrate and invade may be crucial for tumour cells to metastasise. As discussed in Chapter 5, tumour cells could follow chemokine gradients as part of the multi-step process of metastatic spread. Direct evidence for chemokines influencing the secondary localisation of cancer has been obtained in a mouse lymphoma model: local CCL2 and CCL5 expression by kidney was implicated in the tendency of ESb-MP lymphoma cells to form kidney metastases in DBA2 mice (451). Work by Muller *et al* showed that breast cancer cells migrate specifically towards lung and liver homogenates (which are sites for breast cancer metastasis) in response to CXCL12 (286).

The invasiveness of the ovarian cancer cell lines may be dependent on the production of matrix metalloproteinases and other matrix-degrading enzymes. *In vivo*, metastasising cancer cells must degrade extracellular matrix and cross basement membranes to reach the lymphatic system and other sites of metastasis. IGROV expressed mRNA for MMP-7, which can degrade gelatin, fibronectin and collagen IV. Both IGROV and CAOV-3 expressed mRNA for MMP-13, which degrades collagen I, II and III. IGROV were also positive for MT3-MMP, which can activate pro-MMP-2. Both cell lines expressed mRNA for TIMP-2 and TIMP-3, which can inhibit the activity of various MMPs, and uPAR, which localises the proteolytic activity of uPA. None of these molecules appeared to be regulated by CXCL12, but further work is required to confirm

this result. It is possible that IGROV and CAOV-3 produce a variety of matrix-degrading enzymes, and that CXCL12 merely gives them a direction in which to move. In solid ovarian tumours, MMP-2 and MMP-9 have been implicated in invasion and metastasis of tumour cells (452, 453). These enzymes do not appear to be important *in vitro*.

Treatment of the ovarian cancer cell lines with CXCL12 also significantly increased the cell surface expression of β_1 integrin. The functional significance of this upregulation remains to be determined. However, previous studies have shown that CXCL12 induced the adhesion of most circulating lymphocytes (454), and $CD34^+$ progenitor cells (455). It is tempting to speculate that tumour cells in ovarian cancer ascites could upregulate β_1 integrin, which would allow increased adhesion to the mesothelium; this might promote the formation of micrometastases on the peritoneal wall. Modulation of adhesion molecules is also required during chemotaxis (175).

The high levels of CXCL12 present in ascitic fluid (see Section 5.3.4.iv.) could also support the growth of tumour cells present in ovarian cancer ascites. CXCL12-stimulation increased the proliferation/survival of both IGROV and CAOV-3 cells *in vitro*. This phenomenon is similar to the growth-promoting effects of CXCL8 and CXCL1/2/3 on melanoma cells, and the effect of CXCL8 and CXCL1 on pancreatic cancer cell lines (277-279). The growth-promoting effects of CXCL12 were inhibited by anti-CXCR4 neutralising antibodies, demonstrating that CXCR4 is necessary for this response. Unlike melanoma cells, this does not appear to be an autocrine mechanism – IGROV and CAOV-3 cells do not produce their own CXCL12 (see Section 5.3.4.iii.) and the anti-CXCR4 neutralising antibody did not reduce growth below the level of control cells. CXCL12 protein was also detected in solid tumours (see Section 5.3.4.ii.), suggesting that this chemokine could have important autocrine or paracrine growth-promoting effects in solid ovarian tumours.

All chemokine receptors belong to the same class of seven transmembrane domain proteins and are coupled to heterotrimeric G proteins. Binding of chemokines to their receptors can activate a variety of signal transduction pathways, which were discussed in Chapter 1 (See section 1.2.3.v.). Stimulation of both IGROV and CAOV-3 cells with CXCL12 caused an intracellular calcium flux, demonstrating that CXCR4 is able to transduce a signal in these cells. In this case, ligation of the receptor probably caused activation of PLC-β_2 and PLC-β_3, which leads to the formation of inositol-1,4,5-trisphosphate and a transient rise in the intracellular free calcium concentration (89).

CXCL12 stimulation also caused internalisation of the cell surface CXCR4. Transient signalling is a common characteristic of chemokine receptors, but requires rapid inactivation. This is achieved through receptor phosphorylation, desensitisation and internalisation (116). Work by Tilton *et al* demonstrated that CXCR4 can stimulate prolonged signalling of Akt/PKB and ERK2 in T cells, despite receptor internalisation – suggesting that CXCR4 remaining on the surface is not desensitised (121).

In IGROV cells, CXCL12 stimulation resulted in the phosphorylation of Akt/PKB on Ser473. This level of phosphorylation was maintained for at least two hours, demonstrating that activation of Akt/PKB is also prolonged in IGROVs. CAOVs did not show a similar activation of Akt/PKB under these conditions. Activation of Akt/PKB is interesting since it has been shown to have roles in chemotaxis and survival (94). Akt/PKB is recruited to the leading edge of the cell during neutrophil chemotaxis (97). In fibroblasts, Akt/PKB can promote survival by blocking Caspase 3 activity (456), while in haematopoietic cells, Akt/PKB can promote the induction of Bcl-2 and thereby inhibit apoptosis. Cheng *et al* demonstrated that Akt/PKB is overexpressed in a small proportion of solid ovarian tumours, suggesting that this proto-oncogene can contribute to the pathogenesis of ovarian cancer (457). In animal models of prostate cancer, increased Akt/PKB activity has also been shown to contribute to tumour

progression, by accelerating tumour growth (95); prostate cancer cell lines overexpressing Akt/PKB had 6-fold greater tumour growth than the parental cell line, and this was related to decreased expression of $p27^{kip1}$ (which is a negative regulator of the cell cycle).

p44/42 MAPK (ERK1/ERK2) were also phosphorylated in IGROV and CAOV-3 cells following CXCL12 stimulation. As for Akt/PKB, the phosphorylation of p44/42 MAPK was prolonged – the increased level of phosphorylation was maintained at two hours. Further work will be required to elucidate the mechanisms involved in this prolonged signalling. Studies by Tilton *et al* suggest that CXCR4 remaining at the cell surface (i.e. not internalised) is not desensitised, but can continue to transduce a signal in response to CXCL12 (121). Receptor recycling could also contribute to this effect. In IGROV cells, the phosphorylation of p44/42 MAPK was biphasic. There was an initial increase in the ratio of phospho-p44/42 MAPK to p44/42 MAPK after 15 minutes. This ratio then decreased back to control levels after 30 minutes, before increasing again after an hour. Recent work by Han *et al* showed biphasic activation of p44/42 MAPK in astrocytes in response to CXCL12 (458). Early activation of p44/42 MAPK was directly due to CXCL12 stimulation; late activation was indirectly mediated by CXCL12-induced TNF-α. A similar mechanism may be responsible for the biphasic response seen in IGROV cells.

The work in this chapter also showed that CXCR4 levels could be regulated by cytokines, being upregulated by TGF-β, a cytokine that is found in ovarian cancer, but downregulated by IFN-γ which is absent from this tumour microenvironment (361). In Chapter 5, CXCR4 mRNA expression was found only on a proportion of the tumour cells in the solid tumour biopsies; regulation by cytokines within the tumour microenvironment could explain this finding. Alternatively, some cells may have acquired constitutive CXCR4 expression during malignant progression; in this respect it

is noted that CXCR4 levels could be regulated by IFN-γ and TGF-β in CAOV-3 but not IGROV cells. Downregulation of CXCR4 expression by IFN-γ has also been reported in human endothelial cells (262). Upregulation of CXCR4 has been observed in Langerhans cells cultured with TGF-β and IL-4 (459) and on synovial T cells by TGF-β (460). Modulation of CXCR4 expression by other factors has been described including upregulation on endothelial cells by VEGF or bFGF (263) and on T cells by IL-4 and dexamethasone (461, 462). As CXCL12 is constitutively expressed in many tissues, CXCR4 regulation is likely to be important in controlling the response to this chemokine.

The host response to a malignant tumour is not a unique mechanism but has many parallels with inflammation and wound healing. There is increasing evidence that the inflammatory cells, cytokines and chemokines found in human tumours are more likely to contribute to tumour growth, progression and immunosuppression than they are to mount an effective immune response [see (245) for review]. Over the past ten years, study of the cytokine and chemokine network has led to the development of a range of cytokine and chemokine antagonists for the treatment of inflammation and allergy. It is possible that such agents may be of benefit in the treatment of malignant disease and there is some rationale for the use of CXCR4 antagonists as part of a biological approach to treatment of ovarian cancer.

Conclusions from this chapter:

- CXCR4 expression by IGROV and CAOV-3 ovarian cancer cell lines is functional.
- IGROV and CAOV-3 can migrate and invade along a CXCL12 gradient.
- CXCL12 stimulation can upregulate β_1 integrin.
- IGROV and CAOV-3 cells show increased proliferation in response to CXCL12 stimulation.
- Ligation of CXCR4 with its ligand leads to downstream signalling events, including calcium flux, internalisation and the prolonged phosphorylation of Akt/PKB and p44/42 MAPK.

Chapter 7. Summary and future plans

7.1. Summary

Chemokines and their receptors may have important roles in the development, growth and spread of human ovarian cancer. Solid human ovarian carcinomas contain a leukocyte infiltrate consisting predominantly of $CD68^+$ macrophages and $CD3^+$, $CD8^+$, $CD45RO^+$ T cells. Ascitic fluid, which is commonly associated with advanced ovarian cancer also contains leukocytes, including variable numbers of macrophages and T cells (which are predominantly $CD4^+$). Chemokines and their receptors are important in leukocyte trafficking in pathological conditions; therefore leukocytes may be attracted to the tumour by local production of chemokines. Negus *et al* (230) in this laboratory showed that expression of the CC chemokine CCL2 correlated with the number of $CD8^+$ and $CD68^+$ cells in ovarian cancer; CCL5 was also correlated with the number of $CD8^+$ cells.

Solid ovarian tumours express a variety of CC chemokines, including CCL2, CCL3, CCL4, CCL5, CCL8 and CCL22. However, expression of CC chemokine receptors is more restricted. Using an RNase protection assay, only CCR1 (one of the receptors for CCL5) was expressed in the majority of solid tumour biopsies. CCR2b, the receptor for CCL2, was rarely expressed. We propose a simple hypothesis that circulating leukocytes expressing CCR2b can be attracted to solid tumours by CCL2. However, once in the tumour microenvironment, CCR2b is downregulated by factors such as TNF-α (180). CCR1 expression by $CD8^+$ T cells and $CD68^+$ macrophages could enable them to migrate within the tumour microenvironment in response to CCL3, 4 and 5, among others.

Of interest is the accumulation of macrophages in areas of chronic hypoxia (necrotic regions). Hypoxia is a potent 'stop' signal for macrophage migration (378, 414) after as

little as 30 minutes exposure. CCR1 is upregulated after 24 hours hypoxia *in vitro*, suggesting that chronic hypoxia may enable macrophages to respond again to CCL3, 4 and 5, and thereby move away again from hypoxic regions.

In contrast to the solid tumour, CC chemokine receptor expression in ascites is similar to that of PBMC, with most receptors being expressed in a majority of samples. CCR1, 2 and 5 were found on T cells and macrophages, and CCL5 correlated with the $CD3^+$ infiltrate. The predominance for $CD4^+$ T cells in ascites could be due to greater CCR5 expression on these cells, compared with $CD8^+$ T cells. The combined effects of various chemokines acting through the variety of receptors expressed on the leukocytes may determine the composition of the leukocyte infiltrate.

Manipulation of the infiltrate could have therapeutic benefit in ovarian cancer. Nesbit *et al* (234) recently showed that low-level secretion of CCL2 in melanoma caused modest infiltration of leukocytes, particularly monocytes. This infiltrate promoted tumour growth by providing growth factors and angiogenic molecules. Conversely, high-level production of CCL2 caused a massive monocyte infiltrate leading to tumour destruction, possibly by allowing the host leukocytes to mount an effective immune response to the tumour. Increasing or reducing the amount of infiltrate in ovarian cancer may therefore reduce tumour growth. CCR1 antagonists such as Met-RANTES are readily available, and evaluating their benefit in animal cancer models would be useful.

More intriguing is the discovery that epithelial ovarian cancer cells can also express chemokine receptors. CXCR4, which is important for development, haematopoiesis, trafficking to secondary lymphoid organs and inflammation, was found on epithelial tumour cells in solid tumour biopsies and ascites, and on ovarian cancer cell lines. This CXCR4 was functional, allowing ovarian cancer cell lines to migrate and invade

towards CXCL12 (the sole ligand for CXCR4). CXCL12 stimulation also promoted growth/survival *in vitro*.

Ovarian cancer spreads predominantly via dissemination throughout the peritoneal cavity. CXCL12 was present at very high concentrations in ascitic fluid, suggesting that it could form a gradient for movement of tumour cells from the solid tumour into the peritoneum. Once there, growth/survival of these cells could be promoted by CXCL12. Preliminary results also demonstrate CXCL12 expression within the solid tumour. These findings suggest that CXCL12 may be more important as an autocrine or paracrine growth factor for the tumour cells, rather than having a role in local or distant spread. Moreover, some endothelial cells within the solid tumour expressed CXCR4, suggesting a role for CXCR4/CXCL12 in angiogenesis.

This work suggests that CXCR4 is a target for therapeutic intervention in ovarian cancer. Owing to its role as a co-receptor for HIV entry, a variety of antagonists have been developed against CXCR4. These antagonists may have beneficial effects in treating ovarian cancer by reducing tumour growth, reducing angiogenesis and preventing metastasis. However, CXCR4 is widely expressed throughout the body by a variety of different cell types and there may be side effects from the use of CXCR4 antagonists. In the bone marrow, CXCR4/CXCL12 may be important for the retention of haematopoietic progenitor cells. It is possible that CXCR4/CXCL12 have a similar role in ovarian cancer – CXCR4 antagonists could abrogate retention of tumour cells within the primary tumour, and promote their dissemination. Further work is required to investigate these possibilities.

7.2. Future plans

There are a number of questions arising from this thesis that I hope will be answered by further work in the Translational Oncology Laboratory. The following sections will outline some of these experiments.

7.2.1. Hypoxic regulation of CCR1

The mechanism responsible for the upregulation of CCR1 in THP-1 cells under hypoxic conditions has not yet been determined. The experiments described below would help to characterise the factors involved, and determine whether this phenomenon is relevant *in vivo*:

- Clone the promoter region of CCR1 and use reporter assays (such as luciferase or chloramphenicol transferase) to investigate the hypoxic upregulation of this receptor in transfected cells. Deletion/mutation of the potential HIF-1 binding sites would reveal whether this transcription factor is important for CCR1 mRNA regulation. Other transcription factors (such as NFκB) could also be important for this response.
- Repeat the investigation of CCR1 mRNA regulation in primary monocytes/macrophages, using alternative techniques for purification. Monocytes derived by positive MACS selection (rather than the negative selection employed here) from PBMC may actually be less activated (Matt Grimshaw, personal communication), and therefore express lower initial levels of CCR1 mRNA. Regulation of CCR1 mRNA by other cytokines known to be present in the ovarian tumour microenvironment could also be examined.
- Investigate the expression of CCR1 protein by flow cytometry after exposure to hypoxia.
- Use the 3-dimensional tumour model (spheroids) to investigate CCR1 regulation by hypoxia in a more physiological environment. First, the necrotic cores of tumour spheroids could be labelled with nitroimidazole-theophylline (NITP) to determine

whether they are hypoxic or not. *In situ* hybridisation could be used to assess CCR1 mRNA expression within the spheroid; RNase protection assay for chemokine receptors would show whether the profile is similar to that seen in solid tumours, e.g. downregulation of CCR2b etc. The efficacy of chemokine receptor antagonists could also be assessed using this *in vitro* model.

7.2.2. CXCR4/CXCL12 system in ovarian cancer

A variety of experiments could be performed to further assess the significance of CXCR4 expression by tumour cells in ovarian cancer. In particular, the therapeutic benefits of CXCR4 antagonists could be assessed:

7.2.2.i. Localisation of CXCR4 and CXCL12 in solid ovarian tumour biopsies

- Use *in situ* hybridisation to assess the expression of CXCR4 and CXCL12 mRNA during the progression of ovarian cancer. mRNA localisation could be studied in normal ovarian epithelium, benign tumours, increasing grades of carcinoma, peritoneal metastases and involved lymph nodes.

7.2.2.ii. CXCR4 protein expression by ovarian cancer cell lines

- CXCR4 protein expression by IGROV and CAOV-3 appears to decrease with increasing passage number. Recent work by Baribaud *et al* (463) has shown that CXCR4 can exist in antigenically distinct conformations; the anti-CXCR4 antibody (clone 12G5) used in this thesis only detects a small proportion of the CXCR4 molecules present on the surface of leukocytes. It is possible that the apparent decline in cell-surface CXCR4 with increasing passage number of the ovarian cancer cell lines is due to a change in the conformational state of the receptor. This could be assessed using a panel of monoclonal anti-CXCR4 antibodies.

7.2.2.iii. Migration/invasion of ovarian cancer cell lines

- Confirm whether CXCR4-neutralising antibodies and CXCR4 antagonists can abrogate CXCL12-stimulated migration and/or invasion of ovarian cancer cell lines.
- Determine whether marimastat (BB2516; a pan-matrix metalloproteinase inhibitor) can prevent invasion of ovarian cancer cell lines. This would verify whether MMPs are required for the invasion of these cells. The up- or downregulation of more matrix-degrading enzymes could be investigated by RT-PCR, real-time PCR or zymography.
- Investigate whether ovarian cancer cell lines can migrate towards ascitic fluid.

7.2.2.iv. Downstream effects of CXCL12 stimulation in ovarian cancer cell lines

- Assess whether CXCL12 stimulation of IGROV and CAOV-3 leads to the up- or downregulation of cytokines and other chemokines using RNase protection assay. Recent work by Han *et al* (458) has shown that CXCL12 can upregulate TNF-α in primary astrocytes; this TNF-α induction was responsible for biphasic activation of ERK1/ERK2, which is similar to the signalling response seen in IGROV.
- Investigate other signalling molecules, such as JAKs and STATs, and NFκB.

7.2.2.v. Proliferation/survival of ovarian cancer cell lines

- Determine whether CXCR4 antagonists can inhibit the increased proliferation of IGROV and CAOV-3 after CXCL12 stimulation.
- Treat the ovarian cancer cell lines with cisplatin (a chemotherapeutic agent known to be effective against ovarian cancer cells) in the presence or absence of CXCL12, to see if CXCL12 protects the cells from apoptosis.
- Investigate whether incubation of the ovarian cancer cell lines with ascitic fluid increases their growth, in the presence or absence of CXCR4 antagonists.

7.2.2.vi. Animal models of ovarian cancer

- IGROV grows as a xenograft in nude mice. The effect of anti-CXCR4 neutralising antibodies or CXCR4 antagonists on growth of the tumour could be assessed. Human and murine CXCR4/CXCL12 are cross-reactive, so xenografts would be a valuable tool for looking at effects on metastasis, angiogenesis and the leukocyte infiltrate.
- CXCR4 expression on IGROV could also be manipulated to assess its effect on tumour growth. A recent paper by Zeelenberg *et al* (464) demonstrated that retention of CXCR4 in the endoplasmic reticulum (ER), using a CXCL12 construct engineered to express an ER retention sequence, blocked the dissemination of a T cell hybridoma. Similar experiments could be performed with IGROV. In addition, the effects of CXCR4 overexpression could be investigated.

7.2.2.vii. CXCR4/CXCL12 in other types of cancer

- The expression of CXCR4 and CXCL12 in other cancers could also be investigated. Our links with the Gynaecological Cancer Research Unit at St. Bartholomew's Hospital, London means that biopsies from other gynaecological cancers, particularly cervical cancer, are available for investigation.

The work presented and the papers reviewed in this thesis suggest that chemokines and their receptors may have important roles in tumour pathology. The chemokine/receptor network may affect tumour growth and spread; it is therefore an appropriate target for intervention. Moreover, understanding this network in ovarian cancer could provide insights into the pathology of other diseases, including other types of cancer, chronic inflammation, allergy and autoimmune diseases. It also demonstrates the common links between cancer biology, inflammation and the process of wound healing.

Publications

Parts of this thesis have been published; reprints are enclosed at the back.

Published work

Scotton, C., Milliken, D., Wilson, J., Raju, S., and Balkwill, F. 2001. Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours. *Br J Cancer* 85:891-897

Scotton, C., Wilson, J., Milliken, D., Stamp, G. and Balkwill, F. 2001. Epithelial cancer cell migration: a role for chemokine receptors? *Cancer Res* **61**: 4961-4965

Scotton, C. and Burke, F. 2000. Northern analysis, RT-PCR, RNase protection, cDNA array systems, and *in situ* hybridization detect cytokine messenger RNA. *Cytokine Molecular Biology: A Practical Approach.* Oxford University Press

Sica, A., Saccani, A., Bottazzi, B., Bernasconi, S., Allavena, P., Gaetano, B., Fei, F., LaRosa, G., **Scotton, C.**, Balkwill, F., et al. 2000. Defective Expression of the Monocyte Chemotactic Protein-1 Receptor CCR2 in Macrophages Associated with Human Ovarian Carcinoma. *J Immunol* 164:733-738.

Turner, L., **Scotton, C.**, Negus, R., and Balkwill, F. 1999. Hypoxia inhibits macrophage migration. *Eur J Immunol* 29:2280-2287.

Submitted for publication

Milliken, D., **Scotton, C.**, Raju, S., Balkwill, F. and Wilson, J. 2001. Analysis of chemokines and chemokine receptor expression in ovarian cancer ascites. Submitted to *Clin Cancer Res.*

Published abstracts

Scotton, C.J., Milliken, T.D.A. and Balkwill, F.R. 1999. Chemokines and their receptors in the ovarian tumour microenvironment. *Cytokine* 11(11): 918-918. Abstract.

Scotton, C.J., Turner, L., Negus, R.P.M. and Balkwill, F.R. 1998. Chemokines and their receptors in the ovarian tumour microenvironment. *Immunology* 95(S1): 123-123. Abstract.

References

1. Deuel, T.F., Keim, P.S., Farmer, M., and Heinrikson, R.L. 1977. Amino acid sequence of human platelet factor 4. *Proc Natl Acad Sci U S A* 74:2256-2258.

2. Begg, G.S., Pepper, D.S., Chesterman, C.N., and Morgan, F.J. 1978. Complete covalent structure of human beta-thromboglobulin. *Biochemistry* 17:1739-1744.

3. Yoshimura, T., Matsushima, K., Tanaka, S., Robinson, E.A., Appella, E., Oppenheim, J.J., and Leonard, E.J. 1987. Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines. *Proc Natl Acad Sci U S A* 84:9233-9237.

4. Schroder, J.M., and Christophers, E. 1986. Identification of C5ades arg and an anionic neutrophil-activating peptide (ANAP) in psoriatic scales. *J Invest Dermatol* 87:53-58.

5. Murphy, P.M., Baggiolini, M., Charo, I.F., Hebert, C.A., Horuk, R., Matsushima, K., Miller, L.H., Oppenheim, J.J., and Power, C.A. 2000. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev* 52:145-176.

6. Nelson, P.J., and Krensky, A.M. 2001. Chemokines, chemokine receptors, and allograft rejection. *Immunity* 14:377-386.

7. Kelner, G.S., Kennedy, J., Bacon, K.B., Kleyensteuber, S., Largaespada, D.A., Jenkins, N.A., Copeland, N.G., Bazan, J.F., Moore, K.W., Schall, T.J., et al. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. *Science* 266:1395-1399.

8. Bazan, J.F., Bacon, K.B., Hardiman, G., Wang, W., Soo, K., Rossi, D., Greaves, D.R., Zlotnik, A., and Schall, T.J. 1997. A new class of membrane-bound chemokine with a cx(3)c motif. *Nature* 385:640-644.

9. Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., et al. 1997. Identification and

molecular characterization of fractalkine receptor CX3CR1, which mediates both leukocyte migration and adhesion. *Cell* 91:521-530.

10. Wilbanks, A., Zondlo, S.C., Murphy, K., Mak, S., Soler, D., Langdon, P., Andrew, D.P., Wu, L., and Briskin, M. 2001. Expression cloning of the STRL33/BONZO/TYMSTRligand reveals elements of CC, CXC, and CX3C chemokines. *J Immunol* 166:5145-5154.

11. Witt, D.P., and Lander, A.D. 1994. Differential binding of chemokines to glycosaminoglycan subpopulations. *Curr Biol* 4:394-400.

12. Tanaka, Y., Adams, D.H., and Shaw, S. 1993. Proteoglycans on endothelial cells present adhesion-inducing cytokines to leukocytes. *Immunol Today* 14:111-115.

13. Zlotnik, A., and Yoshie, O. 2000. Chemokines: A new classification system and their role in immunity. *Immunity* 12:121-127.

14. Committee, I.W.N. 2001. Chemokine/Chemokine Receptor Nomenclature. *J Leukoc Biol* 70:465-466.

15. Hebert, C.A., Vitangcol, R.V., and Baker, J.B. 1991. Scanning mutagenesis of interleukin-8 identifies a cluster of residues required for receptor binding. *J Biol Chem* 266:18989-18994.

16. Maione, T.E., Gray, G.S., Petro, J., Hunt, A.J., Donner, A.L., Bauer, S.I., Carson, H.F., and Sharpe, R.J. 1990. Inhibition of angiogenesis by recombinant human platelet factor-4 and related peptides. *Science* 247:77-79.

17. Chung, C.W., Cooke, R.M., Proudfoot, A.E., and Wells, T.N. 1995. The three-dimensional solution structure of RANTES. *Biochemistry* 34:9307-9314.

18. Clore, G.M., Appella, E., Yamada, M., Matsushima, K., and Gronenborn, A.M. 1990. Three-dimensional structure of interleukin 8 in solution. *Biochemistry* 29:1689-1696.

19. Crump, M.P., Gong, J.H., Loetscher, P., Rajarathnam, K., Amara, A., Arenzana-Seisdedos, F., Virelizier, J.L., Baggiolini, M., Sykes, B.D., and Clark-Lewis, I. 1997. Solution structure and basis for functional activity of stromal cell-derived factor-1;

dissociation of CXCR4 activation from binding and inhibition of HIV-1. *Embo J* 16:6996-7007.

20. Crump, M.P., Rajarathnam, K., Kim, K.S., Clark-Lewis, I., and Sykes, B.D. 1998. Solution structure of eotaxin, a chemokine that selectively recruits eosinophils in allergic inflammation. *J Biol Chem* 273:22471-22479.

21. Handel, T.M., and Domaille, P.J. 1996. Heteronuclear (1H, 13C, 15N) NMR assignments and solution structure of the monocyte chemoattractant protein-1 (MCP-1) dimer. *Biochemistry* 35:6569-6584.

22. Lodi, P.J., Garrett, D.S., Kuszewski, J., Tsang, M.L., Weatherbee, J.A., Leonard, W.J., Gronenborn, A.M., and Clore, G.M. 1994. High-resolution solution structure of the beta chemokine hMIP-1 beta by multidimensional NMR. *Science* 263:1762-1767.

23. Mizoue, L.S., Bazan, J.F., Johnson, E.C., and Handel, T.M. 1999. Solution structure and dynamics of the CX3C chemokine domain of fractalkine and its interaction with an N-terminal fragment of CX3CR1. *Biochemistry* 38:1402-1414.

24. Zhang, X., Chen, L., Bancroft, D.P., Lai, C.K., and Maione, T.E. 1994. Crystal structure of recombinant human platelet factor 4. *Biochemistry* 33:8361-8366.

25. Schwarz, M.K., and Wells, T.N.C. 1999. Interfering with chemokine networks - the hope for new therapeutics. *Curr Opin Chem Biol* 3:407-417.

26. Rollins, B.J. 1997. Chemokines. *Blood* 90:909-928.

27. Sticht, H., Escher, S.E., Schweimer, K., Forssmann, W.G., Rosch, P., and Adermann, K. 1999. Solution structure of the human CC chemokine 2: A monomeric representative of the CC chemokine subtype. *Biochemistry* 38:5995-6002.

28. Keizer, D.W., Crump, M.P., Lee, T.W., Slupsky, C.M., Clark-Lewis, I., and Sykes, B.D. 2000. Human CC chemokine I-309, structural consequences of the additional disulfide bond. *Biochemistry* 39:6053-6059.

29. Rajarathnam, K., Sykes, B.D., Kay, C.M., Dewald, B., Geiser, T., Baggiolini, M., and Clark-Lewis, I. 1994. Neutrophil activation by monomeric interleukin-8. *Science* 264:90-92.

30. Rajarathnam, K., Kay, C.M., Dewald, B., Wolf, M., Baggiolini, M., Clark-Lewis, I., and Sykes, B.D. 1997. Neutrophil-activating peptide-2 and melanoma growth-stimulatory activity are functional as monomers for neutrophil activation. *J Biol Chem* 272:1725-1729.

31. Laurence, J.S., Blanpain, C., Burgner, J.W., Parmentier, M., and LiWang, P.J. 2000. CC chemokine MIP-1 beta can function as a monomer and depends on Phe13 for receptor binding. *Biochemistry* 39:3401-3409.

32. Paavola, C.D., Hemmerich, S., Grunberger, D., Polsky, I., Bloom, A., Freedman, R., Mulkins, M., Bhakta, S., McCarley, D., Wiesent, L., et al. 1998. Monomeric monocyte chemoattractant protein-1 (mcp-1) binds and activates the mcp-1 receptor ccr2b. *J Biol Chem* 273:33157-33165.

33. Zhang, Y., and Rollins, B.J. 1995. A dominant negative inhibitor indicates that monocyte chemoattractant protein 1 functions as a dimer. *Molecular & Cellular Biology* 15:4851-4855.

34. Moser, B., Dewald, B., Barella, L., Schumacher, C., Baggiolini, M., and Clark-Lewis, I. 1993. Interleukin-8 antagonists generated by N-terminal modification. *J Biol Chem* 268:7125-7128.

35. Holmes, W.D., Consler, T.G., Dallas, W.S., Rocque, W.J., and Willard, D.H. 2001. Solution studies of recombinant human stromal-cell-derived factor-1. *Protein Expr Purif* 21:367-377.

36. Hoogewerf, A.J., Kuschert, G.S., Proudfoot, A.E., Borlat, F., Clark-Lewis, I., Power, C.A., and Wells, T.N. 1997. Glycosaminoglycans mediate cell surface oligomerization of chemokines. *Biochemistry* 36:13570-13578.

37. Thelen, M., Peveri, P., Kernen, P., von Tscharner, V., Walz, A., and Baggiolini, M. 1988. Mechanism of neutrophil activation by NAF, a novel monocyte-derived peptide agonist. *Faseb J* 2:2702-2706.

38. Schumacher, C., Clark-Lewis, I., Baggiolini, M., and Moser, B. 1992. High- and low-affinity binding of GRO alpha and neutrophil-activating peptide 2 to interleukin 8 receptors on human neutrophils. *Proc Natl Acad Sci U S A* 89:10542-10546.

39. Oppenheim, J.J., Zachariae, C.O., Mukaida, N., and Matsushima, K. 1991. Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu Rev Immunol* 9:617-648.

40. Holmes, W.E., Lee, J., Kuang, W.J., Rice, G.C., and Wood, W.I. 1991. Structure and functional expression of a human interleukin-8 receptor. *Science* 253:1278-1280.

41. Murphy, P.M., and Tiffany, H.L. 1991. Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* 253:1280-1283.

42. Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J., and Coughlin, S.R. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc Natl Acad Sci U S A* 91:2752-2756.

43. Szabo, M.C., Soo, K.S., Zlotnik, A., and Schall, T.J. 1995. Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor. *J Biol Chem* 270:25348-25351.

44. Neote, K., Darbonne, W., Ogez, J., Horuk, R., and Schall, T.J. 1993. Identification of a promiscuous inflammatory peptide receptor on the surface of red blood cells. *J Biol Chem* 268:12247-12249.

45. Chaudhuri, A., Rodriguez, M., Zbrzezna, V., Pogo, A.O., and Banerjee, D. 1998. The induction of duffy antigen receptor for chemokines in human endothelial-cells. *Mol Biol Cell* 9:2165-2165.

46. Hadley, T.J., Lu, Z.H., Wasniowska, K., Martin, A.W., Peiper, S.C., Hesselgesser, J., and Horuk, R. 1994. Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy blood group antigen. *J Clin Invest* 94:985-991.

47. Nibbs, R.J., Wylie, S.M., Yang, J., Landau, N.R., and Graham, G.J. 1997. Cloning and characterization of a novel promiscuous human beta- chemokine receptor D6. *J Biol Chem* 272:32078-32083.

48. Khoja, H., Wang, G.L., Ng, C.T.L., Tucker, J., Brown, T., and Shyamala, V. 2000. Cloning of CCRL1, an orphan seven transmembrane receptor related to chemokine receptors, expressed abundantly in the heart. *Gene* 246:229-238.

49. Fan, P., Kyaw, H., Su, K., Zeng, Z.Z., Augustus, M., Carter, K.C., and Li, Y. 1998. Cloning and characterization of a novel human chemokine receptor. *Biochem Biophys Res Commun* 243:264-268.

50. Ahuja, S.K., and Murphy, P.M. 1993. Molecular piracy of mammalian interleukin-8 receptor type B by herpesvirus saimiri. *J Biol Chem* 268:20691-20694.

51. Gao, J.L., and Murphy, P.M. 1994. Human cytomegalovirus open reading frame US28 encodes a functional beta chemokine receptor. *J Biol Chem* 269:28539-28542.

52. Gao, J.L., and Murphy, P.M. 1995. Cloning and differential tissue-specific expression of three mouse beta chemokine receptor-like genes, including the gene for a functional macrophage inflammatory protein-1 alpha receptor. *J Biol Chem* 270:17494-17501.

53. Ahuja, S.K., Ozcelik, T., Milatovitch, A., Francke, U., and Murphy, P.M. 1992. Molecular evolution of the human interleukin-8 receptor gene cluster. *Nat Genet* 2:31-36.

54. Boulay, F., Tardif, M., Brouchon, L., and Vignais, P. 1990. Synthesis and use of a novel N-formyl peptide derivative to isolate a human N-formyl peptide receptor cDNA. *Biochem Biophys Res Commun* 168:1103-1109.

55. Ahuja, S.K., Gao, J.L., and Murphy, P.M. 1994. Chemokine receptors and molecular mimicry. *Immunol Today* 15:281-287.

56. Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., et al. 2000. Crystal structure of rhodopsin: A G protein-coupled receptor. *Science* 289:739-745.

57. Gerard, C., and Gerard, N.P. 1994. C5A anaphylatoxin and its seven transmembrane-segment receptor. *Annu Rev Immunol* 12:775-808.

58. Fraser, C.M., Wang, C.D., Robinson, D.A., Gocayne, J.D., and Venter, J.C. 1989. Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. *Mol Pharmacol* 36:840-847.

59. Farzan, M., Choe, H., Martin, K.A., Sun, Y., Sidelko, M., Mackay, C.R., Gerard, N.P., Sodroski, J., and Gerard, C. 1997. HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5. *J Biol Chem* 272:6854-6857.

60. Lefkowitz, R.J. 1993. G protein-coupled receptor kinases. *Cell* 74:409-412.

61. Richardson, R.M., DuBose, R.A., Ali, H., Tomhave, E.D., Haribabu, B., and Snyderman, R. 1995. Regulation of human interleukin-8 receptor A: identification of a phosphorylation site involved in modulating receptor functions. *Biochemistry* 34:14193-14201.

62. Gayle, R.B., 3rd, Sleath, P.R., Srinivason, S., Birks, C.W., Weerawarna, K.S., Cerretti, D.P., Kozlosky, C.J., Nelson, N., Vanden Bos, T., and Beckmann, M.P. 1993. Importance of the amino terminus of the interleukin-8 receptor in ligand interactions. *J Biol Chem* 268:7283-7289.

63. Ahuja, S.K., Lee, J.C., and Murphy, P.M. 1996. CXC chemokines bind to unique sets of selectivity determinants that can function independently and are broadly distributed on multiple domains of human interleukin-8 receptor B. Determinants of high affinity binding and receptor activation are distinct. *Journal of Biological Chemistry* 271:225-232.

