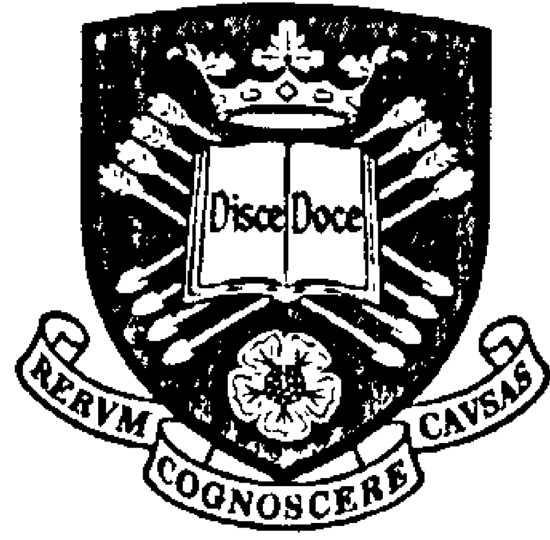

The University Of Sheffield.

The Chemokine Receptors XCR1, CXCR1 AND CXCR2 Regulate Oral Epithelial Cell Behaviour

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ABSTRACT

Chemokines are chemoattractant cytokines which act on specific receptors and play an important role in tumour biology. The aim of this project was to determine whether the chemokine receptors XCR1, CXCR1 and CXCR2 and their respective ligands lymphotactin, IL-8 (CXCR1&2) and GRO-α regulate the behaviour of normal and malignant oral epithelial cells.

XCR1, CXCR1 and CXCR2 mRNA and surface protein expression was detected in normal and oral cancer cell lines. Lymphotactin, IL-8 and GRO-α facilitated intracellular activation of ERK1/2 signaling pathway and stimulated migration, invasion and proliferation of all cells. These effects were mediated through XCR1 for lymphotactin, CXCR1 and CXCR2 for IL-8 and CXCR2 for GRO-α. The cancer cells showed a greater response than normal cells and a direct relationship between receptor expression and migration, invasion and proliferation was observed.

XCR1 but not lymphotactin was expressed by epithelial cells in normal oral mucosa *in vivo* and both were expressed and up-regulated in inflammation and cancer. Constitutive expression of both XCR1 and lymphotactin was found in regional lymph nodes and on metastatic tumours.

Lymphotactin mRNA and constitutive intracellular protein was detected in normal and cancerous oral cells. Exposure of normal cells to lymphotactin resulted in increased adhesion to fibronectin but not collagen and stimulated MMP-2 and -9 release whereas exposure of cancer cells resulted in increased adhesion to both collagen and fibronectin and stimulated MMP-2, 9 and MMP-7 release.

These findings show for the first time that XCR1 and its ligand lymphotactin are expressed by epithelial cells in a range of oral conditions and strongly suggest that they play an important role in regulating the behaviour of normal and malignant epithelial cells. Similarly CXCR1 and CXCR2 are up-regulated on malignant oral cells *in vitro* and may be important in the biology of oral cancer.

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ABBREVIATIONS

Abbreviation	Stands for:
ABC	Avidin biotin complex
BLAST	Basic local alignment search tool
Bp	Base pairs
BSA	Bovine serum albumin
DPX	Distrene, Plasticiser, Xylene (mounting medium)
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ERK1/2	Extracellular signal-related kinases 1 and 2
FBS	Foetal bovine serum
FITC	Fluorescein isothiocyanate
FLS	Fibroblast like synoviocytes
GRO-α	Growth related oncogene-α
HGF	Human gingival fibroblasts
HNSCC	Head and neck squamous cell carcinoma
IEL	Intraepithelial lymphocytes
IL-1β	Interleukin-1β
IL8	Interleukin 8
KGM	Keratinocyte growth medium
LPS	Lipopolysaccharide
MEK	MAPK/ERK kinase
MMPs	Matrix metalloproteinases
MNCs	Mononuclear cells
mRNA	Messenger RNA
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethophenyl)-2- (4-sulfophenyl)- 2H- tetrazolium
NK cells	Natural killer cells
NOK	Normal oral keratinocytes
NSK	Normal skin keratinocytes

(continued)

Abbreviation	Stands for:
OCCL	Oral cancer cell lines
OSCC	Oral squamous cell carcinoma
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristat 13-acetate
RA-	Rheumatoid arthritis
RT-	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
SCID-	Severe combined immunodeficiency
SCM-1	Single cell motif-1
SD	Standard deviation
TAE	Tris base, acetic acid EDTA
TECK	Thymus expressed chemokine
TIMPs	Tissue inhibitors of metalloproteinases
TNF-α	Tumour necrosis factor-α

CHAPTER 1

Introduction

and

Literature Review

1.1 INTRODUCTION

Oral cancer is a significant cause of morbidity and mortality and has a worldwide distribution. The term *'oral'* includes the lips and all other intra-oral sites corresponding to the ICD-10 codes (International classification of disease-version 10) C00-C14 (W.H.O., 1996). It is the sixth most common mortality-related cancer in the world and accounts for approximately 4% of all cancers and 2% of all cancer deaths world-wide (Kademani 2007; Parkin, Pisani, and Ferlay 1993; Parkin, Pisani, and Ferlay 1999).

Since majority of oral cancers are squamous cell carcinomas (OSCC), they will be referred to as *'oral cancer'* in this thesis. Oral cancer constitutes about 2% of all malignant tumours in UK and USA but its incidence is variable and in India and Sri Lanka it rises to 40%. Approximately 30,000 new cases are registered during one calendar year in USA and 4,400 in UK (Cancer Research UK, 2001) (Conway *et al.*, 2006; Davies and Welch 2006; Johnson and Warnakulasuriya 1991; Johnson and Warnakulasuriya 1993; Parkin, Pisani, and Ferlay 1999).

The aetiology of oral cancer is not entirely clear with a multitude of factors being held responsible, including smoking (Llewellyn *et al.*, 2004; Llewellyn, Johnson, and Warnakulasuriya 2001; Warnakulasuriya, Sutherland, and Scully 2005), alcohol (Altieri *et al.*, 2004; Llewellyn *et al.*, 2004), viruses (Cox, Scully, and Maitland 1991; Scully, Prime, and Maitland 1985), malnutrition, betel (pan) chewing (Jacob *et al.*, 2004; Warnakulasuriya, Sutherland, and Scully 2005) and genetic factors (Mackenzie *et al.*, 2000; Scully and Bedi 2000).

Despite the increased knowledge of mechanisms involved in the development and behaviour of oral carcinoma, understanding of the underlying cellular processes is incomplete. The most frequent sites affected include the lower lip, tongue, floor of the mouth and lingual aspect of the alveolar margin. These areas constitute only about 20% of the oral cavity but more than 70% of oral cancers are concentrated there.

Metastasis of oral cancer is primarily to the regional lymph nodes through lymphatics. As most of the cancers arise in the lower part of the mouth, the sub-mandibular and

jugulo-digastric nodes are most commonly involved. Oral cancers spread preferentially down the jugular lymphatic chain and usually do not involve the supra-clavicular lymph nodes until a late stage.

The precise mechanism utilized by oral cancer cells to metastasize to the submental, submandibular, and deep cervical lymph nodes is not known. Recent reports suggest that chemokines and chemokine receptors may facilitate cancer cell migration, proliferation and invasion. This may contribute to their ability to escape surveillance and may partially explain preferential patterns of metastasis to sites such as lymph nodes, skin and lung.

1.2 LITERATURE REVIEW

1.2.1 CHEMOKINES

Chemokines are small (8-14 kDa), structurally related molecules that regulate trafficking and localization of different types of leukocytes through interaction with specific G-protein-coupled receptors. They are structurally and functionally related to growth factors (Baggiolini, Dewald, and Moser 1997). Many novel chemokines and chemokine receptors have been identified during the last few years as a result of large-scale sequencing projects bringing the number up to 46 chemokine ligands and 19 functionally signaling chemokine receptors (Zlotnik, Yoshie, and Nomiyama 2006).

When initially identified, these proteins were characterized by their ability to induce chemotaxis of leukocytes. However, it is now appreciated they have a critical role that extends beyond the regulation of lymphocyte development and migration. Expression of chemokines and their receptors has been reported in a wide range of cells (haematopoietic as well as non-haematopoietic) and tissues where they participate in many physiological and pathological processes.

1.2.1.1 Classification

Chemokines have been subdivided into four families on the basis of the relative position of their conserved two N-terminal cysteine residues (Baggiolini, Dewald, and Moser 1997). These include CXC (α), CC (β), C (γ), and CXXXC or CX3C (δ) (Table 1.1). In the α family, one amino acid separates the first two cysteine residues (cysteine-X amino acid-cysteine, or CXC). In the β family the first two cysteine residues are adjacent to each other (cysteine-cysteine, or CC). Two chemokines that do not fit into this classification are *'lymphotactin'* and *'fractalkine'*. Lymphotactin contains only two cysteines (XCL1) (Kelner *et al.*, 1994; Kennedy *et al.*, 1995) whereas fractalkine has the first two cysteine residues separated by three amino acids (CX3CL1) (Bazan *et al.*, 1997).

The genes encoding for α- chemokines cluster on chromosome 4 and are further subdivided into two groups. Those that contain the sequence 'glutamic acid-leucine-

arginine' (ELR) near the N-terminal (preceding the CXC sequence) are chemotactic for neutrophils in addition to being potent angiogenic factors e.g. Interleukin-8 (IL-8). Those not containing the ELR sequence act on lymphocytes and are angiostatic e.g. Interferon Inducible Protein–10 (IP-10) and Monokine induced by Interferon–γ (MIG) attract activated T lymphocytes and stromal cell-derived factor-1 (SDF-1) acts on resting lymphocytes (Luster 1998; Keane *et al.*, 1998).

The genes encoding for β-chemokines cluster on chromosome 14 and in general, do not act on neutrophils but attract monocytes, eosinophils, basophils and lymphocytes (Luster 1998). They can be subdivided into two families; the monocyte-chemoattractant-protein-eotaxin family containing the five monocyte chemoattractant proteins and eotaxin, and all other β-chemokines (Luster and Rothenberg 1997). Important members include 'monocyte chemoattractant protein–1' (MCP-1), 'macrophage inflammatory protein-1α' (MIP-1α), 'regulated upon activation normal T-cell expressed and secreted' (RANTES) and eotaxin.

Lymphotactin (Lptn/XCL1) and XCL2 are the only members of the γ–(C) chemokine family. Lymphotactin was considered out of the ordinary upon discovery as it possessed a number of conserved amino acid residues present in the CXC and CC chemokines, including two cysteines corresponding to cysteines 2 and 4, but was missing cysteines 1 and 3 (Kelner *et al.*, 1994; Kennedy *et al.*, 1995) (Table 1.1).

Systematic Name	Synonym	Receptor(s)
CXC Chemokines		
CXCL1	Groα	CXCR2
CXCL2	Groβ	CXCR2
CXCL3	Groγ	CXCR2
CXCL4	PF4	CXCR3B
CXCL5	ENA-78	CXCR2
CXCL6	GCP-2	CXCR1, CXCR2
CXCL7	NAP-2	Unknown
CXCL8	IL-8	CXCR1, CXCR2
CXCL9	MIG	CXCR3, CXCR3B
CXCL10	IP-10	CXCR3, CXCR3B
CXCL11	I-TAC	CXCR3, CXCR3B
CXCL12	SDF-1α/β	CXCR4

Systematic Name	Synonym	Receptor(s)
CXC Chemokines (continued)		
CXCL13	BLC, BCA-1	CXCR5
CXCL14	BRAK, Bolekine	Unknown
CXCL16		CXCR6
CXCL17	DMC	Unknown
CC Chemokines		
CCL1	I-309	CCR8
CCL2	MCP-1	CCR2
CCL3	MIP-1α, LD78α	CCR1, CCR5
CCL3L1	LD-78β	CCR1, CCR5
CCL3L3	LD-78β	CCR1, CCR5
CCL4	MIP-1β	CCR5
CCL4L1	AT744.2	CCR5
CCL4L2		CCR5
CCL5	RANTES	CCR1, CCR3, CCR5
CCL7	MCP-3	CCR1, CCR2, CCR3
CCL8	MCP-2	CCR1, CCR2, CCR3, CCR5
CCL11	Eotaxin	CCR3
CCL13	MCP-4	CCR1, CCR2, CCR3
CCL14	HCC-1	CCR1
CCL15	HCC-2	CCR1, CCR3
CCL16	HCC-4 LEC	CCR1, CCR2, CCR5,
CCL17	TARC	CCR4
CCL18	PARC	Unknown
CCL19	MIP-3β, ELC	CCR7
CCL20	MIP-3α, LARC	CCR6
CCL21	SLC	CCR7
CCL22	MDC	CCR4
CCL23	MPIF-1	CCR1
CCL24	Eotaxin 2	CCR3
CCL25	TECK	CCR9
CCL26	Eotaxin 3	CCR3
CCL27	CTACK, ILC	CCR10
CCL28	MEC	CCR10, CCR3

Systematic Name	Synonym	Receptor(s)
<u>Other Chemokines</u>		
XCL1	Lymphotactin, SCM-1α	XCR1
XCL2	SCM-1β	XCR1
CX3CL1	Fractalkine	CX3CR1

Table 1.1. Human chemokines and their receptors.

1.2.2 CHEMOKINE RECEPTORS

Chemokines induce cell migration and activation by binding to specific G-protein-coupled (seven transmembrane) cell-surface receptors on target cells (Figure 1.1). Migration of cells takes place down a chemokine gradient and involves changes in chemokine concentrations which are detected by the receptors. As a result, directional cell movement takes place through re-arrangements of cell cytoskeleton as well as interactions with the extra-cellular matrix (Sanchez-Madrid and del Pozo 1999).

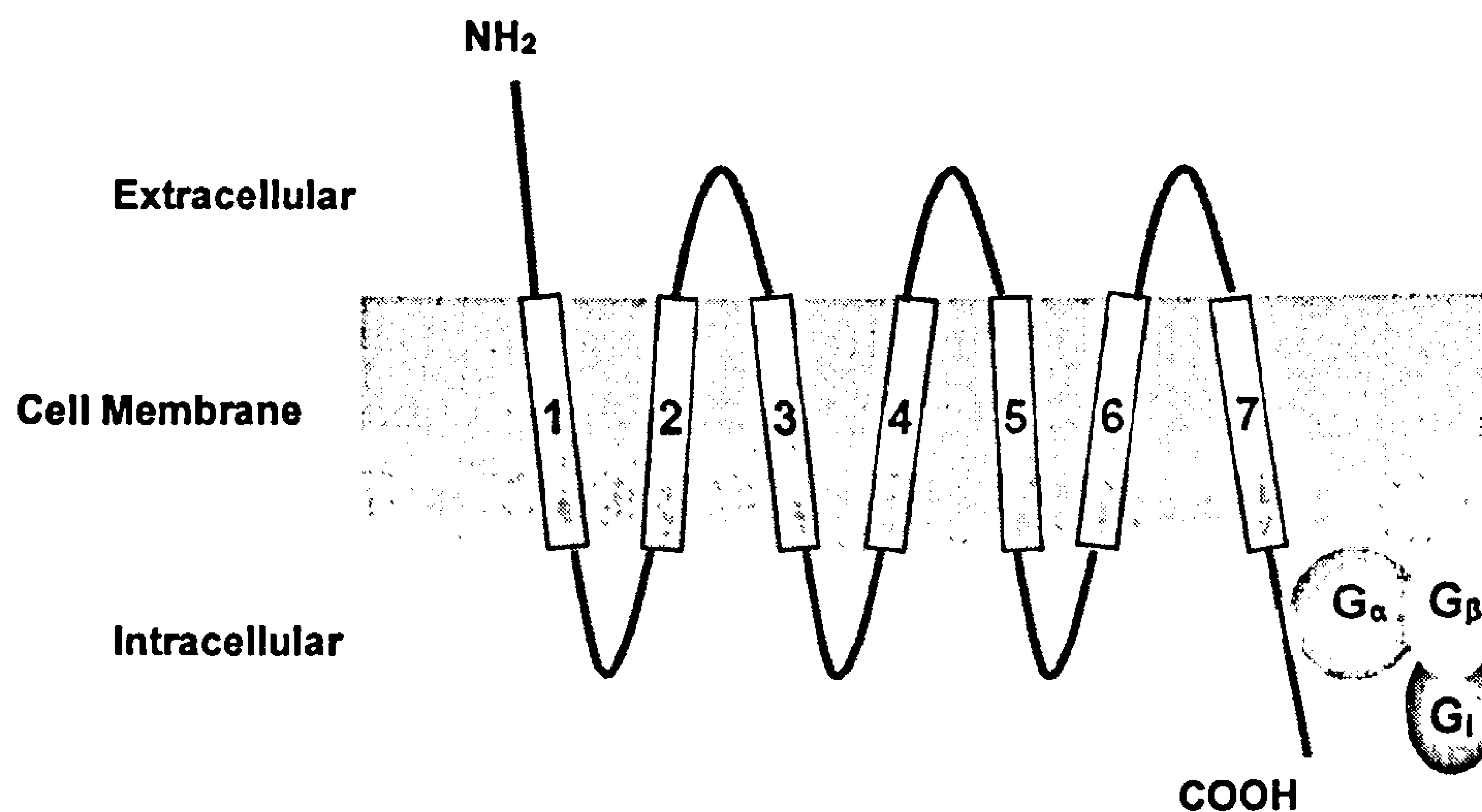

Figure 1.1. Typical structure of a chemokine receptor.

The CXC chemokine receptors selectively bind CXC chemokines and include chemokine receptors CXCR1-7. The CC receptor family (CCR) currently consists of ten receptors, CCR1-10. Receptors for fractalkine (CX3CR1) and lymphotactin (XCR1) have also been identified recently. Chemokines also interact with two types

of non-signaling molecules. The first one is known as the *'Duffy antigen receptor for chemokines'* (DARC) and has been known since 1950s as the determinant of Duffy blood groups. It is expressed on erythrocytes and endothelial cells and has been demonstrated to bind promiscuously to both CXC and CC chemokines (Neote *et al.*, 1993). The second type is a group of *'Heparan sulfate proteoglycans'*. Chemokines are basic proteins and bind avidly to negatively charged heparan and heparan sulfate. Together they serve to establish a local concentration gradient (Luster 1998).

1.2.2.1 Signaling

Chemokine receptor binding initiates a cascade of intracellular events that culminate in the expression of biological effects. Chemokine receptors are functionally linked to phospholipases through G-proteins (Bokoch 1995). Ligation of the receptor by its high affinity ligand induces a conformational change leading to the dissociation of the receptor associated G proteins into α and βγ subunits. The *'Bordtella Pertussis'* toxin causes inhibition of a number of these signaling events, indicating the relationship between the chemokine receptors and the G proteins of the G_i class. The G-protein subunits can then activate various effector enzymes, including phospholipases, which lead to inositol phosphate production, increase in intracellular Ca^{+2} and activation of protein kinases. GTP binding proteins of the *Ras* and *Rho* families are also activated by such signaling. This signal transduction cascade not only leads to the activation of chemotaxis by modulating actin-dependent cellular processes and up-regulating adhesion proteins, but also of a wide range of intracellular functions.

1.2.2.2 Expression

Chemokine receptors are expressed on different types of leukocytes. Some receptors are restricted to certain cells (e.g. CXCR1 and CXCR2 are usually restricted to neutrophils), whereas others are more widely expressed (e.g. CCR2 is expressed on monocytes, T cells, natural killer cells, dendritic cells and basophils). Chemokine receptors are constitutively expressed on some cells, whereas they are inducible on others. CCR1 and CCR2 are constitutively expressed on monocytes but are expressed on lymphocytes only after stimulation by interleukin-2 (Loetscher *et al.*, 1996). Some of the constitutive chemokine receptors can be down-regulated e.g. CCR2 is down-

regulated by lipopolysaccharide, making the cells unresponsive to MCP-1 (Monocyte chemoattractant protein, which activates only this receptor), but it remains responsive to MIP-1α (Macrophage inflammatory protein 1-α which also activates CCR1 and CCR5) (Sica *et al.*, 1997).

1.2.3 Functions of Chemokines

Along with the accelerated rate of discovery of chemokines has come the realization that these molecules not only control haematopoietic cell migration, but are also involved in a number of other physiological and pathological processes (Table 1.2).

Chemokine	Lymphocyte effects
C chemokines	
▪ Lymphotactin	Specifically chemotactic for T and B lymphocytes
C-C chemokines	
▪ RANTES	Activation of T lymphocytes, activation of NK cells; chemotaxis of T lymphocytes and NK cells
▪ MIP-1α	Chemotaxis of naïve T cells, B cells and NK cells; Inhibition of T-cell proliferation and IL-2 production; activation of NK cells
▪ MIP-1β	Chemotaxis of naïve T cells, B cells and NK cells; activation of NK cells
▪ MCP-1	Chemotaxis of NK cells; activation of NK cells
▪ MCP-2	Chemotaxis of NK cells; activation of NK cells
▪ MCP-3	Chemotaxis of NK cells; activation of NK cells
C-X-C chemokines	
▪ IP-10	Chemotaxis of T cells and NK cells
▪ IP-9	B-cell growth and regulation; chemotaxis of T cells and NK cells
▪ Mig	Specifically chemotactic for activated T cells
▪ SDF-1β	B-cell growth factor

Table 1.2. Actions of chemokines on haematopoietic cells.

Chemokines and their receptors have a wide scope of functions extending from development of lymphocytes, maturation of dendritic cells, development of Th1/Th2 responses, lymphoid trafficking, cell recruitment, inflammation, organogenesis and angiogenesis to tumour growth and metastases. Chemokines can facilitate as well as inhibit tumour growth and angiogenesis e.g. PF-4/CXCL-4 and IP-10 inhibit neo-vascularization, tumour growth and metastases (Yamaguchi *et al.*, 2005; Sato *et al.*, 2007), whereas IL-8 promotes angiogenesis and metastases (Yuan *et al.*, 2005; Varney, Johansson, and Singh 2006) (Figure 1.2).

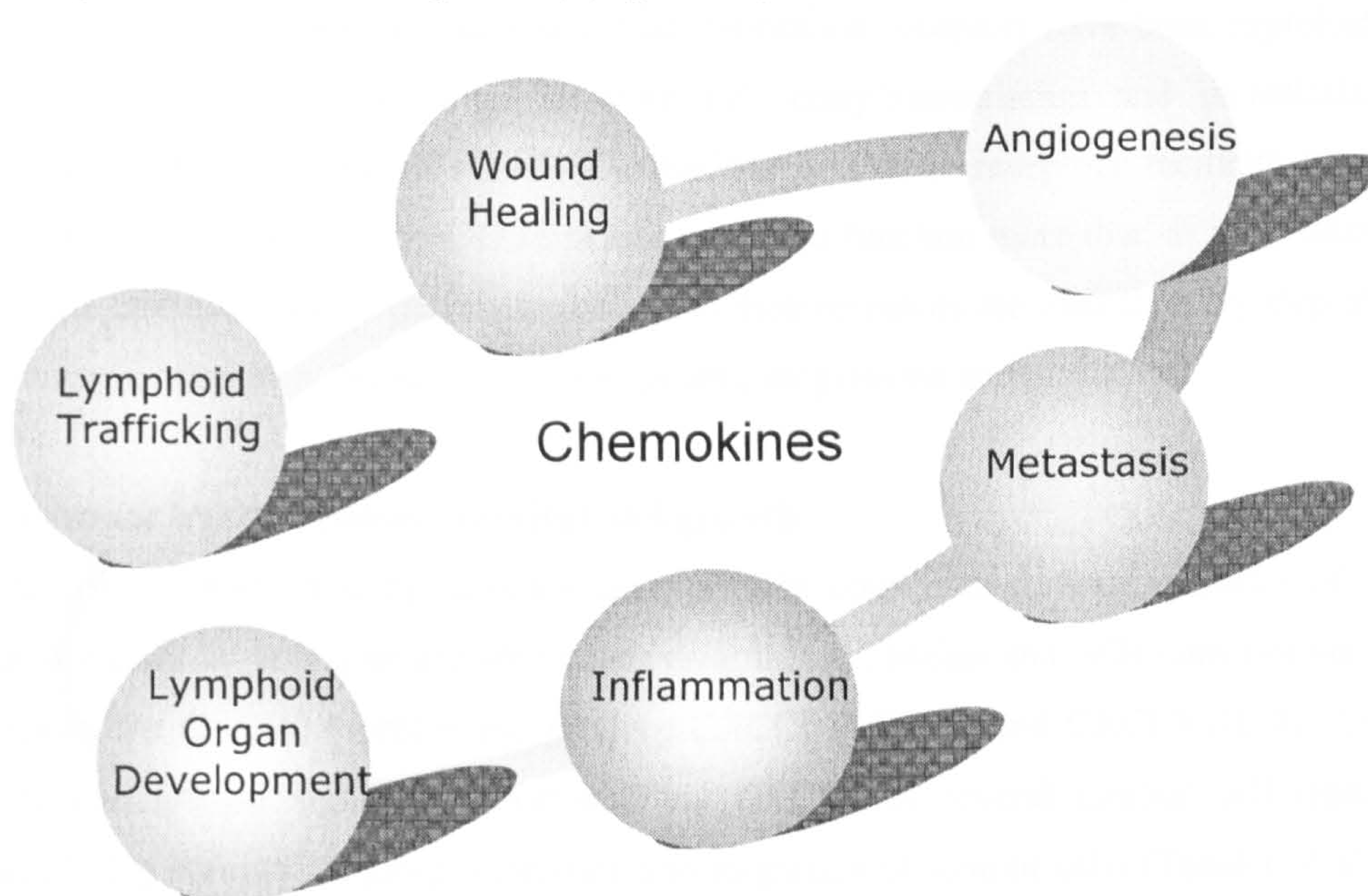

Figure 1.2. Major biological functions of chemokines. Chemokines play an important role in a range of physiological process including lymphoid trafficiking and lymphoid organ development, inflammation and wound healing. They have also been shown to facilitate epithelial tumour growth, angiogenesis and metastasis.

During inflammation, there is a significant increase in the selective recruitment of leukocytes because of increased secretion of chemokines at the site of injury. Chemokines have been associated with inflammation in a number of organs such as skin (Fierro *et al.*, 2006; Tanaka *et al.*, 2006; Homey *et al.*, 2006), joints (Blaschke *et al.*, 2003; Dai *et al.*, 2007; Aggarwal, Agarwal, and Misra 2007), lungs (Garingo *et al.*, 2007; Meyts *et al.*, 2006), kidneys (Krensky and Ahn 2007; Durkan *et al.*, 2007), gastrointestinal tract (Schmitz *et al.*, 2007; Yoshino *et al.*, 2005; Bodger *et al.*, 2001)

and periodontium (Silva *et al.*, 2005). It is known now that most cells can secrete chemokines when provided the apt stimulus. Pro-inflammatory cytokines (IL-1, TNF, bacterial products) act as main stimuli for chemokine release.

