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*Enrico Gurattini*  
The Open University, UK

— Advanced School of Pharmacology —  
Dean, Enrico Gurattini M D

Mario Negri Institute for  
Pharmacological Research

2/10/2006

# Effects of tumour hypoxia on cell migration

A thesis submitted for the degree of Doctor of Philosophy  
at The Open University

February 2006

Istituto di Ricerche Farmacologiche  
"Mario Negri"

AUTHOR NO: W2692142  
DATE OF SUBMISSION: 28 FEBRUARY 2006  
DATE OF AWARD: 13 SEPTEMBER 2006

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## Abstract

Cell adaptation to hypoxia requires activation of transcriptional programs that coordinate expression of genes involved in oxygen delivery (via angiogenesis) and metabolic adaptation (via glycolysis). During migration and invasion of normal and pathological tissues, cells may encounter different oxygen levels, due to poor or altered vascularization, and recent evidence has suggested that chemotaxis is a cell function which may be affected by oxygen availability.

This thesis describes how oxygen availability is a determinant parameter in the setting of chemotactic responsiveness to Stromal-Derived Factor 1 (SDF-1, CXCL12). Low oxygen concentration induces high expression of the CXCL12 receptor CXCR4, in different cell types (monocytes, monocyte-derived macrophages, tumor associated macrophages, endothelial cells, cancer cells and dendritic cells) as both mRNA and protein expression, which is paralleled by increased chemotactic responsiveness to its specific ligand. Furthermore, preliminary results on dendritic cells (DC) show that hypoxia may affect their maturation ( $CCR7^{low}/CCR5^{high}$ ) and functions. In particular, hypoxia-derived DC do not migrate in response to the CCR7 ligand CCL19, while they do express higher levels of pro-inflammatory cytokines (IL-12, TNF- $\alpha$ ), as compared to normoxia-derived DC. CXCR4 induction by hypoxia is dependent on both activation of hypoxia-inducible factor 1 (HIF-1 $\alpha$ ) and transcript stabilization. Our data identify the hypoxia/HIF-1/CXCR4 pathway as a relevant molecular circuit in the functional tuning of the chemokine system and provide novel insights into the mechanisms controlling cell migration in hypoxic regions, with potential relevance in the pathogenesis of human diseases, including chronic inflammatory diseases and cancer.

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## Aknowledgements

It's really impossible for me to list all the people that helped me either directly or indirectly during my PhD, but this doesn't mean that I don't recognize their support and their value.

First I would like to thank my director of studies, Antonio Sica, for giving me the opportunity to do a PhD in the first place, for his support and advices during the five years at Mario Negri Institute.

Second I would like to thank my external supervisors, Frances Balkwill and James Pease, for accepting me as PhD student and helping me during these three years with big discussions and correction of my English.

I would like to thank everyone (past and present) in the Department of Immunology and Cell Biology and in particular in the laboratory of Molecular Immunology for giving me advice, for teaching me experimental techniques, joining me for very good lunches or dinners and for friendship but they are so many that I cannot mention all of them (it could be another thesis) so don't worry I will prepare a big cake to thank everybody!!

Extra special thanks go to: Alessandra Sacconi for her teaching and friendship and in particular for hearing me to repeat my presentation for congresses or seminars; Nadia Polentarutti for her teaching and advices not only about experimental problems, and for being as a "mother" for me in these years in Milan without Lucio. I would also like to thank Andrea Doni for helping me with confocal analysis, dendritic cells and also for our good dinner at Cascina Caremma; Manuela Nebuloni for her help with immunoistochemistry and section cutting; Elena Riboldi for the purification of plasmacytoid dendritic cells and Chiara Buracchi for murine dendritic cells; Sergio Bernasconi for reading my few samples at the cytofluorimeter (I think that I gave him

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more than 1000 samples!!!); a particular memory to Walter Luini who unfortunately is not anymore with us. He taught me everything about chemotaxis assay.

I cannot forget to thank all the people that contributed to my PhD fellowship “Pignatelli”. It was very nice to interact with them and I really appreciate the possibility that they gave me to show them my results (we had very nice dining-seminars, too!).

I must thank all people that I have known in Milan and that offered me a kind and precious friendship: Nicola, Max, Elena, Samantha, Cristina, Lorenzo, Marcella, Sergio, Paola, Riccardo, Federica, Daniela, Andrea, Patrick, Charlotte, Ruth, Pere.

My Dad, Mum and my brother Daniele for supporting me always at school as in other more important moments of my life. Thanks to my grandfather for his fantastic poetry (I hope for another one for my PhD).

Finally, a huge thank you goes to Lucio. Thanks for giving me the possibility to do this PhD, to support these three years alone in Dublin and now in London. Thanks for your patience with my few informatic knowledges and for tolerating me when I’m so nervous. Now we are together.....

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*This thesis is dedicated to a special person: my husband*

*Lucio*

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# Chapter 1. Introduction

## 1.1. Chemokines

### 1.1.1. Introduction

From the genesis of human life to birth and beyond cell migration is a complex, extremely important process that is not completely understood by researchers. During embryogenesis, cells move in sheets or loosely attached populations to create complex tissues. In adults, cell motility is crucial to maintain immunity, or repair damaged tissues. The recruitment of leukocytes in normal and pathological conditions is mainly regulated by chemokines and their receptors. However, cell migration is not only regulated by chemokines, but also by other factors such as the complement component C5a, lipid derivatives leukotrienes and platelet-activating factor, and bacterial N-formylmethionyl-peptides.

Chemokines have been recognized as a family of functionally related small-secreted molecules named “chemo-kine” because of leukocyte chemoattractant and cytokine-like activities. The capability to induce migration identifies molecules belonging to this family. The first chemokines to be discovered were Platelet Factor 4 (now called CXCL4) and  $\beta$ -thromboglobulin in the late 1970s (Deuel, Keim et al. 1977; Begg, Pepper et al. 1978). However, chemokines were not recognized as chemotactic molecules until the characterization of interleukin 8 (IL-8, now called CXCL8) in 1987 (Yoshimura, Matsushima et al. 1987).

The early chemokines were discovered because of their association with inflammatory responses, although, recently, genome sequencing has added numerous members with additional roles.

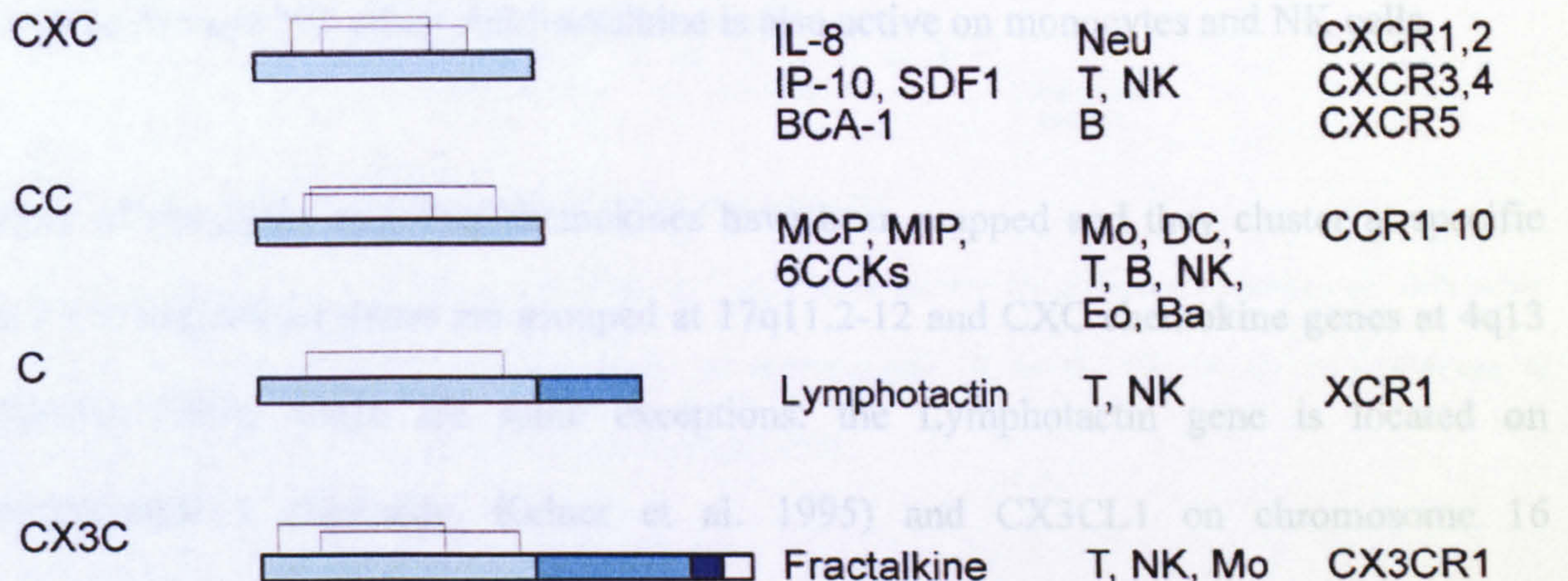
In little more than a decade since the discovery of CXCL8 about 50 human chemokines and nearly 20 receptors have been identified and characterized. Research in this field has



dramatically changed our understanding of leukocyte traffic in inflammation and immunity.

### 1.1.2. Chemokine classification

Chemokines are composed of single polypeptide chains 70-100 amino acids in length, with 20-95% sequence identity to each other including conserved cysteine residues. The position of these conserved cysteines has been used for subfamilies definition and nomenclature (Vicari, Ait-Yahia et al. 2000) (Figure 1.1).



**Figure 1.1. Chemokine subfamilies**

Sub families of chemokines are classified following the disposition of the cysteine residues at the N-terminal domain of the molecules. Example of members, producing cells and receptors are listed. (light cyan: chemokine domain; dark cyan: transmembrane domain; blue: mucin-like stalk; white: cytoplasmic domain).

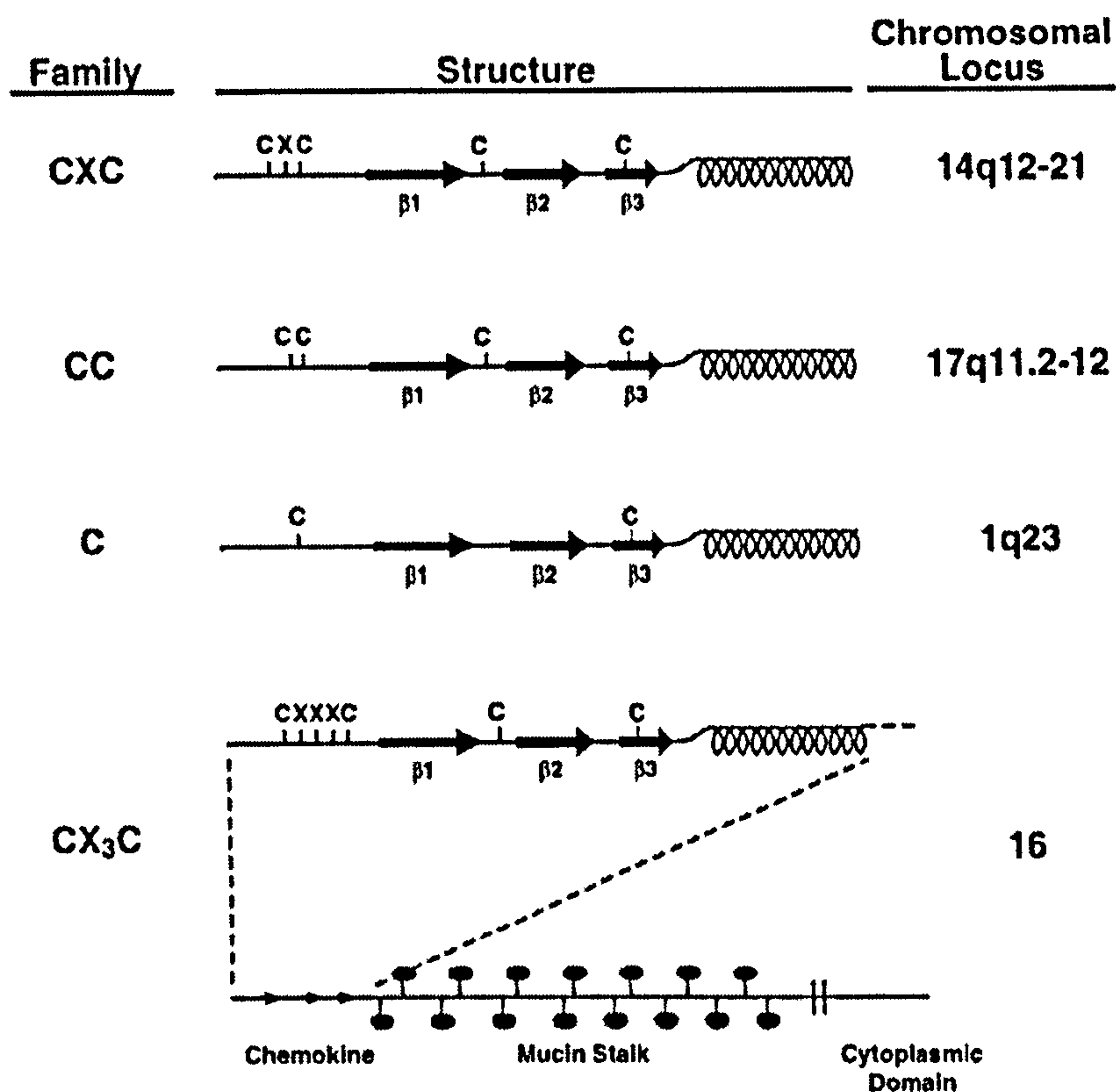
Two main sub-families, CXC and CC chemokines, are distinguished according to the position of the first two cysteines, which are separated by one amino acid (CXC) or adjacent to each other (CC). The cysteines form two disulfide bonds (Cys1→Cys3 and Cys2→Cys4), which confer to the chemokine their characteristic three-dimensional folding (Lodi, Garrett et al. 1994). CXC chemokines are active on neutrophils (PMN) and lymphocytes while CC chemokines exert their action on multiple leukocyte subtypes,



including monocytes, basophils, eosinophils, T lymphocytes, dendritic cells and NK cells, but they are generally inactive on PMN. Eotaxins represent the chemokines with the most restricted spectrum of action being selectively active on eosinophils, basophils and Th2 mast cells. A third subfamily includes molecules with only two conserved cysteine residues (C chemokines) and comprises only one member lymphotactin (Kennedy, Kelner et al. 1995). Finally, the fourth family is composed only by fractalkine. This is a membrane-bound mucin bearing an amino-terminal chemokine-like domain with three amino acids between the first two cysteines (CX3C motif) (Bazan, Bacon et al. 1997); (Pan, Lloyd et al. 1997). These two chemokines are both active on lymphoid cells (T lymphocytes and NK cells) and fractalkine is also active on monocytes and NK cells.

Many of the genes encoding chemokines have been mapped and they cluster at specific loci. CC chemokine genes are grouped at 17q11.2-12 and CXC chemokine genes at 4q13 (Rollins 1997). There are some exceptions: the Lymphotactin gene is located on chromosome 1 (Kennedy, Kelner et al. 1995) and CX3CL1 on chromosome 16 (Nomiya, Imai et al. 1998) (Figure 1.2).

This suggests that some chemokines arose by duplication and divergence from a primordial chemokine gene.



**Figure 1.2. Localization of chemokine genes on chromosome**

Chemokines are divided in four sub-families and each gene cluster on different chromosome with some exception: in the CXC family CXCL12 maps to chromosome 10, in the CC family CCL19 maps to chromosome 9 and CCL20 to chromosome 2 (Rollins 1997).

Chemokine genes were designated long ago, as SCY (for small, secreted cytokines) and numbered chronologically. Whilst the systematic nomenclature has been generally adopted for the receptors, chemokines are still mostly designated by their traditional names. A systematic nomenclature for chemokines and chemokine receptors became necessary as more and more new molecules were found. This classification (Zlotnik and Yoshie 2000) relies on the principle established for the receptors at the 1996 Gordon Conference on Chemotactic Cytokines. The receptors are defined as CXC, CC, XC, and CX<sub>3</sub>C followed by R and a number. The chemokines are defined by the same structure-related acronyms followed by L (for ligand) and the number of their genes (Table 1.1). This new nomenclature will be used throughout this thesis.

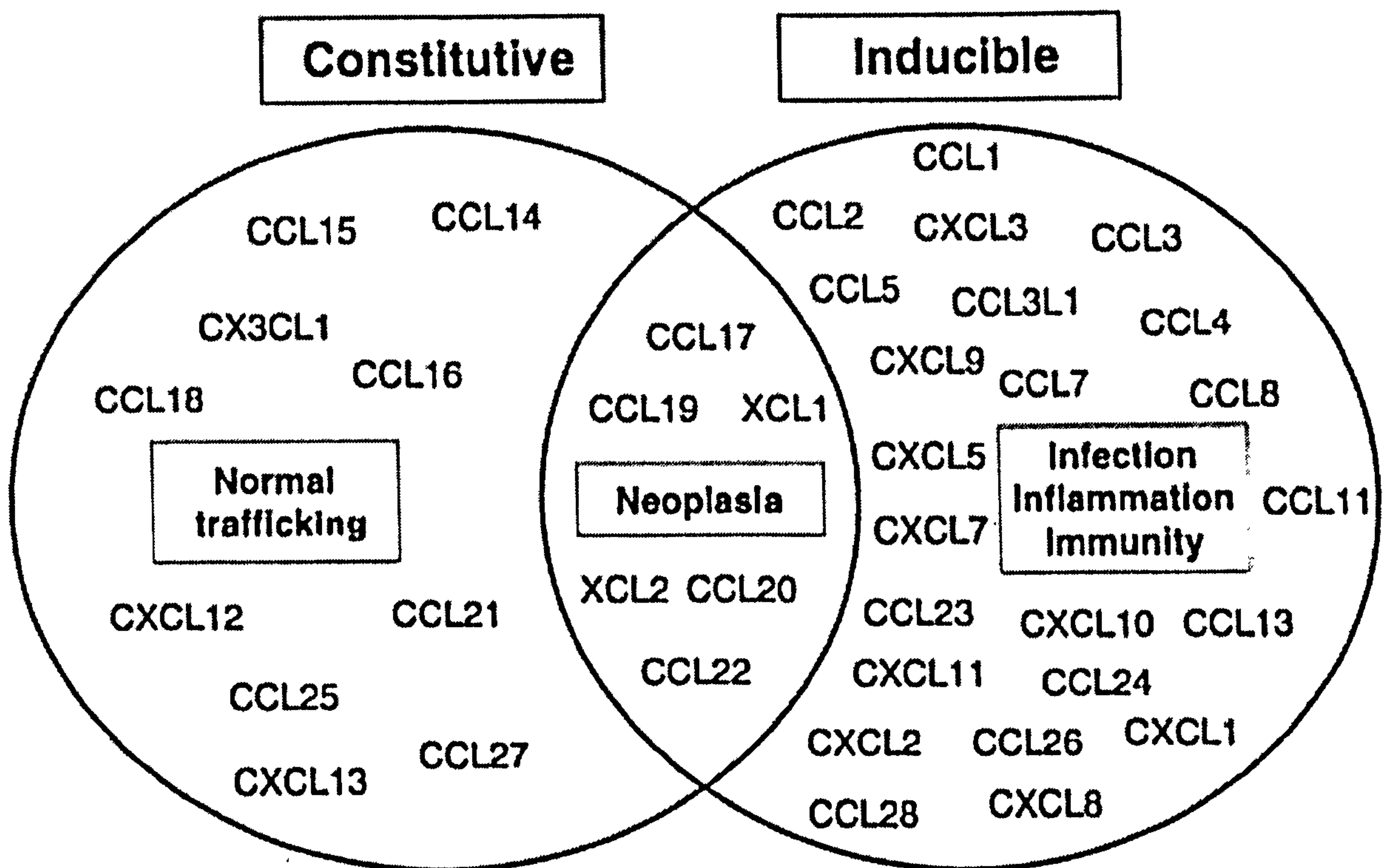


Systematic name	Human chromosome	Human ligand	Mouse ligand
<b>CXC chemokines</b>			
CXCL1	4q21.1.	GRO $\alpha$ /MGSA- $\alpha$	GRO1/KC/MIP-2
CXCL2	4q21.1	GRO $\beta$ /MGSA- $\beta$	GRO1/KC/MIP-2
CXCL3	4q21.1	GRO $\gamma$ /MGSA- $\gamma$	GRO1/KC/MIP-2
CXCL4	4q21.1	PF4	PF4
CXCL5	4q21.1	ENA-78	LIX
CXCL6	4q21.1	GCP-2	CK $\alpha$ -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-1 $\alpha/\beta$	SDF-1
CXCL13	4q21.1	BCA-1	BLC
CXCL14	5q31.1	BRAK/bolekine	BRAK
(CXCL15)	Unknown	Unknown	Lungkine
CXCL16	17p13	SR-PSOX	SR-PSOX
<b>C Chemokines</b>			
XCL1	1q24.2	Lymphotactin/SCM-1 $\alpha$	Lymphotactin
XCL2	1q24.2	SCM-1 $\beta$	Unknown
<b>CX3C chemokines</b>			
CX3CL1	16q13	Fractalkine	Neurotactin
<b>CC chemokines</b>			
CCL1	17q11.2	I-309	TCA-3
CCL2	17q11.2	MCP-1/MCAF/TDCF	JE
CCL3	17q12	MIP-1 $\alpha$ /LD78 $\alpha$	MIP-1 $\alpha$
CCL4	17q12	MIP-1 $\beta$	MIP-1 $\beta$
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-1 $\delta$	Unknown
CCL16	17q12	HCC-4/LEC	LCC-1
CCL17	16q13	TARC	TARC
CCL18	17q12	DC-CK1/PARC	Unknown
CCL19	9p13.3	MIP-3 $\beta$ /ELC/Exodus-3	MIP-3 $\beta$ /ELC
CCL20	2q36.3	MIP-3 $\alpha$ /LARC/Exodus-1	MIP-3 $\alpha$ /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/CK $\beta$ 6/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 human homologue has not yet been discovered. Adapted from IUIS/WHO Subcommittee (2003).

Chemokines were also grouped in two different subfamilies: inflammatory (also called inducible chemokines) or homeostatic (or constitutive) chemokines (Figure 1.3). Inflammatory chemokines control the recruitment of effector leukocytes in infection, inflammation, tissue injury and tumours. Homeostatic chemokines, by contrast, navigate leukocytes during hematopoiesis in the bone marrow and thymus, and during initiation of adaptive immune responses in the spleen and lymph nodes (Mantovani 1999; Moser, Wolf et al. 2004).



**Figure 1.3. Constitutive and inducible chemokines: two overlapping realms**

Constitutive chemokines are produced in a tonic fashion in lymphoid and/or nonlymphoid organs: they probably direct the normal traffic of leukocytes under physiological conditions. Inducible chemokines are produced in response to microbial, inflammatory or immune signals and account for increased recruitment of leukocytes under these conditions. The distinction between constitutive and inducible chemokines is useful but schematic because the two realms overlap in terms of pathology and molecules. In neoplasia, inducible chemokines such as CCL2 are made constitutively. Moreover, certain constitutive chemokines expressed in lymphoid organs in the absence of deliberate stimulation, such as CCL22, are also produced in an inducible way. Adapted from (Locati, Otero et al. 2002).



CXC chemokines can be further classified according to the presence of the tripeptide motif glutamic acid-leucine-arginine (ELR) in the NH<sub>2</sub>-terminal region. ELR<sup>+</sup> chemokines are specific for myeloid cells, are angiogenic and the first angiogenic chemokine to be described was CXCL8/IL-8 in 1992 (Koch, Polverini et al. 1992). On the other hand ELR<sup>-</sup> chemokines attract a variety of leukocytes without a clear specificity and they are anti-angiogenic chemokines. The first member of this family to be identified was CXCL4/PF4. It is a weak attractant for neutrophils (Deuel, Senior et al. 1981) but it is an inhibitor of angiogenesis, probably by inhibiting endothelial cell proliferation (Maione, Gray et al. 1990).

## **1.2. Chemokine receptors**

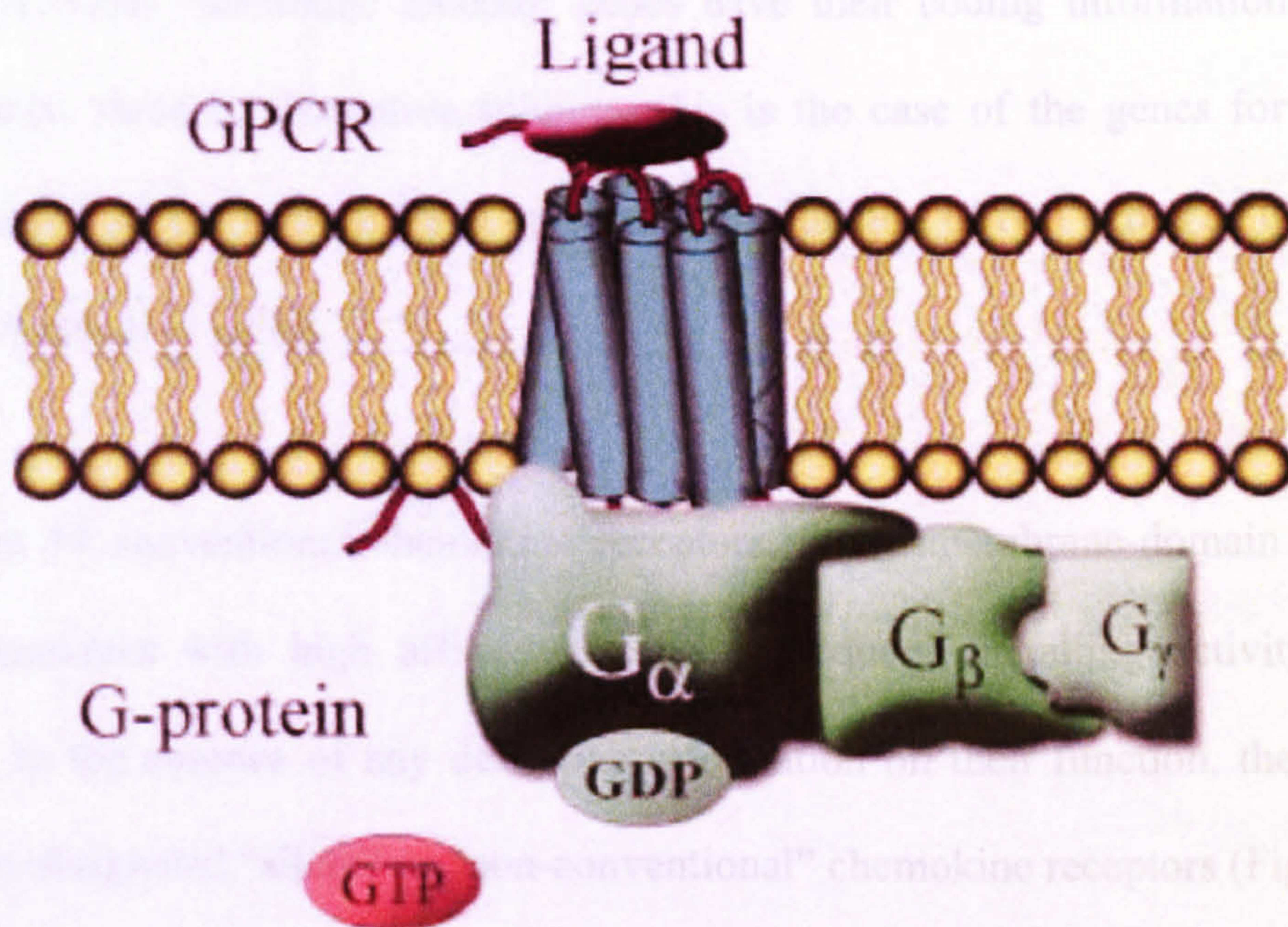
### **1.2.1. Introduction**

Biochemical and functional analysis with CXCL8 and related chemokines demonstrated that neutrophils express two types of CXCL8 receptor, one with selectivity for CXCL8 and GCP-2/CXCL6, and another with promiscuous binding of CXCL8 and numerous other related CXC chemokines (Baggiolini, Dewald et al. 1994). The subsequent cloning of the corresponding receptor cDNAs (called CXCR1 and CXCR2) confirmed these early observations and, in addition, provided molecular tools for the subsequent cloning of novel chemokine receptor genes. Currently, 20 human chemokine receptors are known, including six CXCRs, ten CCRs, one XCR, and one CX3CR, and they are known to bind a total of more than 50 human chemokines.



### 1.2.2. Chemokine receptor nomenclature

Chemokines act through the binding to chemokine receptors which are members of a distinct subfamily in the rhodopsin-like G protein-coupled receptors. (Figure 1.4) (Thelen 2001; Murphy 2002; Moser, Wolf et al. 2004).



**Figure 1.4. G protein coupled receptors structure**

The G protein coupled receptors structure consists of a single polypeptide chain that threads back and forth seven times across the phospholipids bilayer that forms the membrane. Downloaded and adapted from the website: [www.mpibp-frankfurt.mpg.de/reinhart](http://www.mpibp-frankfurt.mpg.de/reinhart).

Experiments with *Bordetella pertussis* toxin indicated that these receptors typically require G proteins of the  $G_i$ -type for signal transduction (Thelen, Peveri et al. 1988).

Chemokine receptors can typically bind several different chemokines, although this rarely occurs between different chemokine families. Therefore they are classified based on the chemokine family that they bind. Chemokine receptors are single polypeptide chains that span the membrane 7 times, with a typically acidic N-terminal extracellular domain and a serine/threonine-rich intracellular C-terminal domain. Two disulfide bonds in between the N-terminal domain and the second extracellular loop and the first and third extracellular



loops are normally required for the definition of the molecular structure. As for ligands, 20-85% amino acid identity to each other is observed, particularly in transmembrane and intracellular domains. The significant identity score and some structural aspects indicate that both ligand and receptor families arose from common ancestors by repetitive gene duplication. Most chemokine receptor genes have their coding information on a single exon. Rarely, there is alternative splicing: this is the case of the genes for CCR2a and CCR2b which appear to be formed by alternative splicing of two exons of the same gene (Charo, Myers et al. 1994).

Other than 18 conventional chemokine receptors, 7-transmembrane-domain proteins that bind chemokines with high affinity and no subsequent signalling activity have been reported. In the absence of any definitive information on their function, these molecules have been designated “silent” or “non-conventional” chemokine receptors (Figure 1.5).



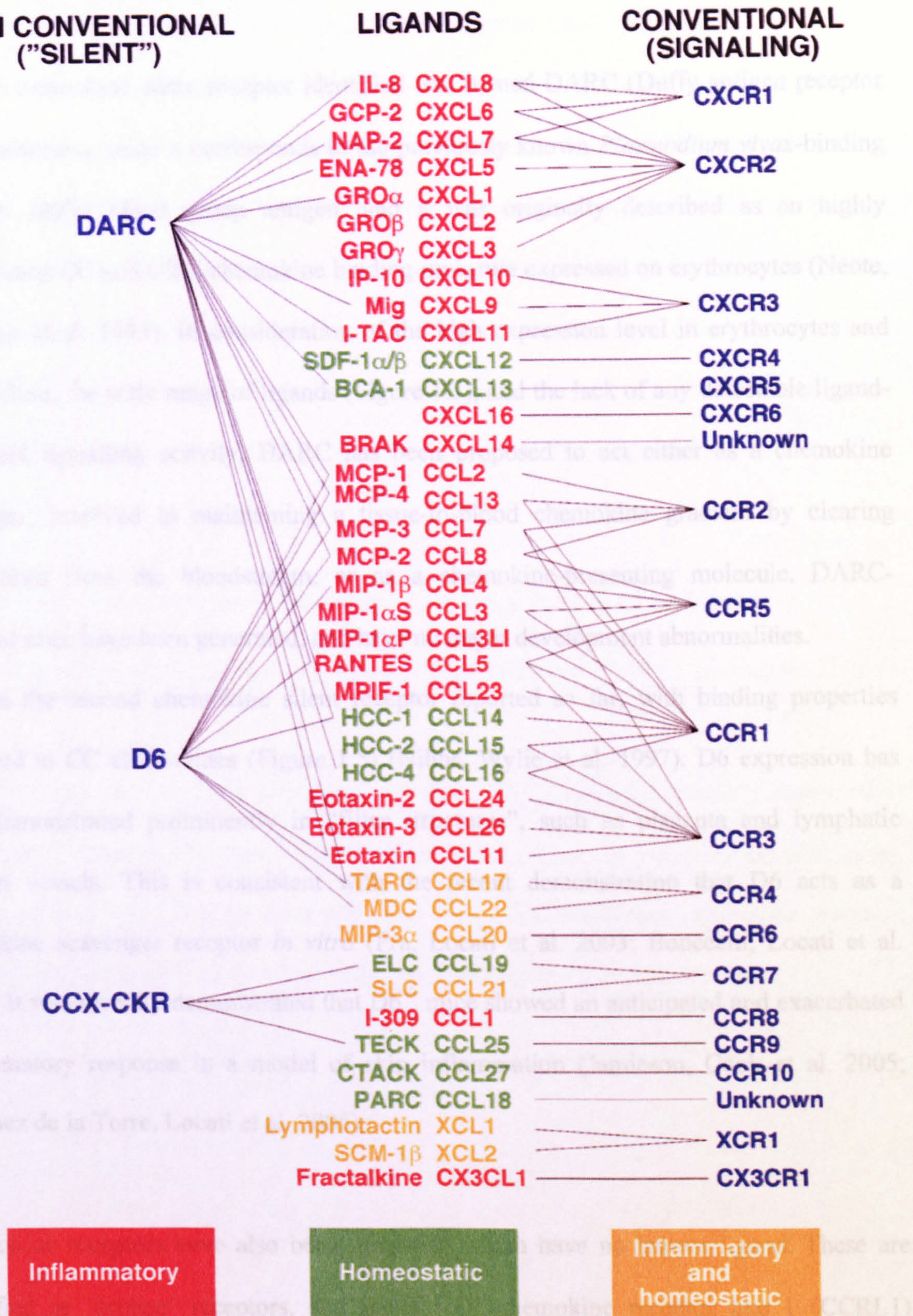


Figure 1.5. Ligand specificity of non conventional and conventional chemokine receptors.

Conventional receptors are listed on the right, "silent" receptors on the left. The central column list chemokines identified with one old acronym and with the new nomenclature in which the first part of the name identifies the family and L stands for "ligand" followed by a progressive number. Adapted from (Mantovani, Sica et al. 2004).



The first chemokine silent receptor identified was named DARC (Duffy antigen receptor for chemokines), since it corresponds to the previously known *Plasmodium vivax*-binding molecule Duffy blood group antigen, and it was originally described as an highly promiscuous CC and CXC chemokine binding molecule expressed on erythrocytes (Neote, Darbonne et al. 1993). In consideration of the high expression level in erythrocytes and endothelium, the wide range of ligands (Figure 1.5), and the lack of any detectable ligand-dependent signalling activity, DARC has been proposed to act either as a chemokine scavenger, involved in maintaining a tissue-to-blood chemokine gradient by clearing chemokines from the bloodstream, or as a chemokine-presenting molecule. DARC-deficient mice have been generated, and have no major development abnormalities.

D6 was the second chemokine silent receptor reported so far, with binding properties restricted to CC chemokines (Figure 1.5) (Nibbs, Wylie et al. 1997). D6 expression has been demonstrated prominently in “filter structures”, such as placenta and lymphatic afferent vessels. This is consistent with the recent demonstration that D6 acts as a chemokine scavenger receptor *in vitro* (Fra, Locati et al. 2003; Bonecchi, Locati et al. 2004). It was recently demonstrated that D6<sup>-/-</sup> mice showed an anticipated and exacerbated inflammatory response in a model of skin inflammation (Jamieson, Cook et al. 2005; Martinez de la Torre, Locati et al. 2005).

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 (Khoja, Wang et al. 2000) and HCR which is expressed in lymphoid tissues such as the spleen, lymph nodes and bone marrow (Fan, Kyaw et al. 1998). Functional chemokine receptor genes have also been found in viruses, for example ECFR3 in Herpesvirus saimiri (Ahuja and Murphy 1993), which has structural



similarities to CXCR1 and CXCR2, and US28 in human CMV (Gao and Murphy 1994) which can bind multiple CC chemokines with high affinity. Some viruses as HHV-8 (Couty and Gershengorn 2004) and EBV (Uchihara, Krensky et al. 2005) might selectively mimic chemokines or chemokine receptors in order to subvert or inactivate the host defence (Nicholas 2005). Virus-encoded chemokines [e.g. vMIP-I, vMIP-II and v-MIP-III; (Murphy 2001)] are either antagonists that block leukocyte recruitment to sites of infection, or agonists that could enhance the recruitment of cells that support viral replication or prevent Th1 anti-viral responses (Alcami and Koszinowski 2000).

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

Chemokine receptors are selectively expressed on different types of leukocytes (Figure 1.6).

Some receptors are restricted to certain cells (e.g., CXCR1 is predominantly restricted to neutrophils), whereas others are widely expressed (e.g., CCR2 is expressed on monocytes, T cells, natural killer cells, dendritic cells, and basophils). In addition, chemokine receptors are constitutively expressed on some cells, whereas they are inducible on others.



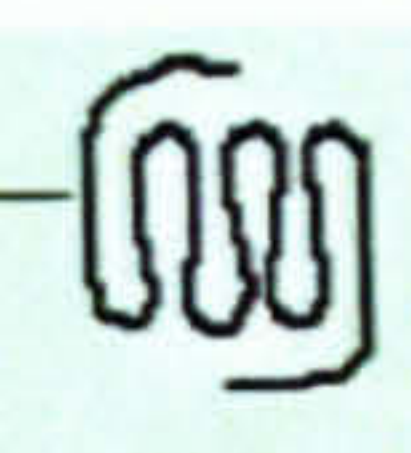








Chemokine	Receptor	Cell Type
		
MCP-3, -4; MIP-1 $\alpha$ ; RANTES MCP-3, -4; eotaxin-1, -2; RANTES	CCR1 CCR3	Eosinophil 
MCP-1, -2, -3, -4, -5 MCP-3, -4; eotaxin-1, -2; RANTES	CCR2 CCR3	Basophil 
MCP-3, -4; MIP-1 $\alpha$ ; RANTES MCP-1, -2, -3, -4, -5 MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES I-309 MDC, HCC-1, TECK	CCR1 CCR2 CCR5 CCR8 ?	Monocyte 
Fractalkine	CX <sub>3</sub> CR1	
SDF-1	CXCR4	
MCP-3, -4; MIP-1 $\alpha$ ; RANTES MCP-1, -2, -3, -4, -5 TARC MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES MIP-3 $\beta$ (ELC) PARC, SLC, 6CKine (Exodus-2)	CCR1 CCR2 CCR4 CCR5 CCR7 ?	Activated T cell 
Fractalkine	CX <sub>3</sub> CR1	
IP-10, MIG, I-TAC	CXCR3	
PARC, DC-CK1 Lymphotactin	? ?	Resting T cell 
SDF-1	CXCR4	
MCP-3, -4; MIP-1 $\alpha$ ; RANTES MCP-1, -2, -3, -4, -5 MCP-3, -4; eotaxin-1, -2; RANTES TARC MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES MIP-3 $\alpha$ (LARC, Exodus-1) MDC, TECK	CCR1 CCR2 CCR3 CCR4 CCR5 CCR6 ?	Dendritic cell 
SDF-1	CXCR4	
Interleukin-8, GCP-2 Interleukin-8, GCP-2; GRO- $\alpha$ , - $\beta$ , - $\gamma$ ; ENA-78; NAP-2; LIX	CXCR1 CXCR2	Neutrophil 
MCP-1, -2, -3, -4, -5 MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES	CCR2 CCR5	
Fractalkine	CX <sub>3</sub> CR1	Natural killer cell 
IP-10, MIG, I-TAC	CXCR3	

Figure 1.6. Cells types that express chemokine receptors

A range of different cells types can express various chemokine receptors (Luster 1998).

Along with agonist production, regulation of chemokine receptors expression is a crucial event for the tuning of the chemokine system (Mantovani 1999). CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes only after



stimulation by IL-2 (Loetscher, Geiser et al. 1994). In addition, some constitutive chemokine receptors can be down-regulated; CCR2 is down-regulated by LPS, making the cells unresponsive to CCL2, but remains responsive to CCL3/4 (Sica, Saccani et al. 1997). Chemokine receptors are also regulated by pro- and anti-inflammatory stimuli with divergent effects: pro-inflammatory stimuli as IFN $\gamma$  downregulate CCR2 receptor while anti-inflammatory stimuli as IL-10 upregulate CCR2 expression (Sozzani, Ghezzi et al. 1998).

In contrast, the expression of other chemokine receptors is restricted to a cell state of activation and differentiation. For example CXCR3 is expressed on activated T lymphocytes of the T helper type 1 (Th1) phenotype, whereas CCR3, is expressed on activated lymphocytes of the T helper type 2 (Th2) phenotype (Sallusto, Mackay et al. 1997; Bonecchi, Bianchi et al. 1998). Some chemokine receptors are also expressed on nonhematopoietic cells, including neurons, astrocytes, epithelial cells, and endothelial cells. This suggests that the chemokine system has other roles in addition to leukocyte chemotaxis.

#### **1.2.4. Chemokine receptor signaling**

There are still many open questions with regard to signalling elements involved in the initiation of leukocyte migration as opposed to those signalling elements controlling migration-unrelated processes. Figure 1.7 gives an overview of signalling via chemokine receptors.

The signal transduction mechanisms activated by chemokines such as CCL2 and CCL5 have been extensively investigated (Sozzani, Molino et al. 1993; Kuang, Wu et al. 1996). 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 (Wu,



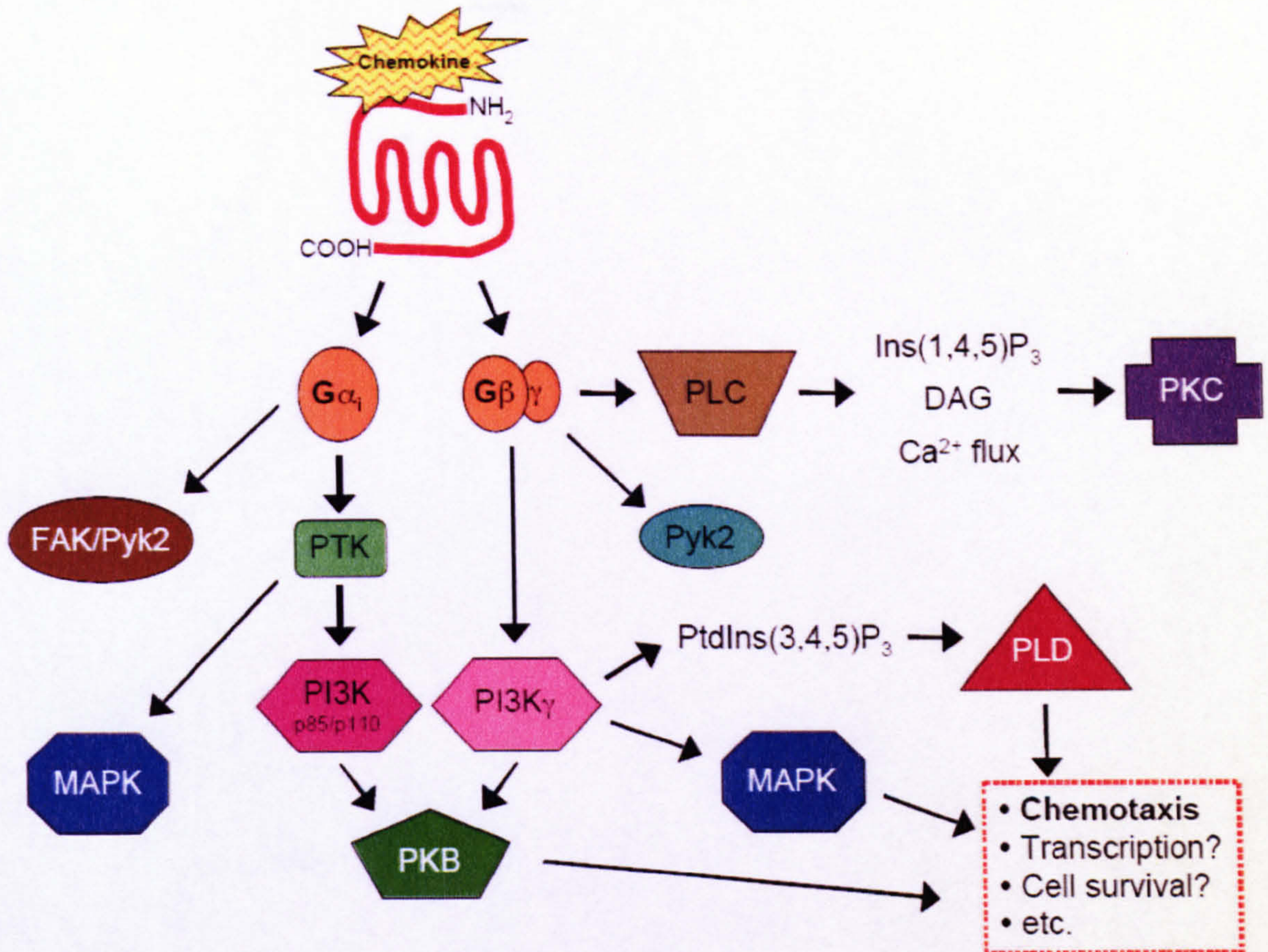
LaRosa et al. 1993); CXCR1 activates phospholipase D (PLD) and causes the formation of superoxide, but CXCR2 does not (Jones, Wolf et al. 1996). The mechanism for receptor-specific signal transduction remains to be elucidated.

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 (Turner, Ward et al. 1995). 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 (Neptune, Iiri et al. 1999). Experiments in *Dictyostelium discoideum* have shown that  $\beta\gamma$  subunits accumulate at the leading edge of migrating amoebae, supporting their role in chemotaxis (Jin, Zhang et al. 2000).

Multiple intracellular second messengers are coupled to chemokine receptor-mediated G protein activation, including phospholipase C $\beta$  (PLC $\beta$ ) isoforms, Ser/Thr-kinases, phosphatidylinositol-3-kinase- $\gamma$  (PI3K $\gamma$ ) and c-Src-related non-receptor tyrosine kinases (Thelen 2001).

The  $G_{\beta\gamma}$  subunit can then activate PLC- $\beta$ 2 and PLC- $\beta$ 3; these isoenzymes are involved in chemokine signal transduction in immune cells (Li, Jiang et al. 2000) and lead to inositol-1,4,5-trisphosphate formation (IP3) and a transient rise in the intracellular free calcium concentration. There is no calcium flux in neutrophils from PLC- $\beta$ 2 and PLC- $\beta$ 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 (Richardson, DuBose et al. 1995). It can also activate the respiratory burst in neutrophils (Li, Jiang et al. 2000).





**Figure 1.7. Signalling through chemokine receptors**

This diagram illustrates some of the potential downstream signalling pathways following chemokine binding to a chemokine receptor. The  $\beta\gamma$  subunits can activate Phospholipase C (PLC) and PI3K $\gamma$ , leading to activation of Protein Kinase C (PKC), Phospholipase 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 (Thelen 2001).

Perhaps most importantly, chemokine stimulation leads to the activation of PI3K $\gamma$  (Turner, Domin et al. 1998).

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 (Hirsch, Katanaev et al. 2000; Sasaki, Irie-Sasaki et al. 2000). 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 (PKB) (Burgering and Coffey 1995). PKB (also known as Akt) can have numerous downstream effects, including promotion of cell survival (Chan, Rittenhouse et al. 1999). In prostate cancer, increased Akt/PKB activity has also been shown to contribute to tumour progression, by accelerating tumour growth (Graff, Konicek et al. 2000). PKB is also involved in chemotaxis and is recruited to the leading edge of cells undergoing migration (Servant, Weiner et al. 2000). Chemokines (including CXCL8, CXCL12, CCL2 and CCL20) have also been shown to activate the MAPK cascade (Sullivan, McGrath et al. 1999; Majka, Ratajczak et al. 2000; Ashida, Arai et al. 2001). The G $\beta\gamma$  subunit leads to activation of PI3K $\gamma$ , and this in turn can activate the MAPK cascade, possibly through its intrinsic protein kinase activity, rather than its ability to phosphorylate lipids (Lopez-Illasaca, Crespo et al. 1997; Bondeva, Pirola et al. 1998). 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 (Boehme, Sullivan et al. 1999; Sotsios, Whittaker et al. 1999). Yet PD98059 does not block the migration of neutrophils to IL-8 (Knall, Worthen et al. 1997).

Phospholipase D (PLD) is also activated in response to chemokines including CXCL8 and CCL5 (Bacon, Flores-Romo et al. 1995; Bacon, Schall et al. 1998) and may be involved in rearrangement of the actin cytoskeleton (Du, Altshuler et al. 2000). 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 (Mellado, Rodriguez-Frade et al. 1998; Vila-Coro, Rodriguez-Frade et al. 1999); ligation of CXCR4 can also activate Pyk2 and NF $\kappa$ B (Ganju, Brubaker et al. 1998) and CCL5 can activate focal adhesion kinase (FAK) (Bacon, Szabo et al. 1996). 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 (Arai, Monteclaro et al. 1997; Guinamard, Signoret et al. 1999; Huttenrauch, Pollok-Kopp et al. 2005; Nasser, Marjoram et al. 2005). 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) (Lefkowitz 1998). GRKs are recruited to the cell membrane by G $\beta\gamma$ , where they can phosphorylate the chemokine receptor; arrestins can then bind to the receptor, sterically inhibit the binding of G proteins and cause internalisation (Barlic, Khandaker et al. 1999; Cheng, Zhao et al. 2000).

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 (Tilton, Ho et al. 2000). 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 function**

In the last ten years it became clear that chemokines not only function as chemoattractants but their interaction with the receptors influences processes such as angiogenesis,

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hematopoiesis, inflammation and T cell differentiation. This section will outline some of these functions. The particular role of CXCR4 and CXCL12 will also be described, since the results chapters concentrate on this chemokine/receptor pair.

In the chemokine/chemokine receptor system there is an apparent redundancy: 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. The importance of chemokine/chemokine receptors is shown by knockout mice. Our understanding of the roles of chemokines in physiological and pathological processes has advanced significantly. It has become clear that in addition to wound healing, metastasis, angiogenesis/angiostasis, cell recruitment, lymphoid organ development, and lymphoid trafficking (Zlotnik and Yoshie 2000) chemokines are fundamental in mediating innate and adaptive immune responses by their ability to activate cells of the immune system (Ward, Bacon et al. 1998). Chemokine gene disruption studies have confirmed most of these biological functions. For example, the CCL3 knockout mouse was the first to be generated. While developmentally normal with no apparent lymphoid or myeloid defects, these mice were reduced in their ability to mount an inflammatory response to influenza infection (Cook, Beck et al. 1995). CCR7-deficient mice exhibit impaired lymphocyte migration, delayed antibody responses and defects in lymphoid architecture signifying an important role for CCR7 signalling in coordinating primary immune responses (Forster, Schubel et al. 1999). Lastly, mutating CCR1, CCR2 or CCR5 in mice impairs monocyte functions such as chemokine-dependent chemotaxis and alters the balance of Th1 or Th2 cytokine responses upon challenge with Th class-specific antigens or pathogens (Boring, Gosling et al. 1997; Gao, Wynn et al. 1997; Gerard, Frossard et al. 1997; Zhou, Kurihara et al. 1998; Sato, Ahuja et al. 2000).