64. Lu, Z.H., Wang, Z.X., Horuk, R., Hesselgesser, J., Lou, Y.C., Hadley, T.J., and Peiper, S.C. 1995. The promiscuous chemokine binding profile of the Duffy antigen/receptor for chemokines is primarily localized to sequences in the amino-terminal domain. *J Biol Chem* 270:26239-26245.

65. Leong, S.R., Kabakoff, R.C., and Hebert, C.A. 1994. Complete mutagenesis of the extracellular domain of interleukin-8 (IL- 8) type A receptor identifies charged residues mediating IL-8 binding and signal transduction. *J Biol Chem* 269:19343-19348.

66. Pease, J.E., Wang, J., Ponath, P.D., and Murphy, P.M. 1998. The n-terminal extracellular segments of the chemokine receptors ccr1 and ccr3 are determinants for mip-1-alpha and eotaxin binding, respectively, but a 2nd domain is essential for efficient receptor activation. *J Biol Chem* 273:19972-19976.

67. Monteclaro, F.S., and Charo, I.F. 1996. The amino-terminal extracellular domain of the MCP-1 receptor, but not the RANTES/MIP-1alpha receptor, confers chemokine selectivity. Evidence for a two-step mechanism for MCP-1 receptor activation. *Journal of Biological Chemistry* 271:19084-19092.

68. Chuntharapai, A., Lee, J., Hebert, C.A., and Kim, K.J. 1994. Monoclonal antibodies detect different distribution patterns of IL-8 receptor A and IL-8 receptor B on human peripheral blood leukocytes. *J Immunol* 153:5682-5688.

69. Ling, K., Wang, P., Zhao, J., Wu, Y.L., Cheng, Z.J., Wu, G.X., Hu, W., Ma, L., and Pei, G. 1999. Five-transmembrane domains appear sufficient for a G protein-coupled receptor: Functional five-transmembrane domain chemokine receptors. *Proc Natl Acad Sci U S A* 96:7922-7927.

70. RodriguezFrade, J.M., VilaCoro, A.J., deAna, A.M., Albar, J.P., MartinezA, C., and Mellado, M. 1999. The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. *Proc Natl Acad Sci U S A* 96:3628-3633.

71. Mellado, M., Rodriguez-Frade, J.M., Vila-Coro, A.J., Fernandez, S., Martin de Ana, A., Jones, D.R., Toran, J.L., and Martinez, A.C. 2001. Chemokine receptor homo- or heterodimerization activates distinct signaling pathways. *Embo J* 20:2497-2507.

72. Mellado, M., Rodriguez-Frade, J.M., Manes, S., and Martinez, A.C. 2001. Chemokine signaling and functional responses: the role of receptor dimerization and TK pathway activation. *Annu Rev Immunol* 19:397-421.

73. Preobrazhensky, A.A., Dragan, S., Kawano, T., Gavrilin, M.A., Gulina, I.V., Chakravarty, L., and Kolattukudy, P.E. 2000. Monocyte chemotactic protein-1 receptor CCR2B is a glycoprotein that has tyrosine sulfation in a conserved extracellular N-terminal region. *J Immunol* 165:5295-5303.

74. Hayes, I.M., Jordan, N.J., Towers, S., Smith, G., Paterson, J.R., Earnshaw, J.J., Roach, A.G., Westwick, J., and Williams, R.J. 1998. Human vascular smooth-muscle cells express receptors for cc chemokines. *Arterioscler Thromb Vasc Biol* 18:397-403.

75. Schecter, A.D., Calderon, T.M., Berman, A.B., McManus, C.M., Fallon, J.T., Rossikhina, M., Zhao, W.X., Christ, G., Berman, J.W., and Taubman, M.B. 2000. Human vascular smooth muscle cells possess functional CCR5. *J Biol Chem* 275:5466-5471.

76. Salcedo, R., Resau, J.H., Halverson, D., Hudson, E.A., Dambach, M., Powell, D., Wasserman, K., and Oppenheim, J.J. 2000. Differential expression and responsiveness of chemokine receptors (CXCR1-3) by human microvascular endothelial cells and umbilical vein endothelial cells. *FASEB J* 14:2055-2064.

77. Volin, M.V., Joseph, E., Shockley, M.S., and Davies, P.F. 1998. Chemokine receptor cxcr4 expression in endothelium. *Biochem Biophys Res Commun* 242:46-53.

78. Jordan, N.J., Kolios, G., Abbot, S.E., Sinai, M.A., Thompson, D.A., Petraki, K., and Westwick, J. 1999. Expression of functional CXCR4 chemokine receptors on human colonic epithelial cells. *J Clin Invest* 104:1061-1069.

79. Stellato, C., Brummet, M.E., Plitt, J.R., Shahabuddin, S., Baroody, F.M., Liu, M.C., Ponath, P.D., and Beck, L.A. 2001. Cutting edge: Expression of the C-C chemokine receptor CCR3 in human airway epithelial cells. *J Immunol* 166:1457-1461.

80. Kleeff, J., Kusama, T., Rossi, D.L., Ishiwata, T., Maruyama, H., Friess, H., Buchler, M.W., Zlotnik, A., and Korc, M. 1999. Detection and localization of Mip-3

alpha/LARC/Exodus, a macrophage proinflammatory chemokine, and its CCR6 receptor in human pancreatic cancer. *Int J Cancer* 81:650-657.

81. Sozzani, S., Molino, M., Locati, M., Luini, W., Cerletti, C., Vecchi, A., and Mantovani, A. 1993. Receptor-activated calcium influx in human monocytes exposed to monocyte chemotactic protein-1 and related cytokines. *J Immunol* 150:1544-1553.

82. Kuang, Y., Wu, Y., Jiang, H., and Wu, D. 1996. Selective G protein coupling by C-C chemokine receptors. *Journal of Biological Chemistry* 271:3975-3978.

83. Wu, D., LaRosa, G.J., and Simon, M.I. 1993. G protein-coupled signal transduction pathways for interleukin-8. *Science* 261:101-103.

84. Jones, S.A., Wolf, M., Qin, S., Mackay, C.R., and Baggiolini, M. 1996. Different functions for the interleukin 8 receptors (IL-8R) of human neutrophil leukocytes: NADPH oxidase and phospholipase D are activated through IL-8R1 but not IL-8R2. *Proc Natl Acad Sci U S A* 93:6682-6686.

85. Turner, L., Ward, S.G., and Westwick, J. 1995. RANTES-activated human T lymphocytes. A role for phosphoinositide 3- kinase. *J Immunol* 155:2437-2444.

86. Neptune, E.R., Iiri, T., and Bourne, H.R. 1999. G alpha(i) is not required for chemotaxis mediated by G(i)-coupled receptors. *J Biol Chem* 274:2824-2828.

87. Jin, T., Zhang, N., Long, Y., Parent, C.A., and Devreotes, P.N. 2000. Localization of the G protein betagamma complex in living cells during chemotaxis. *Science* 287:1034-1036.

88. Thelen, M. 2001. Dancing to the tune of chemokines. *Nat Immunol* 2:129-134.

89. Li, Z., Jiang, H.P., Xie, W., Zhang, Z.C., Smrcka, A.V., and Wu, D.Q. 2000. Roles of PLC-beta 2 and -beta 3 and PI3K gamma in chemoattractant-mediated signal transduction. *Science* 287:1046-1049.

90. Turner, S.J., Domin, J., Waterfield, M.D., Ward, S.G., and Westwick, J. 1998. The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K- C2alpha. *J Biol Chem* 273:25987-25995.

91. Sasaki, T., Irie-Sasaki, J., Jones, R.G., Oliveira-dos-Santos, A.J., Stanford, W.L., Bolon, B., Wakeham, A., Itie, A., Bouchard, D., Kozieradzki, I., et al. 2000. Function of PI3Kgamma in thymocyte development, T cell activation, and neutrophil migration. *Science* 287:1040-1046.

92. Hirsch, E., Katanaev, V.L., Garlanda, C., Azzolino, O., Pirola, L., Silengo, L., Sozzani, S., Mantovani, A., Altruda, F., and Wymann, M.P. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. *Science* 287:1049-1053.

93. Burgering, B.M., and Coffer, P.J. 1995. Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* 376:599-602.

94. Chan, T.O., Rittenhouse, S.E., and Tsichlis, P.N. 1999. AKT/PKB and other D3 phosphoinositide-regulated kinases: kinase activation by phosphoinositide-dependent phosphorylation. *Annu Rev Biochem* 68:965-1014.

95. Graff, J.R., Konicek, B.W., McNulty, A.M., Wang, Z., Houck, K., Allen, S., Paul, J.D., Hbaiu, A., Goode, R.G., Sandusky, G.E., et al. 2000. Increased AKT activity contributes to prostate cancer progression by dramatically accelerating prostate tumor growth and diminishing p27Kip1 expression. *J Biol Chem* 275:24500-24505.

96. Meili, R., Ellsworth, C., Lee, S., Reddy, T.B., Ma, H., and Firtel, R.A. 1999. Chemoattractant-mediated transient activation and membrane localization of Akt/PKB is required for efficient chemotaxis to cAMP in Dictyostelium. *Embo J* 18:2092-2105.

97. Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. 2000. Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287:1037-1040.

98. Knall, C., Young, S., Nick, J.A., Buhl, A.M., Worthen, G.S., and Johnson, G.L. 1996. Interleukin-8 regulation of the Ras/Raf/mitogen-activated protein kinase pathway in human neutrophils. *J Biol Chem* 271:2832-2838.

99. Ashida, N., Arai, H., Yamasaki, M., and Kita, T. 2001. Distinct signaling pathways for MCP-1-dependent integrin activation and chemotaxis. *J Biol Chem* 276:16555-16560.

100. Majka, M., Ratajczak, J., Kowalska, M.A., and Ratajczak, M.Z. 2000. Binding of stromal derived factor-1alpha (SDF-1alpha) to CXCR4 chemokine receptor in normal human megakaryoblasts but not in platelets induces phosphorylation of mitogen-activated protein kinase p42/44 (MAPK), ELK-1 transcription factor and serine/threonine kinase AKT. *Eur J Haematol* 64:164-172.

101. Sullivan, S.K., McGrath, D.A., Liao, F., Boehme, S.A., Farber, J.M., and Bacon, K.B. 1999. MIP-3 alpha induces human eosinophil migration and activation of the mitogen-activated protein kinases (p42/p44 MAPK). *J Leukoc Biol* 66:674-682.

102. Lopez-Ilasaca, M., Crespo, P., Pellici, P.G., Gutkind, J.S., and Wetzker, R. 1997. Linkage of G protein-coupled receptors to the MAPK signaling pathway through PI 3-kinase gamma. *Science* 275:394-397.

103. Bondeva, T., Pirola, L., Bulgarelli-Leva, G., Rubio, I., Wetzker, R., and Wymann, M.P. 1998. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. *Science* 282:293-296.

104. Sotsios, Y., Whittaker, G.C., Westwick, J., and Ward, S.G. 1999. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J Immunol* 163:5954-5963.

105. Boehme, S.A., Sullivan, S.K., Crowe, P.D., Santos, M., Conlon, P.J., Sriramarao, P., and Bacon, K.B. 1999. Activation of mitogen-activated protein kinase regulates eotaxin-induced eosinophil migration. *J Immunol* 163:1611-1618.

106. Knall, C., Worthen, G.S., and Johnson, G.L. 1997. Interleukin 8-stimulated phosphatidylinositol-3-kinase activity regulates the migration of human neutrophils independent of extracellular signal-regulated kinase and p38 mitogen-activated protein kinases. *Proc Natl Acad Sci U S A* 94:3052-3057.

107. Bacon, K.B., Flores-Romo, L., Life, P.F., Taub, D.D., Premack, B.A., Arkinstall, S.J., Wells, T.N., Schall, T.J., and Power, C.A. 1995. IL-8-induced signal transduction in T lymphocytes involves receptor- mediated activation of phospholipases C and D. *J Immunol* 154:3654-3666.

108. Bacon, K.B., Schall, T.J., and Dairaghi, D.J. 1998. Rantes activation of phospholipase-d in jurkat T-cells - requirement of GTP-binding proteins arf and rhoa. *J Immunol* 160:1894-1900.

109. Du, G., Altshuller, Y.M., Kim, Y., Han, J.M., Ryu, S.H., Morris, A.J., and Frohman, M.A. 2000. Dual requirement for rho and protein kinase C in direct activation of phospholipase D1 through G protein-coupled receptor signaling. *Mol Biol Cell* 11:4359-4368.

110. VilaCoro, A.J., RodriguezFrade, J.M., DeAna, A.M., MorenoOrtiz, M.C., Martinez, C., and Mellado, M. 1999. The chemokine SDF-1 alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J* 13:1699-1710.

111. Mellado, M., Rodriguezfrade, J.M., Aragay, A., Delreal, G., Martin, A.M., Vilacoro, A.J., Serrano, A., Mayor, F., and Martineza, C. 1998. The chemokine monocyte chemotactic protein-1 triggers janus kinase-2 activation and tyrosine phosphorylation of the ccr2b receptor. *J Immunol* 161:805-813.

112. Ganju, R.K., Brubaker, S.A., Meyer, J., Dutt, P., Yang, Y.M., Qin, S.X., Newman, W., and Groopman, J.E. 1998. The alpha-chemokine, stromal cell-derived factor-1-alpha, binds to the transmembrane g-protein-coupled cxcr-4 receptor and activates multiple signal-transduction pathways. *J Biol Chem* 273:23169-23175.

113. Bacon, K.B., Szabo, M.C., Yssel, H., Bolen, J.B., and Schall, T.J. 1996. Rantes induces tyrosine kinase-activity of stably complexed p125(fak) and zap-70 in human T-cells. *J Exp Med* 184:873-882.

114. Mack, M., Luckow, B., Nelson, P.J., Cihak, J., Simmons, G., Clapham, P.R., Signoret, N., Marsh, M., Stangassinger, M., Borlat, F., et al. 1998. Aminooxypentane-

rantes induces ccr5 internalization but inhibits recycling - a novel inhibitory mechanism of HIV infectivity. *J Exp Med* 187:1215-1224.

115. Arai, H., Monteclaro, F.S., Tsou, C.L., Franci, C., and Charo, I.F. 1997. Dissociation of chemotaxis from agonist-induced receptor internalization in a lymphocyte cell-line transfected with ccr2b - evidence that directed migration does not require rapid modulation of signaling at the receptor level. *J Biol Chem* 272:25037-25042.

116. Guinamard, R., Signoret, N., Masamichi, I., Marsh, M., Kurosaki, T., and Ravetch, J.V. 1999. B cell antigen receptor engagement inhibits stromal cell-derived factor (SDF)-1 alpha chemotaxis and promotes protein kinase C (PKC)-induced internalization of CXCR4. *J Exp Med* 189:1461-1466.

117. FenigerBarish, R., Ran, M., Zaslaver, A., and BenBaruch, A. 1999. Differential modes of regulation of CXC chemokine-induced internalization and recycling of human CXCR1 and CXCR2. *Cytokine* 11:996-1009.

118. Lefkowitz, R.J. 1998. G protein-coupled receptors. III. New roles for receptor kinases and beta-arrestins in receptor signaling and desensitization. *J Biol Chem* 273:18677-18680.

119. Barlic, J., Khandaker, M.H., Mahon, E., Andrews, J., DeVries, M.E., Mitchell, G.B., Rahimpour, R., Tan, C.M., Ferguson, S.S.G., and Kelvin, D.J. 1999. beta-Arrestins regulate interleukin-8-induced CXCR1 internalization. *J Biol Chem* 274:16287-16294.

120. Cheng, Z.J., Zhao, J., Sun, Y., Hu, W., Wu, Y.L., Cen, B., Wu, G.X., and Pei, G. 2000. beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem* 275:2479-2485.

121. Tilton, B., Ho, L., Oberlin, E., Loetscher, P., Baleux, F., Clark-Lewis, I., and Thelen, M. 2000. Signal transduction by CXC chemokine receptor 4. Stromal cell-

derived factor 1 stimulates prolonged protein kinase B and extracellular signal-regulated kinase 2 activation in T lymphocytes. *J Exp Med* 192:313-324.

122. Kurihara, T., Warr, G., Loy, J., and Bravo, R. 1997. Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* 186:1757-1762.

123. Zhou, Y.H., Kurihara, T., Ryseck, R.P., Yang, Y., Ryan, C., Loy, J., Warr, G., and Bravo, R. 1998. Impaired macrophage function and enhanced T-cell-dependent immune-response in mice lacking ccr5, the mouse homolog of the major HIV-1 coreceptor. *J Immunol* 160:4018-4025.

124. Chvatchko, Y., Hoogewerf, A.J., Meyer, A., Alouani, S., Juillard, P., Buser, R., Conquet, F., Proudfoot, A.E., Wells, T.N., and Power, C.A. 2000. A key role for CC chemokine receptor 4 in lipopolysaccharide-induced endotoxic shock. *J Exp Med* 191:1755-1764.

125. Cacalano, G., Lee, J., Kikly, K., Ryan, A.M., Pitts-Meek, S., Hultgren, B., Wood, W.I., and Moore, M.W. 1994. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science* 265:682-684.

126. Lu, B., Rutledge, B.J., Gu, L., Fiorillo, J., Lukacs, N.W., Kunkel, S.L., North, R., Gerard, C., and Rollins, B.J. 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* 187:601-608.

127. Gao, J.L., Wynn, T.A., Chang, Y., Lee, E.J., Broxmeyer, H.E., Cooper, S., Tiffany, H.L., Westphal, H., Kwon-Chung, J., and Murphy, P.M. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* 185:1959-1968.

128. Gerard, C., Frossard, J.L., Bhatia, M., Saluja, A., Gerard, N.P., Lu, B., and Steer, M. 1997. Targeted disruption of the beta-chemokine receptor CCR1 protects against pancreatitis-associated lung injury. *J Clin Invest* 100:2022-2027.

129. Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., RennerMuller, I., Wolf, E., and Lipp, M. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99:23-33.

130. Karpus, W.J., and Kennedy, K.J. 1997. MIP-1alpha and MCP-1 differentially regulate acute and relapsing autoimmune encephalomyelitis as well as Th1/Th2 lymphocyte differentiation. *J Leukoc Biol* 62:681-687.

131. Sanchez-Madrid, F., and del Pozo, M.A. 1999. Leukocyte polarization in cell migration and immune interactions. *Embo J* 18:501-511.

132. Sallusto, F., and Lanzavecchia, A. 2000. Understanding dendritic cell and T-lymphocyte traffic through the analysis of chemokine receptor expression. *Immunol Rev* 177:134-140.

133. Sozzani, S., Sallusto, F., Luini, W., Zhou, D., Piemonti, L., Allavena, P., Van Damme, J., Valitutti, S., Lanzavecchia, A., and Mantovani, A. 1995. Migration of dendritic cells in response to formyl peptides, C5a, and a distinct set of chemokines. *J Immunol* 155:3292-3295.

134. Ayehunie, S., Garcia-Zepeda, E.A., Hoxie, J.A., Horuk, R., Kupper, T.S., Luster, A.D., and Ruprecht, R.M. 1997. Human immunodeficiency virus-1 entry into purified blood dendritic cells through CC and CXC chemokine coreceptors. *Blood* 90:1379-1386.

135. Sozzani, S., Allavena, P., Vecchi, A., and Mantovani, A. 1999. The role of chemokines in the regulation of dendritic cell trafficking. *J Leukoc Biol* 66:1-9.

136. Banchereau, J., and Steinman, R.M. 1998. Dendritic cells and the control of immunity. *Nature* 392:245-252.

137. Austyn, J.M. 1996. New insights into the mobilization and phagocytic activity of dendritic cells. *J Exp Med* 183:1287-1292.

138. Foti, M., Granucci, F., Aggujaro, D., Liboi, E., Luini, W., Minardi, S., Mantovani, A., Sozzani, S., and RicciardiCastagnoli, P. 1999. Upon dendritic cell (DC)

activation chemokines and chemokine receptor expression are rapidly regulated for recruitment and maintenance of DC at the inflammatory site. *Int Immunol* 11:979-986.

139. Sozzani, S., Allavena, P., Damico, G., Luini, W., Bianchi, G., Kataura, M., Imai, T., Yoshie, O., Bonecchi, R., and Mantovani, A. 1998. Cutting edge - differential regulation of chemokine receptors during dendritic cell maturation - a model for their trafficking properties. *J Immunol* 161:1083-1086.

140. Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Aityahia, S., Briere, F., Zlotnik, A., Lebecque, S., and Caux, C. 1998. Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 188:373-386.

141. Sallusto, F., Schaerli, P., Loetscher, P., Schaniel, C., Lenig, D., MacKay, C.R., Qin, S.X., and Lanzavecchia, A. 1998. Rapid and coordinated switch in chemokine receptor expression during dendritic cell maturation. *Eur J Immunol* 28:2760-2769.

142. Willimann, K., Legler, D.F., Loetscher, M., Roos, R.S., Delgado, M.B., Clarklewis, I., Baggiolini, M., and Moser, B. 1998. The chemokine slc is expressed in T-cell areas of lymph-nodes and mucosal lymphoid-tissues and attracts activated T-cells via ccr7. *Eur J Immunol* 28:2025-2034.

143. Gunn, M.D., Kyuwa, S., Tam, C., Kakiuchi, T., Matsuzawa, A., Williams, L.T., and Nakano, H. 1999. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* 189:451-460.

144. Sallusto, F., Lenig, D., MacKay, C.R., and Lanzavecchia, A. 1998. Flexible programs of chemokine receptor expression on human polarized t-helper-1 and t-helper-2 lymphocytes. *J Exp Med* 187:875-883.

145. Bonecchi, R., Bianchi, G., Bordignon, P.P., D'Ambrosio, D., Lang, R., Borsatti, A., Sozzani, S., Allavena, P., Gray, P.A., Mantovani, A., et al. 1998. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J Exp Med* 187:129-134.

146. Roos, R.S., Loetscher, M., Legler, D.F., Clark-Lewis, I., Baggiolini, M., and Moser, B. 1997. Identification of CCR8, the receptor for the human CC chemokine I-309. *J Biol Chem* 272:17251-17254.

147. Campbell, J.J., Haraldsen, G., Pan, J., Rottman, J., Qin, S., Ponath, P., Andrew, D.P., Warnke, R., Ruffing, N., Kassam, N., et al. 1999. The chemokine receptor CCR4 in vascular recognition by cutaneous but not intestinal memory T cells. *Nature* 400:776-780.

148. Sallusto, F., Lenig, D., Forster, R., Lipp, M., and Lanzavecchia, A. 1999. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 401:708-712.

149. Forster, R., Emrich, T., Kremmer, E., and Lipp, M. 1994. Expression of the G-protein--coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood* 84:830-840.

150. Cyster, J.G. 1999. Chemokines - Chemokines and cell migration in secondary lymphoid organs. *Science* 286:2098-2102.

151. Kaiser, E., Forster, R., Wolf, I., Ebensperger, C., Kuehl, W.M., and Lipp, M. 1993. The G protein-coupled receptor BLR1 is involved in murine B cell differentiation and is also expressed in neuronal tissues. *Eur J Immunol* 23:2532-2539.

152. Hargreaves, D.C., Hyman, P.L., Lu, T.T., Ngo, V.N., Bidgol, A., Suzuki, G., Zou, Y.R., Littman, D.R., and Cyster, J.G. 2001. A coordinated change in chemokine responsiveness guides plasma cell movements. *J Exp Med* 194:45-56.

153. Youn, B.S., Mantel, C., and Broxmeyer, H.E. 2000. Chemokines, chemokine receptors and hematopoiesis. *Immunol Rev* 177:150-174.

154. Broxmeyer, H.E., and Kim, C.H. 1999. Regulation of hematopoiesis in a sea of chemokine family members with a plethora of redundant activities. *Exp Hematol* 27:1113-1123.

155. Reid, S., Ritchie, A., Boring, L., Gosling, J., Cooper, S., Hangoc, G., Charo, I.F., and Broxmeyer, H.E. 1999. Enhanced myeloid progenitor cell cycling and apoptosis in mice lacking the chemokine receptor, CCR2. *Blood* 93:1524-1533.

156. Haneline, L.S., Broxmeyer, H.E., Cooper, S., Hangoc, G., Carreau, M., Buchwald, M., and Clapp, D.W. 1998. Multiple inhibitory cytokines induce deregulated progenitor growth and apoptosis in hematopoietic-cells from fac(-/-) mice. *Blood* 91:4092-4098.

157. Kim, K.W., Bae, S.K., Lee, O.H., Bae, M.H., Lee, M.J., and Park, B.C. 1998. Insulin-like growth factor II induced by hypoxia may contribute to angiogenesis of human hepatocellular carcinoma. *Cancer Res* 58:348-351.

158. Aiuti, A., Webb, I.J., Bleul, C., Springer, T., and Gutierrez-Ramos, J.C. 1997. The chemokine SDF-1 is a chemoattractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. *J Exp Med* 185:111-120.

159. Zou, Y.R., Kottmann, A.H., Kuroda, M., Taniuchi, I., and Littman, D.R. 1998. Function of the chemokine receptor cxcr4 in hematopoiesis and in cerebellar development. *Nature* 393:595-599.

160. D'Apuzzo, M., Rolink, A., Loetscher, M., Hoxie, J.A., Clark-Lewis, I., Melchers, F., Baggiolini, M., and Moser, B. 1997. The chemokine SDF-1, stromal cell-derived factor 1, attracts early stage B cell precursors via the chemokine receptor CXCR4. *Eur J Immunol* 27:1788-1793.

161. Karpus, W.J., Lukacs, N.W., Kennedy, K.J., Smith, W.S., Hurst, S.D., and Barrett, T.A. 1997. Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J Immunol* 158:4129-4136.

162. Sallusto, F., Lanzavecchia, A., and MacKay, C.R. 1998. Chemokines and chemokine receptors in T-cell priming and th1/th2-mediated responses. *Immunol Today* 19:568-574.

163. Chensue, S.W., Warmington, K.S., Ruth, J.H., Sanghi, P.S., Lincoln, P., and Kunkel, S.L. 1996. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. *J Immunol* 157:4602-4608.

164. Zou, W., Borvak, J., Marches, F., Wei, S., Galanaud, P., Emilie, D., and Curiel, T.J. 2000. Macrophage-derived dendritic cells have strong Th1-polarizing potential mediated by beta-chemokines rather than IL-12. *J Immunol* 165:4388-4396.

165. Gu, L., Tseng, S., Horner, R.M., Tam, C., Loda, M., and Rollins, B.J. 2000. Control of T(H)2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 404:407-411.

166. Traynor, T.R., Kuziel, W.A., Toews, G.B., and Huffnagle, G.B. 2000. CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection. *J Immunol* 164:2021-2027.

167. Cook, D.N., Beck, M.A., Coffman, T.M., Kirby, S.L., Sheridan, J.F., Pragnell, I.B., and Smithies, O. 1995. Requirement of MIP-1 alpha for an inflammatory response to viral infection. *Science* 269:1583-1585.

168. Blease, K., Mehrad, B., Standiford, T.J., Lukacs, N.W., Gosling, J., Boring, L., Charo, I.F., Kunkel, S.L., and Hogaboam, C.M. 2000. Enhanced pulmonary allergic responses to Aspergillus in CCR2-/- mice. *J Immunol* 165:2603-2611.

169. Ebnet, K., Kaldjian, E.P., Anderson, A.O., and Shaw, S. 1996. Orchestrated information transfer underlying leukocyte endothelial interactions. *Annu Rev Immunol* 14:155-177.

170. Middleton, J., Neil, S., Wintle, J., Clark-Lewis, I., Moore, H., Lam, C., Auer, M., Hub, E., and Rot, A. 1997. Transcytosis and surface presentation of IL-8 by venular endothelial cells. *Cell* 91:385-395.

171. Baekkevold, E.S., Yamanaka, T., Palframan, R.T., Carlsen, H.S., Reinholt, F.P., von Andrian, U.H., Brandtzaeg, P., and Haraldsen, G. 2001. The CCR7 ligand elc

(CCL19) is transcytosed in high endothelial venules and mediates T cell recruitment. *J Exp Med* 193:1105-1112.

172. Panes, J., and Granger, D.N. 1998. Leukocyte-endothelial cell-interactions - molecular mechanisms and implications in gastrointestinal-disease. *Gastroenterology* 114:1066-1090.

173. Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J.Y., Butcher, E.C., and Laudanna, C. 2000. Chemokines trigger immediate beta 2 integrin affinity and mobility changes: Differential regulation and roles in lymphocyte arrest under flow. *Immunity* 13:759-769.

174. Grabovsky, V., Feigelson, S., Chen, C., Bleijs, D.A., Peled, A., Cinamon, G., Baleux, F., ArenzanaSeisdedos, F., Lapidot, T., vanKooyk, Y., et al. 2000. Subsecond induction of alpha 4 integrin clustering by immobilized chemokines stimulates leukocyte tethering and rolling on endothelial vascular cell adhesion molecule 1 under flow conditions. *J Exp Med* 192:495-505.

175. Campbell, J.J., Qin, S.X., Bacon, K.B., MacKay, C.R., and Butcher, E.C. 1996. Biology of chemokine and classical chemoattractant receptors - differential requirements for adhesion-triggering versus chemotactic responses in lymphoid-cells. *J Cell Biol* 134:255-266.

176. Cinamon, G., Grabovsky, V., Winter, E., Franitza, S., Feigelson, S., Shamri, R., Dwir, O., and Alon, R. 2001. Novel chemokine functions in lymphocyte migration through vascular endothelium under shear flow. *J Leukoc Biol* 69:860-866.

177. Jiang, Y., Beller, D.I., Frendl, G., and Graves, D.T. 1992. Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J Immunol* 148:2423-2428.

178. Detmers, P.A., Powell, D.E., Walz, A., Clark-Lewis, I., Baggiolini, M., and Cohn, Z.A. 1991. Differential effects of neutrophil-activating peptide 1/IL-8 and its homologues on leukocyte adhesion and phagocytosis. *J Immunol* 147:4211-4217.

179. Sica, A., Saccani, A., Borsatti, A., Power, C.A., Wells, T.N., Luini, W., Polentarutti, N., Sozzani, S., and Mantovani, A. 1997. Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *Journal of Experimental Medicine* 185:969-974.

180. Sica, A., Saccani, A., Bottazzi, B., Bernasconi, S., Allavena, P., Gaetano, B., Fei, F., LaRosa, G., Scotton, C., Balkwill, F., et al. 2000. Defective Expression of the Monocyte Chemotactic Protein-1 Receptor CCR2 in Macrophages Associated with Human Ovarian Carcinoma. *J Immunol* 164:733-738.

181. Saccani, A., Saccani, S., Orlando, S., Sironi, M., Bernasconi, S., Ghezzi, P., Mantovani, A., and Sica, A. 2000. Redox regulation of chemokine receptor expression. *Proc Natl Acad Sci U S A* 97:2761-2766.

182. Owen, C. 2001. Chemokine receptors in airway disease: which receptors to target? *Pulm Pharmacol Ther* 14:193-202.

183. Jose, P.J., Griffiths-Johnson, D.A., Collins, P.D., Walsh, D.T., Moqbel, R., Totty, N.F., Truong, O., Hsuan, J.J., and Williams, T.J. 1994. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J Exp Med* 179:881-887.

184. Rankin, S.M., Conroy, D.M., and Williams, T.J. 2000. Eotaxin and eosinophil recruitment: implications for human disease. *Mol Med Today* 6:20-27.

185. Ying, S., Robinson, D.S., Meng, Q., Rottman, J., Kennedy, R., Ringler, D.J., Mackay, C.R., Daugherty, B.L., Springer, M.S., Durham, S.R., et al. 1997. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co- localization of eotaxin mRNA to bronchial epithelial and endothelial cells. *Eur J Immunol* 27:3507-3516.

186. Uguccioni, M., Mackay, C.R., Ochensberger, B., Loetscher, P., Rhis, S., LaRosa, G.J., Rao, P., Ponath, P.D., Baggiolini, M., and Dahinden, C.A. 1997. High expression of the chemokine receptor CCR3 in human blood basophils. Role in activation by eotaxin, MCP-4, and other chemokines. *J Clin Invest* 100:1137-1143.

187. Quackenbush, E.J., Wershil, B.K., Aguirre, V., and Gutierrezramos, J.C. 1998. Eotaxin modulates myelopoiesis and mast-cell development from embryonic hematopoietic progenitors. *Blood* 92:1887-1897.

188. Nickel, R., Beck, L.A., Stellato, C., and Schleimer, R.P. 1999. Chemokines and allergic disease. *J Allergy Clin Immunol* 104:723-742.

189. Gonzalo, J.A., Lloyd, C.M., Wen, D.Y., Albar, J.P., Wells, T.N.C., Proudfoot, A., Martinez, C., Dorf, M., Bjerke, T., Coyle, A.J., et al. 1998. The coordinated action of cc chemokines in the lung orchestrates allergic inflammation and airway hyperresponsiveness. *J Exp Med* 188:157-167.

190. Elsner, J., Petering, H., Hochstetter, R., Kimmig, D., Wells, T.N., Kapp, A., and Proudfoot, A.E. 1997. The CC chemokine antagonist Met-RANTES inhibits eosinophil effector functions through the chemokine receptors CCR1 and CCR3. *Eur J Immunol* 27:2892-2898.

191. Dabbagh, K., Xiao, Y., Smith, C., Stepick-Biek, P., Kim, S.G., Lamm, W.J., Liggitt, D.H., and Lewis, D.B. 2000. Local blockade of allergic airway hyperreactivity and inflammation by the poxvirus-derived pan-CC-chemokine inhibitor vCCI. *J Immunol* 165:3418-3422.

192. Hickey, W.F., and Kimura, H. 1988. Perivascular microglial cells of the CNS are bone marrow-derived and present antigen in vivo. *Science* 239:290-292.

193. Sorensen, T.L., and Ransohoff, R.M. 1998. Etiology and pathogenesis of multiple sclerosis. *Semin Neurol* 18:287-294.

194. Ransohoff, R.M., and Bacon, K.B. 2000. Chemokine receptor antagonism as a new therapy for multiple sclerosis. *Exp Opin Invest Drugs* 9:1079-1097.

195. Cannella, B., and Raine, C.S. 1995. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol* 37:424-435.

196. McManus, C., Berman, J.W., Brett, F.M., Staunton, H., Farrell, M., and Brosnan, C.F. 1998. Mcp-1, mcp-2 and mcp-3 expression in multiple-sclerosis lesions - an immunohistochemical and in-situ hybridization study. *J Neuroimmunol* 86:20-29.

197. Simpson, J.E., Newcombe, J., Cuzner, M.L., and Woodroofe, M.N. 1998. Expression of monocyte chemoattractant protein-1 and other beta- chemokines by resident glia and inflammatory cells in multiple sclerosis lesions. *J Neuroimmunol* 84:238-249.

198. Balashov, K.E., Rottman, J.B., Weiner, H.L., and Hancock, W.W. 1999. CCR5(+) and CXCR3(+) T cells are increased in multiple sclerosis and their ligands MIP-1 alpha and IP-10 are expressed in demyelinating brain lesions. *Proc Natl Acad Sci U S A* 96:6873-6878.

199. Sorensen, T.L., Tani, M., Jensen, J., Pierce, V., Lucchinetti, C., Folcik, V.A., Qin, S.X., Rottman, J., Sellebjerg, F., Strieter, R.M., et al. 1999. Expression of specific chemokines and chemokine receptors in the central nervous system of multiple sclerosis patients. *J Clin Invest* 103:807-815.

200. Barcellos, L.F., Schito, A.M., Rimmler, J.B., Vittinghoff, E., Shih, A., Lincoln, R., Callier, S., Elkins, M.K., Goodkin, D.E., Haines, J.L., et al. 2000. CC-chemokine receptor 5 polymorphism and age of onset in familial multiple sclerosis. Multiple Sclerosis Genetics Group. *Immunogenetics* 51:281-288.