1.2.4 CHEMOKINES/CHEMOKINE RECEPTORS IN TUMOUR BIOLOGY

Chemokines are a central part of the host defence system and facilitate directional movement of leukocytes in addition to performing a wide range of other physiological functions. It is perhaps not surprising that chemokine receptors have been exploited by pathogens and tumours in assisting cell entry/transmission and metastasis. According to some recent reports chemokines and their receptors facilitate site-specific metastasis. However, chemokines appear to function more than as attractants and the interactions between chemokines and their receptors are vital in every step of tumour development, including tumour growth, progression and metastasis.

i) Tumour transformation, survival and growth

Chemokine production by tumours may directly contribute to transformation of tumour cells by acting as growth and survival factors. Melanoma cells constitutively express the CXCR2 receptor and produce CXCL1 (GRO-α) and CXCL8 (IL-8). An autocrine growth signal is transmitted via CXCR2 in several tumour cell types facilitating survival, angio-proliferation and migration of tumour cells (Tanaka *et al.*, 2005). Blocking either the ligands or the receptor, results in diminished tumour growth due to decreased autocrine stimulation by these chemokines (Dhawan and Richmond 2002; Luan *et al.*, 1997).

Chemokines can also trigger cell growth (Figure 1.9). Exposure to GRO-α and IL-8 facilitates growth of pancreatic cell lines (Takamori *et al.*, 2000). These cells also proliferate in response to the chemokine CCL20 (MIP-3α) since they express the CCR6 receptor (Kleeff *et al.*, 1999). Interactions between CXCL12 (SDF-1) and its receptor CXCR4 also stimulate proliferation of prostate cancer cells (Sun *et al.*, 2003; Taichman *et al.*, 2002), glioblastoma cells (Zhou *et al.*, 2002) and malignant plasma cells of multiple myeloma (Alsayed *et al.*, 2007).

In addition, chemokines provide survival signals to tumour cells in some instances. For example, SDF-1α can enhance the survival of CXCR4 expressing glioma cells by preventing apoptosis in sub-optimal conditions, such as low serum concentration (Zhou *et al.*, 2002).

ii) Angiogenesis/Angiostasis

Chemokines can indirectly affect tumour growth by promoting or inhibiting angiogenesis. CXC chemokines bearing an ELR (Glu-Leu-Arg) motif at their NH2 terminus are angiogenic, whereas those lacking the ELR motif are angiostatic. Significant levels of IL-8 are observed in prostate cancer cells, but not in normal or benign hyperplastic cells (Ferrer *et al.*, 1998). Anti-IL-8 antibody effectively inhibits the tumour-related angiogenesis of an IL-8-producing prostate cancer cell line, PC-3, in severe combined immunodeficiency (SCID) mice (Moore *et al.*, 1999).

CXCL10 (IP-10) is an angiostatic chemokine produced at high levels by human non-small cell lung cancer cells. In SCID mice, IP-10 production is inversely related to the tumour progression, and an intratumoral injection of IP-10 results in retardation of growth and neo-vascularization of the tumour (Arenberg *et al.*, 1996b).

iii) Local invasion

The ability of tumour cells to secrete metalloproteinases (MMPs) and other protease enzymes aids invasiveness through the extracellular matrix (ECM) (Vihinen, la-aho, and Kahari 2005). IL-8 expression by human melanoma cells induces transcriptional activation of the gene encoding MMP-2, leading to increased invasiveness through the ECM (Bar-Eli 1999; Luca *et al.*, 1997). IL-8 over-expression in prostate cancer also induces increased MMP-9 expression, which facilitates increased local tumour invasion in a mouse model (Inoue *et al.*, 2000).

Tumour invasion and metastasis are very important aspects of tumour biology. Chemokines may be important in both and this is discussed in greater detail below.

1.2.5 METASTASIS

1.2.5.1 Mechanism of metastasis

Metastasis is a complex process and involves a multitude of sequential steps. The clonal expansion, growth and angiogenesis of the tumour cells facilitate their adhesion to, and invasion of the basement membrane. After breaching the basement membrane, these metastatic cells traverse the extracellular matrix (ECM) by breaking it down, finally gaining access to the lymphatics or circulation. The same cycle is repeated when these tumour cells extravasate at distant sites. This enables the cancer cells to migrate to distant sites in the body resulting in metastasis.

i. Invasion

The first step in the metastatic cascade is the loosening of intercellular junctions in the tumour cells. There is an associated change in the pattern of expression of cell adhesion molecules (Bissell and Radisky 2001).

The second step involves attachment of tumour cells to ECM proteins such as fibronectin and laminin and is mandatory for invasion and metastasis. Tumour cells have a more widespread receptor expression for these constituents compared to normal epithelial cells. An altered pattern of integrin expression also facilitates invasion.

The third step involves degradation of the basement membrane and interstitial connective tissue. Tumour cells may secrete proteolytic enzymes on their own or induce the host cells to perform the same function (Bissell and Radisky 2001; Lynch and Matrisian 2002). The ECM can be degraded by proteinases which are classified into four separate groups, serine-, aspartate-, cysteine- and metalloproteinases. The serine and metalloproteinases play a predominant role in ECM turnover. The matrix metalloproteinases (MMPs) are a family of Zn^{2+} and Ca^{2+} dependent proteinases, which can catabolise most of the macromolecular components of the ECM. Expression of MMPs and the tissue inhibitors of metalloproteinases (TIMPs) is regulated by many cytokines/growth factors, mechanical properties of the ECM/actin cytoskeleton and cell/matrix interactions. They have also been documented to possess

a vital role in tumour invasion and metastasis by facilitating ECM degradation (Kalluri 2003; Lynch and Matrisian 2002).

The final phase of invasion involves movement of the tumour cells through the degraded ECM. This migration may be facilitated by chemokines in addition to some of the cleavage products of matrix components (e.g. collagen, laminin) and growth factors.

ii. Metastasis

Tumour cells finally enter the circulation via extravasation through the vascular endothelium or through lymphatics. The site of extravasation and the resulting metastasis are generally related to the primary tumour location and its vascular or lymphatic drainage. However, this is not always the case and the location/drainage of the primary site may not readily explain metastasis.

1.2.6 THEORIES OF METASTASIS

Metastasis is not a random process and different cancers have different, yet specific metastatic sites. In 1889, the concept of *'seed'* (tumour cell) and *'soil'* (specific organ) was first described to explain the non-random metastasis of breast cancer to specific organs (Paget 1989). This theory suggests that different organs provide growth conditions optimised for specific cancers. A second concept is that endothelial cells in the vascular beds of certain organs express adhesion molecules that specifically trap circulating tumour cells (Nicolson 1993a). A *'chemo-attraction'* or *'homing'* theory suggests that organ-specific attractant molecules enter the circulation, stimulating the migrating tumour cells to adhere to and invade through the walls of vessels and thus enter the organs (Nicolson 1993a; Nicolson 1993b). However, the molecules involved in the metastasis of tumour cells to specific sites must have some special properties. First, the chemoattractant should be constitutively expressed at the site of metastasis. Second, the tumour cells should express receptors for the chemoattractant. Third, adhesion molecules should be present on tumour cells allowing them to bind to the endothelium and last, the chemoattractant should aid the invasion of cells into tissues and facilitate their survival at the site of penetration.

Chemokines and their receptors as well as adhesion molecules play a vital role in leukocyte trafficking and homeostasis, and fulfil many of these criteria important in the chemoattractant theory of tumour metastasis.

1.2.7 EXPERIMENTS OF MÜLLER *et al.*, 2001

Muller *et al.* fashioned a series of experiments to study whether chemokine/chemokine receptor interactions are important in the metastasis of breast cancer. They found that amongst 17 different chemokine receptor genes, CXCR4 and CCR7 were significantly up-regulated in human breast cancer cell lines, malignant breast tumours and metastases compared with normal breast epithelial cells. They screened a range of normal human organs for the ligands of these receptors (CXCL12 and CXCL21) and found peak levels of ligand expression in organs preferred for breast cancer metastasis (i.e. lymph nodes, lungs and bone marrow) (Figure 1.13). *In vitro*, these ligands stimulated pseudopodia formation and directional migration in cells and this chemotactic activity could be neutralized by an anti-CXCR4 antibody. The same group also found that melanoma cell lines express the receptors CCR7 and CCR10 and that skin and lymph nodes, the two major sites of metastatic melanoma, selectively expressed ligands for both these receptors (Muller *et al.*, 2001).

CXCR4 has now been implicated in a wide range of cancers including acute myeloid and lymphoblastic leukemia, chronic lymphocytic leukemia, non-Hodgkin B-cell lymphoma, and pancreatic cancer (Muller *et al.*, 2001), melanoma (Kim *et al.*, 2006); (Mori *et al.*, 2005; Scala *et al.*, 2006), non-small cell lung cancer (Phillips *et al.*, 2003), ovarian cancer (Jiang *et al.*, 2006; Scotton *et al.*, 2001), prostate cancer (Arya *et al.*, 2004; rash-Yahana *et al.*, 2004; Taichman *et al.*, 2002), kidney cancer (Schrader *et al.*, 2002) and oral cancer (Muller *et al.*, 2006). Up-regulation of CXCR4 compared to their normal counterparts has been shown in these neoplastic conditions and this up-regulation is associated with tumour progression and metastasis.

Other chemokine receptors have also been implicated in chemokine-dependent tumour cell attraction to certain tissues. CXCR3 promotes colon cancer metastasis to lymph nodes (Kawada *et al.* 2004; Kawada *et al.* 2007). Functional expression of the CCR7 receptor, which is a receptor for two major chemokines, CCL19 (ELC) and

CCL21 (SLC), enhances the metastasis of B16 murine melanoma cells. This metastasis is blocked by neutralizing anti-CCL21 antibodies (Wiley *et al.*, 2001) CCR10 is expressed in melanoma cells, and its ligand, CCL27 (CTACK), is produced constitutively by keratinocytes in the skin, to which melanomas often metastasize (Mori *et al.*, 2005). CCR4 is often expressed in T-cell leukemias that preferentially invade the skin, where one of the CCR4 ligands CCL17 (TARC) is expressed by endothelial cells (Ishida *et al.*, 2003). CCR3 is expressed in cutaneous lymphomas and its ligand CCL11 (Eotaxin) is often expressed in tumour cells and tumour associated skin lesions (Kleinhans *et al.*, 2003; Kouno *et al.*, 2004).

Chemotaxis has been suggested as a possible mechanism for this organ-specific metastasis. However, it is questionable that tumour metastasis over a long distance, is governed by the concentration gradient of a single chemokine, since it is a highly complex phenomenon. The magnitude of the importance of this chemokine-receptor interaction, therefore, stands unknown.

1.2.8 CHEMOKINES/CHEMOKINE RECEPTORS IN ORAL CANCER

A number of groups have tried to emulate the work of Muller (Muller *et al.*, 2001) by studying the role of chemokines and chemokine receptors in oral cancer. The clinico-pathological significance of expression of the CXCR4 receptor has been investigated in detail.

i) CXCR4 in OSCC

A significant correlation between CXCR4 expression and regional lymph-node metastasis has been reported (Almofti *et al.*, 2004; Ishikawa *et al.*, 2006). Primary tumour cells in OSCC have stronger CXCR4 expression compared to normal epithelium whereas expression intensity is even stronger in metastatic tumour cells in regional lymph nodes (Delilbasi *et al.*, 2004; Ishikawa *et al.*, 2006; Uchida *et al.*, 2003). Expression of its ligand SDF-1α. is occasionally detected on metatastatic tumour cells but not on normal or primary tumour cells. CXCR4 expression has also been shown on oral cancer cells *in vitro* and exposure of these cells to SDF-1α facilitates an up-regulation in intracellular calcium mobilization, chemotaxis, ERK1/2

phosphorylation, adhesion to extracellular matrix components and MMP-9 secretion (Samara *et al.* 2004; Uchida *et al.*, 2003). All of these effects can be blocked by an antibody against CXCR4 or an inhibitor of SDF-1α suggesting that the response is specific. Expression of CXCR4 is also up-regulated on lymph node metastatic cells (HNt and B88) compared with normal or primary oral cancer cells. Exposure of oral cancer cells B88 and HNt to SDF-1α results in downregulation of epithelial markers E-cadherin and β-catenin, and up-regulation of mesenchymal markers vimentin and snail (Onoue *et al.*, 2006). This suggests an important role for CXCR4 and SDF-1α in epithelial-mesenchymal transition which is observed during tumour progression and facilitates invasion and metastasis.

Uchida *et al.*, observed a significant increase in metastases to cervical lymph nodes after inoculation of CXCR4-transfected cells in nude mice compared with untransfected cells. However, no metastases were observed in lungs even after 90 days which indicates a preferential metastasis of oral cancer cells to cervical lymph nodes (Uchida *et al.*, 2004). However, transfection of SDF-1α into B88 cells (expressing functional CXCR4) yielded very interesting results. Expression of CXCR4 and SDF-1α by the same cells provides an autocrine mechanism of activation whereas B88 cells expressing only CXCR4 can only be activated in a paracrine manner. Inoculation of CXCR4/SDF-1α co-expressing cells into nude mice results in increased frequency and more aggressive metastatic foci in the regional lymph nodes compared with cells expressing only CXCR4. In addition, the CXCR4/SDF-1α coexpressing cells show a significant increase in distant metastases to the lung whereas no such effect is observed with cells expressing CXCR4 on its own (Uchida *et al.*, 2007). Administration of a CXCR4 antagonist significantly decreases distant lung metastases and improves the survival rate of the mice. This suggests that autocrine CXCR4/SDF-1α signaling plays a more important role in distant OSCC mestastases than paracrine signaling (Uchida *et al.*,2007).

The ligand for CXCR4 is SDF-1α and is expressed in abundance in lymphoid tissues, lung, liver and bone. However, its expression has not been studied as extensively as CXCR4. Uchida *et al.*, reported that expression of SDF-1α in metastatic tumours in regional lymph nodes is significantly higher than the primary tumour and is correlated with a poor prognosis (Uchida *et al.*, 2007). Almofti *et al.*, could not find a significant

correlation between SDF-1 expression and clinico-pathological factors of OSCC including age, sex, lymph node metastasis, invasion, recurrence and prognosis (Almofti *et al.*, 2004).

All these findings suggest an important role for CXCR4/ SDF-1α interaction in the biology of oral cancer and in local and distant metastasis. However, metastasis is a highly complex phenomenon and the concentration gradient of a single chemokine over a longer distance may not be enough on its own and may require influence of other factors.

ii) Other chemokine receptors and chemokines in OSCC

a) CCR7

Expression of CCR7 has also been associated with tumour progression and poor prognosis in OSCC. CCR7 binds to two chemokines, CCL19/ELC/MIP-3β and CCL21/SLC/6Ckine. CCR7 expression is observed in primary tumours as well as metastatic deposits in regional lymph nodes and a significant correlation exists between CCR7 expression and prognostic factors such as metastasis to cervical lymph nodes, tumour size, clinical stage, local recurrence and mortality (Tsuzuki *et al.*, 2006). In addition, a correlation between tumour cell proliferation (PCNA expression) and CCR7 expression also exists (Xu *et al.*, 2007). Since the two ligands for CCR7 are abundantly expressed in lymph nodes, it is possible that this interaction may facilitate tumour cell proliferation, migration, invasion and metastasis to cervical lymph nodes. However, the extent of the involvement of CCR7 and its ligands in the pathogenesis of OSCC needs to be studied further.

b) CCL20

Expression of CCL20 (MIP-3α) mRNA has also been detected in OSCC *in vitro* and *in vivo* and this expression is up-regulated by infection with *Actinobacillus actinomycetemcomitans* and by stimulation with lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α). Increase in MIP-3α is also correlated with the number of Langerhans cells suggesting a regulatory role on the growth of cells in OSCC (Abiko *et al.*, 2003). However, conflicting results were reported by Wang *et al.* who showed down-regulation of CCR6 (the only known receptor for MIP-3α) in metastatic OSCC cells and tissues compared with normal mucosa (Wang *et al.*, 2005). Further work

needs to be done to establish the precise role of CCL20 and CCR6 in the pathogenesis of OSCC.

c) IL-8 and IL-6

IL-8 and IL-6 have been studied in patients with OSCC as potential biomarkers. IL-8 concentration in saliva and IL-6 concentration in serum are significantly increased at both RNA and protein levels in OSCC patients compared with negative controls (disease free patients). However, no association is observed with sex, age, alcohol or tobacco use (St John *et al.*, 2004). Expression and role of the IL-8 receptors CXCR1 and CXCR2 has also been reported in OSCC and will be discussed later on in section 1.2.13.5.

d) Eotaxin

The chemokine eotaxin exerts its effects through the CCR3 receptor. Expression of eotaxin has been reported in normal as well as neoplastic oral mucosa with and without an intense eosinophilic inflammatory infiltrate. Staining is observed on kearatinocytes, neutrophils, fibroblasts and plasma cells but is much stronger on eosinophils associated with OSCC (Lorena *et al.*, 2003). This suggests a potential mechanism for eosinophil recruitment which may influence adhesion and angiogenesis of tumour cells.

e) CXCL14

CXCL14 (BRAK) is constitutively expressed in certain normal tissues and is absent from many cancers. Abundant BRAK expression is observed in normal tongue mucosa whereas consistent absence is seen in tongue SCC at both mRNA and protein levels. It also acts as a potent inhibitor of angiogenesis and chemotaxis, suggesting that loss or down-regulation of BRAK may lead to tumour neo-vascularization and metastasis (Shellenberger *et al.*, 2004).

f) CXCL10

CXCL10 (IP-10) is a chemoattractant for activated T-cells expressing the CXCR3 receptor. Consistent expression of IP-10 is observed in nasopharyngeal carcinoma (NPC)-derived cell lines as well as tissue biopsies of OSCC of tongue (Teichmann *et*

al., 2005). These results are unusual since IP-10 has been shown to be angiostatic *in vitro* (and is regarded as an anti-neoplastic chemokine). This endogenous expression of IP-10 by tumours suggests that it might not exert an anti-neoplastic effect *in vivo* and needs to be studied further.

All these findings indicate that chemokines and their receptors may have a potential role in the pathogenesis of oral cancer.

1.2.9 PRELIMINARY STUDIES

As part of a previous study on the role of chemokine receptors in oral cancer, a microarray analysis of normal oral epithelial cells and several oral cancer cell lines was carried out in our research group (J. Collier- personal communication). Two findings were of interest. First, mRNA for XCR1 was observed in both normal and cancerous oral epithelial cells. This was surprising as XCR1 expression appears to be almost exclusively restricted to the immune system and expression by epithelial cells has not been shown to date. Second, CXCR1 mRNA appeared to be significantly increased in oral cancer compared with normal epithelial cells. This preliminary data suggests XCR1 and CXCR1 may have some role in the biology of oral cancer and these receptors and their ligands (lymphotactin and IL-8) will be discussed in greater detail.

1.2.10 XCR1/GPR5

1.2.10.1 Discovery

XCR1 is the sole C family chemokine receptor and is specific for the chemokines lymphotactin (XCL1/SCM-1α) and XCL2/SCM-1β. XCR1 was first cloned and mapped by Heiber *et al.* in 1995. They isolated a novel human gene encoding an orphan G protein-coupled receptor and named it GPR5. The entire coding region for this gene was contained on a single exon and was localized to chromosome 3 (p21.3-p21.1) (Heiber *et al.*, 1995).

GPR5 was identified as XCR1 by Yoshida *et al.* in 1998. By stably expressing eight known chemokine receptors and three orphan receptors in murine L1.2 cells and

testing their responsiveness, they found that the orphan receptor GPR5 functioned as a high affinity receptor for SCM-1/lymphotactin (Yoshida *et al.*, 1998). SCM-1α and SCM-1β mediated efficient chemotactic responses and calcium mobilization and this response was completely suppressed by pre-treatment with pertussis toxin, indicating coupling of XCR1 with a Gαi class of G-proteins in murine L1.2 cells. Lymphotactin bound specifically to GPR5-expressing L1.2 cells and northern blot analysis showed GPR5 mRNA in placenta, spleen and thymus. Later on, Yoshida *et al.* isolated and cloned the murine homologue of XCR1 (Yoshida *et al.*, 1999b) by screening a mouse liver genomic library using XCR1 cDNA as a probe. It was named mXCR1.

1.2.10.2 Structure

Like other chemokine receptors, XCR1 also belongs to the seven transmembrane domain G-protein-coupled receptor superfamily. Phylogenic depiction of the evolutional relation of the various chemokine receptors shows XCR1 to be on a branch containing human CC chemokine receptors (Yoshida *et al.*, 1998). Its gene is mapped to chromosome 3p21.3-p21.1 close to other CC chemokine receptor genes clustered at 3p21 (Heiber *et al.*, 1995). This is consistent with the close phylogenic relationship of lymphotactin to CC chemokines (Yoshida *et al.*, 1995).

1.2.10.3 Expression

XCR1 expression in mouse tissues has been detected in spleen and lung. However it is not known precisely which cells express XCR1 in these tissues (Yoshida *et al.*, 1999b). XCR1 mRNA is expressed by CD8+ T cells and natural killer (NK) cells which is striking because these cells are also the main producers of lymphotactin when stimulated (Yoshida *et al.*, 1999a). XCR1 expression has also been detected in tissue macrophages in a murine model of listeriosis (Dorner *et al.*, 2002).

Among human tissues, XCR1 expression is not as widespread as some of its chemokine receptor counterparts. Its mRNA is expressed in selected tissues including placenta, spleen, and thymus (Yoshida *et al.*, 1998; Yoshida *et al.*, 1999b) but it is not known precisely which cells in these tissues express XCR1. T and B lymphocytes and neutrophils express XCR1 and respond to lymphotactin by chemotaxis (Huang *et al.*,

2001b). Recently, XCR1 expression has been demonstrated in fibroblast-like synoviocytes in rheumatoid synovium (Blaschke *et al.*, 2003) and melanocytic lesions (Seidl *et al.*, 2007). Strong XCR1 staining is observed on melanocytes and nevus cells in deeper parts of the dermis whereas staining is weaker in nests adjacent to the epidermis. However, no correlation between XCR1 expression and disease progression is observed in melanocytic lesions and Seidl *et al.* did not compare expression to normal tissue. Therefore, the significance of this expression is not known.

Expression of XCR1 by epithelial cells has not been shown to date.

XCR1 Expression in Mouse	XCR1 Expression in Human
Cells/Tissues	Cells/Tissues
T lymphocytes	T lymphocytes
NK cells	B Lymphocyte
Spleen	Neutrophils
Lung	Placenta
Macrophages	Spleen
(after IFN-γ exposure)	
	Thymus
	Fibroblast-like Synoviocytes
	Melanocytic lesions

Table 1.3. Summary of cells/tissues expressing XCR1.

1.2.11 LIGAND FOR XCR1: LYMPHOTACTIN (SCM-1α/ATAC/Lptn/XCL1)

1.2.11.1 Discovery

In 1994, Kelner *et al.*, tried to establish the cytokine-producing profile of murine progenitor T cells (Kelner *et al.*, 1994). During screening of a mouse pro-T cell cDNA library, they came across a clone, the protein translation of which consistently matched a short COOH-terminal segment of CC chemokine protein chains in *'Basic local alignment search tool'* (BLAST) searches of protein and nucleic acid databases. A weaker similarity in this region was also noted with CXC chemokine sequences. On the basis of its specific chemotactic activity for lymphocytes (considered a unique trait at that time), this molecule was designated **lymphotactin (Ltn).**

The human analogue of lymphotactin was discovered simultaneously and isolated by three independent groups i.e. Kennedy *et al.* (Kennedy *et al.*, 1995), Yoshida *et al.*, (who called it **Single C Motif-1/SCM-1**) (Yoshida *et al.*, 1995) and Muller *et al.*, named it **Activation-induced, T cell-derived, and Chemokine-related molecule (ATAC)** (Muller *et al.*, 1995). It was later realized that the three groups had actually identified the same chemokine and the name lymphotactin was designated.

Lymphotactin was deemed interesting as it possessed a number of the conserved amino acid residues present in CXC and CC chemokines, including two cysteines corresponding to cysteine 2 and 4, but was missing cysteines 1 and 3. Data from chromosomal mapping provided further evidence for identification of a new class of chemokine. All the previously described CC chemokines map to human chromosome 17 and mouse chromosome 11. The CXC chemokines map to human chromosome 4 and to mouse chromosome 5. Lymphotactin is unique as it maps to the distal region of chromosome 1 in both human and mouse (Kelner *et al.*, 1995).

Taken together, the structure, biologic activity and chromosomal location of lymphotactin merited it to be classified as a distinct class of the chemokine superfamily.

1.2.11.2 Structure

Human lymphotactin encodes a 114 amino acid residue precursor protein with a 21 amino acid residue predicted signal peptide (Kennedy *et al.*, 1995). It maintains the secondary and tertiary architecture of classical chemokines in spite of missing two cysteines. Lymphotactin consists of an amino-terminal domain attached to the rest of the molecule by one disulfide bond (Hedrick and Zlotnik 1998).

Studies on lymphotactin structure by molecular dynamics have proved that the lack of one disulfide bond and the extension of the C-terminus in human lymphotactin are complementary to each other. The role of the extended C-terminus is most likely to stabilize the molecule enabling it to function with a single disulfide bond however, it may also be responsible for other functions (Buyong *et al.*, 2000).

1.2.11.3 Expression

i) Expression in lymphoid cells

Expression of lymphotactin in both mouse and humans is observed in activated CD8+ T cells and activated NK cells (Dorner *et al.*, 1997; Hedrick *et al.*, 1997; Kelner *et al.*, 1995; Muller *et al.*, 1995). Lymphotactin expression has been shown in activated pro-T cells (Kelner *et al.*, 1994; Kelner and Zlotnik 1995). Low levels of mRNA expression have also been observed in mouse spleen and thymus tissue (Kelner *et al.*, 1994). However, the exact cells expressing lymphotactin in spleen and thymus have not been documented so far although it is likely that they are lymphocytes. Activated murine epidermal dendritic T cells and intraepithelial γδ T cells have also been shown to express lymphotactin (Boismenu *et al.*, 1996).

Lymphotactin expression in human tissues is more widespread than in the mouse but is still predominant in lymphoid tissues including thymus, spleen, and peripheral blood lymphocytes. However, the expression of lymphotactin by lymphocytes is dependent upon activation (activated CD8+ T cells express more lymphotactin than activated CD4+ T cells) (Hedrick and Zlotnik 1997; Hedrick and Zlotnik 1998; Kennedy *et al.*, 1995; Muller *et al.*, 1995). Recently, CD8+ αβ+ T cells that lack surface CD5 antigen expression have been described as a major cellular source of lymphotactin in peripheral blood (Stievano *et al.*, 2003). Expression has also been shown in activated NK cells (Giancarlo *et al.*, 1996; Hedrick *et al.*, 1997; Hedrick and Zlotnik 1997; Maghazachi 1999), mature dendritic cells (Middel *et al.*, 2001), mast cells (Rumsaeng *et al.*, 1997) and α/β thymocytes (Kelner *et al.*, 1994). A significant increase in lymphotactin expression has been reported in intraepithelial lymphocytes (IEL) in patients suffering from Crohn's disease compared with those having a non-inflammatory gut disorder (Middel *et al.*, 2001). This suggests a role for lymphotactin in inflammatory conditions.

ii) Expression in non-lymphoid cells

Low levels of lymphotactin mRNA are present in small intestine, colon, lung, ovary, and testes tissue (Kelner *et al.*, 1994; Kennedy *et al.*, 1995; Muller *et al.*, 1995; Yoshida *et al.*, 1995). The cells expressing lymphotactin in these tissues have not been reported precisely but are most likely to be lymphocytes and NK cells. It is also

possible that the epithelial cells in these tissues may also contain mRNA for lymphotactin. Glomerular mesangial and vascular endothelial cells express lymphotactin mRNA after stimulation with IL-1β in an experimental murine model of glomerulonephritis (Natori *et al.*, 1998), but the significance this expression is unclear.