Aspects of these different functions will be discussed briefly below.



### 1.3.1. Role in normal physiology

#### 1.3.1.i. Chemotaxis

Chemotaxis can be defined as the directed migration towards a gradient of soluble extracellular agents. Cells showing positive chemotaxis move towards areas with higher concentration of these agents, whilst those showing negative chemotaxis move away from these areas. This behaviour is in marked contrast to the reactions elicited by some other compounds, known as chemokinesis, which is characterized essentially by an undirected movement of cells. Dose dependent migration of cells induced by gradients of substratum-bound substances has been called 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.

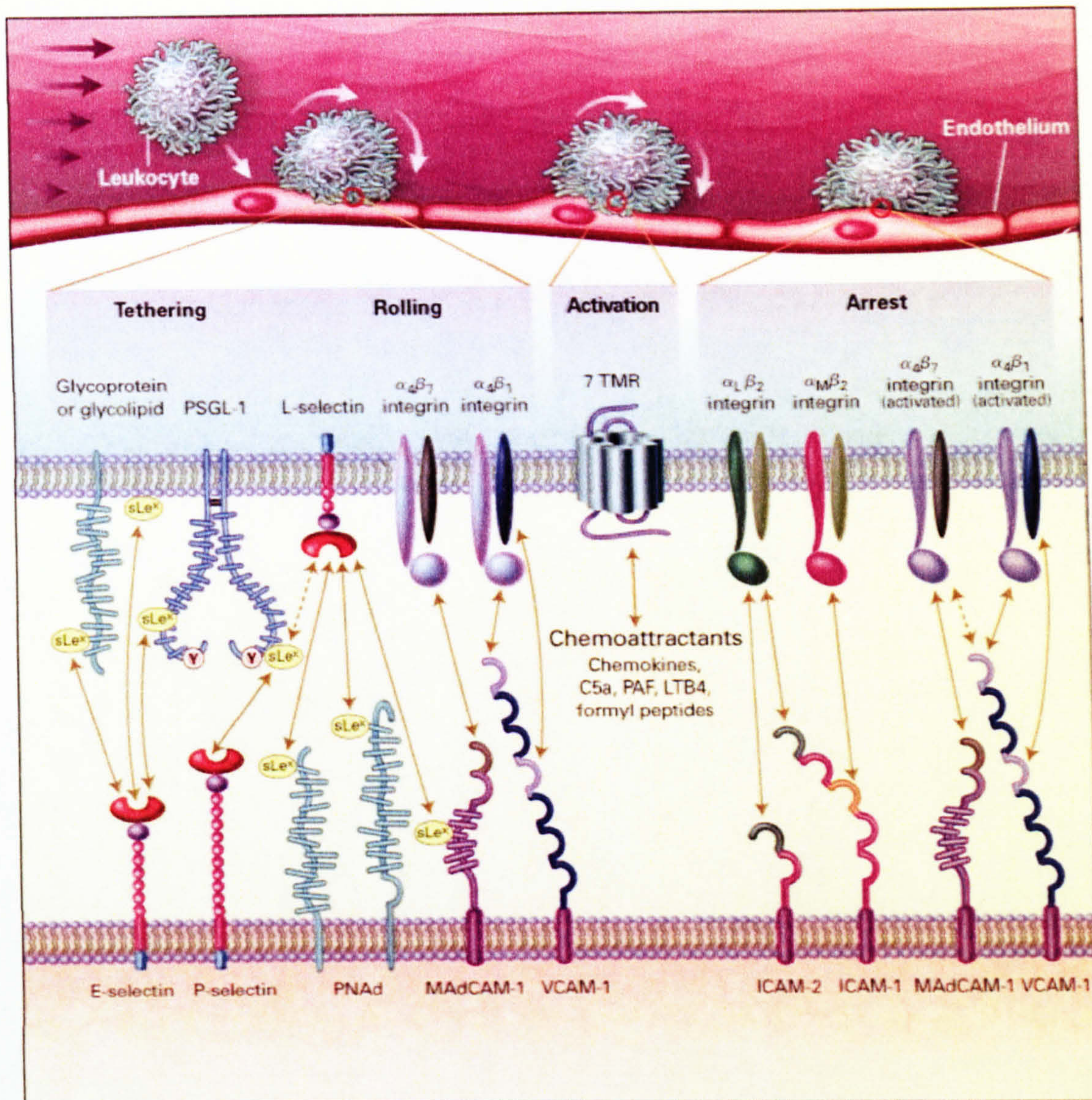
The process of migration includes cell polarization, the formation of lamellipodia and filipodia and the attachment of cell to the surface on which it is migrating (Balkwill 1998). This process is driven by actin filaments while surface integrins are thought to guide the cell (Parsey and Lewis 1993).

The capability of chemokines to guide cell migration is fundamental to their role in normal and pathological physiology.

#### 1.3.1.ii. Leukocyte trafficking.

Chemokines are thought to provide the directional cues for the movement of leukocytes in development, homeostasis, and inflammation. Leukocyte extravasion from the blood into the tissues is a regulated multistep process involving a series of coordinated interactions between leukocytes and endothelial cells (Figure 1.8) (Springer 1994).





**Figure 1.8. Leukocyte extravasation from the blood into the tissues**

Leukocytes in the bloodstream become tethered to endothelial cells and roll slowly downstream. Tethering is greatly facilitated by leukocyte receptors, whereas subsequent rolling is not influenced by the topography of adhesion receptors. Rolling leukocytes respond to chemoattractants on endothelial cells and the activating signal induces rapid activation of  $\beta_2$  and  $\alpha_4$  integrins. The  $\alpha_4$  integrins can mediate activation-independent rolling interactions as well as arrest rolling leukocytes. From (von Andrian and Mackay 2000).

Several families of molecular regulators, such as selectins, integrins, and chemokines, are thought to control different aspects of this process. Selectins facilitate the movement of leukocytes along the surface of endothelial cells (rolling). Chemokines are thought to provide the signals that convert the low affinity, selectin-mediated interaction into the



higher-affinity, integrin mediated interaction that leads to extravasion of leukocytes (Luster 1998). Chemokines are believed to control the homeostatic circulation of leukocytes through tissues. The continuous recirculation of lymphocytes through the blood, tissue, and lymphatics in an organized manner brings naïve lymphocytes into the lymph nodes, where they encounter antigen and are transformed in memory lymphocytes that migrate into inflamed tissue to ensure immunity. Macrophages, eosinophils, and mast cells also migrate into tissues (Ono, Nakamura et al. 2003). Although these cells are produced in the bone marrow, they reside primarily in other tissues. The role of chemokines in regulating the movement of cells into tissues has begun to be elucidated on the basis of studies in mice deficient in a particular chemokine. For example, CXCL12 is critical for the migration of myeloid precursors from the fetal liver to the bone marrow (Nagasawa, Hirota et al. 1996), and CCL11 is important in the recruitment of eosinophils into tissues (Rothenberg, MacLean et al. 1997). Therefore, chemokines and chemokine receptors play a vital role in the trafficking of leukocytes to and from secondary lymphoid organs, and this function is important 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 (Youn, Mantel et al. 2000; Lataillade, Domenech et al. 2004). Members of the chemokine family, including CXC, CC and C chemokines, have been shown to suppress haematopoiesis (Broxmeyer and Kim 1999). Work by Reid *et al* (Reid, Ritchie et al. 1999) and Haneline *et al* (Haneline, L.S., 1998).



*Blood* 91:4092-4098) 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 (Youn, Mantel et al. 2000).

HPC can express CXCR4 and CCR7 and migrate towards CXCL12, CCL19 and CCL21 (Kim, Bae et al. 1998). 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 (D'Apuzzo, Rolink et al. 1997; Zou, Kottmann et al. 1998).

#### 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* (Karpus, Lukacs et al. 1997; Sallusto, Lanzavecchia et al. 1998). CCL2 addition to macrophages *in vitro* can also decrease IL-12 (a Th1 cytokine) expression (Chensue, Warmington et al. 1996). 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- $\gamma$ -producing cells (Karpus, Lukacs et al. 1997) and hence Th1 differentiation. Similarly, CCL3, 4 and 5 production by monocyte-derived DC can promote the development of IFN- $\gamma$ -producing cells (Zou, Borvak et al. 2000). 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 (Cook, Beck et al. 1995; Gao, Wynn et al. 1997; Gu, Tseng et al. 2000; Traynor, Kuziel et al. 2000). For example, CCL2-deficient mice have increased *Leishmania* resistance, indicating a shift from Th2 to Th1 (Gu, Tseng et al. 2000), while CCR2-deficient mice have diminished IFN- $\gamma$  production, increased Th2-type cytokines and higher IgE, suggesting a shift from Th 1 to Th 2 (Blease, Mehrad et al. 2000; Traynor, Kuziel et al. 2000). 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**

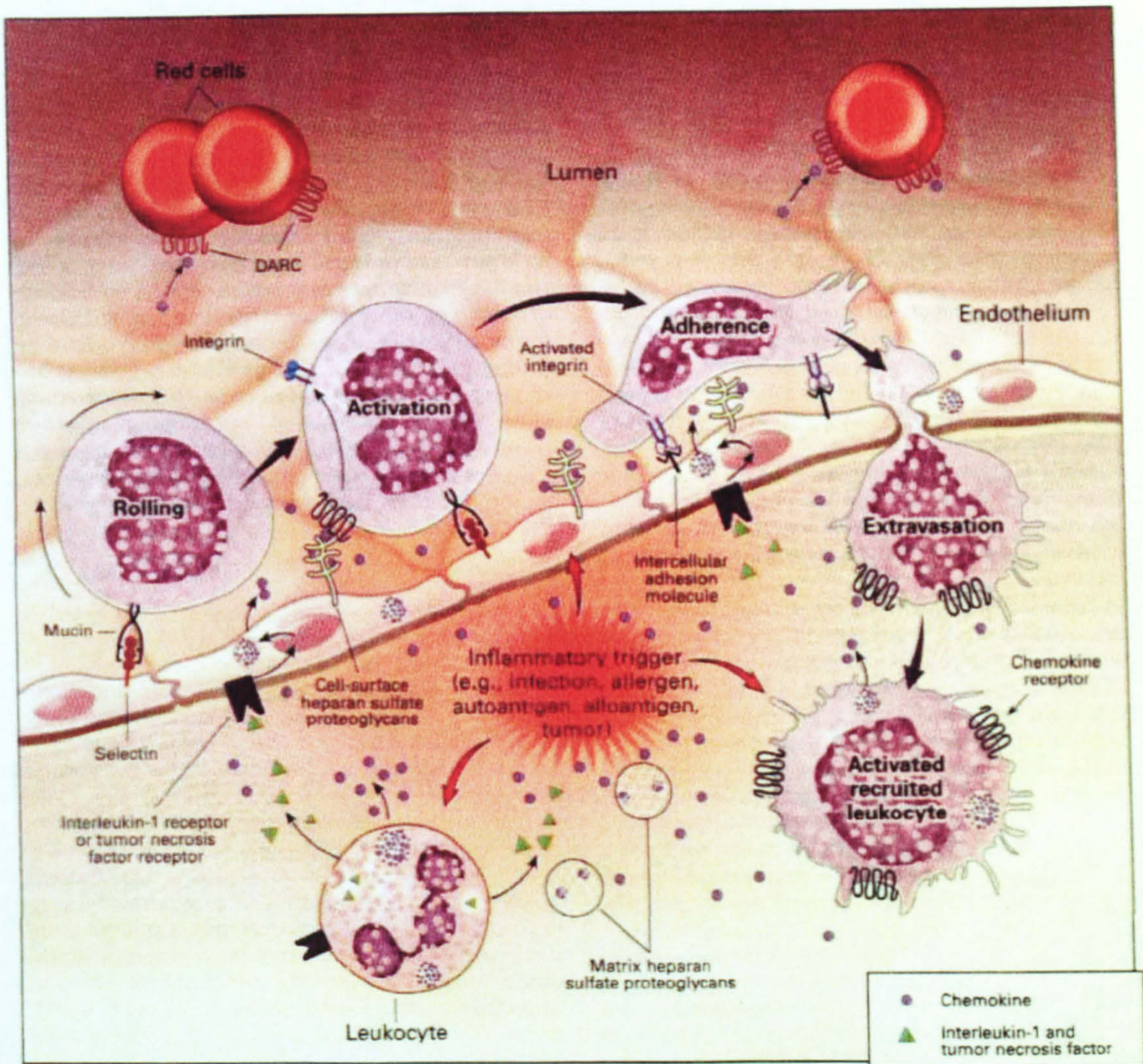
The vital role of chemokines in directing leukocyte traffic during immune responses necessarily involves chemokines/receptors in infection, as well as inflammation and various other disease states. Thus, viral infection, autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, allergy,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

#### **1.3.2.i. Tissue recruitment of leukocytes**

The dramatic increase in the secretion of chemokines during inflammation results in the selective recruitment of leukocytes into inflamed tissue. Chemokines are produced during inflammation in most organs as for example skin, brain, joints, and lungs. Leukocyte



infiltration in inflammatory sites would be a special case of this general model of leukocyte movement (Figure 1.9).



**Figure 1.9. 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. From (Luster 1998).

Production of chemokines by sentinel cells at an inflammatory focus may be responsible for inducing strong adhesive interactions between rolling leukocytes and the endothelium. Diapedesing cells are then attracted into the inflammatory site by the chemokine concentration gradient (Rollins 1997).



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 (Ebnet, Kaldjian et al. 1996), but work by Middleton *et al* (Middleton, Neil et al. 1997) 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 (Baekkevold, Yamanaka et al. 2001).

During the multi-step process of transendothelial migration (Figure 1.9), 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 (Panes and Granger 1998). Then the increased avidity of leukocyte integrins leads to leukocyte arrest and firm adhesion (Constantin, Majeed et al. 2000; Grabovsky, Feigelson et al. 2000). These integrins include VLA-4, which captures lymphocytes under shear flow conditions but only in response to immobilised chemokines, not soluble chemokines (Cinamon, Grabovsky et al. 2001), and  $\beta 2$  integrins on monocytes and neutrophils (Detmers, Powell et al. 1991; Jiang, Beller et al. 1992).

Subsequently, leukocytes undergo transendothelial migration, which may only initiate and progress under conditions of shear flow (Cinamon, Grabovsky et al. 2001), 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- $\alpha$  may serve to retain leukocytes in the tissue (Sica, Saccani et al. 1997; Sica, Saccani et al. 2000).



### 1.3.2.ii. Allergic rhinitis and asthma

Recently, chemokines have been implicated in contributing to allergic airway inflammation such as allergic rhinitis and asthma (Lukacs, Strieter et al. 1996; Griffiths-Johnson, Collins et al. 1997). 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 (Owen 2001; D'Ambrosio 2005). Various chemokine receptors are expressed on the leukocytes associated with asthma, including CCR1, 2, 3, 4, 7 and 8 (Owen 2001). Of these, CCR3 may be particularly important since it is the receptor for CCL11; this chemokine was first described due to its ability to attract eosinophils (Jose, Griffiths-Johnson et al. 1994) which are closely correlated with lung dysfunction clinically (Rankin, Conroy et al. 2000). Protein and mRNA expression of CCR3 are elevated in the bronchial mucosa and skin of patients with asthma (Ying, Robinson et al. 1997); CCR3 is expressed on eosinophils, basophils, mast cells and airway epithelial cells (Uguccioni, Mackay et al. 1997; Quackenbush, Wershil et al. 1998; Stellato, Brummet et al. 2001) and can bind CCL5, 7, 11, 13, 24 and 26. Airway epithelial cells are a major source of CC and CXC chemokines in asthma (Nickel, Beck et al. 1999), and CCR3 expression by these cells could modulate several aspects of epithelial function, including production of more chemokines, activation, proliferation and apoptosis.

Another leukocyte population prominent in the pathogenesis of allergic diseases is the T cells with a Th2 phenotype (Gavett, Chen et al. 1994). Th2 cells preferentially express CCR4 and CCR8 (Zingoni, Soto et al. 1998). The ligands for these chemokine receptors (CCL17, CCL22 and CCL1) are potent chemoattractants for Th2 cells. These observations



suggest that both CCR4 and CCR8 play a role in the control of Th2 responses and may represent potential targets for the treatment of allergic diseases (Murdoch and Finn 2000).

The functional importance of CC-chemokines in rodent asthma models has been demonstrated in studies using neutralising antibodies to CC chemokines, aminoterminal modified CC-chemokines that act as receptor antagonists and a pan-CC chemokine antagonist protein from pox virus (Gonzalo, Lloyd et al. 1998; Dabbagh, Xiao et al. 2000).

Chemokine receptor antagonists may therefore be useful for blocking the inflammatory infiltrate seen in this disease.

### 1.3.2.iii. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease eventually leading to joint destruction (Harris 1990).

One of the cardinal features of rheumatoid synovitis is the recruitment and retention of inflammatory cells (Th1 cells, neutrophils, monocytes) in the sublining region. This process is mediated by chemokines as well as adhesion molecules on vascular endothelium and counter-receptors on circulating leukocytes that draw cells to sites of inflammation. Synovial fluid from inflamed joints contains several chemokines, including CCL2, CCL3, CCL5, CXCL8 and CXCL10 (Godessart and Kunkel 2001; Patel, Zachariah et al. 2001) that are produced by resident synovial cells as well as by infiltrating leukocytes. CCR5 and CXCR3 are expressed by T cells infiltrating the synovial tissue (Qin, Rottman et al. 1998; Ruth, Rottman et al. 2001). CXCR1 on neutrophils, CCR1 and CCR2 on monocytes and CXCR6 on T cells have also been involved in the pathogenesis of the disease (Konig, Krenn et al. 2000; Hayashida, Nanki et al. 2001; Kim, Kunkel et al. 2001). Recently it was demonstrated that CXCL16/CXCR6 pathway has a pathogenic role in RA and that using CXCL16 monoclonal antibody significantly reduced infiltration of



inflammatory cells and bone marrow destruction in mouse model (Nanki, Shimaoka et al. 2005).

It is known that CXCL12 expressed in the rheumatoid synovium, is involved in the recruitment of CD4<sup>+</sup> memory T cells but may also play a role in the migration of dendritic cells (DC) from the blood into the rheumatoid synovium (Penna, Sozzani et al. 2001). Local production of CCL19 and CCL21 could play a role in the trafficking of mature DC from the blood into the rheumatoid sinovium (Cravens and Lipsky 2002).

The temporal expression of CXCL5, CCL2 and CCL3 was assessed during the course of rat AIA, a model for human RA, to understand which chemokines are involved in the early events and later stages of the disease (Szekanecz, Kim et al. 2003). CCL3 was associated with early inflammatory events, including leukocytosis and the production of acute phase reactants, while CCL2 seems to be involved mostly in the later phase of AIA (Kasama, Strieter et al. 1995).

Chemokine production in the RA joint could be targeted indirectly, through the inhibition of cytokine production, or directly. Anti- TNF- $\alpha$  and anti-IL-1 strategies have been shown to downregulate chemokine synthesis (Szekanecz, Kim et al. 2003).

In mouse models of arthritis, neutralizing antibodies to CCL2 reduced the severity of disease (Gong, Ratkay et al. 1997) diminishing the monocyte recruitment and disease development. An antagonist of CCR1 and CCR5, met-CCL5 was also effective in reducing inflammation in experimental mouse models of arthritis (Plater-Zyberk, Hoogewerf et al. 1997; Bruhl, Cihak et al. 2001). These experiments are not only useful for the development of future therapeutic strategies, but provide insights into the pathogenic action of these chemokines.



### 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 (Robey, Safai et al. 1985). 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 (Deng, Liu et al. 1996; Doranz, Rucker et al. 1996; Dragic, Litwin et al. 1996; Feng, Broder et al. 1996). 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 (Berger, Murphy et al. 1999). 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 (Simmons, Reeves et al. 2000) but so far only CXCR4 and CCR5 have been shown to act as co-receptors *in vivo* [hence X4 tropic and R5 tropic viruses (Berger, Doms et al. 1998)]. The co-receptor usage also determines the cellular tropism: R5 virus tends to be macrophage tropic (Collman, Hassan et al. 1989) while X4 virus tends to be T cell tropic, despite the fact that macrophages can express CXCR4 (McKnight, Wilkinson et al. 1997). 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 (Wu, Gerard et al. 1996; Turner and Summers 1999). In a recent work, Tian et al. have demonstrated that many residues of CXCR4 and extracellular loop 2 domains are specifically involved in interaction with HIV-1 gp120 (Tian, Choi et al. 2005).

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<sup>+</sup> T-cell-derived HIV inhibitory activity (Cocchi, DeVico et al. 1995)



and individuals with high levels of these chemokines have reduced HIV infectability (Paxton, Liu et al. 1998).

Also, individuals homozygous for the  $\Delta 32$  allele of CCR5, who are deficient in cell surface CCR5 expression, can remain uninfected despite exposure to HIV (Benkirane, Jin et al. 1997). These observations suggest that chemokines and their receptors which are implicated in 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 (Simmons, Clapham et al. 1997; Donzella, Schols et al. 1998).

### 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 (Mantovani, Bottazzi et al. 1992). Analysis of carcinomas of the colon, lung, breast and stomach showed that the infiltrating mononuclear cells were predominantly in the stroma (Svennevig and Svaar 1979).

In ovarian cancer, leukocytes can be found both within the supporting stroma and the tumour areas (Negus, Stamp et al. 1997).

Leukocytes can infiltrate tumours in response to tumour-derived chemokines, including CCL2 (Negus, Stamp et al. 1995; Negus, Stamp et al. 1997). These chemokines are produced by tumour cells, resident and infiltrating stromal cells (Bottazzi, Ghezzi et al. 1985; Walter, Bottazzi et al. 1991; Negus, Stamp et al. 1997). 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 (Mantovani, Bottazzi et al. 1992). 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 (Nesbit, Schaidler et al. 2001). However, low levels of CCL2 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 (Lin, Nguyen et al. 2001). In breast cancer, macrophage infiltration has been correlated with vascularity, suggesting that leukocytes may promote tumour angiogenesis and hence survival (Leek, Lewis et al. 1996).

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 (Rollins and Sunday 1991; Luster and Leder 1993; Laning, Kawasaki et al. 1994; Dilloo, Bacon et al. 1996; Mule, Custer et al. 1996; Fushimi, Kojima et al. 2000; Vicari, Ait-Yahia et al. 2000).

Vicari *et al* transduced a colon carcinoma cell line with CCL21; these cells showed a reduction in tumorigenicity in both immunocompetent and nude mice, possibly due to angiostatic mechanisms and anti-tumour immunity (Vicari, Ait-Yahia et al. 2000). The CCL21-transduced tumours had an increased infiltrate, which included immature dendritic cells and CD8<sup>+</sup> 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<sup>+</sup> T cell infiltrate showed proliferative activity and IFN- $\gamma$  secretion and this infiltrate



(rather than the peritumoural T cell infiltrate) was correlated with a good prognosis in both squamous cell and adenocarcinomas (Schumacher, Haensch et al. 2001).

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 (Balkwill and Mantovani 2001). In addition, reactive oxygen species released by TAM can damage DNA, causing mutation and hence tumour progression (Hagen, Huang et al. 1994).

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 (Leibovich and Wiseman 1988). Tumour growth is also dependent on angiogenesis (Folkman 1990); 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 (Weidner, Semple et al. 1991).

A variety of pro-angiogenic factors, including vascular endothelial growth factor (VEGF), are produced by the tumour and the leukocytic infiltrate (Folkman and Klagsbrun 1987). TAMs in particular have been correlated with tumour vascularity and prognosis (Leek, Landers et al. 1999). Chemokines may also have a role in tumour angiogenesis (Arenberg, Polverini et al. 1997). CXC chemokines can be both pro- and anti-angiogenic, depending on the presence of a tripeptide motif (glutamate-leucine-arginine: the ELR motif) within the N-terminus of the protein (Belperio, Keane et al. 2000). ELR+ CXC chemokines are



potent pro-angiogenic factors, while ELR- CXC chemokines are angiostatic (Strieter, Polverini et al. 1995). 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* (Strieter, Polverini et al. 1995; Strieter, Addison et al. 1999). 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* (Addison, Daniel et al. 2000) 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 (Luster and Ravetch 1987; Farber 1993; Cole, Strick et al. 1998). 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 (Romagnani, Annunziato et al. 2001). 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 (Gupta, Lysko et al. 1998) and can induce neovascularisation *in vivo* (Salcedo, Wasserman et al. 1999), yet it can antagonise the pro-angiogenic activity of ELR+ chemokines and VEGF (Arenberg, Polverini et al. 1997).

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* (Salcedo, Ponce et al. 2000).

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 (Luan, Shattuck-Brandt et al. 1997;



Owen, Strieter et al. 1997). CXCL8 contributes to angiogenesis in models of non-small cell lung cancer (NSCLC) *in vivo* (Arenberg, Kunkel et al. 1996), while CXCL5 correlates with the degree of vascularity in human NSCLC biopsies (Arenberg, Keane et al. 1998). Work by Yoneda *et al* (Yoneda, Kuniyasu et al. 1998) 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 (Engl T. et al. Life Sci. 2005 Epub ahead of print), gastric cancer (Kitadai, Haruma et al. 1998) and pancreatic cancer (Shi, Abbruzzese et al. 1999).

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 (Sgadari, Angiolillo et al. 1996). Production of CXCL10 from adenocarcinoma or squamous cell carcinoma cell lines inoculated in SCID mice was inversely correlated with tumour growth (Arenberg, Kunkel et al. 1996). 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 (Richmond, Lawson et al. 1985; Richmond, Balentien et al. 1988). MGSA can stimulate the proliferation of melanoma cell lines and also pancreatic cell lines (Takamori, Oades et al. 2000). 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



(Schadendorf, Moller et al. 1993). This chemokine can also stimulate the proliferation of some ovarian cancer cell lines (Xu and Fidler 2000), pancreatic cell lines (Takamori, Oades et al. 2000) and colon carcinoma cell lines (Brew, Erikson et al. 2000). 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 (di Celle, Carbone et al. 1994). 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 (Richards, Eisma et al. 1997). 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 (Paget 1889). 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 (Nicolson 1993). It is also apparent that metastasis is not a random process and that different tumours have preferential metastatic sites (Nicolson 1993). Breast cancer frequently spreads to regional lymph nodes, bone marrow, lung and liver (Muller, Homey et al. 2001); malignant melanoma is similar, but also has frequent skin metastases (Moore 2001). 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' (Opdenakker and Van Damme 1992).

A number of different tumour cells have been shown to migrate towards chemokines, or to express chemokine receptors. Breast cancer cells and melanoma express CXCR4 and CCR7, which bind to CXCL12 and CCL19/CCL21, respectively, which are expressed at major sites of metastasis, skin, bone marrow, lung, liver and lymph nodes (Muller, Homey et al. 2001). 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 (Youngs, Ali et al. 1997); binding studies indicated that MCF-7 possess binding sites for these chemokines, indicating the presence of multiple chemokine receptors. Muller et al. have shown that this cell line expresses high level of CXCR4, CCR7 and CXCR2 (Muller, Homey et al. 2001). The prostate cancer cell line PC3 expresses CXCR2 and can migrate and invade in response to CXCL1 and CXCL8 (Reiland, Furcht et al. 1999). The ovarian cancer cell line CAOV-3 expresses CXCR4 and migrate to CXCL12 (Scotton, Wilson et al. 2002). Scotton et al. showed that CXCL12 was present in ascitic fluid and that CXCR4 was expressed more strongly by tumour cells in ascites concluding that this chemokine receptor/ligand interaction could be involved in peritoneal spread of this cancer (Scotton, Wilson et al. 2002). Many pancreatic tumour cell lines express CXCR4 and it was recently shown that this receptor was expressed most prominently in cell lines derived from metastatic tumours (Marchesi, Monti et al. 2004). Functional CXCR4 expression has been demonstrated on a variety of leukaemic cells, including acute myelomonocytic and lymphoblastic leukaemia (Mohle, Schittenhelm et al. 2000), enabling these cells to migrate towards CXCL12. CXCR4 can also be expressed by a colonic epithelial cell line (Jordan, Kolios et al. 1999). Many in vivo studies have demonstrated that injection of tumour cells transfected with CXCR4 are more aggressive with increased migration, invasion and metastasis formation (Balkwill 2004).



These results suggest that chemokine receptors may play a role in tumour metastasis and that in particular the chemokine receptor CXCR4 seems to play a crucial role in this process.

### 1.3.4. CXCR4 and CXCL12

#### 1.3.4.i. Introduction

CXCR4 was originally identified by different groups as an orphan chemokine receptor (Federspiel, Melhado et al. 1993; Loetscher, Geiser et al. 1994) and they called it LESTR or fusin. It is expressed by most leukocyte populations including the majority of T-lymphocyte subsets, all B cells and monocytes, but is only weakly expressed on natural killer cells (Hori, Sakaida et al. 1998). LESTR/fusin was then identified as HIV-1 coreceptor (Feng, Broder et al. 1996) and the CXC chemokine SDF-1 was identified as a potent ligand and agonist of this receptor (Bleul, Farzan et al. 1996; Oberlin, Amara et al. 1996). From this moment LESTR/fusin was reclassified as chemokine receptor CXCR4. CXCR4 and CXCL12 are now known to have important roles in leukocyte trafficking, haematopoiesis, organogenesis, vascularisation and embryogenesis (Murdoch 2000).

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 (Bleul, Fuhlbrigge et al. 1996).

Shirozu *et al* investigated CXCL12 mRNA expression in human organs (Shirozu, Nakano et al. 1995). Almost all organs tested resulted 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 90% identical compared with other chemokines, while the CXCR4 proteins are



approximately 91% identical; therefore this chemokine/receptor pair is highly conserved between species.

#### 1.3.4.ii. CXCR4/CXCL12 deficient mice

CXCR4- and CXCL12-deficient mice have been generated (Nagasawa, Hirota et al. 1996; Ma, Jones et al. 1998; Tachibana, Hirota et al. 1998; Zou, Kottmann et al. 1998), 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 (Nagasawa, Hirota et al. 1996), and a disorganised cerebellum (Ma, Jones et al. 1998).

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 (Ma, Jones et al. 1998). Recently it was demonstrated that mice deficient for CXCR4 exhibit impaired limb innervation and myogenesis (Odemis, Lamp et al. 2005). 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 (Tachibana, Hirota et al. 1998). The near identical phenotypes of the



CXCR4- and CXCL12-deficient mice suggest a monogamous relationship where CXCL12 only acts through CXCR4.

#### *1.3.4.iii. Role of CXCR4/CXCL12 in development*

Studies on CXCR4 and CXCL12 knock out mice have demonstrated their importance in development. The involvement in ontogeny of CXCR4 came from experiments examining rat embryonic development. Jazin et al. have found elevated mRNA levels of rat CXCR4 in the thymus and brain at embryonic day 9 (E9) (Jazin, Soderstrom et al. 1997).

As well as being involved in brain development, CXCR4 has a role in early embryogenesis. CXCR4 mRNA was detected in embryonic mesodermal cells by day seventh of embryonic development (E7.2). It was demonstrated that during organogenesis the CXCL12 expression pattern is always broader than that of CXCR4.

The CXCL12 and CXCR4 research work performed on mice confirms a key role for this chemokine and its receptor in multiple embryonic and developmental events. Recently their importance in development and germ cell migration was demonstrated using zebrafish model (Dumstrei, Mennecke et al. 2004; Sapede, Rossel et al. 2005).

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. CXCR4/CXCL12 in haematopoiesis and leukocyte trafficking*

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<sup>+</sup> CD8<sup>+</sup>) stage of T cell development - at which point CXCR4 expression is downregulated (Suzuki, Nakata et al. 1998). A central and unique role for the chemokine CXCL12 and its receptor CXCR4 in hematopoietic stem cell (HSCs) trafficking to the bone marrow has now been elucidated. CXCL12 is constitutively expressed by stromal cells in the bone marrow and is found on the endothelium of bone marrow microvessels (Peled, Grabovsky et al. 1999), and it promoted chemotaxis of HSC and progenitor cells in human and mice (Kim and Broxmeyer 1998; Wright, Bowman et al. 2002; Dalakas, Newsome et al. 2005).

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 (Ma, Jones et al. 1998). Peled *et al* have also shown a requirement for CXCR4/CXCL12 in murine bone marrow engraftment by human SCID-repopulating stem cells (CD34<sup>+</sup> cells derived from cord blood). CXCR4 antibodies prevented engraftment in the bone marrow of SCID mice. In addition, treatment of CD34<sup>+</sup> cells with IL-6 and Stem Cell Factor (SCF) upregulated CXCR4 expression and enhanced engraftment (Peled, Petit et al. 1999). This may have clinical implications with regard to therapeutic stem cell transplantation.

In conclusion, CXCR4 and CXCL12 have the usual functions attributed to 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.



## 1.4. HYPOXIA

### 1.4.1. Introduction

Hypoxia is a physiological and physiopathological condition characterized by low oxygen tension.

Hypoxia can be caused by a number of factors, such as: 1) low O<sub>2</sub> partial pressure (O<sub>2</sub> tension) in arterial blood due to, e.g., pulmonary diseases or high altitude; 2) reduced ability of blood to carry O<sub>2</sub> as a result of anemia, methemoglobin formation, or carbon monoxide poisoning; 3) reduced tissue perfusion, generalized or local; 4) deterioration of the diffusion geometry; or 5) inability of cells to use O<sub>2</sub> because of intoxication, as cyanide poisoning (cytotoxic hypoxia) (Hockel and Vaupel 2001).

Hypoxia is characteristic of infected tissues, wounds, rheumatic joints, and parts of tumours but also of normal spleen and joints. All these are infiltrated by leukocytes, major cell types of the innate immune system. As local tissue hypoxia might not result from tissue damage but might reflect the normal tissue microenvironment and vasculature. Immune cells, during their life, are exposed to different oxygen concentrations ranging from 16% (~159 mm Hg) in the pulmonary alveoli to less than 6% (4-34 mm Hg) in most other organs of the body and to less than 1% (~0.8-6 mm Hg) in pathological sites, such as tumours (Semenza 2000; Sitkovsky and Lukashev 2005).

The ability to maintain oxygen homeostasis is essential to the survival of all vertebrate species. Physiological systems have evolved to ensure the optimal oxygenation of all cells in each organism and this happens for the evolution of a complex physiological infrastructure for O<sub>2</sub> delivery that includes an entry (lungs), transport vehicle (erythrocytes), a highway and secondary road system (vasculature), and a propulsion device (heart) (Michiels 2004). Specialized chemoreceptor cells that regulate cardiovascular and ventilatory rates regulate changes in O<sub>2</sub> supply. In response to reduced



O<sub>2</sub> availability cells rapidly activate specific transcription mechanism to induce transcription of hypoxia responsive genes involved in oxygen homeostasis.

During their life span, cells of the innate immune system may encounter environments characterized by different oxygen levels, ranging from normoxia (e. g. blood stream) to hypoxia (e. g. poorly vascularized tissues). Moreover, pathological sites are often characterized by poor oxygen tension, particularly evident in tumours, wounds lesions and joints inflammation (e. g. reumathoid arthritis). Cells localizing in hypoxic sites undergo to a metabolic adaptation program leading to the expression of specific genes, products of which are involved in the maintenance of O<sub>2</sub> homeostasis.

Homeostatic responses to hypoxia can be classified as systemic, local, or cellular in nature. Systemic changes include increased erythropoiesis, ventilation and cardiac output. Local responses include changes in vascular tone and neovascularization. Cellular responses include the transition from oxidative phosphorylation to glycolysis pathway for ATP generation.

#### **1.4.2. Generation of cellular energy**

Oxygen plays a major role in the energy metabolism of aerobic living organism. Under aerobic conditions, the source of cellular energy is glycolysis coupled to oxidative phosphorylation (also known as respiration) (Figure 1.10).

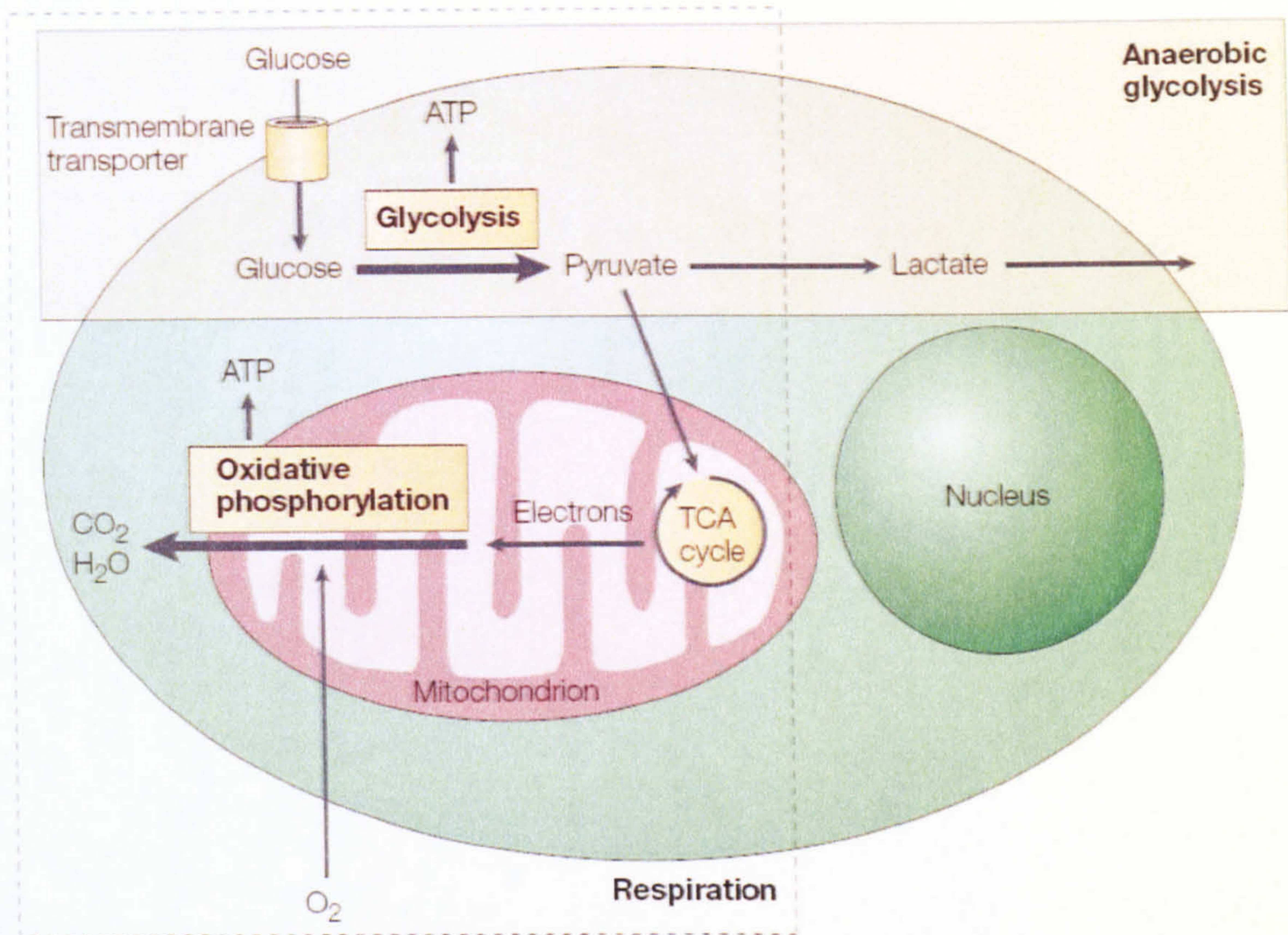
In this process glucose is converted to pyruvate, with ATP being concomitantly produced as a result of substrate-level phosphorylation of ADP using high-energy phosphate groups from intermediates of glucose conversion. After this, pyruvate enters the tricarboxylic acid (TCA) cycle in mitochondria. In the TCA cycle we have the production of electrons that are transferred to oxygen that represents the terminal electron acceptor (Sitkovsky and Lukashev 2005). At this step acts the ATP synthase that transforms the electrochemical gradient created by the electron-transport pathway into energy-carrying ATP.



When there is insufficient oxygen supply, anaerobic glycolysis takes place, and this results in the accumulation of a large amount of pyruvate, which cannot be used by the TCA cycle. In this case we have the production and subsequently the release of lactate.

Several definitions of hypoxia exist, and depend on the experimental endpoint or detection methods. Although no sharp threshold exists between normoxia and hypoxia, the literature shows that median  $pO_2$  levels below 10 mm Hg generally result in intracellular acidosis, ATP depletion, and a drop in energy supply. Oxidative phosphorylation for ATP production measured *in vitro* continues to 0.5-10 mm Hg depending on the cell line and the experimental setup (Hockel and Vaupel 2001). Cell cycle progression is affected (prolonged) at 0.2-1 mmHg, and hypoxia may induce transcriptional or posttranslational changes resulting either in cell death or malignant progression in the range 1-15 mm Hg (Hockel and Vaupel 2001).





**Figure 1.10. Simplified schema of glycolysis and oxidative phosphorylation.**

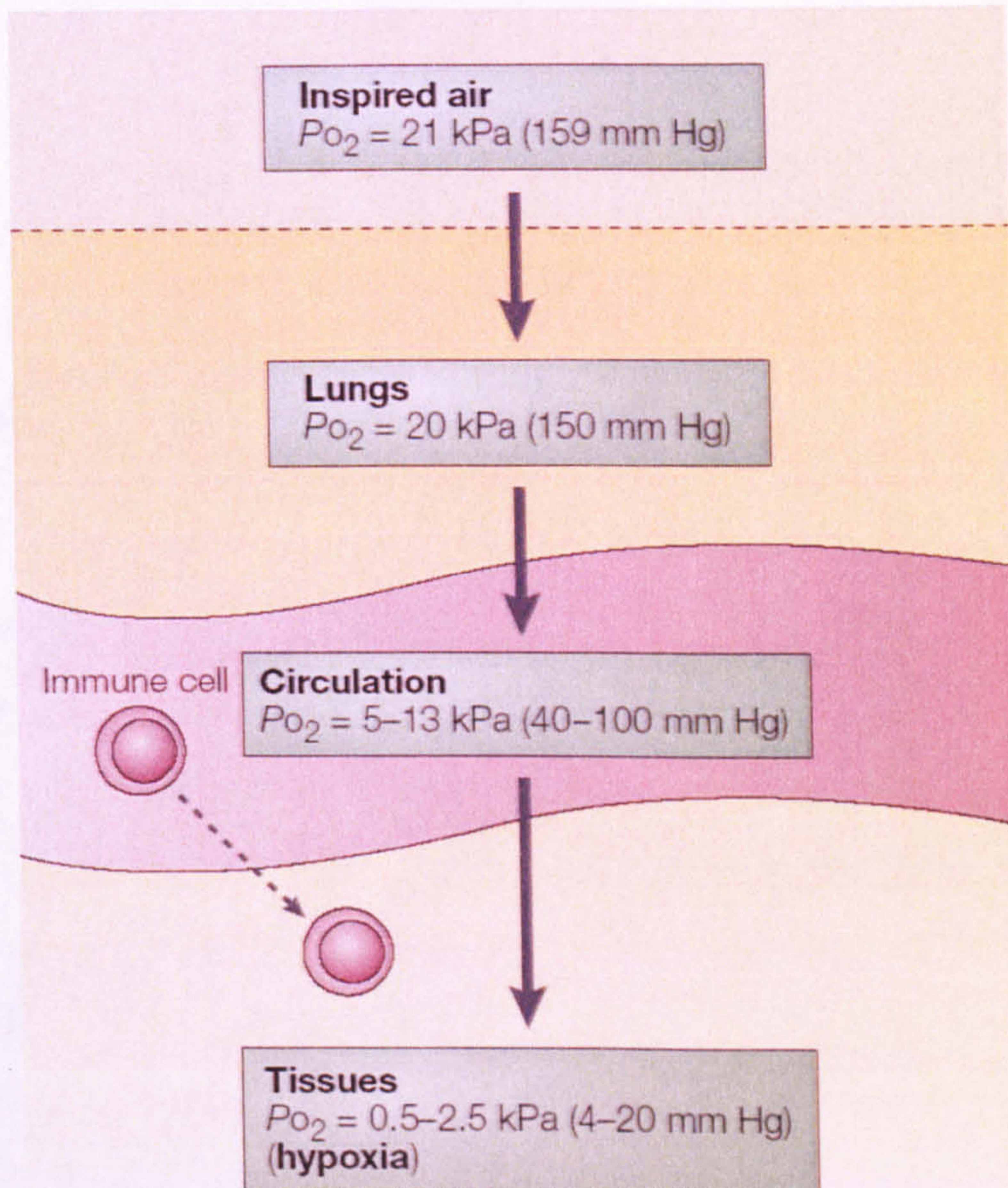
Glucose is converted to pyruvate during glycolysis, with concomitant production of ATP. Pyruvate enters the tricarboxylic acid (TCA) cycle in mitochondria, where electrons are released in a series of TCA-cycle reactions. These electrons enter the electron-transport chain, which creates an electrical gradient at the mitochondrial membrane. To equalize charge on both sides of the membrane, protons move across the membrane, and this is coupled to the generation of ATP as a result of oxidative phosphorylation. In the absence of oxygen ( $O_2$ ), anaerobic glycolysis results in the reduction of pyruvate to lactate, which is then released from the cell.  $CO_2$ , carbon dioxide;  $H_2O$ , water. From (Sitkovsky and Lukashev 2005).

### 1.4.3. Hypoxic microenvironments in tissues

Usually local-tissue hypoxia is not due to tissue damage but might reflect the normal tissue microenvironment, vasculature and geometry. Each tissue compartment is different in vascularization and therefore in the level of oxygenation. In normal situations, the oxygen tension in tissues is proportional to the distance from the end of the nearest capillary. Many groups measured the oxygen level in different tissue and they have found that is different



from air: the  $pO_2$  of inspired air is 21 % (159 mmHg), however it was shown that the  $pO_2$  is 1.3 % (10 mmHg) in the thymus and 2.1 % (16 mmHg) in the spleen (Vaupel, Braunbeck et al. 1973; Braun, Lanzen et al. 2001). The  $pO_2$  was found to be highest near the artery and to gradually decrease with distance from the artery (Figure 1.11) (Sitkovsky and Lukashev 2005).



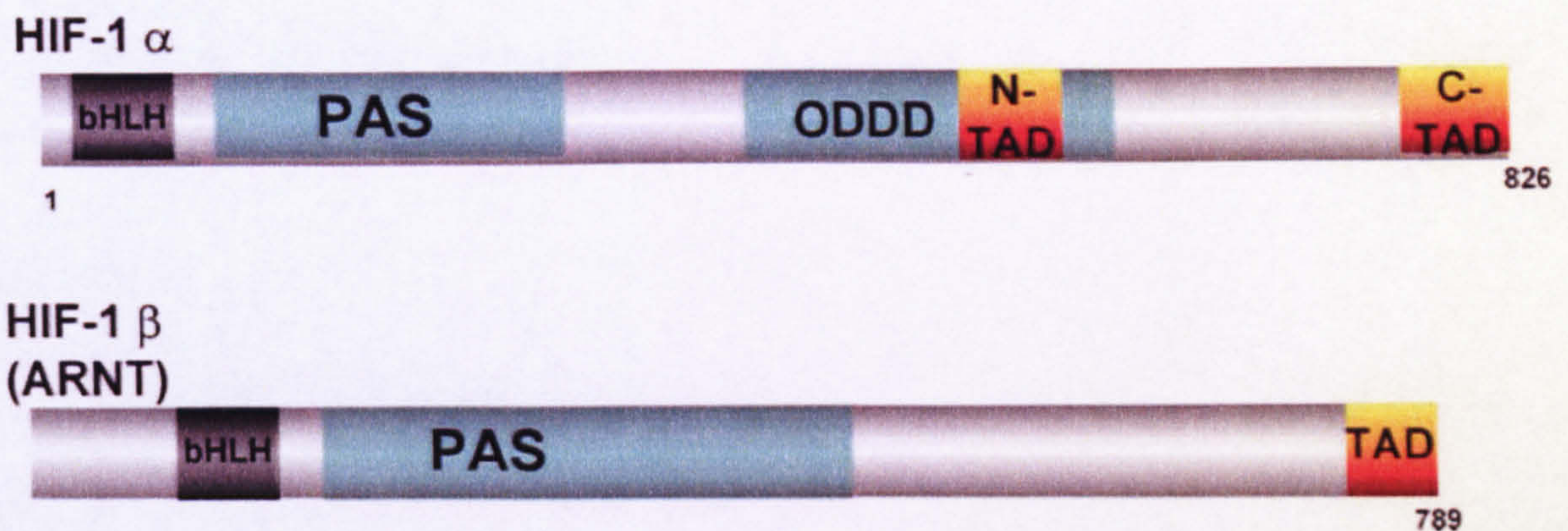
**Figure 1.11. Low oxygen concentration in tissue environments**

Oxygen is inspired from the air; the oxygen tension ( $pO_2$ ) in air is 21 kPa (159 mm Hg), which is a concentration of 21%. When warmed air arrives in the lungs, it loses some pressure:  $pO_2 = 20 \%$  (150 mm Hg). The oxygen pressure continues to decrease in the circulation:  $pO_2 = 13 \%$  (100 mm Hg) in alveoli and 5 % (40 mm Hg) in veins that reach the heart. The oxygen pressure in the tissues then decreases with increasing distance from the blood vessels:  $pO_2 = 0.5-2.5 \%$  (4-20 mm Hg). From (Sitkovsky and Lukashev 2005).



#### 1.4.4. HIF-1

HIF-1 is the master regulator of oxygen homeostasis. HIF-1 was identified as a hypoxia-inducible HRE (hypoxia responsive element)-binding activity (Semenza and Wang 1992). The Semenza group used the HIF-1 binding site in the erythropoietin (EPO) HRE and they purified HIF-1 $\alpha$  and HIF-1 $\beta$  subunits by DNA affinity chromatography (Wang and Semenza 1995). cDNA cloning revealed that both subunits were basic helix-loop-helix (bHLH)-PAS proteins: HIF-1 $\alpha$  was a novel protein, whereas HIF-1 $\beta$  was identical to the aryl hydrocarbon nuclear translocator (ARNT) protein (Wang, Jiang et al. 1995) (Figure 1.12).



**Figure 1.12. Domain structure of the human HIF-1 $\alpha$  and HIF-1 $\beta$ .**

Proteins show a high degree of homology with similar structural features including basic helix loop helix (bHLH), PAS, and NH<sub>2</sub>- and COOH-terminal transactivation domains (TAD-N and TAD-C). Adapted from (Brahimi-Horn and Pouyssegur 2005).

HIF-1 $\alpha$  is encoded by the HIF1A gene, HIF-1 $\beta$  is encoded by the ARNT gene. Hif1a and HIF1A mapped to mouse chromosome 12 and human chromosome 14, respectively (Semenza, Rue et al. 1996).