201. Ransohoff, R.M., Tani, M., Glabinski, A.R., Chernosky, A., Krivacic, K., Peterson, J.W., Chien, H.F., and Trapp, B.D. 1997. Chemokines and chemokine receptors in model neurological pathologies: molecular and immunocytochemical approaches. *Methods Enzymol* 287:319-348.

202. Godiska, R., Chantry, D., Dietsch, G.N., and Gray, P.W. 1995. Chemokine expression in murine experimental allergic encephalomyelitis. *J Neuroimmunol* 58:167-176.

203. Ransohoff, R.M., Hamilton, T.A., Tani, M., Stoler, M.H., Shick, H.E., Major, J.A., Estes, M.L., Thomas, D.M., and Tuohy, V.K. 1993. Astrocyte expression of mRNA encoding cytokines IP-10 and JE/MCP-1 in experimental autoimmune encephalomyelitis. *Faseb J* 7:592-600.

204. Glabinski, A.R., Tani, M., Strieter, R.M., Tuohy, V.K., and Ransohoff, R.M. 1997. Synchronous synthesis of alpha- and beta-chemokines by cells of diverse lineage in the central nervous system of mice with relapses of chronic experimental autoimmune encephalomyelitis. *Am J Pathol* 150:617-630.

205. Fife, B.T., Huffnagle, G.B., Kuziel, W.A., and Karpus, W.J. 2000. CC chemokine receptor 2 is critical for induction of experimental autoimmune encephalomyelitis. *J Exp Med* 192:899-905.

206. Izikson, L., Klein, R.S., Charo, I.F., Weiner, H.L., and Luster, A.D. 2000. Resistance to experimental autoimmune encephalomyelitis in mice lacking the CC chemokine receptor (CCR)2. *J Exp Med* 192:1075-1080.

207. Gerard, C., and Rollins, B.J. 2001. Chemokines and disease. *Nat Immunol* 2:108-115.

208. Arimilli, S., Ferlin, W., Solvason, N., Deshpande, S., Howard, M., and Mocci, S. 2000. Chemokines in autoimmune diseases. *Immunol Rev* 177:43-51.

209. Robey, W.G., Safai, B., Oroszlan, S., Arthur, L.O., Gonda, M.A., Gallo, R.C., and Fischinger, P.J. 1985. Characterization of envelope and core structural gene products of HTLV- III with sera from AIDS patients. *Science* 228:593-595.

210. Choe, H., Farzan, M., Sun, Y., Sullivan, N., Rollins, B., Ponath, P.D., Wu, L., Mackay, C.R., LaRosa, G., Newman, W., et al. 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85:1135-1148.

211. Deng, H., Liu, R., Ellmeier, W., Choe, S., Unutmaz, D., Burkhart, M., Di Marzio, P., Marmon, S., Sutton, R.E., Hill, C.M., et al. 1996. Identification of a major co-receptor for primary isolates of HIV-1. *Nature* 381:661-666.

212. Doranz, B.J., Rucker, J., Yi, Y., Smyth, R.J., Samson, M., Peiper, S.C., Parmentier, M., Collman, R.G., and Doms, R.W. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta- chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* 85:1149-1158.

213. Dragic, T., Litwin, V., Allaway, G.P., Martin, S.R., Huang, Y.X., Nagashima, K.A., Cayanan, C., Maddon, P.J., Koup, R.A., Moore, J.P., et al. 1996. HIV-1 entry into CD4(+) cells is mediated by the chemokine receptor cc-ckr-5. *Nature* 381:667-673.

214. Feng, Y., Broder, C.C., Kennedy, P.E., and Berger, E.A. 1996. HIV-1 entry cofactor: functional cDNA cloning of a seven-transmembrane, G protein-coupled receptor. *Science* 272:872-877.

215. Berger, E.A., Murphy, P.M., and Farber, J.M. 1999. Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism, and disease. *Annu Rev Immunol* 17:657-700.

216. Simmons, G., Reeves, J.D., Hibbitts, S., Stine, J.T., Gray, P.W., Proudfoot, A.E.I., and Clapham, P.R. 2000. Co-receptor use by HIV and inhibition of HIV infection by chemokine receptor ligands. *Immunol Rev* 177:112-126.

217. Berger, E.A., Doms, R.W., Fenyo, E.M., Korber, B.T., Littman, D.R., Moore, J.P., Sattentau, Q.J., Schuitemaker, H., Sodroski, J., and Weiss, R.A. 1998. A new classification for HIV-1. *Nature* 391:240.

218. Collman, R., Hassan, N.F., Walker, R., Godfrey, B., Cutilli, J., Hastings, J.C., Friedman, H., Douglas, S.D., and Nathanson, N. 1989. Infection of monocyte-derived macrophages with human immunodeficiency virus type 1 (HIV-1). Monocyte-tropic and lymphocyte-tropic strains of HIV-1 show distinctive patterns of replication in a panel of cell types. *J Exp Med* 170:1149-1163.

219. McKnight, A., Wilkinson, D., Simmons, G., Talbot, S., Picard, L., Ahuja, M., Marsh, M., Hoxie, J.A., and Clapham, P.R. 1997. Inhibition of human immunodeficiency virus fusion by a monoclonal antibody to a coreceptor (CXCR4) is both cell type and virus strain dependent. *J Virol* 71:1692-1696.

220. Wu, L., Gerard, N.P., Wyatt, R., Choe, H., Parolin, C., Ruffing, N., Borsetti, A., Cardoso, A.A., Desjardin, E., Newman, W., et al. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. *Nature* 384:179-183.

221. Trkola, A., Dragic, T., Arthos, J., Binley, J.M., Olson, W.C., Allaway, G.P., Cheng-Mayer, C., Robinson, J., Maddon, P.J., and Moore, J.P. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co- receptor CCR-5. *Nature* 384:184-187.

222. Turner, B.G., and Summers, M.F. 1999. Structural biology of HIV. *J Mol Biol* 285:1-32.

223. Cocchi, F., DeVico, A.L., Garzino-Demo, A., Arya, S.K., Gallo, R.C., and Lusso, P. 1995. Identification of RANTES, MIP-1 alpha, and MIP-1 beta as the major HIV- suppressive factors produced by CD8+ T cells. *Science* 270:1811-1815.

224. Paxton, W.A., Liu, R., Kang, S., Wu, L., Gingeras, T.R., Landau, N.R., Mackay, C.R., and Koup, R.A. 1998. Reduced HIV-1 infectability of CD4+ lymphocytes from exposed-uninfected individuals: association with low expression of CCR5 and high production of beta-chemokines. *Virology* 244:66-73.

225. Benkirane, M., Jin, D.Y., Chun, R.F., Koup, R.A., and Jeang, K.T. 1997. Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. *J Biol Chem* 272:30603-30606.

226. Donzella, G.A., Schols, D., Lin, S.W., Este, J.A., Nagashima, K.A., Maddon, P.J., Allaway, G.P., Sakmar, T.P., Henson, G., De Clercq, E., et al. 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co- receptor. *Nat Med* 4:72-77.

227. Simmons, G., Clapham, P.R., Picard, L., Offord, R.E., Rosenkilde, M.M., Schwartz, T.W., Buser, R., Wells, T.N., and Proudfoot, A.E. 1997. Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* 276:276-279.

228. Mantovani, A., Bottazzi, B., Colotta, F., Sozzani, S., and Ruco, L. 1992. The origin and function of tumor-associated macrophages. *Immunol Today* 13:265-270.

229. Svennevig, J.L., and Svaar, H. 1979. Content and distribution of macrophages and lymphocytes in solid malignant human tumours. *Int J Cancer* 24:754-758.

230. Negus, R.P., Stamp, G.W., Hadley, J., and Balkwill, F.R. 1997. Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *Am J Pathol* 150:1723-1734.

231. Negus, R.P., Stamp, G.W., Relf, M.G., Burke, F., Malik, S.T., Bernasconi, S., Allavena, P., Sozzani, S., Mantovani, A., and Balkwill, F.R. 1995. The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *Journal of Clinical Investigation* 95:2391-2396.

232. Bottazzi, B., Ghezzi, P., Taraboletti, G., Salmona, M., Colombo, N., Bonazzi, C., Mangioni, C., and Mantovani, A. 1985. Tumor-derived chemotactic factor(s) from human ovarian carcinoma: evidence for a role in the regulation of macrophage content of neoplastic tissues. *Int J Cancer* 36:167-173.

233. Walter, S., Bottazzi, B., Govoni, D., Colotta, F., and Mantovani, A. 1991. Macrophage infiltration and growth of sarcoma clones expressing different amounts of monocyte chemotactic protein/JE. *Int J Cancer* 49:431-435.

234. Nesbit, M., Schaider, H., Miller, T.H., and Herlyn, M. 2001. Low-level monocyte chemoattractant protein-1 stimulation of monocytes leads to tumor formation in nontumorigenic melanoma cells. *J Immunol* 166:6483-6490.

235. Lin, E.Y., Nguyen, A.V., Russell, R.G., and Pollard, J.W. 2001. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. *J Exp Med* 193:727-740.

236. Leek, R.D., Lewis, C.E., Whitehouse, R., Greenall, M., Clarke, J., and Harris, A.L. 1996. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res* 56:4625-4629.

237. Laning, J., Kawasaki, H., Tanaka, E., Luo, Y., and Dorf, M.E. 1994. Inhibition of in vivo tumor growth by the beta chemokine, TCA3. *J Immunol* 153:4625-4635.

238. Rollins, B.J., and Sunday, M.E. 1991. Suppression of tumor formation in vivo by expression of the JE gene in malignant cells. *Mol Cell Biol* 11:3125-3131.

239. Mule, J.J., Custer, M., Averbook, B., Yang, J.C., Weber, J.S., Goeddel, D.V., Rosenberg, S.A., and Schall, T.J. 1996. Rantes secretion by gene-modified tumor-cells results in loss of tumorigenicity in-vivo - role of immune cell subpopulations. *Hum Gene Ther* 7:1545-1553.

240. Fushimi, T., Kojima, A., Moore, M.A., and Crystal, R.G. 2000. Macrophage inflammatory protein 3alpha transgene attracts dendritic cells to established murine tumors and suppresses tumor growth. *J Clin Invest* 105:1383-1393.

241. Luster, A.D., and Leder, P. 1993. IP-10, a -C-X-C- chemokine, elicits a potent thymus-dependent antitumor response in vivo. *J Exp Med* 178:1057-1065.

242. Dilloo, D., Bacon, K., Holden, W., Zhong, W., Burdach, S., Zlotnik, A., and Brenner, M. 1996. Combined chemokine and cytokine gene transfer enhances antitumor immunity. *Nat Med* 2:1090-1095.

243. Vicari, A.P., Ait-Yahia, S., Chemin, K., Mueller, A., Zlotnik, A., and Caux, C. 2000. Antitumor effects of the mouse chemokine 6Ckine/SLC through angiostatic and immunological mechanisms. *J Immunol* 165:1992-2000.

244. Schumacher, K., Haensch, W., Roefzaad, C., and Schlag, P.M. 2001. Prognostic significance of activated CD8(+) T cell infiltrations within esophageal carcinomas. *Cancer Res* 61:3932-3936.

245. Balkwill, F., and Mantovani, A. 2001. Inflammation and cancer: back to Virchow? *Lancet* 357:539-545.

246. Hagen, T.M., Huang, S., Curnutte, J., Fowler, P., Martinez, V., Wehr, C.M., Ames, B.N., and Chisari, F.V. 1994. Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 91:12808-12812.

247. Leibovich, S.J., and Wiseman, D.M. 1988. Macrophages, wound repair and angiogenesis. *Prog Clin Biol Res* 266:131-145.

248. Folkman, J. 1990. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* 82:4-6.

249. Weidner, N., Semple, J.P., Welch, W.R., and Folkman, J. 1991. Tumor angiogenesis and metastasis--correlation in invasive breast carcinoma. *N Engl J Med* 324:1-8.

250. Folkman, J., and Klagsburn, M. 1987. Angiogenic factors. *Science* 235:442-447.

251. Polverini, P.J., and Leibovich, S.J. 1984. Induction of neovascularization in vivo and endothelial proliferation in vitro by tumor-associated macrophages. *Lab Invest* 51:635-642.

252. Leek, R.D., Landers, R.J., Harris, A.L., and Lewis, C.E. 1999. Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. *Br J Cancer* 79:991-995.

253. Arenberg, D.A., Polverini, P.J., Kunkel, S.L., Shanafelt, A., Hesselgesser, J., Horuk, R., and Strieter, R.M. 1997. The role of CXC chemokines in the regulation of angiogenesis in non- small cell lung cancer. *J Leukoc Biol* 62:554-562.

254. Belperio, J.A., Keane, M.P., Arenberg, D.A., Addison, C.L., Ehlert, J.E., Burdick, M.D., and Strieter, R.M. 2000. CXC chemokines in angiogenesis. *J Leukoc Biol* 68:1-8.

255. Strieter, R.M., Polverini, P.J., Kunkel, S.L., Arenberg, D.A., Burdick, M.D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Marriott, D., et al. 1995. The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J Biol Chem* 270:27348-27357.

256. Strieter, R.M., Addison, C.L., Ehlert, J.E., Keane, M.P., Belperio, J.A., Burdick, M.D., and Arenberg, D.A. 1999. Use of Immunodeficient Mice for the Evaluation of CXC Chemokines in the Regulation of Tumor-associated Angiogenesis. *Ilar J* 40:175-182.

257. Addison, C.L., Daniel, T.O., Burdick, M.D., Liu, H., Ehlert, J.E., Xue, Y.Y., Buechi, L., Walz, A., Richmond, A., and Strieter, R.M. 2000. The CXC chemokine receptor 2, CXCR2, is the putative receptor for ELR+ CXC chemokine-induced angiogenic activity. *J Immunol* 165:5269-5277.

258. Luster, A.D., and Ravetch, J.V. 1987. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med* 166:1084-1097.

259. Farber, J.M. 1993. HuMig: a new human member of the chemokine family of cytokines. *Biochem Biophys Res Commun* 192:223-230.

260. Cole, K.E., Strick, C.A., Paradis, T.J., Ogborne, K.T., Loetscher, M., Gladue, R.P., Lin, W., Boyd, J.G., Moser, B., Wood, D.E., et al. 1998. Interferon-inducible T-cell alpha-chemoattractant (i-tac) - a novel non-elr cxc chemokine with potent activity on activated T-cells through selective high-affinity binding to cxcr3. *J Exp Med* 187:2009-2021.

261. Romagnani, P., Annunziato, F., Lasagni, L., Lazzeri, E., Beltrame, C., Francalanci, M., Uguccioni, M., Galli, G., Cosmi, L., Maurenzig, L., et al. 2001. Cell cycle-dependent expression of CXC chemokine receptor 3 by endothelial cells mediates angiostatic activity. *J Clin Invest* 107:53-63.

262. Gupta, S.K., Lysko, P.G., Pillarisetti, K., Ohlstein, E., and Stadel, J.M. 1998. Chemokine receptors in human endothelial-cells - functional expression of cxcr4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem* 273:4282-4287.

263. Salcedo, R., Wasserman, K., Young, H.A., Grimm, M.C., Howard, O.M.Z., Anver, M.R., Kleinman, H.K., Murphy, W.J., and Oppenheim, J.J. 1999. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells - In vivo neovascularization induced by stromal-derived factor-1 alpha. *Am J Pathol* 154:1125-1135.

264. Arenberg, D.A., Polverini, P.J., Kunkel, S.L., Shanafelt, A., and Strieter, R.M. 1997. In vitro and in vivo systems to assess role of C-X-C chemokines in regulation of angiogenesis. *Methods Enzymol* 288:190-220.

265. Salcedo, R., Ponce, M.L., Young, H.A., Wasserman, K., Ward, J.M., Kleinman, H.K., Oppenheim, J.J., and Murphy, W.J. 2000. Human endothelial cells express CCR2 and respond to MCP-1: direct role of MCP-1 in angiogenesis and tumor progression. *Blood* 96:34-40.

266. Luan, J., Shattuck-Brandt, R., Haghnegahdar, H., Owen, J.D., Strieter, R., Burdick, M., Nirodi, C., Beauchamp, D., Johnson, K.N., and Richmond, A. 1997. Mechanism and biological significance of constitutive expression of MGSA/GRO chemokines in malignant melanoma tumor progression. *J Leukoc Biol* 62:588-597.

267. Owen, J.D., Strieter, R., Burdick, M., Haghnegahdar, H., Nanney, L., Shattuck-Brandlt, R., and Richmond, A. 1997. Enhanced tumor-forming capacity for immortalized melanocytes expressing melanoma growth stimulatory activity/growth-regulated cytokine beta and gamma proteins. *Int J Cancer* 73:94-103.

268. Arenberg, D.A., Kunkel, S.L., Polverini, P.J., Glass, M., Burdick, M.D., and Strieter, R.M. 1996. Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice. *J Clin Invest* 97:2792-2802.

269. Arenberg, D.A., Keane, M.P., DiGiovine, B., Kunkel, S.L., Morris, S.B., Xue, Y.Y., Burdick, M.D., Glass, M.C., Iannettoni, M.D., and Strieter, R.M. 1998. Epithelial-neutrophil activating peptide (ENA-78) is an important angiogenic factor in non-small cell lung cancer. *J Clin Invest* 102:465-472.

270. Yoneda, J., Kuniyasu, H., Crispens, M.A., Price, J.E., Bucana, C.D., and Fidler, I.J. 1998. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J Natl Cancer Inst* 90:447-454.

271. Moore, B.B., Arenberg, D.A., Stoy, K., Morgan, T., Addison, C.L., Morris, S.B., Glass, M., Wilke, C., Xue, Y.Y., Sitterding, S., et al. 1999. Distinct CXC chemokines mediate tumorigenicity of prostate cancer cells. *Am J Pathol* 154:1503-1512.

272. Kitadai, Y., Haruma, K., Sumii, K., Yamamoto, S., Ue, T., Yokozaki, H., Yasui, W., Ohmoto, Y., Kajiyama, G., Fidler, I.J., et al. 1998. Expression of interleukin-8 correlates with vascularity in human gastric carcinomas. *Am J Pathol* 152:93-100.

273. Shi, Q., Abbruzzese, J.L., Huang, S.Y., Fidler, I.J., Xiong, Q.H., and Xie, K.P. 1999. Constitutive and inducible interleukin 8 expression by hypoxia and acidosis

renders human pancreatic cancer cells more tumorigenic and metastatic. *Clin Cancer Res* 5:3711-3721.

274. Sgadari, C., Angiolillo, A.L., Cherney, B.W., Pike, S.E., Farber, J.M., Koniaris, L.G., Vanguri, P., Burd, P.R., Sheikh, N., Gupta, G., et al. 1996. Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proc Natl Acad Sci U S A* 93:13791-13796.

275. Arenberg, D.A., Kunkel, S.L., Polverini, P.J., Morris, S.B., Burdick, M.D., Glass, M.C., Taub, D.T., Iannettoni, M.D., Whyte, R.I., and Strieter, R.M. 1996. Interferon-gamma-inducible protein 10 (IP-10) is an angiostatic factor that inhibits human non-small cell lung cancer (NSCLC) tumorigenesis and spontaneous metastases. *J Exp Med* 184:981-992.

276. Richmond, A., Lawson, D.H., Nixon, D.W., and Chawla, R.K. 1985. Characterization of autostimulatory and transforming growth factors from human melanoma cells. *Cancer Res* 45:6390-6394.

277. Richmond, A., Balentien, E., Thomas, H.G., Flaggs, G., Barton, D.E., Spiess, J., Bordoni, R., Francke, U., and Derynck, R. 1988. Molecular characterization and chromosomal mapping of melanoma growth stimulatory activity, a growth factor structurally related to beta- thromboglobulin. *Embo J* 7:2025-2033.

278. Takamori, H., Oades, Z.G., Hoch, O.C., Burger, M., and Schraufstatter, I.U. 2000. Autocrine growth effect of IL-8 and GROalpha on a human pancreatic cancer cell line, Capan-1. *Pancreas* 21:52-56.

279. Schadendorf, D., Moller, A., Algermissen, B., Worm, M., Sticherling, M., and Czarnetzki, B.M. 1993. IL-8 produced by human malignant melanoma cells in vitro is an essential autocrine growth factor. *J Immunol* 151:2667-2675.

280. Xu, L., and Fidler, I.J. 2000. Interleukin 8: An autocrine growth factor for human ovarian cancer. *Oncol Res* 12:97-106.

281. Brew, R., Erikson, J.S., West, D.C., Kinsella, A.R., Slavin, J., and Christmas, S.E. 2000. Interleukin-8 as an autocrine growth factor for human colon carcinoma cells in vitro. *Cytokine* 12:78-85.

282. di Celle, P.F., Carbone, A., Marchis, D., Zhou, D., Sozzani, S., Zupo, S., Pini, M., Mantovani, A., and Foa, R. 1994. Cytokine gene expression in B-cell chronic lymphocytic leukemia: evidence of constitutive interleukin-8 (IL-8) mRNA expression and secretion of biologically active IL-8 protein. *Blood* 84:220-228.

283. Richards, B.L., Eisma, R.J., Spiro, J.D., Lindquist, R.L., and Kreutzer, D.L. 1997. Coexpression of interleukin-8 receptors in head and neck squamous cell carcinoma. *Am J Surg* 174:507-512.

284. Paget, S. 1889. The distribution of secondary growths in cancer of the breast. *Lancet* 1:571-573.

285. Nicolson, G.L. 1993. Paracrine and autocrine growth mechanisms in tumor metastasis to specific sites with particular emphasis on brain and lung metastasis. *Cancer Metastasis Rev* 12:325-343.

286. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature* 410:50-56.

287. Moore, M.A. 2001. The role of chemoattraction in cancer metastases. *Bioessays* 23:674-676.

288. Opdenakker, G., and Van Damme, J. 1992. Chemotactic factors, passive invasion and metastasis of cancer cells. *Immunol Today* 13:463-464.

289. Youngs, S.J., Ali, S.A., Taub, D.D., and Rees, R.C. 1997. Chemokines induce migrational responses in human breast carcinoma cell lines. *Int J Cancer* 71:257-266.

290. Reiland, J., Furcht, L.T., and McCarthy, J.B. 1999. CXC-chemokines stimulate invasion and chemotaxis in prostate carcinoma cells through the CXCR2 receptor. *Prostate* 41:78-88.

291. Venkatakrishnan, G., Salgia, R., and Groopman, J.E. 2000. Chemokine receptors CXCR-1/2 activate mitogen-activated protein kinase via the epidermal growth factor receptor in ovarian cancer cells. *J Biol Chem* 275:6868-6875.

292. Mohle, R., Schittenhelm, M., Failenschmid, C., Bautz, F., KratzAlbers, K., Serve, H., Brugger, W., and Kanz, L. 2000. Functional response of leukaemic blasts to stromal cell-derived factor-1 correlates with preferential expression of the chemokine receptor CXCR4 in acute myelomonocytic and lymphoblastic leukaemia. *Br J Haematol* 110:563-572.

293. Koshiba, T., Hosotani, R., Miyamoto, Y., Ida, J., Tsuji, S., Nakajima, S., Kawaguchi, M., Kobayashi, H., Doi, R., Hori, T., et al. 2000. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: A possible role for tumor progression. *Clin Cancer Res* 6:3530-3535.

294. Murdoch, C. 2000. CXCR4: chemokine receptor extraordinaire. *Immunol Rev* 177:175-184.

295. Bleul, C.C., Fuhlbrigge, R.C., Casasnovas, J.M., Aiuti, A., and Springer, T.A. 1996. A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 184:1101-1109.

296. Shirozu, M., Nakano, T., Inazawa, J., Tashiro, K., Tada, H., Shinohara, T., and Honjo, T. 1995. Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. *Genomics* 28:495-500.

297. Nagasawa, T., Hirota, S., Tachibana, K., Takakura, N., Nishikawa, S., Kitamura, Y., Yoshida, N., Kikutani, H., and Kishimoto, T. 1996. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature* 382:635-638.

298. Tachibana, K., Hirota, S., Iizasa, H., Yoshida, H., Kawabata, K., Kataoka, Y., Kitamura, Y., Matsushima, K., Yoshida, N., Nishikawa, S., et al. 1998. The chemokine receptor cxcr4 is essential for vascularization of the gastrointestinal-tract. *Nature* 393:591-594.

299. Ma, Q., Jones, D., Borghesani, P.R., Segal, R.A., Nagasawa, T., Kishimoto, T., Bronson, R.T., and Springer, T.A. 1998. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in cxcr4-deficient and sdf-1-deficient mice. *Proc Natl Acad Sci U S A* 95:9448-9453.

300. Jazin, E.E., Soderstrom, S., Ebendal, T., and Larhammar, D. 1997. Embryonic expression of the mRNA for the rat homologue of the fusin/CXCR-4 HIV-1 co-receptor. *J Neuroimmunol* 79:148-154.

301. McGrath, K.E., Koniski, A.D., Maltby, K.M., McGann, J.K., and Palis, J. 1999. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev Biol* 213:442-456.

302. Suzuki, G., Nakata, Y., Dan, Y., Uzawa, A., Nakagawa, K., Saito, T., Mita, K., and Shirasawa, T. 1998. Loss of sdf-1 receptor expression during positive selection in the thymus. *Int Immunol* 10:1049-1056.

303. Kim, C.H., Pelus, L.M., and Broxmeyer, H.E. 1998. Regulation of trafficking and proliferation of myeloid progenitors by ccr7 ligands - slc/6ckine/exodus2 and ck-beta-11/mip-3-beta/elc. *Blood* 92:1523-1523.

304. Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., et al. 1999. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science* 283:845-848.

305. Parkin, D.M., Pisani, P., and Ferlay, J. 1999. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 80:827-841.

306. La Vecchia, C., Lucchini, F., Negri, E., Boyle, P., and Levi, F. 1993. Trends in cancer mortality, 1955-1989: Asia, Africa and Oceania. *Eur J Cancer* 15:2168-2211.

307. La Vecchia, C., Negri, E., Levi, F., Decarli, A., and Boyle, P. 1998. Cancer mortality in Europe: effects of age, cohort of birth and period of death. *Eur J Cancer* 34:118-141.

308. Tarone, R.E., and Chu, K.C. 2000. Age-period-cohort analyses of breast-, ovarian-, endometrial- and cervical-cancer mortality rates for Caucasian women in the USA. *J Epidemiol Biostat* 5:221-231.

309. Landis, S.H., Murray, T., Bolden, S., and Wingo, P.A. 1998. Cancer statistics, 1998. *CA Cancer J Clin* 48:6-29.

310. Parazzini, F., Franceschi, S., La Vecchia, C., and Fasoli, M. 1991. The epidemiology of ovarian cancer. *Gynecol Oncol* 43:9-23.

311. Risch, H.A. 1998. Hormonal etiology of epithelial ovarian cancer, with a hypothesis concerning the role of androgens and progesterone. *J Natl Cancer Inst* 90:1774-1786.

312. Derman, S.G., and Adashi, E.Y. 1994. Adverse effects of fertility drugs. *Drug Saf* 11:408-421.

313. Booth, M., Beral, V., and Smith, P. 1989. Risk factors for ovarian cancer: a case-control study. *Br J Cancer* 60:592-598.

314. Rosenblatt, K.A., and Thomas, D.B. 1993. Lactation and the risk of epithelial ovarian cancer. The WHO Collaborative Study of Neoplasia and Steroid Contraceptives. *Int J Epidemiol* 22:192-197.

315. Chiaffarino, F., Pelucchi, C., Parazzini, F., Negri, E., Franceschi, S., Talamini, R., Conti, E., Montella, M., and La Vecchia, C. 2001. Reproductive and hormonal factors and ovarian cancer. *Ann Oncol* 12:337-341.

316. Piver, M.S., Mettlin, C.J., Tsukada, Y., Nasca, P., Greenwald, P., and McPhee, M.E. 1984. Familial Ovarian Cancer Registry. *Obstet Gynecol* 64:195-199.

317. Eby, N., Chang-Claude, J., and Bishop, D.T. 1994. Familial risk and genetic susceptibility for breast cancer. *Cancer Causes Control* 5:458-470.

318. Gayther, S.A., and Ponder, B.A. 1997. Mutations of the BRCA1 and BRCA2 genes and the possibilities for predictive testing. *Mol Med Today* 3:168-174.

319. La Vecchia, C., Parazzini, F., Negri, E., Del Pino, D., and Franceschi, S. 1996. Family history and risk of ovarian cancer. *Int J Cancer* 67:903-904.

320. Tavani, A., Negri, E., Franceschi, S., Parazzini, F., and La Vecchia, C. 1993. Risk factors for epithelial ovarian cancer in women under age 45. *Eur J Cancer* 9:1297-1301.

321. Cramer, D.W. 1990. Epidemiologic aspects of early menopause and ovarian cancer. *Ann N Y Acad Sci* 592:363-375.

322. Moller, H., Mellemgaard, A., Lindvig, K., and Olsen, J.H. 1994. Obesity and cancer risk: a Danish record-linkage study. *Eur J Cancer* 3:344-350.

323. Bosetti, C., Negri, E., Franceschi, S., Pelucchi, C., Talamini, R., Montella, M., Conti, E., and La Vecchia, C. 2001. Diet and ovarian cancer risk: A case-control study in Italy. *Int J Cancer* 93:911-915.

324. Cook, L.S., Kamb, M.L., and Weiss, N.S. 1997. Perineal powder exposure and the risk of ovarian cancer. *Am J Epidemiol* 145:459-465.

325. Cramer, D.W., Liberman, R.F., Titus-Ernstoff, L., Welch, W.R., Greenberg, E.R., Baron, J.A., and Harlow, B.L. 1999. Genital talc exposure and risk of ovarian cancer. *Int J Cancer* 81:351-356.

326. Ness, R.B., and Cottreau, C. 1999. Possible role of ovarian epithelial inflammation in ovarian cancer. *J Natl Cancer Inst* 91:1459-1467.

327. Dreher, D., and Junod, A.F. 1996. Role of oxygen free radicals in cancer development. *Eur J Cancer* 32A:30-38.

328. Auersperg, N., Edelson, M.I., Mok, S.C., Johnson, S.W., and Hamilton, T.C. 1998. The biology of ovarian cancer. *Semin Oncol* 25:281-304.

329. Subbaramaiah, K., Zakim, D., Weksler, B.B., and Dannenberg, A.J. 1997. Inhibition of cyclooxygenase: a novel approach to cancer prevention. *Proc Soc Exp Biol Med* 216:201-210.

330. Chen, S.S., and Lee, L. 1983. Incidence of para-aortic and pelvic lymph node metastases in epithelial carcinoma of the ovary. *Gynecol Oncol* 16:95-100.

331. Berek, J.S., and Hacker, H.F. 1994. *Practical gynecologic oncology*: Williams and Wilkins.

332. Hirabayashi, K., and Graham, J. 1970. Genesis of ascites in ovarian cancer. *Am J Obstet Gynecol* 106:492-497.

333. Nagy, J.A., Herzberg, K.T., Dvorak, J.M., and Dvorak, H.F. 1993. Pathogenesis of malignant ascites formation: initiating events that lead to fluid accumulation. *Cancer Res* 53:2631-2643.

334. Garrison, R.N., Galloway, R.H., and Heuser, L.S. 1987. Mechanisms of malignant ascites production. *J Surg Res* 42:126-132.

335. Nagy, J.A., Masse, E.M., Herzberg, K.T., Meyers, M.S., Yeo, K.T., Yeo, T.K., Sioussat, T.M., and Dvorak, H.F. 1995. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res* 55:360-368.

336. Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S., and Dvorak, H.F. 1983. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* 219:983-985.

337. Nagy, J.A., Herzberg, K.T., Masse, E.M., Zientara, G.P., and Dvorak, H.F. 1989. Exchange of macromolecules between plasma and peritoneal cavity in ascites tumor-bearing, normal, and serotonin-injected mice. *Cancer Res* 49:5448-5458.

338. Feldman, G.B. 1975. Lymphatic obstruction in carcinomatous ascites. *Cancer Res* 35:325-332.

339. Menon, U., and Jacobs, I.J. 2000. Recent developments in ovarian cancer screening. *Curr Opin Obstet Gynecol* 12:39-42.

340. Schwartz, P.E., Chambers, J.T., and Taylor, K.J. 1995. Early detection and screening for ovarian cancer. *J Cell Biochem Suppl* 23:233-237.

341. Bast, R.C., Jr., Klug, T.L., St John, E., Jenison, E., Niloff, J.M., Lazarus, H., Berkowitz, R.S., Leavitt, T., Griffiths, C.T., Parker, L., et al. 1983. A radioimmunoassay using a monoclonal antibody to monitor the course of epithelial ovarian cancer. *N Engl J Med* 309:883-887.

342. Auersperg, N., Wong, A.S., Choi, K.C., Kang, S.K., and Leung, P.C. 2001. Ovarian surface epithelium: biology, endocrinology, and pathology. *Endocr Rev* 22:255-288.

343. Young, R.H., Gilks, C.B., and Scully, R.E. 1991. Mucinous tumors of the appendix associated with mucinous tumors of the ovary and pseudomyxoma peritonei. A clinicopathological analysis of 22 cases supporting an origin in the appendix. *Am J Surg Pathol* 15:415-429.

344. Markman, M., and Hoskins, W.J. 1993. *Cancer of the ovary*: Raven Press.

345. Cotran, R.S., Kumar, V., and Robbins, S.L. 1989. *Robbins pathologic basis of disease*: W. B. Saunders Company.

346. Katso, R.M., Manek, S., O'Byrne, K., Playford, M.P., Le Meuth, V., and Ganesan, T.S. 1997. Molecular approaches to diagnosis and management of ovarian cancer. *Cancer Metastasis Rev* 16:81-107.

347. Tashiro, H., Miyazaki, K., Okamura, H., Iwai, A., and Fukumoto, M. 1992. c-myc over-expression in human primary ovarian tumours: its relevance to tumour progression. *Int J Cancer* 50:828-833.

348. Enomoto, T., Weghorst, C.M., Inoue, M., Tanizawa, O., and Rice, J.M. 1991. K-ras activation occurs frequently in mucinous adenocarcinomas and rarely in other common epithelial tumors of the human ovary. *Am J Pathol* 139:777-785.

349. Berchuck, A., Kamel, A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J.T., Dodge, R., Clarke-Pearson, D.L., Marks, P., et al. 1990. Overexpression of HER-2/neu is associated with poor survival in advanced epithelial ovarian cancer. *Cancer Res* 50:4087-4091.

350. Shayesteh, L., Lu, Y., Kuo, W.L., Baldocchi, R., Godfrey, T., Collins, C., Pinkel, D., Powell, B., Mills, G.B., and Gray, J.W. 1999. PIK3CA is implicated as an oncogene in ovarian cancer. *Nat Genet* 21:99-102.

351. Shelling, A.N., Cooke, I.E., and Ganesan, T.S. 1995. The genetic analysis of ovarian cancer. *Br J Cancer* 72:521-527.

352. Marks, J.R., Davidoff, A.M., Kerns, B.J., Humphrey, P.A., Pence, J.C., Dodge, R.K., Clarke-Pearson, D.L., Iglehart, J.D., Bast, R.C., Jr., and Berchuck, A. 1991. Overexpression and mutation of p53 in epithelial ovarian cancer. *Cancer Res* 51:2979-2984.

353. Berchuck, A., Kohler, M.F., Hopkins, M.P., Humphrey, P.A., Robboy, S.J., Rodriguez, G.C., Soper, J.T., Clarke-Pearson, D.L., and Bast, R.C. 1994. Overexpression of p53 is not a feature of benign and early-stage borderline epithelial ovarian tumors. *Gynecol Oncol* 52:232-236.

354. Yu, Y.H., Xu, F.J., Peng, H.Q., Fang, X.J., Zhao, S.L., Li, Y., Cuevas, B., Kuo, W.L., Gray, J.W., Siciliano, M., et al. 1999. NOEY2 (ARHI), an imprinted putative tumor suppressor gene in ovarian and breast carcinomas. *Proc Natl Acad Sci U S A* 96:214-219.