Lymphotactin Expression (Mouse) (only after activation)	Lymphotactin Expression (Human) (only after activation)
T cells	T cells
NK cells	NK cells
Spleen	Spleen
Thymus	Thymus
Dendritic cells	Dendritic cells
Thymocytes	Colon
Epidermal Intraepithelial T cells	Small intestine
Glomerular mesangial and vascular endothelial cells (after IL-1β exposure) in glomerulonephritis	Lung
	Ovary
	Testes
	Mast cells
	Synovial fluid in Rheumatoid Arthritis

Table 1.4. Summary of cells/tissues expressing lymphotactin.

Recently, lymphotactin expression has been demonstrated in the synovium of Rheumatoid Arthritis (RA) patients (Blaschke *et al.*, 2003). Expression of lymphotactin by epithelial cells has not been shown so far.

1.2.11.4 Functions of lymphotactin

As expression of lymphotactin and its receptor XCR1 has been predominantly observed in the lymphoid system, the scope of its known functions is largely in the same vicinity.

i) Chemotaxis of lymphoid cells

Lymphotactin was originally reported to be specifically chemotactic for CD4+ and CD8+ T lymphocytes in both mouse and human (Kelner *et al.*, 1994; Kennedy *et al.*, 1995) and this finding was corroborated by other groups (Dorner *et al.*, 1997). Later on, it was discovered that mouse and human NK cells also respond to lymphotactin (Giancarlo *et al.*, 1996; Hedrick *et al.*, 1997; Maghazachi *et al.*, 1997).

ii) Role in Immunity

Lymphotactin enhances antigen-specific immune responses suggesting an important role in mucosal immunity. Even low doses of lymphotactin up-regulate antibody levels against a specific antigen as well as supplementing the systemic antibody responses (Lillard, Jr. *et al.*, 1999). Therefore, lymphotactin appears to act as an innate mucosal adjuvant for induction of adaptive immunity.

There is also some evidence lymphotactin may augment antigen specific immune responses. Antigen-specific T cell activation is characterized by an alteration in expression of several cell surface molecules including a decrease in CD62L (L-Selectin). CD62L is utilized by naïve T cells to enter high endothelial venules in lymph nodes. Down-regulation of CD62L following T cell activation is therefore associated with an increased ability to arrive at tertiary inflammatory sites. Lymphotactin facilitates the preferential recruitment of $CD62L^{lo}$ (with low L-Selectin expression) over $CD62L^{hi}$ (with high L-Selectin expression) cells *in vitro,* making it the first chemokine to preferentially recruit recently activated antigen-specific T cells towards the site of inflammation (Kurt *et al.*, 2001).

iii) Role in disease

There is evidence that lymphotactin plays a role in Th-1 mediated diseases. Lymphotactin has been reported to be induced by T-cell receptor stimulation in Th1 but not in Th2 cells and it has also been found to be co-secreted with IFN-γ at a higher level by activated Th1 cells (Dorner *et al.*, 2002; Zhang *et al.*, 2000). Lymphotactin is produced by islet-specific Th1 cells in autoimmune diabetes, and is detected in peripheral blood mononuclear cells (PBMCs) from patients with multiple sclerosis (Bradley *et al.*, 1999; Jalonen *et al.*, 2002). However the precise role of lymphotactin in the biology of these diseases remains unknown.

The *in vitro* functional properties of lymphotactin suggest an important role in the recruitment of T cells in the pathogenesis of chronic inflammatory disorders. A significant increase in lymphotactin expression compared to normal has been observed in Crohn's disease. The cells which express lymphotactin have been identified as T cells, mast cells and dendritic cells and are present throughout the inflamed bowel wall (Middel *et al.*, 2001). Lymphotactin mRNA expression along with MCP-1 and RANTES, is up-regulated in sarcoidosis and is associated with disease progression (Petrek *et al.*, 2002). This suggests that lymphotactin may be involved in lymphocyte migration to the lung *in vivo*. However, the significance of this role needs to be studied in detail.

Lymphotactin also plays an important role in Rheumatoid Arthritis (RA). Lymphotactin mRNA and protein is expressed by CD4+ CD28- T cells in the peripheral blood as well as in CD4+ and CD8+ T cells in the synovial infiltrate from RA patients. Strong lymphotactin expression is observed in lymphocytic infiltrate and a noticeable relationship exists between the expression of lymphotactin and the density of the mononuclear cell infiltrate (Blaschke *et al.*, 2003). As a result, levels of lymphotactin are significantly higher in the synovial fluid of patients suffering from RA compared with disease free patients (Wang *et al.*, 2004). Exposure of fibroblast-like synoviocytes (expressing XCR1) to lymphotactin causes down-regulation of MMP-2 secretion suggesting an important role for lymphotactin/XCR1 interaction in the pathogenesis of RA. All these findings indicate that lymphotactin may act as an immunomodulatory influence in chronic inflammatory disorders.

iv) Role as an anti-tumour agent

Lymphotactin is potentially an attractive anti-tumour agent because of its ability to attract the effector cells (CD8+ T cells and NK cells) which eliminate tumours. This potential has been studied and lymphotactin has been shown to be effective in tumour vaccines in a number of experimental tumour models.

Subcutaneous injections of a vaccine containing lymphotactin in conjunction with interleukin-2 (IL-2) in neuroblastoma patients results in tumour infiltration by CD4+ and CD8+ T cells, NK cells, eosinophils and langerhans cells. This is associated with an increase in circulating IgG antibodies and most tumours are either partially or

completely destroyed (Rousseau *et al.*, 2003). However, this study contained only 21 patients and variable responses were seen in some. Transfection of lymphotactin into a mouse myeloma model does not inhibit tumour development but tumour growth is retarded compared with controls due to infiltration of CD4+ and CD8+ T cells and neutrophils (Cairns *et al.*, 2001).

A dendritoma of gene-modified dendritic cells (DCs) (containing lymphotactin) and mouse hepatocellular carcinoma cell line H-22 attracts T cells and NK cells in chemotactic assays and successfully enhances proliferative and cytotoxic activity (Zhang *et al.*, 2004). DCs containing just lymphotactin fail to elicit a similar response which suggests that lymphotactin requires some sort of carrier to exert its effects. However, this study focussed on *in vitro* studies only and different results might have been obtained *in vivo*. Pre-existing tumours and their metastases are significantly decreased when treated with lymphotactin gene-modified dendritic cells in tumour models of lung carcinoma and melanoma (Cao *et al.*, 1998; Zhang *et al.*, 1999). Co-transfection of lymphotactin and melanoma antigen gp100 in murine dendritic cells results in increased cytotoxicity of lymphocytes and NK cells and increased production of IL-2 and IFN-γ, thus leading to an improved anti-tumour response (Xia *et al.*, 2002).

All these findings suggest that lymphotactin has the ability to attract immune effector cells to tumour sites. It can also act in harmony and facilitate release of various cytokines and has natural adjuvant activities that result in augmented antitumor immune responses.

v) Other biological functions

Lymphotactin wields various biological functions in addition to its chemotaxis.

a. Role as type 1 cytokine

Lymphotactin along with MIP-1α, MIP-1β and RANTES, has been shown to be co-secreted with IFN-γ from NK cells and T cells. It acts as a type 1 cytokine by up-regulating CD40, IL-12 and TNF-α in macrophages in a murine model of listeriosis. This suggests that lymphotactin may play a key role in Th1 mediated immunity (Dorner *et al.*, 2002).

b. Effects on T cell proliferation

Lymphotactin has differential proliferative effects on CD4+ and CD8+ T cells. It inhibits CD4+ T-cell proliferation *in vitro* through a diminished production of Th1 (IL-2, IFN-γ) cytokines. In contrast, lymphotactin directly stimulates proliferation of CD8+ T-cells and IL-2 secretion (Cerdan, Serfling, and Olive 2000).

c. Role in T cell regulation

Lymphotactin acts as a direct inhibitor of CD4+ T cells and co-stimulates their apoptosis. Conversely, it increases CD8+ T cell activation and is the first chemokine known to exert a direct differential regulation on CD4+ and CD8+ T-cell activation (Cerdan *et al.*, 2001). The extent of this role still needs to be elucidated.

1.2.11.5 Mechanism of XCR1/lymphotactin signaling and function

Coupling of lymphotactin and XCR1 mobilizes intracellular calcium permitting the chemokine to exert its functions. Pertussis toxin (PT) was used to determine the coupling of lymphotactin to G-proteins in natural killer cells. It was found that lymphotactin induces the chemotaxis and mobilization of intracellular calcium through pertussis toxin sensitive and -insensitive heterotrimeric G-proteins (Gαi, Gαo and Gαq) (Maghazachi *et al.*, 1997; Maghazachi 1999). The role of phosphoinositide-3γ kinases (PI-3K) was also studied which revealed that lymphotactin signaling within NK cells is mediated by a ternary complex of proteins, including G-protein, pleckstrin and PI-3γ kinase (al-Aoukaty, Rolstad, and Maghazachi 1999).

To summarize, XCR1 and its ligand lymphotactin are expressed predominantly within the lymphoid system. Lymphocytes and neutrophils express high levels of XCR1 and the principal function of lymphotactin appears to be chemoattraction of these cells. Recent reports have shown expression of XCR1 in melanocytes of melanocytic lesions and in rheumatoid synovium, but the importance of this expression is unknown. XCR1 expression on epithelial cells has not been shown to date.

1.2.12 CXCR1 and CXCR2
(IL-8RA/IL-8R1 and IL-8RB/IL-8R2)

In addition to XCR1, preliminary microarray results showed significant difference in CXCR1 mRNA expression between normal and malignant oral epithelial cells. The principle ligand for CXCR1 is IL-8 and the interactions of IL-8 with its receptors CXCR1 and CXCR2 appear to play an important role in the biology of cancer. An outline of their roles is discussed below.

1.2.12.1 Discovery

CXCR1 was first described by several groups as a high affinity receptor for IL-8 on neutrophils (Besemer, Hujber, and Kuhn 1989; Grob *et al.*, 1990; Moser *et al.*, 1991; Samanta, Oppenheim, and Matsushima 1990; Thomas, Taylor, and Navarro 1991). However cross-linking experiments revealed that two proteins of 44 kDa (p44) and 70 kDa (p70) became specifically labelled with radio-iodinated IL-8, GRO-α and NAP-2 (Moser *et al.*, 1991; Schumacher *et al.*, 1992). IL-8 bound to both proteins with high affinity, whereas GRO-α and NAP-2 had much higher affinity for p70 (*Kd* 30 nM) compared to p44 (*Kd* 0.3-0.7 nM) (Lee *et al.*, 1992; Schnitzel *et al.*, 1991; Schumacher *et al.*, 1992). These results suggested that all three cytokines (IL-8, GRO-α and NAP-2) acted on neutrophils through common receptors.

The two interleukin-8 receptors were initially called IL-8 RA (IL-8 R1) and IL-8 RB (IL-8 R2) but were renamed CXCR1 and CXCR2 based on a new classification (Zlotnik and Yoshie 2000). The cDNA of CXCR1 was first cloned by Murphy and Tiffany in 1991 using L-60 neutrophils (Murphy and Tiffany 1991). Holmes and colleagues cloned the cDNA of CXCR2 in 1991 (Holmes *et al.*, 1991).

1.2.12.2 Structure and expression

Both CXCR1 and CXCR2 are transmembrane G-protein-associated receptors and belong to the CXC family and map to chromosome 2. Both receptors bind to IL-8 with high affinity. CXCR1 is specific for IL-8 and Granulocyte chemotactic protein-2 (GCP-2), whereas CXCR2 can interact with a range of other CXC chemokines possessing the N-terminal ELR motif with similar affinity (CXCL1-CXCL7).

Patterns of expression of CXCR1 and CXCR2 appear to differ between different leukocytes. In 1993 Moser *et al.* studied the expression of both IL-8 receptors in myeloid, lymphoid and other tissue cells using northern-blot analysis and reverse transcription polymerase chain reaction (RT-PCR). They found that CXCR1 mRNA is expressed at much higher levels in neutrophils compared to blood monocytes and myeloid cell lines and no CXCR1 mRNA is present in lymphocytes and lymphoid cell lines. CXCR2 mRNA is expressed in neutrophils, monocytes but in contrast to CXCR1 also in lymphocytes (Moser *et al.*, 1993). This suggests different roles for the two receptors.

Expression of both CXCR1 and 2 has since been reported in several other cell types including epidermal keratinocytes (Michel *et al.*, 1992), mast cells (Lippert *et al.*, 1998), endothelial cells (Li *et al.*, 2003), bronchial epithelial cells (Farkas *et al.*, 2005) and oesophageal mucosa (Isomoto *et al.*, 2005). Human gastric epithelial cells express CXCR1 and CXCR2 after exposure to *Helicobacter pylori* infection (Backhed *et al.*, 2003). Expression on gingival keratinocytes has also been reported *in vitro* and *in vivo* (Sfakianakis, Barr, and Kreutzer 2002). Sfakianakis *et al.* also reported an up-regulation of CXCR1 and CXCR2 in periodontitis compared to normal gingival epithelium and suggested that this upregulation facilitates IL-8 mediated migration and proliferation of gingival keratinocytes. However, their conclusions seem speculative as they only showed cell surface expression of CXCR1 and CXCR2. None of their results show a significant difference in expression of CXCR1 and CXCR2 expression between normal and inflamed tissue and they did not study the role of these receptors in cell migration and proliferation.

1.2.13 INTERLEUKIN-8 (IL-8/CXCL-8/NAF/MDNCF/NAP-1/MONAP)

1.2.13.1 Discovery

The principle ligand for CXCR1, interleukin-8, was one of the first chemokines to be discovered and was reported in 1987-88 by a number of groups simultaneously. Yoshimura, Matsushima and colleagues (1987) identified it from LPS-stimulated human monocyte culture supernatants (Matsushima *et al.*, 1988; Yoshimura *et al.*,

1987a; Yoshimura *et al.*, 1987b). It was named **'monocyte-derived neutrophil chemotactic factor'** (MDNCF) and was chemoattractant for neutrophils but not for monocytes/macrophages. Walz *et al.* reported that human blood monocytes stimulated with *E.coli* LPS, phytohaemagglutinin or concanavalin A produced a novel **'neutrophil-activating factor'** (or NAF) (Walz *et al.*, 1987). Stimulation of human peripheral blood monocytes with LPS and phorbol myristate acetate (PMA) lead to production of a chemoattractant which facilitated neutrophil chemotaxis. It was named **'monocyte-derived neutrophil-activating peptide'** (MONAP) (Schroder *et al.*, 1987).

Later on, the name **Interleukin-8 (IL-8)** was assigned and it was discovered that IL-8 exerts a range of actions including induction of lysosomal enzyme release from neutrophils (Walz *et al.*, 1987), expression of adhesion molecules on neutrophils (Detmers *et al.*, 1990), stimulation of histamine release from basophils (White *et al.*, 1989) and chemoattraction of T lymphocytes (Larsen *et al.*, 1989).

1.2.13.2 Structure

IL-8 is an 8kD protein generated as a 99-amino acid precursor with a characteristic leader sequence of 22 amino acids (Matsushima *et al.*, 1988). Several mature forms have been recognized (Gregory *et al.*, 1988; Lindley *et al.*, 1988; Yoshimura *et al.*, 1989) and the major form consists of 72 amino acids (Lindley *et al.*, 1988).

IL-8 has considerable sequence homology with platelet derived peptides such as platelet basic protein (PBP), connective tissue activating peptide III and platelet factor 4. It also possesses both structural and biological similarities with macrophage inflammatory protein-2 (MIP-2) (Matsushima *et al.*, 1988).

1.2.13.3 Expression

IL-8 was originally described as a product of mononuclear phagocytes (Peveri *et al.*, 1988; Schroder, Mrowietz, and Christophers 1988; Van *et al.*, 1988; Walz *et al.*, 1987; Yoshimura *et al.*, 1987a; Yoshimura *et al.*, 1987b) but was later found to be expressed by a variety of cells. The stimulus for expression appears to vary between

cell types. IL-8 expression is induced in fibroblasts (Strieter *et al.*, 1989b), epithelial cells and hepatocytes (Thornton *et al.*, 1990) in response to IL-1α, IL-1β and TNF-α, while monocytes (Peveri *et al.*, 1988; Yoshimura *et al.*, 1987b) and endothelial cells (Schroder and Christophers 1989; Strieter *et al.*, 1989a) express it after stimulation with IL-6, IL-2, LPS and type I or II interferons (IFNs) (Walz *et al.*, 1987).

IL-8 expression has also been shown in oral keratinocytes (Bickel *et al.*, 1996; Zehnder *et al.*, 1999). Constitutive expression of IL-8 mRNA is seen in primary oral keratinocytes and keratinocyte cell lines and stimulation with IL-1β increases this expression (Bickel *et al.*, 1996). However this group did not study IL-8 secretion by oral keratinocytes in culture or *in vivo*. IL-8 is expressed *in vivo* in gingival keratinocytes, microvascular endothelial cells (MVEC) and leukocytes within the gingival epithelium (Sfakianakis, Barr, and Kreutzer 2002). Fibroblasts from nasopharyngeal mucosa constitutively express IL-8 *in vitro* and *in vivo* (Knerer *et al.*, 1999). Nasal epithelial cells also express IL-8 after stimulation with TNF-α in culture (Rudack *et al.*, 2003).

1.2.13.4 Functions of IL-8

i) Actions on neutrophils

The biological profile of newly discovered IL-8 was thought to be restricted to neutrophil chemotaxis. However, subsequent studies have demonstrated a multitude of *in vitro* effects on neutrophils including shape change, production of superoxide and hydrogen peroxide (Thelen *et al.*, 1988), release of lysosomal enzymes (Peveri *et al.*, 1988), induction of respiratory burst (Schroder *et al.*, 1987), generation of bioactive lipids (Schroder 1989) and increased expression of adhesion molecules on neutrophils (Detmers *et al.*, 1990; Paccaud, Schifferli, and Baggiolini 1990).

IL-8 is not species specific, and its effects *in vivo* have been studied with various laboratory animals. Injection of IL-8 into the skin of rabbits results in plasma exudation and a long lasting massive neutrophil infiltration (Colditz *et al.*, 1989). Intradermal injection of human IL-8 causes a transient and dose-dependent neutrophil infiltration in various animal species including rats, mice, guinea pigs, dogs and humans (Colditz *et al.*, 1989; Larsen *et al.*, 1989; Leonard *et al.*,1991).

ii) Signaling

IL-8 exerts its functions by eliciting a transient and rapid intracellular increase in cytosolic calcium (Peveri *et al.*, 1988; Thelen *et al.*, 1988). All IL-8 mediated responses, including the calcium changes, are abrogated by pre-treatment of cells with *pertussis toxin*, indicating that IL-8 signaling is dependent on a GTP-binding protein. IL-8 mediated respiratory burst is also inhibited by Genistein (a protein tyrosine kinase inhibitor), indicating a role for protein kinase C (Thelen *et al.*, 1988). Like a number of other chemokines, IL-8 has also been shown to signal transduce through the ERK1/2 signaling cascade (Shyamala and Khoja 1998).

iii) Chemotaxis of other haematopoietic cells

The chemotactic effects of IL-8 are not restricted to neutrophils and other granulocytes and monocytes also respond to it. IL-8 facilitates *in vitro* and *in vivo* chemotaxis of CD4+ and CD8+ human peripheral T lymphocytes (Larsen *et al.*, 1989). It is also chemotactic for basophils (White *et al.*, 1989) and IL-3 or GMCSF-primed eosinophils (Warringa *et al.*, 1991). However, the pathophysiological relevance of the effects of IL-8 on basophils and eosinophils is not completely understood.

iv) Actions on non-haematopoietic cells

In addition to its effects on leucocytes, IL-8 also acts on non-haematopoietic cells such as epithelial cells, fibroblasts, melanoma cells and endothelial cells which express the receptors for IL-8. It has a variety of effects and these are discussed below.

a) Neovascularization and angiogenesis

IL-8 possesses an ELR motif before the first cysteine residues like other CXC chemokines which appears to confer angiogenic activity. IL-8 has been shown to mediate angiogenesis of endothelial cells (Koch *et al.*, 1992; Li *et al.*, 2003; Li *et al.*, 2005). Addition of human recombinant IL-8 results in a significant increase in angiogenesis of human umbilical vein endothelial cells (HUVECs). In addition, administration of a blocking antibody against IL-8 significantly inhibits the angiogenic activity present in the conditioned media of inflamed human rheumatoid synovial tissue macrophages or LPS-stimulated blood monocytes (Koch *et al.*, 1992).

IL-8 also facilitates angiogenesis in a range of neoplastic conditions such as prostate cancer (Araki *et al.*, 2007; Inoue *et al.*, 2000; Kim *et al.*, 2001), melanoma (Bar-Eli 1999) and pancreatic cancer (Wente *et al.*, 2006). Administration of anti-IL-8 antibody decreases tumourigenicity and neovascularization of bronchogenic cancer cell lines in SCID mice (Arenberg *et al.*, 1996a).

All these findings suggest that IL-8 production by tumours may regulate neovascularization and in due course tumour growth and metastasis. Additionally, tissue injury mediated local IL-8 production may facilitate wound healing through angiogenesis.

b) Role as an autocrine growth factor

Endothelial cells are a major source of IL-8 and this expression is regulated by inflammation, infection, stress and interaction with tumours (Liang *et al.*, 2002; Ramjeesingh, Leung, and Siu 2003; Sica *et al.*, 1990; Vadeboncoeur *et al.*, 2003). Several reports have demonstrated that IL-8 directly modulates endothelial cell proliferation and migration and regulates angiogenesis *in vitro* and *in vivo* in a paracrine manner (Koch *et al.*, 1992; Masood *et al.*, 2001). Neutralizing antibody to IL-8 results in inhibition of endothelial cell proliferation and MMP-2 production compared to controls (Li *et al.*, 2005). In addition, IL-8 can also act as a growth factor for tumour cells in pancreatic and lung cancer (Kamohara *et al.*, 2007; Zhu *et al.*, 2004; Zhu and Woll 2005).

Melanoma cells produce IL-8 *in vitro* (Schadendorf *et al.*, 1993) and possess specific receptors for IL-8 (Varney *et al.*, 2003). It also enhances proliferation of some melanoma and lung cancer cell lines (Norgauer, Metzner, & Schraufstatter 1996; Schadendorf *et al.*, 1993; Zhu *et al.*, 2004) suggesting that it can act as an autocrine growth factor in tumour progression.

c) Role in health and disease

IL-8 appears to play a role in a variety of diseases that are characterised by neutrophil accumulation. IL-8 has been demonstrated in a number of inflammatory conditions such as psoriasis, scleroderma and Crohn's disease (Gijsbers *et al.*, 2004; Kreuter *et al.*, 2006; Ozawa, Terui, and Tagami 2005). Stimulation of synoviocytes in

rheumatoid arthritis leads to up-regulation of IL-8 release (Cho *et al.*, 2007; Hwang *et al.*, 2004; Nanki *et al.*, 2001). This local IL-8 production facilitates neutrophil accumulation which is a major source of cartilage-degrading enzymes.

A role for IL-8 has been reported in adult respiratory distress syndrome (ARDS). An increase in the bronchoalveolar fluid level of IL-8 has been shown in ARDS patients (Miller *et al.*, 1992). This level correlates with the incidence of ARDS development in patient groups at risk (Donnelly *et al.*, 1993). IL-8 also plays an important role in the pathogenesis of glomerulonephritis. Urine IL-8 levels are elevated and immunoreactive IL-8 protein is detected in infiltrating mononuclear cells in glomeruli. Anti-IL8 antibody reduces neutrophil infiltration into glomeruli and abrogates the impairment of renal function (Wada *et al.*, 1994a; Wada *et al.*, 1994b).

An up-regulation of IL-8 serum level is seen in recurrent aphthous ulcers of the oral cavity and in lichen planus suggesting its use as a marker for these diseases (Sun *et al.*, 2004; Sun *et al.*, 2005). However, no IL-8 protein expression on oral mucosal keratinocytes in oral lichen planus (OLP) or oral lichenoid reactions (OLR) is seen *in vivo* (Little *et al.*, 2003). On the contrary, suprabasal epidermal keratinocytes express IL-8 in active regions of psoriatic plaques. A possible explanation for this may be the difference in inflammatory reaction between these lesions. In psoriasis, the inflammatory infiltrate predominantly consists of neutrophils whereas that is not the case in OLP or OLR. Since IL-8 is a potent neutrophil chemoattractant, this would explain IL-8 expression in psoriasis and not in OLP.

IL-8 also appears to play an important role in wound healing. IL-8 expression is induced in the superficial wound bed within 24 hours and is associated with neutrophil infiltration. A strong correlation exists between IL-8 expression, angiogenesis and keratinocyte migration. This IL-8 expression starts decreasing with wound re-epithelialization and becomes undetectable after wound closure at four days. However, neutrophils consistently express IL-8 throughout the process (Engelhardt *et al.*, 1998).

Sfakianakis *et al.* compared expression of normal gingival epithelium with inflamed tissue and suggested that IL-8 expression by gingival keratinocytes is up-regulated by

microbial pathogens and influences polymorphonuclear cell chemotaxis, angiogenesis and epithelial proliferation via specific receptors in periodontitis (Sfakianakis, Barr, and Kreutzer 2002). However, their study was a small one in which they only showed expression of IL-8 in both normal and inflamed oral tissue. No significant difference in IL-8 expression between normal and inflamed tissue is noticeable from their results and no experiments were conducted to study the role of IL-8 in chemotaxis, angiogenesis or epithelial proliferation. Huang *et al.* also studied the role of IL-8 in periodontitis and showed an up-regulation of IL-8 and ICAM-1 expression after stimulation with *A. actinomycetemcomitans* or *F. nucleatum* but not with *P. gingivalis* which down-regulated IL-8 secretion from oral epithelial cells. However, this decrease in IL-8 production was only seen with adherent strains of *P. gingivalis* 381 and 33277. Poorly invasive and non-adherent *P. gingivalis* strains W50 and W83 did not have a similar effect suggesting that attachment and invasion of *P. gingivalis* are mandatory for IL-8 down-regulation (Huang *et al.*, 1998; Huang *et al.*, 2001a; Huang, Haake, and Park 1998).

1.2.13.5 Expression of CXCR1, CXCR2 and IL-8 in tumours

Expression of both CXCR1 and CXCR2 has been observed in a wide variety of tumours including gastric carcinoma (Eck *et al.*, 2003a), nasopharyngeal carcinoma (Horikawa *et al.*, 2005) lung cancer (Zhu *et al.*, 2004), colon carcinoma cell lines Caco-2, KM12C and KM12L4 (Li, Varney, and Singh 2001), malignant melanoma (Varney, Johansson, and Singh 2006) and melanoma-derived cell lines A375P, A375SM and SBC-2 (Varney *et al.*, 2003). In addition, CXCR2 is expressed consistently in acute lymphomblastic leukaemias of B-lineage (Corcione *et al.*, 2006).

IL-8 is also produced by a variety of tumours. Tumours such as human transitional cell carcinoma and renal cell carcinoma express IL-8 both constitutively and in response to cytokines (Abruzzo *et al.*, 1992). IL-8 is expressed *in vivo* in primary gastric carcinoma (Eck *et al.*, 2003b) as well as in colon carcinoma cell lines Caco2, KM12C and KM12L4 (Li, Varney, and Singh 2001). Expression in non-small lung cancer (Wang et al. 1996), breast cancer (Miller *et al.*, 1998) pancreatic (Kamohara *et al.*, 2007) and prostate cancer cells (Inoue *et al.*, 2000) has also been reported.