Mouse and rat HIF-1 $\alpha$  amino acid sequences share 95% identity with the human sequence, and in particular it was found that this protein is highly conserved from *Caenorhabditis elegans* via *Drosophila melanogaster* to *Homo sapiens* providing evolutionary evidence



for the functional importance of HIF-1 $\alpha$  (Li, Ko et al. 1996; Wenger, Rolfs et al. 1996; Luo, Gu et al. 1997; Kietzmann and Gorlach 2005).

HIF-1 $\alpha$  and HIF-1 $\beta$  mRNAs are expressed ubiquitously in human and rodent tissues (Wenger, Rolfs et al. 1996; Wiener, Booth et al. 1996; Wenger, Rolfs et al. 1998).

The basic domain and the COOH-terminal half of PAS are specifically required for DNA binding of HIF-1, whereas the HLH domain and the NH<sub>2</sub>-terminal half of the PAS domains are responsible for the formation of the HIF-1 $\alpha$ /HIF-1 $\beta$  heterodimer capable of DNA binding (Jiang, Rue et al. 1996).

HIF-1 $\alpha$  also contains two transactivation domains (TADs). The main function of the TADs is to recruit and interact with coactivators, which are crucial for the transcriptional activation of target genes (Hopfl, Ogunshola et al. 2004). These domains are also important because HIF-1 $\alpha$  undergoes posttranslational regulation mediated through hydroxylation, phosphorylation, acetylation, and/or redox modifications of these two TAD domains (Richard, Berra et al. 1999; Bruick and McKnight 2001; Jeong, Bae et al. 2002). It was demonstrated by different groups that cells cultured under hypoxic conditions (1% O<sub>2</sub>) show an increase of HIF-1 $\alpha$  protein levels without concomitant elevation in mRNA expression (Hopfl, Ogunshola et al. 2004) suggesting that the main regulation pathways rely on oxygen-dependent protein stabilization (Gradin, McGuire et al. 1996; Huang, Arany et al. 1996). HIF-1 $\alpha$  also comprises a sequence termed oxygen-dependent degradation domain (ODD domain) that allows regulation of protein stability as a function of the O<sub>2</sub> concentration (Huang, Gu et al. 1998).

Hypoxic HIF-1 $\alpha$  translocation and nuclear accumulation still occur in HIF-1 $\beta$ /ARNT-deficient hypoxic cells, demonstrating that these events are ARNT independent (Hopfl, Ogunshola et al. 2004). HIF-1 $\beta$  is a nuclear protein (Chilov, Camenisch et al. 1999) and it is constitutively expressed.



Gassmann's group have shown that HIF-1 $\alpha$  protein is present in the nucleus after < 2 min of exposure to hypoxia or anoxia and that the reoxygenation reduced HIF-1 DNA binding and nuclear HIF-1 $\alpha$  protein levels within 4-8 min, suggesting a protein half life of ~5 min (Wang, Jiang et al. 1995; Jewell, Kvietikova et al. 2001).

#### 1.4.5. Other members of the HIF family

To date, another two member of the HIF family have been cloned: HIF-2 $\alpha$  and HIF-3 $\alpha$  (Figure 1.13).

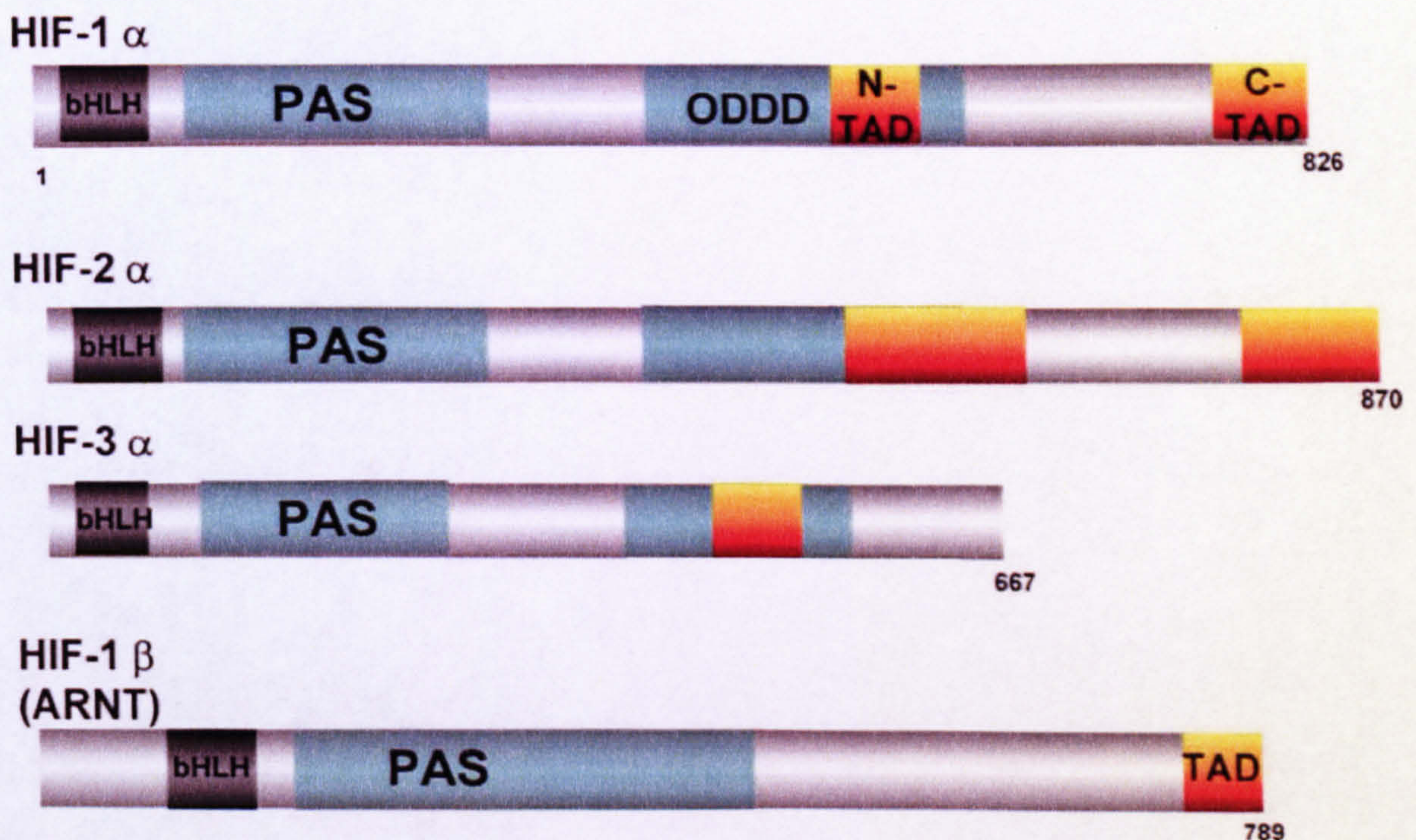


Figure 1.13. Domain structure of the human HIF family.

The HIF-1 $\alpha$  and HIF-2 $\alpha$  subunits contain two transactivation domains, the N- and C- terminal activation domain (N-TAD and C-TAD) while the  $\beta$  subunit contains only one TAD. Of note is the lack of TAD-C in HIF-3 $\alpha$ . Adapted from (Brahimi-Horn and Pouyssegur 2005).

These proteins share several characteristics with HIF-1 $\alpha$ , such as hypoxic protein stabilization, heterodimerization with ARNT(s), DNA-recognition/binding, and reporter gene transactivation (Hopfl, Ogunshola et al. 2004). Eckardt's group has demonstrated that



HIF-2 $\alpha$  is expressed in a complementary but not overlapping pattern to HIF-1 $\alpha$  in specific cells of most organs after systemic hypoxic exposure (Wiesener, Jurgensen et al. 2003). HIF-2 $\alpha$  is expressed in a variety of tumours at different levels, but mostly its expression is associated with stromal cells, especially with tumour-associated macrophages (Talks, Turley et al. 2000; Leek, Talks et al. 2002). HIF-2 $\alpha$  levels have been correlated with tumour grade in non-Hodgkin lymphoma (Stewart, Talks et al. 2002) and bladder cancers (Onita, Ji et al. 2002). MacDonald's group has demonstrated that there are different levels of expression of HIF-1 $\alpha$  and HIF-2 $\alpha$  in the astrocytomas: we can find similar levels of HIF-1 $\alpha$  in high- and low grade astrocytomas but HIF-2 $\alpha$  is overexpressed in high grade astrocytomas (Khatua, Peterson et al. 2003). They have also shown that the HIF-2 $\alpha$  overexpression is related to a dysregulation of the epidermal growth factor/PI3K pathway.

HIF-2 $\alpha$  may also upregulate a different set of target genes involved in neoangiogenesis than HIF-1 $\alpha$ : VEGF receptor 2 and angiopoietin receptor tie-2 (Tian, McKnight et al. 1997; Kappel, Ronicke et al. 1999).

HIF-1 $\alpha$  and HIF-2 $\alpha$  bind to the same HREs; but for example the VEGF promoter sequence is more inducible by HIF-2 $\alpha$  than by HIF-1 $\alpha$  (Camenisch, Stroka et al. 2001; Hopfl, Ogunshola et al. 2004).

A third protein called HIF-3 $\alpha$  was cloned. It is characterized by the loss of a TAD-C (Figure 1.13) but presents a considerable sequence homology with HIF-1 $\alpha$  and HIF-2 $\alpha$ . (Gu, Moran et al. 1998; Hopfl, Ogunshola et al. 2004). At the moment HIF-3 $\alpha$  was found to be expressed in the distal tubules of the kidney (Hara, Hamada et al. 2001) but its role has not been well defined. This third member produces multiple splice variants (Makino, Kanopka et al. 2002; Maynard, Qi et al. 2003) that contain extra DNA binding elements and protein-protein interaction motifs not found in HIF-1 $\alpha$  and HIF-2 $\alpha$ . In particular Maynard MA et al. have found that the HIF-3 $\alpha$  variant HIF-3 $\alpha$ 4 attenuates the ability of



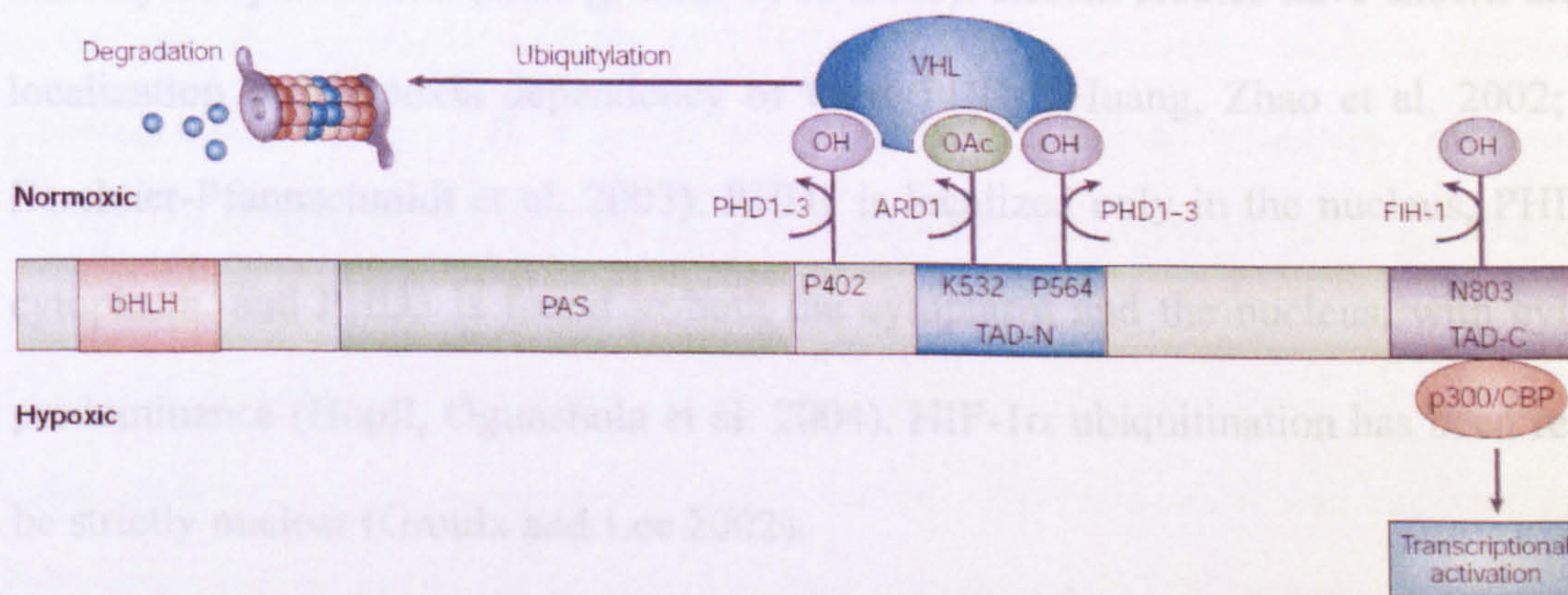
HIF-1 to bind HRE of HIF target genes. They also demonstrated that his expression is down-regulated in renal cell carcinoma (Maynard, Evans et al. 2005).

All these observations suggest that members of HIF family are involved in many processes and in particular that they play a crucial role in the tumour pathology becoming a good target for new therapeutic strategies.

#### 1.4.6. HIF-1 $\alpha$ degradation and oxygen sensing

For many years the oxygen sensor mechanism remained unknown but more recently two different groups identified an oxygen-dependent enzymatic modification of the ODD domain of HIF-1 $\alpha$  (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001).

They have found that there are three new prolyl-4-hydroxylases that are able to hydroxylate two HIF-1 $\alpha$  proline residues (Pro<sup>402</sup> and Pro<sup>564</sup>) in the presence of oxygen, inducing its degradation by the proteasome (Figure 1.14).



**Figure 1.14. O<sub>2</sub>-dependent regulation of HIF-1 $\alpha$  activity.**

O<sub>2</sub> regulates the rate at which HIF-1 $\alpha$  protein is degraded. In normoxic conditions, O<sub>2</sub>-dependent hydroxylation of proline (P) residues 402 and 564 in HIF-1 $\alpha$  by the enzymes PHD (prolyl hydroxylase-domain protein) 1–3 is required for the binding of the von Hippel–Lindau (VHL) tumour-suppressor protein, which is the recognition component of an E3 ubiquitin-protein ligase. VHL binding is also promoted by acetylation of lysine (K) residue 532 by the ARD1 acetyltransferase. Ubiquitylation of HIF-1 $\alpha$  targets the protein for degradation by the 26S proteasome. O<sub>2</sub> also regulates the interaction of HIF-1 $\alpha$  with transcriptional co-activators. O<sub>2</sub>-dependent hydroxylation of asparagine (N) residue 803 in HIF-1 $\alpha$  by the



enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1 $\alpha$  and therefore inhibits HIF-1-mediated gene transcription. Under hypoxic conditions, the rate of asparagine and proline hydroxylation decreases. VHL cannot bind to HIF-1 $\alpha$  that is not prolyl-hydroxylated, resulting in a decreased rate of HIF-1 $\alpha$  degradation. By contrast, p300 and CBP can bind to HIF-1 $\alpha$  that is not asparaginyl-hydroxylated, allowing transcriptional activation of HIF-1 target genes. (Semenza 2003)

PHDs hydroxylate specific prolines recognizing a strongly conserved LXXLAP motif (where X indicates any amino acid and P indicates the hydroxyacceptor proline), requiring oxygen and 2-oxoglutarate as co-substrates as well as iron and ascorbate (Hopfl, Ogunshola et al. 2004).

HIF-1 $\alpha$  can be stabilized also by hypoxia-mimicking elements such as iron chelators or transition metals such as cobalt because they suppress the hydroxylation of the proline residues (Jaakkola, Mole et al. 2001; Hopfl, Ogunshola et al. 2004).

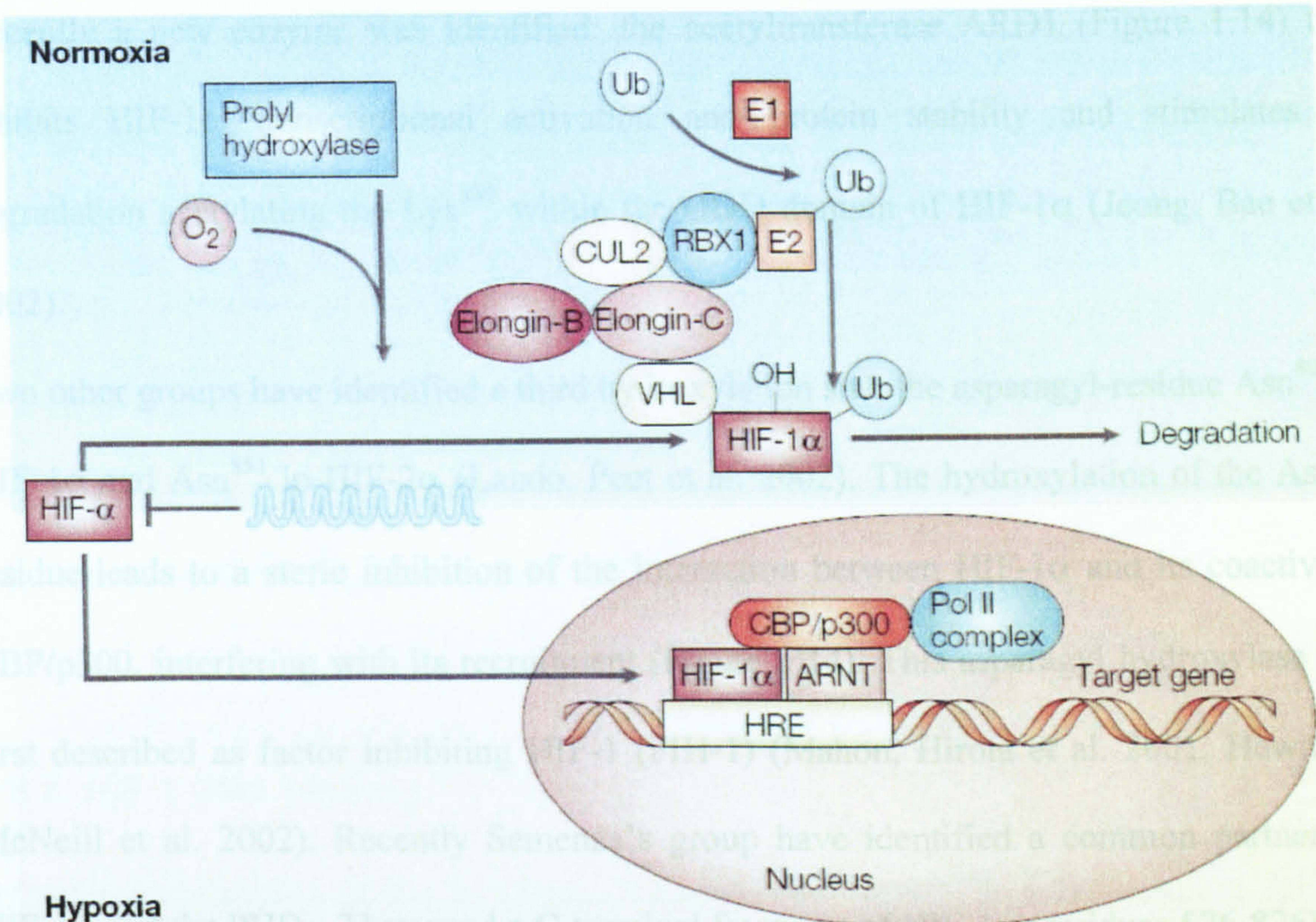
It was recently demonstrated that PHD2 has the highest specific activity toward HIF-1 $\alpha$ 's main hydroxylation site (Huang, Zhao et al. 2002). Recent studies have shown the cellular localization and hypoxia dependency of these PHDs (Huang, Zhao et al. 2002; Metzen, Berchner-Pfannschmidt et al. 2003). PHD1 is localized only in the nucleus, PHD2 in the cytoplasm, and PHD3 is found in both the cytoplasm and the nucleus, with cytoplasmic predominance (Hopfl, Ogunshola et al. 2004). HIF-1 $\alpha$  ubiquitination has been reported to be strictly nuclear (Groulx and Lee 2002).

The most important part of its degradation is the prolyl hydroxylation; HIF-1 $\alpha$  is constitutively expressed, but it is difficult to detect it under normoxic conditions (Jewell, Kvietikova et al. 2001; Hopfl, Ogunshola et al. 2004).

The degradation pathway starts with the binding of the von Hippel-Lindau tumour suppressor protein (pVHL) to the hydroxylated ODD domain of HIF-1 $\alpha$  (Ivan, Kondo et al. 2001; Jaakkola, Mole et al. 2001). pVHL is part of the E3 ubiquitin-ligase complex



(elongin B, elongin C, cullin 2 and RBX1) that targets key regulatory proteins for ubiquitin-mediated proteolysis in the proteasome (Krek 2000) (Figure 1.15).



**Figure 1.15. HIF-1 pathway.**

In the presence of oxygen (O<sub>2</sub>), prolyl hydroxylase post-translationally modifies hypoxia-inducible transcription factor (HIF)-1α, allowing it to interact with the von Hippel-Lindau (VHL) complex. VHL is a part of a larger complex that includes elongin-B, elongin-C, CUL2, RBX1 and a ubiquitin-conjugating enzyme (E2). This complex, together with an ubiquitin-activating enzyme (E1), mediates the ubiquitylation of HIF-1α. In the absence of oxygen, prolyl hydroxylase cannot modify HIF-1α, and the protein remains stable. Stabilized HIF-1α is translocated to the nucleus, where it interacts with cofactors such as aryl hydrocarbon receptor nuclear translocator (ARNT), CBP/p300 and the DNA polymerase II (Pol II) complex to bind to hypoxia-responsive element (HREs) and activate transcription of target genes. (Harris 2002)

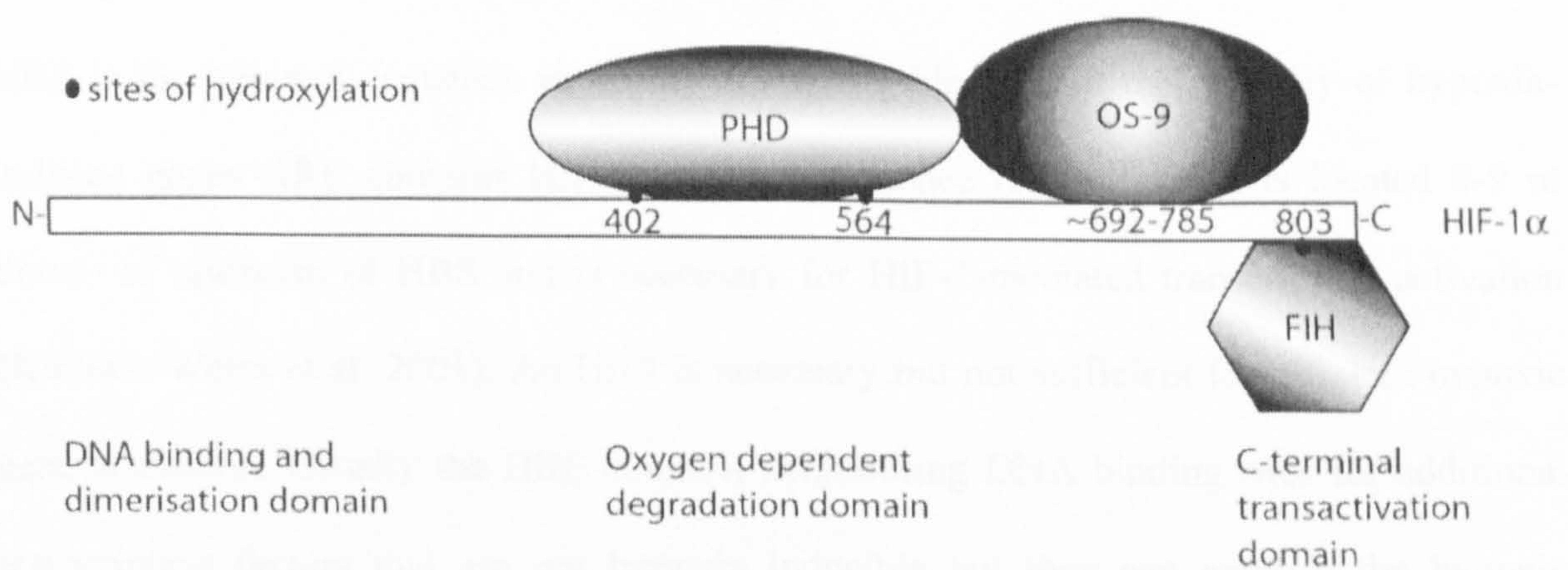
The loss or mutation of pVHL *in vivo* stabilizes the HIF-1α protein under normoxic conditions and may lead to the VHL hereditary cancer syndrome (Cockman, Masson et al. 2000; Krieg, Haas et al. 2000; Ohh, Park et al. 2000; Yu, White et al. 2001) characterized by the development of vascular tumors of the central nervous system and retina, clear cell



renal carcinoma, pheochromocytomas, pancreatic islet cell tumors, endolymphatic sac tumors and benign cyst affecting a variety of organs.

Recently a new enzyme was identified: the acetyltransferase ARD1 (Figure 1.14) that inhibits HIF-1 $\alpha$  transcriptional activation and protein stability and stimulates its degradation acetylating the Lys<sup>532</sup> within the ODD domain of HIF-1 $\alpha$  (Jeong, Bae et al. 2002).

Two other groups have identified a third hydroxylation site, the asparagyl-residue Asn<sup>803</sup> in HIF-1 $\alpha$  and Asn<sup>851</sup> in HIF-2 $\alpha$  (Lando, Peet et al. 2002). The hydroxylation of the Asn<sup>803</sup> residue leads to a steric inhibition of the interaction between HIF-1 $\alpha$  and its coactivator CBP/p300, interfering with its recruitment (Figure 1.14). This asparagyl hydroxylase was first described as factor inhibiting HIF-1 (FIH-1) (Mahon, Hirota et al. 2001; Hewitson, McNeill et al. 2002). Recently Semenza's group have identified a common partner for HIF-1 $\alpha$  and the PHDs. They used a C-terminal fragment of HIF-1 $\alpha$  (residues 576-826) in a yeast two-hybrid screen led to the identification of OS-9 as a HIF-1 $\alpha$  binding protein (Baek, Mahon et al. 2005).



**Figure 1.16. Proposed role for OS-9 in the hypoxic response.**

The figure shows HIF-1 $\alpha$  and HIF hydroxylases (PHD and FIH) with a possible role for OS-9 in binding to both HIF-1 $\alpha$  and the PHD enzymes. (Flashman, McDonough et al. 2005)



By immunoprecipitation analyses they demonstrated that OS-9-HIF-1 $\alpha$  interaction occurs in both hypoxic and normoxic cells and that OS-9 also binds to PHD2 and PHD3. Interestingly, OS-9 and PHD2 were shown to bind to separate regions of HIF-1 $\alpha$  (Figure 1.16). Baek et al. therefore suggest that OS-9 is a critical factor in the oxygen-mediated regulation of HIF-1 $\alpha$ . OS-9 is ubiquitous in human tissue and is overexpressed in tumour cells (Baek, Mahon et al. 2005).

Cytoskeletal proteins also regulate HIF-1 $\alpha$  levels: small GTPases such as Rac1, Rho and Cdc42 are upregulated by hypoxia and are important for HIF-1 $\alpha$  activation (Hirota and Semenza 2001; Turcotte, Desrosiers et al. 2003).

#### **1.4.7. HIF-1 DNA binding**

After the stabilization of the  $\alpha$  subunit, nuclear translocation, and dimerization, HIF-1 $\alpha$  binds to its consensus binding site (HBS, HIF-1 binding site) within the hypoxia response element (HRE). The core sequence of HBS is (A/G)CGTG. HRE may be located within either promoter or enhancer regions of target genes (Table 1.2).

HBS is the minimal sequence necessary for HIF-1 binding. In the majority of hypoxia-induced genes HRE contains HIF-1 ancillary sequence (HAS), which is located 8-9 nt down- or upstream of HBS and is necessary for HIF-1-mediated transcription activation (Kimura, Weisz et al. 2001). An HBS is necessary but not sufficient for efficient hypoxic gene activation. Usually the HRE contains neighboring DNA binding sites for additional transcription factors that are not hypoxia inducible but they can amplify the hypoxic response (Wenger 2002).



Core HRE sequence	Location	Hypoxia-inducible gene	Species	DB	TA	References
<b>Oxygen supply</b>						
gccctACGTGctgtctca	3'FS +3065/+3082	Erythropoietin (erythropoiesis)	Human	+	+	(Wang and Semenza 1993)
gccctACGTGctgctcg	3'FS +359/+376	Erythropoietin (erythropoiesis)	Mouse	+	+	(Firth, Ebert et al. 1994)
gaaatACGTGcgctttgt	5'FS -201/-184	Transferrin (iron transport)	Human	+	+	(Rolfs, Kvietikova et al. 1997)
agcgtACGTGcctcagga	5'FS -93/-76	Transferrin receptor (iron transport)	Human	+	+	(Tacchini, Bianchi et al. 1999)
tctgtACGTGaccacact	5'FS -3639/-3544	Ceruloplasmin (iron oxidase)	Human	+	+	(Mukhopadhyay, Mazumder et al. 2000)
tgcatACGTGggctcca	5'FS -978/-961	VEGF (angiogenesis)	Human	+	+	(Forsythe, Jiang et al. 1996)
tgcatACGTGggctcca	5'FS +61/+78	VEGF (angiogenesis)	Rat	+	+	(Levy, Levy et al. 1995)
gaacaACGTGgaattagt	5'FS +1408/+1425	Flt-1/VEGF-R1 (angiogenesis)	M/(H)	-	+	(Gerber, Condorelli et al. 1997)
agtggGCGTGggaaccg	5'FS -124/-107	Flk-1/VEGF-R2 (angiogenesis; HIF-2)	Mouse	+	+	(Elvert, Kappel et al. 2003)
agcggACGTGctggcgtg	5'FS +322/+339	Heme oxygenase 1 (vessel diameter)	Mouse	+	+	(Lee, Jiang et al. 1997)
<b>Cellular metabolism</b>						
ctcggACGTGactcggac	5'FS -184/-201	Aldolase A (glycolysis)	Human	+	+	(Semenza, Jiang et al. 1996)
gccggACGTGacaaacgg	5'FS -206/-189	Phosphoglycerate kinase 1 (glycolysis)	Human	+	-	(Semenza, Jiang et al. 1996)
gtgagACGTGctactcc	5'FS -184/-201	Phosphoglycerate kinase 1 (glycolysis)	Mouse	+	-	(Okino, Chichester et al. 1998)
gccggACGTGgggcccga	5'FS -413/-396	Enolase 1 (glycolysis)	Human	+	-	(Semenza, Jiang et al. 1996)
cctacACGTGggtcccg	5'FS -89/-72	Lactate dehydrogenase A (glycolysis)	Mouse	+	+	(Firth, Ebert et al. 1995)
ctgatGCGTGtcagacgg	5'FS +300/+283	Glucose transporter 1 (glucose uptake)	Mouse	+	+	(Okino, Chichester et al. 1998)
<b>Cell growth and apoptosis</b>						
aggacACGTGgagaggct	5'FS -6280/-6263	TGF- $\beta$ 3 (placenta development)	Mouse	+	+	(Schaffer, Scheid et al. 2003)
aggctGCGTGcgctggte	5'FS -89/-72	TGF- $\beta$ 3 (placenta development)	Human	+	+	(Nishi, Nakada et al. 2004)
tgtccACGTGggggcctg	5'UT +348/+331	Endoglin (TGF- $\beta$ coreceptor)	Human	+	+	(Sanchez-Elsner, Botella et al. 2002)
cagggACGTGtccccagg	5'FS -1243/-1226	SDF-1 (CXCL12 chemokine)	Human	-	+	(Ceradini, Kulkarni et al. 2004)
gaggeGCGTGcgtcgga	5'FS -1292/-1309	CXCR4 (chemokine receptor)	Human	+	+	(Staller, Sulitkova et al. 2003)
agcgcGCGTGtgggaagg	5'FS +142/+159	<i>met</i> (protooncogene)	Human	-	+	(Pennacchiotti, Michieli et al. 2003)
agagcACGTGgcgggcca	5'FS -222/-205	<i>Wt1</i> (Wilms' tumor suppressor)	Mouse	+	+	(Wagner, Wagner et al. 2003)
ttcccACGTGgcggaggg	5'FS -169/-152	TERT (telomerase)	Human	+	+	(Yatabe, Kyo et al. 2004)
<b>Others</b>						
tgtgcGCGTGgtgccata	5'FS -1197/-1184	Cited2/p35srj (transcriptional cofactor)	Human	+	+	(Bhattacharya, Michels et al. 1999)
cgaatGCGTGcgtgggtg	5'FS -729/-712	ID2 (transcriptional repressor)	Human	+	+	(Lofstedt, Jogi et al. 2004)
gccagACGTGcctggagt	5'FS -461/-444	DEC1 (transcription factor)	Human	+	+	(Miyazaki, Kawamoto et al. 2002)
agctcACGTGcggaacgt	5'FS -297/-314	DEC2 (transcription factor)	Human	+	+	(Miyazaki, Kawamoto et al. 2002)
tggagACGTGcgcgtcct	5'FS -235/-218	Human Herpesvirus 8 ORF34	Viral	+	+	(Haque, Davis et al. 2003)
tctagACGTGaccaggg	5'UT -5/+13	CD18 (leukocyte adhesion)	Human	-	+	(Kong, Eltzschig et al. 2004)
tcgctACGTGcgctcagt	5'FS -75/-92	Collagen prolyl 4-hydroxylase (I)	Rat	+	+	(Takahashi, Takahashi et al. 2000)
gggtgACGTGcagagcgc	5'FS -417/-400	PHD2 (O <sub>2</sub> sensing)	Human	+	+	(Metzen, Stiehl et al. 2005)
gggctACGTGcgtcgtg	IVS1 +12583/+12600	PHD3 (O <sub>2</sub> sensing)	Human	+	+	(Pescador, Cuevas et al. 2005)

**Table 1.2. Compilation of some known HIF target genes**

Examples of only those genes were included in which binding of HIF to target DNA sequence is a DNA binding (DB) assay or functional transactivation (TA) of reporter gene expression, or both, have been shown. FS, flanking sequence; IVS, intervening sequence; UT, untranslated region. For abbreviation of gene name, refer to references. Adapted from (Wenger, Stiehl et al. 2005)



### 1.4.8. HIF-1 activation in normoxic conditions

Recently it was demonstrated that HIF-1 $\alpha$  protein can be stabilized already under normoxic conditions *in vivo* (Stroka, Burkhardt et al. 2001). It appears that posttranslational modifications especially phosphorylation play a major role in HIF-1 $\alpha$  activation. Many oncogenes and tumour suppressor genes can modulate phosphorylation cascades and so they affect HIF-1 $\alpha$  expression levels independent of oxygen levels. HIF-1 $\alpha$  expression and activity are regulated by major signal transduction pathways including those involving phosphatidylinositol 3-kinase (PI3K) and ERK/MAPK.

Different pathways involved in the HIF-1 activation in normoxic conditions will be discussed briefly below.

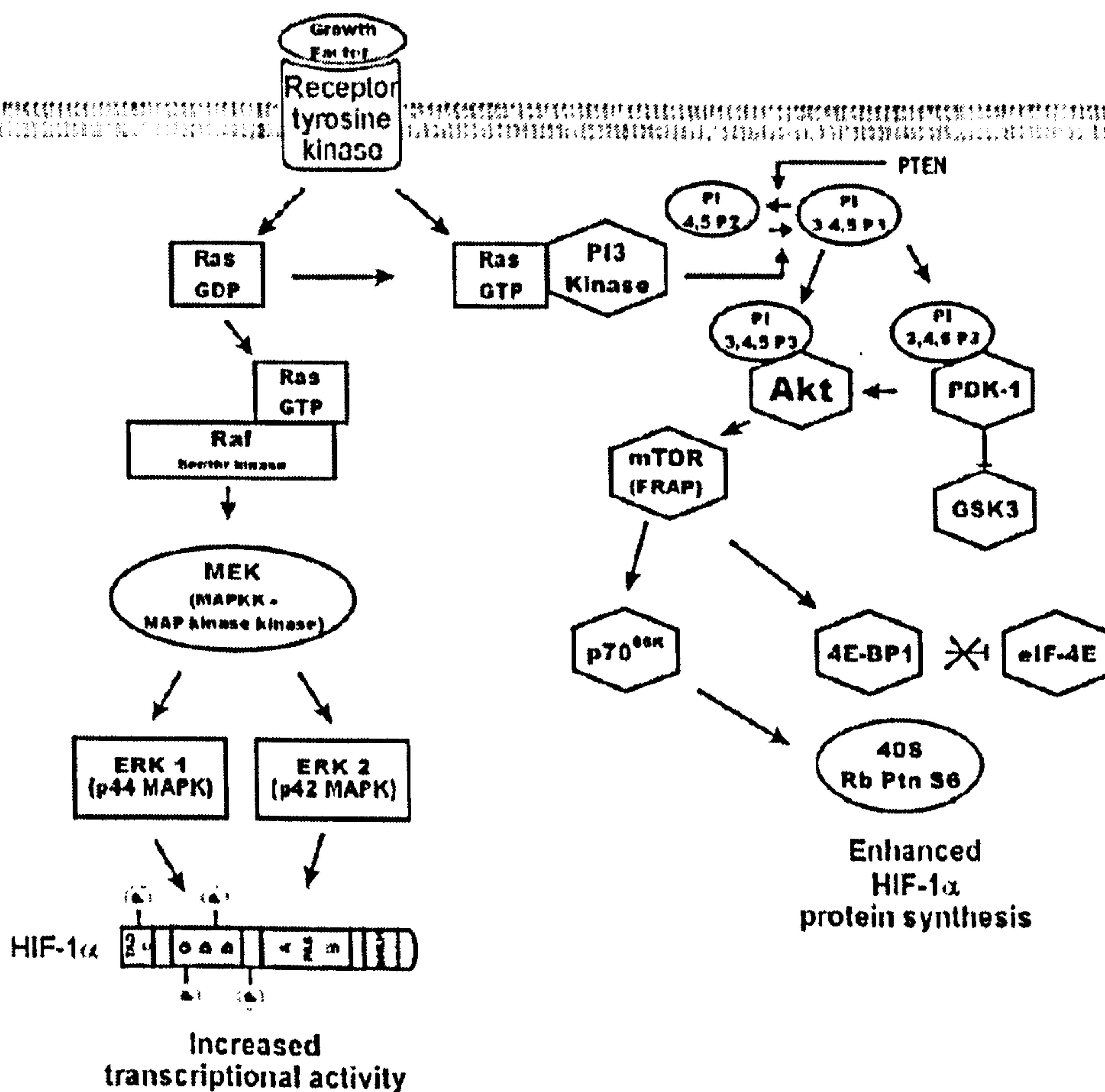


Figure 1.17. Phosphorylation of HIF-1 $\alpha$ .

The two main pathways leading to phosphorylation of HIF-1 $\alpha$ . Adapted from (Hopfl, Ogunshola et al. 2004)



### ***Signaling of the PI3K-AKT-FRAP pathway to HIF-1 $\alpha$***

Stimulation of cells with a variety of growth factors and cytokines (EGF, FGF-2, heregulin, IL-1 $\beta$ ) induces the expression of HIF-1 $\alpha$  protein, HIF-1 DNA-binding activity, and HIF-1 target gene expression under non hypoxic conditions (Feldser, Agani et al. 1999; Hellwig-Burgel, Rutkowski et al. 1999; Laughner, Taghavi et al. 2001). Binding of these factors to their tyrosine receptor induces many pathways including PI3K. The downstream serine-threonine protein kinases AKT has several targets involved in apoptosis, cell cycle, and growth as well as translation (Vivanco and Sawyers 2002). One of these target is FRAP (FKBP12/rapamycin-associated protein). The only known targets of FRAP are p70 s6 Kinase and eIF-4E binding protein, both of which function as regulators of translation (Semenza 2002). P70s6k is a kinase that enhances the translation of mRNAs that have 5'-polypyrimidine tracts as can be found in HIF-1 $\alpha$  mRNA (Iyer, Leung et al. 1998). This pathway is also negatively regulated by the tumour suppressor gene PTEN [phosphatase and tensin homologue deleted on chromosome 10; (Vivanco and Sawyers 2002)] PTEN loss of function is associated with angiogenesis and tumour progression in gliomas and prostate cancers. Forced overexpression of recombinant PTEN in glioma cells results in a dramatic reduction in HIF-1 $\alpha$  expression (Zundel, Schindler et al. 2000) suggesting that the presence or not of PTEN activity influences the stabilization of HIF-1 $\alpha$ .

### ***Signaling of the MAP kinase pathway to HIF-1***

Receptor tyrosine kinase activity also leads to signaling via ERK (p42 and p44) and p38 MAP kinase pathways. (Richard, Berra et al. 1999; Alfranca, Gutierrez et al. 2002; Gao, Jiang et al. 2002; Shemirani and Crowe 2002).



One of the best studied MAPK pathways involved in HIF-1 $\alpha$  regulation leads to the activation of ERK1-2 (p44/p42) after activation of the upstream molecules Ras/Raf-1/MEK-1/ERK1-2 (Hopfl, Ogunshola et al. 2004). It was demonstrated that the MAP kinase inhibitor PD-98059 attenuated HIF-1 $\alpha$  protein modifications and trans-activation ability but not protein stabilization and DNA-binding activity, demonstrating the importance of the MAPK pathway for the functionality of HIF-1 $\alpha$  (Hofer, Desbaillets et al. 2001). Recently it was also suggested that MAPK signalling affects the transactivation activity of p300, possibly by regulating the interaction between p300 and TAD-C (Sang, Stiehl et al. 2003).

### *Oncogenic Ras pathway*

Ras is a multifunctional oncogene that can stimulate both the MAPK- and the PI3K-pathways (Rodriguez-Viciano, Warne et al. 1994; Pouyssegur, Volmat et al. 2002). H-Ras transformation has been shown to increase HIF-1 $\alpha$  protein levels and target gene activation independent of oxygen through the PI3K pathway (Chen, Pore et al. 2001). Giaccia's group have demonstrated that increased VEGF gene transcription in H-RAS-transformed cells is dependent upon the presence of an intact HIF-1 binding site in the VEGF promoter (Mazure, Chen et al. 1997) and it was also demonstrated that loss of HIF-1 $\alpha$  negatively affects tumour growth in these cells (Ryan, Poloni et al. 2000). Thus many essential signal transduction pathways modulate HIF-1 activity.

### *ROS*

When O<sub>2</sub> is not completely reduced to water, the electron transfer results in the production of oxygen-containing free radicals and subsequently also of other reactive compounds, which are referred to as reactive oxygen species



(ROS). High amounts of ROS are known to cause damage to proteins, DNA and lipids (Kietzmann and Gorchach 2005). ROS appear to play an important role in both the hypoxic and non-hypoxic signalling processes which control the activity of HIFs. Under normoxia, enhanced ROS levels in response to many agonists appear to serve as signalling molecules to upregulate HIF-1 $\alpha$  in a variety of cell types, possibly by modulating upstream signalling pathways such as hydroxylases or kinases and phosphatases. In contrast, the role of ROS in the regulation of HIF-1 $\alpha$  under hypoxia is less well understood.

Another reactive molecule that seems to have a role in the HIF-1 $\alpha$  stabilization is nitric oxide (NO). There are contradictory reports: one group has found that NO stabilizes HIF-1 $\alpha$  (Sandau, Fandrey et al. 2001); but there is also evidence that treatment with NO abrogates HIF-1 activity (Huang, Willmore et al. 1999; Yin, Yang et al. 2000). These different results may be because treatment of cells for short time with NO induces activation, but for long time an abrogation. In particular these results were obtained by *in vitro* experiment and they could not reflect the physiological situation *in vivo* (Wenger 2002).

### *Pro-inflammatory stimuli*

Inflammatory cytokines, including TNF- $\alpha$  have been described previously to induce HIF-1 DNA-binding concomitant with increase in either HIF-1 $\alpha$  mRNA or protein, although the published results are somewhat contradictory. Thus Thornton et al. (Thornton, Lane et al. 2000) have reported that brief exposure of gingival and synovial fibroblasts to TNF- $\alpha$  resulted in elevated HIF-1 $\alpha$  mRNA level and DNA-binding activity, whereas Hellwig-Burgel et al (Hellwig-Burgel, Rutkowski et al. 1999)



reported no change in HIF-1 $\alpha$  mRNA or protein level after treatment of HepG2 cells by TNF. Nevertheless, these investigators confirmed that TNF- $\alpha$  induced HIF DNA binding. Zhou J et al. have shown that TNF- $\alpha$  favored accumulation of a ubiquitinated form of HIF-1 $\alpha$  and that NF- $\kappa$ B is involved in this TNF- $\alpha$  response (Zhou, Schmid et al. 2003).

Another cytokine that induce the expression of HIF-1 $\alpha$  in normoxic condition is IL-1 $\beta$ . Ferrara et al. have demonstrated that IL-1 $\beta$  increase mRNA levels of HIF-1 $\alpha$  in several cell lines (Ferrara and Davis-Smyth 1997). Hellwig-Burgel et al. showed that IL-1 $\beta$ , as TNF- $\alpha$ , caused a moderate activation of HIF-1 DNA binding but only IL-1 $\beta$  increased HIF-1 $\alpha$  protein levels (Hellwig-Burgel, Rutkowski et al. 1999).

Interestingly, treatment of macrophages with lipopolysaccharide (LPS) can increase the expression of a number of hypoxic genes (Camhi, Alam et al. 1995; Xiong, Elson et al. 1998). Blouin CC et al. have shown that LPS increases HIF-1 $\alpha$  expression inducing an active form of HIF-1, which binds specific HIF-1 DNA target sequences (Blouin, Page et al. 2004).

The ability of proinflammatory stimuli in inducing HIF-1 activation suggests a central role of this transcriptional factor during inflammatory processes, as recently demonstrated in HIF-1 conditional KO mice (Cramer, Yamanishi et al. 2003).

However other studies are needed to understand the molecular mechanism regulating these pathways.



Stimulus	References
<b>Proteins</b>	
Insulin	(Treins, Giorgetti-Peraldi et al. 2002)
IGF-1	(Fukuda, Hirota et al. 2002)
IGF-2	(Feldser, Agani et al. 1999)
bFGF	(Shi, Wang et al. 2005)
EGF	(Jiang, Jiang et al. 2001)
HGF	(Tacchini, Dansi et al. 2001)
PDGF	(Richard, Berra et al. 2000)
TGF- $\beta$	(Shih and Claffey 2001)
Il-1 $\beta$	(Stiehl, Jelkmann et al. 2002)
TNF- $\alpha$	(Haddad and Land 2001)
Angiotensin II	(Page, Robitaille et al. 2002)
Endothelin-1	(Spinella, Rosano et al. 2002)
Thrombin	(Richard, Berra et al. 2000)
Heregulin	(Laughner, Taghavi et al. 2001)
Nur77	(Yoo, Yeo et al. 2004)
Antigens (T cell receptor)	(Nakamura, Makino et al. 2005)
<b>Small molecules</b>	
Androgens	(Mabjeesh, Willard et al. 2003)
Thyroid hormone	(Ma, Freitag et al. 2004)
Acetylcholine	(Kakinuma, Ando et al. 2005)
FSH	(Alam, Maizels et al. 2004)
Serotonin	(Richard, Berra et al. 2000)
NO	(Sandau, Fandrey et al. 2001)
ROS (junD <sup>-/-</sup> cells)	(Gerald, Berra et al. 2004)
LPS	(Blouin, Page et al. 2004)
<b>Viral infection</b>	
Epstein-Barr virus	(Wakisaka, Kondo et al. 2004)
Hepatitis B virus	(Yoo, Oh et al. 2003)
Herpes virus 8	(Sodhi, Montaner et al. 2000)
<b>Physical stimuli</b>	
Heat	(Katschinski, Le et al. 2002)
Mechanical stress	(Chang, Shyu et al. 2003)
<b>Ions</b>	
Fe <sup>3+</sup> chelation	(Wang and Semenza 1993)
Co <sup>2+</sup>	(Wang and Semenza 1993)
Ni <sup>2+</sup>	(Salnikow, Donald et al. 2004)
Ca <sup>2+</sup>	(Mottet, Michel et al. 2003)
Cu <sup>2+</sup>	(Martin, Linden et al. 2005)
Zn <sup>2+</sup>	(Chun, Choi et al. 2000)
As <sup>+III</sup>	(Duyndam, Hulscher et al. 2001)
V <sup>+V</sup>	(Gao, Ding et al. 2002)
Cr <sup>+VI</sup>	(Gao, Jiang et al. 2002)
H <sup>+</sup> (pH)	(Mekhail, Gunaratnam et al. 2004)

**Table 1.3. Stimuli that induce HIF-1 $\alpha$  protein abundance, HIF transactivation activity, or both independently of hypoxia.**

Many proteins, peptides, and small molecules are known to induce HIF-1 $\alpha$  under normoxic conditions. Growth factors usually increase cell growth, and small-molecule ligands, viruses, and various environmental stimuli often elicit specific cell responses, all of which are associated with increased metabolism and oxygen consumption, finally leading to hypoxia. On the other hand, some stimuli, such as NO, ROS, and certain cations, might directly interact with the PHD oxygen sensors, leading to HIF-1 $\alpha$  stabilization. From (Wenger, Stiehl et al. 2005)



### 1.4.9. Involvement of HIF-1 in embryogenesis

The essential role of HIF-1 in embryonic development has been demonstrated by the analysis of mice that lack expression of HIF-1 $\alpha$  (Iyer, Kotch et al. 1998) or ARNT/HIF-1 $\beta$  (Kozak, Abbott et al. 1997; Maltepe, Schmidt et al. 1997).

*ARNT*<sup>-/-</sup> mice manifested developmental delay, neural tube defects, abnormal vascularization of the yolk sac, embryo, and placenta, and died by embryonic day (E) 10.5 (Kozak, Abbott et al. 1997; Maltepe, Schmidt et al. 1997). ARNT could dimerize with other bHLH-PAS transcription factors in addition to HIF-1 $\alpha$  and the loss of activity of one or more of these factors could therefore contribute to the observed phenotype (Semenza 1998). In fact it was not surprising that HIF-1 $\alpha$  and ARNT (HIF-1 $\beta$ ) deficiency resulted in overlapping but distinct embryonic phenotypes. HIF-1 $\alpha$ <sup>-/-</sup> embryos suffered developmental arrest at E8.5-8.75 and died at E10.5 (Iyer, Kotch et al. 1998). Analysis of viable embryos at E9.75-E10.0 showed a failure of neural tube closure with massive death of cephalic mesenchymal cells and the appearance of markedly enlarged/dilated vascular structures. Vascularization and development at E8.5-8.75 appeared normal, whereas one day later the entire vascular system of the branchial/cephalic region was disrupted. In contrast to this, the presumptive myocardium of HIF-1 $\alpha$ <sup>-/-</sup> embryos was strikingly hyperplastic, resulting in obliteration of the heart lumen. Neither the cephalic nor myocardial defects were observed in *ARNT*<sup>-/-</sup> embryos (Semenza 1998). There was no differences at the level of the yolk sac between HIF-1 $\alpha$ <sup>-/-</sup> and HIF-1 $\alpha$ <sup>+/+</sup> demonstrated that HIF-1 is required for the establishment of the embryonic cardiovascular system.