355. Young, R.C., Walton, L.A., Ellenberg, S.S., Homesley, H.D., Wilbanks, G.D., Decker, D.G., Miller, A., Park, R., and Major, F., Jr. 1990. Adjuvant therapy in stage I and stage II epithelial ovarian cancer. Results of two prospective randomized trials. *N Engl J Med* 322:1021-1027.

356. Young, R.C., Chabner, B.A., Hubbard, S.P., Fisher, R.I., Bender, R.A., Anderson, T., Simon, R.M., Canellos, G.P., and DeVita, V.T., Jr. 1978. Advanced ovarian adenocarcinoma. A prospective clinical trial of melphalan (L-PAM) versus combination chemotherapy. *N Engl J Med* 299:1261-1266.

357. Omura, G., Blessing, J.A., Ehrlich, C.E., Miller, A., Yordan, E., Creasman, W.T., and Homesley, H.D. 1986. A randomized trial of cyclophosphamide and doxorubicin with or without cisplatin in advanced ovarian carcinoma. A Gynecologic Oncology Group Study. *Cancer* 57:1725-1730.

358. Partridge, E.E., and Barnes, M.N. 1999. Epithelial ovarian cancer: prevention, diagnosis, and treatment. *CA Cancer J Clin* 49:297-320.

359. Lanciano, R.M., and Randall, M. 1991. Update on the role of radiotherapy in ovarian cancer. *Semin Oncol* 18:233-247.

360. Balkwill, F.R. 1994. Cytokine therapy of cancer. The importance of knowing the context. *Eur Cytokine Netw* 5:379-385.

361. Burke, F., Relf, M., Negus, R., and Balkwill, F. 1996. A cytokine profile of normal and malignant ovary. *Cytokine* 8:578-585.

362. Baserga, R., Sell, C., Porcu, P., and Rubini, M. 1994. The role of the IGF-I receptor in the growth and transformation of mammalian cells. *Cell Prolif* 27:63-71.

363. Stromberg, K., Collins, T.J.t., Gordon, A.W., Jackson, C.L., and Johnson, G.R. 1992. Transforming growth factor-alpha acts as an autocrine growth factor in ovarian carcinoma cell lines. *Cancer Res* 52:341-347.

364. Olson, T.A., Mohanraj, D., Carson, L.F., and Ramakrishnan, S. 1994. Vascular permeability factor gene expression in normal and neoplastic human ovaries. *Cancer Res* 54:276-280.

365. Yamamoto, S., Konishi, I., Mandai, M., Kuroda, H., Komatsu, T., Nanbu, K., Sakahara, H., and Mori, T. 1997. Expression of vascular endothelial growth factor (VEGF) in epithelial ovarian neoplasms: correlation with clinicopathology and patient survival, and analysis of serum VEGF levels. *Br J Cancer* 76:1221-1227.

366. Reynolds, K., Farzaneh, F., Collins, W.P., Campbell, S., Bourne, T.H., Lawton, F., Moghaddam, A., Harris, A.L., and Bicknell, R. 1994. Association of ovarian malignancy with expression of platelet-derived endothelial cell growth factor. *J Natl Cancer Inst* 86:1234-1238.

367. Xu, L., Xie, K.P., Mukaida, N., Matsushima, K., and Fidler, I.J. 1999. Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res* 59:5822-5829.

368. Naylor, M.S., Stamp, G.W., Foulkes, W.D., Eccles, D., and Balkwill, F.R. 1993. Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. *J Clin Invest* 91:2194-2206.

369. Moore, R.J., Owens, D.M., Stamp, G., Arnott, C., Burke, F., East, N., Holdsworth, H., Turner, L., Rollins, B., Pasparakis, M., et al. 1999. Mice deficient in tumor necrosis factor-alpha are resistant to skin carcinogenesis. *Nat Med* 5:828-831.

370. Malik, S.T., Griffin, D.B., Fiers, W., and Balkwill, F.R. 1989. Paradoxical effects of tumour necrosis factor in experimental ovarian cancer. *Int J Cancer* 44:918-925.

371. Offner, F.A., Obrist, P., Stadlmann, S., Feichtinger, H., Klingler, P., Herold, M., Zwierzina, H., Hittmair, A., Mikuz, G., Abendstein, B., et al. 1995. IL-6 secretion by human peritoneal mesothelial and ovarian cancer cells. *Cytokine* 7:542-547.

372. Obata, N.H., Tamakoshi, K., Shibata, K., Kikkawa, F., and Tomoda, Y. 1997. Effects of interleukin-6 on in vitro cell attachment, migration and invasion of human ovarian carcinoma. *Anticancer Res* 17:337-342.

373. Nash, M.A., Ferrandina, G., Gordinier, M., Loercher, A., and Freedman, R.S. 1999. The role of cytokines in both the normal and malignant ovary. *Endocr Relat Cancer* 6:93-107.

374. Hockel, M., and Vaupel, P. 2001. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 93:266-276.

375. Sutherland, R.M. 1998. Tumor hypoxia and gene expression--implications for malignant progression and therapy. *Acta Oncol* 37:567-574.

376. Vaupel, P., Thews, O., Kelleher, D.K., and Hoeckel, M. 1998. Current status of knowledge and critical issues in tumor oxygenation. Results from 25 years research in tumor pathophysiology. *Adv Exp Med Biol* 454:591-602.

377. Negus, R.P., Turner, L., Burke, F., and Balkwill, F.R. 1998. Hypoxia down-regulates MCP-1 expression: implications for macrophage distribution in tumors. *J Leukoc Biol* 63:758-765.

378. Turner, L., Scotton, C., Negus, R., and Balkwill, F. 1999. Hypoxia inhibits macrophage migration. *Eur J Immunol* 29:2280-2287.

379. Kallinowski, F., Zander, R., Hoeckel, M., and Vaupel, P. 1990. Tumor tissue oxygenation as evaluated by computerized-pO2-histography. *Int J Radiat Oncol Biol Phys* 19:953-961.

380. Hodgkiss, R.J., Webster, L., and Wilson, G.D. 1997. Measurement of hypoxia in vivo using a 2-nitroimidazole (NITP). *Adv Exp Med Biol* 428:61-67.

381. McCoy, C.L., McIntyre, D.J., Robinson, S.P., Aboagye, E.O., and Griffiths, J.R. 1996. Magnetic resonance spectroscopy and imaging methods for measuring tumour and tissue oxygenation. *Br J Cancer Suppl* 27:S226-231.

382. Amellem, O., Loffler, M., and Pettersen, E.O. 1994. Regulation of cell proliferation under extreme and moderate hypoxia: the role of pyrimidine (deoxy)nucleotides. *Br J Cancer* 70:857-866.

383. Riva, C., Chauvin, C., Pison, C., and Leverve, X. 1998. Cellular physiology and molecular events in hypoxia-induced apoptosis. *Anticancer Res* 18:4729-4736.

384. Prehn, R.T. 1991. The inhibition of tumor growth by tumor mass. *Cancer Res* 51:2-4.

385. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. 1992. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843-845.

386. Kuwabara, K., Ogawa, S., Matsumoto, M., Koga, S., Clauss, M., Pinsky, D.J., Lyn, P., Leavy, J., Witte, L., Joseph-Silverstein, J., et al. 1995. Hypoxia-mediated induction of acidic/basic fibroblast growth factor and platelet-derived growth factor in mononuclear phagocytes stimulates growth of hypoxic endothelial cells. *Proc Natl Acad Sci U S A* 92:4606-4610.

387. Graham, C.H., Forsdike, J., Fitzgerald, C.J., and MacdonaldGoodfellow, S. 1999. Hypoxia-mediated stimulation of carcinoma cell invasiveness via upregulation of urokinase receptor expression. *Int J Cancer* 80:617-623.

388. Dachs, G.U., and Tozer, G.M. 2000. Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. *Eur J Cancer* 36:1649-1660.

389. Koong, A.C., Chen, E.Y., Mivechi, N.F., Denko, N.C., Stambrook, P., and Giaccia, A.J. 1994. Hypoxic activation of nuclear factor-kappa B is mediated by a Ras and Raf signaling pathway and does not involve MAP kinase (ERK1 or ERK2). *Cancer Res* 54:5273-5279.

390. Young, S.D., Marshall, R.S., and Hill, R.P. 1988. Hypoxia induces DNA overreplication and enhances metastatic potential of murine tumor cells. *Proc Natl Acad Sci U S A* 85:9533-9537.

391. Reynolds, T.Y., Rockwell, S., and Glazer, P.M. 1996. Genetic instability induced by the tumor microenvironment. *Cancer Res* 56:5754-5757.

392. Nordsmark, M., Alsner, J., Keller, J., Nielsen, O.S., Jensen, O.M., Horsman, M.R., and Overgaard, J. 2001. Hypoxia in human soft tissue sarcomas: adverse impact on survival and no association with p53 mutations. *Br J Cancer* 84:1070-1075.

393. Hockel, M., Schlenger, K., Aral, B., Mitze, M., Schaffer, U., and Vaupel, P. 1996. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 56:4509-4515.

394. Birner, P., Schindl, M., Obermair, A., Plank, C., Breitenecker, G., and Oberhuber, G. 2000. Overexpression of hypoxia-inducible factor 1alpha is a marker for an unfavorable prognosis in early-stage invasive cervical cancer. *Cancer Res* 60:4693-4696.

395. Birner, P., Schindl, M., Obermair, A., Breitenecker, G., and Oberhuber, G. 2001. Expression of hypoxia-inducible factor 1alpha in epithelial ovarian tumors: its impact on prognosis and on response to chemotherapy. *Clin Cancer Res* 7:1661-1668.

396. Hosaka, S., Akahoshi, T., Wada, C., and Kondo, H. 1994. Expression of the chemokine superfamily in rheumatoid arthritis. *Clin Exp Immunol* 97:451-457.

397. Mattei, S., Colombo, M.P., Melani, C., Silvani, A., Parmiani, G., and Herlyn, M. 1994. Expression of cytokine/growth factors and their receptors in human melanoma and melanocytes. *Int J Cancer* 56:853-857.

398. Van Coillie, E., Froyen, G., Nomiyama, H., Miura, R., Fiten, P., Van Aelst, I., Van Damme, J., and Opdenakker, G. 1997. Human monocyte chemotactic protein-2: cDNA cloning and regulated expression of mRNA in mesenchymal cells. *Biochem Biophys Res Commun* 231:726-730.

399. Naylor, M.S., Malik, S.T., Stamp, G.W., Jobling, T., and Balkwill, F.R. 1990. In situ detection of tumour necrosis factor in human ovarian cancer specimens. *Eur J Cancer* 26:1027-1030.

400. Cox, K.H., DeLeon, D.V., Angerer, L.M., and Angerer, R.C. 1984. Detection of mrnas in sea urchin embryos by in situ hybridization using asymmetric RNA probes. *Dev Biol* 101:485-502.

401. Altman, D.G. 1991. *Practical statistics for medical research*: Chapman and Hall.

402. Haskill, S., Becker, S., Fowler, W., and Walton, L. 1982. Mononuclear-cell infiltration in ovarian cancer. I. Inflammatory-cell infiltrates from tumour and ascites material. *Br J Cancer* 45:728-736.

403. Dietl, J., Horny, H.P., Ruck, P., and Kaiserling, E. 1993. Dysgerminoma of the ovary. An immunohistochemical study of tumor- infiltrating lymphoreticular cells and tumor cells. *Cancer* 71:2562-2568.

404. Kabawat, S.E., Bast, R.C., Jr., Welch, W.R., Knapp, R.C., and Bhan, A.K. 1983. Expression of major histocompatibility antigens and nature of inflammatory cellular infiltrate in ovarian neoplasms. *Int J Cancer* 32:547-554.

405. Yoong, K.F., Afford, S.C., Jones, R., Aujla, P., Qin, S., Price, K., Hubscher, S.G., and Adams, D.H. 1999. Expression and function of CXC and CC chemokines in human malignant liver tumors: a role for human monokine induced by gamma-interferon in lymphocyte recruitment to hepatocellular carcinoma. *Hepatology* 30:100-111.

406. Colantonio, L., Iellem, A., Clissi, B., Pardi, R., Rogge, L., Sinigaglia, F., and Dambrosio, D. 1999. Upregulation of integrin alpha 6/beta 1 and chemokine receptor

CCR1 by interleukin-12 promotes the migration of human type 1 helper T cells. *Blood* 94:2981-2989.

407. Bonecchi, R., Polentarutti, N., Luini, W., Borsatti, A., Bernasconi, S., Locati, M., Power, C., Proudfoot, A., Wells, T.N.C., Mackay, C., et al. 1999. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-gamma in human neutrophils. *J Immunol* 162:474-479.

408. Zella, D., Barabitskaja, O., Casareto, L., Romerio, F., Secchiero, P., Reitz, M.S., Gallo, R.C., and Weichold, F.F. 1999. Recombinant IFN-alpha (2b) increases the expression of apoptosis receptor CD95 and chemokine receptors CCR1 and CCR3 in monocytoid cells. *J Immunol* 163:3169-3175.

409. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. 1995. MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* 23:4878-4884.

410. Forsythe, J.A., Jiang, B.H., Iyer, N.V., Agani, F., Leung, S.W., Koos, R.D., and Semenza, G.L. 1996. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol-Cell-Biol* 16:4604-4613 issn: 0270-7306.

411. Maxwell, P., and Ratcliffe, P. 1998. Regulation of expression of the erythropoietin gene. *Curr Opin Hematol* 5:166-170.

412. Semenza, G.L., Roth, P.H., Fang, H.M., and Wang, G.L. 1994. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J Biol Chem* 269:23757-23763.

413. Mueller-Klieser, W. 1997. Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am J Physiol* 273:C1109-1123.

414. Grimshaw, M.J., and Balkwill, F.R. 2001. Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation - a potential mechanism. *Eur J Immunol* 31:480-489.

415. Mantovani, A., Allavena, P., Vecchi, A., and Sozzani, S. 1998. Chemokines and chemokine receptors during activation and deactivation of monocytes and dendritic cells and in amplification of th1 versus th2 responses. *Int J Clin Lab Res* 28:77-82.

416. Signoret, N., Rosenkilde, M.M., Klasse, P.J., Schwartz, T.W., Malim, M.H., Hoxie, J.A., and Marsh, M. 1998. Differential regulation of cxcr4 and ccr5 endocytosis. *J Cell Sci* 111:2819-2830.

417. Hockel, M., Schlenger, K., Hockel, S., Aral, B., Schaffer, U., and Vaupel, P. 1998. Tumor hypoxia in pelvic recurrences of cervical cancer. *Int J Cancer* 79:365-369.

418. Grutkoski, P.S., Graeber, C.T., Damico, R., Keeping, H., and Simms, H.H. 1999. Regulation of IL-8RA (CXCR1) expression in polymorphonuclear leukocytes by hypoxia/reoxygenation. *J Leukoc Biol* 65:171-178.

419. Griffiths, L., Binley, K., Iqball, S., Kan, O., Maxwell, P., Ratcliffe, P., Lewis, C., Harris, A., Kingsman, S., and Naylor, S. 2000. The macrophage - a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther* 7:255-262.

420. Mantovani, A. 1994. Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. *Lab Invest* 71:5-16.

421. Gao, W., Topham, P.S., King, J.A., Smiley, S.T., Csizmadia, V., Lu, B., Gerard, C.J., and Hancock, W.W. 2000. Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J Clin Invest* 105:35-44.

422. Sheid, B. 1992. Angiogenic effects of macrophages isolated from ascitic fluid aspirated from women with advanced ovarian cancer. *Cancer Lett* 62:153-158.

423. Veatch, A.L., Carson, L.F., and Ramakrishnan, S. 1994. Differential expression of the cell-cell adhesion molecule E-cadherin in ascites and solid human ovarian tumor cells. *Int J Cancer* 58:393-399.

424. Li, B.Y., Mohanraj, D., Olson, M.C., Moradi, M., Twiggs, L., Carson, L.F., and Ramakrishnan, S. 1992. Human ovarian epithelial cancer cells cultures in vitro express both interleukin 1 alpha and beta genes. *Cancer Res* 52:2248-2252.

425. Mills, G.B., May, C., Hill, M., Campbell, S., Shaw, P., and Marks, A. 1990. Ascitic fluid from human ovarian cancer patients contains growth factors necessary for intraperitoneal growth of human ovarian adenocarcinoma cells. *J Clin Invest* 86:851-855.

426. Punnonen, R., Teisala, K., Kuoppala, T., Bennett, B., and Punnonen, J. 1998. Cytokine production profiles in the peritoneal fluids of patients with malignant or benign gynecologic tumors. *Cancer* 83:788-796.

427. Hellstrom, I., Goodman, G., Pullman, J., Yang, Y., and Hellstrom, K.E. 2001. Overexpression of HER-2 in ovarian carcinomas. *Cancer Res* 61:2420-2423.

428. Kameyoshi, Y., Dorschner, A., Mallet, A.I., Christophers, E., and Schroder, J.M. 1992. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 176:587-592.

429. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature* 392:565-568.

430. Kunz, M., Hartmann, A., Flory, E., Toksoy, A., Koczan, D., Thiesen, H.J., Mukaida, N., Neumann, M., Rapp, U.R., Brocker, E.B., et al. 1999. Anoxia-induced up-regulation of interleukin-8 in human malignant melanoma. A potential mechanism for high tumor aggressiveness. *Am J Pathol* 155:753-763.

431. Adams, D.H., and Lloyd, A.R. 1997. Chemokines: leucocyte recruitment and activation cytokines. *Lancet* 349:490-495.

432. Poznansky, M.C., Olszak, I.T., Foxall, R., Evans, R.H., Luster, A.D., and Scadden, D.T. 2000. Active movement of T cells away from a chemokine. *Nat Med* 6:543-548.

433. Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., LifshitzMercer, B., Chaitchik, S., Keydar, I., and BenBaruch, A. 1999. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. *Cancer Res* 59:4681-4687.

434. Bottazzi, B., Walter, S., Govoni, D., Colotta, F., and Mantovani, A. 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration,

growth, and susceptibility to IL-2 therapy of a murine melanoma. *J Immunol* 148:1280-1285.

435. Rossi, D., and Zlotnik, A. 2000. The biology of chemokines and their receptors. *Annu Rev Immunol* 18:217-243.

436. Coulomb-L'Hermin, A., Amara, A., Schiff, C., Durand-Gasselin, I., Foussat, A., Delaunay, T., Chaouat, G., Capron, F., Ledee, N., Galanaud, P., et al. 1999. Stromal cell-derived factor 1 (SDF-1) and antenatal human B cell lymphopoiesis: expression of SDF-1 by mesothelial cells and biliary ductal plate epithelial cells. *Proc Natl Acad Sci U S A* 96:8585-8590.

437. Hodohara, K., Fujii, N., Yamamoto, N., and Kaushansky, K. 2000. Stromal cell-derived factor-1 (SDF1) acts together with thrombopoietin to enhance the development of megakaryocytic progenitor cells (CFU-MK). *Blood* 95:769-775.

438. Bleul, C.C., Wu, L., Hoxie, J.A., Springer, T.A., and Mackay, C.R. 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. *Proc Natl Acad Sci U S A* 94:1925-1930.

439. Zaitseva, M., Blauvelt, A., Lee, S., Lapham, C.K., Klaus-Kovtun, V., Mostowski, H., Manischewitz, J., and Golding, H. 1997. Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. *Nat Med* 3:1369-1375.

440. Dwinell, M.B., Eckmann, L., Leopard, J.D., Varki, N.M., and Kagnoff, M.F. 1999. Chemokine receptor expression by human intestinal epithelial cells. *Gastroenterology* 117:359-367.

441. Murdoch, C., Monk, P.N., and Finn, A. 1999. Functional expression of chemokine receptor CXCR4 on human epithelial cells. *Immunology* 98:36-41.

442. Bajetto, A., Bonavia, R., Barbero, S., Florio, T., and Schettini, G. 2001. Chemokines and their receptors in the central nervous system. *Front Neuroendocrinol* 22:147-184.

443. Allavena, P., Sica, A., Vecchi, A., Locati, M., Sozzani, S., and Mantovani, A. 2000. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol Rev* 177:141-149.

444. Rempel, S.A., Dudas, S., Ge, S., and Gutierrez, J.A. 2000. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. *Clin Cancer Res* 6:102-111.

445. Sood, A.K., Seftor, E.A., Fletcher, M.S., Gardner, L.M., Heidger, P.M., Buller, R.E., Seftor, R.E., and Hendrix, M.J. 2001. Molecular determinants of ovarian cancer plasticity. *Am J Pathol* 158:1279-1288.

446. Haghnegahdar, H., Du, J., Wang, D., Strieter, R.M., Burdick, M.D., Nanney, L.B., Cardwell, N., Luan, J., Shattuck-Brandt, R., and Richmond, A. 2000. The tumorigenic and angiogenic effects of MGSA/GRO proteins in melanoma. *J Leukoc Biol* 67:53-62.

447. Prest, S.J., Rees, R.C., Murdoch, C., Marshall, J.F., Cooper, P.A., Bibby, M., Li, G., and Ali, S.A. 1999. Chemokines induce the cellular migration of MCF-7 human breast carcinoma cells: Subpopulations of tumour cells display positive and negative chemotaxis and differential in vivo growth potentials. *Clin Exp Metastasis* 17:389-396.

448. Curran, S., and Murray, G.I. 1999. Matrix metalloproteinases in tumour invasion and metastasis. *J Pathol* 189:300-308.

449. Irigoyen, J.P., Munoz-Canoves, P., Montero, L., Koziczak, M., and Nagamine, Y. 1999. The plasminogen activator system: biology and regulation. *Cell Mol Life Sci* 56:104-132.

450. Sotsios, Y., and Ward, S.G. 2000. Phosphoinositide 3-kinase: a key biochemical signal for cell migration in response to chemokines. *Immunol Rev* 177:217-235.

451. Wang, J.M., Chertov, O., Proost, P., Li, J.J., Menton, P., Xu, L., Sozzani, S., Mantovani, A., Gong, W., Schirrmacher, V., et al. 1998. Purification and identification of chemokines potentially involved in kidney-specific metastasis by a murine

lymphoma variant: induction of migration and NFkappaB activation. *Int J Cancer* 75:900-907.

452. Fishman, D.A., Bafetti, L.M., and Stack, M.S. 1996. Membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation in primary human ovarian epithelial carcinoma cells. *Invasion Metastasis* 16:150-159.

453. Autio-Harmainen, H., Karttunen, T., Hurskainen, T., Hoyhtya, M., Kauppila, A., and Tryggvason, K. 1993. Expression of 72 kilodalton type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. *Lab Invest* 69:312-321.

454. Campbell, J.J., Hedrick, J., Zlotnik, A., Siani, M.A., Thompson, D.A., and Butcher, E.C. 1998. Chemokines and the arrest of lymphocytes rolling under flow conditions. *Science* 279:381-384.

455. Peled, A., Grabovsky, V., Habler, L., Sandbank, J., ArenzanaSeisdedos, F., Petit, I., BenHur, H., Lapidot, T., and Alon, R. 1999. The chemokine SDF-1 stimulates integrin-mediated arrest of CD34(+) cells on vascular endothelium under shear flow. *J Clin Invest* 104:1199-1211.

456. Kennedy, S.G., Wagner, A.J., Conzen, S.D., Jordan, J., Bellacosa, A., Tsichlis, P.N., and Hay, N. 1997. The PI 3-kinase/Akt signaling pathway delivers an anti-apoptotic signal. *Genes Dev* 11:701-713.

457. Cheng, J.Q., Godwin, A.K., Bellacosa, A., Taguchi, T., Franke, T.F., Hamilton, T.C., Tsichlis, P.N., and Testa, J.R. 1992. AKT2, a putative oncogene encoding a member of a subfamily of protein- serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci U S A* 89:9267-9271.

458. Han, Y., He, T., Huang, D.R., Pardo, C.A., and Ransohoff, R.M. 2001. TNF-alpha mediates SDF-1 alpha-induced NF-kappa B activation and cytotoxic effects in primary astrocytes. *J Clin Invest* 108:425-435.

459. Zoeteweij, J.P., Golding, H., Mostowski, H., and Blauvelt, A. 1998. Cytokines regulate expression and function of the HIV coreceptor CXCR4 on human mature dendritic cells. *J Immunol* 161:3219-3223.

460. Buckley, C.D., Amft, N., Bradfield, P.F., Pilling, D., Ross, E., Arenzana-Seisdedos, F., Amara, A., Curnow, S.J., Lord, J.M., Scheel-Toellner, D., et al. 2000. Persistent induction of the chemokine receptor CXCR4 by TGF-beta 1 on synovial T cells contributes to their accumulation within the rheumatoid synovium. *J Immunol* 165:3423-3429.

461. Wang, J., Harada, A., Matsushita, S., Matsumi, S., Zhang, Y., Shioda, T., Nagai, Y., and Matsushima, K. 1998. IL-4 and a glucocorticoid up-regulate CXCR4 expression on human CD4+ T lymphocytes and enhance HIV-1 replication. *J Leukoc Biol* 64:642-649.

462. Jourdan, P., Abbal, C., Noraz, N., Hori, T., Uchiyama, T., Vendrell, J.P., Bousquet, J., Taylor, N., Pene, J., Yssel, H., et al. 1998. IL-4 induces functional cell-surface expression of CXCR4 on human T cells. *J Immunol* 160:4153-4157.

463. Baribaud, F., Edwards, T.G., Sharron, M., Brelot, A., Heveker, N., Price, K., Mortari, F., Alizon, M., Tsang, M., and Doms, R.W. 2001. Antigenically distinct conformations of cxcr4. *J Virol* 75:8957-8967.

464. Zeelenberg, I.S., Ruuls-Van Stalle, L., and Roos, E. 2001. Retention of CXCR4 in the endoplasmic reticulum blocks dissemination of a T cell hybridoma. *J Clin Invest* 108:269-277.

British Journal of Cancer (2001) **85**(6), 891–897

doi: 10.1054/ bjoc.2001.2020, available online at http://www.idealibrary.com on IDEAL®

Analysis of CC chemokine and chemokine receptor expression in solid ovarian tumours

C Scotton[1], D Milliken[2], J Wilson[1], S Raju[2] and F Balkwill[1]

[1]ICRF Translational Oncology Laboratory, St Bartholomew's and Royal London School of Medicine and Dentistry, Science Building, Charterhouse Square, London EC1M 6BQ, UK; [2]Department of Obstetrics and Gynaecology, Guy's and St Thomas' Hospital Trust, Lambeth Palace Road, London SE1 7EH, UK

Summary To understand the chemokine network in a tissue, both chemokine and chemokine receptor expression should be studied. Human epithelial ovarian tumours express a range of chemokines but little is known about the expression and localisation of chemokine receptors. With the aim of understanding chemokine action in this cancer, we investigated receptors for CC–chemokines and their ligands in 25 biopsies of human ovarian cancer. CC–chemokine receptor mRNA was generally absent from solid tumours, the exception being CCR1 which was detected in samples from 75% of patients. CCR1 mRNA localised to macrophages and lymphocytes and there was a correlation between numbers of $CD8^+$ and CCR1 expressing cells ($P = 0.031$). mRNA for 6 CC-chemokines was expressed in a majority of tumour samples. In a monocytic cell line in vitro, we found that CCR1 mRNA expression was increased 5-fold by hypoxia. We suggest that the CC-chemokine network in ovarian cancer is controlled at the level of CC-chemokine receptors and this may account for the phenotypes of infiltrating cells found in these tumours. The leukocyte infiltrate may contribute to tumour growth and spread by providing growth survival factors and matrix metalloproteases. Thus, CCR1 may be a novel therapeutic target in ovarian cancer.

Keywords: chemokine; ovarian cancer; CCR1; hypoxia

Chemokines are chemoattractant cytokines, characterised by the presence of a conserved cysteine motif adjacent to the N-terminus. They are subdivided into 2 major groups: the CC chemokines and the CXC chemokines, in which the first 2 cysteine residues are either adjacent or separated by one amino acid, respectively (Clore and Gronenborn, 1995). Individual chemokines selectively attract leukocyte subsets through chemoattraction and by activating leukocyte integrins to bind their adhesion receptors on endothelial cells (Ebnet and Vestweber, 1999). The CXC chemokines act predominantly on neutrophils and T-lymphocytes, while the CC chemokines are active on various cell types, including monocytes and lymphocytes. Chemokines exert their effects by binding to 7 transmembrane domain G protein-coupled receptors. 11 receptors for the CC chemokines (CCR1-11) and 5 receptors for the CXC chemokines have been identified (Wang et al, 1998; Schweickart et al, 2000; Zlotnik and Yoshie, 2000). Their ligands bind to the extracellular N-terminus, leading to phosphorylation of serine/threonine residues on the cytoplasmic C-terminus, signalling and receptor desensitisation (Turner et al, 1998). There is apparent redundancy in the system; each receptor can respond to more than one chemokine; most chemokines can use more than one receptor, and each leukocyte subset may express several receptors. Yet the importance of individual chemokines is shown in transgenic and knockout mice (Gao et al, 1997; Kurihara et al, 1997; Lu et al, 1998). Specific chemokines are associated with distinct inflammatory infiltrates in a number of diseases, and chemokine antagonists have activity in experimental models (Takeya et al, 1993; Car et al, 1994; Baggiolini and Moser, 1997; Howard et al, 1999). However, in most tissues there is likely to be a complex chemokine/receptor network.

We have been studying the chemokine network in human epithelial ovarian cancer. This tumour microenvironment is a variable mixture of epithelial tumour cells, stromal fibroblasts, endothelial cells and infiltrating leukocytes, which are mainly $CD68^+$ macrophages and $CD8^+$ lymphocytes (Negus et al, 1997). The cytokine context of these tumours generally comprises inflammatory cytokines, growth factors and chemokines, but there is a lack of lymphocyte-associated cytokines (Burke et al, 1996).

The presence of macrophages and lymphocytes in this tumour microenvironment is related to local production of chemokines (Bottazzi et al, 1985; Yoong et al, 1999). We reported a relationship between lymphocyte and macrophage counts in ovarian tumours and mRNA expression of the CC chemokine CCL2 (MCP-1) by tumour cells and macrophages (Negus et al, 1997). The cytokine TNF-α, also present in the epithelial tumour islands may regulate this CCL2 production (Negus et al, 1995). We also found that mRNA for other CC chemokines, CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES), localised to leukocytes in the tumour (Negus et al, 1997), suggesting that a chemokine network existed. However, it was not clear how this network was controlled. Changes in the profile of chemokine receptors expressed by individual cells can inhibit cell migration or change their path. Thus, to understand the chemokine network in a tissue, both chemokine and chemokine receptor expression must be studied.

In this paper we have compared CC chemokine receptor mRNA expression in solid tumours with the expression of CC chemokine mRNA. In spite of abundant CC chemokine expression in ovarian tumours, we have found that CC chemokine receptor expression is weak or absent in the solid tumour microenvironment. CCR1

Received 19 March 2001
Revised 6 June 2001
Accepted 11 June 2001

Correspondence to: F Balkwill

(whose ligands include CCL3, CCL4, CCL5 and CCL7 (MCP-3)) was the only chemokine receptor consistently expressed in the solid tumours. This receptor localised to infiltrating $CD68^+$ macrophages and $CD8^+$ lymphocytes. There was no evidence that the CC chemokine receptors studied were expressed by epithelial tumour cells. The microenvironment of solid tumours may down-regulate expression of some chemokine receptors and we present preliminary evidence that hypoxia, which is a common feature of solid tumours (Vaupel et al, 1998), may up-regulate CCR1.

MATERIALS AND METHODS

Samples

25 biopsies from human ovarian tumours were obtained at operation and snap frozen into liquid nitrogen. These were classified as serous adenocarcinoma (19), clear cell carcinoma (2), mucinous adenocarcinoma (1), anaplastic carcinoma (1), signet ring carcinoma (1) and endometrioid carcinoma (1). Sections for in situ hybridisation were mounted on baked glass slides coated with 3-aminopropyl-triethoxy-silane, air dried, and stored at –70°C. Serial sections were cut onto poly-L-lysine-coated slides and stored at -70°C. Control peripheral blood mononuclear cells (PBMC) were isolated from heparinised venous blood or cytophoresis buffy coats from normal volunteers by Ficoll-Hypaque density centrifugation (Lymphoprep™, Nycomed, Birmingham, UK).

RNA extraction and RT-PCR

Total RNA was prepared from all samples using Tri Reagent™ (Sigma, Poole, UK). Solid tumour biopsies were homogenised in Tri Reagent™ using an Ultra-turrax T25 tissue homogeniser (Janke & Kunkel, Staufen, Germany). For RT-PCR and RNase protection analysis, total RNA was DNase-treated to remove contaminating genomic DNA, using RNase-free DNase I (Pharmacia Biotech, St Albans, UK). cDNA was synthesised from DNase-treated total RNA using the Ready-to-Go™ T-primed First Strand kit (Pharmacia Biotech, UK). The primers for CCL2, CCL22 (MDC) and CCR1 were designed from sequences submitted to Genbank, using Primer 3.0. CCL3 and CCL4, CCL5 and CCL8 primer sequences were from (Hosaka et al, 1994), (Mattei et al, 1994) and (Van Coillie et al, 1997) respectively. The primer sequences and product sizes for CCL2, CCL22 and CCR1 are:

CCL2	For	CAAACTGAAGCTCGCACTCTCGCC
	Rev	ATTCTTGGGTTGTGGAGTGAGTGTTCA
	Product = 354 bp	
CCL22	For	CCCTACCTCCCTGCCATTAT
	Rev	CAGGGAGCTAGAACCCAACA
	Product = 338 bp	
CCR1	For	AAAGCCTACGAGAGTGGAAGC
	Rev	AGAGGAAGGGGAGCCATTTA
	Product = 426 bp	

25 µl volume per sample was used, containing 200 ng cDNA, 1 U AmpliTaq DNA polymerase, GeneAmp PCR buffer, GeneAmp dNTPs (all from Perkin Elmer, Beaconsfield, UK) and 4µM each primer. The following protocol was used in a GeneAmp® PCR System 9700 thermal cycler: 94°C (5 min); 35 cycles 94°C (30 s), 60°C (30 s), 72°C (30 s); 72°C (7 min). PCR products were electrophoresed through 1.2% agarose gel and visualised by ethidium bromide. 123 bp markers (Gibco BRL, Paisley, UK) were used to estimate band sizes. PCR products were gel extracted and sequenced to confirm their identity.

RNase protection assay (RPA)

The hCR5 template set from Pharmingen (Becton Dickinson, Oxford, UK) contained DNA templates for CCR1, CCR2, CCR2a, CCR2b, CCR3, CCR4, CCR5, CCR8, GAPDH and L32. RPA was carried out using [α^{35}S]UTP (Amersham International plc, Aylesbury, UK) instead of [α^{32}P]UTP. The RNase-protected fragments were run out on an acrylamide-urea sequencing gel (BioRad Laboratories Ltd, Hemel Hempstead, UK), which was then adsorbed to filter paper and dried under vacuum. Autoradiography was subsequently carried out using Kodak Biomax MS film with a Transcreen LE-intensifying screen (Sigma).

In situ hybridisation (ISH)

[α^{35}S]UTP-labelled antisense and sense riboprobes were generated from 1100 bp fragments of CCR1 and CCR4 cDNA cloned in pcDNA1 (Stratagene, Cambridge, UK), using Sp6 and T7 RNA polymerases (Promega Ltd, Southampton, UK). These cDNAs were a kind gift from Antonio Sica (Mario Negri Institute, Milan, Italy). Antisense β-actin was used as a positive control in all experiments. In situ hybridisation was carried out using the method described in (Naylor et al, 1990). Image capture was with Image Grabber PCI (Neotech Ltd, London, UK).