1.2.13.6 Role of CXCR1, CXCR2 and IL-8 in tumour biology

As mentioned earlier, IL-8 can act as an angiogenic agent in tumours. In addition, the interaction of IL-8 with CXCR1 and CXCR2 on cancer cells can yield a number of other responses.

i) Proliferation

IL-8 mediates *in vitro* proliferation of epithelial cells in a number of tumours including prostate (Araki *et al.*, 2007), melanoma (Varney *et al.*, 2003), non small cell lung carcinoma (Luppi *et al.*, 2006), colon (Li, Varney, and Singh 2001), breast (Yao *et al.*, 2007), hepatocellular (Akiba *et al.*, 2001) and epidermoid cancer cells (Metzner *et al.*, 1999). Individual roles for CXCR1 and CXCR2 in IL-8 mediated proliferation have also been studied. IL-8 increases proliferation in melanoma and prostate cancer through both CXCR1 and CXCR2 as addition of antibodies to either receptor significantly decreases proliferation (Murphy *et al.*, 2005; Varney *et al.*, 2003). However, another study showed that CXCR1 plays a more important role in IL-8 mediated proliferation in prostate cancer than CXCR2 (Araki *et al.*, 2007). The findings of Metzner *et al.*, indicate that CXCR2 is the predominant receptor in IL-8 mediated proliferation in epidermoid carcinoma compared with CXCR1 (Metzner *et al.*,1999). This suggests that different cells may respond in a different manner to IL-8 and that signaling through CXCR1 or CXCR2 may vary depending upon the cell type or the response.

ii) Migration and invasion

Exposure to IL-8 causes migration and invasion of a variety of cancer cells *in vitro* including ovarian (So *et al.*, 2004), breast (Kim *et al.*, 2005; Yao *et al.*, 2007) colon (Li, Varney, and Singh 2001), gastric (Kitadai *et al.*, 2000) pancreatic (Kuwada *et al.*, 2003) and hepatocellular cancer (Kubo *et al.*, 2005). Some data exists regarding the individual roles of CXCR1 and CXCR2 in IL-8 induced cell migration. Migration of colon carcinoma cells is mediated through CXCR1 and not CXCR2 (Bates, DeLeo, III, and Mercurio 2004). In contrast, CXCR2 and not CXCR1 appears to mediate migration of HUVECs and human dermal microvascular endothelial cells (HMECs) towards IL-8 (Li *et al.*, 2005). However the individual roles of CXCR1 and CXCR2 in cancer cell migration and invasion need to be characterized further.

1.2.13.7 Expression and role of CXCR1, CXCR2 and IL-8 in oral cancer

Expression of both IL-8 receptors CXCR1 and CXCR2 has been reported in OSCC *in vitro* and *in vivo*. Head and neck squamous cell carcinoma (HNSCC) cells and associated microvascular endothelial cells (MVEC) express both receptors and IL-8 *in vivo* and staining intensity is much higher in well-differentiated cells within the tumour compared to basal layer and normal oral epithelium (Richards *et al.*, 1997). However this group did not perform any *in vitro* studies and did not provide any comparison of staining intensity or pattern between normal and cancerous oral epithelial cells even though controls were included in the study.

IL-8 expression has been reported in fresh tumour homogenates as well as primary cultures of HNSCC and continuous HNSCC cell lines. Stimulation of both primary culture and cell lines with IL-1 and TNF significantly up-regulates IL-8 expression while no such effect is observed with other cytokines (Cohen *et al.*, 1995). This suggests that IL-8 expression in HNSCC may be regulated by cytokines such as IL-1 and TNF.

IL-8 is detected in the supernatants of HNSCC cultures derived from cell lines as well as primary tumours. Serum concentration of IL-8 is significantly higher in OSCC patients compared with healthy controls and a correlation exists between IL-8 serum concentration and primary tumour volume (Chen *et al.*, 1999). An association between IL-8 gene polymorphism and OSCC risk has also been shown. Polymorphism of IL-8 (-251 A/T) which influences IL-8 gene expression, is directly proportional to increased OSCC risk (Vairaktaris *et al.*, 2007). This suggests a role for IL-8 in the biology of OSCC and it may hold potential as a biomarker

IL-8 expression is induced in oral and pharyngeal tumour cells *in vitro* after stimulation with fibrin while fibrinogen, thrombin and collagen are unable to do so (Lalla *et al.*, 2001). IL-8 expression is also observed in the cytoplasm of OSCC tissue sections adjacent to fibrin deposition *in vivo* again suggesting a possible association between fibrin and IL-8 in OSCC (Lalla *et al.*, 2003). It appears that fibrin can stimulate production of tumourigenic agents such as IL-8 from the tumour cells. Since IL-8 is a potent angiogenic factor, another possibility is that new blood vessel

formation facilitates increased fibrinogen outflow into the tumour microenvironment. This fibrinogen (after conversion into fibrin) can cause a further increase in IL-8 production. However, this interaction needs to be studied in much more detail to establish the precise relationship between fibrin and IL-8 in OSCC.

In SCID mice, secretion of IL-8 by endothelial cells in OSCC overexpressing BCl-2 is up-regulated and directly induces angiogenesis and proliferation. Addition of an anti-IL-8 antibody significantly reduces both (Nor *et al.*, 2001). In addition, systemic treatment of these SCID mice with anti-IL-8 antibody significantly reduces tumour weight and volume whereas no such effect is observed in the control group. Administration of an anti-IL-8 antibody also facilitates a significant decrease in tumour neovascularization compared with controls. However, this group did not study the expression or role of CXCR1 and CXCR2 in their experimental model and therefore it is not known which receptor is more important in mediating these effects.

Constitutive *in vitro* expression of CXCR1, CXCR2 and IL-8 is observed in the HSC-4 and NA oral cancer cell lines (Watanabe *et al.*, 2002). CXCR1 expression is higher in these cells lines compared with CXCR2 and this expression is not affected by addition of TNF-α and IL-1β. However, IL-8 expression is significantly up-regulated after stimulation with TNF-α and IL-1β suggesting that cytokines in the tumour microenvironment may regulate IL-8 release by OSCC cells. Exposure to IL-8 causes migration and invasion of NA and HSC-4 cells *in vitro* in a dose dependent manner. Administration of both CXCR1 and CXCR2 blocking antibodies partially inhibits IL-8 mediated migration but does not reduce it to control levels. The migrational/invasive capacity of oral cancer cells appears proportional to the expression levels of IL-8 receptors as HSC-4 cells expressing higher levels of CXCR1 and CXCR2 migrate and invade more than the NA cells (Watanabe *et al.*, 2002). However this study has a number of shortcomings. All of their experiments were performed *in vitro* and no *in vivo* studies were carried out to corroborate the *in vitro* findings. Expression of CXCR1 and CXCR2 was not studied at mRNA level. Also, they did not compare expression of CXCR1 and CXCR2 on oral cancer cells to that on normal oral keratinocytes which may have provided some valuable insight into the difference in expression and behaviour between normal and cancerous oral epithelial

cells. Furthermore, they did not use blocking antibodies against CXCR1 and CXCR2 to study their individual roles in IL-8 mediated cell invasion.

To summarize, IL-8 and its receptors CXCR1 and CXCR2 are expressed in malignant oral epithelial cells. IL-8 appears to mediate angiogenesis, proliferation, migration and invasion in OSCC but whether this is mediated by CXCR1 or CXCR2 is not well characterized. Migration appears to be mediated through both CXCR1 and CXCR2 whereas the roles of the receptors in angiogenesis, proliferation or invasion are not known. Overall, these findings suggest an important role for IL-8 in the biology of OSCC.

1.2.14 AIMS AND OBJECTIVES

As mentioned previously in Section 1.2.8, the preliminary microarray results suggested that mRNA for XCR1 is present in both normal oral epithelial cells as well as oral cancer cell lines. However it is not known whether XCR1 is expressed on the surface of these cells and if so whether the oral cells respond to its ligand lymphotactin.

The micro-array data also suggested that CXCR1 mRNA appears to be up-regulated in oral cancer cell lines compared with normal oral epithelial cells. However it is not known whether normal and malignant oral epithelial cells differ in their response to IL-8, the ligand for CXCR1, or if its effects are mediated through CXCR2, the alternate IL-8 receptor.

1.2.14.1 Aim

The overall aim of this study is to investigate the roles of XCR1 and its ligand lymphotactin and CXCR1 and CXCR2, receptors for IL-8 in the regulation of both normal and malignant oral epithelial cell behaviour.

1.2.14.2 General Objectives

1. To confirm the preliminary data and determine whether XCR1, CXCR1 and

CXCR2 are expressed by normal and malignant oral epithelial cells.

2. To determine whether these receptors are functional on the surface of normal and cancer cells and respond to their respective ligands.

1.2.14.3 Specific Objectives

1. To determine whether mRNA for XCR1, CXCR1 and CXCR2 is present in normal oral keratinocytes (NOK) and oral cancer cell lines (OCCL).
2. To determine whether XCR1, CXCR1 and CXCR2 are expressed on the surface of NOK and OCCL.
3. To determine whether XCR1, CXCR1 and CXCR2 are functional on the surface of these cells and can respond via signal transduction, migration, invasion and proliferation.
4. If these receptors are functional, to find out whether there is any difference in response of normal and malignant oral epithelial cells.

1.2.15 EXPERIMENTAL DESIGN

The following techniques will be used to determine whether NOK express XCR1, CXCR1 and CXCR2 receptors *in vitro.*

1. RT-PCR will be employed to study receptor expression at mRNA level.
2. Immunocytochemistry and flow cytometry will be used to study receptor expression at the protein level.
3. A cell-based ELISA will be performed to study signal transduction in oral epithelial cells in response to the ligands for the receptors.
4. Migration/chemotaxis and invasion assays will be performed to study effect on cell migration and invasion.
5. Proliferation assays will be performed to study the role of these receptors and their ligands in cell proliferation.

For this purpose, primary oral keratinocytes and gingival fibroblasts will be grown along with a range of oral cancer cell lines (derived from oral squamous cell carcinoma) for comparison. The melanoma cell line A375P and isolated neutrophils

will be used as positive controls for CXCR1 and CXCR2 as they have been shown to express CXCR1 and CXCR2 previously (Moser *et al.*, 1993; Varney *et al.*, Singh 2003). The T-cell leukaemia cell line Jurkat and isolated neutrophils will be used as a positive controls for XCR1 (Blaschke *et al.*, 2003; Huang *et al.*, 2001b).

CHAPTER 2

Expression of XCR1, CXCR1 and CXCR2 by Oral Epithelial Cells

CHAPTER 2a

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mRNA Expression

2.1 INTRODUCTION

A number of factors have to be taken into consideration while studying the physiological or pathological significance of a cell surface receptor. First, it is essential to show that the mRNA for the receptor is present. Second, the receptor should be expressed on the cell surface, and third, stimulation of the receptor should result in a functional response.

Therefore, first of all mRNA expression of XCR1, CXCR1 and CXCR2 was studied to confirm expression in normal and cancerous oral epithelial cells.

2.2 AIM

The aim of this chapter was to establish whether the mRNA for XCR1, CXCR1 and CXCR2 is present in normal and malignant oral cells.

2.3 EXPERIMENTAL PROTOCOL

RT-PCR was used for subjective analysis of mRNA expression. The following methods were used in conjunction:

- Normal oral keratinocytes (NOK), normal skin keratinocytes (NSK) and oral cancer cell lines (OCCL) (H357, TR146, SCC4, SCC25, CAL27, FADU) were cultured along with human gingival fibroblasts (HGF).
- A375P cell line was used as a positive control for CXCR1 and CXCR2 as it has been previously shown to express these two receptors (Varney *et al.*, 2003).
- Commercially available neutrophil RNA (Ambion) was used as positive control for XCR1 as well as CXCR1 and CXCR2.
- RNA was isolated from the cultured cells to be used for RT-PCR. Isolated RNA was quantified and its quality checked using gel electrophoresis and spectrophotometry.
- Primers were designed for all receptors (sense and anti-sense).
- DNase-treatment of all RNA samples was carried out to eliminate the risk of false positive results due to genomic DNA contamination.
- RT-PCR was carried out at least three times for XCR1, CXCR1 and CXCR2.

2.4 MATERIALS AND METHODS

2.4.1 CELL CULTURE

2.4.1.1 Cell types

The following cells were used in this study.

1. Primary cells (Non-cancerous)

i. Primary human gingival fibroblasts (HGF)

Primary human gingival fibroblasts (HGF) were obtained from the department archive of frozen cells (Department of Oral Pathology, University of Sheffield). These cells were originally obtained from clinically healthy gingival tissue removed during third molar extraction and originally frozen in 1999.

ii. Primary Normal oral keratinocytes (NOK)

Normal oral keratinocytes (NOK) were a gift from Jonathan Collier (Barts and The London). These cells were obtained from clinically healthy tissue removed during third molar extraction and frozen down after second passage.

iii. Primary normal skin keratinocytes (NSK)

Normal skin keratinocytes (NSK) were a gift from Jonathan Collier (Barts and The London). They were established from normal skin removed at the time of breast reduction surgery and frozen at the time of first passage in 2002.

2. Established cancer cell lines

i. H357

The cell line H357 was a gift from Prof. Steve Prime (Bristol) and is derived from a well differentiated oral squamous cell carcinoma from the tongue of a 74 year old male (Prime *et al.*, 1990). The original tumour was less than 4cm in diameter with regional lymph node involvement but no distant metastases.

ii. TR146

The continuous cell line TR146 is derived from a human neck metastasis originating from a buccal carcinoma (Rupniak *et al.*, 1985).

iii. SCC4

The SCC4 cell line (ATCC, CRL-1624) is derived from a squamous cell carcinoma of the tongue of a 55 year old male (Rheinwald and Beckett 1981).

iv. SCC25

The SCC25 cell line (ATCC, CRL-1628) is derived from a squamous cell carcinoma of the tongue of a 70 year old male (Rheinwald and Beckett 1981).

v. CAL27

The CAL27 cell line (ATCC, CRL-2095) was established in 1982 from tissue taken prior to treatment from a 56 year old caucasian male with OSCC of the middle of the tongue (Gioanni *et al.*, 1988). CAL27 cells are epithelial, polygonal with a highly granular cytoplasm.

vi. FaDu

The FADU cell line (ATCC, HTB-43) was established in 1968 from a hypo-pharyngeal tumour removed from a 56 year old patient (Rangan 1972). The established line contains bundles of tono-filaments in the cell cytoplasm and desmosomal regions are prominent at cell boundaries.

vii. A375P

The A375P cell line, a variant of A375 cell line (ATCC, CRL-1619) is derived from malignant melanoma of a 53 year old female (Giard *et al.*, 1973).

viii. JURKAT

The Jurkat cell line (ATCC, TIB-152) was established from the peripheral blood of a 14 year old boy and expressed characteristics of leukaemic T cells in addition to complement receptors (Schneider, Schwenk, and Bornkamm 1977).

2.4.1.2 Media for Cells

NOK and H357 cell line were cultured in keratinocyte growth medium (KGM) (Formanek *et al.*, 1996) independent of fibroblast feeder cells (Appendix 10.1.1).

CAL27, FADU and Jurkat cells were cultured in RPMI-1640 with 25mM Hepes and L-Glutamine (Invitrogen, Carlsbad, CA, USA) (Appendix 10.1.4).

HGF, TR146 and A375P cells were grown in Dulbecco's Modified Eagle Medium (Invitrogen) (Appendix 10.1.2). Medium for SCC4 and SCC25 cells was prepared using DMEM:F12 (1:1 Mix) with 15mM Hepes and L-Glutamine (Invitrogen) (Appendix 10.1.3). All media contained 10% foetal bovine serum (Invitrogen), penicillin/streptomycin (Invitrogen) and fungizone/Amphotericin B (Invitrogen) unless otherwise specified.

2.4.1.3 Culture of cells

Cells were cultured in 12ml of their respective media in T75 cm^2 flasks (Nunc GmbH, Wiesbaden, Germany) at 37°C in 5% CO_2 and 99% humidity. They were fed every third day.

2.4.1.4 Passaging of cells

All cell lines were cultured in T75cm^2 tissue culture flasks until confluent and passaged. Medium was removed from the flasks by aspiration and 5ml of sterile phosphate buffered saline (PBS) used to wash the cells. 3ml trypsin/EDTA (Invitrogen) was introduced in the flask after PBS removal. The flask was incubated in a CO_2 incubator until the cells could be dislodged by gentle agitation. 3ml of medium was added to neutralize the trypsin. This cell suspension was transferred to a 15ml conical centrifuge tube and centrifuged for 5min at 1000rpm. Medium was removed by aspiration taking special care to avoid removing the cell pellet at the base of the tube. Cells were re-suspended in 2ml of their respective fresh medium and 1ml of this added to two new T75cm^2 flasks containing 11ml of fresh medium. The flasks were labelled and placed in a CO_2 incubator at 37 °C in 99% humidity.

The Jurkat cell line grows in suspension and does not require trypsin for removal from the tissue culture flask. Cell suspension was removed from the flask, added to a conical centrifuge tube and centrifuged as described previously. Medium was

aspirated and cells were re-suspended in fresh medium before being added to a new flask.

2.4.2 RNA EXTRACTION AND ISOLATION

2.4.2.1 RNA Extraction

RNA extraction was carried out when the cells were 80-100% confluent. Trizol (Invitrogen) which is a reagent for total RNA isolation was used for this purpose.

Medium was removed from the T75cm^2 flasks and 1ml of Trizol added to each flask. The entire growth surface of the flask was thoroughly wetted with Trizol and a cell scraper used to harvest the cells. This mixture of Trizol and cells (cell lysate) was transferred to a 2ml micro-centrifuge tube. Samples were homogenized by vigorous pipetting, allowed to stand for 10min and archived at -80° C to be isolated later.

2.4.2.2 RNA Isolation

A range of materials were used for isolation and analysis of RNA (Appendix 10.2.1 and 10.2.2).

Cell lysates were allowed to warm to ambient temperature after retrieval from the -80°C archive and 200µl of chloroform (Sigma, Dorset, UK) was added to each sample. The sample was covered, shaken vigorously for 20sec and incubated at room temperature (RT) for 10min. The mixture was then centrifuged at ≥ 10,000xg for 15min at 4°C. This separated the mixture into three phases i.e. the colourless upper aqueous phase (containing the RNA), a semi-solid interphase (containing most of the DNA), and a lower organic phase. The upper aqueous phase was carefully transferred into a clean RNase-free tube without disrupting the interphase, followed by the addition of 1ml of isopropanol (Sigma), mixed well and incubated at RT for 10min. The samples were then centrifuged at ≥ 10,000xg for 15min at 4°C to pellet the RNA. The supernatant was decanted and the pellet washed with 1ml of cold 75% ethanol (Sigma) by vortexing. Samples were centrifuged at ≥ 7,500xg for 5min at 4°C. The supernatant was discarded and the pellet air dried for 10min. RNA was re-suspended

in 100µl of RNase-free water (Sigma). Brief vortexing was carried out to aid re-suspension.

RNA was extracted from identically prepared samples from separate experiments. All dilutions and reagents were prepared in RNase-free water.

2.4.2.3 DNase Treatment of RNA

A final RNA concentration of 10µg was used in a 50µl solution. The following reagents were used:

- RNase-free DNase (Promega, Madison, WI, USA) — 10 µl (1 µl per µg of RNA)
- Buffer (Promega) — 5 µl
- RNase-free Water — variable

Samples were mixed and incubated at 37°C for 30 min.

2.4.2.4 RNA Re-isolation

A 50/50 volume mixture of phenol and chloroform was prepared. 50µl of this solution was added to the DNase-treated samples and mixed by vortexing. Samples were then centrifuged at 10,000 rpm for 5min. The upper aqeous phase was removed and sodium acetate (0.1 vol.) (Sigma) and ethanol (2.2 vol.) (Sigma) added. Samples were placed in a freezer for one hour to facilitate RNA precipitation. Centrifugation at 4°C at 10,000 rpm was performed after removal from the freezer to pellet the RNA. The supernatant was discarded and the pellet washed with 1ml of cold 75% ethanol for 5min. The supernatant was decanted and the pellet air dried for 5-10 min. RNA was re-suspended in 50µl RNase-free water. Brief vortexing was carried out to aid re-suspension.

2.4.3 ANALYSIS OF ISOLATED RNA

2.4.3.1 Spectrophotometry

A 1:100 dilution of all purified RNA samples was made (1µl sample + 99µl of RNase-free water). 100µl RNase-free water was used to blank the spectrophotometer. Water was discarded, the cuvette dried and the diluted sample read at 260nm (A1) and 280nm (A2). A1, A2 readings and the A1:A2 ratios were recorded and amount of isolated RNA calculated. Spectrophotometric analysis was performed on all RNA samples (Appendix 10.2.3.1).

2.4.3.2 RNA gel electrophoresis

A 1% agarose gel was prepared by heating 1g of RNase-free agarose (Sigma) in 72ml RNase-free water until dissolved, then cooled to 60°C. 10ml of MOPS running buffer (10x) (Eppendorf, Hamburg, Germany) and 18ml of 37% formaldehyde (12.3 M) (Sigma) were added to this mixture.

10x MOPS running buffer contains:

- 0.4 M MOPS, pH 7.0
- 0.1 M sodium acetate
- 0.01 M EDTA

The gel was poured after placing a teflon comb and enough 1x MOPS running buffer added to cover the gel by a few millimetres. The comb was removed and RNA samples were prepared by adding 0.5 x sample loading buffer (Sigma) to 1-3µl of RNA. Samples were heat denatured at 65-70°C for 5min. The gel was loaded and run at 90mV for 2/3rd of the length of the gel. The 28S and 18S bands for RNA were visualized on a UV transilluminator and a visual record was made using GeneSnap software (Syngene, Cambridge, UK) (Appendix 10.2.3.2).

2.4.4 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

2.4.4.1 Primer design

Primers for XCR1, CXCR1 and CXCR2 were designed using Primer3 which is a web-based primer designing application (Rozen and Skaletsky). Specificity of the primers was tested using the Basic Local Search Alignment Tool (BLAST) (Altschul *et al.*, 1997).

Following sequences were used:

i. XCR1

F/Sense: AGCTGGGGTCCCTACAACTT

R/Anti-sense: GACCCCCACGAAGACATAGA

i. CXCR1

F/Sense: AGCTTCTGTTCCGTGCTTGT

R/Anti-sense: TGTGGACAAAGGGATCTTCC

ii. CXCR2

F/Sense: ACAGCTACTTGGGAGGCTGA

R/Anti-sense: ATGGGTTCAAGTCCCTGCTT

2.4.4.2 RT reaction

The RT master mix per reaction was prepared as follows:

- $MgCl_2$ Buffer (Promega) — 4μl
- dNTPs — 1μl
- RNase inhibitor (Promega) — 0.5μl
- AmV RT buffer (Promega) — 0.5μl

- Oligo dT (Promega) 0.5μl

6.5μl of master mix was transferred to individual reaction tubes. 1μg/ml of RNA for each sample was added and the total volume made up to 20μl using RNase-free water. All samples were placed in a DYAD PCR machine and the following program was used:

- 42 °C for one hour
- 95 °C for 5 min
- 4°C forever

2.4.4.3 PCR

The PCR master mix per reaction was prepared as follows:

- $MgCl_2$ free buffer (Promega) 2.5μl
- dNTPs 1μl
- $MgCl_2$ (Promega) 2μl
- Taq polymerase (Promega) 0.2μl
- H_2O 17.3μl
- Sense/forward primer 1μl
- Antisense/reverse primer 1μl

24μl of this mix was transferred to respective PCR tubes and 1μl of cDNA was added. Samples were placed in a DYAD PCR machine and the following program was used:

- 94 °C for 1 min (Denaturation)
- 60 °C for 2 min (Annealing)
- 72 °C for 3 min (Extention)

After 35 cycles of the above mentioned program, the following steps were carried out:

- 72 °C for 7 min (Final extention step)

- 4 °C forever (cooling cycle)

2.4.4.4 Gel for PCR products

A 1% agarose gel was prepared by adding 1.5g agarose (Sigma) to 150ml of 1xTAE buffer (Tris base, acetic acid EDTA) (Invitrogen) and dissolved by heating in a microwave. After allowing the gel to cool, 1μl of ethidium bromide (Sigma) was added and mixed. The gel was then poured into the gel-casting tray containing a 1mm 16-space teflon comb. A mixture of the PCR sample (12μl) and loading dye (2μl) (Sigma) was loaded in each well and separated by running in TAE buffer at 90V for 40-60min along with 4μl DNA hyper ladder IV (Bioline, Taunton, MA, USA). The gel was viewed using a UV-transilluminator and a photographic record was made.

2.5 RESULTS

2.5.1 mRNA EXPRESSION OF XCR1

All tested cells showed mRNA for XCR1. A band for XCR1 mRNA was seen at 169 base pairs (bp) in the positive control (neutrophil RNA). A similar band was observed in NOK, NSK and HGF. XCR1 mRNA was also seen in all tested OCCL and surprisingly, A375P cells (positive control for CXCR1 and CXCR2) (Figure 2.1).

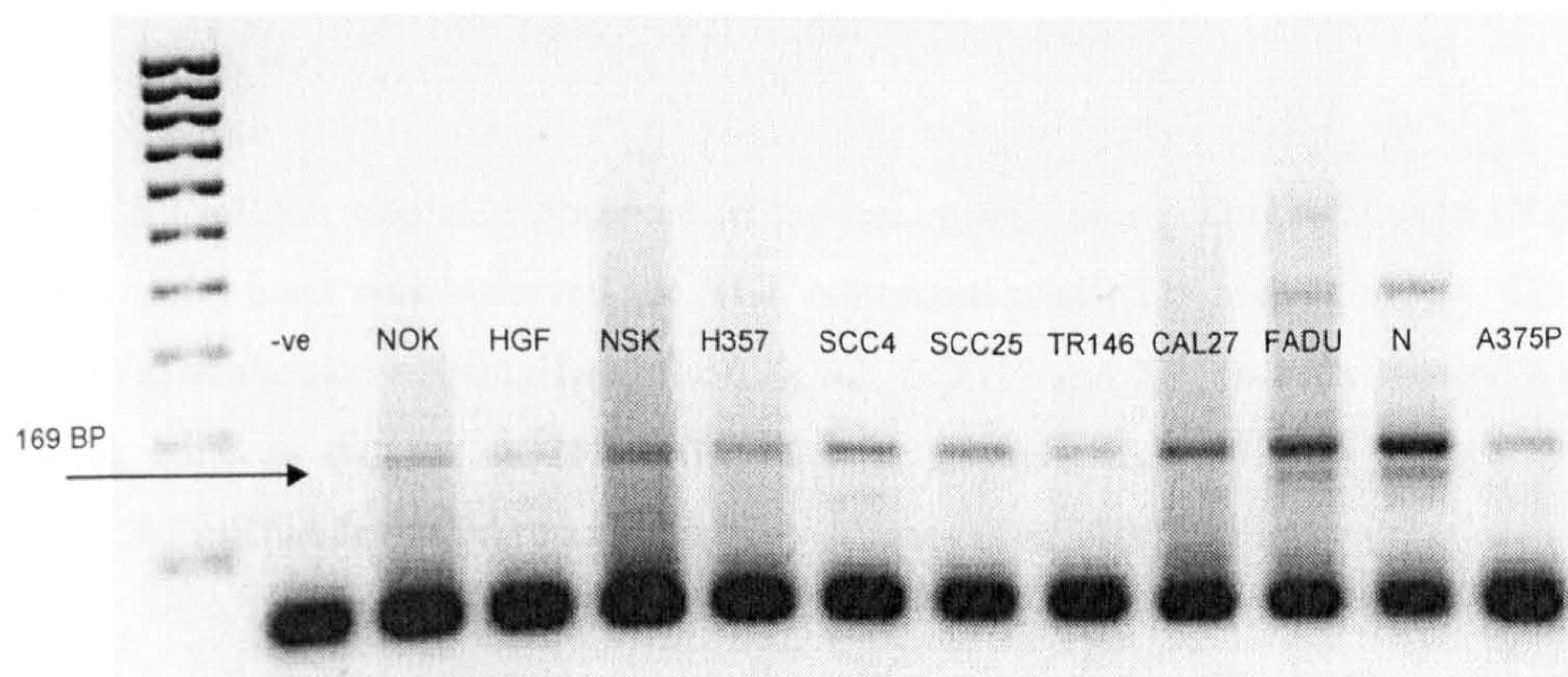


Figure 2.1. mRNA Expression of XCR1 (Where N denotes Neutrophil RNA) (Representative picture). Arrow indicates the PCR product of 169bp. Bands were strongest for neutrophils, CAL27, FaDu whereas NOK, HGF, TR146 and A375P showed weak bands.