### 1.4.10. Tumour hypoxia

Tumour hypoxia is the net result of an imbalance between the O<sub>2</sub> supply and (metabolic) demand in stroma, endothelial cells, and the tumour cells. Tumour progression is



associated with both increased microvascular density and intratumoural hypoxia (Hockel and Vaupel 2001). Tissue hypoxia due to inadequate blood supply is supposed to occur very early during tumour development beginning at a tumour diameter of a few millimeters (Folkman 1990; Folkman and Shing 1992; Helmlinger, Yuan et al. 1997).

In response to this low level of oxygen, tumour cells adapt their metabolism by increasing glucose transport and glycolysis in order to maintain ATP production and this switch is mediated by HIF-1. Tumours influence the function and positioning of immune cells and in particular it is known that tumour-associated macrophages (TAM) localize preferentially in hypoxic areas. Such environment promotes TAM adaptation to hypoxia, which is achieved by the increased expression of hypoxia inducible and pro-angiogenic genes, such as VEGF, bFGF and CXCL8, as well as glycolytic enzymes, whose transcription is controlled by the transcription factors HIF-1 and HIF-2 (Talks, Turley et al. 2000). The *in vivo* relevance of this metabolic adaptation to hypoxia by macrophages was recently demonstrated by Cramer et al (Cramer, Yamanishi et al. 2003). Ablation of the HIF-1 $\alpha$  resulted in impaired macrophage motility and cytotoxicity, in low oxygen conditions.

In chapter 3 of this thesis we analyzed the effects of hypoxia on different immune cells and tumour cells in order to understand how it might influence their functions and expression of chemokine receptors as they are the principal regulator of cell migration.

Immunohistochemical analysis of human tumour biopsies revealed dramatic overexpression of HIF-1 $\alpha$  in common cancers (Zhong, De Marzo et al. 1999; Talks, Turley et al. 2000). HIF-1 $\alpha$  overexpression is also a marker of highly aggressive disease in several different tumour types (Table 1.4) but the correlation between HIF-1 $\alpha$  overexpression and adverse clinical outcome is not always demonstrated (Semenza 2002).



Tumor type	Association
Breast cancer, lymph-node negative	Bad progression
Cervical cancer, early-stage	Bad progression
Oligodendroglioma	Bad progression
Oropharyngeal squamous cell carcinoma	Treatment failure, Bad progression
Esophageal cancer, early-stage, BCL2-positive	Treatment failure
Ovarian cancer, p53 mutant	Bad progression

**Table 1.4. Association of HIF-1 $\alpha$  overexpression with adverse clinical outcome.**

Adapted from (Semenza 2002).

Tumour hypoxia selects for gene mutations in tumour cells, in particular in genes involved in the process of apoptosis (Kunz and Ibrahim 2003).

In malignant tumours the rate of apoptosis is high in undervascularized areas but the mechanisms by which hypoxia induces it are poorly understood (Barnhill, Piepkorn et al. 1998; Saikumar, Dong et al. 1998).

It was demonstrated in ovarian cancer that the overexpression of HIF-1 $\alpha$  is correlated with apoptosis in tumour expressing wild type p53, whereas it is not correlated with apoptosis in tumours expressing mutant p53, and the combination of mutant p53 and HIF-1 $\alpha$  overexpression is associated with a highly increased risk of patient mortality (Semenza 2002).

It's well known that low oxygen tension confers resistance of tumours to irradiation therapy and may thereby contribute to tumour aggressiveness (Harris 2002). Hypoxia is a stress factor for rapidly growing tumour cells and in fact it was demonstrated that hypoxia activates the JNK/SAPK and p38 stress kinases in human squamous carcinoma cells *in vitro* (Laderoute, Mendonca et al. 1999) and in low aggressive melanoma cells (Kunz, Bloss et al. 2002).

The HIF-1 complex is also involved in tumourigenesis. Mouse hepatoma cell lines that express mutated forms of ARNT form much smaller tumours that express only low levels of VEGF and do not become highly vascularized (Maxwell, Dachs et al. 1997).



Different groups have demonstrated that HIF-1 $\alpha$  is overexpressed in various solid tumours: colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate and renal carcinomas, and that is associated with cell proliferation (Zhong, De Marzo et al. 1999; Talks, Turley et al. 2000).

All these evidences highlight the relevance that the hypoxia-HIF-1 pathway may play in the recruitment and activation of TAM in solid tumours and may represent a target for therapeutic strategies against tumours.

#### **1.4.11. HIF-1 target genes**

Over 60 HIF-1 target genes have been identified thus far; these encode proteins that play key roles in critical developmental and physiological processes including angiogenesis/vascular remodelling, erythropoiesis, glucose transport, glycolysis, iron transport, and cell proliferation/survival (Figure 1.18).

Role of some of these proteins in different processes will be discussed briefly below.

##### ***Angiogenesis.***

Vascular endothelial cell growth factor (VEGF) is one of the major HIF-1 target genes; it specifically recruits endothelial cells into hypoxic and avascular areas and stimulates their proliferation. VEGF is the most potent endothelial-specific mitogen and is known to directly participate in angiogenesis (Harris 2000; Josko, Gwozdz et al. 2000; Conway, Collen et al. 2001). It was also shown that hypoxia induces the expression of VEGF mRNA and protein, suggesting that hypoxia is a stimulus of angiogenesis through the up-regulation of the VEGF expression (Harris 2000; Josko, Gwozdz et al. 2000). The induction of angiogenesis leads to an increase in vascular density and hence a decrease in the oxygen diffusion distances (Lee, Bae et al. 2004). However, local blood flow under pathophysiological



conditions is controlled by modulation of the vascular tone through production of inducible nitric oxide synthase (NO), heme oxygenase 1 (CO), endothelin 1, adrenomedulin, or activation of the  $\alpha_{1B}$ -adrenergic receptor, all of which are HIF-1 target genes (Eckhart, Yang et al. 1997; Lee, Jiang et al. 1997; Hu, Discher et al. 1998; Palmer, Semenza et al. 1998; Nguyen and Claycomb 1999).

### *Cell proliferation/survival*

Hypoxia-induced growth factors are known to promote cell proliferation and survival. Several growth factors, most notably insulin-like growth factor-2 (IGF-2) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) are also HIF-1 target genes (Feldser, Agani et al. 1999; Krishnamachary, Berg-Dixon et al. 2003). Binding of these factors to their cognate receptors, the insulin-like growth factor 1 receptor (IGFIR) and epidermal growth factor receptor (EGFR), respectively activates signal transduction pathways that lead both to HIF-1 $\alpha$  expression and to cell proliferation/survival (Semenza 2003).

### *Glucose metabolism*

Under hypoxic conditions, cells switch to the oxygen-independent metabolic pathway, and they use anaerobic glycolysis as a primary mechanism of ATP production (Sitkovsky and Lukashev 2005). Many genes involved in glucose uptake and glycolysis were identified as HIF-1 target genes (Wenger 2000). HIF-1 regulates expression of all enzymes in the glycolytic pathway, as well as expression of the glucose transporters GLUT1 and GLUT3, which mediate cellular glucose uptake (Chen, Pore et al. 2001).



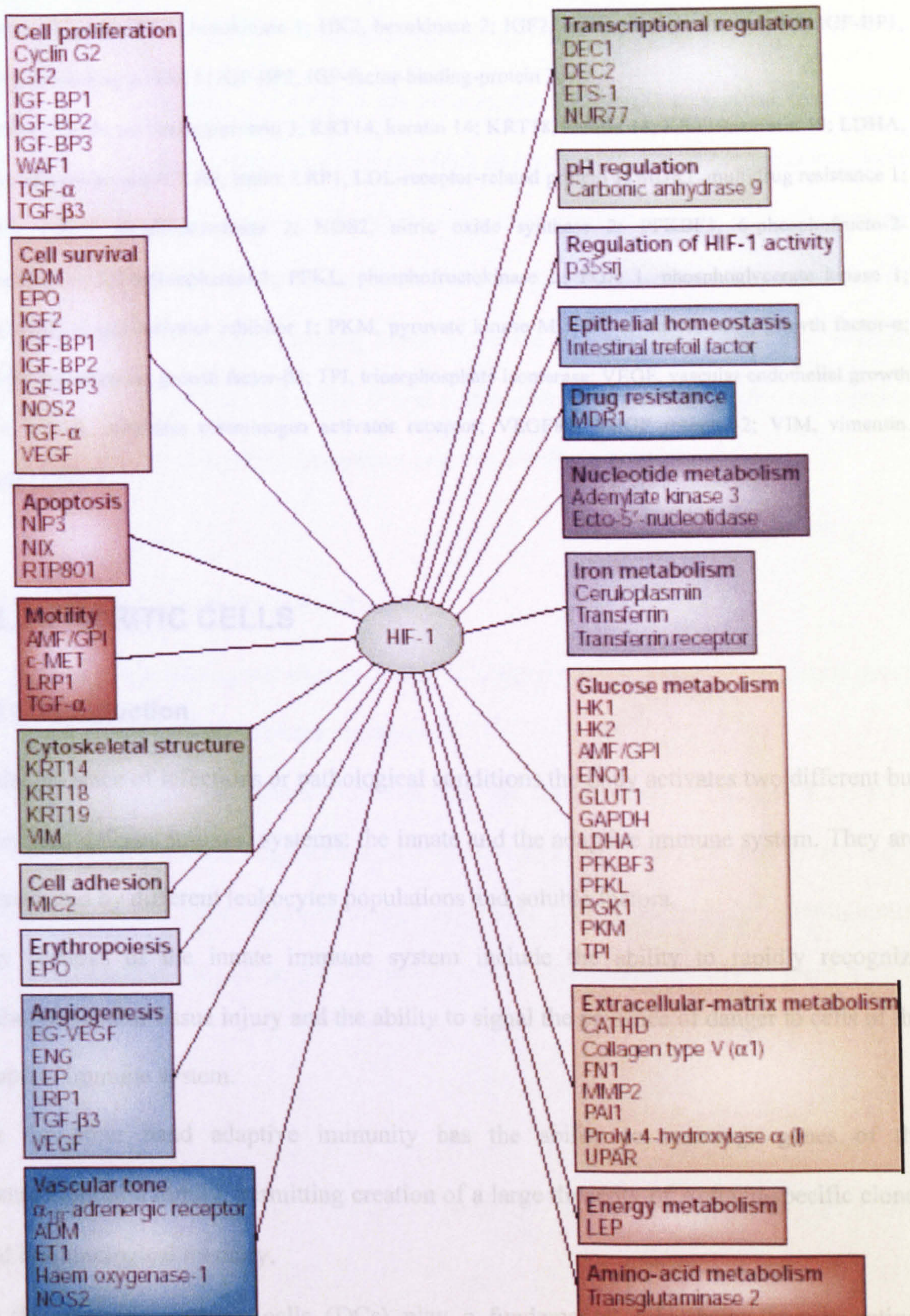
Upregulation of certain glycolytic enzymes is instrumental to the pyrimidine/purine pathway during glycolysis, and to cell proliferation by enhancing the supply of DNA precursors (Griffiths, McSheehy et al. 2002). The end product of anaerobic glycolysis is lactate. The enhanced production of this metabolite, possibly in association with the HIF-1-mediated upregulation of carbonic anhydrases (CA) could be a major cause for the acidic pH usually found in tumours (Stubbs, McSheehy et al. 2000) and these two factors are related to tumour invasiveness and they can be used as marker of tumour hypoxia (Hopfl, Ogunshola et al. 2004).

### *Iron metabolism*

Hypoxia has found to increase the expression of transferrin, probably to enhance iron transport to erythroid tissues (Rolfs, Kvietikova et al. 1997). The transferrin receptor is a hypoxia-inducible HIF-1 target gene, enabling cellular transferrin uptake (Tacchini, Bianchi et al. 1999). Ceruloplasmin, also known as a ferroxidase, was reported to be a HIF-1 target gene (Mukhopadhyay, Mazumder et al. 2000) and is required to oxidize ferrous to ferric iron (Lee, Bae et al. 2004).

Wenger's group have demonstrated that hypoxia increases the expression of transferrin, probably to enhance the iron transport to erythroid tissues (Rolfs, Kvietikova et al. 1997; Wenger 2002).





**Figure 1.18. Genes that are transcriptionally activated by HIF-1.**

Genes that are involved in many processes are transcriptionally activated by HIF-1. ADM, adrenomedullin; ALDA, aldolase A; ALDC, aldolase C; AMF, autocrine motility factor; CATHD, cathepsin D; EG-VEGF, endocrine gland-derived VEGF; ENG, endoglin; ET1, endothelin-1; ENO1, enolase 1; EPO, erythropoietin;



FN1, fibronectin 1; GLUT1, glucose transporter 1; GLUT3, glucose transporter 3; GAPDH, glyceraldehyde-3-P-dehydrogenase; HK1, hexokinase 1; HK2, hexokinase 2; IGF2, insulin-like growth-factor 2; IGF-BP1, IGF-factor-binding-protein 1; IGF-BP2, IGF-factor-binding-protein 2; IGF-BP3, IGF-factor-binding-protein 3; KRT14, keratin 14; KRT18, keratin 18; KRT19, keratin 19; LDHA, lactate dehydrogenase A; LEP, leptin; LRP1, LDL-receptor-related protein 1; MDR1, multidrug resistance 1; MMP2, matrix metalloproteinase 2; NOS2, nitric oxide synthase 2; PFKBF3, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase-3; PFKL, phosphofructokinase L; PGK 1, phosphoglycerate kinase 1; PAI1, plasminogen-activator inhibitor 1; PKM, pyruvate kinase M; TGF- $\alpha$ , transforming growth factor- $\alpha$ ; TGF- $\beta$ 3, transforming growth factor- $\beta$ 3; TPI, triosephosphate isomerase; VEGF, vascular endothelial growth factor; UPAR, urokinase plasminogen activator receptor; VEGFR2, VEGF receptor-2; VIM, vimentin. (Semenza 2003)

## 1.5. DENDRITIC CELLS

### 1.5.1. Introduction

In the presence of infections or pathological conditions the body activates two different but connected defence immune systems: the innate and the adaptive immune system. They are coordinated by different leukocytes populations and soluble factors.

Key features of the innate immune system include the ability to rapidly recognize pathogens and/or tissue injury and the ability to signal the presence of danger to cells of the adaptive immune system.

On the other hand adaptive immunity has the ability to rearrange genes of the immunoglobulin family, permitting creation of a large diversity of Antigen-specific clones and immunological memory.

In this system, dendritic cells (DCs) play a fundamental role, being unique antigen presenting cells (APC) able to efficiently activate a specific immune response and to establish immunological memory (Banchereau, Briere et al. 2000).



Moreover, different DC subsets and activation states, may play different and opposite roles in the balance between immune responsiveness and immune tolerance.

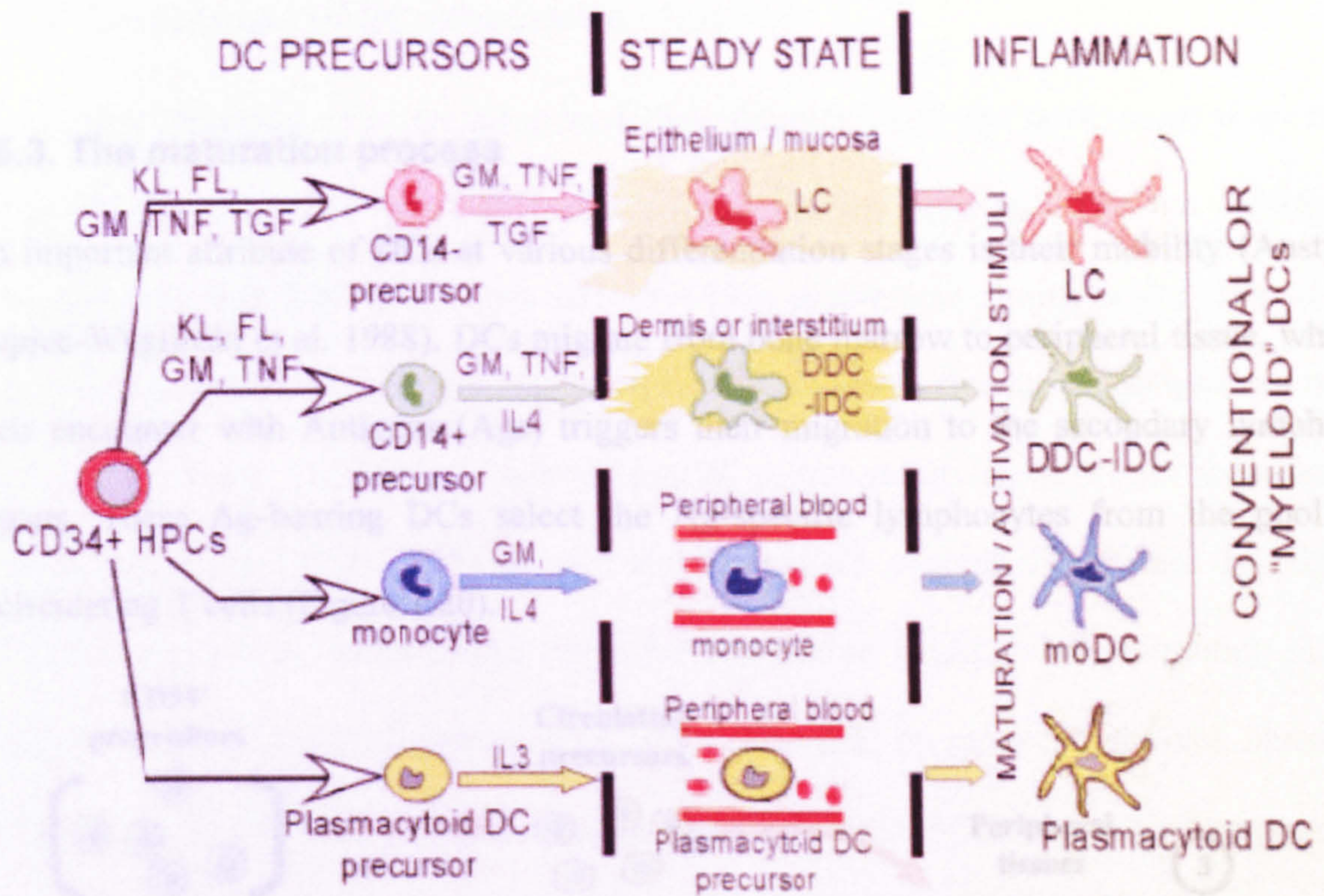
Immature DC precursors, generated in the bone marrow, home to peripheral sites where they reside. Here, these immature DC have a high phagocytic ability and express moderate levels of surface MHC Class II. However, upon encountering antigen or inflammatory stimuli, immature DC undergo a number of changes: downregulation and upregulation of some chemokine receptors enabling them to home to secondary lymphoid organs, upregulation of antigen-loaded MHC II and costimulatory molecules on the cell surface and increased ability to present the antigen to naïve T cells in lymph organs.

### **1.5.2. Different dendritic cell subsets**

Dendritic cells are heterogeneous populations of antigen presenting cells highly specialized in priming T-cell-dependent immune responses.

In the past there has been much confusion over the classification of DC into subclasses; however, recent evidence suggests that in both mice and men there exist at least two major subsets of DC: myeloid-related (including Langerhans' cells, interstitial DC, interdigitating DC, blood myeloid DC, monocyte-derived DC and CD34<sup>+</sup>-derived DC) and plasmacytoid DC (Figure 1.19).





**Figure 1.19. Development of human DC subsets.**

Precursors in blood and bone marrow (left section) can give rise to four types of DCs under cytokine driven conditions *ex vivo*. Resident populations of immature, nonactivated DCs (middle section) are normally found in the steady state in the periphery and/or the circulation. Semimature DCs continuously present self-Ags, and probably harmless non-self-Ags, in secondary lymphoid organs to maintain peripheral tolerance and anergy. Counterparts for these various DC types develop *in vitro* in the presence of the indicated cytokines. Harmful pathogens or other dangerous insults to the steady state can lead to inflammation with full maturation and activation of each DC subset (right section). From (Rossi and Young 2005)

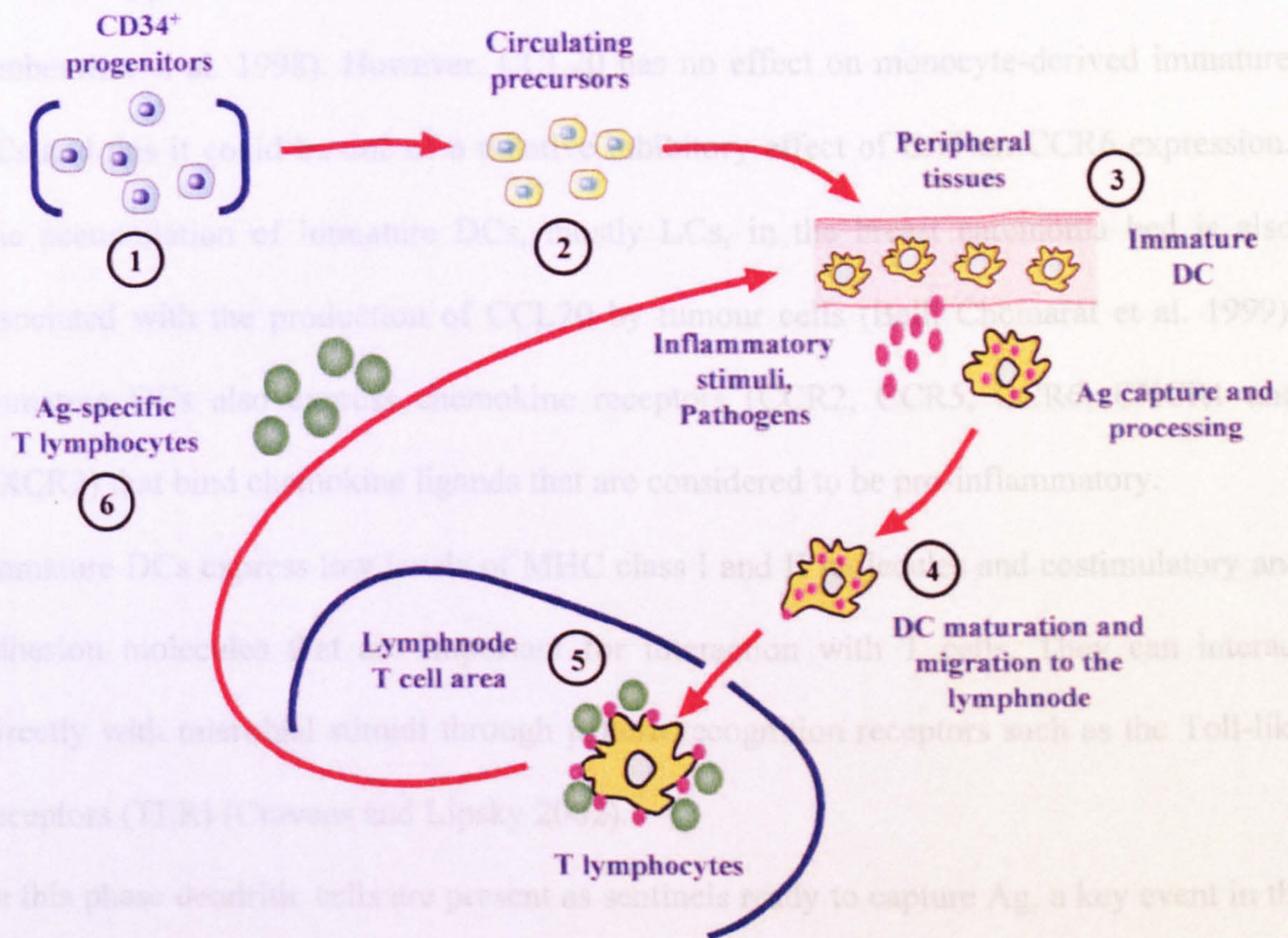
Myeloid related DCs are the classical T cell priming subset; plasmacytoid DC (pDC) play an important role in the body defence against pathogens and it was shown that they are able to produce large quantities of type I interferons (Vicari, Treilleux et al. 2004). The level of heterogeneity reflected by anatomical localization includes skin epidermal Langerhans cells (LCs), dermal (interstitial) DCs (intDCs), splenic marginal DCs, T zone interdigitating cells, germinal-center DCs, thymic DCs, liver DCs, and blood DCs (Banchereau, Briere et al. 2000). Plasmacytoid DCs are found most abundantly in the blood, but also in the liver and bone marrow.



### 1.5.A. Recruitment of DC precursors

#### 1.5.3. The maturation process

An important attribute of DCs at various differentiation stages is their mobility (Austyn, Kupiec-Weglinski et al. 1988). DCs migrate from bone marrow to peripheral tissue, where their encounter with Antigens (Ags) triggers their migration to the secondary lymphoid organs. There Ag-bearing DCs select the Ag-specific lymphocytes from the pool of recirculating T cells (Figure 1.20).



**Figure 1.20. The life cycle of dendritic cells (DC).**

(1) DCs originate from CD34<sup>+</sup> bone marrow precursors. (2) DC progenitors reach peripheral tissues via blood vessels. (3) Immature DC reside in peripheral tissue in a resting state, but upon the encounter with Ag, they efficiently capture and process it and initiate a process of maturation (4) and migration to regional lymph nodes. (5) In the T cell areas of lymph nodes, DCs present the Ag to T lymphocytes. (6) Activated T cell effectors will leave the lymph node to reach the peripheral site and fight the invading pathogens.



#### 1.5.4. Recruitment of DC precursors

When new dendritic cells are generated they migrate from the bone marrow to non-lymphoid tissues, where they eventually become resident cells and they are considered immature DC with high phagocytic capacity. DCs accumulate rapidly at the sites of Ag deposition and this accumulation is presumably due to the production of chemokines upon local inflammation. Different DC subsets display unique sensitivity to certain chemokines. For example the CD34<sup>+</sup> HPC-derived immature DCs express CCR6 and his ligand (CCL20) appears to be the most powerful chemokine guiding their migration (Dieu, Vanbervliet et al. 1998). However, CCL20 has no effect on monocyte-derived immature DCs and this it could be due of a putative inhibitory effect of IL-4 on CCR6 expression. The accumulation of immature DCs, mostly LCs, in the breast carcinoma bed is also associated with the production of CCL20 by tumour cells (Bell, Chomarat et al. 1999). Immature DCs also express chemokine receptors (CCR2, CCR5, CCR6, CXCR1 and CXCR2) that bind chemokine ligands that are considered to be pro-inflammatory.

Immature DCs express low levels of MHC class I and II molecules and costimulatory and adhesion molecules that are important for interaction with T cells. They can interact directly with microbial stimuli through pattern recognition receptors such as the Toll-like receptors (TLR) (Cravens and Lipsky 2002).

In this phase dendritic cells are present as sentinels ready to capture Ag, a key event in the induction of immunity, with the following maturation and mobilization of DC.

#### 1.5.5. Antigen capture

Immature DCs are very efficient at Ag capture and can use several pathways, such as macropinocytosis, receptor-mediated endocytosis via C-type lectin receptors or Fcγ receptors and phagocytosis.



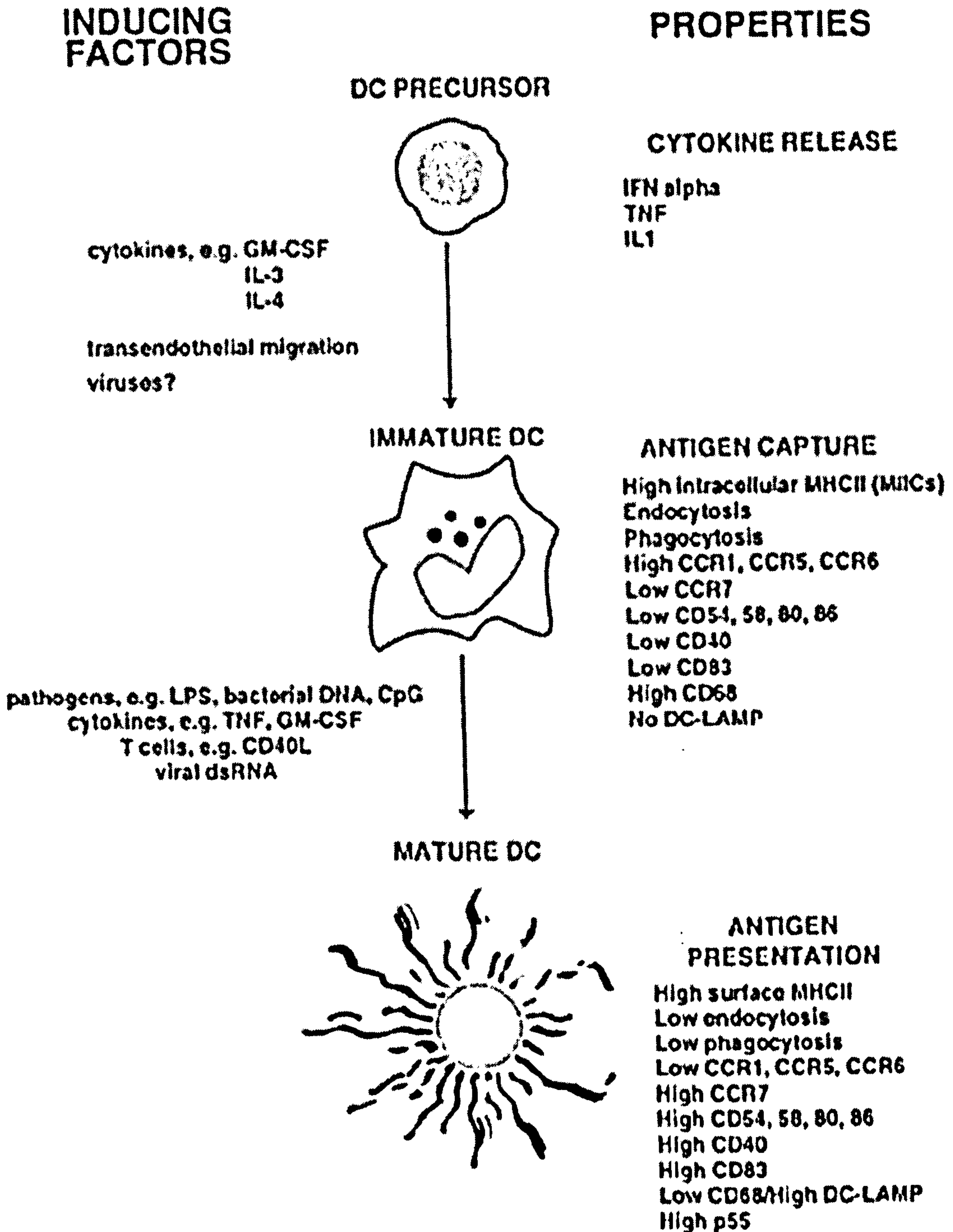
### 1.5.6. Dendritic cell activation and maturation

The antigen/pathogen induces the immature DC to undergo phenotypic and functional changes that culminate in the complete transition from Ag-capturing cell to APC. DC maturation is intimately linked with their migration from the peripheral tissue to the draining lymphoid organs (Banchereau, Briere et al. 2000).

Numerous factors induce and/or regulate DC maturation including pathogen-related molecules such as LPS (Rescigno, Granucci et al. 1999), bacterial DNA (Hartmann, Weiner et al. 1999) and dsRNA (Cella, Salio et al. 1999); the balance between proinflammatory and anti inflammatory signals in the local microenvironment, including TNF- $\alpha$ , IL-1, IL-6, IL-10, TGF- $\beta$  and prostaglandin; T cell-derived signals. The maturation process is also associated with several coordinated events such as loss of endocytic/phagocytic receptors; upregulation of costimulatory molecules CD40, CD58, CD80, and CD86; synthesis and translocation of the MHC II-peptide complexes to the cell surface.

DC maturation induces also morphological changes, as loss of adhesive structures, cytoskeleton reorganization, and acquisition of high cellular motility (Winzler, Rovere et al. 1997) that is the most important characteristic of these cells.





**Figure 1.21.**Maturation of Dendritic cells (DCs).

The left side of the scheme shows the factors inducing progression from one stage to another (GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; dsRNA, double-stranded RNA); the right side shows the main properties of each differentiation/maturation stage (IFN, interferon; MHCII, major histocompatibility complex II; MIIC, MHCII-rich compartment; LAMP, lysosome-associated membrane protein). From (Banchereau, Briere et al. 2000)



### **1.5.7. Migration of Antigen-Bearing Dendritic Cells**

It is well demonstrated that DCs leave the non lymphoid organs through the afferent lymph (Kripke, Munn et al. 1990; Larsen, Steinman et al. 1990).

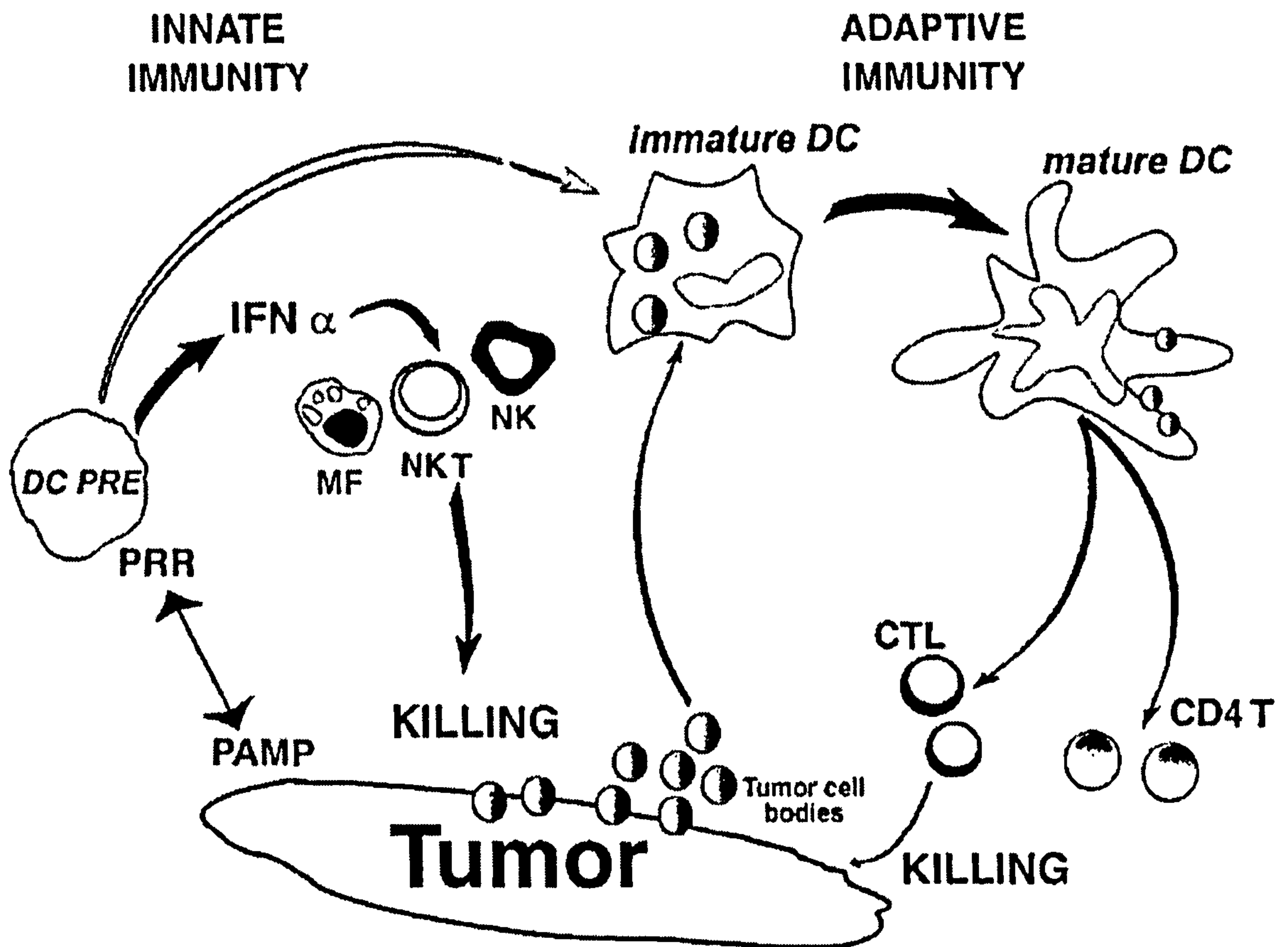
The migration of maturing DCs involves a coordinated action of several chemokines. Upon maturation DCs upregulate chemokine receptor, CCR7 and subsequently acquire responsiveness to CCL19 and CCL21. Consequently, maturing DCs will leave the inflamed tissues and enter the lymph stream, potentially directed by CCL21 that is expressed on lymphatic vessels (Gunn, Tangemann et al. 1998; Saeki, Moore et al. 1999). Mature DC arrived near the T cell zone become themselves a source of CCL19 and CCL20 (Dieu, Vanbervliet et al. 1998; Ngo, Tang et al. 1998; Sallusto, Schaerli et al. 1998), allowing an amplification and or a persistence of the chemotactic signal.

### **1.5.8. Tumour and dendritic cells**

The immune system has the potential to eliminate neoplastic cells, as evidenced by occasional spontaneous remission in renal-cell carcinomas and melanomas (Bell, Young et al. 1999; Timmerman and Levy 1999).

The induction of tumour immunity can be initiated by the effectors of innate immunity and further developed by cells of adaptive immunity where DCs play a central regulatory role (Figure 1.22).





**Figure 1.22. Dendritic cells in tumour immunology.**

Dendritic cells as a link between innate immunity and adaptive immunity in antitumor immune responses (a hypothesis). Precursor DCs recognize tumor pathogen associated molecular patterns (PAMP) through their pattern recognition receptors (PRR). Consequently, DCs release interferon (IFN)  $\alpha$ , which activates macrophages (MF), natural killer (NK) T cells, and NK cells that kill tumors leading to the release of tumor cell bodies. The cell bodies are captured by immature DCs (which may be the progeny of the initial precursor), which will mature and display tumor antigens for selection of tumor specific lymphocytes. CD8 T cells will further directly kill the tumor while selected CD4 T cells will activate macrophages, NK cells, and eosinophils. The tumor may affect this process at various stages, by either preventing DC maturation or skewing the T cell responses towards the type 2. From (Banchereau, Briere et al. 2000)

Tumour immunity can be viewed as a three-step process that includes (i) presentation of tumour-associated Ags (TAAs); (ii) selection and activation of TAA-specific T cells as well as non-Ag-specific effectors, and (iii) homing of TAA-specific T cells to the tumour site and elimination of tumour cells (Bell, Chomarat et al. 1999). Tumours may escape immune surveillance owing to alterations at each of these steps. Tumour can prevent DC



differentiation and/or APC function producing cytokines such as IL-6, IL-10, M-CSF, VEGF. Recent publications demonstrated that hypoxia, which is characteristic of solid tumours, may affect functions and maturation process of DC (Qu, Yang et al. 2005; Zhao, Darmanin et al. 2005). This may be an example of how tumours may subvert immune cells functions.

Analysis of DCs distribution in breast carcinoma revealed two levels of heterogeneity: a) immature CD1a<sup>+</sup>DCs, mostly of the LC type are retained within the tumour bed, b) mature DCs are confined to peritumoural areas (Banchereau, Briere et al. 2000). Scarpino S. et al. have found that in papillary carcinoma in thyroid most dendritic cells present an immature phenotype and are located at the invasion edge of the tumour while macrophages are evenly distributed throughout the tumour (Scarpino, Stoppacciaro et al. 2000).

It was recently demonstrated that also pDCs are present in tumours [reviewed in (Vicari, Treilleux et al. 2004)] and their infiltration in ovarian tumours is paralleled by the expression of CXCL12, to which they respond (Zou, Machelon et al. 2001).

Many chemokines active on immature DC (CCL2, CCL3, CCL4, CCL7) are secreted under inflammatory conditions and by tumours [as reported by histological analysis (Negus, Stamp et al. 1997; Scarpino, Stoppacciaro et al. 2000; Tang, Tan et al. 2001)] and their activity appears to be restricted to specific DC subsets.

The significance of the recruitment of DC in tumours is an important issue not properly investigated yet.

New studies are requested to clarify the mechanisms by which microenvironmental signals affect DC functions.

## 1.6. Aims

The aim of this thesis is to investigate the effects of tumour hypoxia on cell migration. It is known that solid tumours are generally characterized by regions of necrosis, associated

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with low oxygen tension and the consequent activation of metabolic adaptation pathways by resident (cancer cells) and infiltrating cells (stroma cells). Based on these observations the first aim was to describe the functional chemokine receptor profile acquired by cancer and stroma cells in hypoxia and to identify the molecular mechanisms promoting this event. The results of this study provide novel insights into the mechanisms governing recruitment and activation of macrophages associated with tumours and identify possible therapeutic targets to prevent their accumulation, as well as their protumoral functions.

The second aim was to investigate the influence of hypoxia on dendritic cells maturation and function. The rationale of this study stems from the observation that DC play an essential role in the activation of specific antitumour immunity and represent an important component of the inflammatory infiltrate of inflammatory diseases characterized by low oxygen tension (e.g. tumours, arthritis rheumatoid). Identification of mechanisms governing DC maturation and functions in hypoxia represents an unexplored field which may eventually lead to the discovery of specific pathways governing activation of immune programmes associated with several pathological conditions.



## Chapter 2. Materials and Methods

### 2.1. Cells and Culture Conditions

#### 2.1.1. Isolation of human monocytes

Human monocytes were separated from the peripheral blood of human healthy donors by Percoll gradient centrifugation (Sica, Sacconi et al. 1997). Briefly, blood was diluted 1:5 with PBS and centrifuged for 10 min at 1000 rpm. The supernatant was eliminated, cells pellet were re-diluted 1:4 in PBS, the Ficoll (Amersham Biosciences) stratificated at the bottom and centrifuged for 25 min at 1650 rpm at room temperature. The mononuclear cell layer was collected and washed thoroughly by resuspension in PBS. After washing, the mononuclear cell layer was diluted in osmolorized medium (285 mOSM) and an equal volume of Percoll (46% in osmolorized medium) was stratificated at the bottom. Following centrifugation for 30 min at 2000 rpm at room temperature, monocytes were collected at the interface between medium and Percoll, while lymphocytes were at the bottom of the tube. Monocytes were >90% pure as assessed by morphology criteria (Giemsa-stained cytopsin preparations, looking at the morphology of the nucleus and FACS analysis using CD14 antibody). The cells were washed thoroughly by resuspension in PBS and finally resuspend at  $10^7$ /ml in RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% FBS (HyClone, Logan, UT), 2 mM glutamine, and antibiotics. All reagents contained <0.125 EU/ml of endotoxin as checked by limulus amebocyte lysate assay (Microbiological Associates, Walkersville, MD).

#### 2.1.2. Generation of monocytes-derived macrophages and dendritic cells

Monocyte-derived macrophages (MDMs) were derived from freshly isolated monocytes ( $3-5 \times 10^6$  cells/ml) after incubation for 5 days in RPMI 1640 medium supplemented with



10% FBS, 2 mM glutamine, antibiotics, and 40% autologous serum on hydrophobic petriperm plates (Sigma-Aldrich), as described previously (Mantovani, Caprioli et al. 1977). The resulting macrophages were washed and resuspended in RPMI 1640 supplemented with 10 % foetal bovine serum (FBS; Gibco BRL) prior to use. The viability of the cell population was assessed by Trypan Blue staining. Macrophage differentiation was assessed by FACS analysis using specific marker CD68.

Human dendritic cells were derived from freshly isolated monocytes, followed by plastic adherence, after incubation for 6 days at  $1 \times 10^6$ /ml in medium RPMI 1640 with 50 ng/ml GM-CSF and 20 ng/ml IL-13. After 6 days of culture, the outcoming population consisted of typical immature dendritic cells which usually expressed CD1a (>90% positive cells), low levels of CD80 and CD86 and was negative for CD83 (<10%) and CD14 (<10%) (Piemonti, Bernasconi et al. 1995; Sozzani, Sallusto et al. 1995; D'Amico, Bianchi et al. 1998).

To induce maturation, LPS (Rescigno, Martino et al. 1998) at different concentration ranging from 0.1 ng/ml to 100 ng/ml was added at day 6 for 24 h in normoxia (20% O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub>) or hypoxia conditions (94% N<sub>2</sub>, 5% CO<sub>2</sub>, and 1% O<sub>2</sub>).

### **2.1.3. Isolation of tumour associated macrophages**

TAMs were obtained from ascitic fluids collected from untreated patients with histologically confirmed epithelial ovarian carcinoma admitted to the Department of Obstetric and Gynecology, S. Gerardo Hospital, Monza, Italy (Sica, Sacconi et al. 2000). All patients had cancer classified as stage II, III, or IV. Ascitic fluid was collected and centrifuged for 10 min at 1500 rpm. Cells pellet was 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 non adherent cells. TAMs were >85% pure as assessed by morphology criteria (Giemsa-stained slide preparation treated as all the other samples). The adherent cells were rested with complete medium overnight at 37°C and subsequently stimulated as indicated in the result chapters of this thesis.

#### **2.1.4. Isolation of human endothelial cells**

Human endothelial cells were obtained from umbilical veins (from normal placenta without hepatitis or HIV contamination). The umbilical vein was cannulated with sterile blunt needles and perfused with PBS to wash out the blood. Then, the vein was filled with collagenase at 1mg/ml (Boehringer) and allowed digestion to proceed for 20 minutes at 37°C. After this period the vein was washed with Hanks buffer (Sigma, Milan) and the supernatant was collected. Cells were pelleted by centrifugation (5 min at 1500 rpm), resuspended in M199+20% FCS without growth factor and put in a flask without gelatine. When cells formed a monolayer, they were trypsinased, resuspended in medium supplemented with growth factor (50µg/ml of endothelial cell growth supplement and 100 µg/ml heparin) and transferred in flasks coated with gelatine at 0.5% (Sigma) (Romano, Sironi et al. 1997). We used routinely confluent cells at second to sixth passages. Cells at the concentration of  $1.5 \times 10^4 / 0.2$  ml were cultured for 24 h in flat-bottomed 96-well plates (Falcon) in M199 medium with 20% FCS, supplemented with 50µg/ml of endothelial cell growth supplement (Collaborative Research Inc.) and 100 µg/ml heparin. Cells were maintained at 37°C in a humidified incubator containing 20%O<sub>2</sub>, 5% CO<sub>2</sub>, and 75% N<sub>2</sub>.



### 2.1.5. Isolation of plasmacytoid dendritic cells

PBMCs were isolated from buffy coats by Ficoll gradient and plasmacytoid dendritic cells (P-DC) were magnetically sorted with blood DC Ag BDCA-4 cell isolation kits (Miltenyi Biotec, Milan), to a purity of 90-98 % (Penna, Sozzani et al. 2001). Blood P-DC ( $10^6$  cells/ml). Cells were then cultured in medium containing 1,000 U/ml recombinant human GM-CSF (Mielogen; Schering-Plough) and 20 ng/ml IL-3 (ProSpec). Cells were matured by 24-h incubation with 100 ng/ml LPS (*Escherichia coli* 055:B5; Sigma-Aldrich), or 20 ng of hemagglutinin/ml inactivated influenza virus strain A/Moscow/10/99 (a gift from T. De Magistris, Istituto Superiore di Sanità, Rome, Italy).

### 2.1.6. Isolation of murine dendritic cells

Mice were sacrificed using CO<sub>2</sub>. The skin mid-back was clipped and the skin from the lower part of the body was removed. All the tissue from legs was removed with scissors and dissected away from body. The remaining tissue was cleaned from the pelvic and femoral bones and separated at knee joint (it is important to remove all the tissue from the bones since cells associated with this can contaminate the marrow preparation). Each end of bone was cut off and using a 25g needle and a 12cc syringe filled with PBS. The bone marrow from both ends of the bone was expelled with a jet of PBS into a 50 ml Falcon tube.

CD34<sup>+</sup> bone marrow cells from femurs and tibias of C57BL/6 mice were prepared by positive immunoselection using MACS microbeads coated with goat anti-rat IgG (Miltenyi Biotec Inc. Auburn, CA), using the rat mAb MEC14.7 (Garlanda, Berthier et al. 1997) to mouse CD34 as selecting agent. Cells were separated following the manufacturer's instructions. CD34<sup>+</sup> positive cells (23105 /mL) were cultured in RPMI 1640 medium with



10% fetal calf serum,  $2 \times 10^{-5}$  2-ME, GM-CSF (40 ng/mL), and Flt3 ligand (100 ng/mL).

Cells were diluted 1:2–1:3 every 2 or 3 days.

Cultured cells were collected after 9 days and used in the different assays. DC were characterized in terms of membrane phenotype (expression of DEC205, MHC class II, CD11c, CD86), pinocytosis, and antigen presentation in allogeneic MLR.

Where specified, DCs were cultured with 20 ng/mL TNF- $\alpha$  or 10-100 ng/mL LPS for the last 24 h of culture in normoxia, hypoxia or desferrioxamine (DFX, 400 $\mu$ M) condition.

### **2.1.7. Cells lines and culture**

Mouse embryonic fibroblasts (MEFs), wild type, and HIF-1 $\alpha$ <sup>-/-</sup> were a gift from G. Semenza (The Johns Hopkins University School of Medicine, Baltimore, MD) and were routinely maintained in DMEM (Invitrogen and Life Technologies, Milan) supplemented with 10% heat-inactivated FBS (Whittaker Bioproducts), 50 IU/ml penicillin, 50  $\mu$ g/ml streptomycin, and 2 mM glutamine (all purchased from Invitrogen and Life Technologies, Milan).

MCF-7 (human breast cancer cells), CAOV3 (human ovarian cancer cell line), 786.0 (Lieubeau-Teillet, Rak et al. 1998), and WT2 human renal cancer cells (Ivanov, Kuzmin et al. 1998) were routinely maintained in RPMI 1640 medium (Whittaker Bioproducts, USA) supplemented with 5% heat-inactivated FBS, 50 IU/ml penicillin, 50 $\mu$ g/ml streptomycin, and 2 mM glutamine. Cells were maintained at 37°C in a humidified incubator containing 20% O<sub>2</sub>, 5% CO<sub>2</sub> in air (referred to as normoxic conditions).

### **2.1.8. Post-trypsinisation recovery of ovarian cancer cell lines**

Prior to their use in chemotaxis or flow cytometry, the adherent ovarian and breast 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 (SIGMA, Milan) at  $0.5-1 \times 10^6$  cells/ml for a minimum of 5 hours.

### **2.1.9. Normoxia and hypoxia treatment**

In this thesis we considered as normoxia the normal cell culture condition where the percentage of O<sub>2</sub> is 21% and it is important to specify that this condition is of supra normoxia as it isn't a physiological normoxia corresponding to 13% of O<sub>2</sub> in the blood vessels (even if used by all the scientist).

Hypoxia treatment was performed by placing cells in a modular incubator chamber (Billups-Rothenberg Inc., Del Mar, California) and flushing them with a mixture of 1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub> for 20 min. The chamber was placed at 37°C.

To induce pharmacological hypoxia we use desferrioxamine (Sigma Aldrich, Milan) as hypoxia mimicking compound at the concentration of 400 µM.