Immunohistochemistry

Cryostat sections were fixed in 4% paraformaldehyde in PBS for 5 minutes. Sections were preincubated with normal rabbit serum (DAKO, Ely, UK) at a 1/25 dilution, for 15 minutes before application of the primary antibody. Sections were then incubated for 30 minutes at room temperature with the anti-CD8 mAb DK25 (DAKO) diluted 1/100; then biotinylated rabbit anti-mouse IgG and avidin-biotin-peroxidase complex (both DAKO). The final incubation was with the chromogen 3,3′-diaminobenzidine tetrahyrdochloride. Toluidine blue was used as the counterstain.

Hypoxic culture

2×10^6 THP-1 cells (purchased from the American Type Culture Collection, Rockville, MD) were cultured in 2 ml serum-free RPMI 1640 supplemented with 0.1 % BSA and 50 µM β-mercaptoethanol, in each well of a 6-well plate. The cells were then incubated for up to 24 h at 37°C under normoxia (5% CO_2, in air) or hypoxia (gassed with 5% CO_2, balanced N_2 until $< 0.1\%$ O_2, unpublished data) in a modular incubation chamber.

Northern analysis

Total RNA (15 µg) was run on a 1% agarose-formaldehyde gel as described in (Turner et al, 1999). Densitometric analysis was carried out using NIH Image 1.61.

Cell counting and statistics

The CCR1 expressing cells in 15 High Power Fields (HPF) were compared with the number of $CD8^+$ cells in 15 HPF in the serial section. 15 HPF corresponded to a total tumour area of 1.095 mm^2,

so the counts were expressed as cells mm^{-2}. As the data were not normally distributed, the nonparametric Spearman's rank correlation was used to calculate *P* values (Altman, 1991).

RESULTS

Chemokine receptor mRNA expression in ovarian epithelial tumours

25 biopsies from human epithelial ovarian cancer were analysed for chemokine receptor mRNA expression by RPA. RPA was performed with a template set containing probes for CCR1, 2, 2a, 2b, 3, 4, 5, 8, and the 'housekeeping' genes *GAPDH* and *L32*. All the CC chemokine receptor mRNAs were expressed by a control PBMC preparation (Figure 1A). In contrast, CC chemokine receptor mRNA was weakly expressed in the solid tumour biopsies. CCR1 was the only CC chemokine receptor present in the majority of the samples, with 75% of the biopsies positive for this receptor mRNA. Less than 15% of the biopsies were positive for the remaining CC chemokine receptor mRNAs (Figure 1A and B).

Leukocytes are likely to be the main source of chemokine receptor mRNA in the tumours and their mRNA would have been diluted by other cells in the tumour microenvironment. To confirm the RPA results, therefore, the more sensitive technique of RT-PCR was used on RNA from the same biopsies. No mRNA was detected for CCR2, 2a, 2b, 3 or 5 in samples that were negative by RPA. CCR1 was detected in those samples positive by RPA (Figure 1C). 72% of the samples also gave a positive signal for CCR4 although these were negative by RPA (Figure 1C).

Characterisation of chemokine receptor mRNA expressing cells in ovarian tumour biopsies

We used in situ hybridisation to mRNA on frozen sections from 11 biopsies to localise CCR1 and CCR4 expression. 9 of the 11 biopsies were positive for CCR1 by RPA and CCR1 could be detected on cells in these biopsies by ISH. Numbers of CCR1 expressing cells ranged from 13.7-83.1 cells mm^{-2}, with a median 41.4 cells mm^{-2}. 2 of the biopsies used for ISH were negative by RPA, yet very occasional cells (mean 2.7 cells mm^{-2}) could be found that were positive for CCR1 mRNA, demonstrating the increased sensitivity of ISH compared with RPA. CCR1 mRNA localised mainly to clusters of cells in the stromal areas of the ovarian tumour biopsies and the distribution was consistent with expression by infiltrating cells (Figure 2). Epithelial tumour cells did not appear to express CCR1 mRNA. As described above, CCR4 mRNA was detected by RT-PCR but not by RPA in RNA from solid tumours. As might be expected, individual cells expressing CCR4 were rare (Figure 2). They were seen in 4/11 biopsies, with only 2-3 positive cells detected in the entire section. This demonstrates the extreme sensitivity of RT-PCR, but suggests that RPA and ISH give more meaningful data on chemokine receptor mRNA expression in tissue samples. Thus a combination of techniques for measuring RNA suggests that expression of CCR 2, 3, 5 and 8 in the solid tumour microenvironment is extremely low both in terms of RNA and number of expressing cells, although cells that might be expected to express these receptors are present.

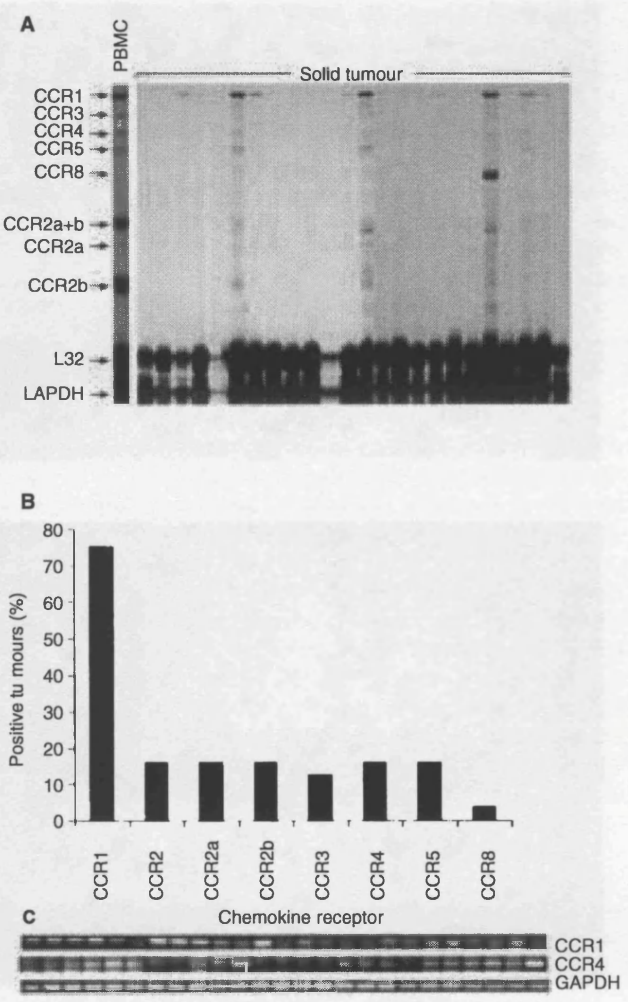


Figure 1 (**A**) RNase protection assay of CC chemokine receptor expression in normal PBMC and solid human ovarian tumour biopsies. (**B**) Percentage of samples expressing CC chemokine receptor mRNA in solid human ovarian tumours, using the data derived from the RPA shown in Figure 1 A. Only CCR1 is expressed by the majority of solid tumours. (**C**) The presence of CCR1 and CCR4 mRNA in the solid tumours was confirmed by RT-PCR. Amplification of GAPDH is shown as a loading control

Correlation of CCR1 expression in solid tumours with CD8 lymphocytes

In a previous publication, we characterised the infiltrating leukocytes in ovarian cancer as $CD3^+/CD8^+/CD45RO^+$ lymphocytes and $CD68^+$ macrophages (Negus et al, 1997). In this study, we found that many of these lymphocytes express CCR1. Immunohistochemistry for CD8 and ISH to CCR1 mRNA was performed on sequential sections. CCR1 expression was often seen at the same location as $CD8^+$ T cells in the sequential section (Figure 3). The number of cells expressing CCR1 was counted in 15 HPF, corresponding to a total tumour area of 1.095 mm^2. Similarly, the number of cells expressing CD8 was counted in 15 HPF in the sequential section; only those cells with obvious nuclei and good cytoplasmic staining were scored. A possible correlation was found between the number of cells expressing CCR1 and the number of infiltrating $CD8^+$ T-cells in individual

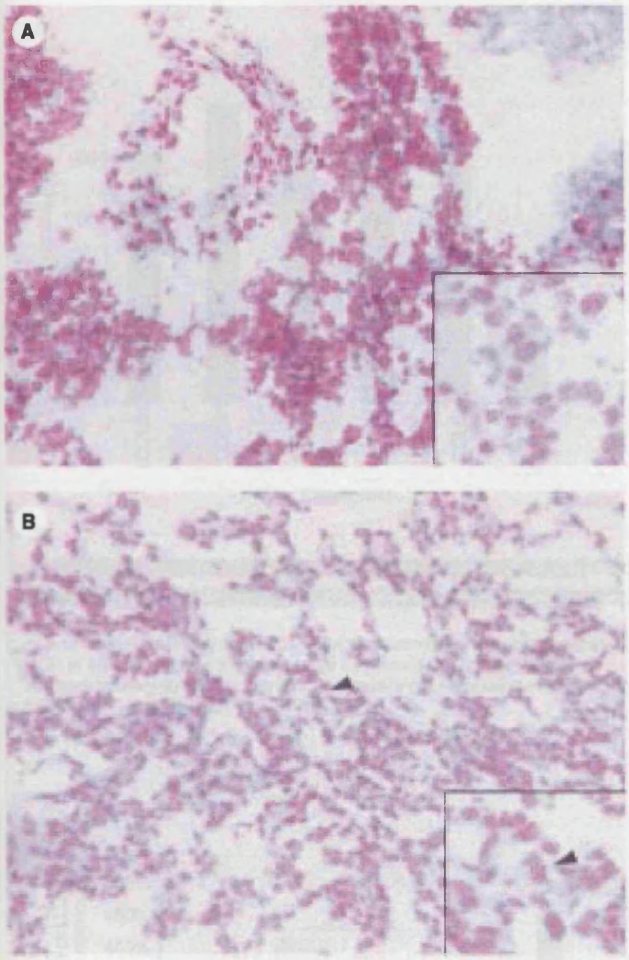


Figure 2 Localisation of CCR1 and CCR4 mRNA by in situ hybridisation. The figure shows a cluster of cells expressing CCR1, ×400 (**A**); a cluster of cells expressing CCR1, ×1000 (inset); a single cell expressing CCR4, ×400 (**B**) and the same cell ×1000 (inset)

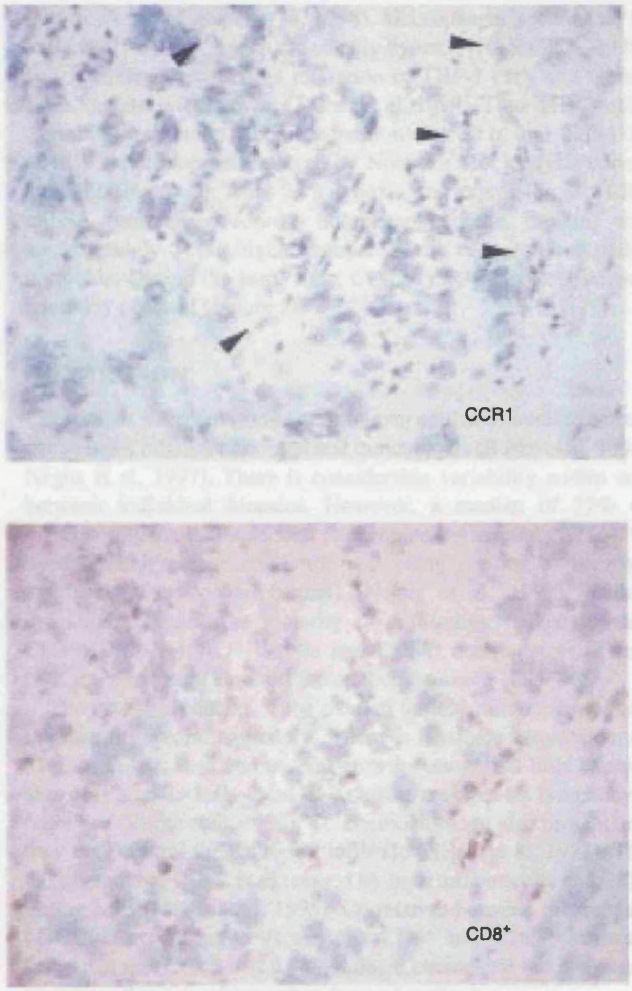


Figure 3 Co-localisation of CCR1 expression and CD8 expression in the same field of view in serial sections (×400). Arrows indicate cells expressing CCR1

tumour sections ($r_s = 0.682$; $P = 0.031$). A proportion of the cells expressing CCR1 also had the morphological characteristics of macrophages. Immunohistochemistry to CD68 was not of sufficient quality to obtain statistical correlation with the ISH results but there were examples of concordance between CD68 positivity and CCR1 expression on the sequential sections (data not shown).

A range of CC chemokine mRNA is detected in ovarian tumours

We used RT-PCR to screen for 6 of the CC chemokines (CCL2, CCL3, CCL4, CCL5, CCL8 and CCL22) that bind to the receptors studied above, in the same biopsy samples. CCL2, CCL3, CCL4, CCL5 and CCL8, were detected in more than 80% of the solid tumour samples, while CCL22 was detected in 6/25 samples (Figure 4). This agrees with previous work from our laboratory where CCL2, 3, 4 and 5 were detected by ISH (Negus et al, 1997). Thus, a range of chemokine mRNA are expressed, despite the variability in mRNA expression of chemokine receptors, suggesting that their action may be controlled at the level of the receptor.

Regulation of CCR1 expression in the tumour microenvironment

A number of agents regulate CCR1 such as LPS (Sica et al, 1997), and the cytokines IL-12, IFN-γ and IFN-α (Bonecchi et al, 1999; Colantonio et al, 1999; Zella et al, 1999). However, these cytokines have not been reported in ovarian tumours (Burke et al, 1996). TNF-α is a predominant cytokine in the ovarian tumour microenvironment (Naylor et al, 1993) and previous reports suggested that the presence of this pro-inflammatory cytokine might down-regulate the CCL2 receptor, CCR2b (Sica et al, 2000). To study the in vitro effect of this cytokine on CCR1, we used the monocytic cell line THP-1 which expresses both CCR1 and CCR2b mRNA. Treatment of THP-1 cells with 1 and 10 ng ml^{-1} TNF-α did not influence CCR1 mRNA expression (data not shown); 100 ng ml^{-1} was toxic to the cells. Another environmental factor likely to influence cell behaviour is intratumoural oxygen tension. We therefore studied the influence of hypoxia on CCR1 and CCR2b expression.

The regulation of CC chemokine receptors by hypoxia

Regions of hypoxia are common in solid tumours due to the chaotic and intermittent blood supply, and the high metabolic rate

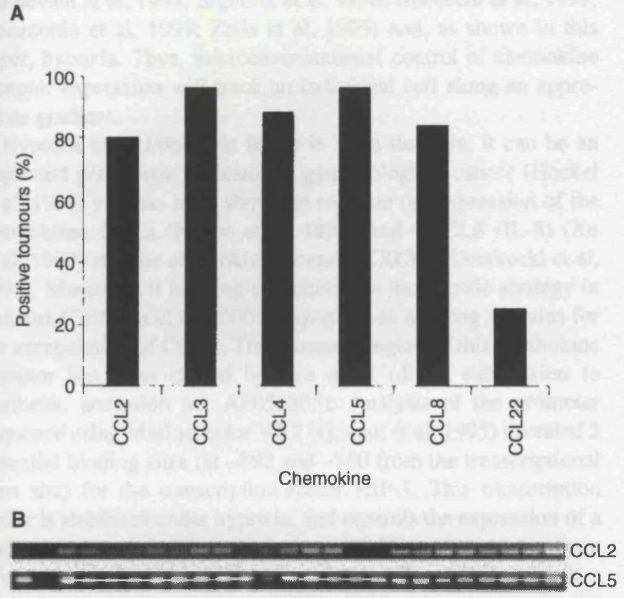


Figure 4 The mRNA expression of 6 CC chemokines was assessed by RT-PCR in 25 solid human ovarian tumour biopsies. (**A**) The percentage of samples positive for each chemokine is shown. (**B**) Representative gels for CCL2 and CCL5 RT-PCR

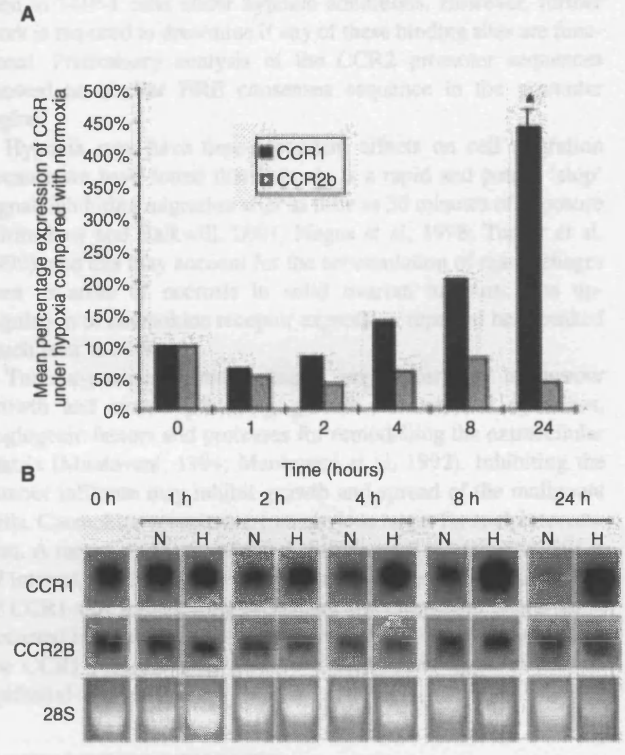


Figure 5 Northern analysis of CCR1 and CCR2b mRNA expression in THP-1 cells. THP-1 cells were cultured under hypoxic conditions for 24 h. (**A**) The expression under hypoxia is shown as a percentage of the expression under normoxia, normalised for the housekeeping gene b-actin. *24 h timepoint includes the SEM (n = 6); all other timepoints were representative of 2 experiments. (**B**) Representative Northern blots for CCR1 and CCR2b showing mRNA expression at each timepoint, with the 28S band from the ethidium bromide stained gel shown as a loading control. N = normoxia; H = hypoxia

of tumour cells (Vaupel et al, 1998). Macrophages accumulate in regions of necrosis, which are usually hypoxic (Negus et al, 1998) and hypoxia can affect the migration of THP-1 cells and monocytes, but not lymphocytes (Turner et al, 1999). Thus THP-1 cells were cultured under hypoxic conditions for 24 h, and CCR1/2b mRNA expression was assayed by Northern blot analysis during this time (Figure 5). After 24 h hypoxia, the expression of CCR1 mRNA (normalised for the housekeeping gene, β-actin) was approximately 5-fold higher compared with cells cultured under normoxia. During the same time, CCR2b mRNA expression was relatively constant (Figure 5).

DISCUSSION

The tumour microenvironment of human epithelial ovarian cancer comprises a mixture of normal and tumour cells (Burke et al, 1996; Negus et al, 1997). There is considerable variability within and between individual biopsies. However, a median of 37% of the area of each tissue section is occupied by stromal cells, 43% by epithelial tumour areas, the rest being regions of necrosis and space (real or artefactual) (Negus et al, 1997). Within the stromal areas, the majority of infiltrating leukocytes are $CD3^+/CD8^+/CD45RO^+$ T cells and $CD68^+$ macrophages. These cells are also found amongst the epithelial tumour cells.

The cytokine context of the ovarian tumour microenvironment is generally proinflammatory. There is frequent expression of TNF-α, IL-1α, IL-6 and several growth factors, but little expression of IFN-γ, IL-2, IL-4 and IL-7 that are required for lymphocyte functions (Burke et al, 1996). CC chemokines are also present and they may control the leukocyte infiltrate (Negus et al, 1997). The CC chemokine CCL2 is expressed by both tumour cells and infiltrating cells (Negus et al, 1995). Correlation between the number of CCL2 expressing cells and the $CD8^+$ and $CD68^+$ infiltrate suggested that CCL2 was a predominant chemokine but it was not clear how the chemokine network was functioning.

In contrast to our findings with CC chemokines, receptor expression is limited in solid tumours, with CCR1 predominating. CCR1 mRNA expression correlated with the $CD8^+$ infiltrate, and CCR1 mRNA also appeared to be expressed by macrophages. CCL2 is not a ligand for this receptor and the major receptor for CCL2, CCR2b, was not detected in the solid tumours, as has previously been reported (Sica et al, 2000). CCR2 may be down-regulated by proinflammatory cytokines present in solid tumours (Sica et al, 1997). We did not find any expression of CC chemokine receptors on ovarian tumour cells, or ovarian cancer cell lines.

The present study demonstrates the importance of studying both chemokines and their receptors in a tissue and leads us to suggest that the CC chemokine network in solid tumours of ovarian cancer is controlled at the level of CC chemokine receptor. We suggest that CC chemokines such as CCL2 attract peripheral leukocytes into the tumour tissue but once there, they lose the ability to respond to this and other CC chemokines because of receptor down-regulation. This traps cells in the tumour microenvironment and changes their chemokine response profile.

Control of the chemokine network by receptor expression makes sense. As a range of chemokines are expressed by the tumours there will be conflicting chemoattractant gradients that an individual cell can follow. These gradients may be difficult to regulate with chemokines being retained by the extracellular matrix. Chemokine receptor expression, in contrast, can be rapidly modulated by LPS, chemokines, cytokines (Sica et al, 1997;

Mantovani et al, 1998; Signoret et al, 1998; Bonecchi et al, 1999; Colantonio et al, 1999; Zella et al, 1999) and, as shown in this paper, hypoxia. Thus, microenvironmental control of chemokine receptor expression will track an individual cell along an appropriate gradient.

Hypoxia is an important factor in solid tumours; it can be an important prognostic indicator in gynaecological cancer (Hockel et al, 1998) and has been shown to regulate the expression of the chemokines CCL2 (Negus et al, 1998) and CXCL8 (IL-8) (Xu et al, 1999) and the chemokine receptor CXCR1 (Grutkoski et al, 1999). Moreover, it is being exploited as a therapeutic strategy in tumours (Griffiths et al, 2000). Hypoxia was a strong stimulus for the upregulation of CCR1. The promoter region of this chemokine receptor has been cloned by Lee et al (direct submission to Genbank, accession no. AF051305). Analysis of the promoter sequence using MatInspector V2.2 (Quandt et al, 1995) revealed 2 potential binding sites (at –892 and –760 from the transcriptional start site) for the transcription factor HIF-1. This transcription factor is stabilised under hypoxia, and controls the expression of a number of target genes including VEGF and erythropoietin (Forsythe et al, 1996; Maxwell and Ratcliffe, 1998) through a hypoxia responsive element (HRE). HREs always contain the sequence RCGTG (where R is a purine) which is critical for HIF-1 binding; the flanking residues are also important, but no strong consensus has been observed. The presence of potential HIF-1-binding sites suggests that CCR1 could be a target for transcriptional regulation by HIF-1, and could account for the upregulation seen in THP-1 cells under hypoxic conditions. However, further work is required to determine if any of these binding sites are functional. Preliminary analysis of the CCR2 promoter sequences showed no similar HRE consensus sequence in the promoter region.

Hypoxia may have time-dependent effects on cell migration because we have found that hypoxia is a rapid and potent 'stop' signal, inhibiting migration after as little as 30 minutes of exposure (Grimshaw and Balkwill, 2001; Negus et al, 1998; Turner et al, 1999), and this may account for the accumulation of macrophages seen in areas of necrosis in solid ovarian tumours. The upregulation of chemokine receptor expression reported here peaked much later at 24 hours.

Tumour-associated macrophages may contribute to tumour growth and spread providing growth and survival cytokines, angiogenic factors and proteases for remodelling the extracellular matrix (Mantovani, 1994; Mantovani et al, 1992). Inhibiting the tumour infiltrate may inhibit growth and spread of the malignant cells. Chemokine receptors are an obvious target for such intervention. A recent study of acute and chronic graft rejection models is of interest. Graft survival in mice with a targeted gene disruption of CCR1 was significantly prolonged and permanent engraftment occurred in some of these mice (Gao et al, 2000). We propose that the CCR1 receptor may also be a therapeutic target in human epithelial ovarian cancer.

ACKNOWLEDGEMENTS

We would like to thank Frances Burke and Matt Grimshaw from our laboratory for help with in situ hybridisation and analysis of the CCR1 promoter; and George Elia and other members of the ICRF histopathology lab.

REFERENCES

Altman DG (1991) *Practical statistics for medical research*. Chapman and Hall: London

Baggiolini M and Moser B (1997) Blocking chemokine receptors. *J Exp Med* **186**: 1189-1191

Bonecchi R, Polentarutti N, Luini W, Borsatti A, Bernasconi S, Locati M, Power C, Proudfoot A, Wells TNC, Mackay C, Mantovani A and Sozzani S (1999) Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-gamma in human neutrophils. *J Immunol* **162**: 474-479

Bottazzi B, Ghezzi P, Taraboletti G, Salmona M, Colombo N, Bonazzi C, Mangioni C and Mantovani A (1985) Tumor-derived chemotactic factor(s) from human ovarian carcinoma: evidence for a role in the regulation of macrophage content of neoplastic tissues. *Int J Cancer* **36**: 167-173

Burke F, Relf M, Negus R and Balkwill F (1996) A cytokine profile of normal and malignant ovary. *Cytokine* **8**: 578-585

Car BD, Meloni F, Luisetti M, Semenzato G, Gialdroni-Grassi G and Walz A (1994) Elevated IL-8 and MCP-1 in the bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Am J Respir Crit Care Med* **149**: 655-659

Clore GM and Gronenborn AM (1995) Three-dimensional structures of alpha and beta chemokines. *Faseb J* **9**: 57-62

Colantonio L, Iellem A, Clissi B, Pardi R, Rogge L, Sinigaglia F and Dambrosio D (1999) Upregulation of integrin alpha 6/beta 1 and chemokine receptor CCR1 by interleukin-12 promotes the migration of human type 1 helper T cells. *Blood* **94**: 2981-2989

Ebnet K and Vestweber D (1999) Molecular mechanisms that control leukocyte extravasation: the selectins and the chemokines. *Histochem Cell Biol* **112**: 1-23

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD and Semenza GL (1996) Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. *Mol-Cell-Biol* **16**: 4604-4613

Gao JL, Wynn TA, Chang Y, Lee EJ, Broxmeyer HE, Cooper S, Tiffany HL, Westphal H, Kwon-Chung J and Murphy PM (1997) Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J Exp Med* **185**: 1959-1968

Gao W, Topham PS, King JA, Smiley ST, Csizmadia V, Lu B, Gerard CJ and Hancock WW (2000) Targeting of the chemokine receptor CCR1 suppresses development of acute and chronic cardiac allograft rejection. *J Clin Invest* **105**: 35-44

Griffiths L, Binley K, Iqball S, Kan O, Maxwell P, Ratcliffe P, Lewis C, Harris A, Kingsman S and Naylor S (2000) The macrophage-a novel system to deliver gene therapy to pathological hypoxia. *Gene Ther* **7**: 255-262

Grimshaw MJ and Balkwill FR (2001) Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation-a potential mechanism. *Eur J Immunol* **31**: 480-489

Grutkoski PS, Graeber CT, Damico R, Keeping H and Simms HH (1999) Regulation of IL-8RA (CXCR1) expression in polymorphonuclear leukocytes by hypoxia/reoxygenation. *J Leukoc Biol* **65**: 171-178

Hockel M, Schlenger K, Hockel S, Aral B, Schaffer U and Vaupel P (1998) Tumor hypoxia in pelvic recurrences of cervical cancer. *Int J Cancer* **79**: 365-369

Hosaka S, Akahoshi T, Wada C and Kondo H (1994) Expression of the chemokine superfamily in rheumatoid arthritis. *Clin Exp Immunol* **97**: 451-457

Howard OMZ, Oppenheim JJ and Wang JM (1999) Chemokines as molecular targets for therapeutic intervention. *J Clin Immunol* **19**: 280-292

Kurihara T, Warr G, Loy J and Bravo R (1997) Defects in macrophage recruitment and host defense in mice lacking the CCR2 chemokine receptor. *J Exp Med* **186**: 1757-1762

Lu B, Rutledge BJ, Gu L Fiorillo J, Lukacs NW, Kunkel SL, North R, Gerard C and Rollins BJ (1998) Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp Med* **187**: 601-608

Mantovani A (1994) Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. *Lab Invest* **71**: 5-16

Mantovani A, Bottazzi B, Colotta F, Sozzani S and Ruco L (1992) The origin and function of tumor-associated macrophages. *Immunol Today*, **13**: 265-270

Mantovani A, Allavena P, Vecchi A and Sozzani S (1998) Chemokines and chemokine receptors during activation and deactivation of monocytes and dendritic cells and in amplification of th1 versus th2 responses. *Int J Clin Lab Res* **28**: 77-82

Mattei S, Colombo MP, Melani C, Silvani A, Parmiani G and Herlyn M (1994) Expression of cytokine/growth factors and their receptors in human melanoma and melanocytes. *Int J Cancer* **56**: 853-857

Maxwell P and Ratcliffe P (1998) Regulation of expression of the erythropoietin gene. *Curr Opin Hematol* **5**: 166-170

Naylor MS, Stamp GW and Balkwill FR (1990) Investigation of cytokine gene expression in human colorectal cancer. *Cancer Res* **50**: 4436-4440

Naylor MS, Stamp GW, Foulkes WD, Eccles D and Balkwill FR (1993) Tumor necrosis factor and its receptors in human ovarian cancer. Potential role in disease progression. *J Clin Invest* **91**: 2194-2206

Negus RP, Stamp GW, Relf MG, Burke F, Malik ST, Bernasconi S, Allavena P, Sozzani S, Mantovani A and Balkwill FR (1995) The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *Journal of Clinical Investigation* **95**: 2391-2396

Negus RP, Stamp GW, Hadley J and Balkwill FR (1997) Quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. *Am J Pathol* **150**: 1723-1734

Negus RP, Turner L, Burke F and Balkwill FR (1998) Hypoxia down-regulates MCP-1 expression: implications for macrophage distribution in tumors. *J Leukoc Biol* **63**: 758-765

Quandt K, Frech K, Karas H, Wingender E and Werner T (1995) MatInd and MatInspector: new fast and versatile tools for detection of consensus matches in nucleotide sequence data. *Nucleic Acids Res* **23**: 4878-4884

Schweickart VL, Epp A, Raport CJ and Gray PW (2000) CCR11 is a functional receptor for the monocyte chemoattractant protein family of chemokines. *J Biol Chem* **275**: 9550-9556

Sica A, Saccani A, Borsatti A, Power CA, Wells TN, Luini W, Polentarutti N, Sozzani S and Mantovani A (1997) Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *Journal of Experimental Medicine*, **185**: 969-974

Sica A, Saccani A, Bottazzi B, Bernasconi S, Allavena P, Gaetano B, Fei F, LaRosa G, Scotton C, Balkwill F and Mantovani A (2000) Defective Expression of the Monocyte Chemotactic Protein-1 Receptor CCR2 in Macrophages Associated with Human Ovarian Carcinoma. *J Immunol* **164**: 733-738

Signoret N, Rosenkilde MM, Klasse PJ, Schwartz TW, Malim MH, Hoxie JA and Marsh M (1998) Differential regulation of cxcr4 and ccr5 endocytosis. *J Cell Sci* **111**: 2819-2830

Takeya M, Yoshimura T, Leonard EJ and Takahashi K (1993) Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. *Hum Pathol* **24**: 534-539

Turner L, Scotton C, Negus R and Balkwill F (1999) Hypoxia inhibits macrophage migration. *Eur J Immunol* **29**: 2280-2287

Turner SJ, Domin J, Waterfield MD, Ward SG and Westwick J (1998) The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II P13K-C2alpha. *J Biol Chem* **273**: 25987-25995

Van Coillie E, Froyen G, Nomiyama H, Miura R, Fiten P, Van Aelst I, Van Damme J and Opdenakker G (1997) Human monocyte chemotactic protein-2: cDNA cloning and regulated expression of mRNA in mesenchymal cells. *Biochem Biophys Res Commun* **231**: 726-730

Vaupel P, Thews O, Kelleher DK and Hoeckel M (1998) Current status of knowledge and critical issues in tumor oxygenation. Results from 25 years research in tumor pathophysiology. *Adv Exp Med Biol* **454**: 591-602

Wang JM, Deng XY, Gong WH and Su SB (1998) Chemokines and their role in tumor-growth and metastasis. *J Immunol Methods* **220**: 1-17

Xu L, Xie K, Mukaida N, Matsushima K and Fidler IJ (1999) Hypoxia-induced elevation in interleukin-8 expression by human ovarian carcinoma cells. *Cancer Res* **59**: 5822-5829

Yoong KF, Afford SC, Jones R, Aujla P, Qin S, Price K, Hubscher SG and Adams DH (1999) Expression and function of CXC and CC chemokines in human malignant liver tumors: a role for human monokine induced by gamma-interferon in lymphocyte recruitment to hepatocellular carcinoma. *Hepatology* **30**: 100-111

Zella D, Barabitskaja O, Casareto L, Romerio F, Secchiero P, Reitz MS, Gallo RC and Weichold FF (1999) Recombinant IFN-alpha (2b) increases the expression of apoptosis receptor CD95 and chemokine receptors CCR1 and CCR3 in monocytoid cells. *J Immunol* **163**: 3169-3175

Zlotnik A and Yoshie O (2000) Chemokines: A new classification system and their role in immunity. *Immunity* **12**: 121-127

[CANCER RESEARCH 61, 4961–4965, July 1, 2001]

Epithelial Cancer Cell Migration: A Role for Chemokine Receptors?

Chris J. Scotton, Julia L. Wilson, David Milliken, Gordon Stamp, and Frances R. Balkwill[1]

Imperial Cancer Research Fund Translational Oncology Laboratory, Barts and the Royal London School of Medicine and Dentistry, London EC1M 6BQ, United Kingdom [C. J. S., J. L. W., D. M., F. R. B.], and Department of Histopathology, Division of Investigative Sciences, Imperial College School of Medicine, Hammersmith Hospital Campus, London W12 ONN, United Kingdom [G. S.]

ABSTRACT

We investigated the possibility that chemokine gradients influence migration of human ovarian epithelial tumor cells. Of 14 chemokine receptors investigated, only CXCR4 was expressed on ovarian cancer cells. CXCR4 mRNA localized to a subpopulation of tumor cells in ovarian cancer biopsies. Ovarian cancer cell lines and cells freshly isolated from ascites expressed CXCR4 protein. The CXCR4 ligand, CXCL12, was found in ascites from 63 patients. CXCL12 elicited intracellular calcium flux and directed migration and changes in integrin expression in ovarian cancer cells. CXCR4 may influence cell migration in the peritoneum, a major route for ovarian cancer spread, and could be a therapeutic target.

INTRODUCTION

Chemokines are small, secreted peptides that control the migration of leukocytes, especially during immune and inflammatory reactions (1, 2). They are divided into two major subfamilies, CC and CXC, based on the position of their NH_2-terminal cysteine residues, and bind to G protein-coupled receptors, whose two major subfamilies are designated CCR and CXCR. Chemokines are also produced by most, if not all, cancers. There is strong evidence that they are major determinants of the macrophage and lymphocyte infiltrate found in melanomas; in carcinomas of the ovary, breast, and cervix; and in sarcomas and gliomas (3–5). However, chemokines may play other roles in cancer. Some are potent angiogenic factors, whereas others can be angiostatic (1, 6). Alterations in the balance between these chemokines may contribute to the development of the tumor vasculature.

Using human epithelial ovarian cancer as an example, we have investigated another role for chemokines in cancer: the possibility that the malignant cells in epithelial tumors may use chemokine gradients as part of the process of metastatic spread. Only 1 of 14 chemokine receptors investigated was expressed on the ovarian tumor cells. We present evidence that interaction of this chemokine receptor, CXCR4, with its ligand, CXCL12, may influence the spread of epithelial ovarian cancer. This observation has implications for the development of new biological therapies for cancer.

MATERIALS AND METHODS

Samples. Human ovarian tumor biopsies were frozen in liquid nitrogen. Sections for *in situ* hybridization were mounted on baked glass slides coated with 3-aminopropyl-triethoxy-silane, air-dried, and stored at −70°C. Red cells were removed from ascites with ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2 EDTA).