2.5.2 mRNA EXPRESSION OF CXCR1 AND CXCR2

A band for CXCR1 mRNA was observed in the positive controls (neutrophil RNA and A375P) at 200bp. However, the band for A375P was very weak even though it has been shown to express CXCR1 previously (Varney *et al.*, 2003). Expression was also observed in NOK and HGF (Figure 2.2). The band for NOK appeared stronger than HGF. CXCR1 mRNA was also detected in OCCL however bands appeared much weaker for SCC25 and TR146 cells compared with other cells. Multiple bands were observed for some cells.

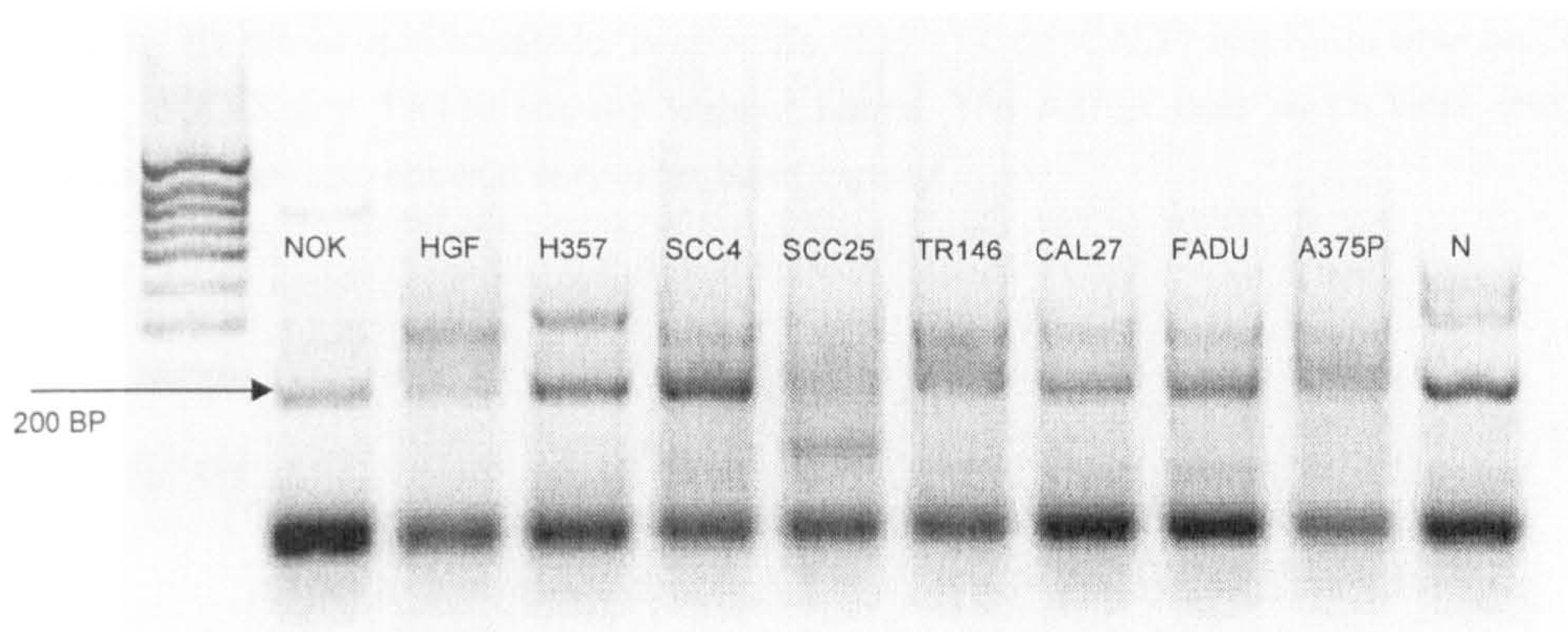


Figure 2.2. mRNA Expression of CXCR1 (Where N denotes Neutrophil RNA) (Representative picture). Bands were strongest for neutrophils, SCC4 and FaDu whereas NOK and TR146 showed weaker bands followed by HGF and SCC25. Surprisingly, the A375P cells which were used as positive control also showed very weak band density.

CXCR2 mRNA was also observed in the neutrophils along with NOK and HGF. A very weak band was observed for HGF compared with NOK. H357, SCC4, CAL27 and FaDu showed a strong band for CXCR2. SCC25 and TR146 cells showed a very weak band as did the A375P cells (used as positive control) even though strong CXCR2 mRNA has been reported before (Varney *et al.*, 2003) (Figure 2.3).

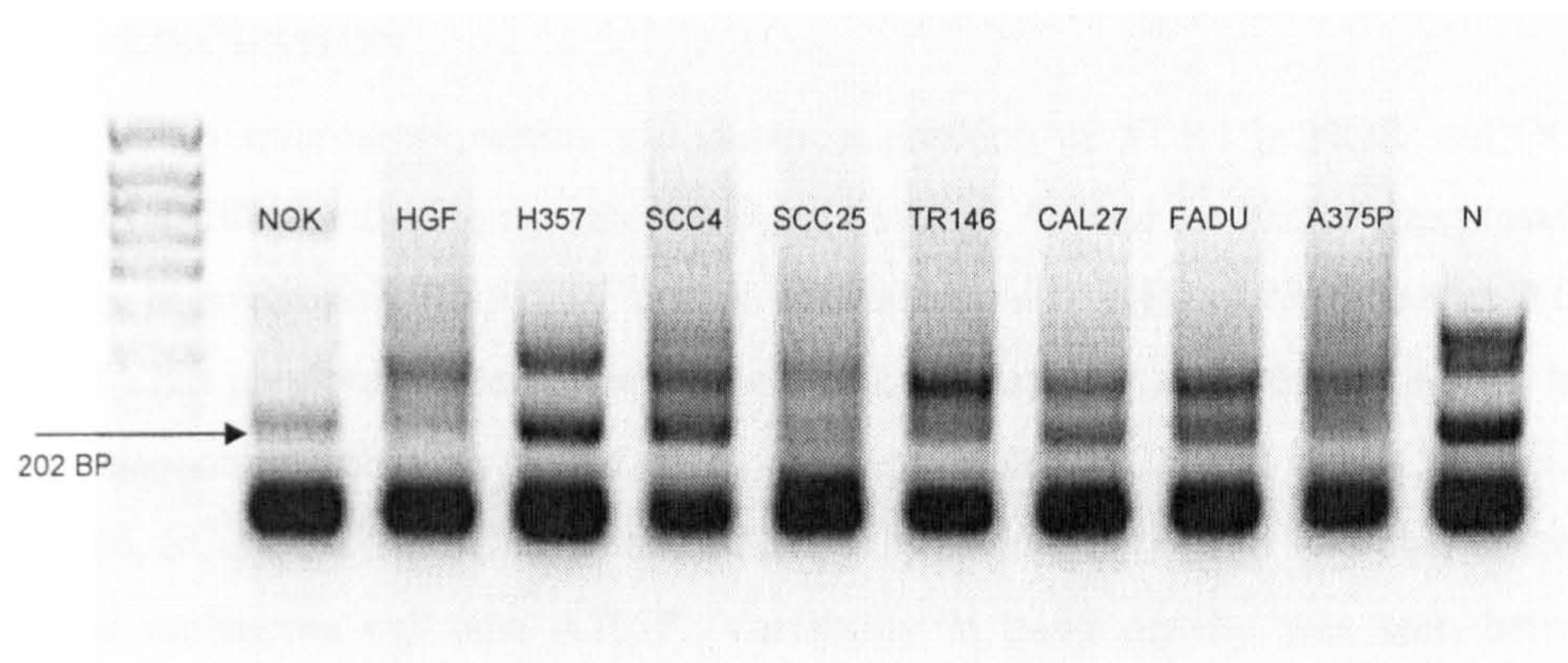


Figure 2.3. mRNA Expression of CXCR2 (Where N denotes Neutrophil RNA) (Representative picture). Bands were strongest for neutrophils, H357, SCC4, CAL27 and FaDu whereas NOK, HGF, SCC25 and TR146 showed weaker bands. The A375P cells which were used as positive control also showed very weak band density.

2.6 DISCUSSION

Preliminary microarray results had shown expression of XCR1 in NOK and OCCL and RT-PCR was used to corroborate these findings. A band for XCR1 was present at 169bp in neutrophil RNA and this is agreement with existing literature as XCR1 mRNA has previously been shown in neutrophils (Huang *et al.*, 2001). A similar band was also observed in all tested cells including NOK, HGF and all OCCL (H357, TR146, SCC4, SCC25, CAL27 and FADU). Interestingly, a band was also observed in the melanoma cell line A375P. Variability in band density was seen between different cell types which could not be quantified as RT-PCR only provides a subjective assessment of mRNA expression. It is therefore not clear whether this variability represents differences in the levels of mRNA.

A band for CXCR1 mRNA was detected in the positive controls (neutrophil RNA and the A375P cell line) at 200bp which is consistent with previous findings (Moser *et al.*, 1993; Varney *et al.*, 2003). A 200bp band was also seen in both normal and cancerous oral cells. Similar results were obtained for CXCR2. A 202bp band for CXCR2 mRNA was observed in OCCL as well as the positive controls (Neutrophils and A375P). Expression in NOK was also detected but HGF appeared negative for CXCR2.

To our knowledge, XCR1 mRNA has predominantly been shown in lymphoid cells (Huang *et al.*, 2001). Recent reports have shown XCR1 mRNA in fibroblast-like synoviocytes (Blaschke *et al.*, 2003) and in melanoma cells lines (Seidl *et al.*, 2007). However, the significance of this expression is not completely understood. Moreover, XCR1 mRNA in epithelial cells has not been reported to date. Expression of CXCR1 and CXCR2 on oral epithelial cells has only been shown at protein level (Sfakianakis, Barr, and Kreutzer 2002; Watanabe *et al.*, 2002). CXCR1 and CXCR2 mRNA was also seen in HGF which has not been reported before.

The RT-PCR results confirmed that the message for XCR1, CXCR1 and CXCR2 is present in both normal and cancerous oral epithelial cells at RNA level. Therefore, the next step was to study protein expression of the receptors.

CHAPTER 2b

Protein Expression

2.7 INTRODUCTION

The RT-PCR results in the previous section showed that mRNA for XCR1, CXCR1 and CXCR2 is present in oral epithelial cells. Therefore, it was important to establish whether this mRNA is translated into protein and expressed on cell surface.

2.8 AIM

The aim of this chapter was to establish whether XCR1, CXCR1 and CXCR2 are expressed on the surface of normal and cancerous oral cells.

2.9 EXPERIMENTAL PROTOCOL

The following methods were used:

1. Cell Culture

- NOK, HGF and OCCL were cultured as previously described.
- A375P cell line and isolated neutrophils were used as positive controls for CXCR1 and CXCR2 (Varney *et al.*, 2003; Moser *et al.*, 1993).
- The T-cell leukaemia cell line Jurkat and isolated neutrophils acted as positive controls for XCR1 (Blaschke *et al.*, 2003; Huang *et al.*, 2001)

2. Immunocytochemistry

- Immunocytochemistry was performed on cultured monolayers of cells for subjective assessment of receptor expression at protein level. Staining was carried out at least three times for each cell type.

3. Flow Cytometry

- Flow cytometry was performed on cultured cells to quantify cell surface expression of XCR1, CXCR1 and CXCR2. Assay was performed three times for each cell type.

2.10 MATERIALS AND METHODS

2.10.1 CELL CULTURE

Cells were grown and maintained as previously described.

2.10.2 IMMUNOCYTOCHEMISTRY

2.10.2.1 Preparation of cultured cell monolayers

Cells were detached from flasks using a non-enzymatic cell dissociation buffer (Sigma). Cells were seeded on BD-Falcon chamber slides (BD Biosciences, Franklin Lakes, NJ, USA) and grown for 48 hours before being washed and fixed with 2% paraformaldehyde for 10min at RT. Fixative was washed and slides were stored in PBS at 4°C to prevent drying.

2.10.2.2 Immunocytochemistry protocol

An indirect streptavidin-biotin immunoperoxidase technique was employed to detect expression in all the cell types. A Vectastain kit was used for this purpose (Vector Laboratories, Burlingame, CA, USA). A pilot study determined the optimum dilutions for the primary antibodies to be 20μg/ml.

Slides recovered from storage were washed three times in PBS and endogenous peroxidase blocking achieved via 10min incubation with ChemMate peroxidase-blocking solution (Dako, Glostrup, Denmark) at RT. Cells were washed three times in PBS and serum block was applied for 30min at RT. 20μg/ml dilutions of primary antibodies were prepared using the blocking serum solution. The following primary antibodies were used:

- Rabbit anti-human XCR1 antibody (Lifespan Biosciences, Seattle, WA, USA)
- Mouse anti-human CXCR1 antibody (R & D Systems, Minneapolis, MN, USA)
- Mouse anti-human CXCR2 antibody (R & D Systems)

Omission of the primary antibody and negative isotype antisera served as negative controls in the staining procedure. Cells were incubated with primary antibodies for

one hour and were washed in PBS three times. Secondary antibodies were prepared (provided with the Vectastain kit) and applied for 30min. ABC solution was prepared alongside secondary antibodies and allowed to stand for 30min before use (as recommended by the kit protocol). After removal of secondary antibodies, cells were washed three times in PBS and incubated with the ABC solution for 30min. After three more washes in PBS, the colouring solution (provided with the kit) was applied for 5min and slides washed with distilled water. Mayers haemotoxylin was used as a counterstain and slides were washed, dehydrated, cleared and mounted in DPX.

Specimens were viewed and photographed using a digital camera. A red-brown staining reaction was considered a positive result when compared to negative controls.

2.10.3 FLOW CYTOMETRY

Single cell suspensions were prepared using a non-enzymatic solution (Sigma, Cat. No. Cat. No. C5914) and washed in staining buffer (PBS+2%FBS+1%BSA) by centrifugation (400xg, 5min, 4°C). The supernatants were discarded and cells re-suspended ($1x10^6$ cells/ml) in ice cold staining buffer. 200µl of cells were aliquoted into polypropylene tubes. The same antibodies as immunocytochemistry were used in this assay. Neutrophils were isolated from the blood of healthy volunteers and used as positive control XCR1, CXCR1 and CXCR2 (Appendix 10.3)

Primary antibodies were added (20µg/ml), mixed by vortexing and incubated for 30min at 4°C. Appropriate negative isotype antisera served as negative controls (Rabbit serum for XCR1, mouse immunoglobulin for CXCR1 and CXCR2) (Dako).

Cells were washed three times in 1ml staining buffer (400xg, 5min, 4°C), and re-suspended. The following FITC-conjugated secondary antibodies were added (10µl/million cells):

- Swine anti-rabbit antibody (for XCR1), (Dako)
- Goat anti-mouse antibody (for CXCR1 and CXCR2), (Dako)

Cells were vortexed and incubated for 30min with secondary antibodies at 4°C in the dark. Cells were washed three times, re-suspended and kept on ice prior to analysis. Data was acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Staining was considered positive when the percentage of positive cells for test antibodies was higher than the negative controls.

2.11 RESULTS

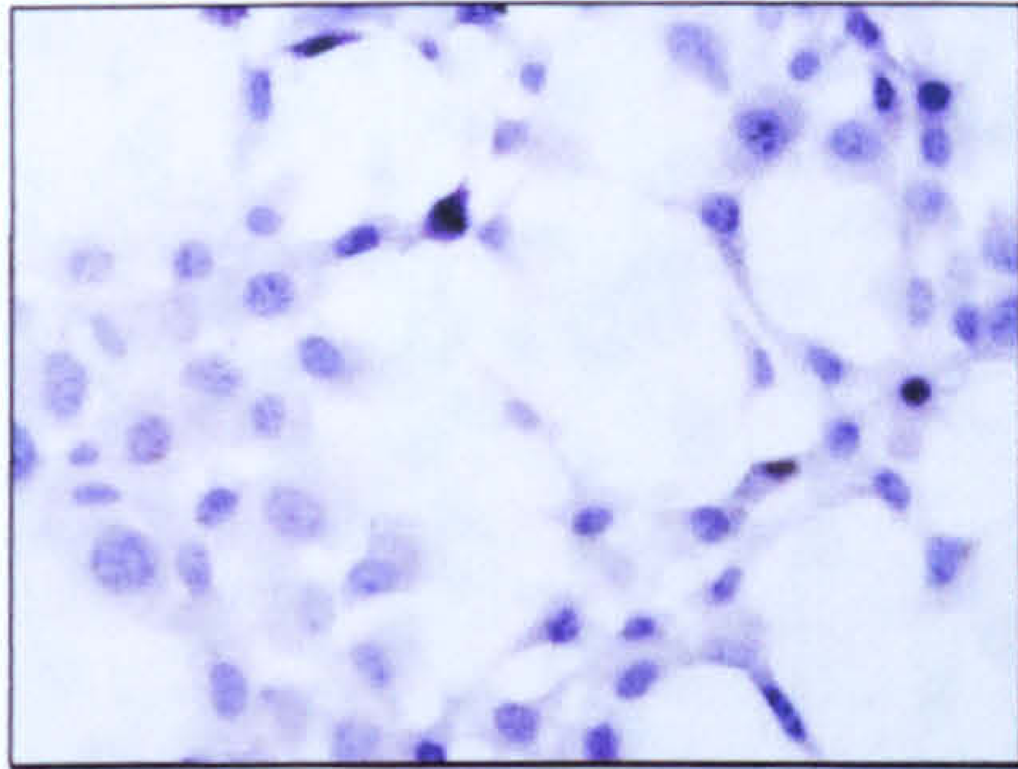
2.11.1 IMMUNOCYTOCHEMISTRY

2.11.1.1 XCR1

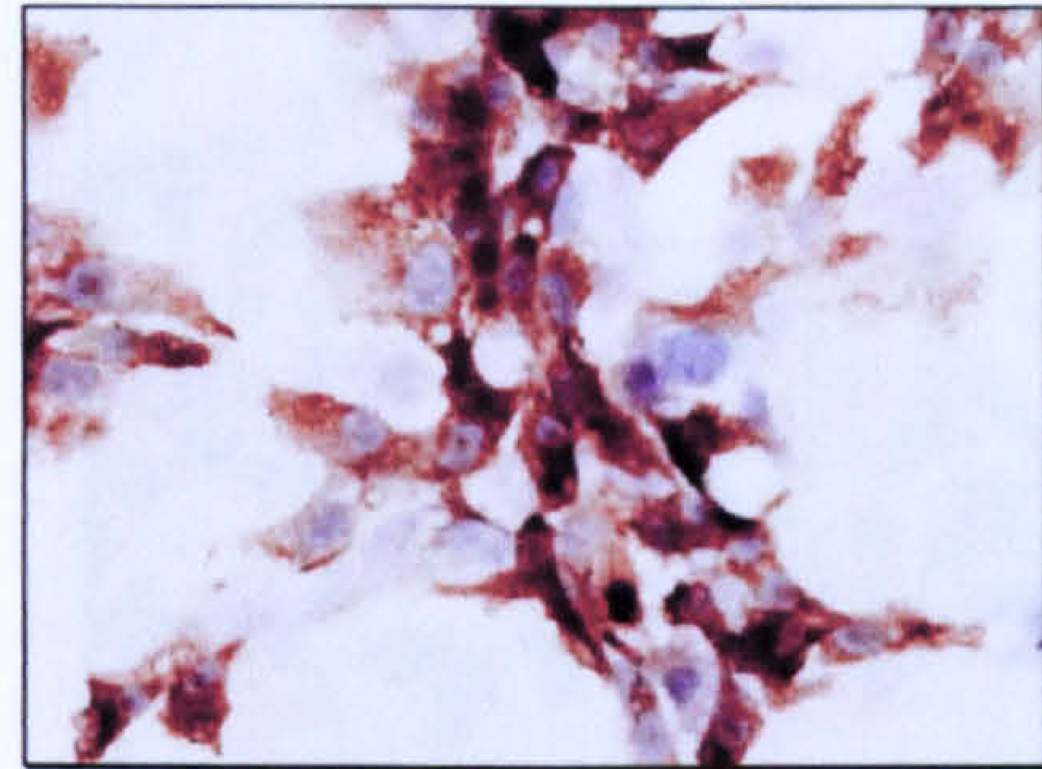
All tested cells stained consistently for XCR1. Staining was seen in NOK as well as HGF. Three distinct cell populations were observed in NOK i.e. a negative population and two positive ones, one with low staining intensity and a second with higher staining intensity. Staining in HGF however, was more uniform. Both NOK and HGF exhibited cell surface as well as cytoplasmic staining (Figure 2.4).

OCCL (H357, TR146, SCC4) also stained for XCR1. In H357 cells (like NOK) three different populations were noticed as some cells showed stronger staining intensity than others and negative cells were also observed. This staining pattern was not observed in TR146 and SCC4 cells. Surprisingly, A375P cells (used as positive control for CXCR1 and CXCR2) also consistently stained positive for XCR1. In all cancer cells, XCR1 staining was seen on the cell surface as well as in the cytoplasm.

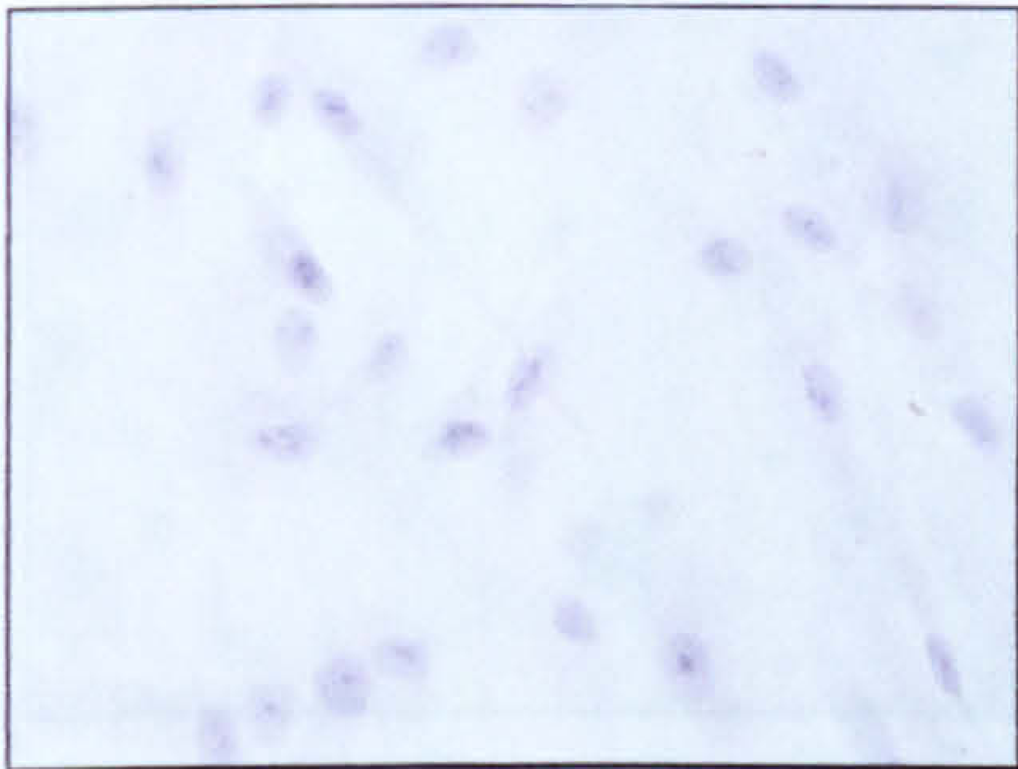
No staining was observed in the negative controls (Figure 2.4). SCC4 and A375P cells appeared to have stronger staining intensity than other cell lines.