## **2.2. Cytokines and Reagents**

Human recombinant CXCL12 and CCL5 were from PeproTech (London, UK). Desferrioxamine (DFX) and actinomycin D (used at 1 µg/ml) were purchased from Sigma-Aldrich (Milan). LPS (*Escherichia coli* 005:B5; Difco, MI) was used from 0.1 ng/ml to 100 ng/ml. Actinomycin D were from Sigma (Milan).

## **2.3. Migration Assay**

Monocyte migration was evaluated using a chemotaxis microchamber technique as described previously (Sica, Saccani et al. 1997). 27 µl of chemoattractant solution or



control medium (RPMI 1640 with 1% FCS) was added to the lower wells of a chemotaxis chamber (Neuroprobe). A polycarbonate filter (5 $\mu$ m pore size; Neuroprobe) was layered onto the wells and covered with a silicon gasket and the top plate. 50  $\mu$ l of cell suspension (1.5x10<sup>6</sup>/ml fresh human monocytes), preincubated for 16 h in the presence of 400 $\mu$ M DFX, were seeded in the upper chamber. The chamber was incubated at 37°C for 90 min. At the end of this period, filters were removed and stained with Diff-Quik (Baxter), and 10 high-power oil immersion fields were counted.

Cancer cell migration was assayed as described previously (Scotton, Wilson et al. 2001) by using Falcon transwells (24-well format, 8- $\mu$ M pore; BD Biosciences). 0.5 ml of media (DMEM plus 1 % BSA) containing 5x10<sup>5</sup> cells was added to the upper chamber, and 0.5 ml of medium alone or media supplemented with CXCL12 (PeproTech) was added to the lower chamber. After overnight incubation in normoxic (5% CO<sub>2</sub>) or hypoxic conditions (1%O<sub>2</sub>), cells on the upper surface of the filter were removed using a cotton wool swab. Migrated cells on the lower surface were stained using DiffQuick (Dade Behring, Düringen, Switzerland). For each transwell, the number of migrated cells in 10 medium-power fields (20X) was counted. Three transwells chambers were used per condition.

For human umbilical vein endothelial cell (HUVEC) migration, polycarbonate filters (5 $\mu$ m pore size, polyvinylpyrrolidone free) were soaked in 0.5 M acetic acid, washed with PBS, incubated for 24 h in 0.01% gelatin (Sigma-Aldrich), and air dried (Colotta, Bussolino et al. 1993). CXCL12 and fibrinogen (Sigma-Aldrich) in M199, containing 1% FCS, were seeded in the lower compartment, and 50 $\mu$ l of HUVECs (2x10<sup>6</sup>/ml) was added to the upper compartment. After 6 h of incubation at 37°C, the upper surface of the filter was scraped with a rubber policeman. The filters were fixed and stained, and five oil immersion fields (lower surface) were counted after coding samples.

Dendritic cells migration was evaluated using a 48-well microchemotaxis chamber technique. Briefly 27  $\mu$ l of chemokine or control medium (RPMI 1640+1%FCS) was



added to the lower wells of the chemotaxis chamber (Neuropore, Pleasanton, CA) (Sozzani, Sallusto et al. 1995) and 50  $\mu$ l of cell suspension ( $1.5 \times 10^6$ /ml) were seeded in the upper chamber. The two compartments were separated by 5  $\mu$ m pore size polyvinylpyrrolidone polycarbonate filter (Neuroprobe). The chamber was incubated for 90 min at 37°C in a humidified atmosphere in the presence of 5% CO<sub>2</sub>.

## **2.4. Methods for analysing RNA expression**

### **2.4.1. Preparation of RNA from tissue samples and cell lines**

Total RNA was prepared from monocytes, TAM, M-DM, endothelial cells and cancer cell line (MCF7 and CAOV3) using Trizol Reagent™ (Invitrogen) according to the manufacturer's instructions.

### **2.4.2. Northern Blot Analysis**

#### *2.4.2.i. Northern blotting*

10  $\mu$ g of total RNA was mixed with cold water to a final volume of 10  $\mu$ l and added 30  $\mu$ l of solution I for RNA (50 % deionised formamide [Merck/BDH, Lutterworth, UK], 6 % formaldehyde [Merck/BDH, Lutterworth, UK], 400 mM MOPS [3-*N*-morpholinopropanesulphonic acid], 100 mM sodium acetate and 10 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 10 minutes, then placed on ice prior to loading. The RNA was subjected to electrophoresis through a 1 % agarose-formaldehyde gel (1 % agarose [Gibco BRL, Paisley, UK], 6 % formaldehyde, 400 mM MOPS, 100 mM sodium acetate, 10 mM EDTA pH 8.0, 0.5  $\mu$ g/ml ethidium bromide) and subsequently blotted by capillary transfer onto



Gene screen Plus membranes (New England Nuclear Boston, MA). After transfer, the membrane was dried in an oven at 80° C for 2h.

#### *2.4.2.ii. 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 (50 % deionised formamide, 10% dextran sulphate, 1% SDS, 1 M NaCl, 100 mg/ml Salmon Sperm DNA made up in distilled water).

cDNA probes were labelled by random priming using a commercial kit (Boehringer) according to the manufacturer's instructions and  $\alpha$ -[<sup>32</sup>P]dCTP (3,000 Ci/mmol; Amersham Biosciences).

Unincorporated [<sup>32</sup>P]CTP nucleotide was removed by passing the radiolabelled probe through a Sephadex G50 column. 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  $1 \times 10^6$  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 three times at room temperature for 10 min in 0.2X SSC (1X SSC = 0.15 M NaCl, 0.015 M of sodium citrate, pH 7.0), 0.1% SDS, and twice at 60° C for 20 min in 0.2X SSC, 0.1% SDS before being autoradiographed using films and intensifier screens at -80° C (XAR-5; Kodak). Densitometric analysis was performed with a scanning densitometer (model GS300; Hoefer Scientific Instruments).

#### *2.4.2.iii. cDNA probes*

CCR1 and CCR5 cDNAs were obtained in our laboratory (Sica A. et al. 2000 J. Immunol. 164: 733-738). CXCR4 cDNA was provided by T.N.C. Wells (Serono Pharmaceutical



Research Institute, Geneva, Switzerland).  $\beta$ -Actin was provided by Dott. Chiesa R. (Mario Negri Institute) (Hanukoglu, Tanese et al. 1983).

Appropriate restriction enzymes were used to cut out each cDNA insert; each digested reaction was added to 5  $\mu$ l of loading buffer (0.25 % w/v bromophenol blue; 0.25 % xylene cyanol [all from Sigma] and 25% Ficoll 400, made up in distilled water) and electrophoresed through a 1 % agarose gel containing 0.5  $\mu$ g/ml ethidium bromide.

Insert cDNA bands were visualised by UV transillumination and gel extracted (QiaQuick Gel Extraction kit, Qiagen). The purified cDNA insert was subsequently used for probe labelling.

### 2.4.3. Real-time PCR

Total RNA from MEFs, 786.0, and WT2 cells was obtained using an RNA mini kit (QIAGEN). REAL TIME RT-PCR was performed using a REAL TIME RT-PCR kit (PE Biosystems) as described previously (Wang and Semenza 1993). To measure the human vascular endothelial growth factor (VEGF), human CXCR4 and mouse CXCR4 expression real-time PCR was performed using a sequence detector (ABI-Prism, model 7700; Applied Biosystems). The following primers were used:

Species	Target	Forward Primer	Reverse Primer
Human	CXCR4	GCATGACGGACAAGTACAGGCT	AAAGTACCAGTTTGCCACGGC
Mouse	CXCR4	TTGTCCACGCCACCAACAGTCA	TGAAACACCACCATCCACAGGC

Detection of 18S rRNA, used as internal control, was performed using premixed reagents from Applied Biosystems. Detection of VEGF and 18S rRNA was performed using a PCR master mix (TaqMan Universal; Applied Biosystems) and CXCR4 detection was also performed using a PCR master mix (SyBr Green; Applied Biosystems). Detection of



CXCL12 expression by the MCF7 and CAOV3 cell lines was performed by using a PCR master mix (SyBr Green; Applied Biosystem), and the following primers were used:

Species	Target	Forward Primer	Reverse Primer
Human	CXCL12	ACACTCCAAACTGTGCCCTTCA	CCACGTCTTTGCCCTTTCATC

## 2.5. Transient Transfection

DNA plasmids were prepared using a commercially available kit (Endofree Maxi-Prep; QIAGEN).

Transfections were performed using effectene transfection reagents (QIAGEN) according to the manufacturer's instructions and the transfection efficiency in all the experiments was > 90%.

Cells were seeded at a concentration of  $5 \times 10^4$  per well in 48-well plates the day before transfection. 24 h after transfection, reagents were removed, and cells were allowed to recover for 8 h before being treated for 16–24 h. Cotransfection experiments were performed using a 1:1 ratio between the reporter plasmid and HIF-1 $\alpha$  expression vector. Luciferase reporter assays were performed in 96-well optiplates (Packard Instrument Co.) using a luciferase assay system (Promega) according to the manufacturer's instructions. Results were normalized for the protein content using the protein assay (Bio-Rad Laboratories). Reporter gene assay pGL–Hyp responsive element (HRE) plasmid contains three copies of the canonical HRE (5'-GTGACTACGTGCTGCCTAG-3') from the inducible nitric oxide synthase promoter (Melillo, Musso et al. 1995). pCXCR4 plasmid, containing a 2.6-kb fragment from the human CXCR4 promoter upstream of the luciferase reporter gene, was obtained from A.J. Caruz [Universidad de Jaen, Madrid, Spain; reference (Caruz, Samsom et al. 1998)]. HIF-1 $\alpha$  (ODD) expression vector was obtained from E. Huang [Brigham and Women's Hospital, Harvard Medical School, Boston, MA;



reference (Huang, Gu et al. 1998)]. The pCMV(HA)–HIF-1 $\alpha$  expression vector was obtained from D. Livingston (Dana Farber Cancer Institute, Boston, MA).

## 2.6. Chromatin Immunoprecipitation Assay (ChIP)

ChIP assays was performed in CAOV3 cells, transiently transfected with the p(HA)HIF-1 $\alpha$  expression vector, and performed as described previously (Saccani, Pantano et al. 2002). In brief,  $4 \times 10^6$  cells were fixed by adding directly to the medium formaldehyde (formaldehyde from a 37% formaldehyde/10% methanol stock; Calbiochem) to a final concentration of 1%. After 10 min, ice-cold PBS was immediately added, plates were transferred on ice and washed three times with PBS, and cells were collected by scraping. After centrifugation at 4°C (1200-1500 rpm for 5 min), cells were lysed for 5 min in L1 buffer (50 mM Tris, pH 8.0, 2 mM EDTA, 0.1% NP-40, and 10% Glycerol) supplemented with protease inhibitors maintaining cells in ice (nuclear membrane remains intact). Nuclei were pelleted at 3,000 rpm in microfuge at 4°C and resuspended in L2 buffer (50 mM Tris, pH 8.0, 1% SDS, and 5 mM EDTA plus inhibitors). Chromatin was sheared by sonication (5x10 s at one fifth of the maximum potency in a Sonics vibracell VC13 equipped with a 3-mm tip), centrifuged to pellet debris (13000 rpm at 8°C), and diluted ten times in dilution buffer (50 mM Tris, pH 8.0, 0.5% NP-40, 0.2 M NaCl, and 0.5 mM EDTA). Extracts were precleared for 2 h (with rotation at 4°C) with 80 $\mu$ l of a 50% suspension of salmon sperm DNA–saturated protein A. Immunoprecipitations were performed at 4° C overnight with 2 $\mu$ g of polyclonal anti–human hemagglutinin antibody (Santa Cruz Biotechnology, Inc.). Immune complexes were collected with salmon sperm DNA–saturated protein A, and washed three times (5 min each) with high salt buffer (washing buffer: 20 mM Tris, pH 8.0, 0.1% SDS, 1% NP-40, 2 mM EDTA, and 500 mM NaCl), two times with a 0.5 M LiCl buffer, and three times with low salt buffer (1X TE). Immune complexes were extracted in



1X TE containing 2% SDS and protein; DNA cross-links were reverted by heating at 65° C for 6 h. After proteinase K digestion (100µg, 1–2 h at 50° C), DNA was extracted by phenol, chloroform, and ethanol precipitated (O/N at -20°C). The DNA was centrifuged; air dried, resuspended it in 30 µl of TE and 1µl of RNAase was added and incubated at 37°C for 20 min. Approximately 1/20 of the immunoprecipitated DNA was used in each PCR. Sequences of promoter-specific primers included the CXCR4 promoter region 1860 to 1578 as follows:

Species	Target	Sense
		Antisense
Human	CXCR4	TCGTGCCAAAGCTTGTCCTG
		GCGGTAACCAATTCGCGAATAGTGC

## 2.7. Immunohistochemistry

### 2.7.1. Tissues

Neoplastic breast tissues from patients with a diagnosis of invasive ductal carcinoma were revised. Tissue had been previously fixed in buffered formalin for 24 hours at room temperature and embedded in paraffin. 3-µm sections were obtained from blocks and stained with Haematoxylin and Eosin for routine histological examination.

### 2.7.2. Antibodies

The following antibodies were used: mouse monoclonal antiserum to HIF-1 alpha (clone H1alpha67, Novus Biologicals) and mouse fluorescein-conjugated monoclonal antiserum to CXCR4 (clone 12G5, R&D System).

### 2.7.3. Immunohistochemical method

2-µm paraffin sections were cut from blocks and dried at 37°C for 10 minutes.



The following steps were then carried out at room temperature:

- 1) Sections were deparaffinised in xylene (3x10 min) and hydrated immersing the slides in 100% ethanol (2x1 min), 90% ethanol (1 min), 90% ethanol with 0.3% H<sub>2</sub>O<sub>2</sub> (to block endogenous peroxidase), 70% ethanol (1 min) and distill water (1min).
- 2) Sections were laid out in a humid chamber
- 3) Sections were washed twice in PBS (5 minutes each)
- 4) Microwave pretreatment (780W, 5 min. twice, in 0.01M citrate buffer, pH 6.0) was performed in order to retrieve antigen only for anti HIF-1 $\alpha$  antiserum
- 5) Sections were washed in PBS for 5 minutes
- 6) 5 % normal human serum (DAKO) was added to each section for 10 min, then drained off.
- 7) To each section, primary antibody (monoclonal antiserum to HIF-1 $\alpha$  at the dilution of 1:50 and mouse fluorescein-conjugated monoclonal antiserum to CXCR4 at the dilution of 1:20) was added. The slides were incubated for 2 hour in a humid chamber at room temperature.
- 8) Sections were washed twice in PBS (5 minutes each)
- 9) For anti HIF-1 $\alpha$  antiserum a double indirect immunoperoxidase staining was used to reveal the immunohistochemical reaction: first, a rabbit anti mouse peroxidase-conjugated secondary antibody (at the dilution of 1:50, Dako, 1 hour incubation) followed by a swine anti-rabbit peroxidased secondary antibody (at the dilution of 1:50, Dako, 1 hour incubation).
- 10) For anti CXCR4 antiserum a double indirect staining was used to reveal the immunoistochemical reaction: a first reaction with monoclonal anti-fluorescein antiserum (1:20, Dako, 1 hour incubation) followed by a double indirect immunoperoxidase technique (a rabbit anti mouse peroxidased secondary antibody,



at the dilution of 1:50, Dako, 1 hour incubation, Dako, and a swine anti rabbit peroxidased secondary antibody, at the dilution of 1:50, Dako, 1 hour incubation)

11) Sections were washed twice in PBS for 5 min

12) The chromogen was prepared as indicated: 250  $\mu$ l 3,3'-diaminobenzidine free base, 10 ml PBS, 10  $\mu$ l H<sub>2</sub>O<sub>2</sub>

13) DAB solution was added to each section and incubated for less than 5 min; the positivity was identified as a brown staining

14) Sections were washed in distilled water

15) Sections were counterstained for 2 minutes in Haematoxylin

16) Sections were dehydrated in taken dow through 70% ethanol (1 min), 90% ethanol (1 min), 100% ethanol (2x1 min) and xylene (3x1 min).

17) Sections were mounted with Eukitt (Sigma)

## 2.8. Methods for analysing protein expression

### 2.8.1. Flow cytometry

#### 2.8.1.i. Monoclonal antibodies

Cell staining was performed using mouse monoclonal anti-human CXCR4 antibody (clone 12G5; BD Biosciences) and an irrelevant isotype-specific control mouse, IgG2a, (UPC10; Sigma-Aldrich) followed by FITC-conjugated, isotype-matched affinity-purified, goat anti-mouse antibody (Southern Biotechnology Associates, Inc.).

Monocytes were stained with mouse monoclonal anti-human CCR1 (clone 53504) and CCR5 (clone 45531) antibodies (all from R&D).

Human dendritic cells were stained for mouse monoclonal anti-human CCR7 (clone 2H4), R-phycoerythrin (R-PE)-conjugated mouse anti-human monoclonal CD80 (clone L307.4),



mouse anti-human CD83 (clone HB15e) (all from BD Biosciences) and MHCII (L243 clone hybridoma from ATCC).

### *2.8.1.ii. Immunofluorescent staining protocol*

For staining, cells were washed in PBS supplemented with 1% of inactivated human serum or FCS respectively for human or murine cells (FACS buffer). Approximately  $5 \times 10^5$  cells were resuspended in 100  $\mu$ l of FACS buffer in a microfuge tube then 10  $\mu$ g of human IgG (Sigma) was added to block Fc receptors. After 15 min incubation at room temperature, primary antibody was added to a final concentration ranging between 2-20  $\mu$ g/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 PE-conjugated antibodies were then ready for flow cytometric analysis. For unconjugated antibodies, cells were resuspended in 100  $\mu$ l of FACS buffer and FITC-conjugated secondary antibody was added (at an appropriate dilution). Following further 30 minutes of incubation in ice, the cells were washed twice as before, then analysed by flow cytometry (FacsCalibur, Becton-Dickinson, Milan).

### **2.8.2. Laser Confocal Microscopic Analysis of CXCR4 Expression**

Cells growing on sterile coverslips were washed with PBS after 16-h incubation in hypoxic or normoxic conditions, as indicated in the text. Non-specific sites were blocked by 10-min incubation at room temperature with wash buffer containing 0.9% wt/vol sodium chloride, 1% vol/vol human serum, and 0.02% wt/vol sodium azide. Thereafter, the cells were stained with mouse anti-human CXCR4 antibodies (BD Biosciences) at a dilution of 1:20 for 30 min at room temperature. The cells were washed twice with 2 ml of wash buffer and



incubated with goat F(ab')<sub>2</sub> anti-mouse Ig-FITC (human adsorbed) at a dilution of 1:20 for another 30 min at room temperature. After two changes in wash buffer, the cells were fixed in 4% wt/vol paraformaldehyde for 15 min, and the coverslips were mounted on glass slides for microscopy. Images were visualized using a system with differential interference contrast (FV500; Olympus, UK).

### **2.8.3. Enzyme-linked immunosorbent assay (ELISA)**

The concentration of hTNF- $\alpha$ , hIL-12, mL-12, mTNF- $\alpha$ , mL-10 and mL-6 levels was measured using ELISA Sandwich purchased by R&D in according to manufacturer's instructions.

### **2.8.4. Statistical method**

All measurement are shown as average  $\pm$  SD. Statistical analysis of data was performed using a two-tailed unpaired t-test. All p values were calculated vs untreated samples unless indicated otherwise. P values of  $<0.05$  were considered as statistically significant.



## **Chapter 3. Expression of chemokine receptors in different cells types after hypoxia treatment**

### **3.1. Introduction**

Regulation of chemokine receptor expression is a crucial level of control for the tuning of the chemokine system. In particular, pro- and anti-inflammatory stimuli have divergent effects on chemokines and chemokine receptors. Original observations by our group demonstrated that pro-inflammatory stimuli, such as LPS and IFN- $\gamma$ , downregulate the expression of CCR2, while upregulate CCL2 expression (Sica, Saccani et al. 1997). In contrast, anti-inflammatory stimuli (e. g. IL-10) upregulate CCR2 and downregulate CCL2 expression (Sozzani, Ghezzi et al. 1998).

The downregulation of certain inflammatory chemokine receptors may serve as stop signal to focus their actions at sites of infection and inflammation. In particular our lab has demonstrated that tumour derived TNF- $\alpha$ , in human ovarian cancer, inhibits CCR2 expression by TAMs (Sica, Saccani et al. 2000). In addition to pro and anti-inflammatory stimuli other microenvironmental stimuli can regulate the chemokine receptors expression. For instance, stress stimuli could induce changes in the expression level of several chemokine and chemokine receptors and reactive oxygen intermediates (ROI) upregulate the expression of CCR5 and CXCR4 (Saccani, Saccani et al. 2000).

### **3.2. Aim of this chapter**

Hypoxia is a feature of several inflammatory diseases as rheumatoid arthritis, atherosclerosis, acute respiratory distress syndrome, psoriasis, etc. Hypoxia also has a fundamental role in tumours and tumour-associated macrophages were reported to localize preferentially in necrotic zones of the tumour. Based on these observations, the initial aim



of this chapter was to examine the effects of microenvironmental hypoxia on the chemokine receptor expression and functions.

### 3.3. Results

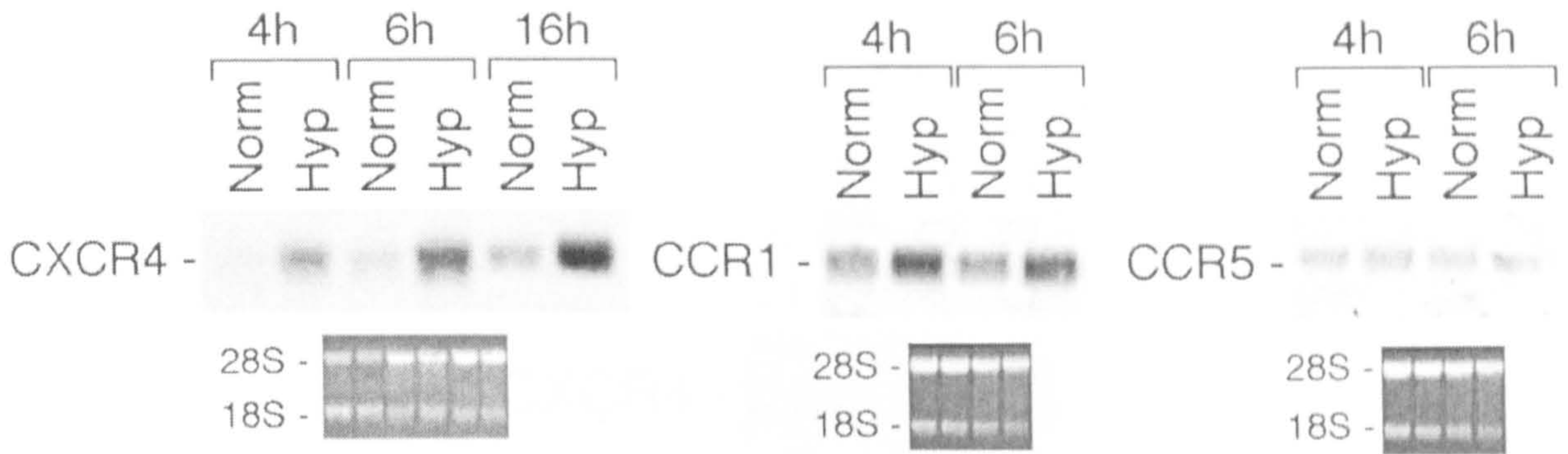
#### 3.3.1. Hypoxia-increased CXCR4 Expression in Mononuclear Phagocytes

As the inspired air has a concentration of 21% of O<sub>2</sub>, we considered normoxia (Norm) the standard cell cultured condition, characterized by 20% of O<sub>2</sub> tension, and hypoxia (Hyp) culture conditions with 1% of O<sub>2</sub>, in line with previous works and with the observation that in hypoxic tissues the O<sub>2</sub> concentration ranges between 0.5 and 2.5%.

Monocytes were cultured in normoxia (20% oxygen) and hypoxia (1% oxygen) and the total RNA was next collected at different time points, mRNA expression of CXCR4, CCR1 and CCR5 was analyzed by Northern blot. As shown in Figure 3.1, human monocytes cultured in hypoxia, for 4 and 6 h, showed a strong increase in the expression of CXCR4 mRNA, as compared with normoxia-cultured monocytes.

Hyp-mediated up-regulation of CXCR4 mRNA was still present at 16 h. In Hyp, a slight increase was also observed for CCR1 mRNA expression, whereas CCR5 mRNA level was unaffected. Interestingly, it was reported that Hyp down-regulates the responsiveness of monocytes to the CCR2 agonists CCL2 (Turner, Scotton et al. 1999; Grimshaw and Balkwill 2001). Based on these results, we focused our attention on CXCR4.





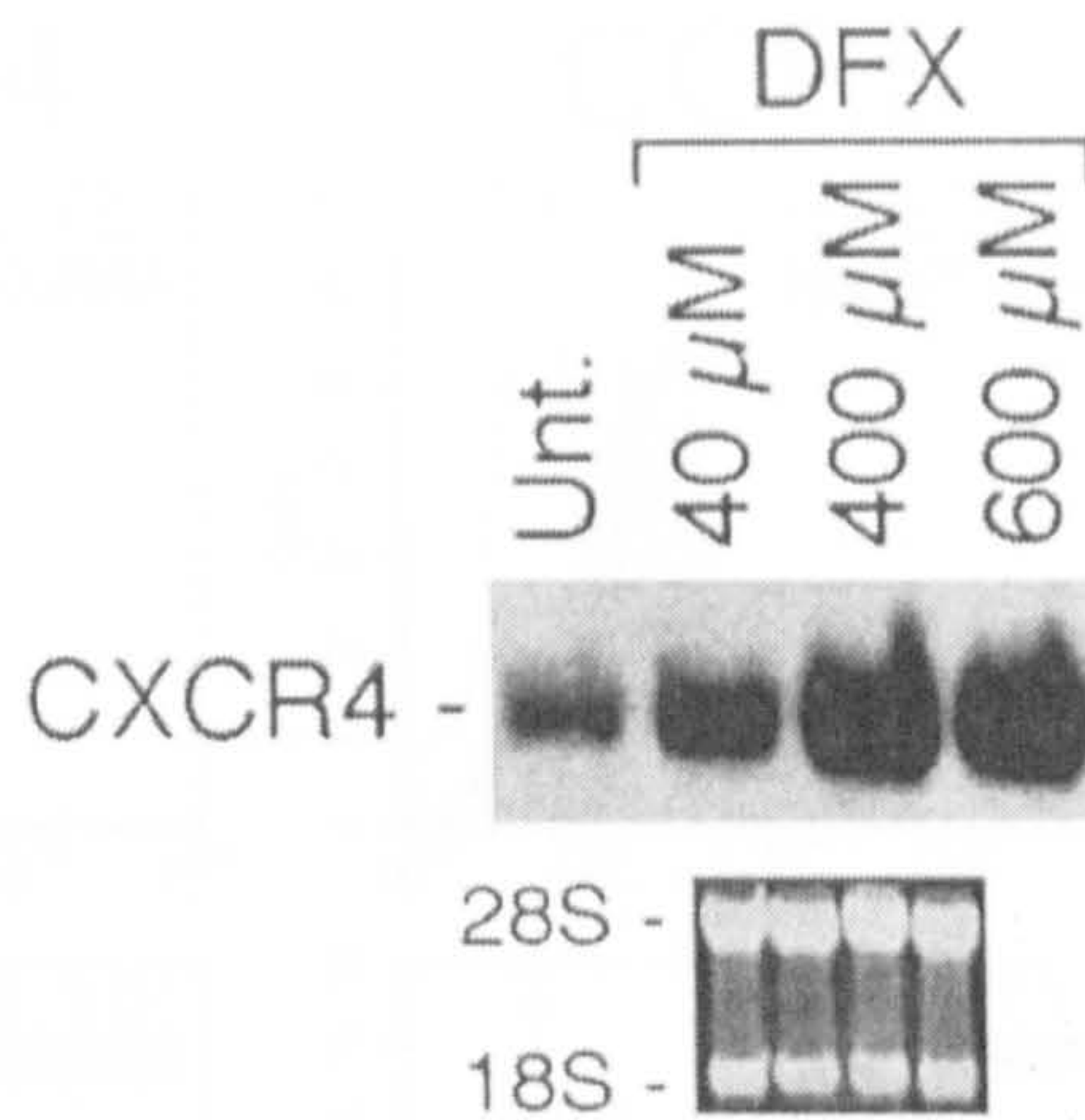
**Figure 3.1. Analysis of chemokine receptors by Northern Blot.**

Fresh human monocytes obtained from peripheral blood of healthy donors were cultured for different times in normoxia (Norm) or hypoxic (Hyp) conditions, as indicated. Total RNA was analyzed by Northern blot for CXCR4, CCR1, and CCR5 mRNA expression. Results are representative of five different experiments. 28S and 18S are shown to demonstrate the equal amount of RNA.

### 3.3.2. The hypoxia mimicking compound desferioxamine induces CXCR4 expression

Desferioxamine (DFX) is an iron chelator recognized as an hypoxia mimicking compound (Wang and Semenza 1993). Fresh human monocytes were incubated for 4 h in the presence of different concentration of DFX and total RNA was analyzed by Northern blot. CXCR4 mRNA levels increased in a dose-dependent manner in response to DFX (Figure 3.2). Together, these results show that CXCR4 mRNA expression is controlled by changes in oxygen levels.





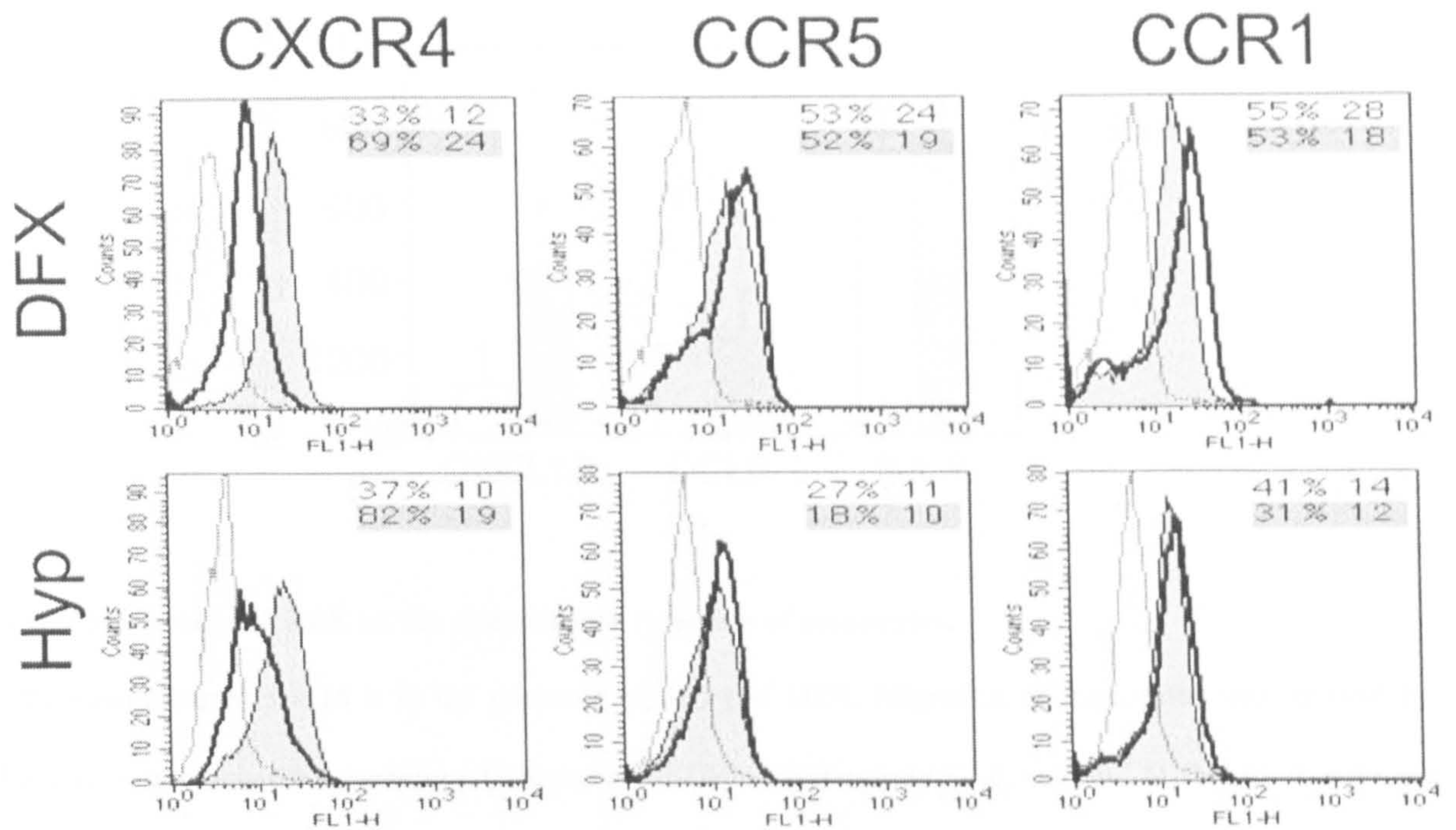
**Figure 3.2. Hypoxia-mimicking compound induces CXCR4 expression.**

Total RNA from fresh human monocytes cultured for 4 h in the presence of increasing concentration of DFX was analyzed by Northern blot. Results are representative of three experiments and the effect was consistent in the different donors analyzed (different donors can have different basal levels of CXCR4).

### 3.3.3. Analysis of chemokine receptor surface expression

Modulation of CXCR4 by Hyp was confirmed at the protein level by flow cytometry. To evaluate the functional effects of Hyp on this chemokine receptor, monocytes were incubated in hypoxic conditions and surface expression of different chemokine receptors was determined. Exposure of monocytes for 16 h to Hyp resulted in a strong increase of CXCR4 surface expression (Figure 3.3). Similarly, cells incubated in the presence of 400 μM DFX for the same duration showed a significant increase of CXCR4 surface expression. In contrast, neither Hyp nor DFX treatment affected CCR1 and CCR5 surface expression (Figure 3.3), confirming the data obtained by Northern blot.





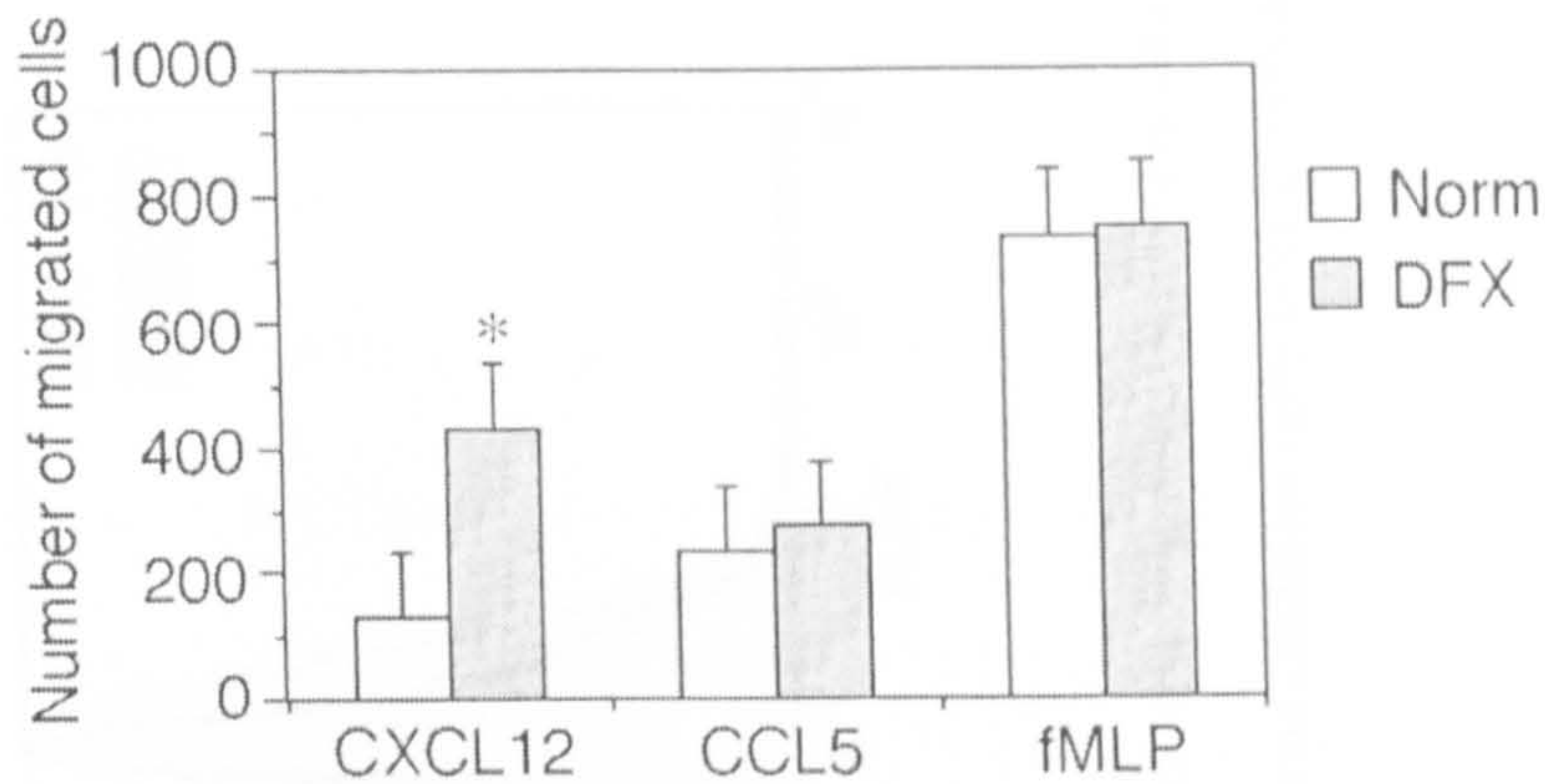
**Figure 3.3.** Hyp- and DFX-induced CXCR4 surface expression in fresh human monocytes.

Cells were cultured for 16 h in the indicated conditions and analyzed for CXCR4 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CXCR4. The results are representative of three independent experiments. In the right corner percentage of positive cells for CXCR4 and mean of fluorescence are shown for normoxia condition (white box) and hypoxia conditions (grey box). (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp or DFX. Results are representative of five different experiments.

### 3.3.4. Migration assay

The observed DFX-dependent increase of CXCR4 surface expression was paralleled by a rise in the number of monocytes migrating in response to CXCL12 (Figure 3.4). The increased chemotactic responsiveness toward CXCL12 was specific, as both CCL5 and fMLP-induced migration were not significantly affected.





**Figure 3.4. Effects of DFX on the chemotactic response of monocytes.**

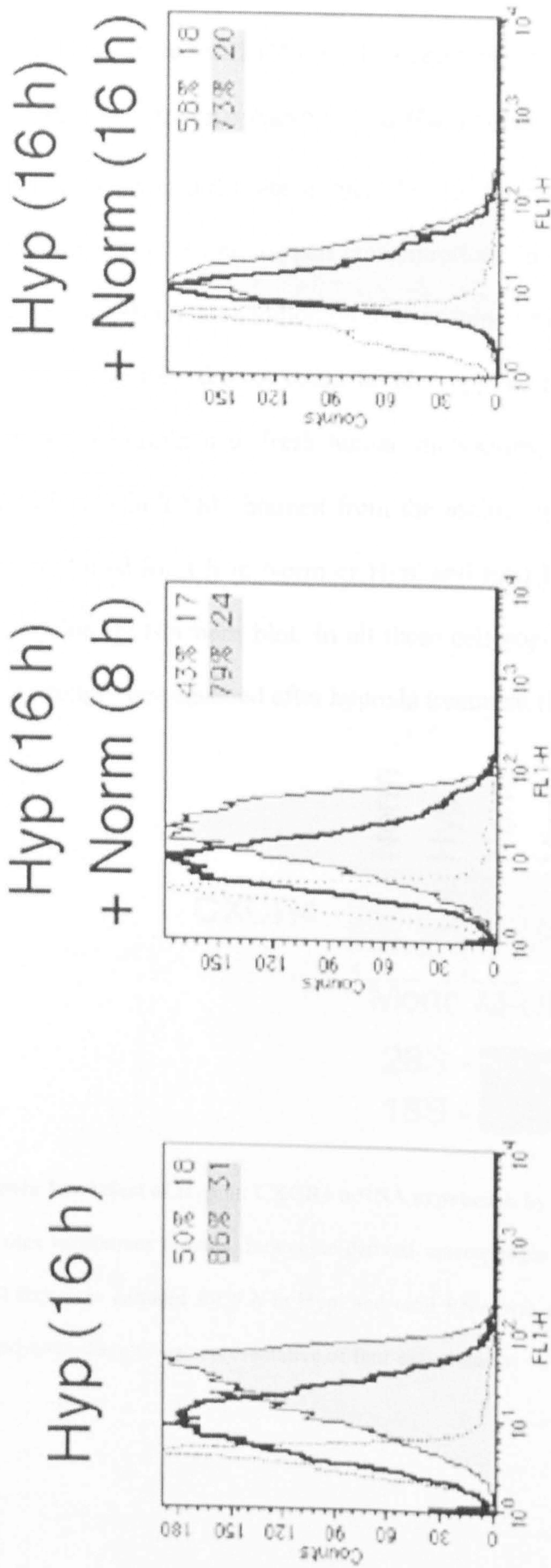
Cells were cultured for 16 h in the presence of 400  $\mu$ M DFX. Migration of monocytes was assayed by chemotaxis microchamber technique (100 ng/ml CXCL12, 100 ng/ml CCL5, and  $10^{-8}$  M fMLP). Results are mean  $\pm$ SD of five experiments. \*,  $P < 0.05$  versus cells cultured in normoxic conditions (paired Student's  $t$  test).

Thus, in human monocytes, low oxygen conditions result in a specific up-regulation of the CXCR4 expression and function.

### 3.3.5. Reoxygenation

Hypoxia and reoxygenation are important pathophysiological conditions that occur during injury, ischemia, reperfusion and stroke. It is known that the response to hypoxia and reoxygenation is mediated by different transcriptional factor (AP-1, NFkB) inducing the expression of several genes (Rupec and Baeuerle 1995). As decreased oxygen availability modifies the expression of CXCR4 it was important to assess the reversibility of this effect following reoxygenation. As shown in Figure 3.5, fresh human monocytes cultured in hypoxic conditions for 16 h and then reoxygenated, retained high levels of CXCR4 for a 8 h. Higher levels than control cells were still present at 16 h (Figure 3.5), while they returned to baseline by 24 h (data not shown).





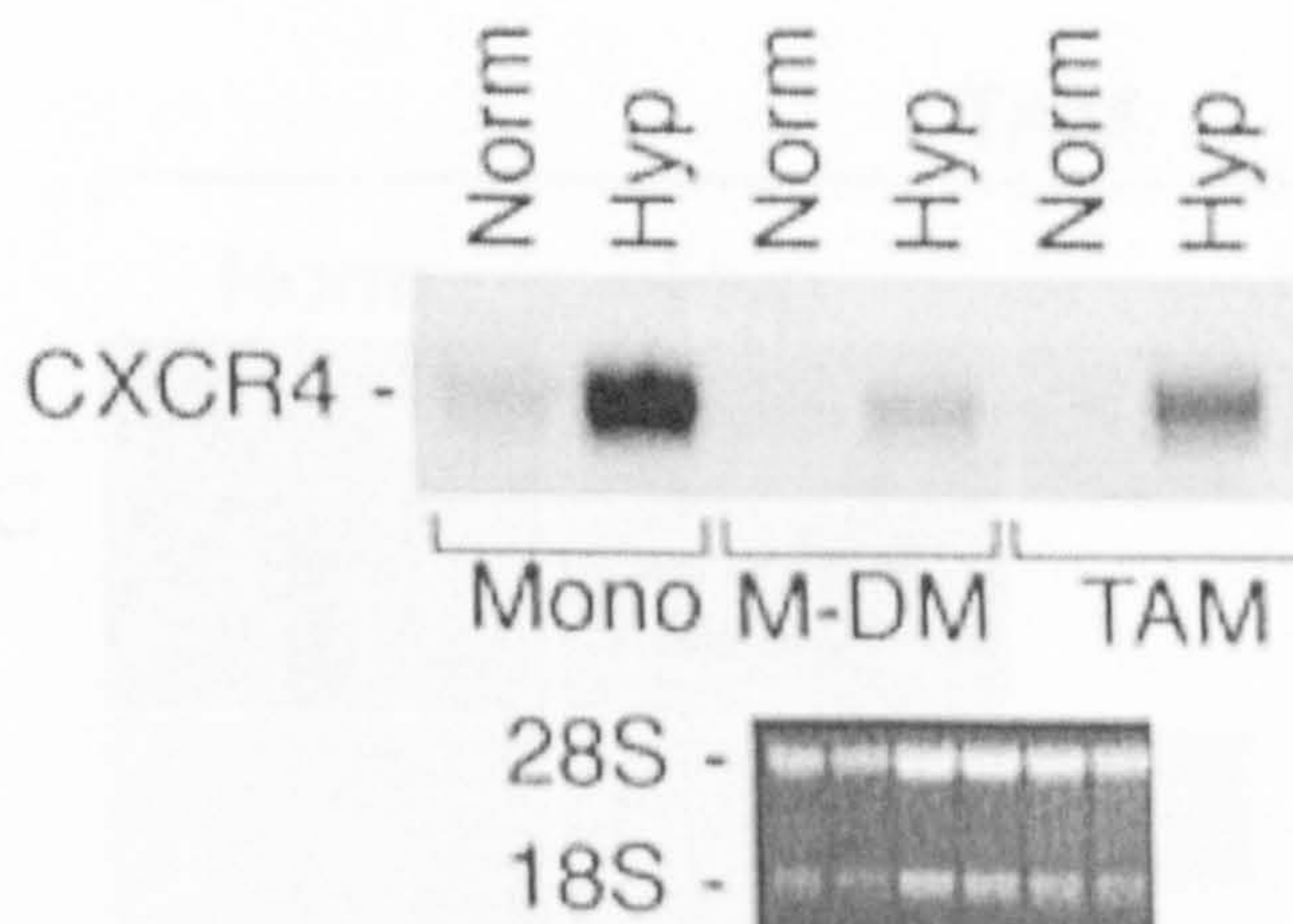
**Figure 3.5.** Effect of reoxygenation on CXCR4 surface expression.

Fresh human monocytes were cultured for 16 h in Hyp and subsequently reexposed to Norm for the indicated times. CXCR4 surface expression was determined by flow cytometry. In the right corner percentage of positive cells for CXCR4 and mean of fluorescence are shown for normoxia condition (white box) and hypoxia conditions (grey box). Results are representative of three experiments



### 3.3.6. Hyp-increased CXCR4 Expression in Monocytes-Derived Macrophages (M-DM) and Tumour Associated Macrophages (TAM)

When monocytes infiltrate tissues they differentiate into macrophages. As tissues are characterized by lower oxygen concentrations in comparison with peripheral circulation (Semenza 2001), it was important to determine whether CXCR4 up-regulation in response to low oxygen conditions could be also applied to macrophages. CXCR4 expression in Hyp was determined in fresh human monocytes, in *in vitro*-differentiated macrophages (MDM) and in TAM obtained from the ascitic fluid of human ovarian carcinoma. Cells were cultured for 4 h in Norm or Hyp, and total RNA was analyzed for CXCR4 mRNA expression by Northern blot. In all these cell populations the CXCR4 mRNA expression was strongly up-regulated after hypoxia treatment (Figure 3.6).

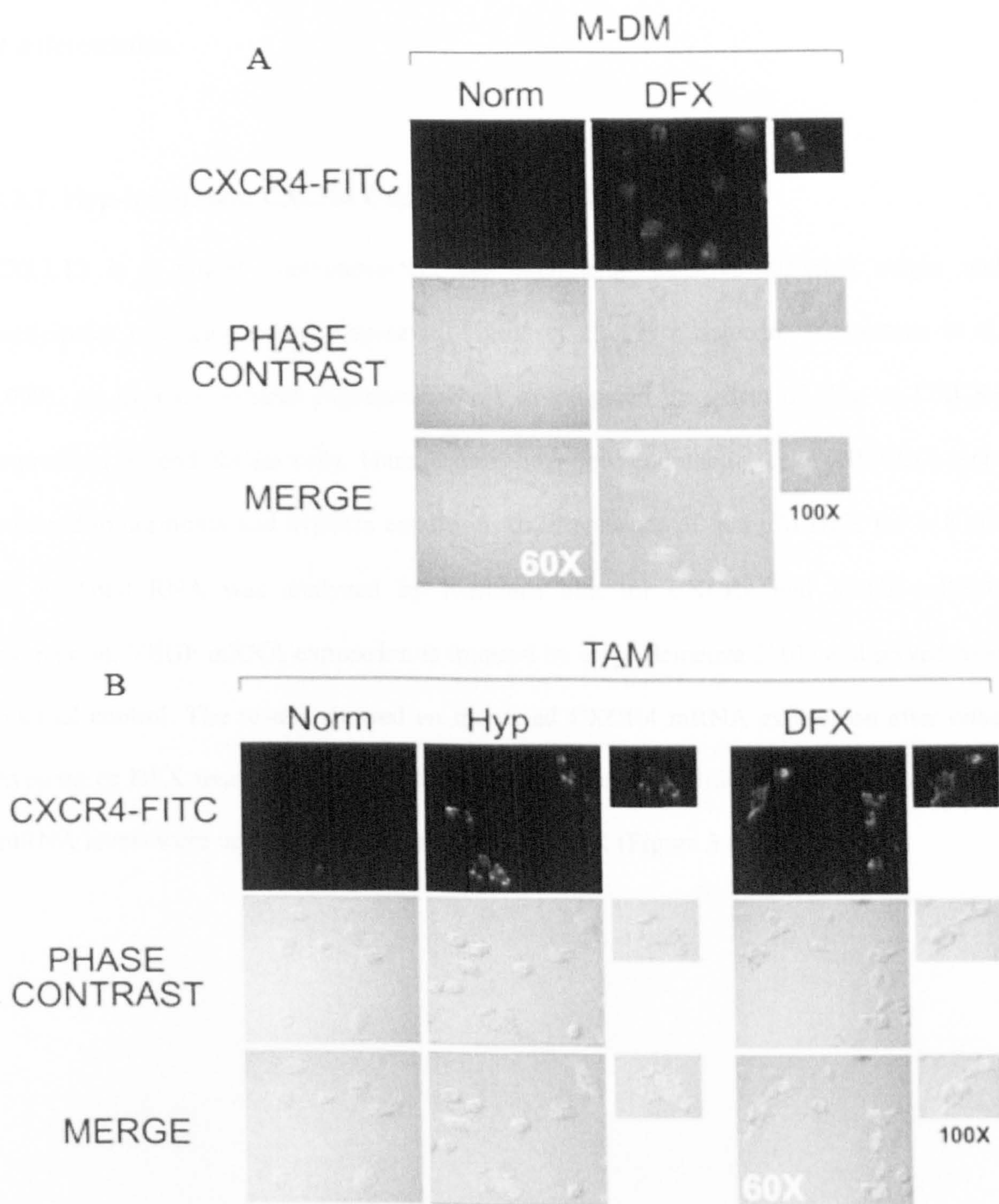


**Figure 3.6. Effect of Hyp on CXCR4 mRNA expression by different cell types.**

Human monocytes (mono), monocyte-derived macrophages (MDMs), and tumor associated macrophages (TAMs) were cultured for 4 h in Hyp, and total RNA was analyzed by Northern blot for CXCR4 mRNA expression. Results are representative of four experiments.