Cell Lines. Ovarian cancer cell lines PEO1 and PEO14 (from S. Langdon, Imperial Cancer Research Fund Oncology Unit, Edinburgh, United Kingdom), OVCAR-3 (purchased from American Type Culture Collection), and IGROV (from J. Bénard) were grown in RPMI 1640 supplemented with 10% FCS and 10 μg/ml insulin (for PEO1 and PEO14); SKOV-3 and CAOV-3 (purchased from American Type Culture Collection) were grown in DMEM supplemented with 10% FCS. Cell lines were cultured in pyrogen-free conditions. To allow recovery of cells after trypsinization, cells were cultured as single cell suspensions in Teflon-coated pots (Tuf Tainer, Perbio Science UK Limited) at 0.5–1 × 10^6 cells/ml.

RNA Extraction and RPA.[2] Total RNA was prepared with Tri Reagent (Sigma Chemical Co., Poole, United Kingdom). Tumor biopsies were homogenized in Tri Reagent using an Ultra-turrax T25 (Janke & Kunkel, Staufen, Germany). Total RNA was treated with RNase-free DNase I (Pharmacia Biotech, St Albans, United Kingdom). RPA using Riboquant hCR5 and hCR6 template sets (BD PharMingen, Oxford, United Kingdom) was carried out using α-^{35}S-labeled UTP (Amersham International plc, Aylesbury, United Kingdom). RNase-protected fragments were run on an acrylamide-urea sequencing gel (Bio-Rad Laboratories Ltd., Hemel Hempstead, United Kingdom), adsorbed to filter paper, and dried under vacuum. Autoradiography was performed using Kodak Biomax MS film with a Transcreen LE intensifying screen (Sigma Chemical Co.).

***In Situ* Hybridization.** Antisense and sense riboprobes labeled with α-^{35}S-labeled UTP were generated from 1100-bp fragments of CXCR4 cDNA cloned in pcDNA3 (Stratagene, Cambridge, United Kingdom) using Sp6 and T7 RNA polymerases (Promega Ltd., Southampton, United Kingdom). The cDNA was a gift from Antonio Sica (Mario Negri Institute, Milan, Italy). Antisense β-actin was used as a positive control in all experiments. *In situ* hybridization was carried out using the method described in Ref. 5. Image capture was performed using Image Grabber PCI (Neotech Ltd., London, United Kingdom).

mAbs and Flow Cytometry. PE-labeled mAbs against CXCR4 (12G5; R&D Systems, United Kingdom), FITC-labeled Her2/Neu (Neu 24.7; BD PharMingen), and isotype-matched labeled controls were used. Unconjugated β_1 antibodies were a gift from Fiona Watt (Imperial Cancer Research Fund, London, United Kingdom). Cells were resuspended in PBS supplemented with 1% heat-inactivated FCS and 0.01% NaN_3. Antibodies diluted in this buffer were used at concentrations between 2 and 20 μg/ml and incubated for 30 min on ice. For unconjugated antibodies, cells were incubated with a 1:200 dilution of PE-conjugated polyclonal secondary antibody (BD PharMingen). After a final wash, labeled cells were fixed with 1% paraformaldehyde solution, and 10,000 cells were analyzed by flow cytometry on a FACScan flow cytometer using Cellquest software (BD PharMingen).

Calcium Flux. Five × 10^5 cells/ml were incubated with 5 μM Fluo-3 (Molecular Probes, Cambridge Biosciences, Cambridge, United Kingdom) in HBSS plus 0.5% BSA, 1 mM Ca^{2+}, 1 mM Mg^{2+}, and 10 μM Zn^{2+} for 30 min at 37°C and then washed. Fluorescence was measured in a PTI fluorometer (excitation wavelength, 485 nm; emission wavelength, 530 nm).

Migration. Chemotaxis was assayed using Falcon Transwells (24-well format, 8-μm pore; BD PharMingen). Media (0.5 ml) containing 5 × 10^5 cells were added to the upper chamber, and 0.5 ml of medium alone or media supplemented with CXCL12 were added to the lower chamber. After overnight incubation at 37°C and 5% CO_2, cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained using DiffQuik (Dade Behring, Düdingen, Switzerland). For each transwell, the number of migrated cells in 10 medium-power fields (×20) was counted.

ELISA for CXCL12 in Ascitic Fluid. Concentration of the chemokine CXCL12 (SDF-1α) in ascitic fluid was measured using Quantikine ELISA kits (R&D Systems, Abingdon, United Kingdom) according to the manufacturer's protocol. The sensitivity of the assay was 18 pg/ml.

Received 3/15/01; accepted 5/9/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom requests for reprints should be addressed, at Imperial Cancer Research Fund Translational Oncology Laboratory, Barts and the Royal London School of Medicine and Dentistry, Charterhouse Square, London, EC1M 6BQ, United Kingdom. Phone: 44-0-20-7882-6106; Fax: 44-0-20-7882-6110; E-mail: f.balkwill@icrf.icnet.uk.

[2] The abbreviations used are: RPA, RNase protection assay; mAb, monoclonal antibody; PE, phycoerythrin.

Statistical Analysis. The results of the migration assays were evaluated using Welch's approximate *t* test (which is a more stringent analysis than Student's *t* test).

RESULTS

Ovarian Tumor Cells Express CXCR4 mRNA. mRNA extracted from six ovarian cancer cell lines (SKOV-3, IGROV, OVCAR-3, CAOV-3, PEO1, and PEO14) was screened for chemokine receptor expression by RPA. The cells did not express CCR1, CCR2a, CCR2b, CCR3, CCR4, CCR5, CCR7, CCR8, CXCR1, CXCR2, CXCR3, CXCR5, or CX_3CR1, but two of the six cell lines (IGROV and CAOV-3) strongly expressed CXCR4 mRNA (Fig. 1, *A* and *B*). PEO1 and PEO14 also had detectable levels of CXCR4 mRNA (Fig. 1*A*). This chemokine receptor was also expressed in biopsies from 8 of 10 primary ovarian tumors and 19 of 20 samples of ovarian cancer ascites (Fig. 1*C*).

CXCR4 Localizes to Tumor and Stromal Cells in Tumor Biopsies. *In situ* hybridization localized CXCR4 mRNA to a proportion of neoplastic cells in 10 of 10 ovarian cancer biopsies studied (Fig. 2, *A–C*). Expression of CXCR4 mRNA was not uniform throughout the tumor. In five of the biopsies, between 5% and 20% of tumor cells were labeled, and in the other five biopsies, 1–5% of tumor cells were positive for CXCR4. There was no correlation between CXCR4 positivity and areas of necrosis or hot spots of angiogenesis. CXCR4 mRNA was also detected in mononuclear and endothelial cells. Tumors with a strong lymphoid infiltrate in the stroma showed highest mononuclear cell labeling for CXCR4 (Fig. 3, *A* and *B*). The presence and distribution of tumor-infiltrating lymphocytes were assessed by H&E staining and CD8 immunostaining, and the CXCR4 labeling pattern was clearly in excess of these populations in every sample. CD8 cells also showed a different distribution from CXCR4-labeled tumor cells. In addition, it was possible to distinguish between neoplastic and tumor-infiltrating lymphocyte cell nuclei. Some endothelial cells within the tumor were also labeled in each biopsy (Fig. 3, *C–F*). CXCR4-expressing endothelial cells were detected in fibrovascular cores of papillary tumors, adjacent to the epithelial tumor cells, or within stromal invaginations (Fig. 3, *C* and *D*). Endothelial cells in tissue adjacent to the tumors were not positive for CXCR4.

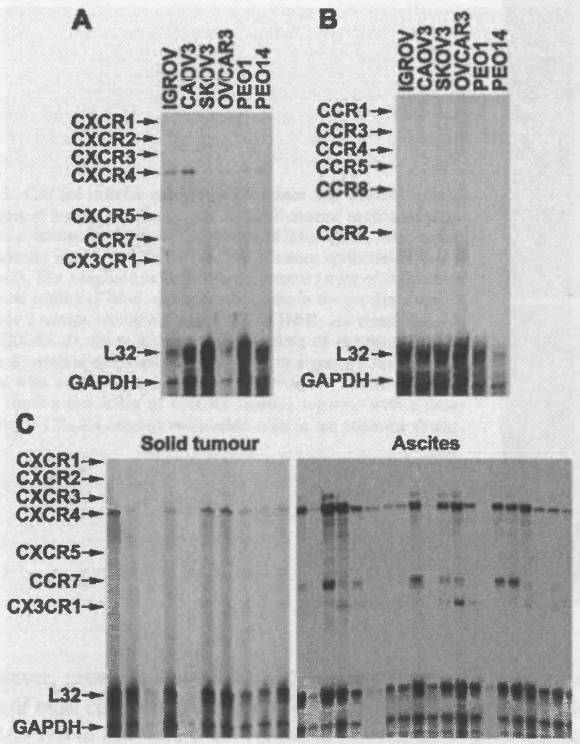


Fig. 1. Chemokine receptor mRNA expression in ovarian cancer. mRNA expression was measured by RPA. *GAPDH* and *L32* are housekeeping genes. *A* and *B*, chemokine receptor expression by ovarian cancer cell lines. *C*, chemokine receptor expression in primary solid tumor biopsies and cells isolated from ovarian cancer ascites.

CXCR4 Protein Can Be Detected on Ovarian Cancer Cells. As shown in Fig. 4*A*, CXCR4 protein could be detected on IGROV and CAOV-3 cells using flow cytometry. Two-color flow cytometric analysis of cells freshly isolated from ovarian cancer ascites revealed CXCR4 surface expression on cells that were also positive for the tumor marker HER2/neu (Fig. 4*B*).

The CXCR4 Receptor Is Functional on Ovarian Cancer Cells. CXCL12 (100 ng/ml) elicited an intracellular calcium flux in both IGROV and CAOV-3 cells (Fig. 4*C*). No release of intracellular calcium was detectable when cells were incubated with 10 ng/ml CXCL12. Both IGROV and CAOV-3 cells demonstrated chemotaxis toward CXCL12 (Fig. 4*D*).

Binding of CXCL12 to its receptor caused internalization of CXCR4 on IGROV cells after approximately 15 min, as determined by flow cytometry (Fig. 5*A*). The receptor was recycled to the cell surface within 30 min. CXCL12 induced significant migration of IGROV and CAOV-3 cells at concentrations of 100 ng/ml (IGROV, $P = 0.0001$; CAOV-3, $P = 0.0002$) and 300 ng/ml (IGROV, $P = 0.0001$; CAOV-3, $P = 0.0003$). Stimulation of CAOV-3 and IGROV cells with CXCL12 at 100 ng/ml for 24 h increased cell surface expression of the β_1 integrin (Fig. 5*B*).

Ascitic Fluid from Ovarian Cancer Contains High Levels of CXCL12. CXCL12 levels were assayed by ELISA in 63 samples of ascitic fluid from patients with ovarian cancer. High concentrations of this chemokine (range, 613-9333 pg/ml; median, 6021 pg/ml) were detected in all of the samples.

DISCUSSION

We provide evidence that chemokine gradients may influence routes of ovarian tumor cell migration. Response to chemokine gradients may be an important component of the multistep process of metastasis of epithelial tumors. Binding of ligand to the only chemokine receptor that was reproducibly expressed by human ovarian cancer cells (CXCR4) induces actions that would permit a role in tumor cell migration.

Only a proportion (between 1% and 20%) of the tumor cells in the ovarian cancer biopsies expressed CXCR4 mRNA. Receptor levels could be regulated by cytokines in the tumor microenvironment. For example, in T cells, CXCR4 can be up-regulated by interleukin 4 (7). We have preliminary evidence that ovarian cancer cell CXCR4 protein is up-regulated by transforming growth factor β, a cytokine that is found in ovarian cancer, but down-regulated by IFN-γ, which is absent from this tumor microenvironment (8). This could explain why a proportion of the tumor cells was positive at any one time. Alternatively, some cells may have acquired constitutive CXCR4 expression during malignant progression. In contrast, all of the HER2/neu-positive tumor cells that had spread into ascites were CXCR4 positive.

The ability of the ovarian cancer cell lines to elicit an intracellular calcium flux and migrate in response to CXCL12 indicates the functionality of the CXCR4 cell surface receptors. It is possible that the high levels of CXCL12 found in ascites could create a chemokine gradient for migration of tumor cells into the peritoneum. Treatment of the ovarian cancer cell lines with CXCL12 significantly increased cell surface expression of β_1 integrin, which may affect peritoneal adhesion of cells. The significance of this remains to be determined.

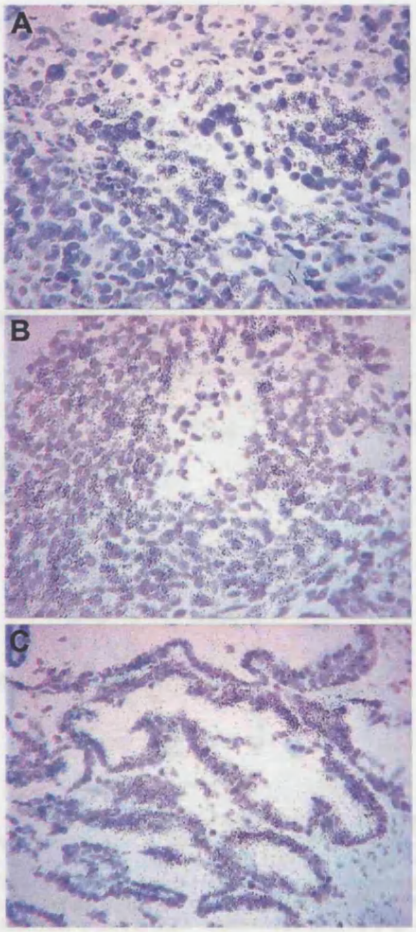


Fig. 2. CXCR4 mRNA expression by epithelial tumor cells in biopsies of human ovarian cancer. *A*, variable degree of labeling of two tumor epithelial cell groups in a serous carcinoma separated by a cellular stroma that is largely unlabeled. *B*, grade 3 serous carcinoma with heterogeneous labeling of tumor epithelial cells and focal weak labeling of some cells in the stroma. *C*, grade 1 serous carcinoma with neoplastic epithelial cells clearly labeled for CXCR4.

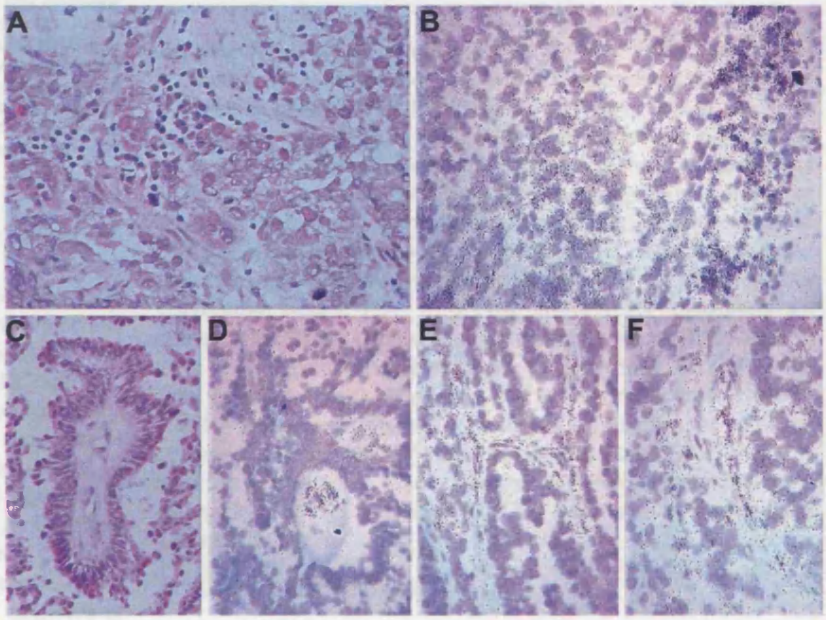


Fig. 3. CXCR4 mRNA expression by tumor and stromal cells in biopsies of human ovarian cancer. *A*, H&E-stained section of grade 3 serous carcinoma with stromal lymphoid infiltrate. *B*, same serous carcinomas in *A* with CXCR4 labeling of tumor epithelial cells (*left of field*). The lymphoid cells within the stroma (*right of field*) show a denser pattern of label. *C*, endothelial cells in the papillary core of a grade 1 serous carcinoma (stained with H&E) are clearly labeled for CXCR4. *D*, there is focal weak labeling of occasional tumor cells. *E*, stromal endothelial cell labeling in a grade 2 serous carcinoma with equivocal epithelial cell labeling. *F*, tumor epithelial cells show a low index of CXCR4 labeling together with a linear pattern of CXCR4-labeled endothelial cells in the adjacent stroma.

However, previous studies have shown that CXCL12 induced adhesion of most circulating lymphocytes and CD34+ progenitor cells (9).

Analysis of mRNA by RPA showed expression of other chemokine receptors in the tumor and ascites samples, which are a mixture of tumor cells, leukocytes, and connective tissue cells. In solid tumors we also detected expression of the CC chemokine receptor CCR1, but this localized to infiltrating leukocytes.[3] More chemokine receptor mRNAs were expressed in cells from ascites, but apart from CXCR4,

[3] C. Scotten, D. Milliken, J. Wilson, S. Raju, and F. R. Balkwill. Chemokine and chemokine receptor expression in solid ovarian tumors, submitted for publication.

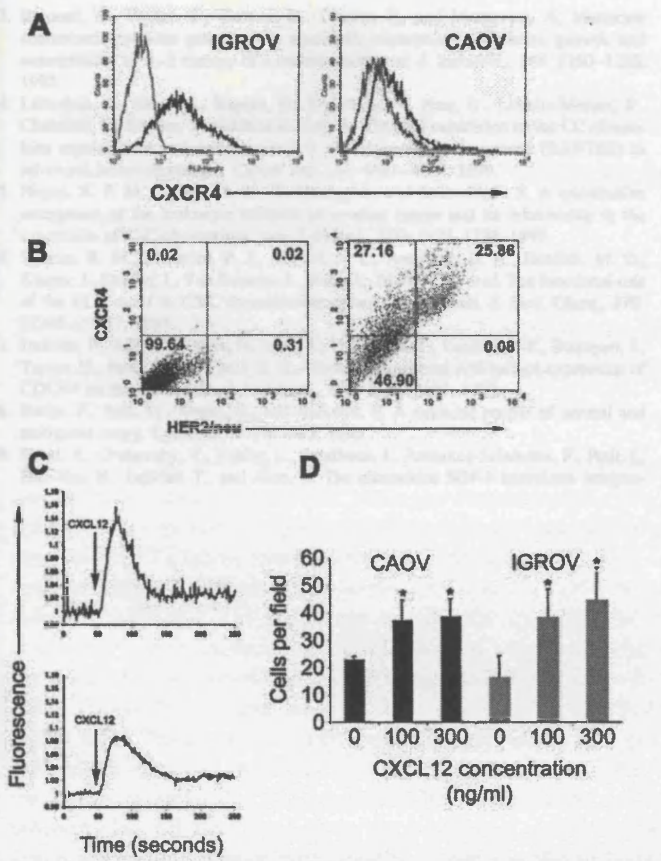


Fig. 4. Functional CXCR4 protein on ovarian cancer cells. *A*, flow cytometry histograms for cell surface expression of CXCR4 on the ovarian cancer cell lines IGROV and CAOV-3. Cells were incubated with IgG2a isotype control mAb (*gray line*) or CXCR4 mAb (*black line*). Results are expressed as mean fluorescence intensity and show one representative experiment of five experiments performed. *B*, two-color flow cytometric analysis of CXCR4 *versus* HER2/neu expression on cells from ovarian ascites. Representative plots are shown with isotype control panel (*left*) and percentages of HER2/neu-positive CXCR4-positive cells (*right*). Cells from five different patients gave similar results. *C*, Ca^{2+} flux in IGROV (*top panel*) and CAOV-3 (*bottom panel*) tumor cell lines in response to CXCL12 stimulation. *Arrows* indicate the point at which the chemokine was added. Results shown are representative of five experiments. *D*, migration of CAOV-3 (■) and IGROV (▨) cell lines to CXCL12. Values are the mean ± SD of 10 determinations from 2 experiments. *, statistically significant results, $P < 0.0005$

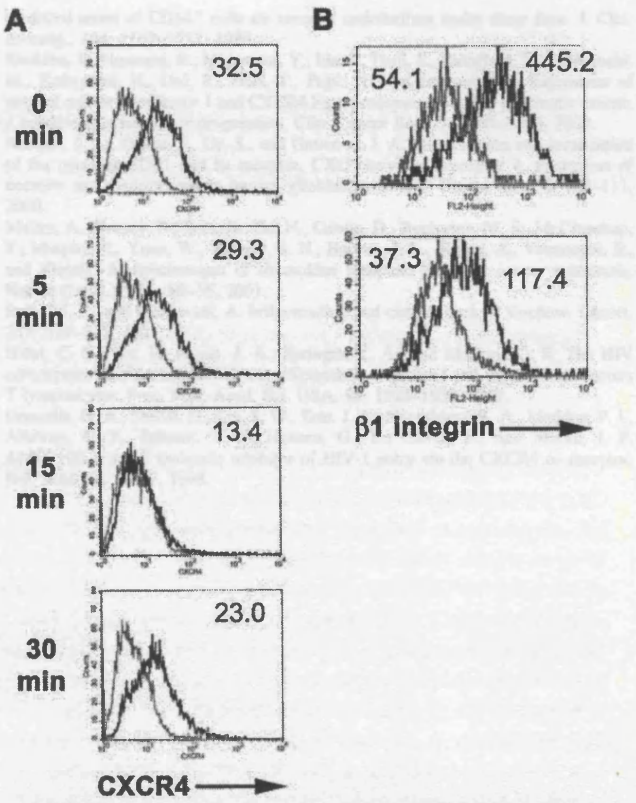


Fig. 5. CXCL12 induced CXCR4 internalization, integrin up-regulation, and CXCL12 expression in ascitic fluid. *A*, the expression/internalization of CXCR4 by IGROV cells at various time points after stimulation with CXCL12 (isotype control, *gray line*; CXCR4, *black line*). *Numbers* on figures represent the mean fluorescent intensity of CXCR4. *B*, up-regulation of the β_1 integrin on IGROV (*left panel*) and CAOV-3 cells (*right panel*) after stimulation with CXCL12. Unstimulated control, *gray line*; CXCL12-stimulated cells, *black line*.

these were also detected on leukocyte but not on tumor cell populations.[4]

Thus the only chemokine receptor we have found on ovarian cancer cells is CXCR4. Two recent studies (10, 11) described tumor and endothelial cell CXCR4 expression in human pancreatic cancer and glioblastoma, but it is not clear whether these tumor cells expressed additional chemokine receptors. In brain tumors, the receptor and its CXCL12 ligand were primarily expressed in regions of angiogenesis and degeneration and were associated with high-grade tumors (11).

Most persuasively, Muller *et al.* (12) recently reported high expression of CXCR4 and CCR7 on human breast cancer cells. As measured by real-time PCR, mRNA levels of the respective ligands CXCL12 and CCL21 were highest at sites of breast cancer metastasis. As we have also shown in this study, the chemokines induced migration of tumor cells. Furthermore, neutralizing antibodies to these chemokine receptors reduced experimental metastasis in a xenograft model of breast cancer. Thus it seems that malignant cells from common tumors express restricted and specific patterns of chemokine receptors.

There is increasing evidence that the inflammatory cells, cytokines, and chemokines found in human tumors are more likely to contribute to tumor growth, progression, and immunosuppression than they are to mount an effective antitumor response (reviewed in Ref. 13). Over the past 10 years, study of the cytokine and chemokine network has led to the development of a range of antagonists for the treatment of inflammation and allergy. We suggest that such agents may also be of benefit in the treatment of malignant disease. The chemokine receptor CXCR4, which is now known to be functional on both breast and ovarian cancer cells, is of particular interest. It is the coreceptor for macrophage trophic HIV virus (14), and CXCR4 antagonists are currently in Phase I clinical trial for the treatment of HIV/AIDS. In view of the expression of this receptor on both tumor and endothelial cells in human ovarian cancers, there is a rationale for the use of CXCR4 antagonists as part of a biological approach to the treatment of ovarian cancer. IGROV cells express functional CXCR4. These cells form i.p. tumors in nude mice and may provide a useful preclinical model to study the action of CXCR4 antagonists in tumor growth and spread (15).

ACKNOWLEDGMENTS

We thank Sue Hoare for performing the CXCL12 ELISA analysis and surgeons at the Department of Obstetrics and Gynecology, Guy's and St Thomas' Hospital for providing patient material.

REFERENCES

1. Rossi, D., and Zlotnik, A. The biology of chemokines and their receptors. Annu. Rev. Immunol., *18:* 217–242, 2000.
2. Zlotnik, A., and Yoshie, O. Chemokines: a new classification system and their role in immunity. Immunity, *12:* 121–127, 2000.

[4] D. Milliken, C., Scotten, S. Raju, F. Balkwill, and J. Wilson. Analysis of chemokines and chemokine receptor expression in ovarian cancer ascites, manuscript in preparation.

3. Bottazzi, B., Walter, S., Govoni, D., Colotta, F., and Mantovani, A. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. J. Immunol., *148:* 1280–1285, 1992.
4. Luboshits, G., Shina, S., Kaplan, O., Engelberg, S., Nass, D., Lifshitz-Mercer, B., Chaitchik, S., Keydar, I., and Ben-Baruch, A. Elevated expression of the CC chemokine regulated on activation, normal T cell expressed and secreted (RANTES) in advanced breast carcinoma. Cancer Res., *59:* 4681–4687, 1999.
5. Negus, R. P. M., Stamp, G. W. H., Hadley, J., and Balkwill, F. R. A quantitative assessment of the leukocyte infiltrate in ovarian cancer and its relationship to the expression of C-C chemokines. Am. J. Pathol., *150:* 1723–1734, 1997.
6. Strieter, R. M., Povlerini, P. J., Kunkel, S. L., Arenberg, D. A., Burdick, M. D., Kasper, J., Dzuiba, J., Van Damme, J., Walz, A., Mariott, D., *et al.* The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. J. Biol. Chem., *270:* 27348–27357, 1995.
7. Jourdan, P., Abbal, C., Nora, N., Hori, T., Uchiyama, T., Vendrell, J-P., Bousquet, J., Taylor, N., Pene, J., and Yssel, H. IL-4 induces functional cell-surface expression of CDCR4 on human T cells. J. Immunol., *160:* 4153–4157, 1998.
8. Burke, F., Relf, M., Negus, R., and Balkwill, F. A cytokine profile of normal and malignant ovary. Cytokine, *8:* 578–585, 1996.
9. Peled, A., Grabovsky, V., Habler, L., Sandbank, J., Arenzana-Seisdedos, F., Petit, I., Ben-Hur, H., Lapidot, T., and Alon, R. The chemokine SDF-1 stimulates integrin-mediated arrest of $CD34^+$ cells on vascular endothelium under shear flow. J. Clin. Investig., *104:* 1199–1211, 1999.
10. Koshiba, T., Hosotani, R., Miyamoto, Y., Ida, J., Tsuji, S., Nakajima, S., Kawaguchi, M., Kobayashi, H., Doi, R., Hori, T., Fujii, N., and Imamura, M. Expression of stromal cell-derived factor 1 and CXCR4 ligand receptor system in pancreatic cancer: a possible role for tumor progression. Clin. Cancer Res., *6:* 3530–3535, 2000.
11. Rempel, S. A., Dudas, S., Ge, S., and Gutierrez, J. A. Identification and localization of the cytokine SDF1 and its receptor, CXC chemokine receptor 4, to regions of necrosis and angiogenesis in human glioblastoma. Clin. Cancer Res., *6:* 102–111, 2000.
12. Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M. E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S. N., Barrera, J. L., Mohar, A., Verastegui, E., and Zlotnik, A. Involvement of chemokine receptors in breast cancer metastasis. Nature (Lond.), *410:* 50–56, 2001.
13. Balkwill, F., and Mantovani, A. Inflammation and cancer: back to Virchow. Lancet, *357:* 539–545, 2001.
14. Bleul, C. C., Wu, L., Hoxie, J. A., Springer, T. A., and Mackay, C. R. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. USA, *94:* 1925–1930, 1997.
15. Donzella, G. A., Schols, D., Lin, S. W., Este, J. A., Nagashima, K. A., Maddon, P. J., Allaway, G. P., Sakmar, T. P., Henson, G., De Clercq, E., and Moore, J. P. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med., *4:* 72–77, 1998.

Defective Expression of the Monocyte Chemotactic Protein-1 Receptor CCR2 in Macrophages Associated with Human Ovarian Carcinoma[1]

Antonio Sica,[2]* Alessandra Saccani,* Barbara Bottazzi,* Sergio Bernasconi,* Paola Allavena,* Brancatelli Gaetano,† Francesca Fei,† Graig LaRosa,‡ Chris Scotton,§ Frances Balkwill,§ and Alberto Mantovani*¶

Monocyte chemotactic protein-1 (MCP-1, CCL2) is an important determinant of macrophage infiltration in tumors, ovarian carcinoma in particular. MCP-1 binds the chemokine receptor CCR2. Recent results indicate that proinflammatory and anti-inflammatory signals regulate chemokine receptor expression in monocytes. The present study was designed to investigate the expression of CCR2 in tumor-associated macrophages (TAM) from ovarian cancer patients. TAM isolated from ascitic or solid ovarian carcinoma displayed defective CCR2 mRNA (Northern blot and PCR) and surface expression and did not migrate in response to MCP-1. The defect was selective for CCR2 in that CCR1 and CCR5 were expressed normally in TAM. *CCR2* gene expression and chemotactic response to MCP-1 were decreased to a lesser extent in blood monocytes from cancer patients. *CCR2* mRNA levels and the chemotactic response to MCP-1 were drastically reduced in fresh monocytes cultured in the presence of tumor ascites from cancer patients. Ab against TNF-α restored the *CCR2* mRNA level in monocytes cultured in the presence of ascitic fluid. The finding of defective *CCR2* expression in TAM, largely dependent on local TNF production, is consistent with previous in vitro data on down-regulation of chemokine receptors by proinflammatory molecules. Receptor inhibition may serve as a mechanism to arrest and retain recruited macrophages and to prevent chemokine scavenging by mononuclear phagocytes at sites of inflammation and tumor growth. In the presence of advanced tumors or chronic inflammation, systemic down-regulation of receptor expression by proinflammatory molecules leaking in the systemic circulation may account for defective chemotaxis and a defective capacity to mount inflammatory responses associated with advanced neoplasia. *The Journal of Immunology,* 2000, 164: 733–738.

Selective accumulation of leukocyte subpopulations is the hallmark of pathological conditions, including allergic and inflammatory reactions and tumors (1). In particular, the presence of macrophages in tumor tissues and/or their periphery has generated broad interest, and analysis of tumor-associated macrophages (TAM)[3] suggests that these cells have the capacity to affect diverse aspects of the immunobiology of neoplastic tissues, including vascularization, growth rate and metastasis, stroma formation, and dissolution (1, 2). On the other hand, cells of the monocyte-macrophage lineage have the potential to express tumoricidal capacity and to elicit tumor-destructive reactions (3, 4). Based on these observations, the "macrophages balance" hypothesis (1) was coined to emphasize the dual potential of TAM to influence tumor growth in opposite directions.

Macrophages represent a major component of the lymphoreticular infiltrate of tumors, and the percentage of TAM for each tumor is usually maintained as a relatively stable "individual" property during tumor growth and upon transplantation in syngeneic hosts (1, 5). The search for tumor-derived chemotactic factors, which may account for recruitment of mononuclear phagocytes in neoplastic tissues, lead to the identification of the monocyte chemotactic protein-1 (MCP-1, CCL2) as well as of other chemokines (1, 2, 6–12).

MCP-1 is a member of a superfamily of cytokines called chemokines. The hallmark of this family is a conserved cysteine residue motif (13–15). According to the relative position of the first two cysteines, it is possible to distinguish two main families: the CXC (or α) chemokines, which are active on neutrophils, T and B lymphocytes (13–15), and the CC (or β) chemokines that exert their action on multiple leukocyte populations, including monocytes, basophils, eosinophils, T lymphocytes, NK, and dendritic cells (13–18). A third type of protein (the C or γ chemokines) was described, which is active on T lymphocytes and NK cells. This protein is characterized by the absence of the first and third cysteines, but shows overall sequence identity with CC chemokines (19, 20). More recently, fractalkine, CX3C (or δ motif), was described as chemotactic for monocytes, T cells, and NK cells (21, 22).

Chemokines, as well as classical chemotactic agonists, such as formylated peptides (of which fMLP is the prototype) and C5a, bind to and activate a family of rhodopsin-like, GTP-binding protein-coupled seven-transmembrane domain receptors (23–25). Nine receptors for CC chemokines, now named CCR1 through 9, have been identified and cloned (13, 14, 23–26).

*Istituto di Ricerche Farmacologiche "Mario Negri," Milan, Italy; †Department of Obstetrics and Gynecology, S. Gerardo Hospital, Monza, Italy; ¶Department of Biotechnology, University of Brescia, Brescia, Italy; ‡Receptor Pharmacology, Leukosite, Inc., Cambridge, MA; and §Biological Therapy Laboratory, Imperial Cancer Research Fund, London, United Kingdom

Received for publication June 15, 1999. Accepted for publication November 4, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by Associazione Italiana Ricerca sul Cancro, Target Project on Biotechnology, Consiglio Nazionale delle Ricerche, Contract 99.00119.PF49, Project Therapy of Tumors, and Ministero Universita' Ricerca Scientifica e Tecnologica. Travel support for F.B. was provided by a special program from Consiglio Nazionale delle Ricerche.

[2] Address correspondence and reprint requests to Dr. Antonio Sica, Istituto di Ricerche Farmacologiche "Mario Negri," via Eritrea 62, 20157 Milan, Italy. E-mail address: Sica@irfmn.mnegri.it

[3] Abbreviations used in this paper: TAM, tumor-associated macrophages; MCP-1, monocyte chemotactic protein-1; MIP-1, macrophage inflammatory protein-1; M-DM, monocyte-derived macrophage.

0022-1767/00/$02.00

MCP-1 interacts with CCR2, of which two isoforms have been cloned and termed A and B (26). In monocytes and NK cells, CCR2 is expressed predominantly as B isoform, with vanishingly low levels of A transcripts (27). In addition to MCP-1, CCR2 recognizes MCP-2 and MCP-3 (28–30). Several lines of evidence, including studies in gene-targeted mice, indicate that MCP-1 and CCR2 are important for monocyte recruitment at the site of inflammation (31, 32). There is also evidence that in a variety of murine and human tumors, including ovarian cancer, MCP-1 is a major determinant of the degree of macrophage infiltration in neoplastic tissue (1, 2, 33–40).

Recent results indicate that proinflammatory and anti-inflammatory signals regulate chemokine receptor expression in human mononuclear phagocytes (41–44). In particular, certain primary proinflammatory signals (e.g., LPS) rapidly inhibit chemokine receptor expression (41). It was speculated that inhibition of chemokine receptor expression may serve as a stop signal to arrest and retain mononuclear phagocytes at sites of infection or inflammation (41). As discussed elsewhere (2), tumors have served as a paradigm of the in vivo function of chemokines in monocyte recruitment with minimal activation. It was therefore important to investigate chemokine receptor expression in TAM, since regulation of receptor expression has emerged as a crucial set point for the action of these molecules. In this study, we report that macrophages isolated from the ascitic fluid or solid tumor from ovarian cancer patients exhibit a drastic and selective defect of expression of the MCP-1 receptor (CCR2), which correlates with the lack of chemotaxis in response to MCP-1. In addition, we identified TNF-α as a protumor factor which may contribute to the negative regulation of CCR2.