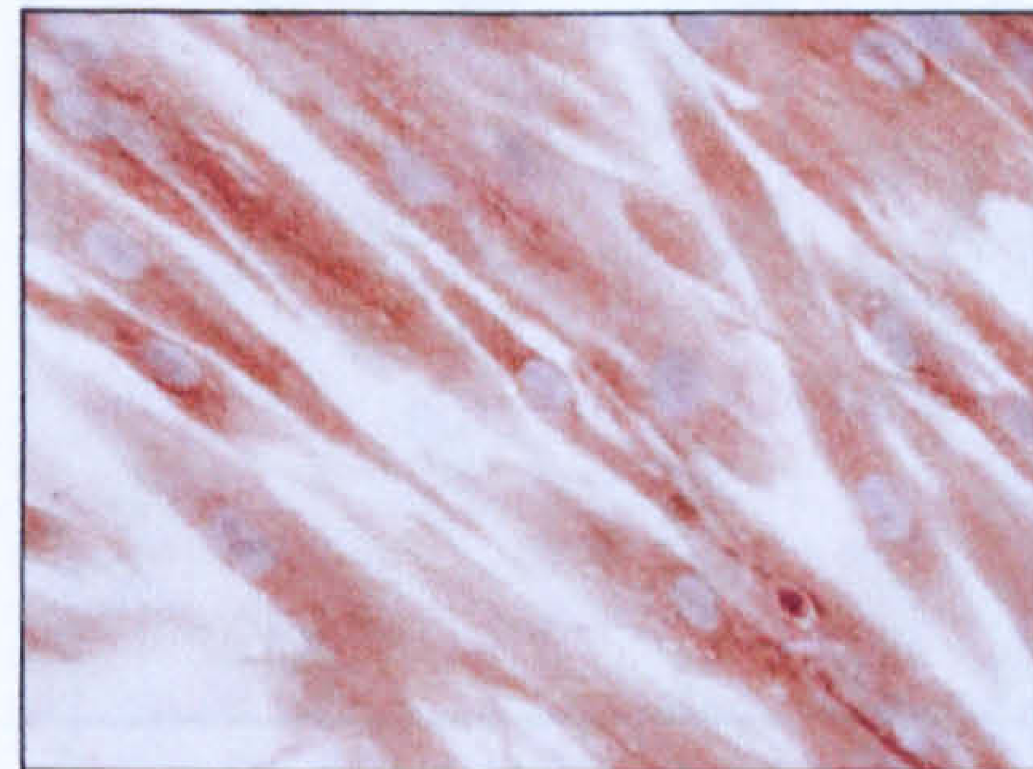
A) NOKs **-ve control**



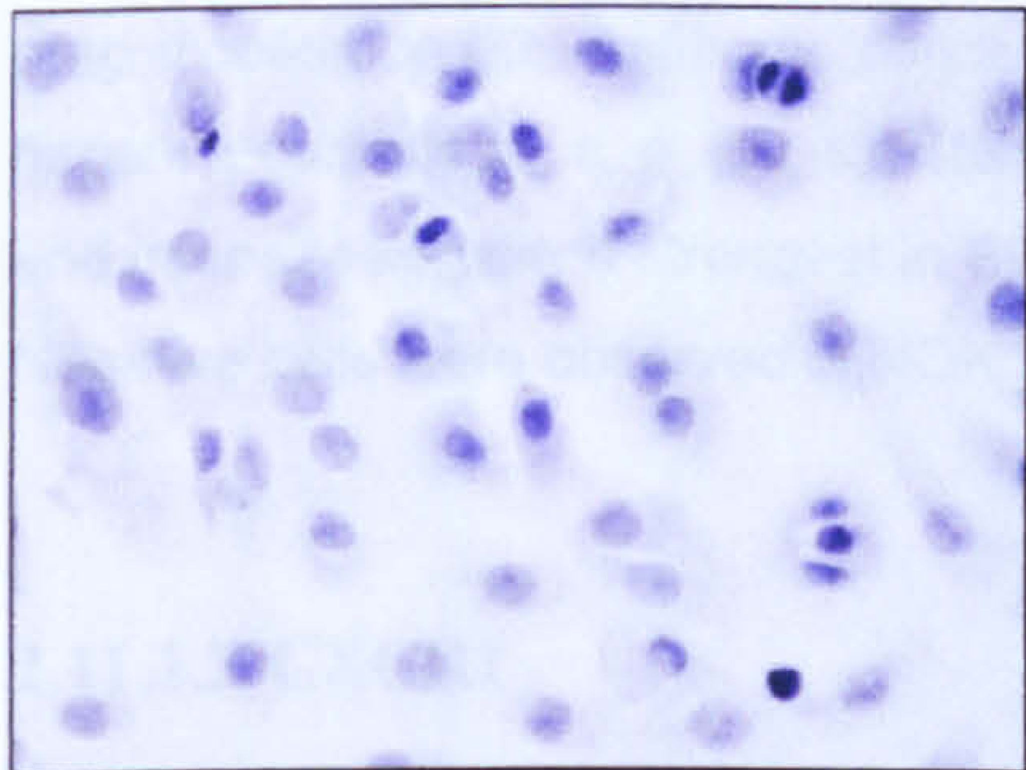
B) NOKs **XCR1**



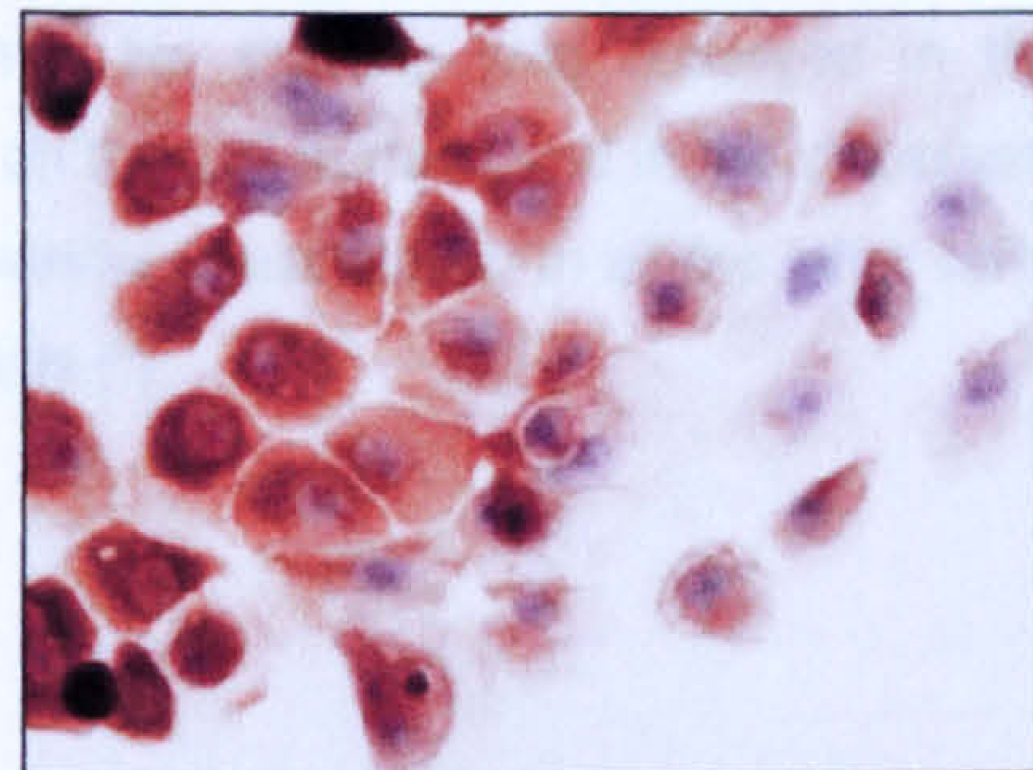
C) HGFs **-ve control**



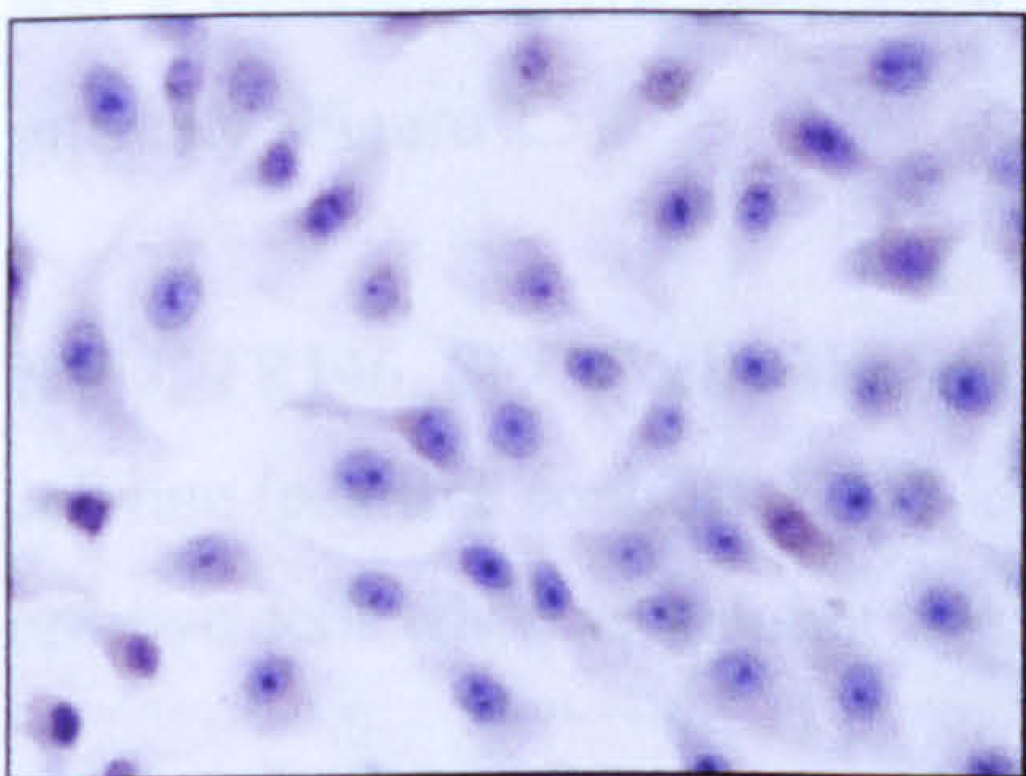
D) HGFs **XCR1**



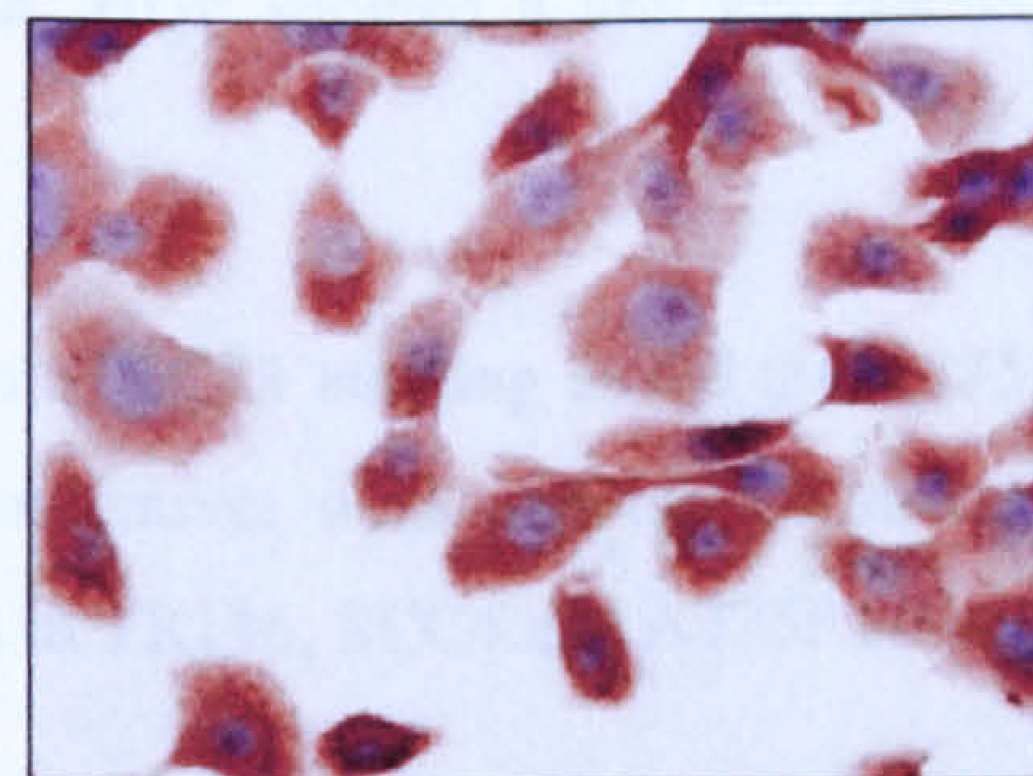
E) H357 cells **-ve control**



F) H357 cells **XCR1**



G) TR146 cells **-ve control**



H) TR146 cells **XCR1**

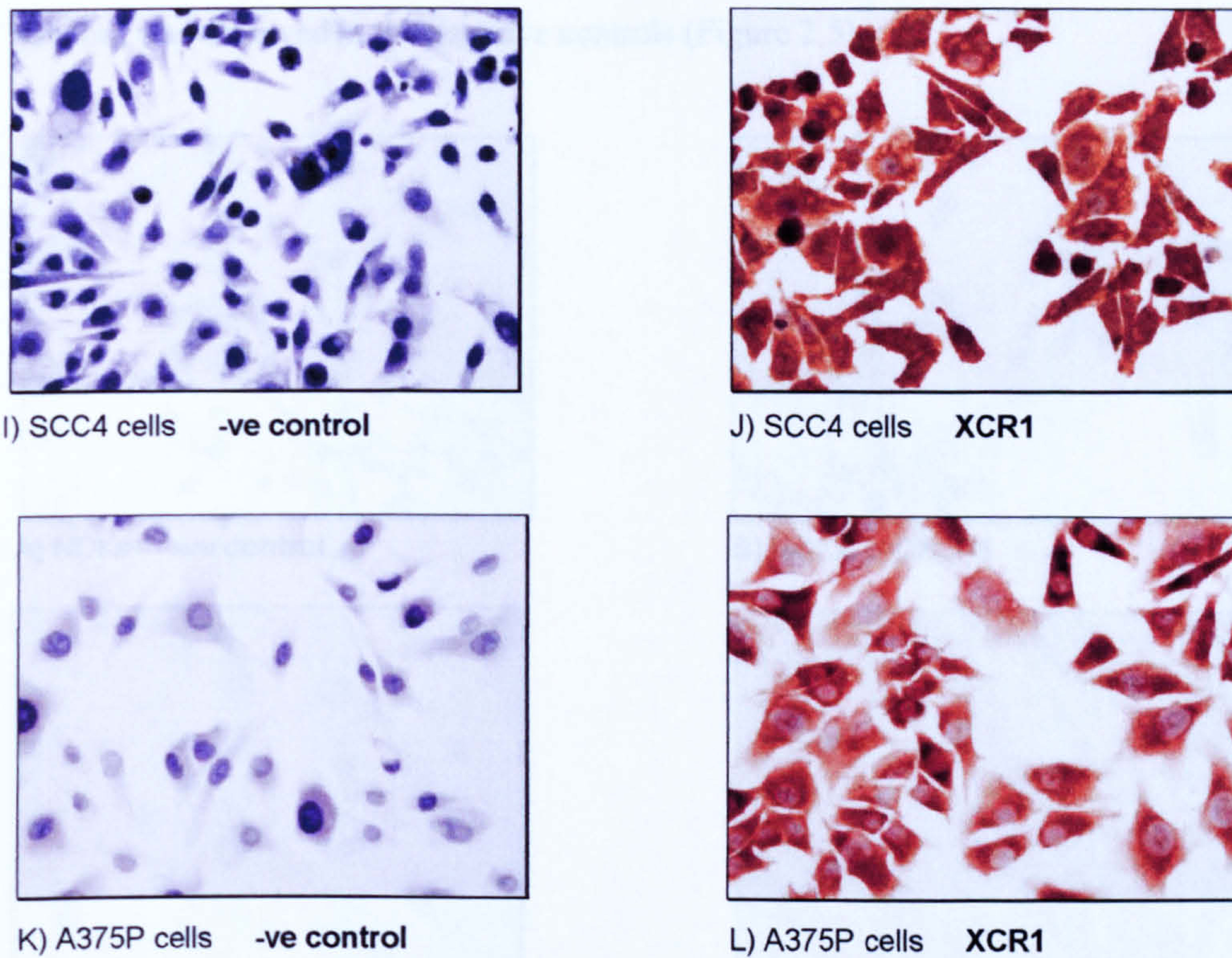


Figure 2.4. Representative pictures showing immunocytochemical XCR1 expression (Original magnification x 60, Antibody concentration 20µg/ml). No staining was observed in the negative controls (a, c, e, g, i, k). Staining was seen on the cell surface and in the cytoplasm and intensity appeared strongest in SCC4 cells (j) whereas it was similar in all the other cells. Negative cells were observed in NOK (b) and H357 cells (f).

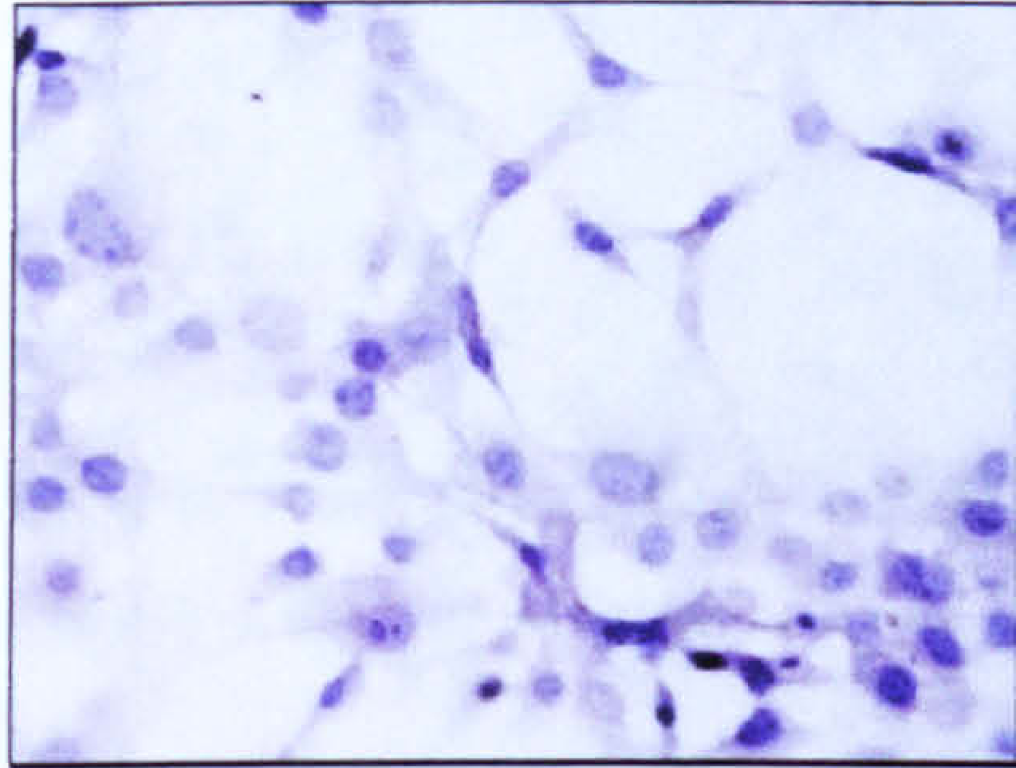
2.11.1.2 CXCR1

NOK and HGF consistently stained positively for CXCR1. NOK showed a staining pattern similar to XCR1 and in addition to some negative cells, a strongly-positive and a weakly-positive population was also seen. HGF showed a uniform staining intensity (Figure 2.5).

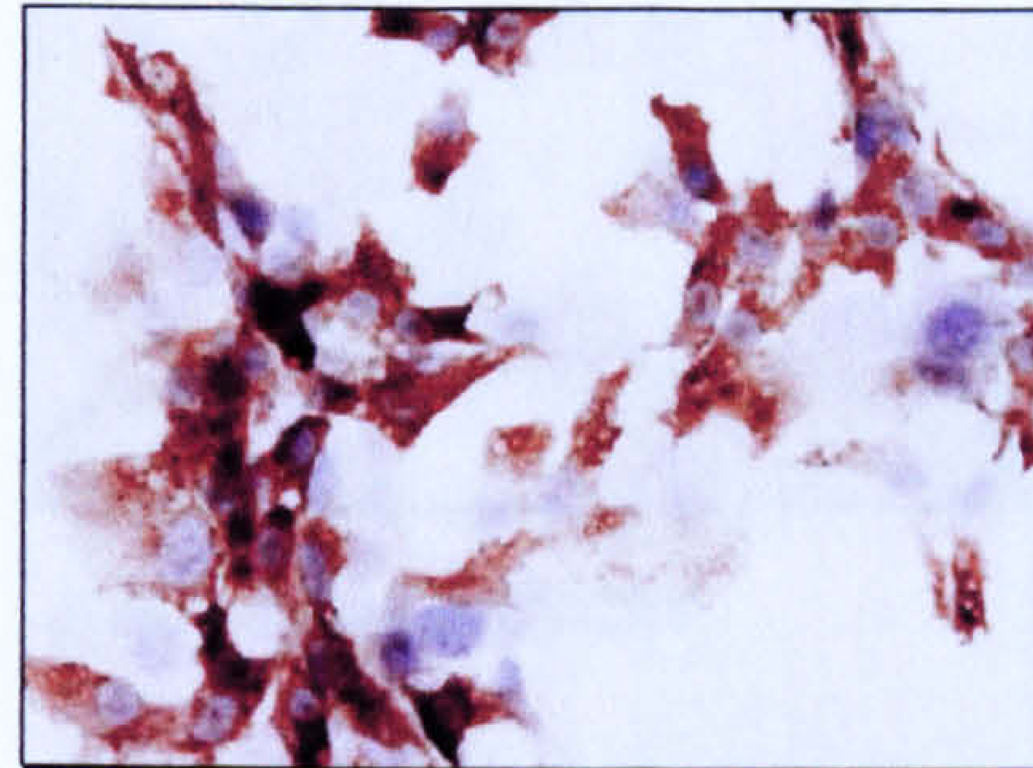
H357, TR146, SCC4 cells also showed consistent CXCR1 staining as did the A375P cell line (positive control). H357 cells exhibited negative, weak-positive and strong-positive cells whereas staining was more uniform in TR146, SCC4 and A375P cells.

CXCR1 expression was noticed on the cell surface as well as in the cytoplasm. No

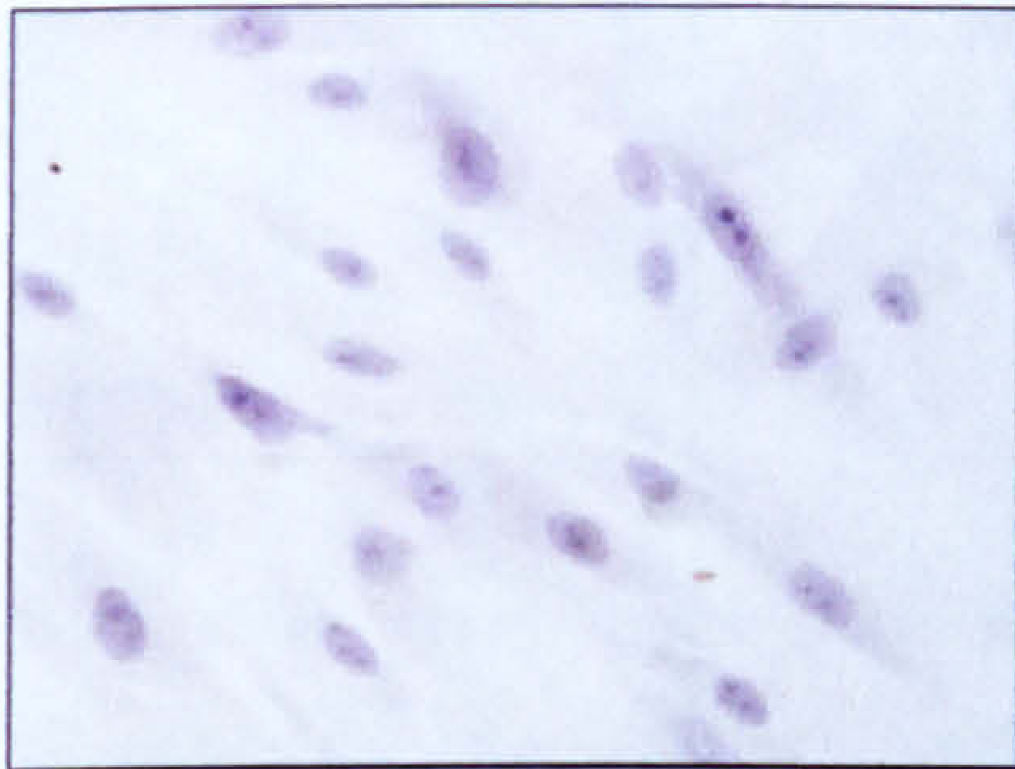
staining was observed in the negative controls (Figure 2.5).