These results were confirmed by laser confocal microscopy Figure 3.7 (A and B), wherein Hyp induced a strong up-regulation of CXCR4 surface expression, in both MDMs and TAMs.



**Figure 3.7. Effect of Hyp or DFX on the CXCR4 surface expression.**

M-DM (A) and TAMs from ascitic fluid of human ovarian cancer (B) were cultured for 16 h in Norm, Hyp, or in the presence of 400  $\mu$ M DFX, as indicated. CXCR4 surface staining was performed and detected by



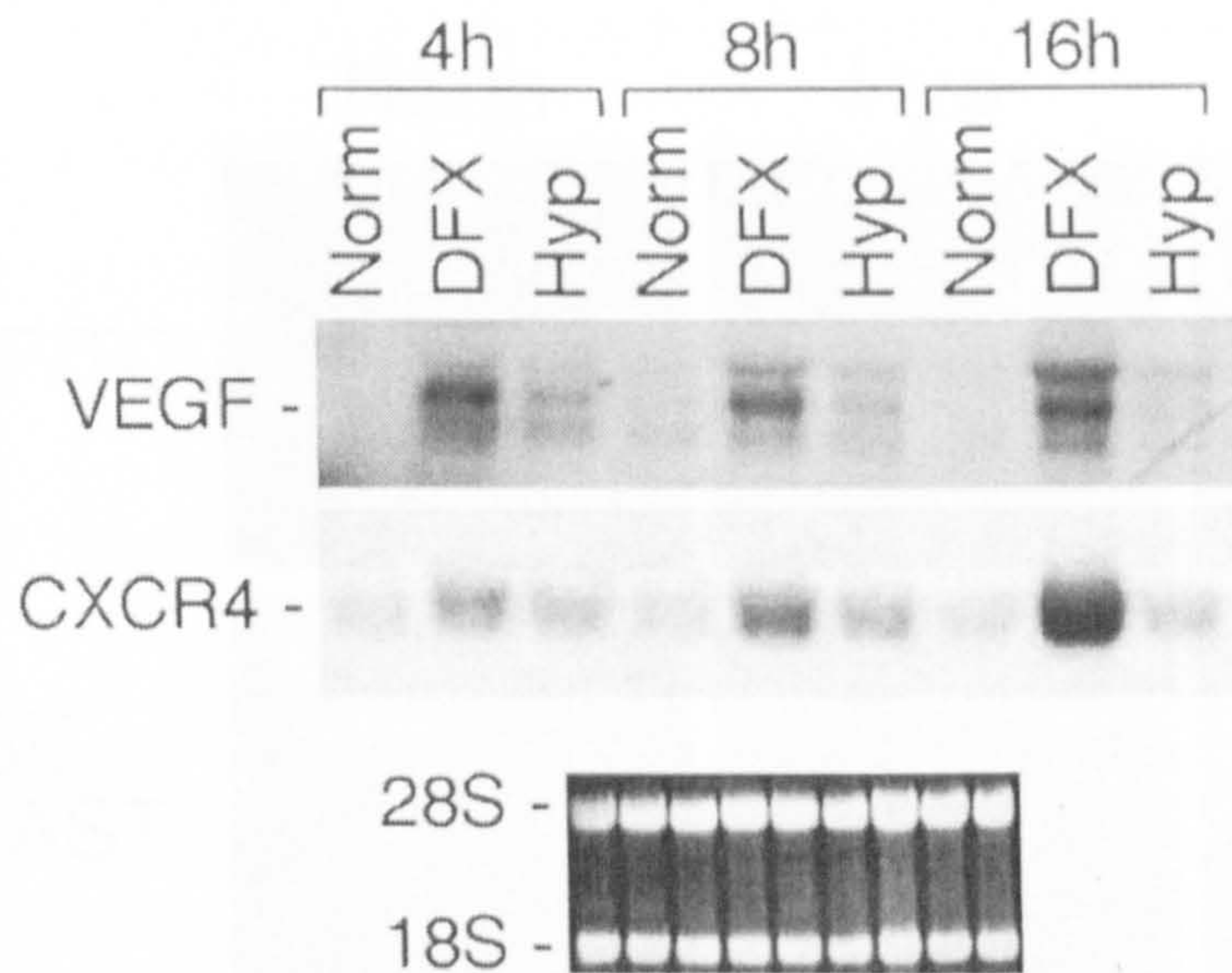
laser confocal microscopy (~70% of CXCR4 positive cells). Staining with both isotype matched control antibodies was done for all samples (not depicted). Results are representative of three experiments.

Thus hypoxia controls CXCR4 expression in mononuclear phagocytes at different stages of differentiation.

### **3.3.7. Hyp-increased CXCR4 Expression in HUVECs**

CXCL12 is a potent chemoattractant for endothelial cells of different origin and participates in angiogenesis (Nagasawa, Hirota et al. 1996; Salcedo, Wasserman et al. 1999). As hypoxia induces angiogenesis, we investigated the effect of Hyp on CXCR4 expression by endothelial cells. Human umbelical vein endothelial cells (HUVEC) were cultured in normoxia and hypoxia conditions or in presence of 400  $\mu$ M DFX for 4, 8 and 16 h. Total RNA was analyzed by Northern blot for CXCR4 and VEGF mRNAs expression. VEGF mRNA expression is induced by Hyp (Semenza 2001) and served as an internal control. The results showed an increased CXCR4 mRNA expression after either hypoxia or DFX treatment, as compared with normoxic conditions. As expected, VEGF mRNA levels were up-regulated either by Hyp or DFX (Figure 3.8).





**Figure 3.8. Effect of Hyp on CXCR4 expression by human endothelial venules (HUVECS).**

Cells were cultured for 4, 8, and 16 h under Norm, Hyp, or in the presence of 400 μM DFX, respectively. Thereafter, total RNA was analyzed by Northern blot for CXCR4 and VEGF mRNAs expression. The different bands present in the northern Blot for VEGF correspond to different splicing forms of VEGF. Results are representative of three experiments.

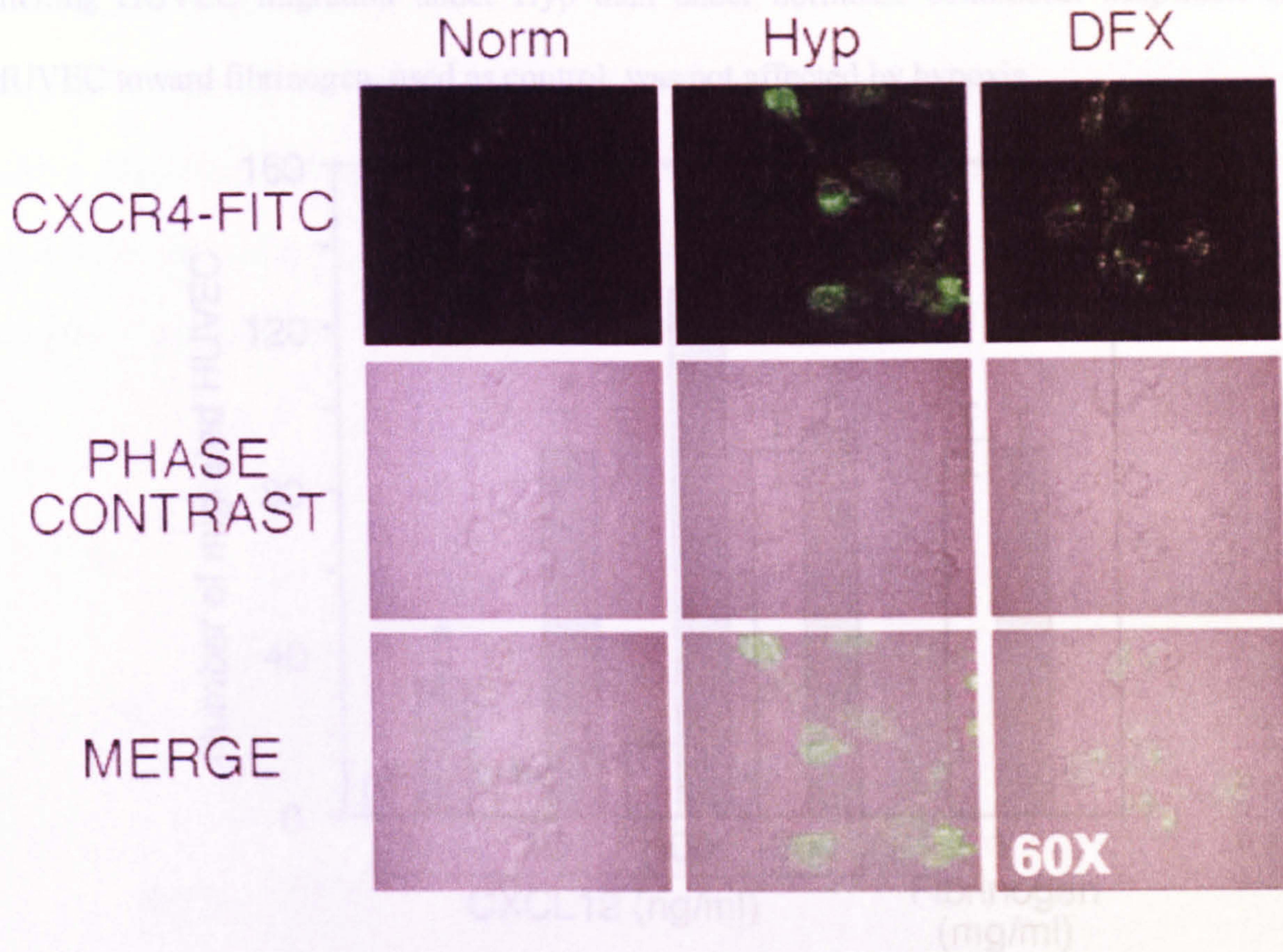
#### 3.3.4. Effect of Dexamethasone on CXCR4 surface expression in HUVECS

These data were then confirmed analyzing the CXCR4 surface expression by laser confocal microscopy. Cells were treated for 16 h in Norm, Hyp and DFX conditions and, as shown in Figure 3.9, CXCR4 surface expression in endothelial cells was strongly increased.

#### 3.3.5 Endothelial cell migration

Endothelial cell migration plays a central role in the angiogenic process and it was therefore important to analyze if CXCR4 could be involved in this process. HUVECS cultured in normoxia or hypoxia were treated for 16 h with Dexamethasone or not. Cells were cultured for 48 h in hypoxia conditions and their level of migration capability to migrate in response to CXCL12 was analyzed. The number of migrated HUVECS in response to CXCL12 was significantly higher in hypoxia conditions compared to normoxia conditions (Figure 3.10). Dexamethasone treatment did not affect the migration of HUVECS in response to CXCL12 in hypoxia conditions (Figure 3.10).





**Figure 3.9. Effect of Hyp or DFX on the CXCR4 surface expression.**

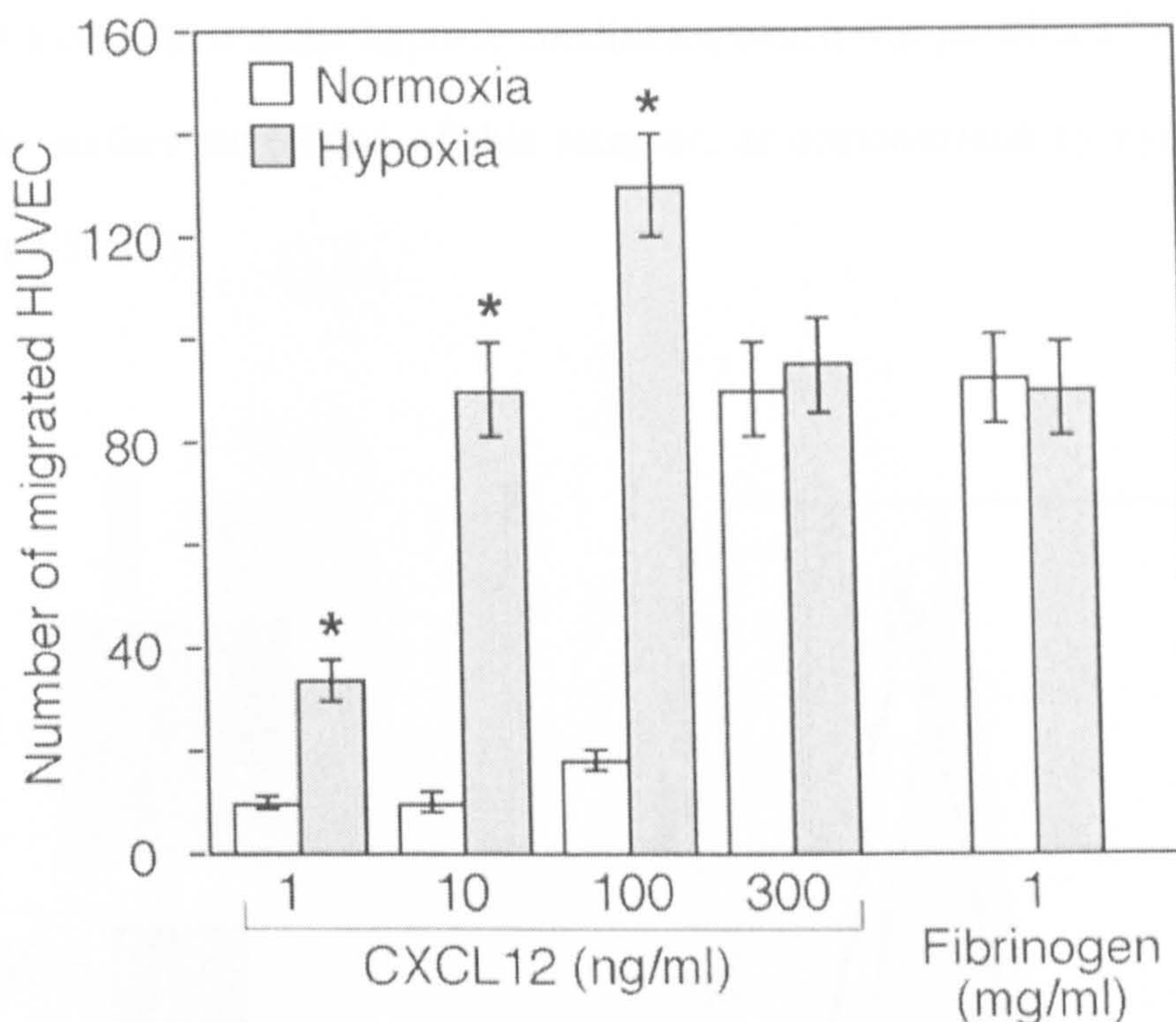
HUVECs were cultured for 16 h in Norm, Hyp, and in the presence of 400  $\mu$ M DFX, as indicated. CXCR4 surface staining was performed and detected by laser confocal microscopy. Results are representative of three experiments.

### 3.3.8. Endothelial cell migration

Endothelial cell recruitment represents an initial step of the angiogenetic process and it was therefore important to evaluate if hypoxia could influence this event. HUVECs cultured in Norm or Hyp were tested for their capability to migrate in response to CXCL12. Cells were cultured for 16 h in hypoxia conditions and then tested for their capability to migrate in response to chemotatic signals. As shown in Figure 3.10, the number of migrated HUVECs in response to CXCL12 was significantly higher in hypoxic conditions, at concentrations ranging from 1 to 100 ng/ml. CXCL12 was  $\sim$ 100-fold more effective at



eliciting HUVEC migration under Hyp than under normoxic conditions. Migration of HUVEC toward fibrinogen, used as control, was not affected by hypoxia.



**Figure 3.10.** Effect of Hyp on the chemotactic response of HUVECs to CXCL12.

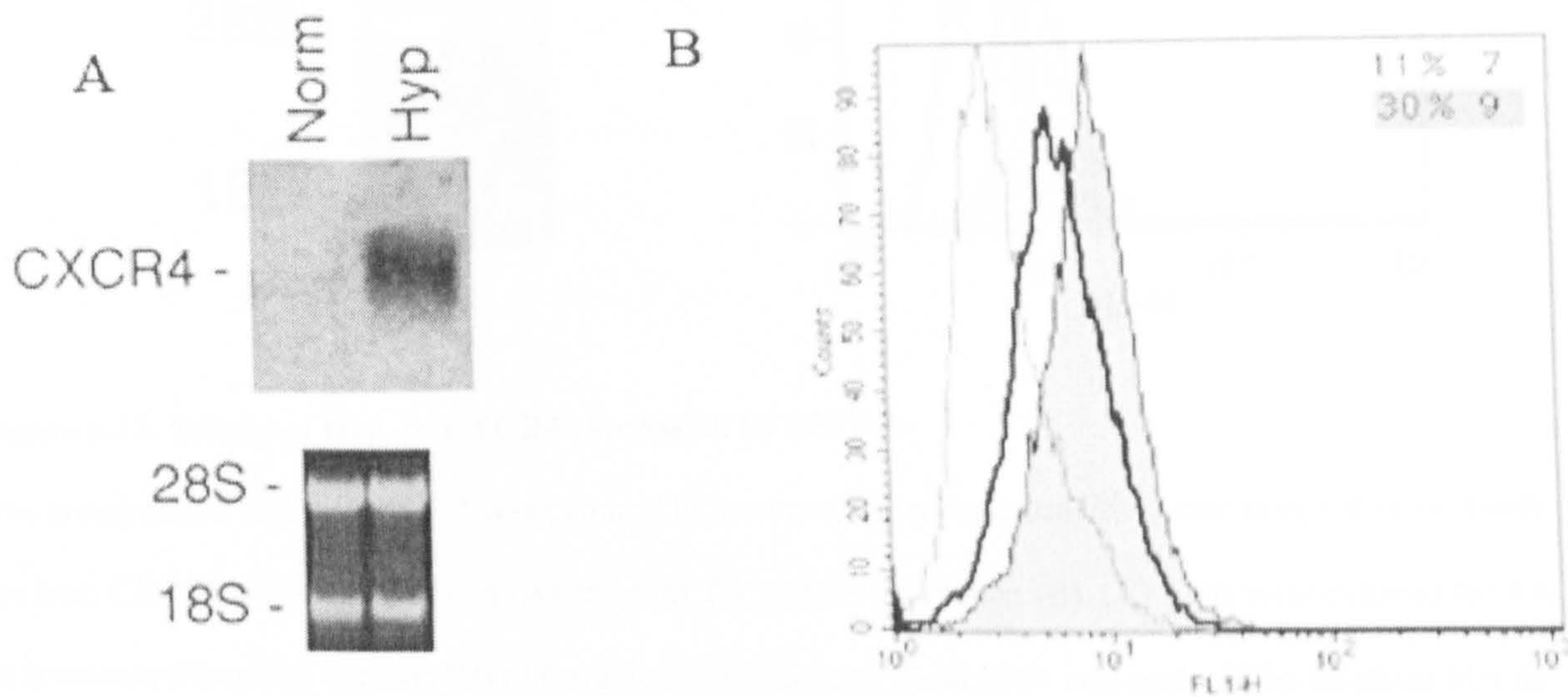
Cells were cultured for 16 h in hypoxic conditions. Fibrinogen was used as a reference attractant. Results are mean SD of three experiments. \*,  $P < 0.05$  versus cells cultured in normoxic conditions (paired Student's *t* test).

### 3.3.9. Hyp increases CXCR4 Expression in Cancer Cells

Chemokine receptors may act as molecular tools exploited by cancer cells which metastasize to target organs (Muller, Homey et al. 2001; Kang, Watkins et al. 2005; Saur, Seidler et al. 2005). In particular, CXCR4 was shown to play a major role in the migration of breast cancer cells from the primary tumor to secondary metastatic sites, such as lung, liver and bone. As solid tumors are often characterized by the presence of necrotic areas with low oxygen tension, hypoxia may be a potential mechanism that up-regulates expression of chemokine receptors in cancer cells localized in poorly vascularized and oxygenated areas. To test this hypothesis, we investigated the level of CXCR4 mRNA



expression in response to Hyp in the ovarian cancer cell line CAOV3 by Northern blot analysis. As shown in Figure 3.11A, CAOV3 cells showed up-regulation of CXCR4 mRNA after 4 h of culture under hypoxic conditions, which was paralleled by a significant increase in the surface expression of this receptor, as demonstrated by cytofluorimetric analysis (Figure 3.11 B).

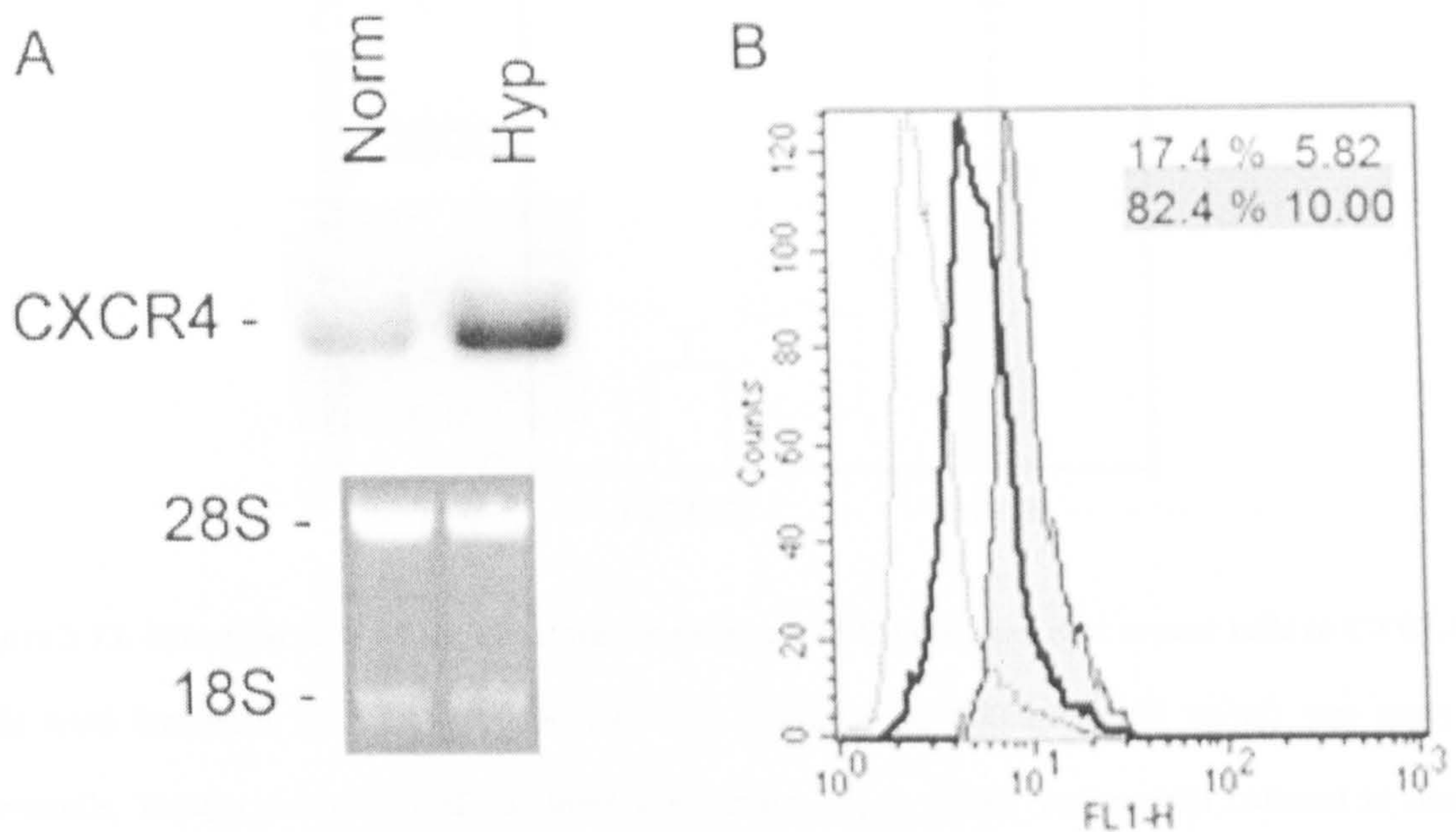


**Figure 3.11. Effects of Hyp on CXCR4 expression by CAOV-3**

The ovarian cancer cell line CAOV3 was cultured in normoxic or hypoxic conditions and analyzed respectively for both CXCR4 mRNA expression (A) and CXCR4 surface expression (B). (A) Cells were cultured for 4 h in normoxia (Norm) or hypoxic (Hyp) conditions, as indicated. Total RNA was analyzed by Northern blot for CXCR4. (B) Cells were cultured for 16 h in Norm or hypoxic (Hyp) conditions. After this period, CXCR4 surface expression was determined by flow cytometry. In the right corner percentage of positive cells for CXCR4 and mean of fluorescence are shown for normoxia condition (white box) and hypoxia conditions (grey box). (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp. Results are representative of five experiments.



Similar results were obtained with the breast cancer cell line MCF-7 (Figure 3.12).



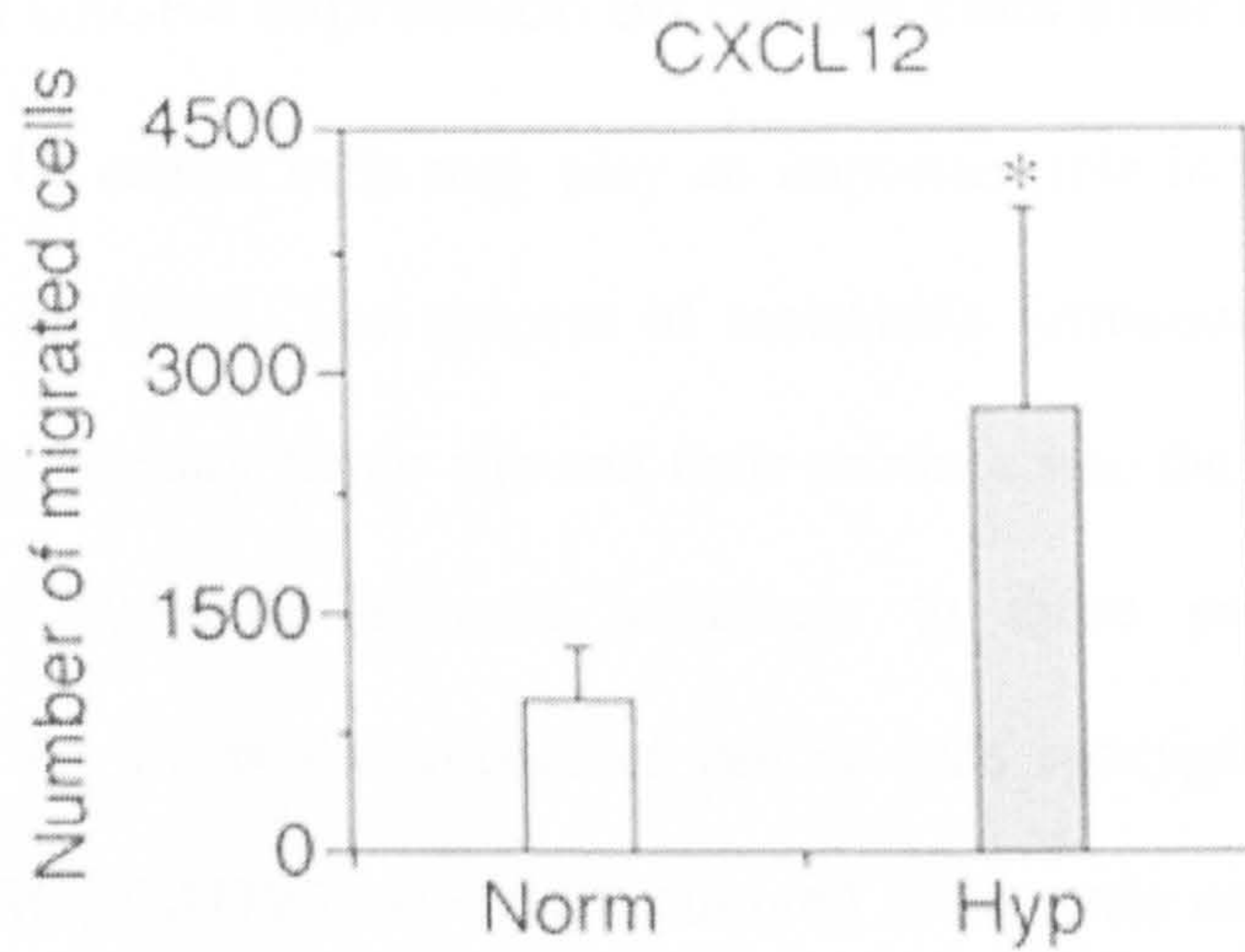
**Figure 3.12. Effects of Hyp on CXCR4 expression by MCF-7.**

The breast cancer cell line MCF-7 was cultured in normoxic or hypoxic conditions and analyzed respectively for both CXCR4 mRNA expression (A) and CXCR4 surface expression (B). (A) Cells were cultured for 4 h in normoxia (Norm) or hypoxic (Hyp) conditions, as indicated. Total RNA was analyzed by Northern blot for CXCR4. (B) Cells were cultured for 16 h in Norm or hypoxic (Hyp) conditions. After this period, CXCR4 surface expression was determined by flow cytometry. In the right corner percentage of positive cells for CXCR4 and mean of fluorescence are shown for normoxia condition (white box) and hypoxia conditions (grey box). (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp. Results are representative of three experiments.

### 3.3.10. Migration of cancer cells

The Hyp-induced expression of CXCR4 in the CAOV3 cells correlated with an increased migration of these cells toward CXCL12 in the chemotaxis assay (Figure 3.13).

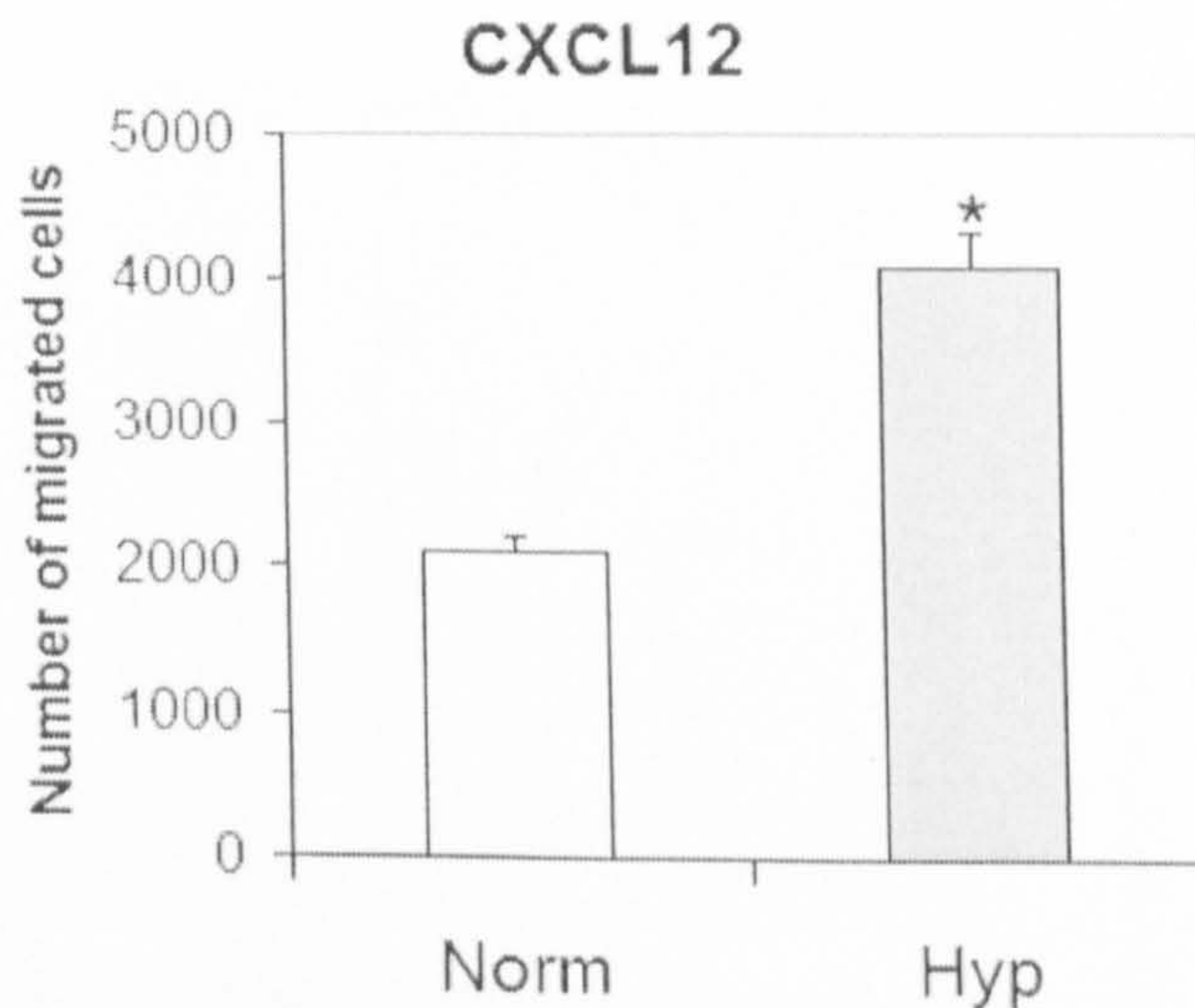




**Figure 3.13.** Effects of Hyp on the chemotactic response of CAOV3 ovarian cancer cells to CXCL12.

Cells were incubated 16 h in Hyp condition and migration to CXCL12 (100 ng/ml) was assayed by Transwells. Results are mean  $\pm$ SD of three experiments. \*,  $P < 0.05$  versus cells cultured in normoxic conditions (Paired Student's  $t$  test).

Similar results were obtained with the MCF-7 breast cancer cell line (Figure 3.14).



**Figure 3.14.** Effects of Hyp on the chemotactic response of MCF-7 breast cancer cells to CXCL12.

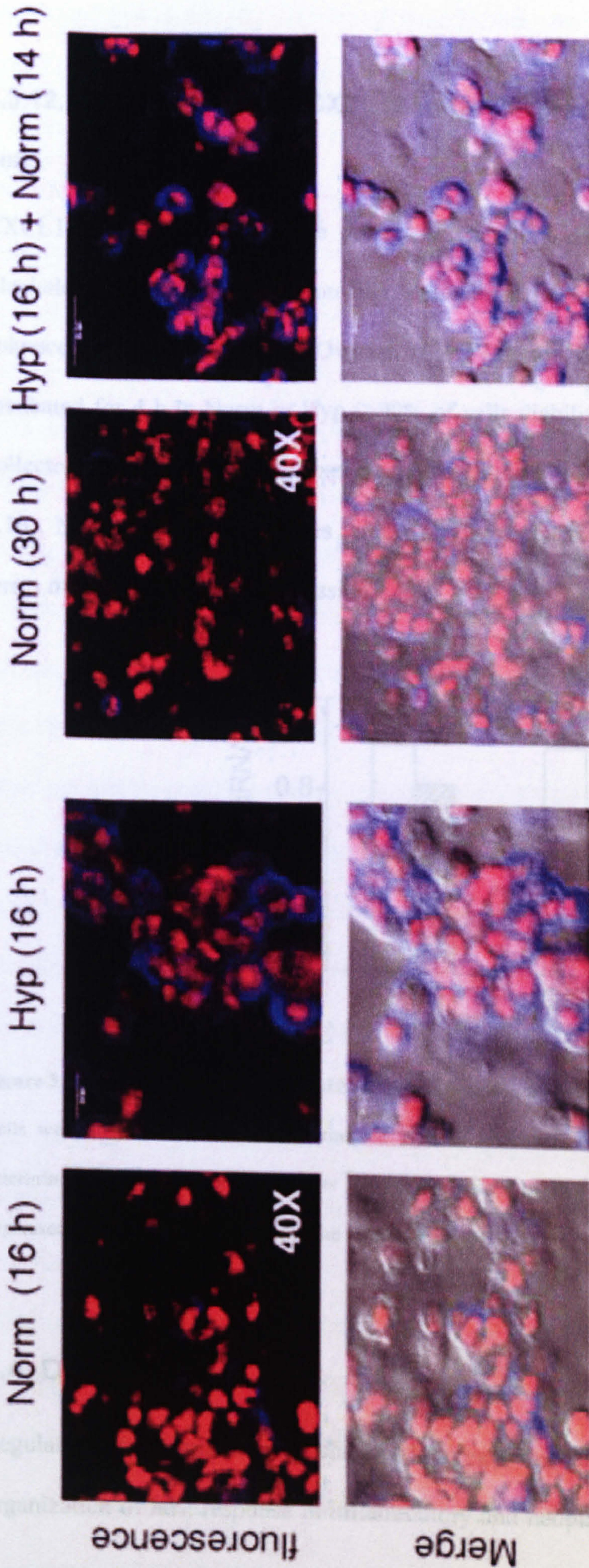
Cells were incubated 16 h in Hyp condition and migration to CXCL12 (100 ng/ml) was assayed by Transwells. Results are mean  $\pm$ SD of three experiments. \*,  $P < 0.05$  versus cells cultured in normoxic conditions (Paired Student's  $t$  test).



### 3.3.11. Effects on CXCR4 expression on cancer cells after reoxygenation

CXCR4 expression by cancer cells may play an important role in the metastatic process (Muller, Homey et al. 2001). The process of metastasis formation includes the exit of cancer cells from the primary tumor site and their entrance into the circulation, where the oxygen levels will markedly increase, relatively to those present in the tumour microenvironment. To assess the impact of cancer cells reoxygenation on the surface expression of CXCR4, CAOV3 cells were cultured in hypoxic conditions for 16 h and subsequently exposed to Norm up to 14 h. As shown in Figure 3.13, hypoxia (16 hrs) increased CXCR4 surface expression in CAOV3 cells and high levels of CXCR4 were retained after reoxygenation for 14 h, while they returned to baseline by 24 h (not shown).





**Figure 3.15. Sustained Hyp-induced CXCR4 expression upon reoxygenation.**

CAOV3 cells were cultured under Norm or Hyp conditions for 16 h. Thereafter, the cells were exposed to Norm for a further 14 h and stained for CXCR4 expression. The figure shows a representative field using confocal microscopy. CAOv3 cells stained for CXCR4 (blue fluorescence) and nuclei (red fluorescence). The bottom panels in each group shows phase-contrast images merged with fluorescence readings. Results are representative of four experiments.



### 3.3.12. Effects of Hyp on CXCL12 mRNA expression by MCF-7 and CAOV3 cells

CXCL12 is highly expressed in some solid tumours, as shown in ovarian cancer (Zou, Machelon et al. 2001). Based on this, we wanted to understand whether hypoxia could enhance CXCL12 expression. Ovarian (CAOV3) and breast (MCF7) cancer cells were incubated for 4 h in Norm or Hyp (>90% of cells viability). Subsequently total RNA was collected and CXCL12 gene expression was determined by real-time RT-PCR (Figure 3.16). No significant differences were observed between normoxic and hypoxic cells in terms of CXCL12 mRNA expression.

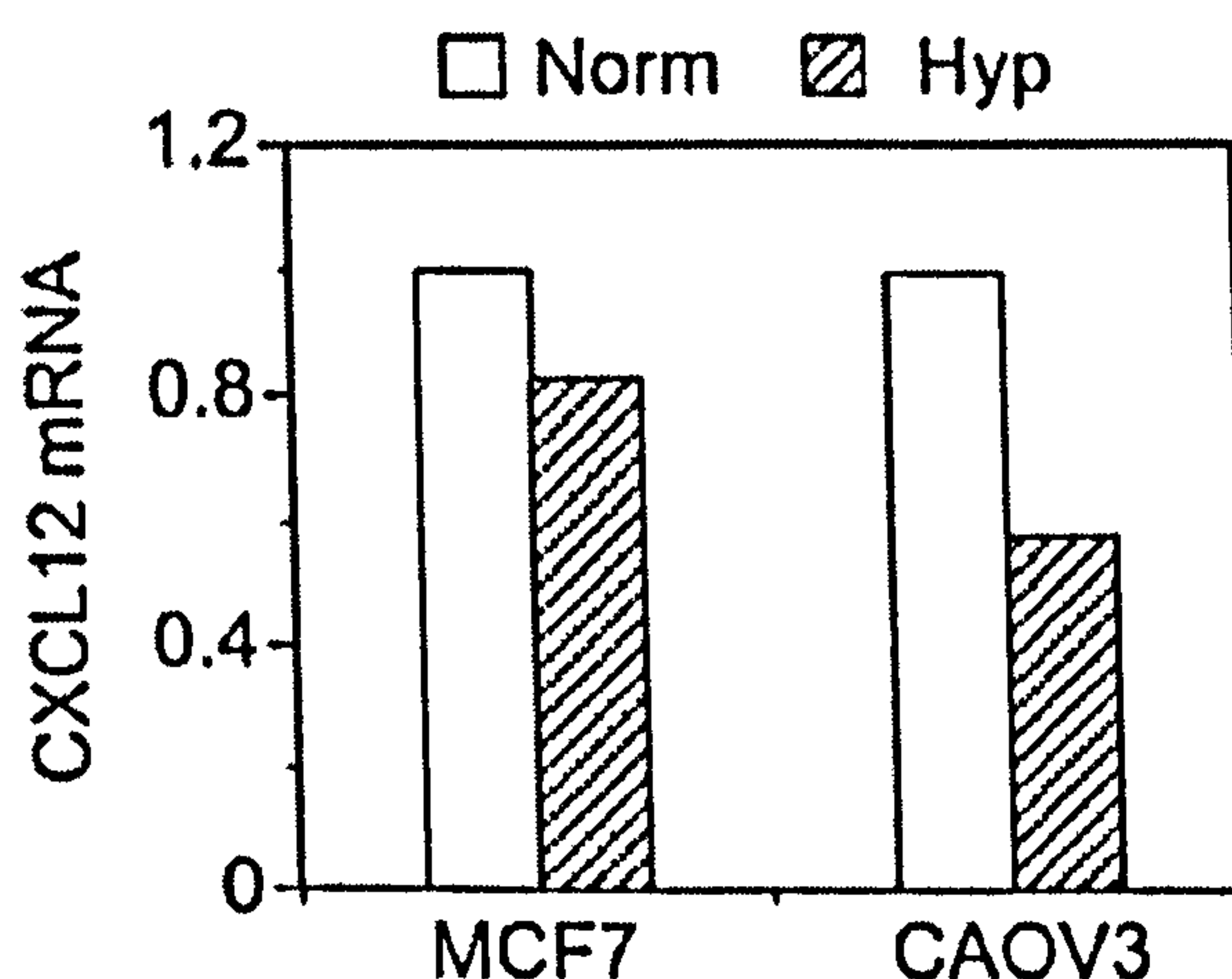


Figure 3.16. Effects of Hyp on CXCL12 mRNA expression by MCF-7 and CAOV3.

Cells were incubated for 4 h in Norm and Hyp as indicated, and CXCR4 gene expression was next determined by real-time PCR. Results are representative of two independent experiments. Values are expressed as fold increases relative to the reference sample (Norm).

## 3.4. Discussion

Regulation of cell migration by changes in oxygen availability is a central event during the organization of host response in inflammatory and neoplastic diseases as it may influence



leukocyte recruitment and activation, angiogenesis, and metastasis formation (Semenza 2002). In this chapter we have demonstrated that Hyp mediates selective up-regulation of CXCR4 in different cell types, including mononuclear phagocytes (monocytes, MDMs, and TAMs), endothelial cells, and cancer cells, and shown that oxygen levels act as an important regulator of CXCR4 receptor expression.

In contrast to standard cell culture conditions, characterized by 20% oxygen concentration, cells in the human body are exposed to much lower oxygen concentrations, ranging from 16% in the pulmonary alveoli to ~6% in most other organs of the body. Moreover, oxygen concentration may even drop to extremely low concentrations, close to anoxia, in the presence of altered vascularization as observed at pathological sites such as tumours (Semenza 2001). As selective accumulation of leukocyte subpopulations is the hallmark in allergy, inflammation, and tumours (Mantovani, Sozzani et al. 2002), it was important to investigate how leukocyte recruitment is affected by changes in oxygen tension. We first observed that in response to Hyp monocytes and MDM increase CXCR4 expression and function, as assessed by surface expression and chemotactic responsiveness to its specific ligand CXCL12. Thus, dynamic regulation of the chemotactic responsiveness of monocytes/macrophages may represent a feature of the pathophysiology of inflammatory diseases associated with Hyp. Interestingly, Hyp is present in the joint microenvironment, because articular cartilage is an avascular tissue that functions at lower oxygen tension than do most tissues. Moreover, in the setting of diseases such as rheumatoid arthritis and osteoarthritis, in which macrophages promote perpetuation of chronic inflammation (Liew and McInnes 2002), a further decrease in synovial fluid oxygen tension may occur (Blake, Winyard et al. 1994; Cernanec, Guilak et al. 2002).

In solid tumours, TAMs represent a prominent component of the mononuclear leukocyte population, which displays an ambivalent relationship with tumours [the “macrophage balance hypothesis”; (Mantovani, Bottazzi et al. 1992)]. Interestingly, TAMs preferentially



localize at the tumour–host tissue interface, in regions often associated with low oxygen tensions. Increasing evidences show that TAMs mainly accumulate in avascular, necrotic/hypoxic areas of tumors where they presumably act to clear necrotic cell debris from this sites (Murdoch, Giannoudis et al. 2004).

Several lines of evidence also indicate that chemokines play a pivotal role in the recruitment of monocytes to neoplastic tissues (Mantovani, Bottazzi et al. 1992; Gerard and Rollins 2001; Mantovani, Sozzani et al. 2002) and a variety of chemokines have been detected as products of cancer cells or tumour stromal elements (Azenshtein, Luboshits et al. 2002; Milliken, Scotton et al. 2002). In particular, CCL2 was proposed as tumor-derived chemotactic factor which plays a major role in the recruitment of macrophages at the tumour site (Mantovani, Sozzani et al. 2002). Receptor expression is a crucial determinant of the spectrum of action of chemokines (Mantovani, Sozzani et al. 2002). It was reported that the capacity of monocytes/macrophages to migrate in response to CCL2 is decreased in low oxygen conditions (Turner, Scotton et al. 1999; Grimshaw and Balkwill 2001). Thus, our observation of Hyp-mediated up-regulation of CXCR4 expression in TAMs may indicate that in regions associated with oxygen decrease, a dynamic change of their receptor profile occurs, with up-regulation of functional CXCR4.

A relay of distinct chemokine–chemokine receptor interactions may regulate initial recruitment, tissue infiltration in hypoxic areas, and in neoplastic and non-neoplastic inflammatory sites in a multistep navigation process (Foxman, Campbell et al. 1997).

Angiogenesis is a prerequisite for the expansion of solid tumors and is often activated during the early, preneoplastic stages of tumor development (Folkman 1995; Hanahan and Folkman 1996). Tumour angiogenesis is controlled by a number of positive and negative regulators produced by cancer cells and tumour-associated leukocytes.

A number of molecules with possible impact on angiogenesis have been shown to be expressed by macrophages in low oxygen conditions, such as VEGF, TNF- $\alpha$ , bFGF, and



CXCL8 (Crowther, Brown et al. 2001). The contribution of chemokines toward angiogenesis is currently a focus of intensive investigation (Hirani, Antonicelli et al. 2001). It was recently demonstrated that in CCL3-deficient mice the tumour angiogenesis diminished, demonstrating its fundamental role (Yang, Lu et al. 2005).

Strikingly, it was recently reported that CXCL12 acts as a potent chemoattractant for endothelial cells of different origins bearing CXCR4 and is a participant in angiogenesis that is regulated at the receptor level by VEGF and bFGF (Strieter, Polverini et al. 1995; Salcedo, Wasserman et al. 1999; Nagasawa 2001; Payne and Cornelius 2002). In agreement with these observations, we observed an increased chemotactic responsiveness of HUVECs toward CXCL12, which may well be part of the angiogenic program induced by hypoxia. Hyp is a well-recognized pathophysiological condition for the induction of angiogenic factors, including but not limited to VEGF (Semenza 2002). In agreement with these observations, our data suggest that the angiogenic program established by hypoxia may rely also on the increased expression of CXCR4 by different cellular components in the tumour microenvironment, including endothelial cells, tumour cells, and TAMs.

The involvement of CXCR4 in cancer metastasis has been proposed by Muller and colleagues, who showed that this receptor and its ligand (CXCL12) together govern the pattern of breast cancer metastasis in a mouse model (Liotta 2001; Muller, Homey et al. 2001). This observation is in support of the “chemoattraction” theory of metastasis, which holds that organ-specific attractant molecules stimulate the migrating tumour cells to invade the walls in blood vessels and enter the organs.

However, this remarkable observation does not clarify the mechanisms of selection by which cancer cells became CXCR4 positive. Our observation that the levels of CXCR4 surface expression induced by Hyp are sustained for several hours after reoxygenation is consistent with the idea that this pathway may confer metastatic potential to cancer cells. Indeed, it was described in *in vivo* models of metastasis that after entering the circulation,



the majority of cancer cells home to target organs in a time-frame ranging from 1 to 24 h (Aoudjit, Potworowski et al. 1998).

The next chapter investigates the possible involvement of the Hypoxia-Inducible Factor (HIF-1) in Hyp-induced expression of CXCR4.



# Chapter 4. Role of HIF-1 $\alpha$ in the regulation of CXCR4 gene expression

## 4.1. Introduction

Hypoxia occurs when oxygen availability/delivery is below the level required to maintain physiological O<sub>2</sub> tensions. Tumour hypoxia is a result of an abnormal vascular formation, which often characterizes neoplastic tissue, and results in poor oxygen and nutrient supply. This supply is mainly achieved through neoangiogenesis, a process by which new blood vessels are formed from pre-existing ones and hypoxia induces the expression of the most important molecule mediating neoangiogenesis: the vascular endothelial growth factor (VEGF). This is only one example of response to hypoxia: hypoxic conditions elicit cellular responses designed to improve cell oxygenation through several mechanisms such as neoangiogenesis, enhanced glycolytic pathway for energy requirement, and upregulation of molecules related to cell survival/apoptosis (Semenza 2002).

The master regulator of the adoptive response to hypoxia is the transcriptional factor Hypoxia Inducible Factor-1 (HIF-1). HIF-1 is a heterodimer that consists of a constitutively expressed HIF-1 $\beta$  subunit and a HIF-1 $\alpha$  subunit, the expression of which is highly regulated. HIF-1 activates the transcription of many genes that encode for proteins involved in angiogenesis, glucose metabolism, cell proliferation/survival and invasion/metastasis.

It was recently demonstrated that in hypoxia several chemokine gene products (e.g. CXCL12 and CXCL8) are induced by HIF-1 activation (Ceradini, Kulkarni et al. 2004; Phillips, Mestas et al. 2005).