Materials and Methods

Monocytes

Human monocytes were separated from peripheral blood of human healthy donors by Percoll gradient centrifugation (45). Monocytes (>98% pure as assessed by morphology) were resuspended at 10^7/ml in RPMI 1640 supplemented with 10% of FBS, 2 mM glutamine, and antibiotics. All reagents contained <0.125 endotoxin units/ml of endotoxin as checked by *Limulus* amebocyte lysate assay (Microbiological Associates, Walkersville, MD). Monocyte-derived macrophages (M-DM) were derived from freshly isolated monocytes (3–5 × 10^6cells/ml) after incubation for 5 days in RPMI 1640 medium supplemented with 10% of FBS, with 2 mM glutamine, antibiotics, and 40% autologous serum on hydrophobic plates (Petriperm Hydrophobic; Heraeus Instruments GmbH, Germany) as described previously (46). PBMC of healthy donors were obtained from buffy coats, whereas PBMC from patients with ovarian carcinoma were obtained from heparinized venous blood. Blood was diluted 1/5 with saline, and 40 ml was then placed on 10 ml of Ficoll (Seromed, Berlin, Germany) in 50-ml conical tubes (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) for centrifugation at 400 × g for 20 min at room temperature. PBMC were collected at the interface, washed with saline, and suspended in complete medium at 2–5 × 10^6 cells/ml in 50-ml conical tubes.

TAM

Blood samples (5) and ascitic fluids (10) were collected from untreated patients with histologically confirmed epithelial ovarian carcinoma admitted to the Department of Obstetrics and Gynecology, San Gerardo Hospital (Monza, Italy). All patients had cancer classified as stage II, III, or IV. Ascitic fluid was collected and centrifuged. Cell pellets were resuspended in RPMI 1640 medium without serum and layered on top of a Ficoll-Hypaque cushion to prepare mononuclear cells. Purification of peritoneal macrophages was further conducted by two subsequent adherence steps for 45 min, each in RPMI 1640 medium without serum. After adherence procedures, cells were repeatedly washed with saline to remove all nonadherent cells. The adherent cells were cultured with complete medium over night at 37°C to rest and then stimulated as indicated above. To purify TAM by flow cytometry and sorting, ascitic fluids (four patients) were incubated with anti-CD68 mAb. A total of 5 × 10^7 cells was washed in saline with 1% human serum and then incubated in 0.5 ml of anti-CD68 diluted 1/5 for 30 min at 4°C. The cells were then washed three times in saline/1% human serum. The second incubation was conducted at 4°C for 30 min with FITC-conjugated goat $F(ab')_2$ anti-mouse Ig. The sorting process was performed with a FACStar^{Plus} apparatus (Becton Dickinson, Mountain View, CA). Purification of TAM from solid tumor was performed as described previously (34).

FACS analysis

Cell staining was performed using human mAb anti-CCR5 (clone 2D7; PharMingen, San Diego, CA) and its irrelevant control mouse, IgG2a, κ (UPC10-Sigma) followed by FITC-conjugated affinity-purified, isotype-specific goat anti-mouse Ab (Southern Biotechnology Associates, Birmingham, AL). For phenotype analysis, indirect immunofluorescence was performed with the human anti-CCR2 mAb Ab (clone LS132.1D9) and PE-labeled goat anti-mouse Ig (Jackson ImmunoResearch, West Grove, PA) using a FACStar (Becton Dickinson).

Cytokines and Abs

Human recombinant IL-1β was obtained through the courtesy of Dr. J. E. Sims (Immunex, Seattle, WA) and was used at 20 ng/ml; TNF-α (BASF/Knoll, Ludwighafen, Germany) was used at 500 U/ml. Human recombinant MCP-1, MCP-2, and RANTES (regulated on activation normal T cell expressed and secreted) were from PeproTech (Rocky Hill, NJ). Human recombinant MCP-3 and macrophage inflammatory protein-1α (MIP-1α) were a kind gift from Dr. A. Minty (Sanofi, Labège, France) and Dr. Czaplewski, respectively. The mAb against TNF-α (B154.2) was a kind gift from Dr. G. Trinchieri (Wistar Institute of Anatomy and Biology, Philadelphia, PA). The mAb against IFN-γ (IFGCP) was purchased from American Type Culture Collection (Manassas, VA). IL-1ra mutant DoB 0039 was kindly donated by Dr. Diana Boraschi (Dompe', L'Aquila, Italy).

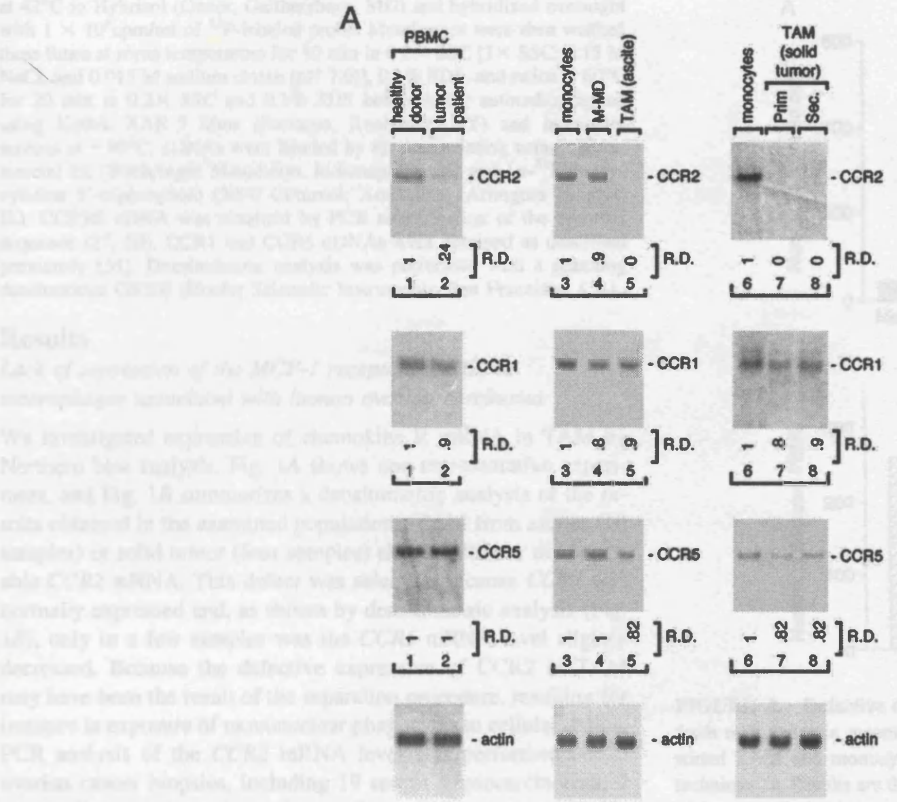
Migration assay

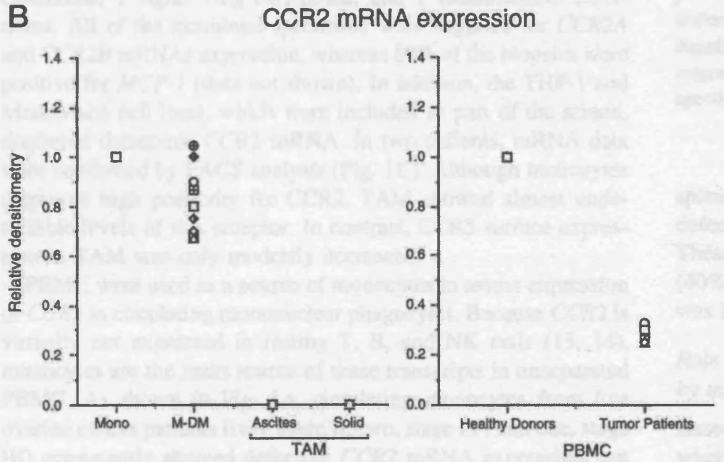
Cell migration was evaluated using a chemotaxis microchamber technique (47) as described previously (48). Twenty-seven microliters of chemoattractant solution or control medium (RPMI 1640 with 1% FCS) was added to the lower wells of a chemotaxis chamber (Neuroprobe, Pleasanton, CA). A polycarbonate filter (5-μm pore size; Neuroprobe) was layered onto the wells and covered with a silicon gasket and the top plate. A total of 50 μl of cell suspension (1.5 × 10^6/ml monocytes in PBMC) was seeded in the upper chamber. The chamber was incubated at 37°C in air with 5% CO_2 for 90 min. At the end of the incubation, filters were removed, stained with Diff-Quik (Baxter S.P.A., Rome, Italy), and five high-power oil-immersion fields were counted.

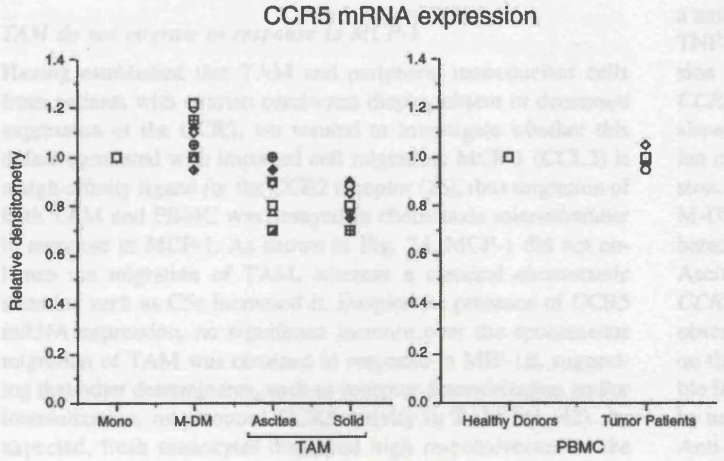
Northern blot analysis

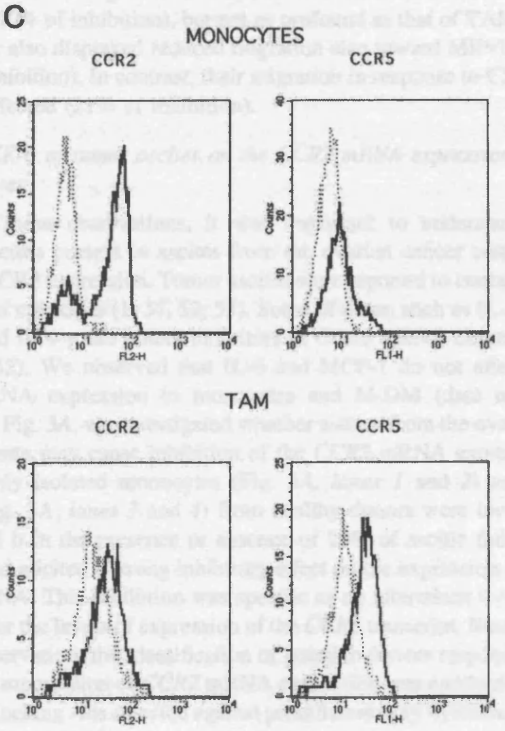
Cells were cultured in medium alone or supplemented with the indicated agents, and total RNA was purified as described (49). Ten micrograms of total RNA from each sample was electrophoresed under denaturing conditions, blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH), and cross-linked by UV irradiation. Membranes were prehybridized

FIGURE 1. Defective expression of CCR2 in TAM. A, Expression of *CCR1, CCR2,* and *CCR5. Lanes 1* and 2, Expression of the *CCR2, CCR1,* and *CCR5* mRNAs in PBMC. PBMC were obtained from peripheral blood of either healthy donors (*lane 1*) or tumor patients (*lane 2*). *Lanes 3–5,* Expression of *CCR1, CCR2,* and *CCR5* mRNAs in monocytes (*lane 3*), M-DM (*lane 4*), and TAM purified from ascites (*lane 5*). *Lanes 6–8,* Lack of *CCR2* mRNA expression in TAM isolated from solid tissue of ovarian carcinoma. *Lane 6,* Fresh monocytes from healthy donors; *lanes 7 and 8,* TAM from ovarium and omentum of cancer patients. Total RNA was purified from cells cultured for 4 h in RPMI 1640 supplemented with 10% of FBS. Ten micrograms of total RNA was next analyzed by Northern blot. The results presented in *A* show one representative experiment for each population. A total of 10, 4, and 5 specimens were analyzed for either TAM (purified from tumor ascites), TAM (purified from solid ovarian carcinoma), or PBMC, respectively. R. D., relative densitometry. *B,* Densitometric analysis was performed on all Northern blot results, and values were normalized for the levels of mRNA expression observed in fresh monocytes (mono) or in PBMC from healthy donors, to which a value of 1.0 was assigned. *C,* Surface expression of CCR2 and CCR5 in TAM and monocytes. Surface expression was determined by flow cytometry using the human anti-CCR2 and anti-CCR2 Abs. Dots, Irrelevant mAbs; continuous line, cells stained with anti-CCR2 or anti-CCR5 as indicated.


A
PBMC
healthy donor
tumor patient
monocytes
M-MD
TAM (ascite)
monocytes
TAM (solid tumor)
Prim
Sec.
- CCR2
- CCR1
- CCR5
- actin
R.D.
1 .24
1 .9 0
1 0 0
1 .9
1 .8 .9
1 .8 .9
1 1
1 1 .82
1 .82 .82
1 2
3 4 5
6 7 8


B
CCR2 mRNA expression
Relative densitometry
Mono
M-DM
Ascites
Solid
TAM
Healthy Donors
Tumor Patients
PBMC


CCR5 mRNA expression
Relative densitometry
Mono
M-DM
Ascites
Solid
TAM
Healthy Donors
Tumor Patients
PBMC


C
MONOCYTES
CCR2
CCR5
TAM
CCR2
CCR5
Counts
FL2-H
FL1-H

at 42°C in Hybrisol (Oncor, Gaithersburg, MD) and hybridized overnight with 1 × 10^6cpm/ml of ^{32}P-labeled probe. Membranes were then washed three times at room temperature for 10 min in 0.2× SSC [1× SSC: 0.15 M NaCl, and 0.015 M sodium citrate (pH 7.0)], 0.1% SDS, and twice at 60°C for 20 min in 0.2× SSC and 0.1% SDS before being autoradiographed using Kodak XAR-5 films (Eastman, Rochester, NY) and intensifier screens at −80°C. cDNAs were labeled by random priming using a commercial kit (Boehringer Mannheim, Indianapolis, IN) and [α-^{32}P]deoxycytidine 5′-triphosphate (3000 Ci/mmol; Amersham, Arlington Heights, IL). CCR2B cDNA was obtained by PCR amplification of the reported sequence (27, 50). CCR1 and CCR5 cDNAs were obtained as described previously (51). Densitometric analysis was performed with a scanning densitometer GS300 (Hoefer Scientific Instruments, San Francisco, CA).

Results

Lack of expression of the MCP-1 receptor (CCR2) in macrophages associated with human ovarian carcinoma

We investigated expression of chemokine R mRNA in TAM by Northern blot analysis. Fig. 1*A* shows one representative experiment, and Fig. 1*B* summarizes a densitometric analysis of the results obtained in the examined populations. TAM from ascites (10 samples) or solid tumor (four samples) showed little or no detectable *CCR2* mRNA. This defect was selective because *CCR1* was normally expressed and, as shown by densitometric analysis (Fig. 1*B*), only in a few samples was the *CCR5* mRNA level slightly decreased. Because the defective expression of CCR2 in TAM may have been the result of the separation procedure, resulting for instance in exposure of mononuclear phagocytes to cellular debris, PCR analysis of the *CCR2* mRNA level was performed on 25 ovarian cancer biopsies, including 19 serous adenocarcinomas, 2 clear cell carcinomas, 1 mucinous adenocarcinoma, 1 anaplastic carcinoma, 1 signet ring carcinoma, and 1 endometrioid carcinoma. All of the examined specimens were negative for *CCR2A* and *CCR2B* mRNAs expression, whereas 80% of the biopsies were positive for *MCP-1* (data not shown). In addition, the THP-1 and MonoMac6 cell lines, which were included as part of the screen, displayed detectable CCR2 mRNA. In two patients, mRNA data were confirmed by FACS analysis (Fig. 1*C*). Although monocytes displayed high positivity for CCR2, TAM showed almost undetectable levels of this receptor. In contrast, CCR5 surface expression in TAM was only modestly decreased.

PBMC were used as a source of monocytes to assess expression of *CCR2* in circulating mononuclear phagocytes. Because *CCR2* is virtually not expressed in resting T, B, and NK cells (13, 14), monocytes are the main source of these transcripts in unseparated PBMC. As shown in Fig. 1*A*, circulating monocytes from five ovarian cancer patients (two, stage II; two, stage IV; and one, stage III) consistently showed defective *CCR2* mRNA expression, but the defect was not as drastic as in TAM.

TAM do not migrate in response to MCP-1

Having established that TAM and peripheral mononuclear cells from patients with ovarian carcinoma display absent or decreased expression of the CCR2, we wanted to investigate whether this defect correlated with impaired cell migration. MCP-1 (CCL2) is a high-affinity ligand for the CCR2 receptor (26), thus migration of both TAM and PBMC was assayed in chemotaxis microchamber in response to MCP-1. As shown in Fig. 2*A*, MCP-1 did not enhance the migration of TAM, whereas a classical chemotactic stimulus such as C5a increased it. Despite the presence of CCR5 mRNA expression, no significant increase over the spontaneous migration of TAM was obtained in response to MIP-1β, suggesting that other determinants, such as receptor desensitization and/or internalization, may control CCR5 activity in TAM (41, 42). As expected, fresh monocytes displayed high responsiveness to the chemoattractants used in the assay. As shown in Fig. 2*B* , in response to MCP-1, migration of PBMC from tumor patients was defective (60% of inhibition), but not as profound as that of TAM. These cells also displayed reduced migration also toward MIP-1β (40% of inhibition). In contrast, their migration in response to C5a was less affected (21% of inhibition).

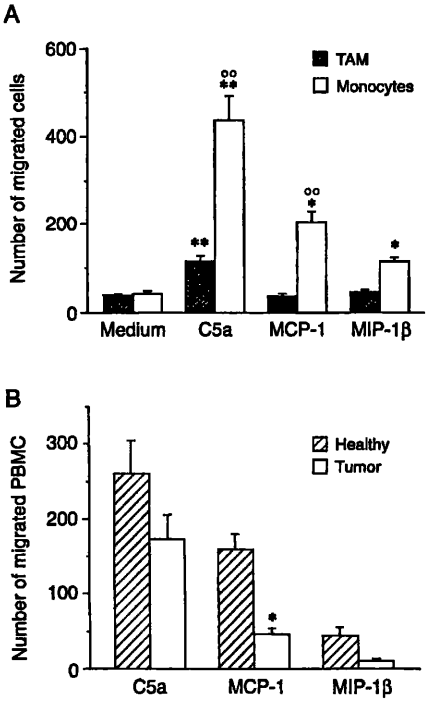


FIGURE 2. Defective chemotaxis of TAM. A, Migration of TAM and fresh monocytes in response to MCP-1, MIP-1β, and C5a. Migration of sorted TAM and monocytes was assayed by chemotaxis microchamber technique. *A*, Results are the number of cells migrated and are means ± SD of four experiments; **, $p < 0.01$; *, $p < 0.05$ vs respective medium; OO, $p < 0.0.1$ vs TAM (Tukey's test). *B*, Migration of PBMC from healthy donors and tumor patients in response to MCP-1, MIP-1β, and C5a. *B*, Results are the number of cells migrated after subtraction of spontaneous migration and are means ± SD of four experiments; *, $p < 0.05$ vs respective healthy donor (paired *t* test).

Role of TNF-α of tumor ascites on the CCR2 mRNA expression by monocytes

Based on these observations, it was important to understand whether factors present in ascites from the ovarian cancer could suppress CCR2 expression. Tumor ascites were reported to contain a number of cytokines (1, 37, 52, 53). Some of these, such as IL-1, TNF-α, and IFN-γ, are potent inhibitors of *CCR2* mRNA expression (41, 42). We observed that IL-6 and MCP-1 do not affect *CCR2* mRNA expression in monocytes and M-DM (data not shown). In Fig. 3*A*, we investigated whether ascites from the ovarian carcinoma may cause inhibition of the *CCR2* mRNA expression. Freshly isolated monocytes (Fig. 3*A*, *lanes 1* and *2*) and M-DM (Fig. 3A, *lanes 3* and *4*) from healthy donors were incubated for 4 h in the presence or absence of 20% of ascitic fluid. Ascitic fluid elicited a strong inhibitory effect on the expression of *CCR2* mRNA. This inhibition was specific as no alterations were observed for the levels of expression of the *CCR5* transcript. Based on this observation, the identification of putative factors responsible for the suppression of *CCR2* mRNA expression was conducted by using blocking Abs directed against proinflammatory cytokines. Anti-IFN-γ, anti-TNF-α Abs, and the IL-1ra were preincubated for 2 h at room temperature with ascitic fluid in an attempt to block the

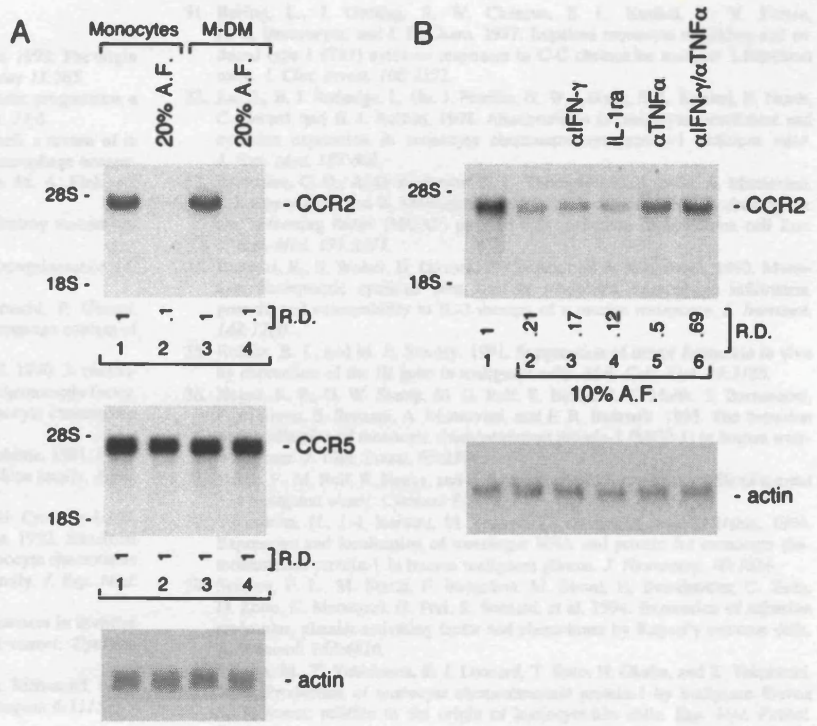


FIGURE 3. Role of cytokines in inhibition of CCR2 expression by ascites. *A*, Effect of ascitic fluid from ovarian carcinoma on the *CCR2* mRNA expression by fresh monocytes (*lanes 1* and *2*) and M-DM (*lanes 3* and *4*). Fresh monocytes and M-DM obtained from healthy donors were cultured for 4 h in the presence or absence of 20% of ascitic fluid as indicated. Results are representative of five experiments. *B*, Restoration of *CCR2* mRNA expression by anti-TNF-α-blocking Ab. RPMI 1640 medium containing 10% of ascitic fluid from ovarian cancer was preincubated for 2 h with anti-IFN-γ, IL-1ra, anti-TNF-α, or anti-IFN-γ/anti-TNF-α, respectively. Freshly isolated monocytes were subsequently cultured for 4 h in RPMI 1640 medium (*lane 1*) or RPMI 1640 containing ascitic fluid (*lanes 2–6*) as indicated. Ten micrograms of total RNA was next analyzed in Northern blot. R. D., relative densitometry.

inhibitory activity for *CCR2*. As shown in Fig. 3*B*, in the presence of ascitic fluid, anti-TNF-α (*lane 5*) was able to efficiently preserve the levels of expression of *CCR2* mRNA, whereas the anti-IFN-γ Ab (*lane 3*) and IL-1ra (*lane 4*) did not. Coincubation with anti-IFN-γ and anti-TNF-α Abs did not synergize (Fig. 3*B*, *lane 6*). These results strongly indicate TNF-α present in the ascites of ovarian carcinoma as a tumor-derived factor responsible for the inhibition of *CCR2* in TAM.

Discussion

The results presented here show that macrophages associated with human ovarian carcinoma display defective expression of CCR2, a receptor for MCPs. Lack of *CCR2* expression was specific and functionally relevant since TAM were unable to respond to MCP-1 chemotactically, but still migrated in response to C5a. This defect was selective in that TAM expressed basically normal levels of *CCR1* and *CCR5*. Moreover, lower *CCR2* expression and function was observed also in the periphery, as PBMC from five tumor patients expressed lower levels of *CCR2* mRNA and poorly migrated in response to MCP-1. Loss of *CCR2* mRNA expression was reproduced in monocytes cultured in the presence of ascitic fluid of ovarian cancer and confirmed in 25 ovarian cancer biopsies.

The selective loss of *CCR2* expression in TAM resembles the specific and selective inhibition operated by LPS and certain proinflammatory cytokines (e.g., IL-1, TNF-α, and IFN-γ) on the *CCR2* expression in monocytes (41, 42). We also reported that the anti-inflammatory cytokine IL-10 induces *CCR2*, *CCR5*, and *CCR1* expression (43), suggesting that proinflammatory and anti-inflammatory signals have opposite and divergent actions on CC chemokine receptor expression in monocytes. In this regard, ovarian cancer is one epithelial malignancy in which there is evidence of a complex network between the tumor microenvironment and the immune system (37, 54), and there is strong evidence that TNF-α is a cytokine which strongly influences the biology of this tumor (54, 55). Despite the expression of chemokine receptors, lack of migration in response to a specific ligand has been reported in cells activated with proinflammatory signals (41–43), a condition likely shared by TAM. This may potentially be the result of a number of mechanisms such as homologous or heterologous receptor desensitization, internalization, and/or inhibition of receptor signaling. In addition, the observed difference in the number of migrated TAM vs migrated monocytes in response to C5a may be at least partially due to cell differentiation.

Previous reports have indicated that ovarian cancer cells significantly express TNF-α (55) and we pointed to this cytokine as a possible tumor-derived inhibitor of the *CCR2* expression. The validity of this hypothesis is substantiated by the observation that inhibition of *CCR2* mRNA expression by tumor ascites is efficiently prevented by an anti-TNF-α Ab. Despite this observation, the levels of TNF-α that we detected in tumor ascites were quite low (data not shown) and may not fully explain the drastic down-regulation of *CCR2* mRNA levels, but rather suggest possible synergism with other proinflammatory signals. Indeed, since ovarian cancer cells are a rich source of cytokines (37), it is likely that other proinflammatory cytokines within the tumor microenvironment may strengthen the inhibitory action of TNF-α. The observed decrease of *CCR2* mRNA levels in PBMC from cancer patients may also imply the possibility that factors released from growing tumors may, beyond a certain size, leak into the systemic circulation to control excessive recruitment and reverse transmigration. Thus, the drastic down-regulation of *CCR2* in comparison to the others receptors studied here may assume particular relevance in view of evidence suggesting MCP-1 as a main determinant of macrophage infiltration in tumors (1, 2) and normal tissues (31, 32).

We previously proposed (41) that reciprocal influences exerted by proinflammatory molecules on chemokine agonist production and receptor expression may represent a crucial set point in the regulation of the chemokine system. Our data provide the first evidence that this emerging paradigm has in vivo relevance in the pathophysiology of tumors and point to TNF-α as a tumor-derived cytokine controlling the chemokine system of macrophages associated with tumors.

References

1. Mantovani, A., B. Bottazzi, F. Colotta, S. Sozzani, and L. Ruco. 1992. The origin and function of tumor-associated macrophages. *Immunol. Today 13:265.*
2. Mantovani, A. 1994. Tumor-associated macrophages in neoplastic progression: a paradigm for the in vivo function of chemokines. *Lab. Invest. 71:5.*
3. Hibbs, J. B. 1976. The macrophage as a tumoricidal effector cell: a review of in vitro and in vivo studies on the mechanism of the activated macrophage nonspecific cytotoxicity reaction. In *The Macrophage in Neoplasie.* M. A. Fink, ed. Academic Press, New York, p. 83.
4. Adams, D. O., and R. Snyderman. 1979. Do macrophages destroy nascent tumors? *J. Natl. Cancer Inst. 62:1341.*
5. Evans, R. 1972. Macrophages in syngeneic animal tumors. *Transplantation 14: 468.*
6. Bottazzi, B., N. Polentarutti, R. Acero, A. Balsari, D. Boraschi, P. Ghezzi, M. Salmona, and A. Mantovani. 1983. Regulation of the macrophage content of neoplasms by chemoattractants. *Science 220:210.*
7. Bottazzi, B, F. Colotta, A. Sica, N. Nobili, and A. Mantovani. 1990. A chemoacttractant expressed in human sarcoma cells (tumor-derived chemotactic factor, TDCF is identical to monocyte chemoattractant protein-1/monocyte chemotactic and activating factor (MCP-1/MCAF). *Int. J. Cancer 45:795.*
8. Oppenheim, J. J., C. O. Zachariae, N. Mukaida, and K. Matsushima. 1991. Properties of the novel proinflammatory supergene "intecrine" cytokine family. *Annu. Rev. Immunol. 9:617.*
9. Schall, T. J. Biology of the RANTES/SIS cytokine family. 1991. *Cytokine 3:165.*
10. Van Damme, J., P. Proost, J. P. Lenaerts, and G. Opdenakker. 1992. Structural and functional identification of two human, tumor-derived monocyte chemotactic proteins (MCP-2 and MCP-3) belonging to the chemokine family. *J. Exp. Med. 176:59.*
11. Opdenakker, G., and J. Van Damme. 1992. Cytokines and proteases in invasive processes: molecular similarities between inflammation and cancer. *Cytokine 4:251.*
12. Balentien, E., B. E. Mufson, R. L. Shattuck, R. Derynck, and A. Richmond. 1991. Effects of MGSA/GRO α on melanocyte transformation. *Oncogene 6:1115.*
13. Roolins, B. J. 1997. Chemokines. *Chemokines 90:909.*
14. Baggiolini, M. 1998. Chemokines and leukocyte traffic. *Nature 392:565.*
15. Mantovani, A. 1999. The chemokine system: redundancy for robust outputs. *Immunol. Today 20:254.*
16. Sozzani, S., M. Locati, D. Zhou, M. Rieppi, W. Luini, G. Lamorte, G. Bianchi, N. Polentarutti, P. Allavena, and A. Mantovani. 1995. Receptors, signal transduction and spectrum of action of monocyte chemotactic protein-1 and related chemokines. *J. Leukocyte Biol. 57:788.*
17. Sozzani, S., F. Sallusto, W. Luini, D. Zhou, L. Piemonti, P. Allavena, J. Van Damme, S. Valitutti, A. Lanzavecchia, and A. Mantovani. 1995. Migration of dendritic cells in response to formyl peptides, C5a and a distinct set of chemokines. *J. Immunol. 155:3292.*
18. Mantovani, A., S. Sozzani, P. Proost, and J. Van Damme. 1996. The monocyte chemoattractant protein family. In *Chemoattractant Ligands and Their Receptors.* R. Horuk, ed. CRC, Boca Raton, FL, p. 169.
19. Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, T. J. Schall, and A. Zlotnik. 1994. Lymphotactin: a cytokine that represents a new class of chemokine. *Science 266:1395.*
20. Bianchi, G., S. Sozzani, A. Zlotnik, A. Mantovani, and P. Allavena. 1996. Migratory response of human NK cells to lymphotactin. *Eur. J. Immunol. 26:3238.*
21. Bazan, J. F., K. B. Bacon, G. Hardiman, W. Wang, K. Soo, D. Rossi, D. R. Greaves, D. Rossi, A. Zlotnik, and T. J. Schall. 1997. A new class of membrane-bound chemokine with a CX3C motif. *Nature 385:640.*
22. Schall, T. 1997. Fractalkine: a strange attractor in the chemokine landscape. *Immunol. Today 18:147.*
23. Horuk, R. 1994. The interleukin-8-receptor family: from chemokines to malaria. *Immunol. Today 15:169.*
24. Murphy, P. M. 1994. The molecular biology of leukocyte chemoattractant receptors. *Annu. Rev. Immunol. 12:593.*
25. Gerard, C., and N. P. Gerard. 1994. C5a anaphylatoxin and its seven transmembrane-segment receptor. *Annu. Rev. Immunol. 12:775.*
26. Charo, I. F., S. J. Myers, A. Herman, C. Franci, A. J. Connolly, and S. R. Coughlin. 1994. Molecular cloning and functional expression of two monocyte chemoattractant protein-1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc. Natl. Acad. Sci. USA 91:2752.*
27. Polentarutti N., Allavena P., Bianchi G., Giardina G., Basile A., Sozzani S., Mantovani A., and M. Introna. 1997. IL-2-regulated expression of the monocyte chemotactic protein-1 receptor (CCR2) in human NK cells. *J. Immunol. 158: 2689.*
28. Franci, C., L. M. Wong, J. Van Damme, P. Proost, and I. F. Charo. 1995. Monocyte chemoattractant protein-3, but not monocyte chemoattractant protein-2, is a functional ligand of the human monocyte chemoattranctant protein-1 receptor. *J. Immunol. 154:6511.*
29. Combadiere, C., S. K. Ahuja, J. Van Damme, H. L. Tiffany, J. L. Gao, and P. M. Murphy. 1995. Monocyte chemoattractant protein-3 is a functional ligand for CC chemokine receptors 1 and 2B. *J. Biol. Chem. 270:29671.*
30. Sozzani, S., D. Zhou, M. Locati, M. Rieppi, P. Proost, M. Magazin, N. Vita, J. Van Damme, and A. Mantovani. 1994. Receptors and transduction pathways for monocyte chemotactic protein-2 and monocyte chemotactic protein-3: similarities and differences with MCP-1. *J. Immunol. 152:3615.*
31. Boring, L., J. Gosling, S. W. Chensue, S. L. Kunkel, R. V. Farese, H. E. Broxmeyer, and I. F. Charo. 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest. 100:2552.*
32. Lu, B., B. J. Rutledge, L. Gu, J. Fiorillo, N. W. Lukacs, S. L. Kunkel, R. North, C. Gerard, and B. J. Rollins. 1998. Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein-1 deficient mice. *J. Exp. Med. 187:601.*
33. Zachariae, C. O., A. O. Anderson, H. L. Thompson, E. Appella, A. Mantovani, J. J. Oppenheim, and K. Matsushima. 1990. Properties of monocyte chemotactic and activating factor (MCAF) purified from a human fibrosarcoma cell line. *J. Exp. Med. 171:2177.*
34. Bottazzi, B., S. Walter, D. Govoni, F. Colotta, and A. Mantovani. 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. *J. Immunol. 148:1280.*
35. Rollins, B. J., and M. E. Sunday. 1991. Suppression of tumor formation in vivo by expression of the JE gene in malignant cells. *Mol. Cell. Biol. 11:3125.*
36. Negus, R. P., G. W. Stamp, M. G. Relf, F. Burke, S. T. Malik, S. Bernasconi, P. Allavena, S. Sozzani, A. Mantovani, and F. R. Balkwill. 1995. The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *J. Clin. Invest. 95:2391.*
37. Burke, F., M. Relf, R. Negus, and F. Balkwill. 1996. A cytokine profile of normal and malignant ovary. *Cytokine 8:578.*
38. Takeshima, H., J.-I. Karatsu, M. Takeya, T. Yoshimura, and Y. Ushio. 1994. Expression and localization of messenger RNA and protein for monocyte chemoattractant protein-1 in human malignant glioma. *J. Neurosurg. 80:1056.*
39. Sciacca, F. L., M. Sturzi, F. Bussolino, M. Sironi, H. Brandstetter, C. Zietz, D. Zhou, C. Matteucci, G. Peri, S. Sozzani, et al. 1994. Expression of adhesion molecules, platelet-activating factor and chemokines by Kaposi's sarcoma cells. *J. Immunol. 153:4816.*
40. Takeya, M., T. Yoshimura, E. J. Leonard, T. Kato, H. Okabe, and K. Takahashi. 1991. Production of monocyte chemoattractant protein-1 by malignant fibrous histiocitoma: relation to the origin of histiocyte-like cells. *Exp. Mol. Pathol. 54:61.*
41. Sica, A., A. Saccani, A. Borsatti, C. A. Power, T. N. C. Wells, W. Luini, N. Polentarutti, S. Sozzani, and A. Mantovani. 1997. Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med. 185:969.*
42. Penton-Rol, G., N. Polentarutti, W. Luini, A. Borsatti, R. Mancinelli, A. Sica, S. Sozzani, and A. Mantovani. 1998. Selective inhibition of expression of the chemokine receptor CCR2 in human monocytes by IFN-γ. *J. Immunol. 160:3869.*
43. Sozzani, S., G. Iannolo, W. Luini, A. Borsatti, N. Polentarutti, A. Sica, C. Mackay, T. N. C. Wells, S. Ghezzi, E. Vincenzi, et al. 1998. Interleukin-10 increases the expression of functional HIV coreceptors in monocytes. *J. Exp. Med. 187:439.*
44. Xu, L., R. Rahimpour, L. Ran, C. Kong, A. Biragyn, J. Andrews, M. Devries, J. M. Wang, and D. J. Kelvin. 1997. Regulation of CCR2 chemokine receptor mRNA stability. *J. Leukocyte Biol. 62:653.*
45. Colotta, F., G. Peri, A. Villa, and A. Mantovani. 1984. Rapid killing of actinomycin D-treated tumor cells by human mononuclear cells. I. Effectors belong to the monocyte-macrophage lineage. *J. Immunol. 132:936.*
46. Mantovani, A., V. Caprioli, P. Gritti, and F. Spreafico. 1977. Human mature macrophages mediate antibody-dependent cellular cytotoxicity on tumor cells. *Transplantation 24:29.*
47. Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well microchemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods 33:239.*
48. Sozzani, S., W. Luini, M. Molino, P. Jflek, B. Bottazzi, C. Cerletti, K. Matsushima, and A. Mantovani. 1991. The signal transduction pathway involved in the migration induced by a monocyte chemotactic cytokine. *J. Immunol. 147:2215.*
49. Sica, A., J. M. Wang, F. Colotta, E. Dejana, A. Mantovani, J. J. Oppenheim, C. G. Larsen, C. O. Zachariae, and K. Matsushima. 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol. 144:3034.*
50. Allavena, P., G. Bianchi, P. Giardina, N. Polentarutti, D. Zhou, M. Introna, S. Sozzani, and A. Mantovani. 1996. Migratory response of human NK cells to monocyte-chemotactic proteins. *Methods 10:145.*
51. Power, C. A., A. E. I. Proudfoot, E. Magnenat, K. B. Bacon, and T. N. C. Wells. 1994. Molecular cloning and characterisation of a neutrophil chemotactic protein from porcine platelets. *Eur. J. Biochem. 227:713.*
52. Erroi, A., M. Sironi, F. Chiaffarino, Z. G. Chen, M. Mengozzi, and A. Mantovani. 1989. IL-1 and IL-6 release by tumor associated macrophages from human ovarian carcinoma. *Int. J. Cancer 44:795.*
53. Economou, J. S., S. D. Colquhoun, T. M. Anderson, W. W. McBride, S. Golub, E. C. Holmes, and D. L. Morton. 1988. Interleukin 1 and tumor necrosis factor production by tumor-associated mononuclear leukocytes and peripheral mononuclear leukocytes in cancer patients. *Int. J. Cancer 42:712.*
54. Balkwill, F. R. 1994. Cytokine therapy of cancer: the importance of knowing the context. *Eur. Cytokine Network 5:379.*
55. Naylor, M. S., G. W. Stamp, W. D. Foulkes, D. Eccles, and F. R. Balkwill. 1993. Tumor necrosis factor and its receptors in human ovarian cancer: potential role in disease progression. *J. Clin. Invest. 91:2194.*