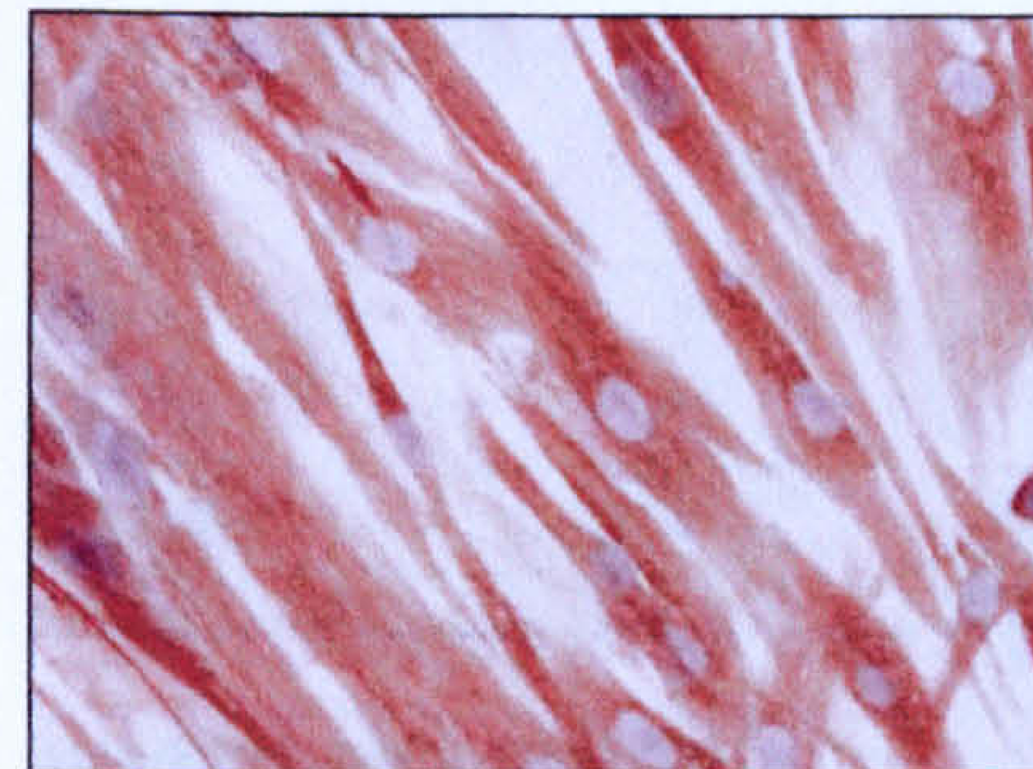
A) NOKs **-ve control**



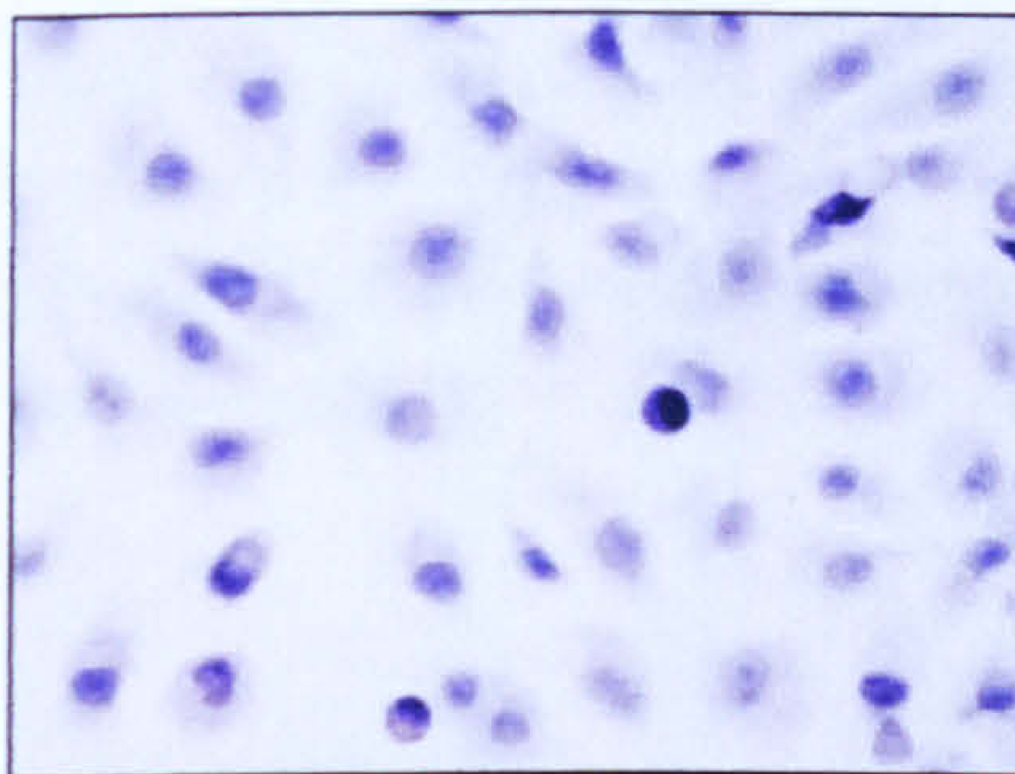
B) NOKs **CXCR1**



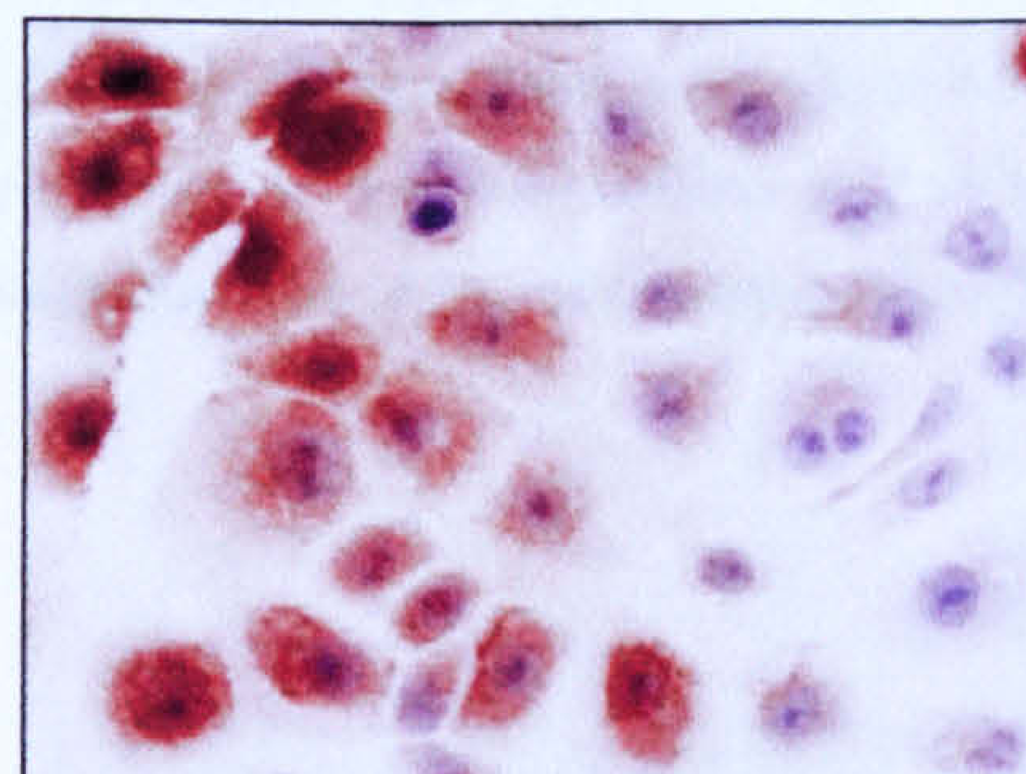
C) HGFs **-ve control**



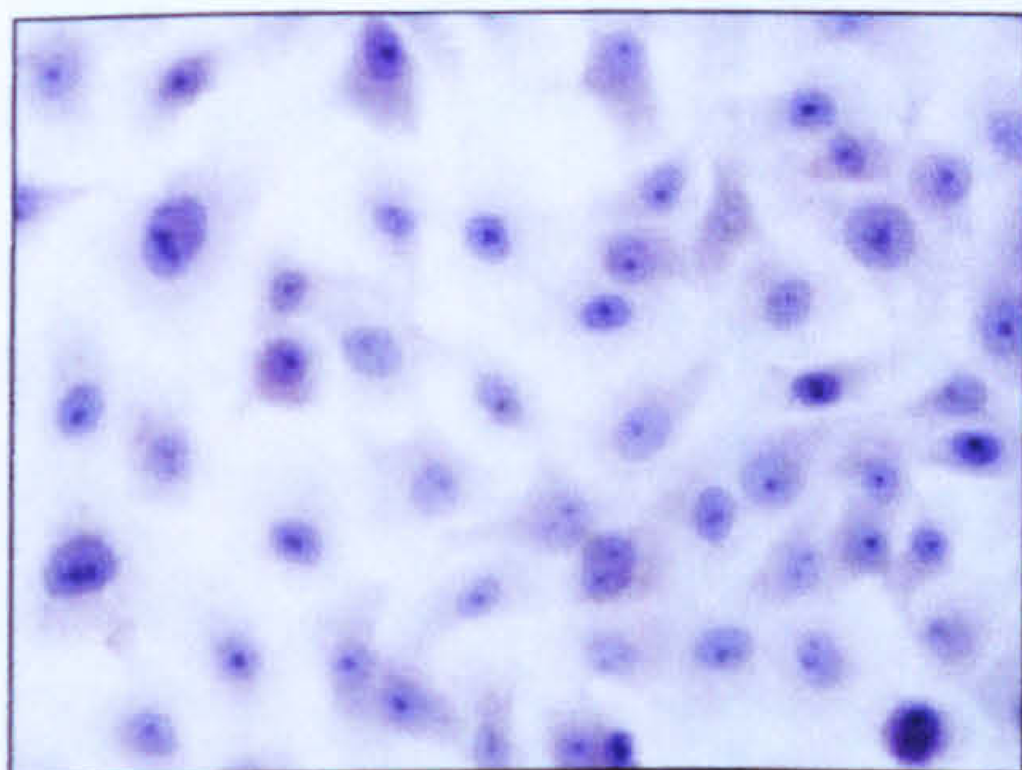
D) HGFs **CXCR1**



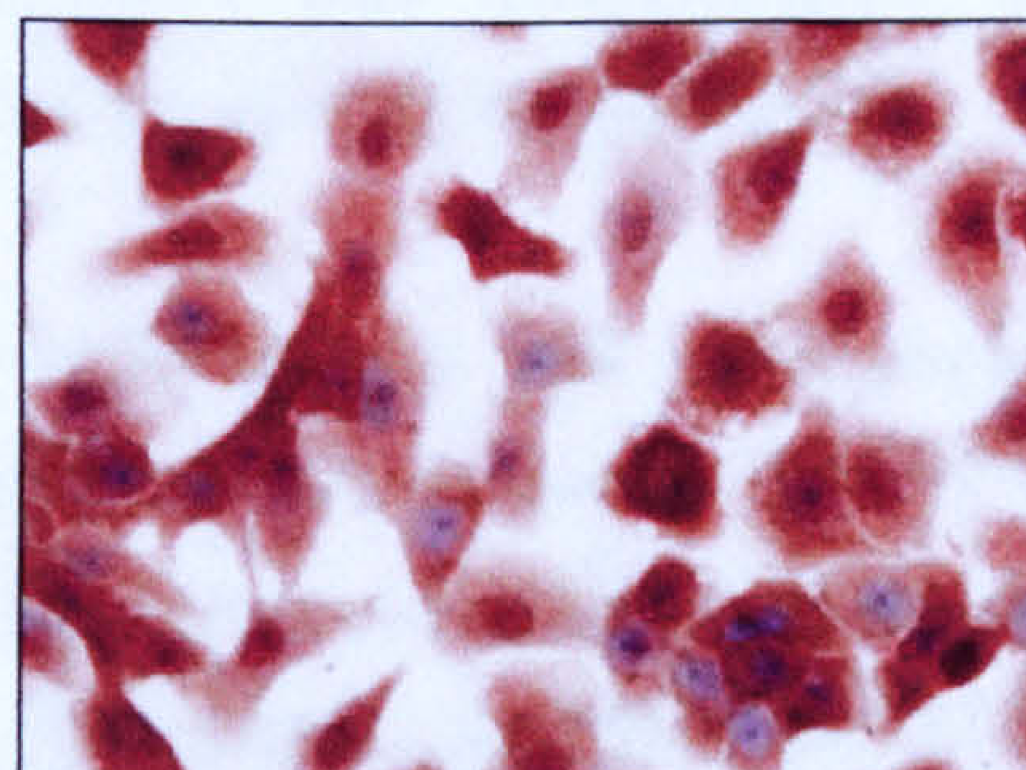
E) H357 cells **-ve control**



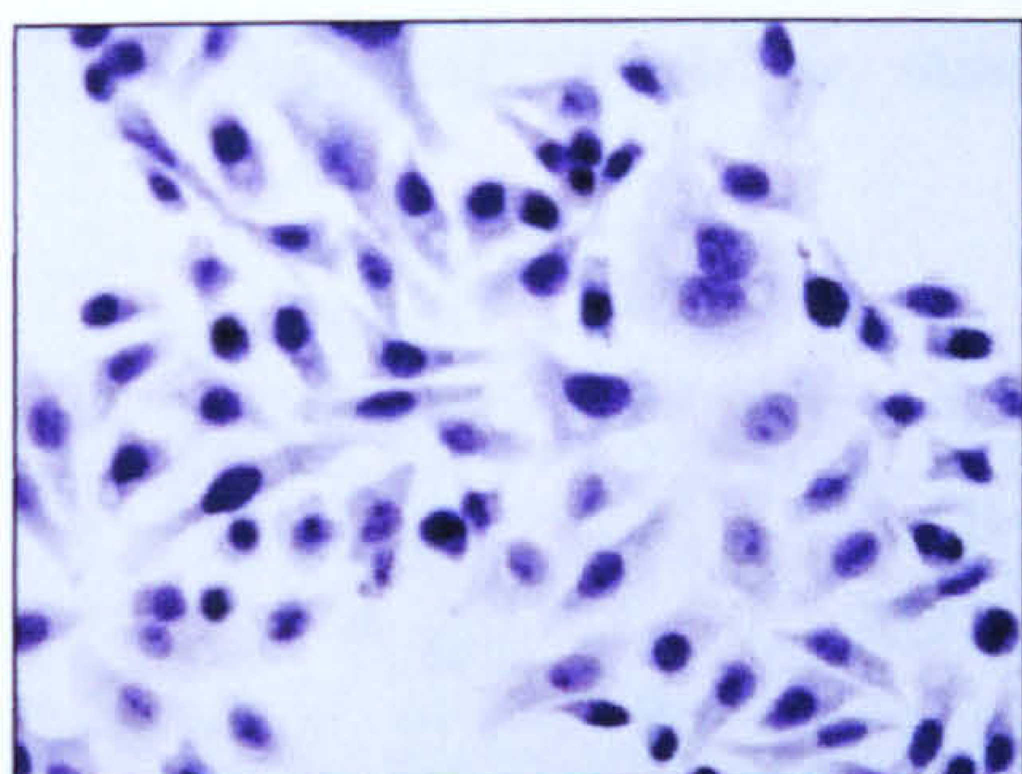
F) H357 cells **CXCR1**



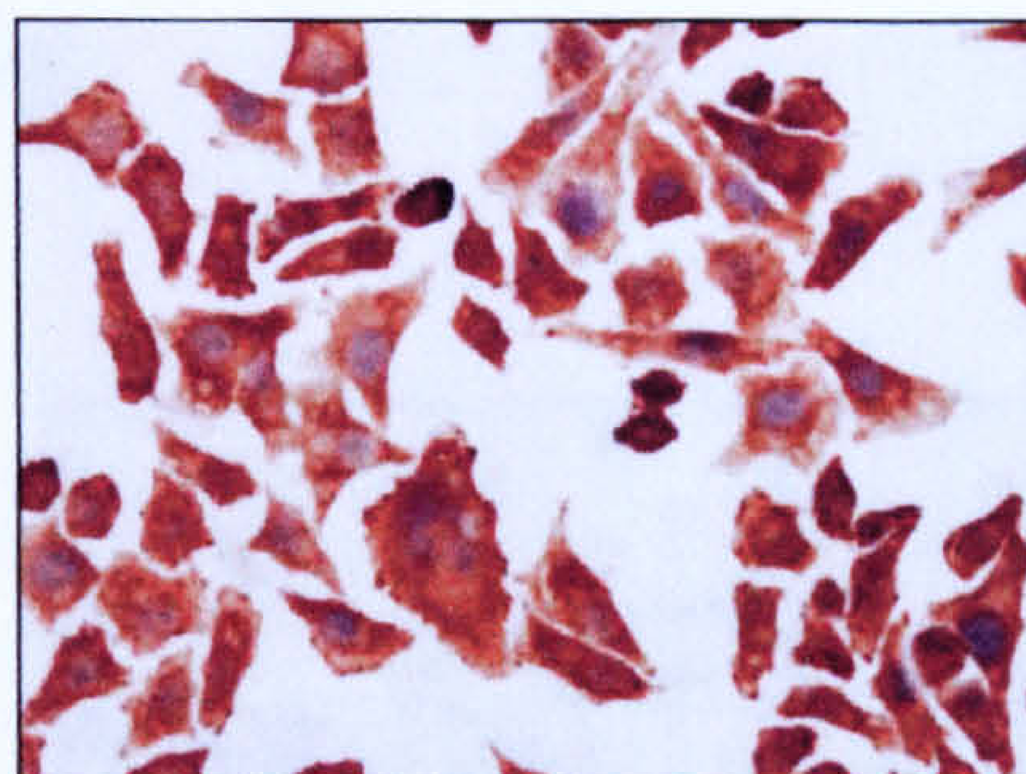
G) TR146 cells **-ve control**



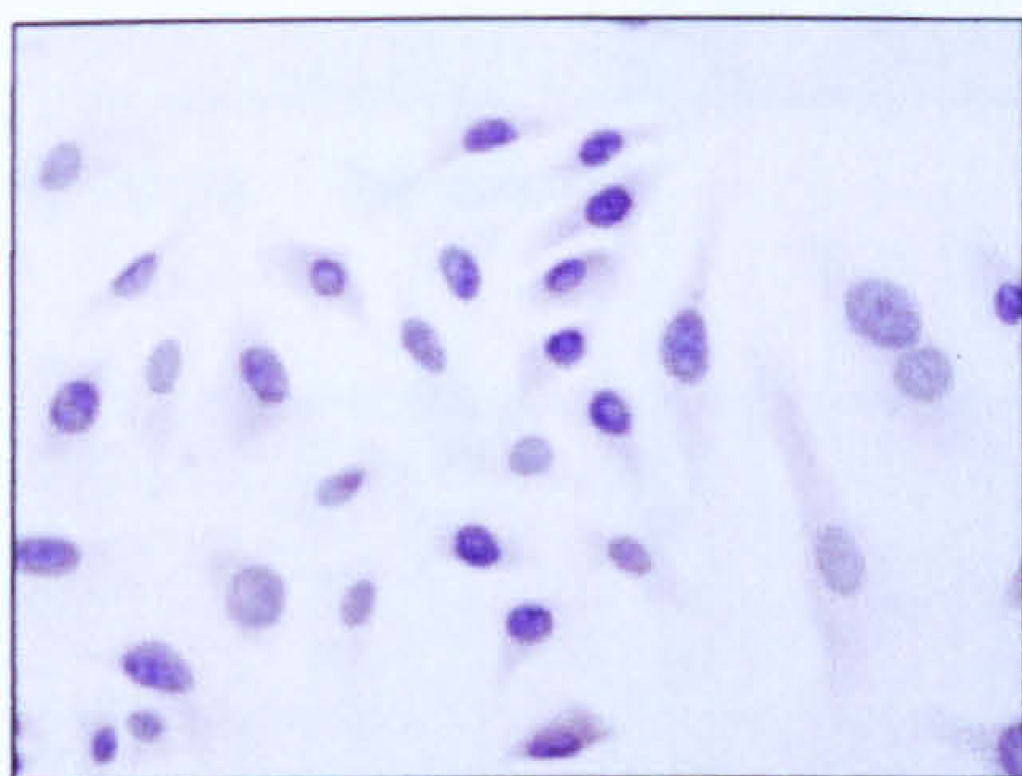
H) TR146 cells **CXCR1**



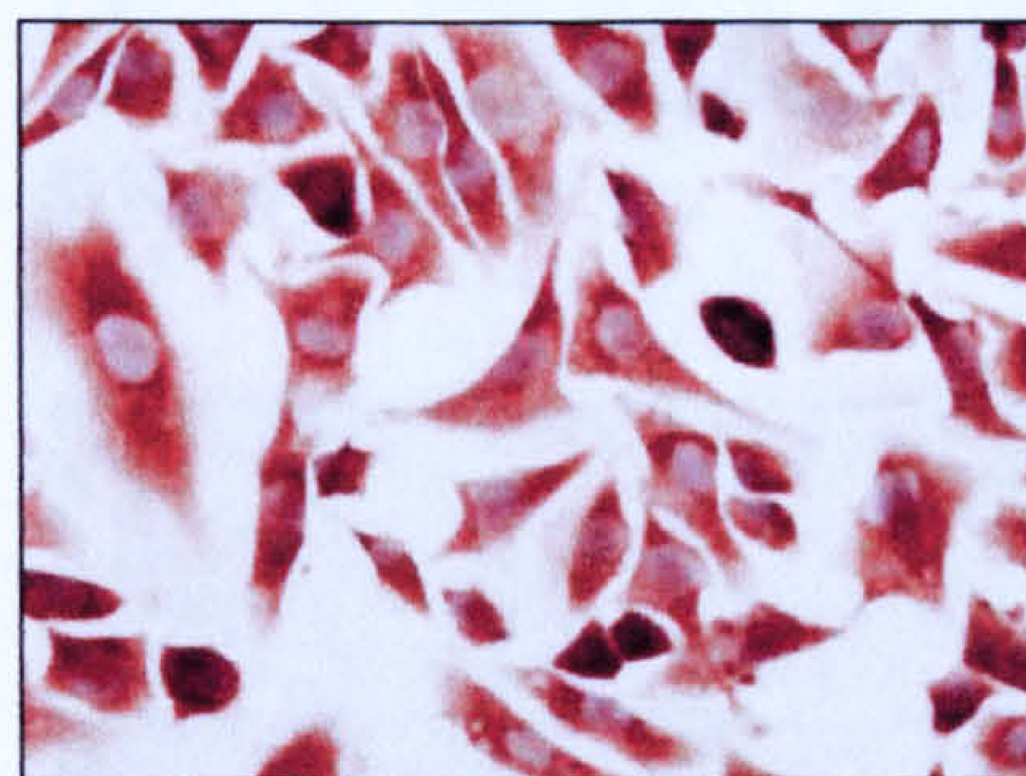
I) SCC4 cells **-ve control**



J) SCC4 cells **CXCR1**



K) A375P cells **-ve control**



L) A375P cells **CXCR1**

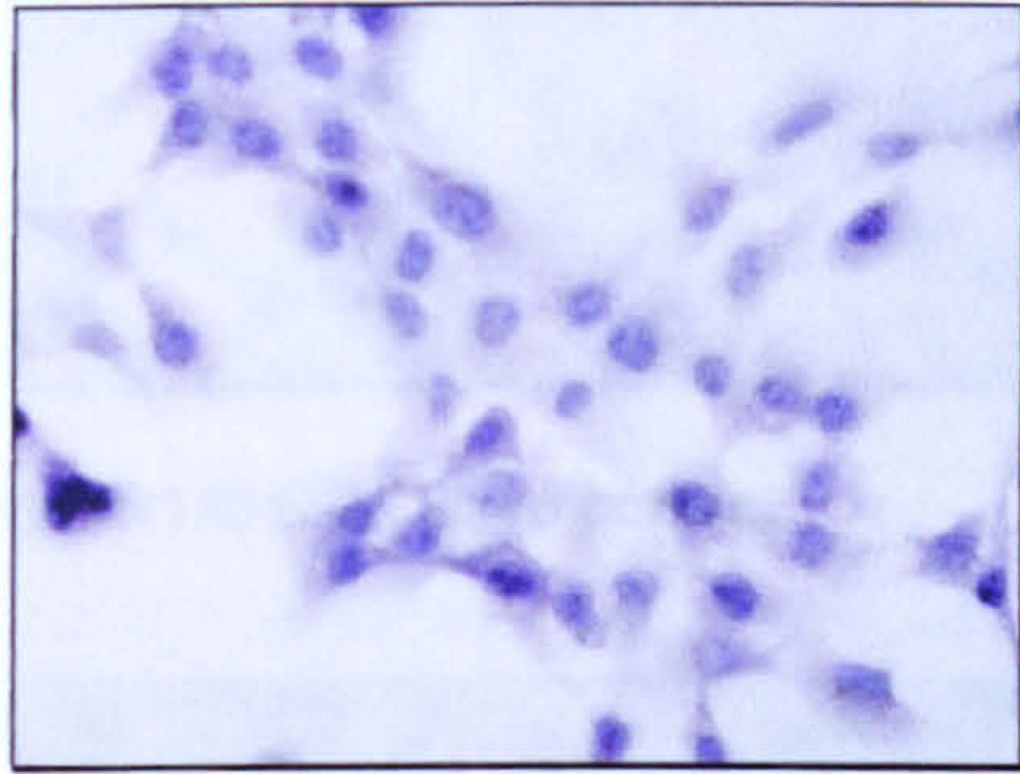
Figure 2.5. Representative pictures showing immunocytochemical CXCR1 expression (Original magnification x 60, Antibody concentration 20 µg/ml). No staining was observed in the negative controls (a, c, e, g, i, k). Staining was seen on the cell surface and in the cytoplasm and intensity appeared similar all cells. Negative cells were observed in NOK (b) and H357 cells (f).

2.11.1.3 CXCR2

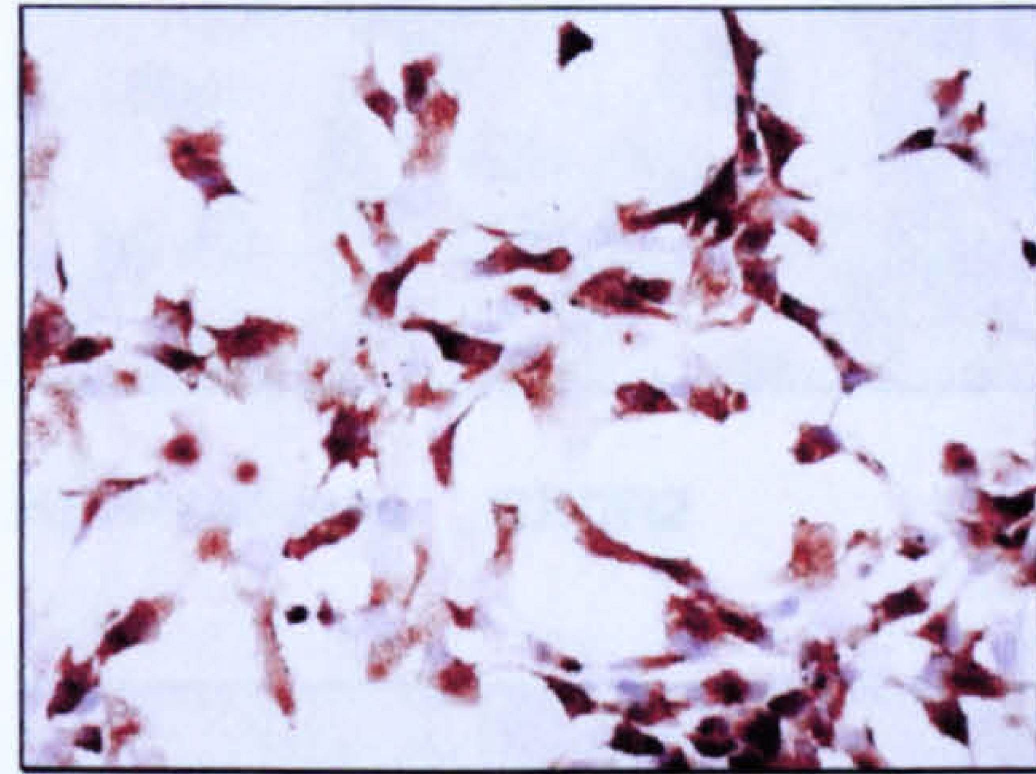
CXCR2 staining was observed in all tested cells except HGF. NOK stained consistently for CXCR2 but the strongly-positive and weakly-positive cell populations were not as distinct as observed for XCR1 and CXCR1 (Figure 2.6).

Staining was also observed on H357, TR146, SCC4 cells as well as the positive control A375P cells. H357 cells showed a different staining pattern for CXCR2 compared with CXCR1 as they exhibited a more uniform staining pattern. SCC4 cells showed the strongest CXCR2 staining intensity while staining on TR146 and A375P cells was more granular and cytoplasmic compared to other cell types. None of the

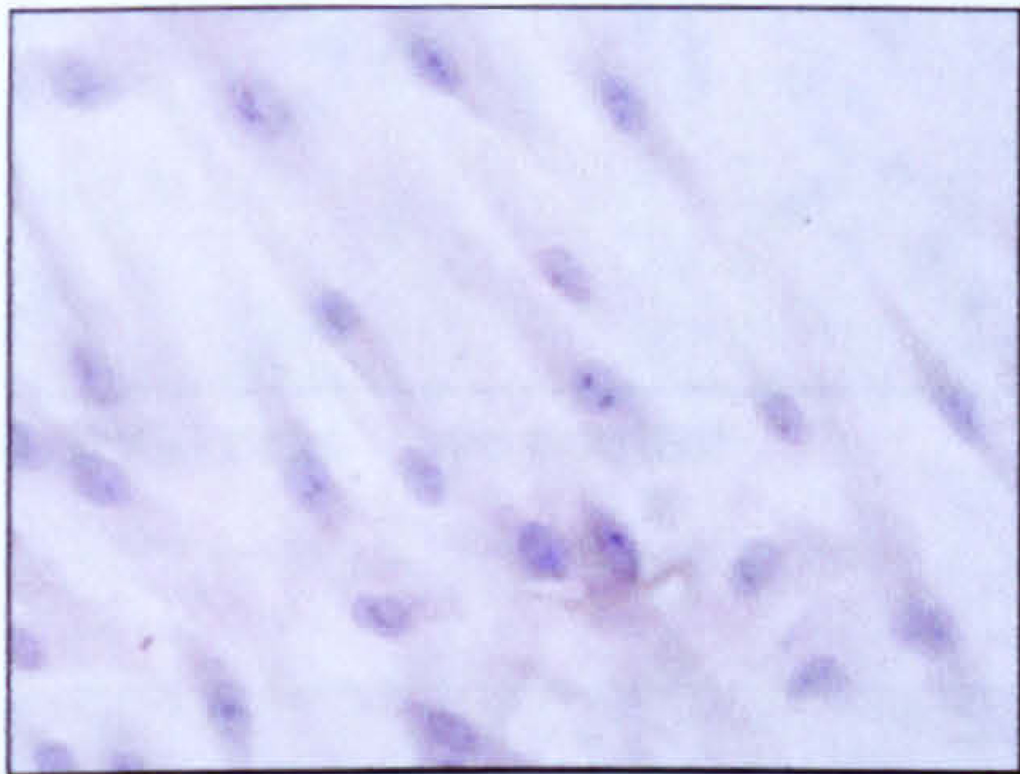
negative controls showed any staining (Figure 2.6).