## 4.2. Aim of this chapter

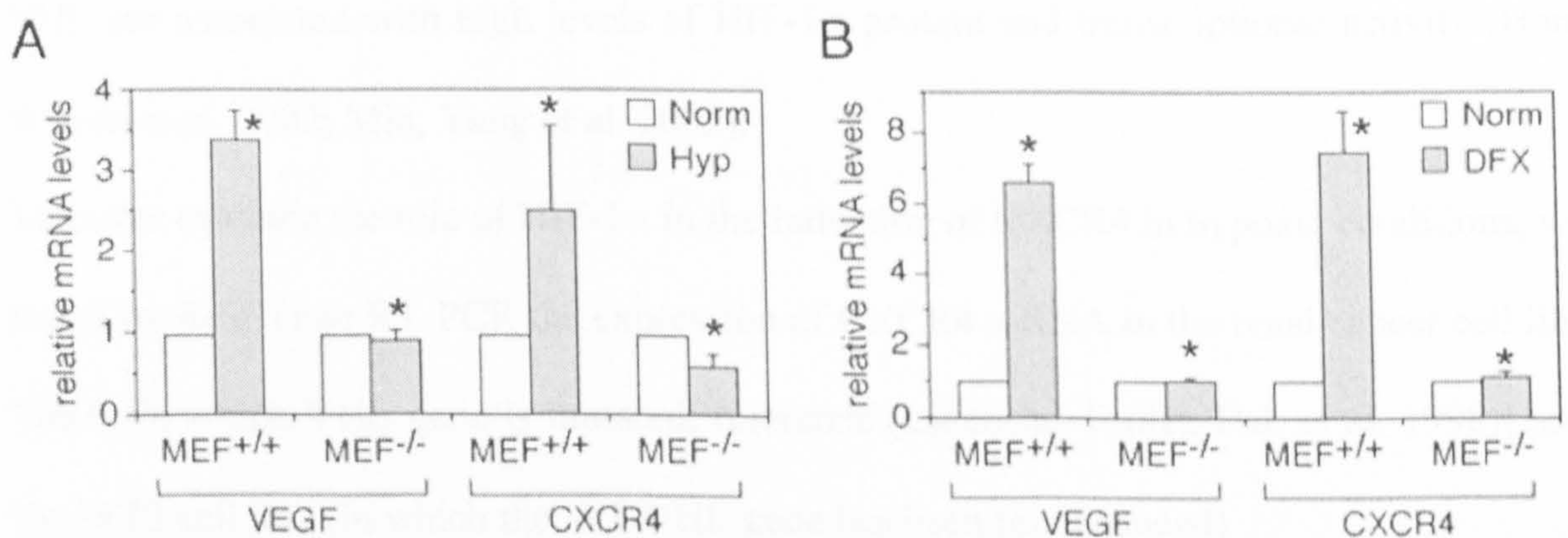
The aim of this chapter was to understand the molecular mechanism promoting CXCR4 upregulation in hypoxic conditions.

This part of the work was done in collaboration with the Laboratory of Tumour hypoxia, lead by Dr. Melillo (National Cancer Institute, Frederick), with the help of Simona Saccani for the Chip analysis and Manuela Nebuloni for the immunohistochemical analysis.

## 4.3. Results

### 4.3.1. Expression of CXCR4 in HIF-1 $\alpha$ KO mouse embryo fibroblast

HIF-1 is a key regulator of the transcriptional response to hypoxia. To confirm our hypothesis we decided to use murine embryonal fibroblasts (MEFs) deficient for the HIF-1 $\alpha$  subunit but not for HIF-2 $\alpha$ , as well as the corresponding parental cell lines. Mouse embryonic fibroblasts were incubated under normoxic or hypoxic conditions for 6 h, and total RNA was tested for VEGF and CXCR4 mRNA levels by Real-Time RT-PCR.



**Figure 4.1. Expression of CXCR4 in HIF-1 $\alpha$  KO mouse embryo fibroblast.**

(A) Mouse embryo fibroblast from wild type (MEF<sup>+/+</sup>) or knockout for the  $\alpha$  subunit of HIF-1 (MEF<sup>-/-</sup>) were incubated under normoxic or hypoxic conditions for 6 h, and total RNA was tested for VEGF and CXCR4 mRNA levels by real-time PCR. (B) DFX was used as Hypoxia inducing agent. Results are the average of



three independent experiments. \*, P<0.05 versus cells cultured in normoxic conditions (Paired Student's *t* test).

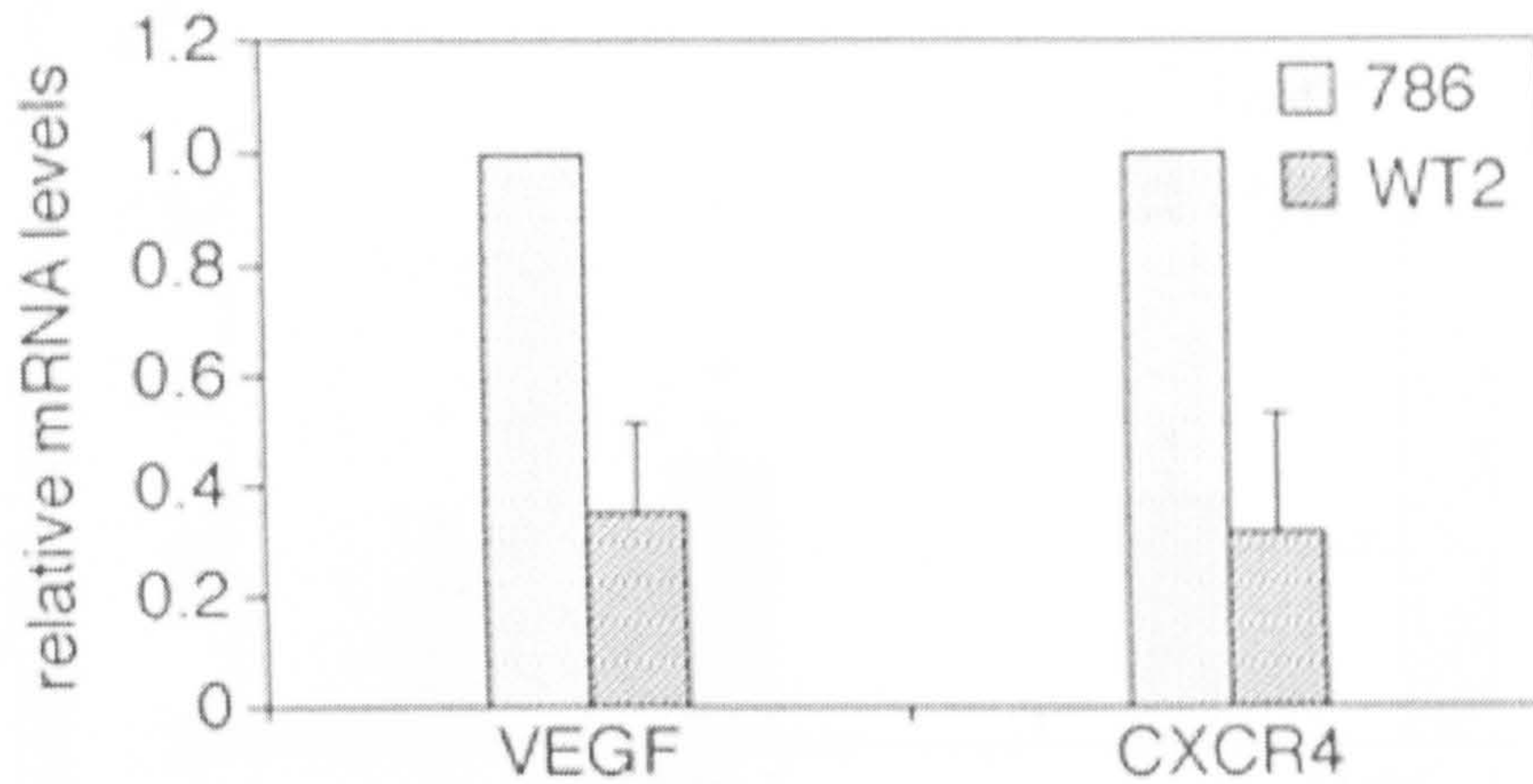
As shown in Figure 4.1 A, MEFs from WT animals, but not from HIF-1 $\alpha$  KO mice, expressed 3.5-fold-higher levels of VEGF mRNA when incubated under hypoxic conditions relative to the levels expressed in normoxic conditions. Likewise, MEFs from WT mice expressed 2.5-fold-higher levels of CXCR4 mRNA when cultured under hypoxic conditions relative to normoxic conditions. In contrast, in MEFs from HIF-1 $\alpha$ <sup>-/-</sup> mice, the hypoxic induction of CXCR4 mRNA was no longer apparent. Furthermore, DFX also induced VEGF and CXCR4 mRNA in HIF-1 WT cells (6.5- and 7.2-fold, respectively) but not in HIF-1 $\alpha$ <sup>-/-</sup> cells (Figure 4.1 B). These data suggest a role for HIF-1 in the induction of CXCR4 mRNA expression by hypoxia.

#### **4.3.2. Expression of CXCR4 in VHL WT and mutated renal carcinoma cells**

The von Hippel–Lindau tumor suppressor protein (VHL) is involved in the degradation of HIF-1 $\alpha$  by the proteasome inducing its ubiquitination in presence of oxygen. Mutations in VHL are associated with high levels of HIF-1 $\alpha$  protein and transcriptional activity (Hon, Wilson et al. 2002; Min, Yang et al. 2002).

To better evaluate the role of HIF-1 $\alpha$  in the induction of CXCR4 in hypoxic conditions, we tested by Real Time RT-PCR the expression of CXCR4 mRNA in the renal cancer cell line 786.0 [in which VHL gene is mutated; reference (Lieubeau-Teillet, Rak et al. 1998)] and the WT2 cell line (in which the WT VHL gene has been reintroduced).





**Figure 4.2. Expression of CXCR4 in VHL WT and mutated renal carcinoma cells.**

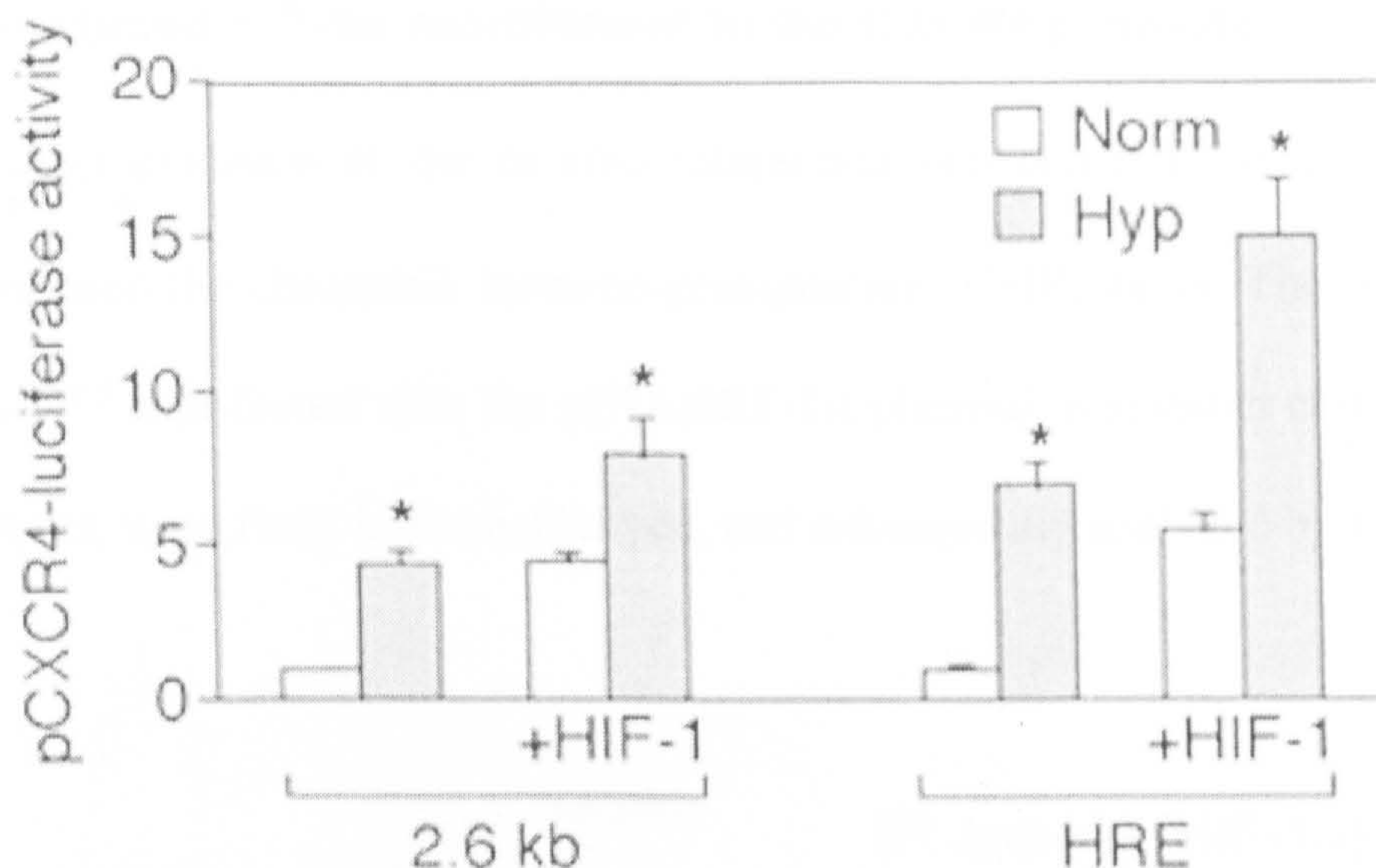
Expression of CXCR4 and VEGF mRNAs was tested by real-time PCR in the renal cancer cell line 786.0 (VHL mutated) and WT2 (in which a WT VHL has been reintroduced). Results are the average of three independent experiments.

These cells have been extensively used in several laboratories as a prototype in which lack of VHL is associated with high levels of expression of HIF-1-inducible genes (Ivanov, Kuzmin et al. 1998). As reported previously (Stratmann, Krieg et al. 1997), 786.0 cells expressed high constitutive levels of VEGF mRNA that were significantly lower (~60%) in WT2 cells (Figure 4.2). Accordingly, 786.0 cells expressed higher levels of CXCR4 mRNA relative to WT2 cells. These data are fully consistent with the involvement of HIF-1 in the hypoxic regulation of CXCR4 gene expression.

#### 4.3.3. HIF-1-dependent transcriptional activation

To investigate the role of HIF-1 on the transcriptional activation of CXCR4 promoter we used the MCF-7 breast carcinoma cell line, in which hypoxia augmented the expression of CXCR4 mRNA (see sections 3.3.9). Cells were transiently transfected with a plasmid containing a 2.6-kb fragment of the CXCR4 promoter linked to the luciferase reporter gene and incubated under normoxic or hypoxic conditions for 24 h.





**Figure 4.3. HIF-1 dependent transcriptional activation of CXCR4 promoter.**

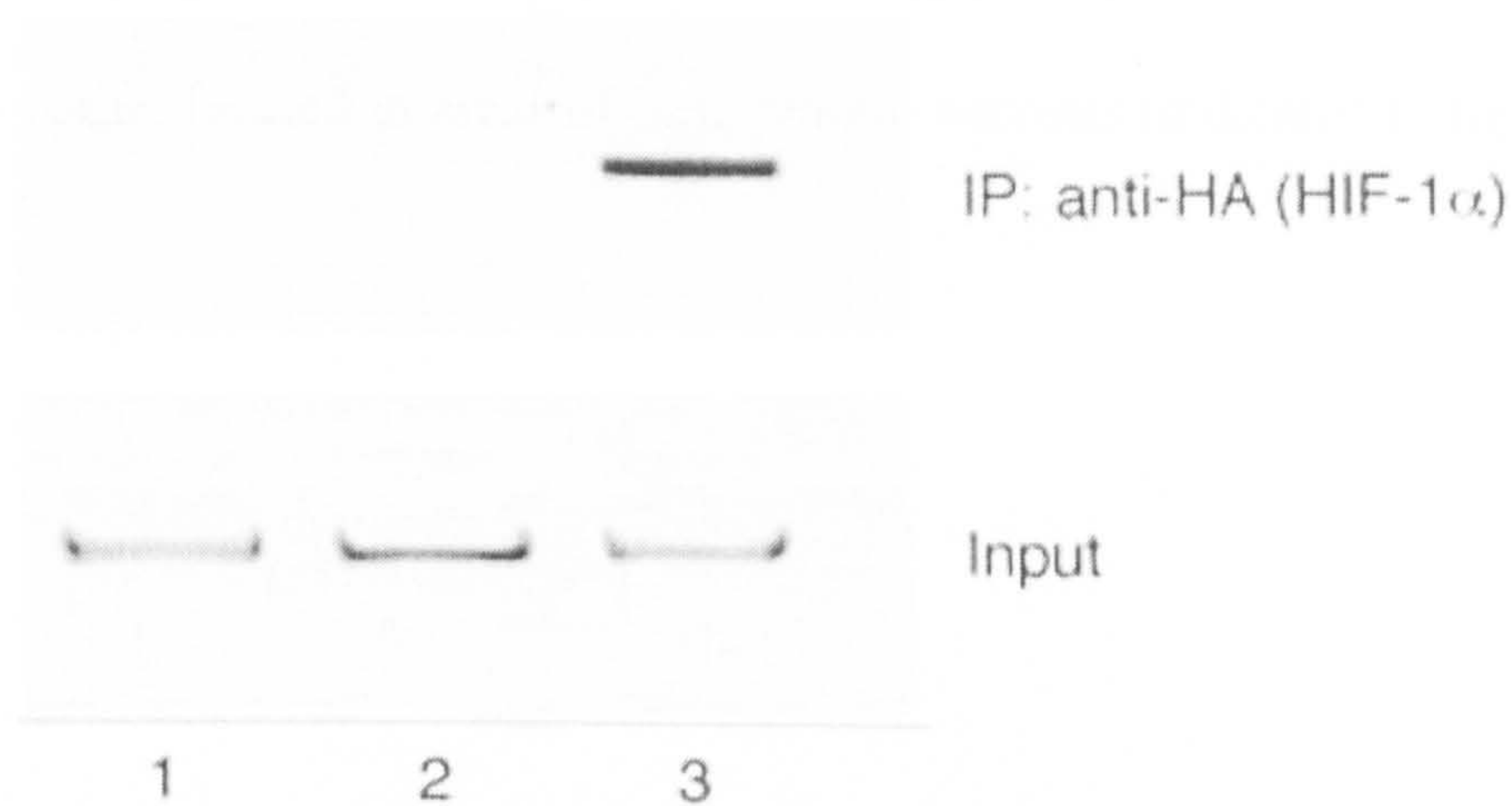
MCF-7 breast carcinoma cells were transiently transfected with a plasmid containing a 2.6 kb fragment of the CXCR4 promoter linked to the luciferase reporter gene, with or without a HIF-1 $\alpha$  expression vector. Cells were incubated under normoxic or hypoxic conditions for 24 h and evaluated for the luciferase activity. Results are the average of three independent experiments. \*,  $P < 0.05$  versus cells cultured in normoxic conditions (Paired Student's *t* test).

As shown in Figure 4.3, Hyp induced four fold induction of the luciferase activity relative to the cells cultured under normoxic conditions. Interestingly, cotransfection of a HIF-1 $\alpha$  expression vector significantly increased luciferase expression driven by the 2.6-kb CXCR4 promoter (four fold relative to control). Hypoxia further increased luciferase expression induced by HIF-1 $\alpha$  cotransfection up to nine fold, compared to untreated normoxic cells. Experiments conducted in parallel using a plasmid containing three copies of a canonical hypoxia responsive element (HRE) upstream of the luciferase reporter gene showed a similar trend of luciferase expression, although levels of induction were substantially higher.



#### 4.3.4. Hyp-induced HIF-1 $\alpha$ recruitment to the CXCR4 promoter

To obtain direct evidence of the *in vivo* interaction between HIF-1 $\alpha$  and the CXCR4 promoter, we used the chromatin immuno-precipitation (ChIP) assay. The ovarian cancer cell line CAOV3 transfected with the p(HA)HIF-1 $\alpha$  plasmid, was either cultured in Norm or Hyp for 4 hrs, were fixed in formaldehyde, and subsequently analyzed by ChIP.



**Figure 4.4. Hyp-induced HIF-1 $\alpha$  recruitment to the CXCR4 promoter.**

CAOV3 cells transfected with the p(HA)HIF-1 $\alpha$  plasmid were cultured for 4 h in normoxic or hypoxic conditions. ChIP was performed to investigate the recruitment of HIF-1 $\alpha$  on the CXCR4 promoter. (lane 1) Untransfected. (lane 2) Norm. (lane 3) Hyp.

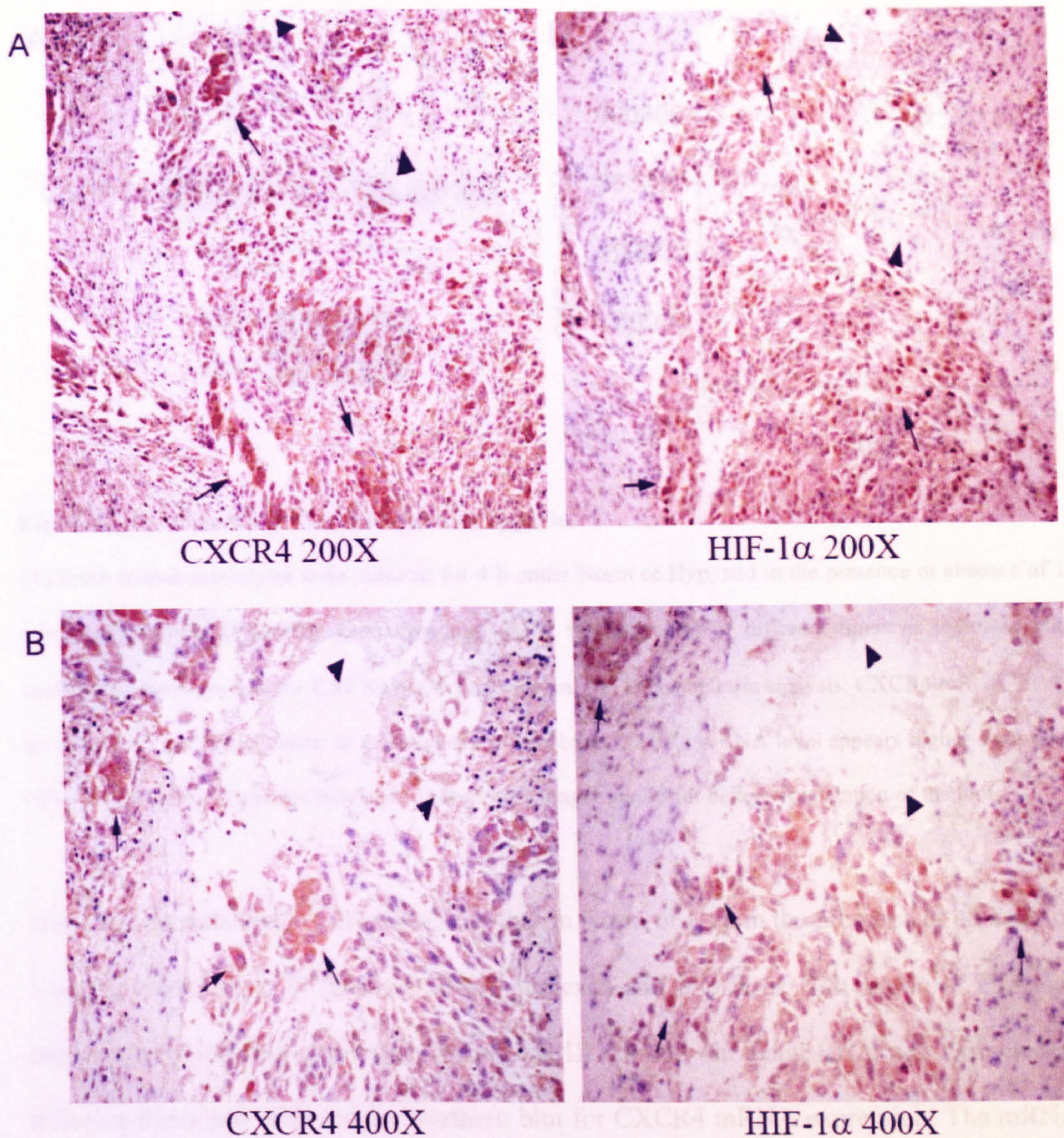
Although no interaction between HIF-1 $\alpha$  and the CXCR4 promoter was observed in Norm, recruitment of HIF-1 $\alpha$  to the CXCR4 promoter was clearly detected at 4 h after Hyp, in the promoter region -1860 to -1578 (Figure 4.4, lane 3). This result is consistent with the functional data obtained in transient transfection experiments (Figure 4.3). Overall these data demonstrate that HIF-1 $\alpha$  is the molecular effect promoting CXCR4 gene transcription in hypoxia.



### **4.3.5. Immunohistochemistry for CXCR4 and HIF-1 $\alpha$ in ductal mammary carcinoma**

To confirm the existence of the HIF-CXCR4 pathway in human tumours we have performed histological analysis of serial sections of ductal mammary carcinoma (n=3). Immunohistochemistry for CXCR4 and HIF-1 $\alpha$  (Figure 4.5: brown colour indicated by a little arrow) displayed colocalization of HIF-1 $\alpha$  and CXCR4 in ductal carcinoma cells from breast tissue, located in areas of intratumoral necrosis (indicated in figure by a head arrow).





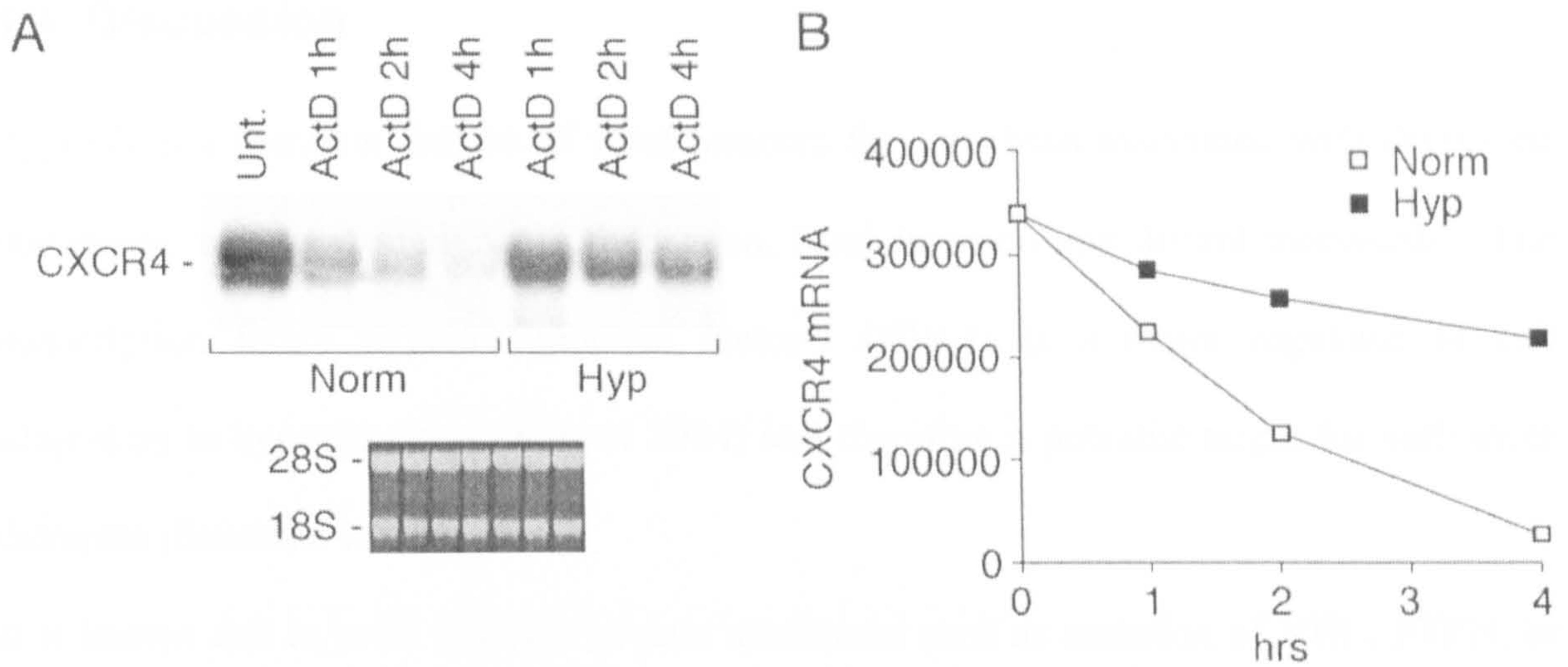
**Figure 4.5. Co-localization of CXCR4 and HIF-1 $\alpha$  in ductal mammary carcinoma**

Immunohistochemistry for CXCR4 and HIF-1 $\alpha$  on a sequential section of ductal mammary carcinoma (n=3) marked by arrows (necrotic areas are marked by arrowheads) 200X (A) 400X (B).

#### 4.3.6. Effect of Hyp and DFX on CXCR4 mRNA Stability

To further investigate the mechanisms of hypoxia induced CXCR4 mRNA expression, we estimated its effects on CXCR4 mRNA stability (Figure 4.6).





**Figure 4.6. Stabilization of CXCR4 mRNA by hypoxia.**

(A) Fresh human monocytes were cultured for 4 h under Norm or Hyp, and in the presence or absence of 1  $\mu$ g/ml actinomycin D (ActD). Thereafter, total RNA was extracted at different times as indicated and analyzed by Northern blot for CXCR4 mRNA expression. (B) Densitometric analysis: CXCR4 mRNA levels are expressed as arbitrary units. In this experiment, the basal CXCR4 mRNA level appears higher compared with other experiments, as the film was exposed for a longer period for better visualization of the blot.

Fresh human monocytes were cultured either in Norm or Hyp in the presence or absence of 1  $\mu$ g/ml actinomycin D (binds to DNA duplexes and interferes with action of enzymes engaged in replication and transcription) for 1h, 2h and 4h. Total RNA was extracted at different times and analyzed by Northern blot for CXCR4 mRNA expression. The mRNA decay observed in Hyp conditions was compared with the rate of mRNA degradation observed in normoxic conditions. The results indicate that Hyp increased CXCR4 mRNA stability, suggesting that Hyp-induced CXCR4 mRNA expression relies on both transcriptional and posttranscriptional mechanisms.

Densitometric analysis shows that the mRNA level of CXCR4 is higher in hypoxia conditions also after 4h (Figure 4.6 B).



## 4.4. Discussion

Hypoxia is a common feature of solid tumours that has been associated with decreased therapeutic response, malignant progression, local invasion and distant metastasis. The transcription factor hypoxia-inducible factor-1 (HIF-1) is a major regulator of cell adaptation to hypoxic stress (Vaupel 2004) and therefore a potential target for anticancer therapies (Semenza 2003).

It is known that in solid tumours genetic alterations such as mutation of VHL, PTEN, or p53 genes that are associated with increased levels of HIF-1 transcriptional activity.

HIF-1 activation in tumor cells activates several mechanisms leading to angiogenesis, glycolysis, inhibition of apoptosis, upregulation of growth factors (eg. PDGF, TGF- $\beta$ , IGF-2, EGF, VEGF) and protein involved in tumor invasion (e.g. urokinase-type plasminogen activator). Moreover, hypoxia downregulates adhesion molecules thus contributing to cancer cell detachment (Koong, Denko et al. 2000; Czekay, Aertgeerts et al. 2003).

Results obtained in this thesis propose that microenvironmental hypoxia may also increase CXCR4 expression and the metastatic potential of cancer cells. In line with this hypothesis, recent evidence has been provided that invasive cancer phenotype is associated with hypoxia and/or HIF-1 $\alpha$  overexpression (Krishnamachary, Berg-Dixon et al. 2003).

HIF-1 activates transcription of genes that mediate adaptive responses to reduced oxygen availability and a number of HIF-1-regulated genes have been identified, whose products play key roles in angiogenesis, vascular reactivity and remodelling, and glucose and energy metabolism (Semenza 2004).

HIF-1 is a heterodimer composed of a HIF-1 $\beta$  subunit that is constitutively expressed and a HIF-1 $\alpha$  subunit that is rapidly degraded by ubiquitination via the proteasomal pathway, a process that is inhibited under hypoxic conditions.

Oxygen-regulated destruction of HIF-1 $\alpha$  requires the von Hippel-Lindau tumor suppressor protein (pVHL) (Hon, Wilson et al. 2002; Min, Yang et al. 2002). pVHL acts as the



recognition component of a ubiquitin E3 ligase complex, which binds HIF-1 $\alpha$ , and loss of pVHL function results in constitutive activation of the hypoxic response (Min, Yang et al. 2002; Pugh and Ratcliffe 2003). Taken together the results of this chapter suggest a role for HIF-1 in the regulation of CXCR4 mRNA expression:

- (a) mouse embryonal fibroblast lacking the  $\alpha$  subunit of HIF-1 had impaired hypoxic induction of CXCR4 mRNA;
- (b) CXCR4 mRNA was differentially expressed in the renal cancer cell lines 786.0 and WT2 bearing a VHL-mutated and WT phenotype, respectively;
- (c) Hyp or cotransfection of a HIF-1 $\alpha$  expression vector induced transcriptional activation of a 2.6-kb CXCR4 promoter luciferase reporter construct;
- (d) ChIP analysis demonstrated that after Hyp, HIF-1 $\alpha$  is specifically recruited to the CXCR4 promoter, in the nucleotide region -1860 to -1578;
- (e) the HIF-1 inhibitor Topotecan inhibited the Hyp-induced expression of CXCR4 in CAOV3 cells.

A sequence homology search of the region -1860 to -1578 of the CXCR4 promoter revealed the presence of a putative HIF-1 binding site at position -1725 (5'-GCGTG-3'). Our data are in line with P. Staller's paper where they have shown the presence of four potential hypoxia-response elements (HRE) at upstream of the transcriptional start of the CXCR4 promoter. In particular they have also demonstrated that mutation of sequence -1.3 Kb upstream of the transcriptional start in the context of the full-length CXCR4 promoter rendered the promoter insensitive to hypoxia and HIF-1 $\alpha$  confirming that CXCR4 is a hypoxia dependent gene (Staller, Sulitkova et al. 2003).

In addition to transcriptional activation, we found that Hyp further contributes to increased CXCR4 gene expression by stabilization of CXCR4 transcripts, suggesting that Hyp-regulated RNA binding factors may interact with and stabilize the CXCR4 mRNA at the posttranscriptional level.



The results of this chapter identify the Hyp–HIF-1–CXCR4 pathway as a relevant molecular circuit in the functional tuning of the chemokine system. The validity of this observation in different cell types (mononuclear phagocytes, HUVECs, fibroblasts, and cancer cells), consistent with the virtually universal expression of the Hyp–HIF-1 pathway in mammalian cells, argues in favour of its potential involvement in the pathophysiology of diverse conditions. In a multistep navigation process, the Hyp–HIF-1 $\alpha$ –CXCR4 pathway may regulate trafficking and localization in hypoxic tissues and represents a target for novel therapeutic strategies.



# Chapter 5. Regulation of maturation of dendritic cells by hypoxia

## 5.1. Introduction

To anticipate and initiate immune responses, dendritic cells (DCs) follow a migratory route from their recruitment as sentinels into tissues, including solid tumours, to secondary lymphoid organs where they profile the immune response. Migratory capacities, and especially chemokine responsiveness, are therefore key elements in dendritic cell biology. DCs express receptors for and respond to both constitutive and inducible chemokines. Immature myeloid DC, generated *in vitro* from monocytes, express a unique repertoire of inflammatory chemokine receptors (CCR1, CCR5, CCR6). Purified circulating DCs were also found to express CCR1, CCR2, CCR3, CCR5 and CXCR4 (Ayehunie, Garcia-Zepeda et al. 1997; Sozzani, Allavena et al. 1999).

A dramatic change in the repertoire of chemokine receptors is promoted by DC activation. This change is responsible for the migration of DC from periphery to the draining lymph nodes. The signals that promote this process include a variety of maturation factors, such as IL-1, TNF and LPS (Sozzani 2005). Many microbial products, such as the activators of Toll-like pattern recognition receptors (TLRs) and some endogenous molecules, like CD40L, expressed on the surface of activated T cells may also promote DC activation. DC activation is usually associated with the acquisition of a mature phenotype consisting in an up-regulation of co-stimulatory and MHC class II molecules. Activation of DC is also associated with down-regulation of chemokine inflammatory receptors and the de novo expression of CCR7, the receptor for CCL19 and CCL21, two chemokines that are expressed at the luminal side of high endothelial cells and in the T cell rich areas of secondary lymphoid organs, like tonsils, spleen and lymph nodes (Dieu, Vanbervliet et al. 1998; Ngo, Tang et al. 1998; Willimann, Legler et al. 1998). Within the T cell area, CCL19



is expressed by mature interdigitating DC, whereas CCL21 is expressed by stromal cells, as indicated by the severe reduction of expression in the lymphotoxin- $\alpha^{-/-}$  stromal cell deficient mice (Ngo, Korner et al. 1999).

It was also shown by Sallusto et al. that when dendritic cells mature there is an upregulation of the CXCR4 chemokine receptor (Sallusto, Schaerli et al. 1998).

DC maturation and function may be impaired by cytokines such as IL-6, IL-10, M-CSF and VEGF (Gabrilovich, Chen et al. 1996; Banchereau, Briere et al. 2000). This is of particular relevance in tumors, where Tumour-associated DC (TADC) generally show an immature phenotype with high CD1a and low costimulatory molecules CD80, CD86 and CD40 (Hillenbrand, Neville et al. 1999; Scarpino, Stoppacciaro et al. 2000). In agreement, while IL-10 has been shown to promote the differentiation of monocytes to mature macrophages and to block their differentiation to DC (Allavena, Sica et al. 2000) a gradient of tumour-derived IL-10 has been observed in various tumours, including papillary carcinoma of the thyroid. Strikingly, in this tumour tumour-associated macrophages (TAM) are evenly distributed throughout the tissue, in contrast to DC which are present in the periphery (Scarpino, Stoppacciaro et al. 2000).

Furthermore, it was demonstrated in breast carcinoma tissue that immature dendritic cells reside within the tumour, whereas mature dendritic cells are located in peritumoral areas (Bell, Chomarat et al. 1999).

These evidences emphasize the concept that tumour microenvironmental signals are determinant of DC differentiation and maturation and may contribute to impair effective antitumour immunity.

## **5.2. Aim of this chapter**

This chapter was aimed to investigate whether tumour hypoxia could influence DC maturation and migration. In particular, the study was designed to provide insights into the expression profile of chemokine/chemokine receptors, cytokines and maturation markers

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acquired by myeloid precursors exposed to DC-inducing maturation signals, under both normoxia and hypoxia conditions. Part of this work was performed in collaboration with Andrea Doni (purification of myeloid precursors and DC maturation), Silvano Sozzani (purification of plasmacytoid DC and phenotypic characterization) and Annunciata Vecchi (maturation of murine DC from myeloid precursors).

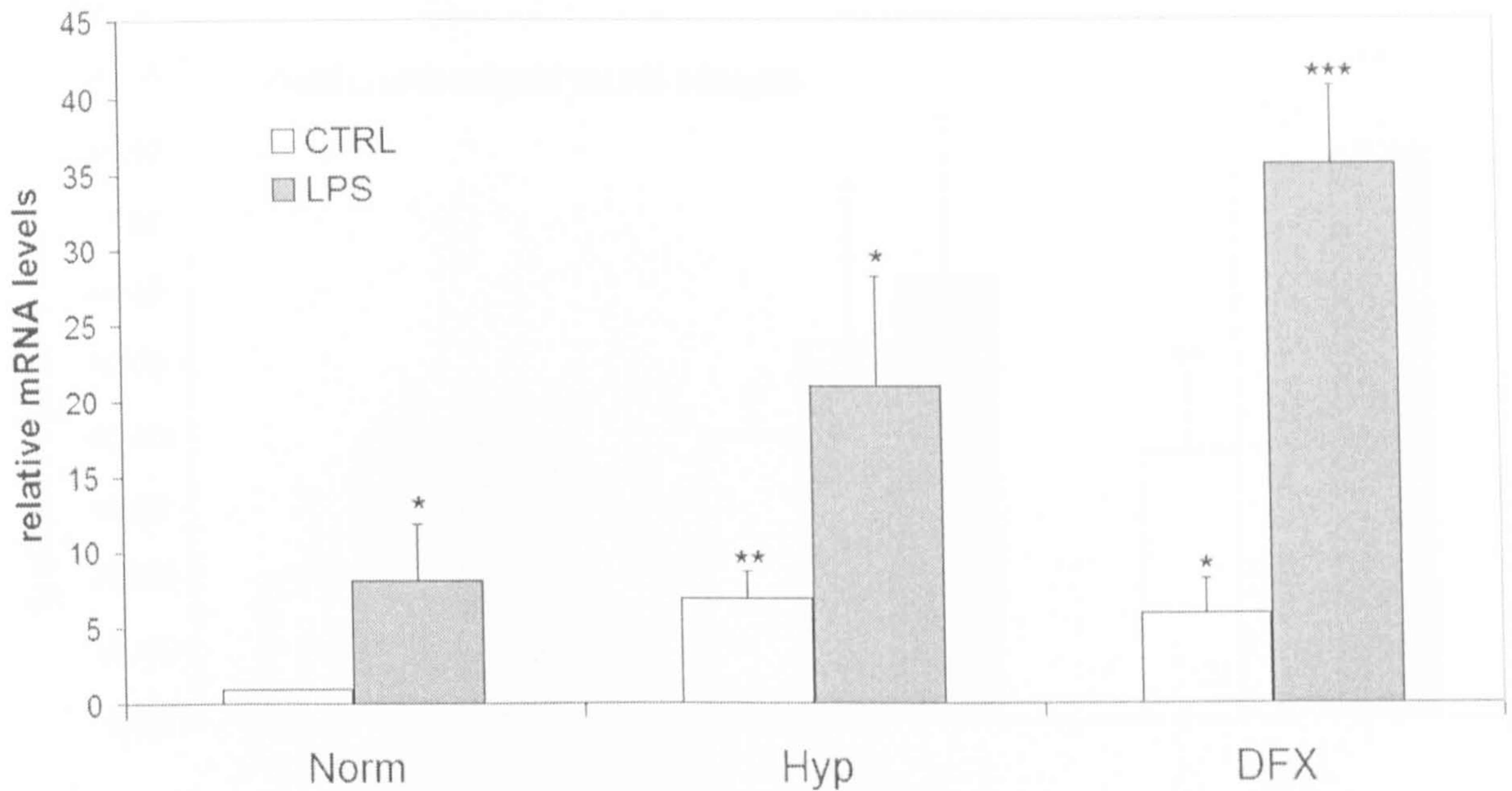
## **5.3. Results**

### **5.3.1. Hypoxia induces CXCR4 mRNA expression in both immature and mature dendritic cells**

It was recently demonstrated that DC maturation is paralleled by increased CXCR4 expression (Sallusto, Schaerli et al. 1998). Monocyte-derived immature dendritic cells were cultured in the presence or absence of maturation stimuli (LPS at 100 ng/ml) either normoxia and hypoxia conditions. DFX was further used as hypoxia mimicking compound. After 24 hours RNA was isolated and analyzed for CXCR4 mRNA levels by real-time RT-PCR.

As shown in Figure 5.1, in the presence of LPS dendritic cells expressed increased levels of CXCR4 mRNA (8.8 fold), as compared to untreated DC. A similar increase of the CXCR4 mRNA level was observed in immature DC cultured in hypoxia or DFX, in the absence of LPS, the LPS-dependent upregulation of CXCR4 was higher in the DFX-treated sample (37 fold) and in hypoxic conditions (23 fold).





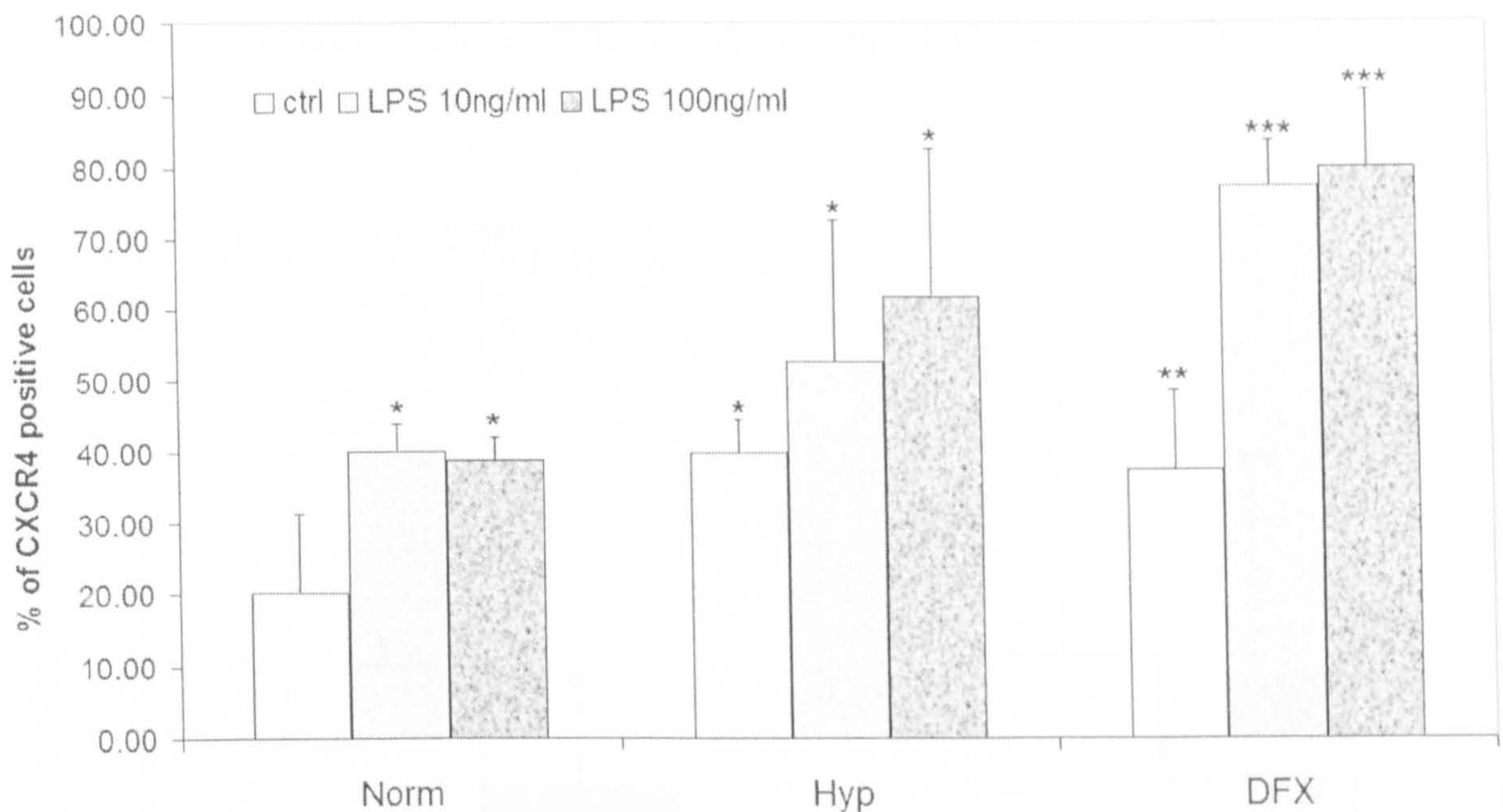
**Figure 5.1. Effects of hypoxia on CXCR4 mRNA expression by dendritic cells**

Human Monocyte-derived dendritic cells were cultured for 24 h in normoxia, hypoxia or DFX conditions with or w/o LPS (100ng/ml). Total RNA was analyzed by Real Time PCR for CXCR4 mRNA expression (quantitative analysis; all the samples were normalized vs the  $\beta$ -actin mRNA expression). Results are mean  $\pm$ SD of three experiments. \*,  $P < 0.03$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P = 0.001$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

### 5.3.2. Modulation of chemokine receptors surface expression in DC by hypoxia

To investigate whether the hypoxia-induced CXCR4 mRNA expression was paralleled at protein level, human Mo-DCs were cultured for 24h with or without LPS at different concentration (10 and 100 ng/ml) in normoxia, hypoxia or in the presence of DFX.





**Figure 5.2. Hypoxia and DFX induce CXCR4 surface expression in immature and mature Mo-DCs**

Cells were cultured for 24 h in the indicated conditions and analyzed for CXCR4 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CXCR4. Results are mean  $\pm$ SD of three experiments. \*,  $P < 0.2$ ; \*\*,  $P < 0.3$ ; \*\*\*,  $P \leq 0.03$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

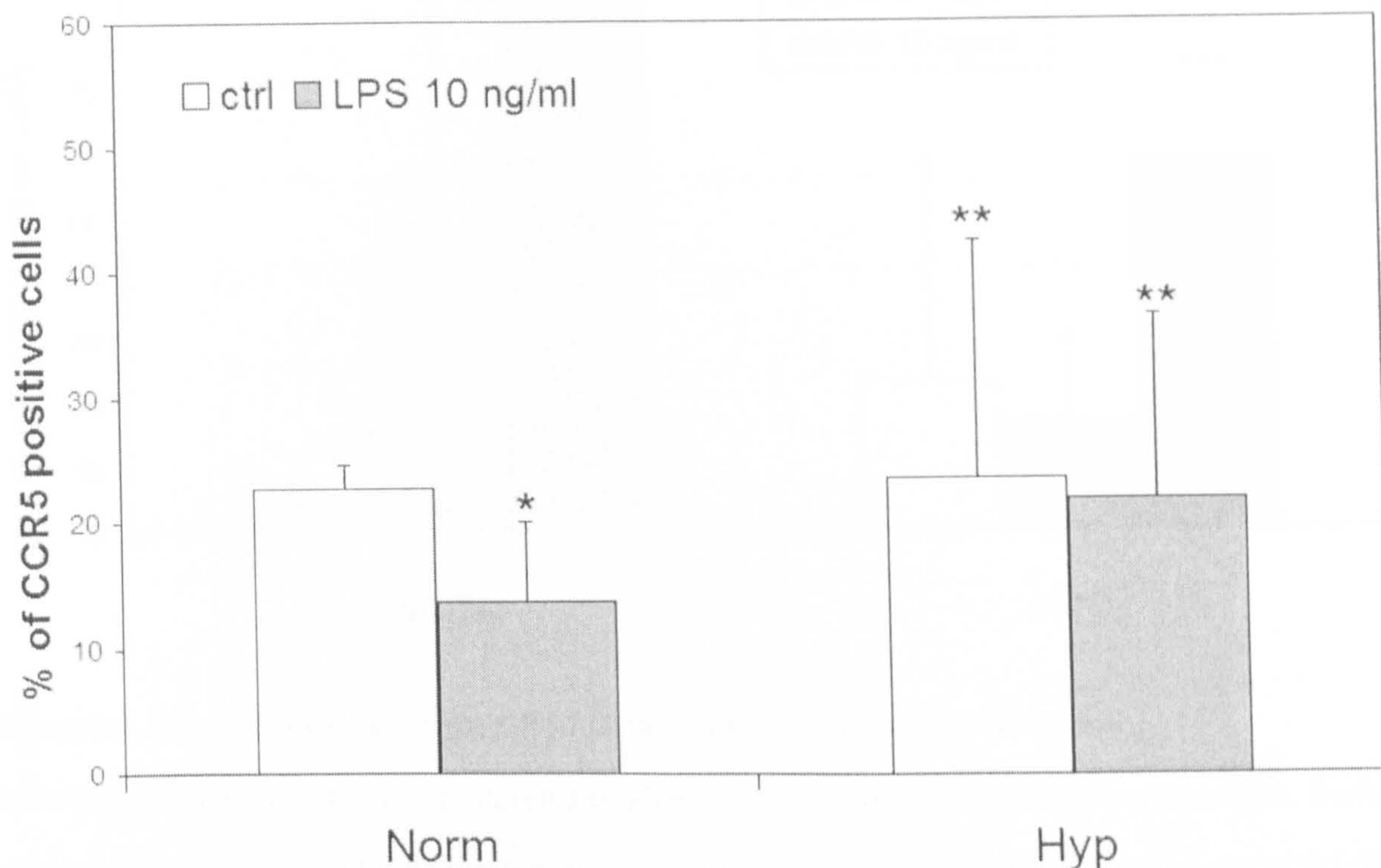
As expected, CXCR4 surface expression was upregulated following DC maturation with LPS. In agreement with the RT-PCR results, its surface expression was higher when dendritic cells were matured in hypoxia or DFX conditions (Figure 5.2). Moreover, CXCR4 surface expression was upregulated in immature DCs treated with hypoxia or DFX.