Hypoxia inhibits macrophage migration

Lynn Turner, Chris Scotton, Rupert Negus and Frances Balkwill

Biological Therapies Laboratory, Imperial Cancer Research Fund, London, GB

The chemokine monocyte chemoattractant protein (MCP)-1 plays a role in regulating the lymphocyte and macrophage infiltrate in ovarian cancer, but macrophages also accumulate in necrotic areas of the tumors where there is little MCP-1 expression (Negus, R. P. M. et al., *Am. J. Pathol.* 1997. **150:** 1723–1734). Necrotic regions are likely to be hypoxic. In this study we show that hypoxia inhibits MCP-1-induced migration of THP-1 monocytic cells and human macrophages. In contrast, lymphocytes from peripheral blood migrate normally to an MCP-1 gradient in hypoxic conditions. The inhibition of monocyte migration by hypoxia is rapid and reversible. At the exposure times studied (30–90 min) hypoxia does not affect expression of the MCP-1 receptor CCR2B and cells exposed to hypoxia still respond to MCP-1 with an elevation of intracellular calcium. Although hypoxia is known to modulate gene expression, the inhibition of migration reported here was not due to the production of soluble factors, and mRNA expression of macrophage migration inhibitory factor was unchanged. Hypoxia-induced inhibition of chemotaxis was not limited to MCP-1. Hypoxia also inhibited the chemotactic response to macrophage inflammatory protein-1α, RANTES and the chemoattractant N-formyl-met-leu-phe, but hypoxic cells were still able to phagocytose opsonized red blood cells. We suggest that inhibition of migration by hypoxia is not due to gene regulation but is a reflection of metabolic changes in the cell. Transient hypoxia may regulate the distribution of macrophages in tumors and other inflammatory conditions.

Received	8/3/99
Accepted	29/4/99

Key words: Hypoxia / Migration / Macrophage / Chemokine / Tumor

1 Introduction

Human ovarian carcinomas contain a leukocyte infiltrate that consists of mainly $CD68^+$ macrophages and $CD8^+$/$CD45RO^+$ T lymphocytes [1]. This accumulation of selected populations of leukocytes is similar to that seen in immune and inflammatory processes and is an orchestrated event involving coordinated expression of adhesion molecules and cell-specific chemokines [2].

One of the chemokines important in regulating the leukocyte infiltrate of ovarian cancer is monocyte-chemoattractant protein (MCP)-1. MCP-1 is a member of the CC class of chemokines and is a potent inducer of monocyte, macrophage and $CD45RO^+$ lymphocyte migration [2]. Chemokines bind to a family of related seven-transmembrane domain G protein-coupled receptors [3]. MCP-1 initiates most of its cellular responses by binding to the CC chemokine receptor 2, CCR2 [4]. Signal transduction pathways activated by MCP-1 involve pertussis toxin-sensitive phospholipase C activation [5], elevation of intracellular calcium levels [6] and phosphatidylinositol 3-kinase activation [7].

Levels of MCP-1 expression correlate with the extent of lymphocyte and macrophage infiltrate in ovarian biopsies [1]. MCP-1 protein has been detected in the ascites of patients with ovarian cancer, while MCP-1 mRNA can be detected in ovarian carcinoma sections and is localized to both tumor cell and stromal areas [8]. However, the highest density of macrophages is found in necrotic regions of the carcinoma, even though MCP-1 expression is not associated with these areas [1]. The necrotic regions are likely to have low oxygen tensions ($< 1\ \% O_2$) which also occur transiently in other tumor areas [9].

We previously reported that chemokine-induced migration of monocytes in PBMC preparations was inhibited by low oxygen tension [10]. In this study we have further examined the relationship between oxygen tension and leukocyte migration.

[I 19486]

Abbreviations: CCR: CC chemokine receptor **MCP-1:** Monocyte chemoattractant protein-1 **MIF:** Macrophage migration inhibitory factor **HIBS:** Heat-inactivated bovine serum **HPF:** High power field **MIP:** Macrophage inflammatory protein **fMLP:** N-formyl-met-leu-phe **HRE:** Hypoxia response element **HIF:** Hypoxia-inducible factor

0014-2980/99/0707-2280$17.50+.50/0

2 Results and discussion

2.1 Hypoxia inhibits MCP-1-induced migration of monocytes and macrophages

To study the influence of hypoxia on chemokine-induced migration, we used the monocytic cell line THP-1, and human macrophages and lymphocytes isolated from peripheral blood.

MCP-1 induced chemotaxis of THP-1 cells with a bell-shaped dose response curve characteristic of chemokine-induced migration. An incubation time of 90 min was found to be optimal (data not shown), with peak chemotactic activity at 10–100 ng/ml MCP-1 (Fig. 1A). To determine the relevance of oxygen tension to MCP-1-induced migration, chemotaxis experiments were performed under hypoxic conditions for the entire duration of the assay. Hypoxia inhibited MCP-1-induced migration of THP-1 cells at all concentrations of chemokine (Fig. 1B). This inhibition of THP-1 migration was used as a control in all other experiments. It was reversible and not due to cell death because THP-1 cells migrated to the same extent after 90 min hypoxia followed by re-oxygenation for 60 min, compared with cells that had been incubated under normoxia for the same period of time [in response to MCP-1, 79 ± 14 cells migrated in five high power fields (HPF) and 62 ± 13 cells migrated in five HPF, respectively].

Macrophages cultured from peripheral blood monocytes also migrated in response to MCP-1 and this migration was inhibited under hypoxic conditions (Fig. 2).

2.2 Hypoxia does not affect MCP-1-induced migration of lymphocytes

Hypoxia did not inhibit MCP-1-induced migration of PBL when six different donors were studied (Fig. 3). While hypoxia had no consistent effect on PBL migration, it did inhibit MCP-1-induced migration of THP-1 cells cultured under hypoxic conditions at the same time.

The mechanism(s) by which hypoxia might inhibit migration of THP-1 cells and macrophages were then investigated.

2.3 Hypoxia does not down-reguiate CCR2B expression

Exposure of THP-1 cells to hypoxia induces the release of cytokines such as TNF-α, IL-1 and IL-8 [11]. Inflammatory cytokines and LPS can down-regulate CCR2 [12]. However, incubation of THP-1 cells for 90 min under hypoxic conditions did not affect CCR2B mRNA levels (Fig.4).

2.4 Hypoxia does not affect early MCP-1-induced signaling

It was still possible that hypoxia affected the levels of CCR2 at the cell surface. We therefore studied the ability of MCP-1 to induce calcium flux after cells had been subjected to hypoxia. MCP-1-induced elevation of intracellular calcium was the same whether cells were incubated under normoxic (Fig. 5A) or hypoxic conditions (Fig. 5B) for 30–90 min after loading with fura-2AM.

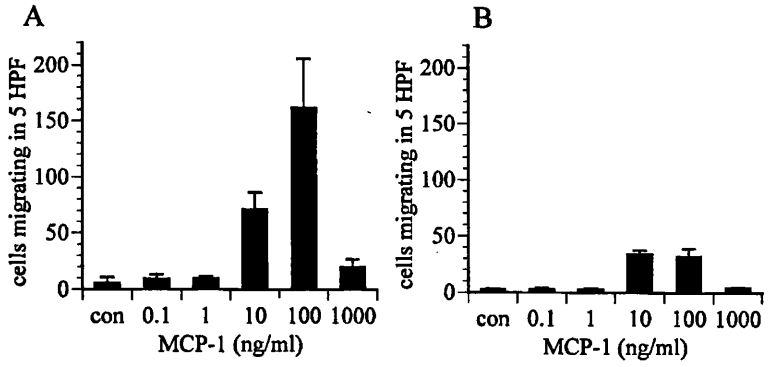


Figure 1. Migration of the monocytic cell line THP-1 in response to control (RPMI/BSA) or MCP-1 in a microchemotaxis assay under normoxic conditions (A) and hypoxic conditions (B). Migration was significantly reduced under hypoxic conditions at all concentrations of MCP-1. Results are representative of four other experiments.

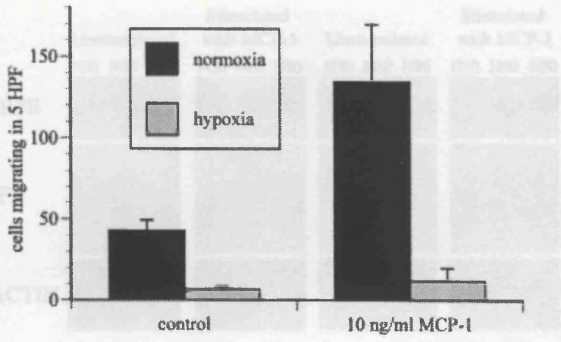


Figure 2. Migration of macrophages cultured from monocytes in response to 10 ng/ml MCP-1 in a microchemotaxis assay. MCP-1-induced migration of macrophages was significantly reduced under hypoxic conditions.

2.5 Hypoxia does not induce migration inhibitory factors

We next investigated the possibility that hypoxia induced a soluble factor that inhibited migration. One candidate would be macrophage migration inhibitory factor (MIF), a secretory product of activated lymphocytes and macrophages, which prevents the migration of macrophages [13]. We could not detect any changes in MIF mRNA after hypoxic incubation of THP-1 cells over the time course used for the chemotaxis assays (Fig. 4).

MIF protein ca be stored intracellularly, therefore we tested the possibility that a migration inhibitory factor may be secreted during hypoxic culture. Supernatants from THP-1 cells incubated under hypoxia for 90 min were added to THP-1 cells in a chemotaxis assay performed under normoxic conditions. The hypoxically cultured supernatants had no effect on MCP-1-induced migration (data not shown). This also suggested that hypoxia does not induce secretion of a chemokine that could destroy the chemotactic gradient established in the chemotaxis assay.

2.6 Hypoxia does not affect phagocytic activity

Cells that accumulate in areas of hypoxia are often observed to phagocytose debris from tissue damage. We therefore tested the influence of hypoxia on phagocytic activity of THP-1 cells under the same conditions that inhibited their migration. As shown in Table 1, normoxic and hypoxic cells had the same ability to phagocytose opsonized sheep RBC.

2.7 Hypoxic inhibition of migration is not limited to MCP-1

Hypoxia-induced inhibition of chemotaxis was not limited to MCP-1. Hypoxia also inhibited the migration of PBMC induced by macrophage inflammatory protein (MIP)-1α, RANTES and N-formyl-met-leu-phe (fMLP), a chemoattractant which signals through a G protein-linked receptor (Fig. 6).

In summary, the inhibitory effect of hypoxia on THP-1 cells and macrophages is fast, reversible, not due to the secretion of a migration inhibitory factor, not linked to receptor down-regulation, does not involve an early signaling event, does not affect phagocytic activity and is not limited to chemokine-induced migration.

This inhibition of migration is similar to that reported with inflammatory cytokines or LPS [12]. Exposure of monocytes to these inflammatory stimuli inhibits chemokine-

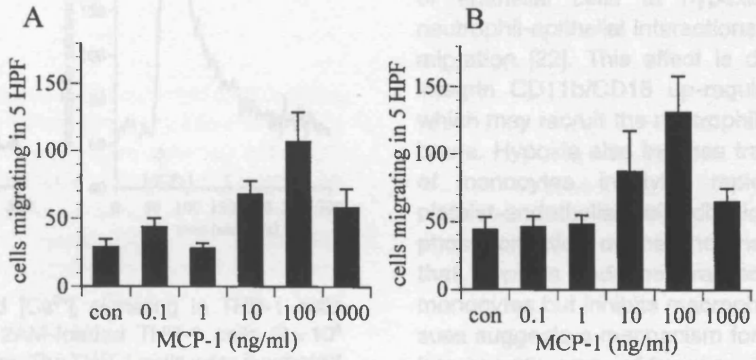


Figure 3. Migration of PBL in response to control (RPMI/BSA) or MCP-1 in a microchemotaxis assay under normoxic conditions (A) and hypoxic conditions (B). Results are representative of five other experiments.

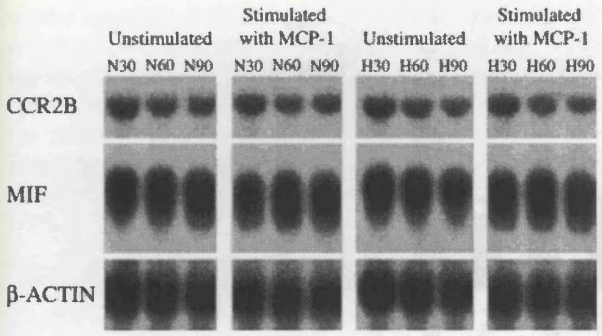


Figure 4. Northern analysis of CCR2B (the receptor for MCP-1) or MIF mRNA expression. THP-1 cells were cultured with or without MCP-1 stimulus, under normoxia (N) or hypoxia (H) for 30, 60 or 90 min. The results are representative of at least one other experiment.

induced migration is as little as 30 min. However, down-regulation of receptor mRNA and chemokine binding is observed later, after about 4 h exposure [12]. Thus hypoxia, inflammatory cytokines and bacterial products may all generate a "stop" signal for monocytes and macrophages that precedes inhibition of chemokine receptor signaling.

Exposure to hypoxic conditions modulates genes that orchestrate adaptive responses such as angiogenesis, erythropoiesis and glycolysis. Genes such as vascular endothelial growth factor (VEGF), IL-1α and IL-8 all contain hypoxia response elements (HRE) [14–16] which are recognized by a heterodimeric complex consisting of hypoxia-inducible factor HIF-1α and the Ah receptor nuclear translocator [17]. HIF-1α protein levels are

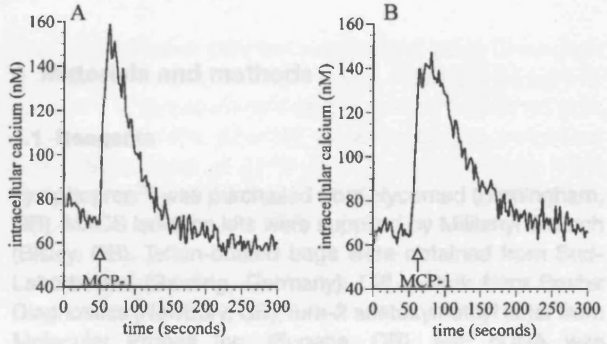


Figure 5. MCP-1-induced $[Ca^{2+}]_i$ signaling in THP-1 cells was determined in fura-2AM-loaded THP-1 cells (2×10^6 cells/ml) using PTI software. The THP-1 cells were incubated under normoxia (A) or hypoxia (B) for 30 min. Data are from a single representative experiment of at least one other experiment.

Table 1. Effect of hypoxia on THP-1 phagocytosis of sheep red blood cells (SRBC)[a)]

	% of THP-1 containing at least one SRBC	Average no. of SRBC per THP-1
Normoxia	88	2.5
Hypoxia	86	2.4

a) THP-1 cells were incubated with 2×10^7 IgG-SRBC under normoxia or hypoxia for 60 min at 37 °C. After non-phagocytosed SRBC were removed by density gradient centrifugation, phagocytosis was determined by Diff-Quik staining and microscopic examination. The results are expressed as the percentage of cells having phagocytosed at least one SRBC and the average number of phagocytosed SRBC per cell. For each parameter, 322 cells were examined.

strongly up-regulated in response to hypoxia due to post-transcriptional stabilization. Nuclear accumulation of HIF-1α is slow compared with other transcription factors [18] and up-regulation of genes containing HRE can take several hours [19].

In the case of hypoxia-induced inhibition of monocyte migration we suggest that this is not due to gene regulation but is a reflection of metabolic changes in the cell. By analogy, several PMN functions such as motility, chemotaxis and phagocytosis are impaired by short-term hypoxia and this reduction in motility may be a result of depletion of ATP stores [20]. Hypoxia can lead to reduced cellular oxidative status even after 30 min, suggesting a mechanism by which intracellular redox balance might regulate the mobility of PMN [21].

The situation is more complicated when transepithelial or transendothelial migration is considered. Pre-exposure of epithelial cells to hypoxia for 48 h modulates neutrophil-epithelial interactions, increasing PMN transmigration [22]. This effect is dependent upon the β2 integrin CD11b/CD18 up-regulation and IL-8 release which may recruit the neutrophils to the epithelial membrane. Hypoxia also induces transendothelial migration of monocytes, involving rapid PKC activation and platelet-endothelial cell adhesion molecule (PECAM)-1 phosphorylation of the endothelial cells [23]. The fact that hypoxia induces transendothelial migration of monocytes but inhibits macrophage migration in the tissues suggests a mechanism for directing macrophages into necrotic areas of tissues.

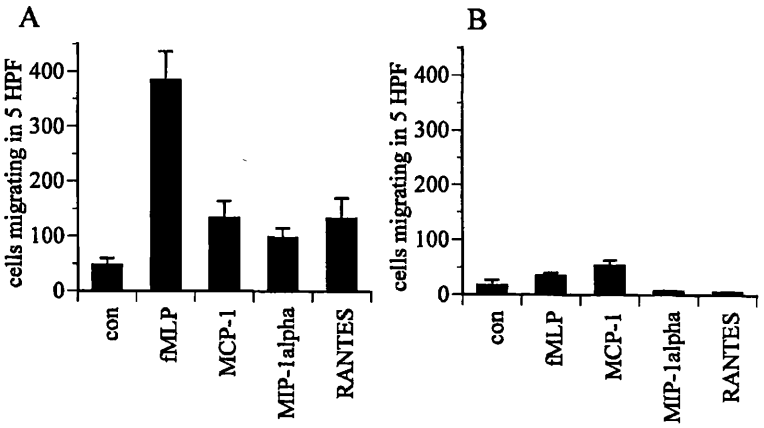


Figure 6. Migration of PBMC in response to control (RPMI/BSA), 10^{-7} M fMLP and 10 ng/ml MCP-1, MIP-1α and RANTES in a microchemotaxis assay under normoxic (A) or hypoxic (B) conditions.

3 Concluding remarks

If hypoxia also inhibits macrophage migration *in vivo* this could explain the high density of macrophages in areas of necrosis, even in the presence of a chemokine gradient. Our findings suggest that macrophages migrate in response to chemokine gradients throughout the tumor until they encounter a necrotic region where they accumulate and function as phagocytes clearing up cell debris. Unlike macrophage migration, PBL migration is not inhibited by hypoxia *in vitro* which could explain why lymphocytes are not clustered in areas of necrosis. This inhibition of macrophage migration by hypoxia is a mechanism by which the distribution of macrophages can be regulated in tumors and in other inflammatory conditions.

4 Materials and methods

4.1 Reagents

Lymphoprep™ was purchased from Nycomed (Birmingham, GB). MACS isolation kits were supplied by Militenyi Biotech (Bisley, GB). Teflon-coated bags were obtained from Sud-Laborbedarf (Gauting, Germany); Diff – Quik from Baxter Diagnostics (Newbury, GB); fura-2 acetoxymethyl ester from Molecular Probes Inc. (Eugene, OR). MIF cDNA was obtained from Graeme Wistow (NIH Bethesda, MD) and human chemokines from Peprotech (London, GB). Gases were purchased from BOC (Manchester, GB); the prime-IT kit from Stratagene (Cambridge, GB) and all other reagents were from Sigma (Poole, GB).

4.2 Cell lines and culture

The monocytic cell line, THP-1, was purchased from the American Type Culture Collection (Rockville, MD), and was grown in a humidified atmosphere at 37 °C (5 % CO_2) under pyrogen-free conditions in RPMI 1640 supplemented with 10 % heat-inactivated FBS (RPMI/HIBS) and 50 μM 2-ME.

4.3 Purification of cell populations from human peripheral blood

PBMC and PBL were prepared from the blood of normal donors, as described previously [24]. Briefly, the heparinized blood was centrifuged on Lymphoprep™ (density gradient of 1.077), and the mononuclear cell layer removed and washed thoroughly. PBMC in RPMI/HIBS were subjected to two-step plastic adherence to obtain purified PBL. Plastic adherence was performed for 1 and 18 h at 37 °C and the nonadherent cells collected. Morphological examination was used to assess the purity of the lymphocyte population (91.5 ± 1.4 %; $n = 4$). Isolation of monocytes from PBMC was performed by negative selection using a MACS monocyte isolation kit. PBMC were incubated with FcR blocking reagent (human Ig), followed by hapten-antibody cocktail (a cocktail of hapten-conjugated CD3, CD7, CD19, CD45RA, CD56 and anti-IgE mAb) and then MACS microbeads conjugated to an anti-hapten mAb. The magnetically labeled cell suspension was washed then passed through a magnetic field. Unlabeled cells were the negative fraction, the enriched monocyte population, which was then evaluated by flow cytometry using FITC-conjugated anti-CD14.

4.4 Culture of macrophages from monocytes

Purified monocytes were cultured in RPMI. 1640 supplemented with 10 % human AB serum for 7 days in teflon-

coated bags [25]. The resulting macrophages were washed and resuspended in RPMI 1640 supplemented with 10 % human AB serum for a further 15 h, then analyzed by flow cytometry using FITC-conjugated anti-CD14. The viability of the cell populations was assessed by trypan blue. All cell populations were resuspended at 2×10^6 cells/ml in RPMI 1640 supplemented with sterile (low endotoxin) BSA (RPMI/BSA) before assay.

4.5 Microchemotaxis assay

Chemotaxis was examined using a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD). The lower wells were filled with 27 µl chemoattractant in RPMI/BSA and covered with a 5-µm pore size polycarbonate membrane. Cell suspension (52 µl) was added to each upper well [26]. Migration under hypoxic conditions was performed by placing the chemotaxis chamber in a modular incubation chamber and gassing with 95 % N_2, 5 % CO_2 for the duration of the assay. After incubation at 37 °C for 90 min, the membrane was removed and the cells attached to the upper surface of the membrane were removed by washing with PBS. The membrane was fixed in methanol and stained with Diff-Quik. Cells attached to the lower surface of the membrane were counted in five HPF (×10 eyepiece, ×100 objective lens) per well.

4.6 Hypoxic culture

THP-1 cells were cultured with or without stimulus for 90 min in a 6-well plate. Cells were incubated at 37 °C under normoxia (5 % CO_2 in air) or hypoxia (5 % CO_2, balanced N_2) in a modular incubation chamber.

4.7 Northern analysis

RNA was prepared from cell suspensions using Tri-reagent, following the manufacturer's protocol. Total RNA (15 µg) was run on a 1 % agarose/formaldehyde gel and transferred onto a nylon membrane. After UV cross-linking, the membrane was prehybridized at 42 °C in 45 % formamide in 0.2 M phosphate buffer, 1 % BSA, 7 % SDS, 1 mM EDTA. cDNA probes were labeled with [^{32}P]CTP by random priming using a commercially available kit (Prime-It). Hybridization was performed overnight at 42 °C after which the membrane was washed twice in 2 × saline sodium citrate (SSC)/0.1 % SDS for 5 min at room temperature, twice in 0.1 × SSC/0.1 % SDS for 15 min at 70 °C, and lastly in 2 × SSC for 10 min at room temperature. The membrane was then exposed to film at –70 °C with an intensifying screen.

4.8 Determination of cytosolic free calcium

Cytosolic free calcium levels $[Ca^{2+}]_i$ were determined in cells that had been in normoxic or hypoxic culture. Cell populations were incubated with 2.5 µM fura-2 acetoxymethyl ester (fura-2AM) for 30 min [27], washed and incubated for 30–90 min under normoxia or hypoxia in a modular incubation chamber at 37 °C. The fluorescence of 2-ml aliquots was analyzed using a PTI fluorimeter with dual excitation wavelengths (340 and 380 nm) and a single emission wavelength (510 nm), before and after stimulation. The $[Ca^{2+}]_i$ was calculated using the ratio method [28] with PTI software.

4.9 Determination of phagocytosis

Sheep red blood cells (SRBC) were opsonized with specific IgG at 1/2000 dilution (IgG-SRBC). IgG-SRBC (2×10^7) were added to 0.5×10^6 cells in RPMI/HIBS and incubated for 60 min at 37 °C under normoxia or hypoxia in a modular incubation chamber [29]. Non-phagocytosed SRBC were removed by density gradient centrifugation. The remaining cells were washed and phagocytosis was determined by Diff-Quik staining and microscopic examination. The results are expressed as the percentage of cells having phagocytosed at least one SRBC and the average number of phagocytosed SRBC per cell. For each parameter, 322 cells were examined.

Acknowledgments: We thank Mr. D. Davies and Mr. A. Rae (Imperial Cancer Research Fund, London) for their help with flow cytometry; Dr. N. Hogg (Imperial Cancer Research Fund, London) for helpful discussions; Dr. P. Bastiaens (Imperial Cancer Research Fund, London) for help with and use of his fluorimeter; Professor Alberto Mantovani (Mario Negri Institute, Milan, Italy) for the gift of CCR2B cDNA and Dr. G. Wistow (NIH Bethesda, MD) for the gift of MIF cDNA.

5 References

1 **Negus, R. P. M., Stamp, G. W. H., Hadley, J. and Balkwill, F. R.,** Quantitative assessment of the leukocyte infiltrate in ovarian-cancer and its relationship to the expression of c-c chemokines. *Am. J. Pathol.* 1997. **150:** 1723–1734.

2 **Sozzani, S., Zhou, D., Locati, M., Bernasconi, S., Luini, W., Mantovani, A. and O'Flaherty, J. T.,** Stimulating properties of 5-oxo-eicosanoids for human monocytes: synergism with monocyte chemotactic protein-1 and -3. *J. Immunol.* 1996. **157:** 4664–4671.

3 **Baggiolini, M. and Moser, B.,** Blocking chemokine receptors. *J. Exp. Med.* 1997. **186:** 1189–1191.

4 **Boring, L., Gosling, J., Chensue, S. W., Kunkel, S. L., Farese, R. V., Broxmeyer, H. E. and Charo, I. F.,** Impaired monocyte migration and reduced type-1 (th1) cytokine responses in c-c chemokine receptor-2 knockout mice. *J. Clin. Invest.* 1997. **100:** 2552–2561.

5 **Kuang, Y., Wu, Y., Jiang, H. and Wu, D.,** Selective G protein coupling by C-C chemokine receptors. *J. Biol. Chem.* 1996. **271:** 3975–3978.

6 **Charo, I. F., Myers, S. J., Herman, A., Franci, C., Connolly, A. J. and Coughlin, S. R.,** Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl-terminal tails. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 2752–2756.

7 **Turner, S. J., Domain, J., Waterfield, M. D., Ward, S. G. and Westwick, J.,** The CC chemokine monocyte chemotactic peptide-1 activates both the class I p85/p110 phosphatidylinositol 3-kinase and the class II PI3K-C2alpha. *J. Biol. Chem.* 1998. **273:** 25987–25995.

8 **Negus, R. P., Stamp, G. W., Relf, M. G., Burke, F., Malik, S. T., Bernasconi, S., Allavena, P., Sozzani, S., Mantovani, A. and Balkwill, F. R.,** The detection and localization of monocyte chemoattractant protein-1 (MCP-1) in human ovarian cancer. *J. Clin. Invest.* 1995. **95:** 2391–2396.

9 **Durand, R. E. and Raleigh, J. A.,** Identification of nonproliferating but viable hypoxic tumor cells *in vivo*. *Cancer Res.* 1998. **58:** 3547–3550.

10 **Negus, R. P., Turner, L., Burke, F. and Balkwill, F. R.,** Hypoxia down-regulates MCP-1 expression: implications for macrophage distribution in tumors. *J. Leukoc. Biol.* 1998. **63:** 758–765.

11 **Scannell, G.,** Leukocyte responses to hypoxic/ischemic conditions. *New Horiz.* 1996. **4:** 179–183.

12 **Sica, A., Saccani, A., Borsatti, A., Power, C. A., Wells, T. N., Luini, W., Polentarutti, N., Sozzani, S. and Mantovani, A.,** Bacterial lipopolysaccharide rapidly inhibits expression of C-C chemokine receptors in human monocytes. *J. Exp. Med.* 1997. **185:** 969–974.

13 **Bernhagen, J., Calandra, T. and Bucala, R.,** Regulation of the immune response by macrophage migration inhibitory factor: biological and structural features. *J. Mol. Med.* 1998. **76:** 151–161.

14 **Bunn, H. F. and Poyton, R. O.,** Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* 1996. **76:** 839–885.

15 **Carmeliet, P., Dor, Y., Herbert, J. M., Fukumura, D., Brusselmans, K., Dewerchin, M., Neeman, M., Bono, F., Abramovitch, R., Maxwell, P., Koch, C. J., Ratcliffe, P., Moons, L., Jain, R. K., Collen, D. and Keshet, E.,** Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 1998. **394:** 485–490.

16 **Sampson, L. E. and Chaplin, D. J.,** The influence of oxygen and carbon dioxide tension on the production of TNF alpha by activated macrophages. *Br. J. Cancer* 1996. **74:** S133–135.

17 **Gradin, K., McGuire, J., Wenger, R. H., Kvietikova, I., Whitelaw, M. L., Toftgard, R., Tora, L., Gassmann, M. and Poellinger, L.,** Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol. Cell. Biol.* 1996. **16:** 5221–5231.

18 **Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L.,** Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *EMBO J.* 1998. **17:** 6573–6586.

19 **Semenza, G. L., Roth, P. H., Fang, H. M. and Wang, G. L.,** Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. Biol. Chem.* 1994. **269:** 23757–23763.

20 **Rotstein, O. D., Fiegel, V. D., Simmons, R. L. and Knighton, D. R.,** The deleterious effect of reduced pH and hypoxia on neutrophil migration *in vitro*. *J. Surg. Res.* 1988. **45:** 298–303.

21 **Montoya, M. C., Lusclnskas, F. W., del Pozo, M. A., Aragones, J. and de Landazuri, M. O.,** Reduced intracellular oxidative metabolism promotes firm adhesion of human polymorphonuclear leukocytes to vascular endothelium under flow conditions. *Eur. J. Immunol.* 1997. **27:** 1942–1951.

22 **Colgan, S. P., Dzus, A. L. and Parkos, C. A.,** Epithelial exposure to hypoxia modulates neutrophil transepithelial migration. *J. Exp. Med.* 1996. **184:** 1003–1015.

23 **Kalra, V. K., Shen, Y., Sultana, C. and Rattan, V.,** Hypoxia induces PECAM-1 phosphorylation and transendothelial migration of monocytes. *Am. J. Physiol.* 1996. **271:** H2025–2034.

24 **Bacon, K. B., Camp, R. D., Cunningham, F. M. and Woollard, P. M.,** Contrasting *in vitro* lymphocyte chemotactic activity of the hydroxyl enantiomers of 12-hydroxy-5,8,10,14-eicosatetraenoic acid. *Br. J. Pharmacol.* 1988. **95:** 966–974.

25 **Landmann, R., Ludwig, C., Obrist, R. and Obrecht, J. P.,** Effect of cytokines and lipopolysaccharide on CD14 antigen expression in human monocytes and macrophages. *J. Cell. Biochem.* 1991. **47:** 317–329.

26 **Turner, L., Ward, S. G. and Westwick, J.,** A role for phospholnositide 3-kinase in RANTES induced chemotaxis of T lymphocytes. *Biochem. Soc. Trans.* 1995. **23:** 283S.

27 **Ward, S. G., Cantrell, D. and Westwick, J.,** Inhibition by staurosporine of mitogen-induced calcium mobilisation in human T lymphoblasts. *FEBS Lett.* 1988. **239:** 363–366.

28 **Grynkiewicz, G., Poenie, M. and Tsien, R. Y.,** A new generation of CA^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 1985. **260:** 3440–3450.

29 **Capo, C., Meconi, S., Sanguedolce, M. V., Bardin, N., Flatau, G., Boquet, P. and Mege, J. L.,** Effect of cytotoxic necrotizing factor-1 on actin cytoskeleton in human monocytes: role in the regulation of integrin-dependent phagocytosis. *J. Immunol.* 1998. **161:** 4301–4308.

Correspondence: Frances Balkwill, Biological Therapies Laboratory, Imperial Cancer Research Fund, 44 Lincoln's Inn Fields, London, WC2A 3PX, GB
Fax: +44-171 269 3094
e-mail: f.balkwill@icrf.icnet.uk