As DC maturation is associated with the downregulation of inflammatory chemokine receptors (e.g. CCR5) and the increased expression of CCR7, we checked the surface expression of these receptors in both normoxia and hypoxia conditions.

Dendritic cells were treated for 24h in presence or absence of LPS (10 ng/ml), both in normoxia and hypoxia conditions and surface expression of CCR5 and CCR7 was



evaluated by FACS analysis. The results showed that LPS-mediated decrease of CCR5 expression was partially inhibited in hypoxia, as compared to normoxia (Figure 5.3).

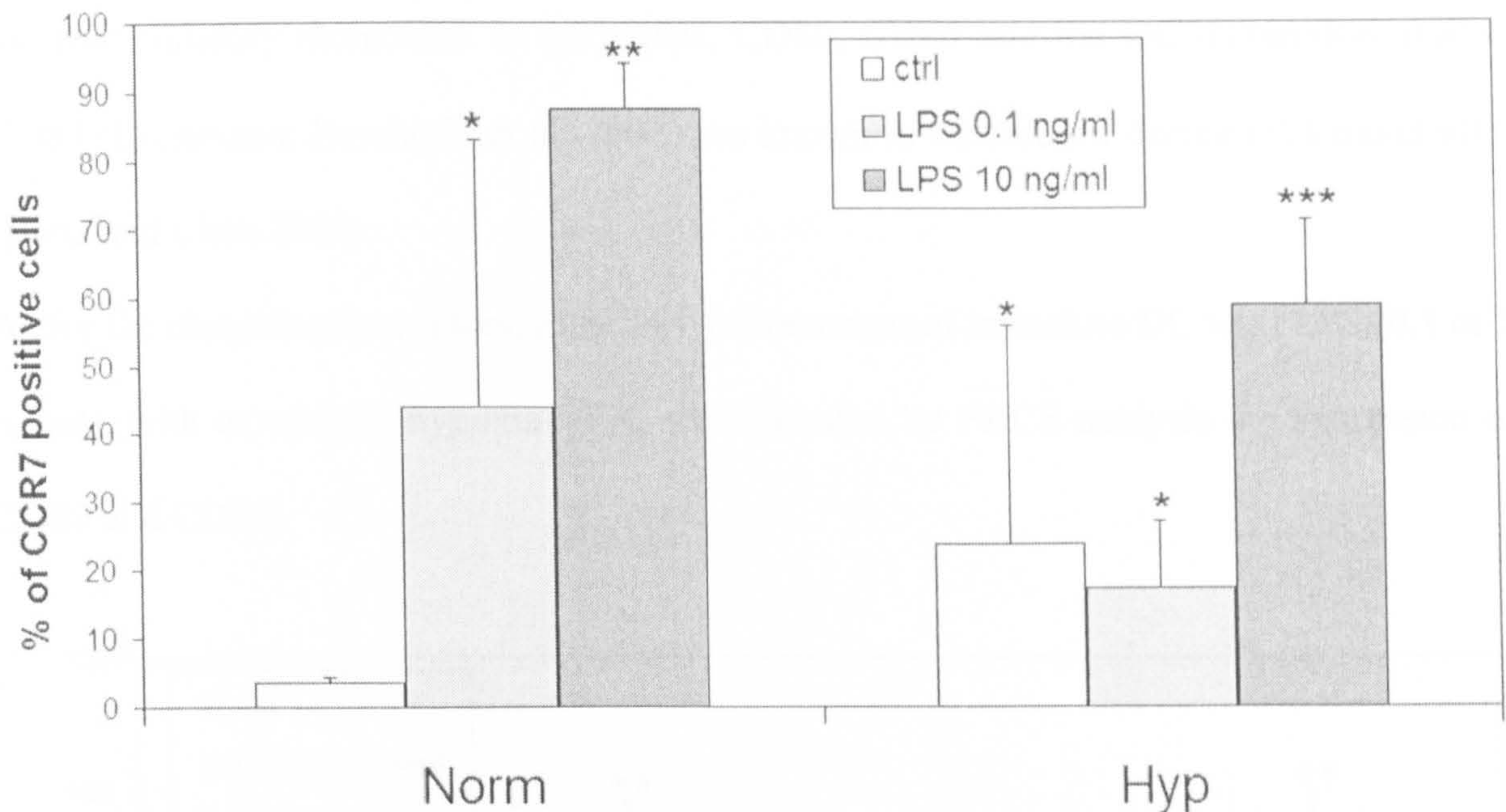


**Figure 5.3. Effects of hypoxia on the CCR5 chemokine receptor surface expression**

Cells were cultured for 24 h in the indicated conditions and analyzed for CCR5 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CCR5. Results are mean  $\pm$ SD of two different experiments. \*,  $P < 0.2$ ; \*\*,  $P < 1$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

In preliminary experiments, hypoxia partially prevented the LPS-mediated induction of CCR7 (Figure 5.4). These results will be confirmed in a larger number of donors.





**Figure 5.4. Effects of hypoxia on the CCR7 chemokine receptor surface expression**

Cells were cultured for 24 h in the indicated conditions and analyzed for CCR7 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CCR7. The results are the average of three independent experiments and represent the % of positive cells for CCR7 receptor. Results are mean  $\pm$ SD of two different experiments. \*,  $P < 0.3$ ; \*\*,  $P < 0.004$ ; \*\*\*,  $P < 0.03$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

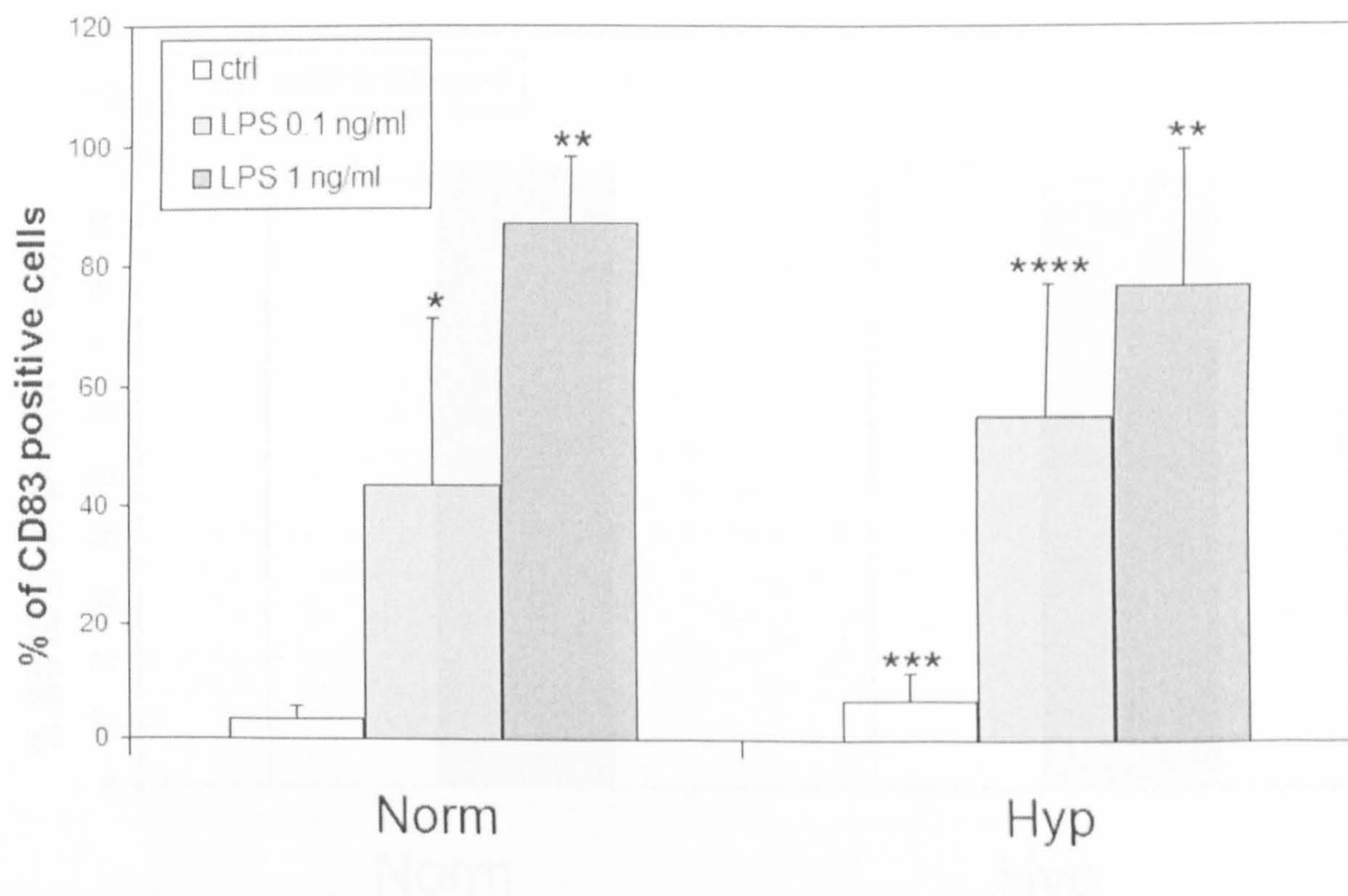
### 5.3.3. Expression of costimulatory molecules by DC, in normoxia and hypoxia

The potent functional capacity of DC to initiate the immune response is related to a high-level expression of major histocompatibility complex (MHC) I/II molecules and constitutive expression of costimulatory molecules (Cella, Salio et al. 1999). Classical costimulatory molecules, expressed primarily on antigen-presenting cells that deliver the 'signal 2' (an antigen non-specific signal) are, for example, B7-1 (CD80) and B7-2 (CD86). B7-1 and B7-2 deliver a co-stimulatory signal through the CD28 receptor that is constitutively expressed on T cells (Rietz and Chen 2004). During maturation dendritic



cells change not only the chemokine receptor expression but also the level of expression of the costimulatory molecules. In particular, CD80, CD86 and the DC maturation marker CD83 (Lechmann, Berchtold et al. 2002) are known to be induced during DCs maturation (Rietz and Chen 2004).

As for the chemokine receptors, after 24 h of treatment of immature DC with LPS (0.1 or 1 ng/ml), with or without hypoxia/DFX, we evaluated by FACS analysis the expression of CD80 and CD83.



**Figure 5.5. Effects of hypoxia on the CD83 co-stimulatory molecules surface expression**

Cells were cultured for 24 h in the indicated conditions and analyzed for CD83 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CD83. Results are mean  $\pm$ SD of four different experiments. \*,  $P < 0.03$ ; \*\*,  $P < 0.0007$ ; \*\*\*,  $P < 0.3$ ; \*\*\*\*,  $P < 0.004$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test)..

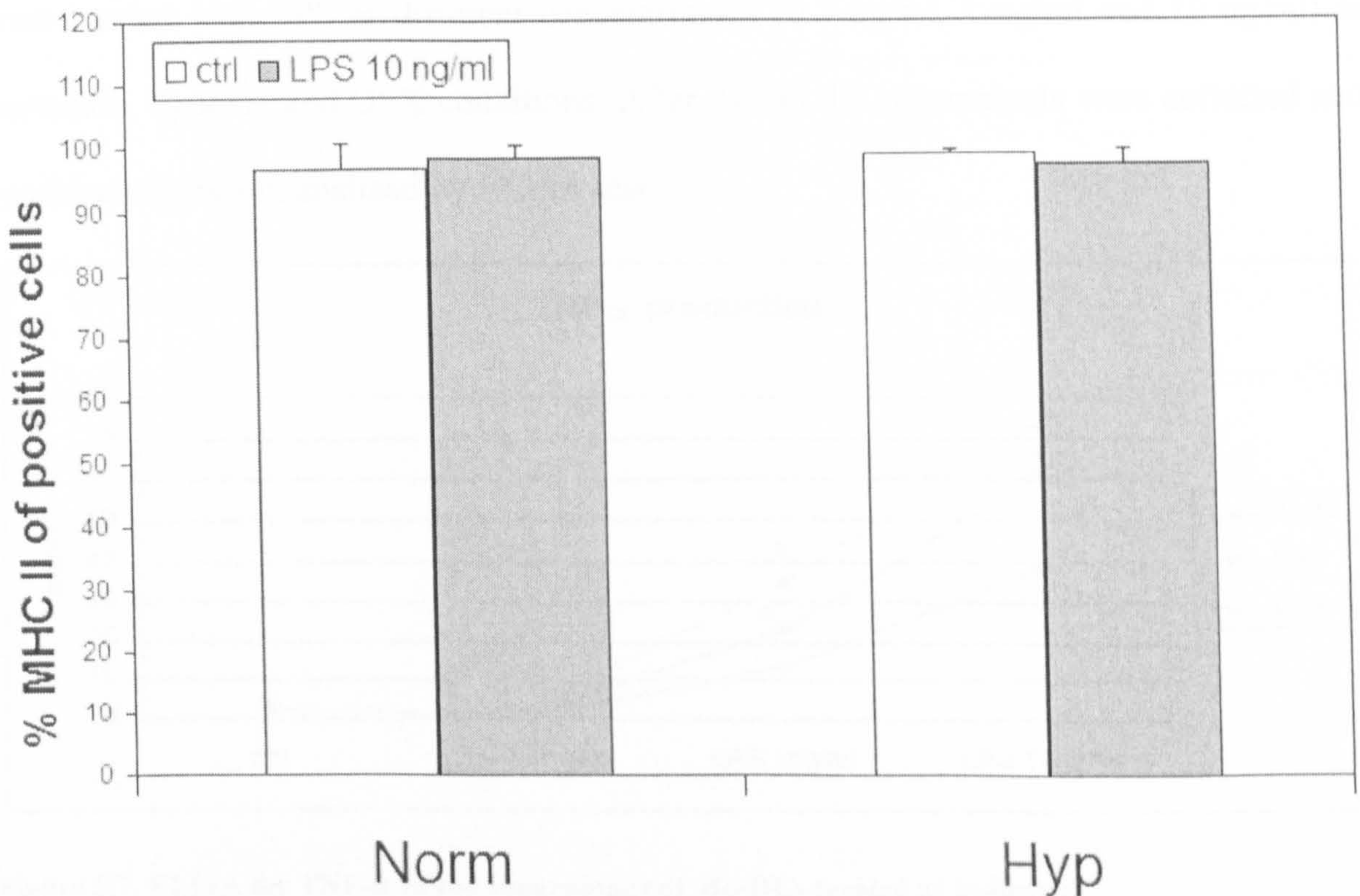
As shown in Figure 5.5 hypoxia did not affect the level of CD83 induced by LPS treatment.



Regarding the expression of CD80, though still preliminary, the results obtained from four experiments did not provide consistent and conclusive evidence. Additional experiments are required to fully clarify this aspect.

Maturation/inflammatory stimuli lead to a burst of class II synthesis and translocation of the MHC II-peptide complexes to the cell surface (Banchereau, Briere et al. 2000).

We treated immature DCs for 24h with LPS (10ng/ml), either in normoxia or hypoxia, and then analyzed by FACS analysis the expression of MHC II molecules.



**Figure 5.6. Effects of hypoxia on the MHC II surface expression**

Cells were cultured for 24 h as indicated and analyzed for MHC II surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human MHC II. Results are mean  $\pm$ SD of four different experiments. The differences between treated cells and cells cultured in normoxic conditions and untreated are not statistically significant.

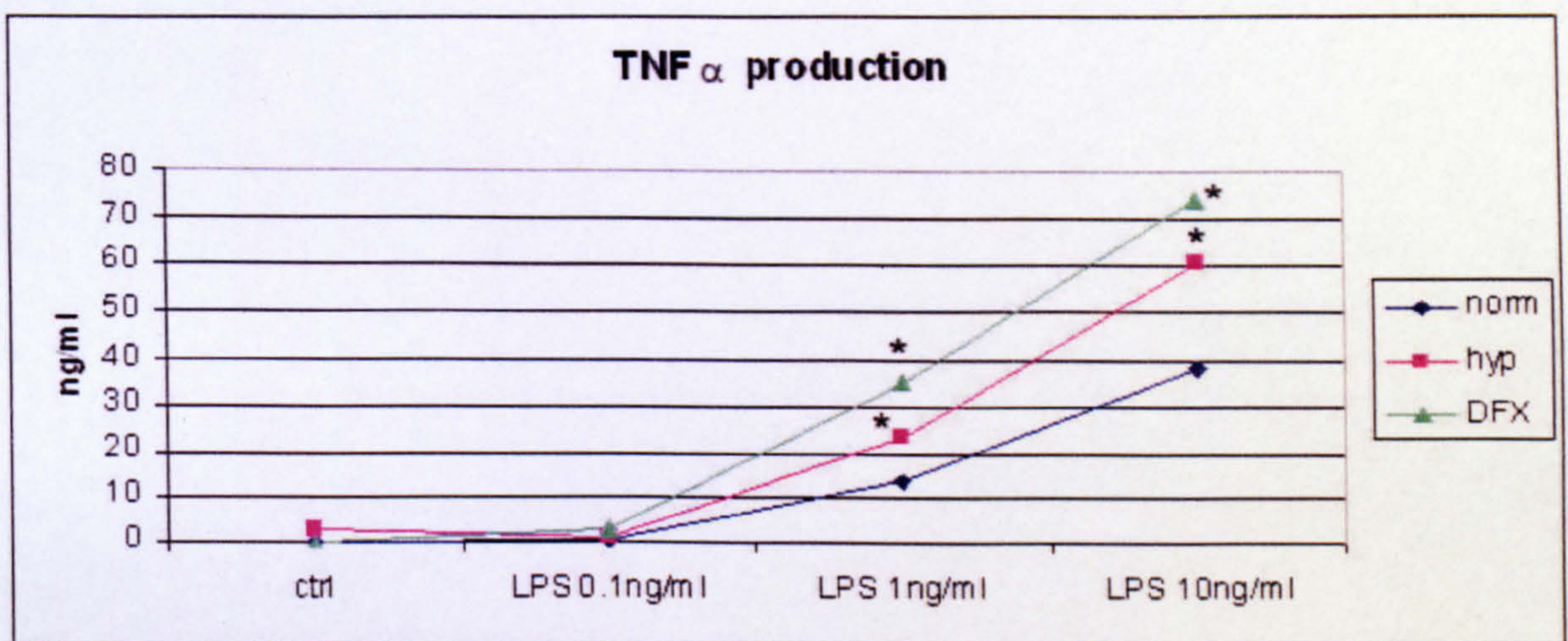
As showed in Figure 5.6 hypoxia did not affect the expression of MHC II in immature or mature DCs.



### 5.3.4. Analysis of cytokine expression by DC

Upon activation DCs mature into potent immunostimulatory cells that can drive T-cell clonal expansion and through production of M1 and/or M2 immunomodulatory cytokines, such as IL12 and IL10 respectively, promote the development of T helper 1 or Th 2 effectors.

To estimate the effects of hypoxia on cytokine production by DC, immature dendritic cells were treated with LPS at different concentrations (0.1 ng/ml, 1 ng/ml and 10 ng/ml) in normoxia, hypoxia and DFX conditions. After 24 hrs the supernatants were collected and cytokine expression analyzed by ELISA test.

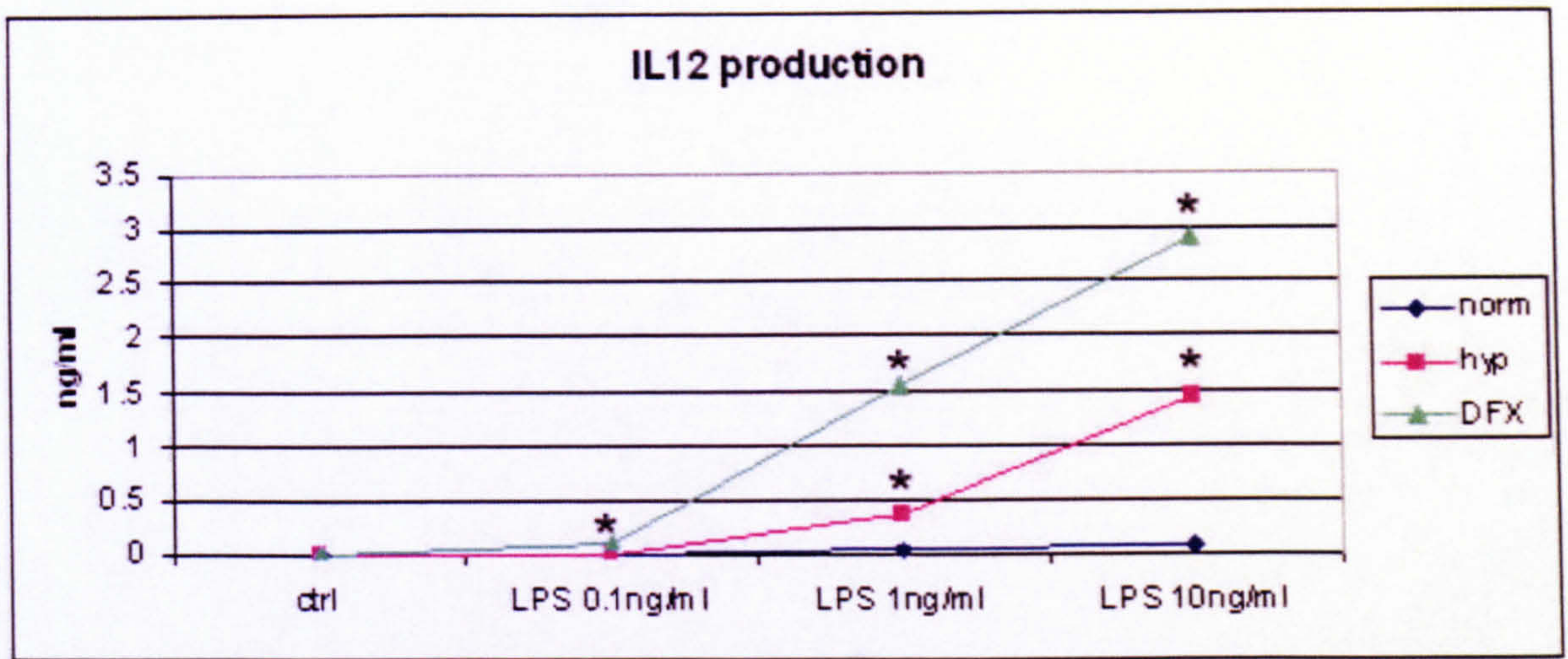


**Figure 5.7. ELISA for TNF- $\alpha$  in the supernatant of Mo-DCs treated as indicated**

Cells were incubated for 24h and supernatant were collected and assayed by ELISA to evaluate TNF- $\alpha$  protein level. \*,  $P < 0.05$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

As expected, LPS upregulated the production of TNF- $\alpha$  in a dose response manner. Strikingly, TNF- $\alpha$  production was further increased when LPS-treated DC were cultured in hypoxia or in the presence of DFX (Figure 5.7).





**Figure 5.8. ELISA for IL-12 in the supernatant of Mo-DCs treated as indicated**

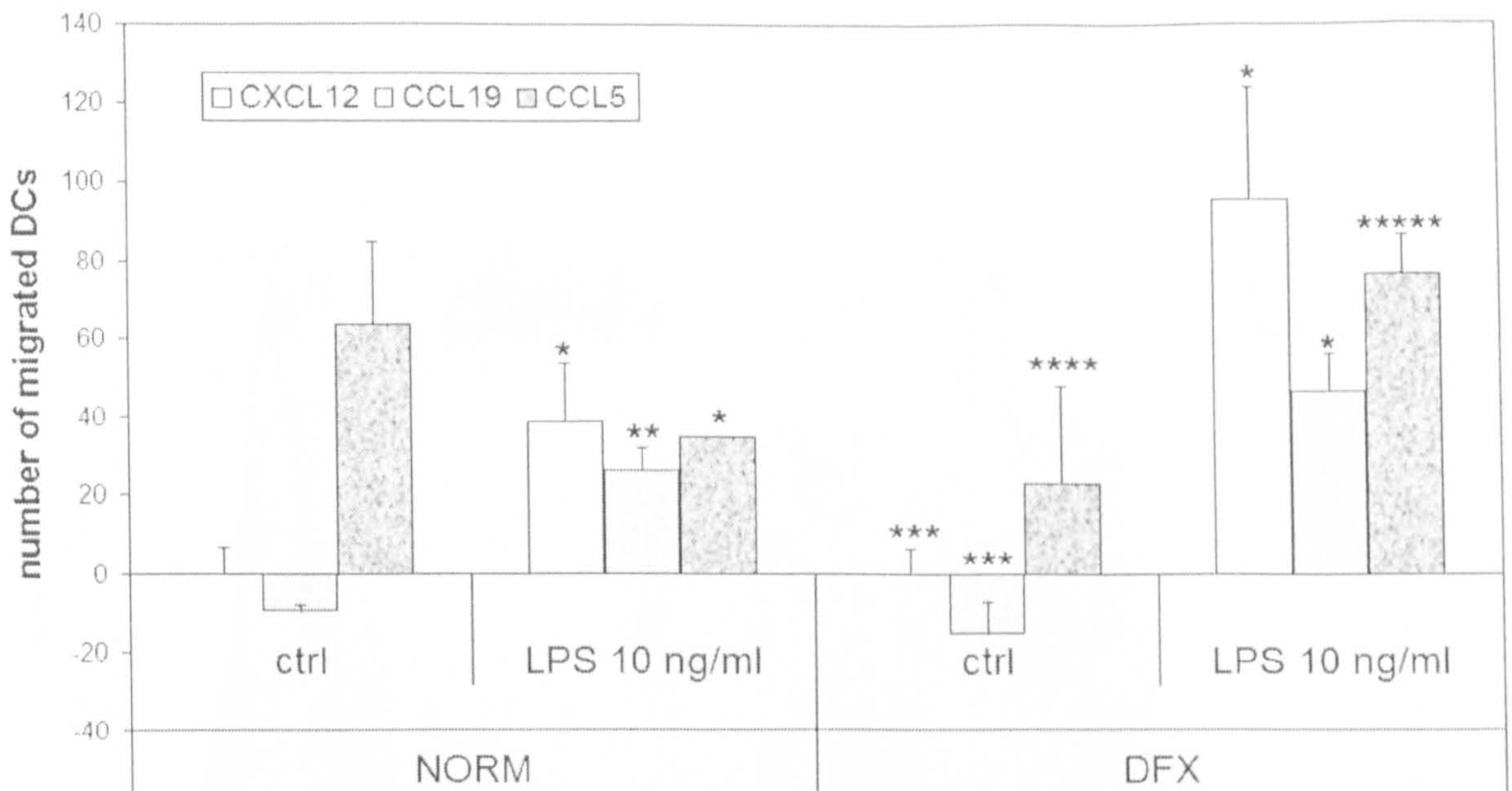
Cells were incubated for 24h and supernatant were collected and assayed by ELISA to evaluate IL-12 protein level. \*,  $P < 0.05$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

We also tested the production of IL-12 (Figure 5.8). As shown, the LPS-induced production of IL-12 by dendritic cells matured in normoxia conditions was very low, while both hypoxia and DFX strongly increased its production in a dose response manner.

### 5.3.5. Chemotaxis

In addition to CXCR4, mature DCs upregulate the chemokine receptors CCR7 thus acquiring responsiveness to CCL19 and CCL21. Consequently, mature DCs may leave the inflamed tissues and enter the lymph stream. As functional CCR7 expression by DC represents an essential event to promote an adaptive immune response, we analyzed the chemotactic responsiveness of LPS-activated DC (10 ng/ml) toward the CXCR4 (CXCL12), CCR5 (CCL5) and CCR7 (CCL19) specific ligands, either in normoxia, hypoxia or in the presence of DFX.





**Figure 5.9. Effects of DFX on the chemotactic response of immature and mature Mo-DCs**

Cells were cultured for 24 h in the presence of 400  $\mu$ M DFX. Migration of Mo-DCs was assayed by chemotaxis microchamber technique. Results are mean  $\pm$ SD of two different experiments. \*,  $P \leq 0.1$ ; \*\*,  $P < 0.02$ ; \*\*\*,  $P \leq 0.001$ ; \*\*\*\*,  $P < 0.0001$ ; \*\*\*\*\*,  $P < 0.00001$  versus cells cultured in normoxic conditions and untreated (Paired Student's *t* test).

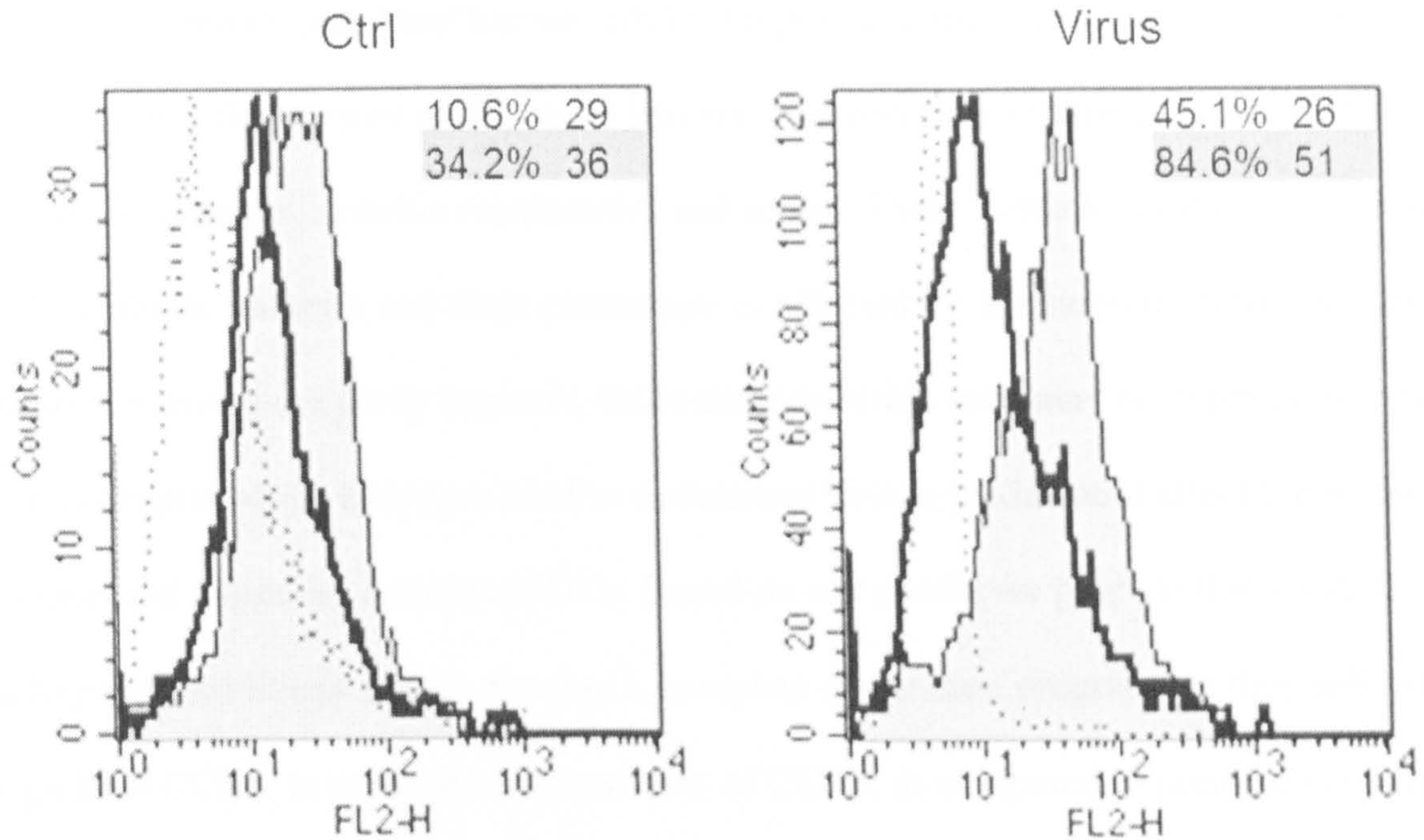
As shown, the number of cells migrated in response to CXCL12 was higher when DC maturation and migration occurred in the presence of DFX. In contrast, no significant changes were observed in response to CCL19, while DFX treatment partially prevented the LPS-mediated inhibition of the chemotactic responsiveness in response to CCL5.

### 5.3.6. Analysis of CXCR4 surface expression in plasmacytoid dendritic cells

It was recently demonstrated that pDCs are present in tumours (Vicari, Treilleux et al. 2004) and that their infiltration in ovarian tumours correlates with the level of CXCL12 expression to which they respond (Zou, Machelon et al. 2001). As tumours are characterized by hypoxic areas, we evaluated the influence of hypoxia on the expression of the CXCR4 chemokine receptor. pDC purified from peripheral blood of healthy donors were cultured for 24 h in normoxia or in the presence of DFX, with or without the inactivated influenza



virus, typical inducers of pDC maturation (Vermi, Riboldi et al. 2005). Next, CXCR4 expression was evaluated by FACS analysis.



**Figure 5.10. Effects of DFX on the CXCR4 chemokine receptor surface expression in pDCs**

Cells were cultured for 24 h in the indicated conditions and analyzed for CXCR4 surface expression. Surface expression was determined by flow cytometry using a mouse monoclonal antibody anti-human CXCR4. In the right corner percentage of positive cells for CXCR4 and mean of fluorescence are shown for normoxia condition (white box) and hypoxia conditions (grey box). (dotted line) Irrelevant antibody. (continuous line) Norm. (shaded region) Hyp. The results are representative of five experiments.

As shown in Figure 5.10 the expression of the CXCR4 chemokine receptor was upregulated by DFX (grey pick), either in the absence or in the presence of the inactivated influenza virus.

## 5.4. Discussion

Dendritic cells are the most efficient type of migrating APC, playing an essential role in the initiation of immune responses. Accumulating data have shown that DC migration is modulated in a complex manner and requires multiple factors, including growth factors, hormones and inflammatory cytokines such as TNF- $\alpha$  and IL-1 $\beta$ , and chemokines



(Cumberbatch, Dearman et al. 1997; Cumberbatch, Bhushan et al. 2003). Recently it was demonstrated that dendritic cell migration is also highly sensitive to microenvironmental changes (Turnbull and MacPherson 2001). Hypoxia, a local decrease in oxygen tension occurring in inflammatory and tumour lesions, is a common feature of many pathological processes: tumours, arthritis rheumatoid, and sepsis. There is much evidence that dendritic cells infiltrate tumours and their phenotype is affected by the tumour microenvironment. As solid tumours are partly hypoxic, infiltrating dendritic cells may be exposed to hypoxia. In this chapter of the thesis we tried to understand how hypoxia could affect the maturation process and migratory activity of DCs. Based on our results we propose that dendritic cells in hypoxic conditions do not develop a complete maturation program, as they still present high level CCR5, in contrast with low level of CCR7, as compared to normoxia conditions. On the other hand, hypoxia-derived DCs display a light upregulation of CD83, while other costimulatory molecules (e.g MHC II) are not affected.

Recent publications have demonstrated that hypoxia inhibits the migratory capacity of human monocyte-derived dendritic cells by regulating the balance between MMP and TIMP gene expression (Qu, Yang et al. 2005; Zhao, Darmanin et al. 2005). These authors have also demonstrated that expression of some chemokine receptors is affected by hypoxia. Nevertheless, the results in these two papers are somewhat controversial. In agreement with our results, Qu X. et al. showed a downregulation of CCR7. In contrast, Zhao W et al. showed the opposite result. We have found that the migratory capacity of mature dendritic cells is affected by hypoxia reducing their capacity to migrate through CCL19 but increasing their capacity to migrate in response to CXCL12 and CCL5.

We also investigated the influence of hypoxia on cytokine production and we observed that LPS-treated DC produce higher amount of TNF- $\alpha$  and IL-12 in hypoxia condition, thus amplifying their inflammatory response. As the expression of both genes is controlled by the transcriptional complex NF- $\kappa$ B, overexpression of these genes is likely the result of a



synergism between NF- $\kappa$ B and the HIF-1 transcriptional factor, a mechanism previously suggested (Jung, Isaacs et al. 2003).

It is demonstrated that plasmacytoid dendritic cells (pDCs) are present in tumours (Vicari, Treilleux et al. 2004) and their infiltration in ovarian tumours is promoted by the expression of CXCL12, to which they respond (Zou, Machelon et al. 2001). Interestingly, we observed that pDC exposed to hypoxia upregulated the expression of CXCR4.

Unfortunately, so far we could not confirm this observation in *in vivo* models, as we faced a lot of problems with the culture of murine dendritic cells. Recently, we discovered that the source of the problem was the new stock of FCS that we recently purchased. At the moment we are controlling the viability of these cells with different FCS (foetal calf serum).

From these preliminary results we can deduce that hypoxia prevents full maturation of DC and amplifies inflammatory functions (increased level of CCR5, IL-12 and TNF- $\alpha$ ) and suggest that hypoxia can promote DC functions associated with high local inflammation.

Our data are also in agreement with previous observation on the inhibitory action that the hypoxia-inducible factor VEGF elicits on DC maturation (Gabrilovich, Chen et al. 1996), while it promotes macrophage differentiation.

Studies on DC functions such as migratory activity in tumour tissue with low oxygen tension may contribute to a deeper understanding of the mechanisms by which tumour cells escape immune surveillance.



## Chapter 6. Summary and future plans

### 6.1. Summary

Migration and motility are essential features of the behavioural repertoire of a cell. During embryogenesis, cells move in sheets or as loosely attached populations to create complex tissues. In the adult, cell motility is crucial to maintain immunity, or to repair damaged tissues. The recruitment of leukocytes in normal and pathological conditions is mainly regulated by chemokines and their receptors. The expression of these molecules is regulated by different stimuli such as LPS, IFN $\gamma$  and IL-10 (Sica, Saccani et al. 1997; Sozzani, Ghezzi et al. 1998).

In this thesis we have demonstrated that hypoxia is a microenvironmental condition influencing chemokine receptor expression and leukocyte migration.

It was demonstrated that TAMs preferentially localize in hypoxic areas (Leek, Talks et al. 1996) and that hypoxia represents a stop signal for macrophage migration (Turner, Scotton et al. 1999; Grimshaw and Balkwill 2001).

In this thesis we have demonstrated that hypoxia induces the expression of CXCR4 in different cell types: monocytes, macrophages; endothelial cells with an amplification of angiogenesis and tumour cells.

The induction of CXCR4 in monocytes and macrophages may increase their localization on hypoxic areas. Chemokines play a fundamental role in the recruitment of monocytes and macrophages and, along with other groups, our laboratory has demonstrated that CCL2 is the most frequent CC chemokine found in tumours, whose level of expression correlates with increased infiltration of leukocytes. It was demonstrated that solid tumours (e.g. ovarian cancer) express the CXCL12 chemokine (Scotton, Wilson et al. 2001), thus supporting our hypothesis that the axis CXCR4/CXCL12 regulates the recruitment of leukocytes in tumours. Our results disclose a possible new mechanism by which the



chemokine system promotes recruitment and positioning of infiltrating macrophages into tumours.

Angiogenesis is an important process for tumour growth and survival and is regulated by various molecules (e.g. VEGF). We have demonstrated that hypoxia induces CXCR4 expression in endothelial cells, thus supposing a contribution to the angiogenic process in low oxygen condition. It was demonstrated that breast cancer cells are positive for CXCR4 and that they migrate to organs such as bone, lung and liver, where high concentrations of CXCL12 have been found (Muller, Homey et al. 2001). We have shown that the expression of CXCR4 is induced by hypoxia, thus increasing the ability of these cells to migrate in response to CXCL12. The hypoxia-induced CXCR4 expression may contribute to increase the aggressiveness and capacity of tumour cells to form metastasis.

The master regulator of the oxygen homeostasis is HIF-1. It regulates the expression of over 60 genes (Semenza 2003), involved in glycolysis to survival and apoptosis. Data obtained in this thesis show that HIF-1 activation mediates the Hyp-dependent upregulation of CXCR4 expression.

It was very interesting to find that hypoxia further contributes to increase CXCR4 gene expression by stabilization of CXCR4 transcript, suggesting that Hyp-regulated RNA binding factors may interact with and stabilize CXCR4 mRNA. Noteworthy, a recent work has shown that HIF-1 activation may play a role in the induction of the CXCR4 ligand, CXCL12 (Ceradini, Kulkarni et al. 2004), further suggesting that HIF-1 $\alpha$  is a central determinant of the leukocyte trafficking in hypoxic environments. This is also supported by recent work by Cramer et al. (Cramer, Yamanishi et al. 2003), which showed that ablation of the hypoxia responsive transcription factor HIF-1 $\alpha$  resulted in impaired macrophage motility and cytotoxicity. These observations together with our results contribute to a better understanding of how cells are recruited into inflammatory sites.



As DC infiltrate solid tumours, which are often characterized by areas of low oxygen tension, we investigated the effects resulting from their metabolic adaptation to hypoxia on DC maturation.

It is also known that in tumours, immature and mature dendritic cells localize in different areas: usually immature DCs migrate into the tumour while mature DC are confined to peritumoral areas (Banchereau, Briere et al. 2000).

Our preliminary results suggest that hypoxia prevents full maturation of DC ( $CCR7^{low}/CCR5^{high}$ ) and amplifies inflammatory functions, thus being likely involved in the amplification of local inflammation. We have observed that hypoxia induces the upregulation of CXCR4 in immature and mature dendritic cells and hypothesize that this could be one factor that influences their localization in tumours.

Recent publications by Zhao et al. and Qu X. et al. (Qu, Yang et al. 2005; Zhao, Darmanin et al. 2005) showed that hypoxia suppress the expression of MMP9 affecting dendritic cells migration, their most important function in response of infections or inflammation. In particular Qu X. et al. demonstrated that hypoxia downregulates the expression of CCR7 affecting dendritic cells maturation.

It is clear, from both our results and recent works by Zhao et al. and Qu X. et al. (Qu, Yang et al. 2005; Zhao, Darmanin et al. 2005) that hypoxia affects dendritic cells maturation and migration by affecting their function, the capability to migrate to the lymph nodes to active T cells.

This work suggests that Hyp-HIF-1 $\alpha$ -CXCR4 pathway may represent a target for new anticancer therapies. At the moment there are many studies using antibody (Ottaviano et al. 2005) or siRNA (Lapteva et al. 2005) against CXCR4 providing an inhibition of cancer metastasis and growth. Moreover the inhibition of HIF-1 $\alpha$  is considered a promising therapeutic approach against cancers (Semenza 2003) and in fact some antitumour agents



currently in clinical trials are specific HIF-1 inhibitors (e.g. farnesyl transferase inhibitors, PI3K inhibitors) (Giaccia, Siim et al. 2003).

Further work is required to validate the hypoxia-HIF-CXCR4 pathway as a therapeutic target in other diseases.

## 6.2. Future plans

There are a number of questions arising from this thesis that I hope will be answered by future works. The following sections outline ongoing experiments.

### 6.2.1. Inhibition of the expression of PTEN and HIF-1 $\alpha$ by siRNA

RNA interference is a mechanism of post transcriptional gene silencing using double strand RNA (dsRNA) (Elbashir, Harborth et al. 2001). RNA interference allows one to determine the function of selected genes by transfecting a target cell with small interference RNA (siRNA) able to promote degradation of specific mRNAs and to prevent their translation into protein. To accomplish this, we are using an expression vector (pSUPERIOR vector, by Oligoengine, Seattle) that expresses siRNA for the PTEN and HIF-1 $\alpha$  genes, under the control of tetracycline (Tet). As PTEN was found to be involved in the regulation of the PI3K-HIF-1 activity it would be interesting to understand how this tumour-suppressor gene influences the expression of CXCR4 in hypoxia.

pSUPERIOR vector is a tetracycline-regulated expression vector that utilizes regulatory elements from the E. Coli Tn10-encoded Tet resistance operon (Hillen and Berens 1994). Tetracycline (Tet) regulation in pSUPERIOR vector is based on the binding of Tet to the Tet repressor, which controls the expression of the gene of interest (Yao, Svensjo et al. 1998).

The siRNA sequence for PTEN (Czuderna, Fechtner et al. 2003) and HIF-1 $\alpha$  (Krishnamachary, Berg-Dixon et al. 2003) are known and we need to insert them in the



pSUPERIOR vector, before transfecting breast cancer (MDA-MB231) and ovarian cancer (TOV21G) cell lines with such constructs.

Here below are listed the steps to obtain a cell line expressing our siRNA:

- 1- to transfect cells with a plasmid expressing the Tet repressor protein. We'll use the pcDNA6/TR by Invitrogen (Milan);
- 2- after blasticidin selection, clones will be screened for the  $\beta$ -galactosidase production. Next, positive clones will be transiently transfected with the LacZ vector (where the gene lacZ is under the control of the tetracycline). Clones expressing high level of Tet repressor (corresponding of high level of  $\beta$ -gal) will be chosen;
- 3- to transfect the selected clone with the pSUPERIOR vector containing the siRNA for HIF-1 $\alpha$  and PTEN;
- 4- to select the clones with higher silencing activity for PTEN and HIF-1 $\alpha$  by Western Blot.

We have just obtained some Tet repressor positive clones for breast cancer cell line. Next we need to transfect cells with the plasmid containing the PTEN and HIF-1 $\alpha$  si RNA and to select for positive clones.

#### *6.2.1.i. Functional and molecular characterization of these cell lines*

After clonal selection, we will check the expression of PTEN, HIF-1 $\alpha$  and CXCR4 in normoxia and hypoxia conditions, and in the absence and presence of tetracycline, by using real time RT-PCR and Western Blot.

The second step will investigate the migratory capability of these cells after silencing of PTEN and HIF-1 $\alpha$ , controlling in particular their capability to migrate towards CXCL12.

This will be done using the transwell system.



### 6.2.1.ii. Metastasis formation

To evaluate the role of HIF-1 $\alpha$  and PTEN in the formation of metastasis clones expressing inducible siRNA against PTEN or HIF-1 $\alpha$  will be injected in nude mice and, following a proper period, metastasis number will be determine. This part will be done in collaboration with Prof. Balkwill's laboratory (Queen's Mary Hospital, London).

### 6.2.2. Hypoxia influences macrophage polarization

In solid tumours TAMs represent a prominent component of the infiltrating mononuclear leukocyte population, which display an ambivalent relationship with tumours. It's known that macrophages localize preferentially to necrotic areas characterized by low oxygen tension. Recently in our lab we defined the phenotype of TAM by using the microarray technology (Biswas, Gangi et al. 2005). The results of this study showed that TAMs have a distinct and unique M2 phenotype. Based on this it could be interesting to study whether hypoxia could influence the M1-M2 polarization of monocytes and macrophages.

To perform this, by using Real Time RT-PCR we started the study of the chemokine/chemokine receptors profile expressed by monocytes in normoxia versus hypoxia conditions, following activation with LPS or IL-4.

Preliminary results have shown that hypoxia upregulates selected M1 genes (e. g. IL-12, CXCL10) in the presence of M1-polarizing signals, such as LPS. Compared with LPS, the M2-polarizing cytokine IL-4 didn't have any appreciable effect on the expression of these genes.

Additional experiments are ongoing in the laboratory to further investigate the effects of hypoxia on the polarization of innate immune functions.



### 6.2.3. Regulation of maturation of DCs by hypoxia.

A variety of experiments could be performed to further assess the influence of hypoxia on dendritic cells maturation after the confirmation of the preliminary data exposed in this thesis. As example we plan to evaluate the activation of T cells in a mixed leukocytes reaction (MLR).

#### 6.2.3.i. Knock down HIF-1 $\alpha$ expression using siRNA.

It will be interesting to study whether HIF-1 $\alpha$  silencing in dendritic cells results in the recovery of their capability to undergo to full maturation. To perform this study we would need to optimize methods to transfect dendritic cells with siRNA.

#### 6.2.3.ii. To characterize the phenotype of murine DCs under hypoxia conditions.

Migration is essential function of dendritic cells as, following maturation, they move to the T cell zones of secondary lymphoid organs where they present Ag and costimulatory signals to recirculating lymphocytes. Our preliminary results suggest that hypoxia affects DC maturation in response to pro-inflammatory signals. In particular, as compared to normoxia-derived DC, DCs matured in hypoxia display higher expression of costimulatory molecules (CD83), lower CCR7, higher CCR5 and increased expression of pro-inflammatory cytokines (IL-12, TNF- $\alpha$ ): a phenotype likely involved in amplification of local inflammation. Our aim is to provide *in vivo* evidence supporting this hypothesis. We performed *in vitro* experiments to investigate whether the functional responses of murine DC to hypoxia were similar to the human counterpart. By taking this approach we did not obtain conclusive results, as we faced several technical problems, relative to culture and differentiation conditions for murine DC.

When the proper experimental conditions for murine dendritic cells will be optimized we will repeat the *in vitro* experimentation, before proceeding with the *in vivo* studies.



In particular, we will perform *in vivo* experiments with murine dendritic cells, treated in normoxia or hypoxia conditions, to evaluate their capacity to migrate to lymph nodes. We will label normoxic or hypoxic DCs with the vital dye 5-(and-6)-carboxyfluorescein diacetate succinimidyl estermixed isomer [5-(6)-CFDA, SE (CFSE)] and inject them subcutaneously into the hind leg footpad. At different time points popliteal lymph nodes will be recovered, disaggregated mechanically and treated with collagenase A (1mg/ml). The enzymatically treated cell suspension will be evaluated by FACS analysis (Del Prete, Vermi et al. 2004).



## Publications

Parts of this thesis have been published; a copy is enclosed at the back.

### Published work

Schioppa T., Uranchimeg B., Saccani A., Biswas SK, Doni A., Rapisarda A., Bernasconi S., Saccani S., Nebuloni M., Vago L., Mantovani A., Melillo G., Sica A. 2003. Regulation of the chemokine receptor CXCR4 by hypoxia. *J. Exp. Med.* 198: 1391-402.



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