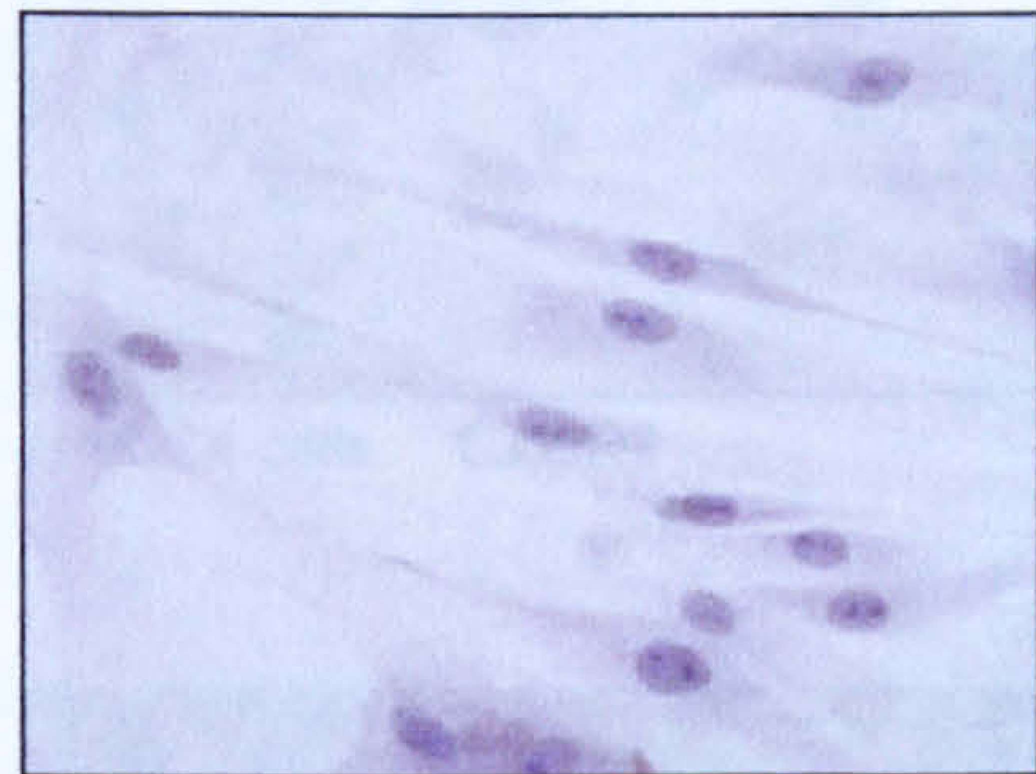
A) NOKs **-ve control**



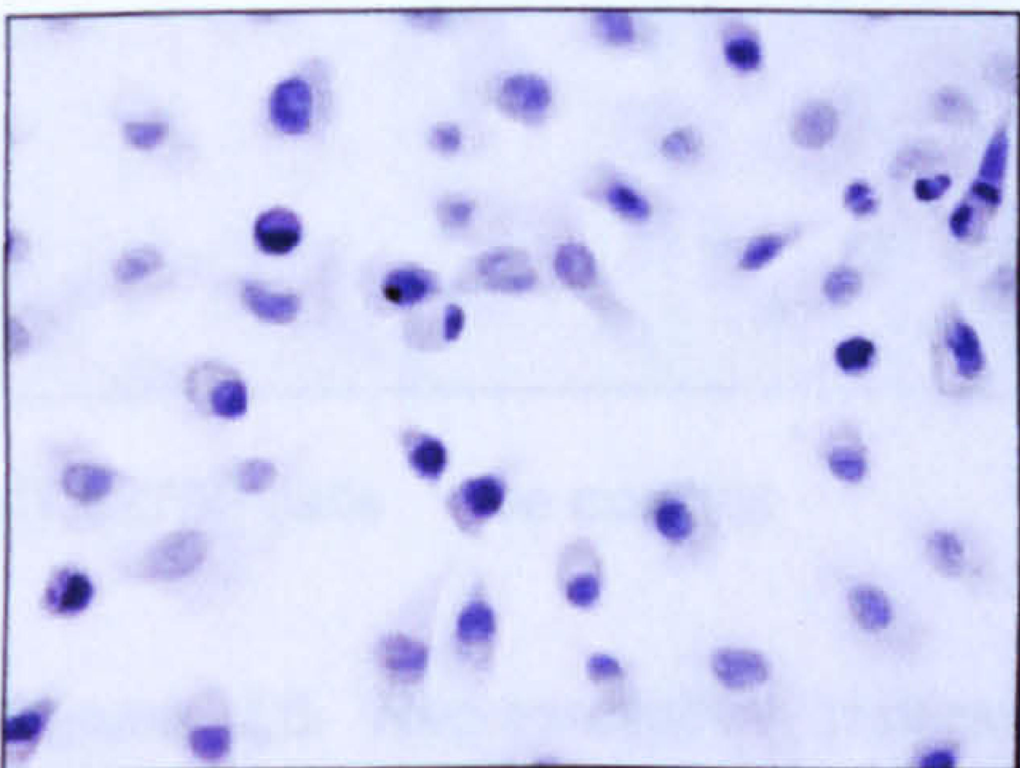
B) NOKs **CXCR2**



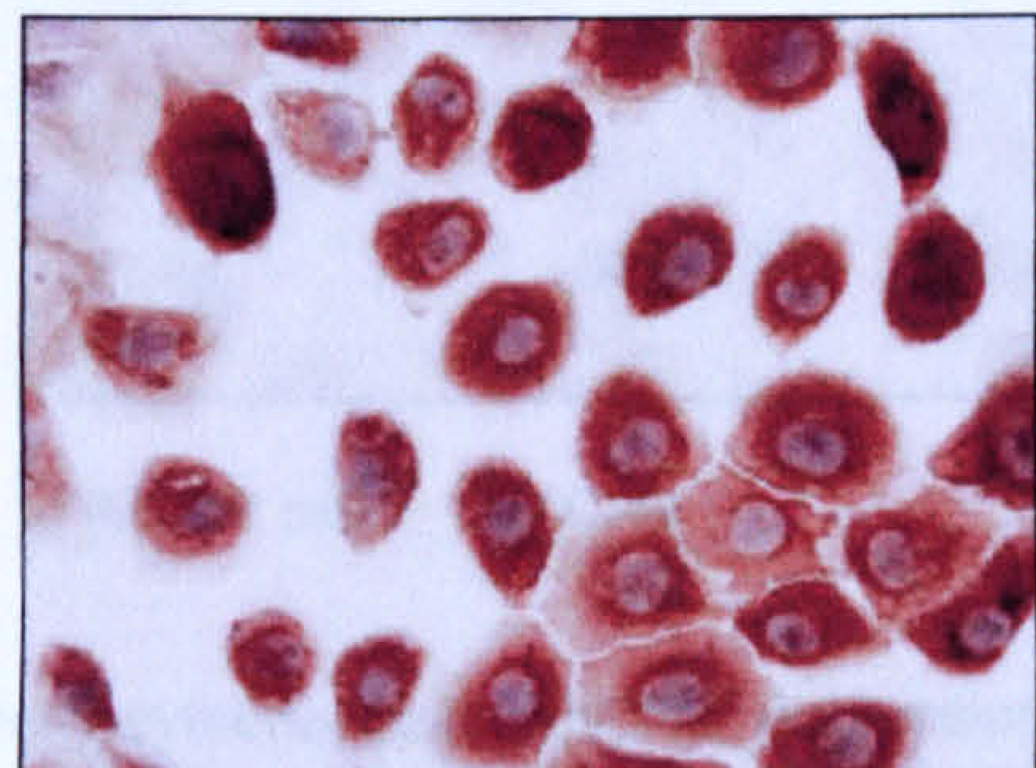
C) HGFs **-ve control**



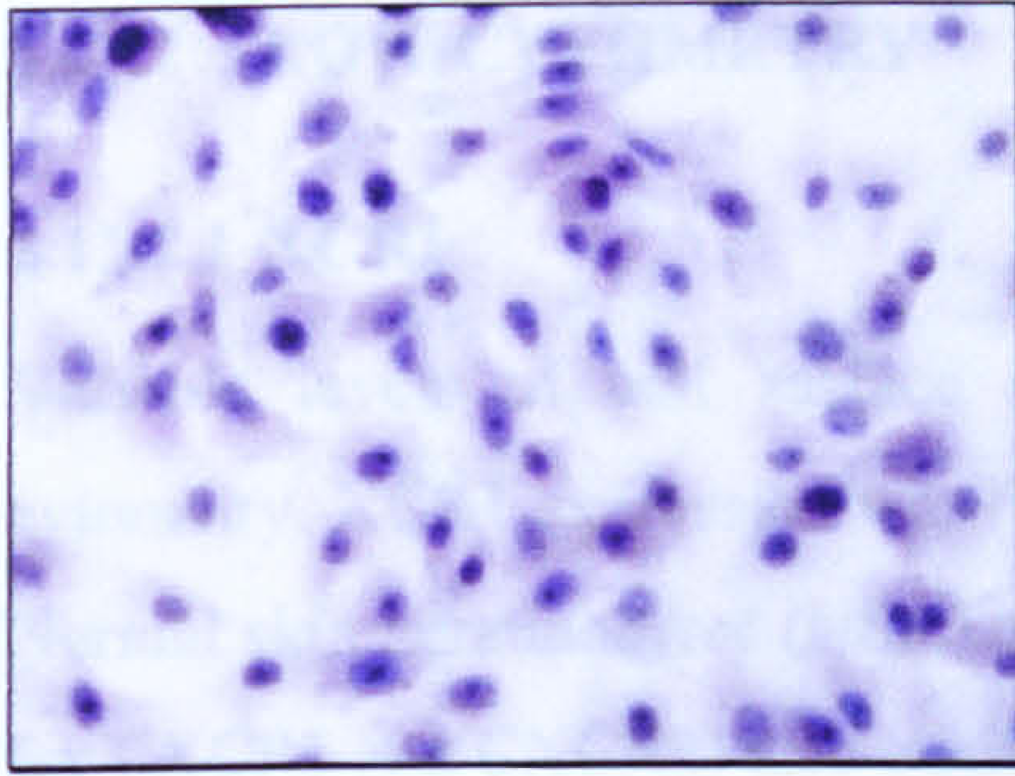
D) HGFs **CXCR2**



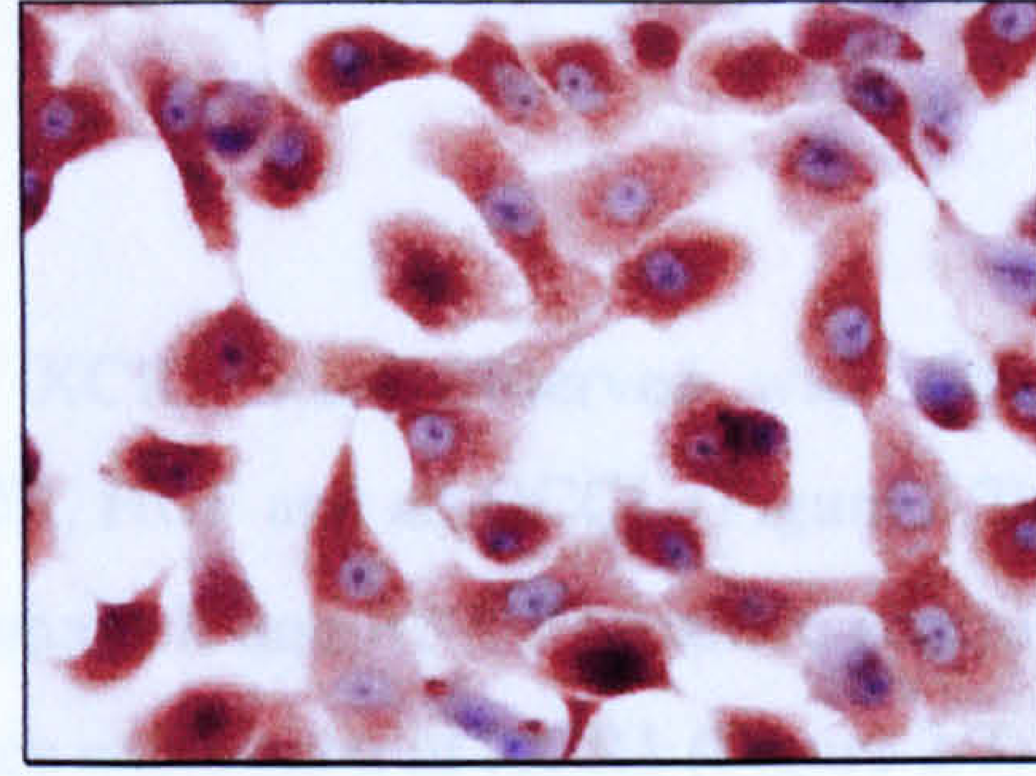
E) H357 cells **-ve control**



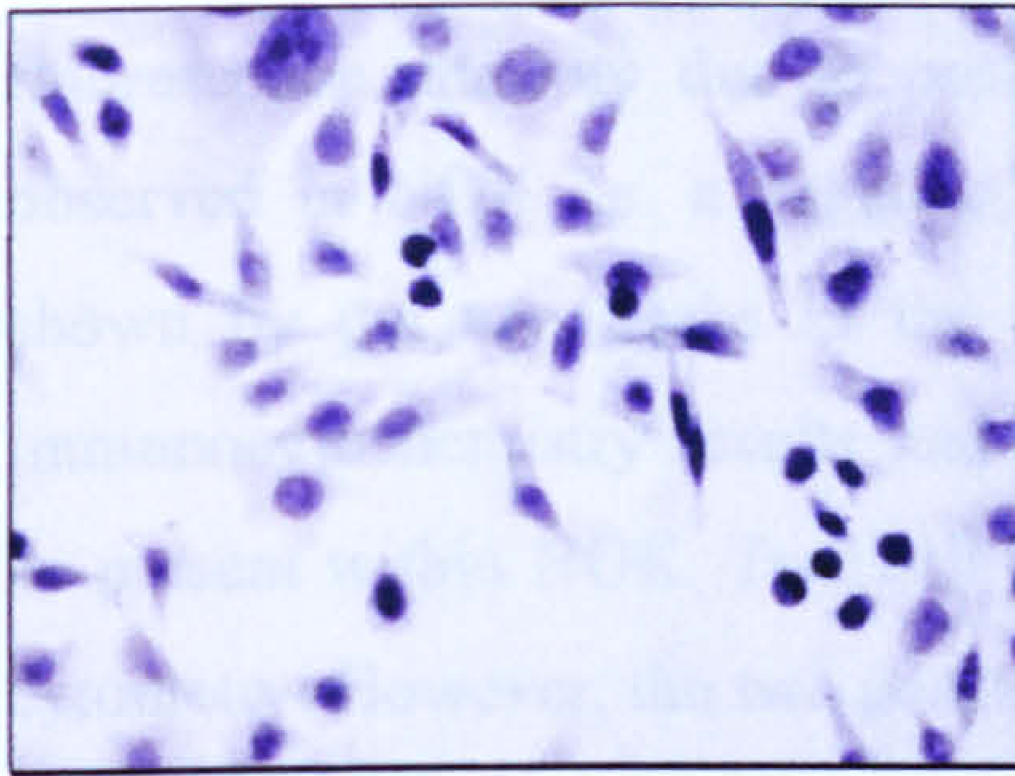
F) H357 cells **CXCR2**



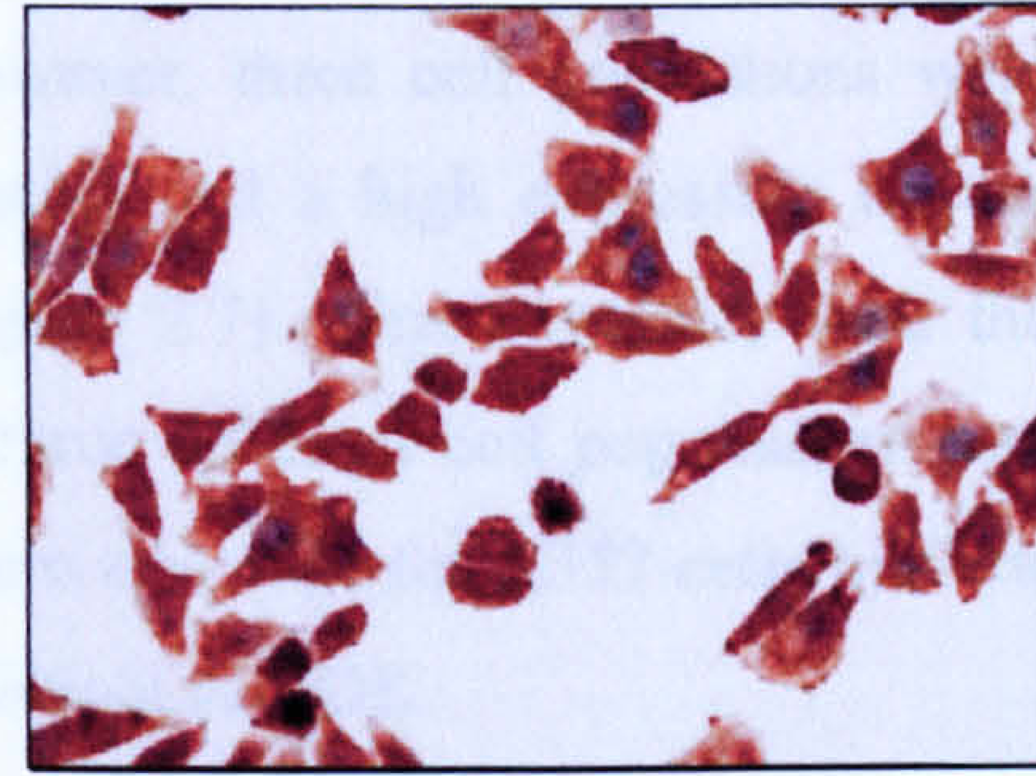
G) TR146 cells **-ve control**



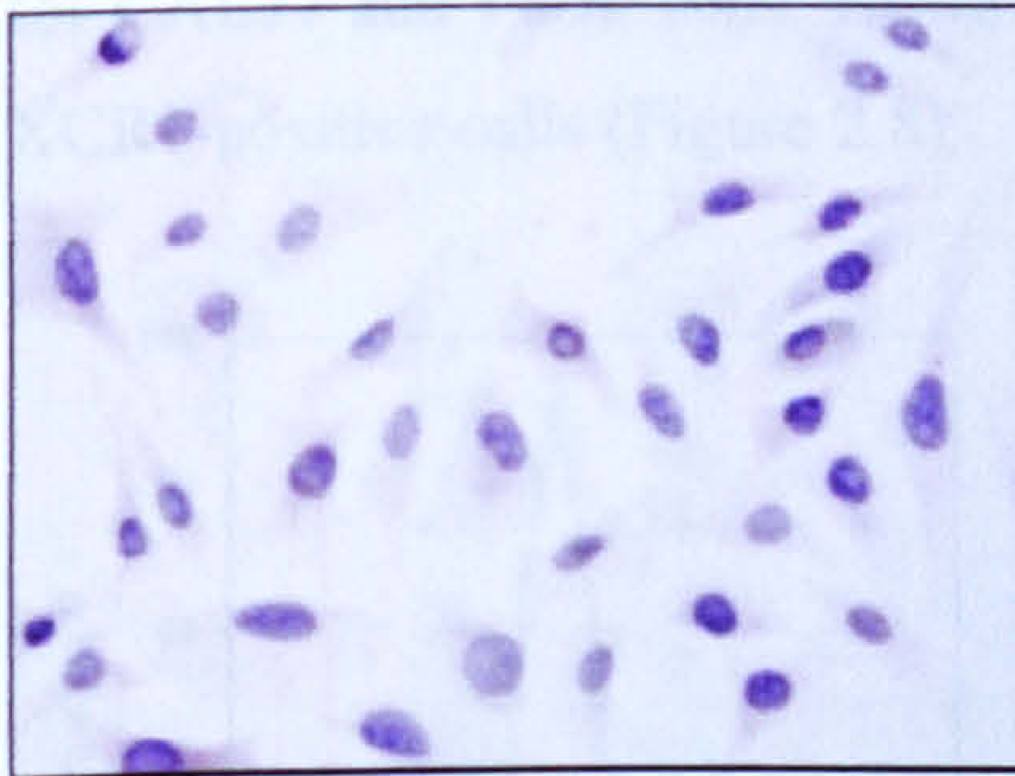
H) TR146 cells **CXCR2**



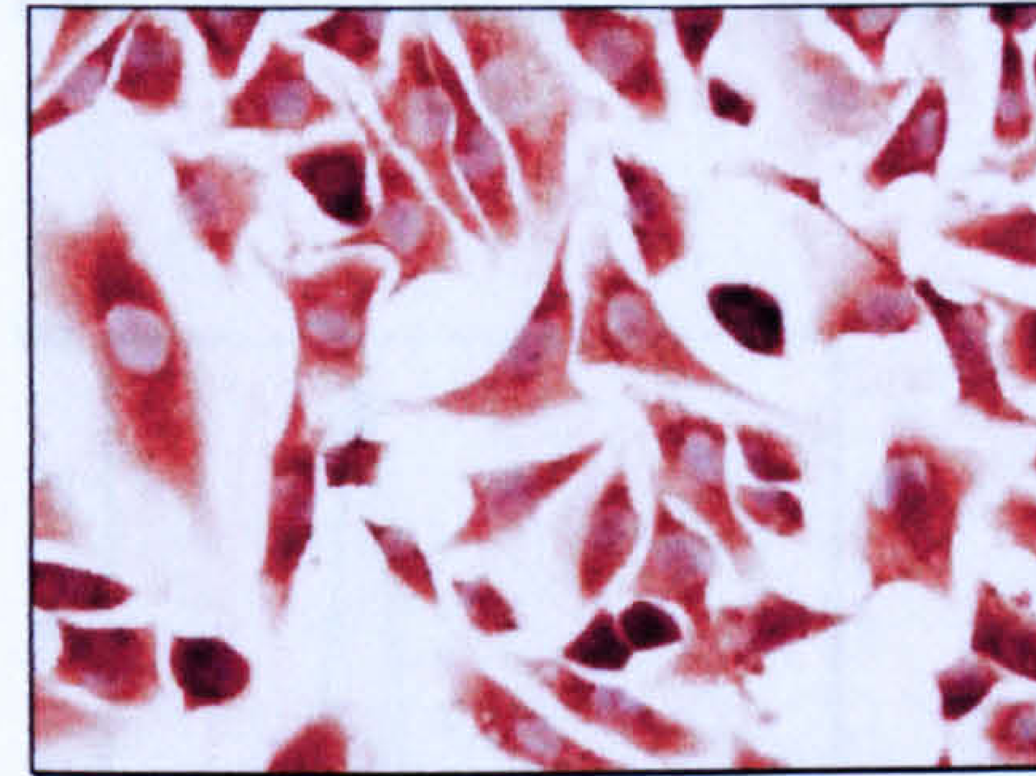
I) SCC4 cells **-ve control**



J) SCC4 cells **CXCR2**



K) A375P cells **-ve control**



L) A375P cells **CXCR2**

Figure 2.6. Representative pictures showing immunocytochemical CXCR2 expression (Original magnification x 60, Antibody concentration 20 μg/ml). No staining was observed in the negative controls (a, c, e, g, i, k). Staining was seen on the cell surface and in the cytoplasm and intensity and HGF did not show any staining (d).

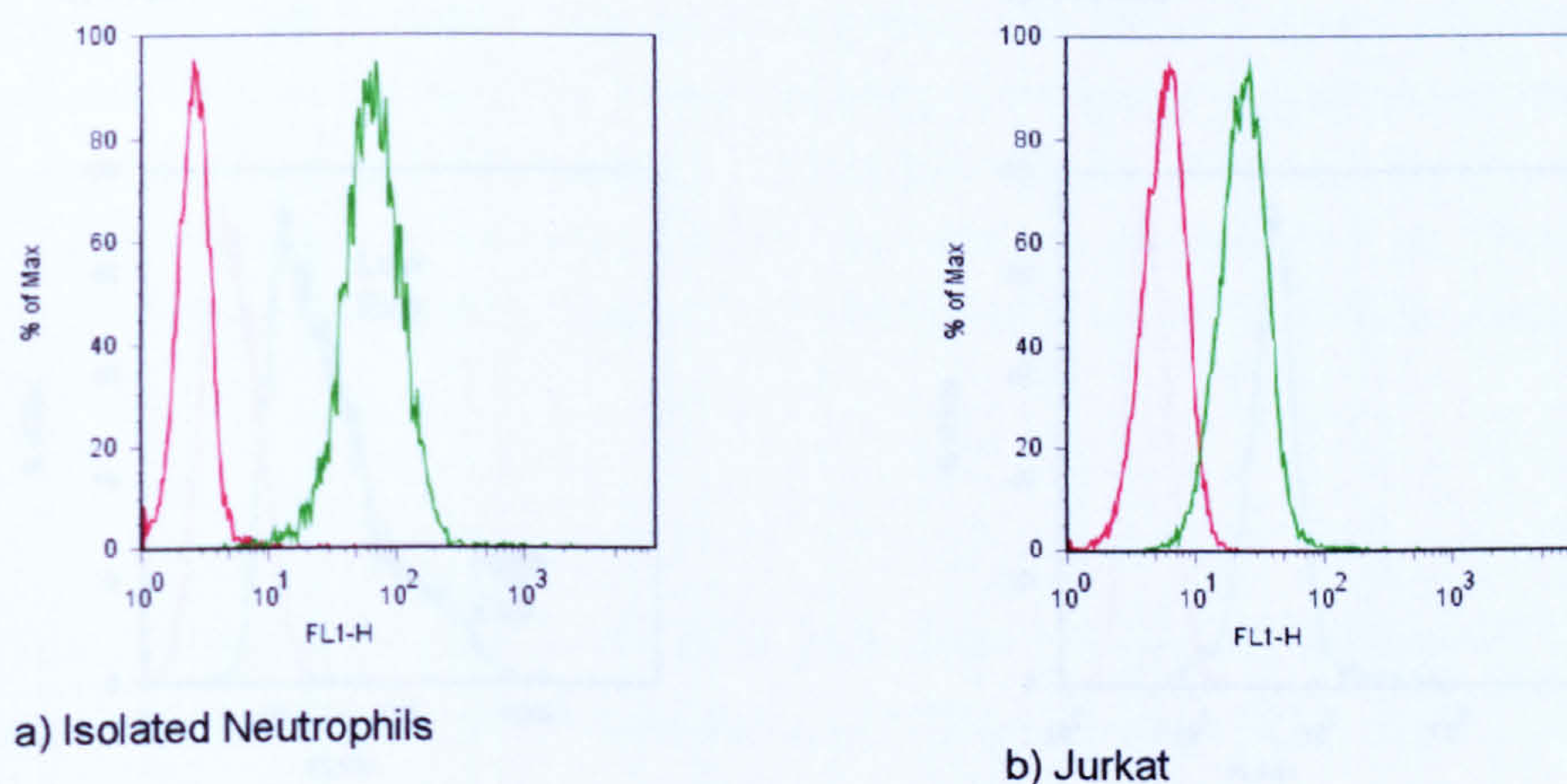
2.11.2 FLOW CYTOMETRY

2.11.2.1 XCR1

Variable levels of cell surface expression of XCR1 were observed with flow cytometry. XCR1 expression was detected on NOK, HGF and all OCCL (Figure 2.7). Normal skin keratinocytes (NSK) and the melanoma cell line A375P (positive control for CXCR1 and CXCR2) also exhibited cell surface expression of XCR1 (Figure 2.7).

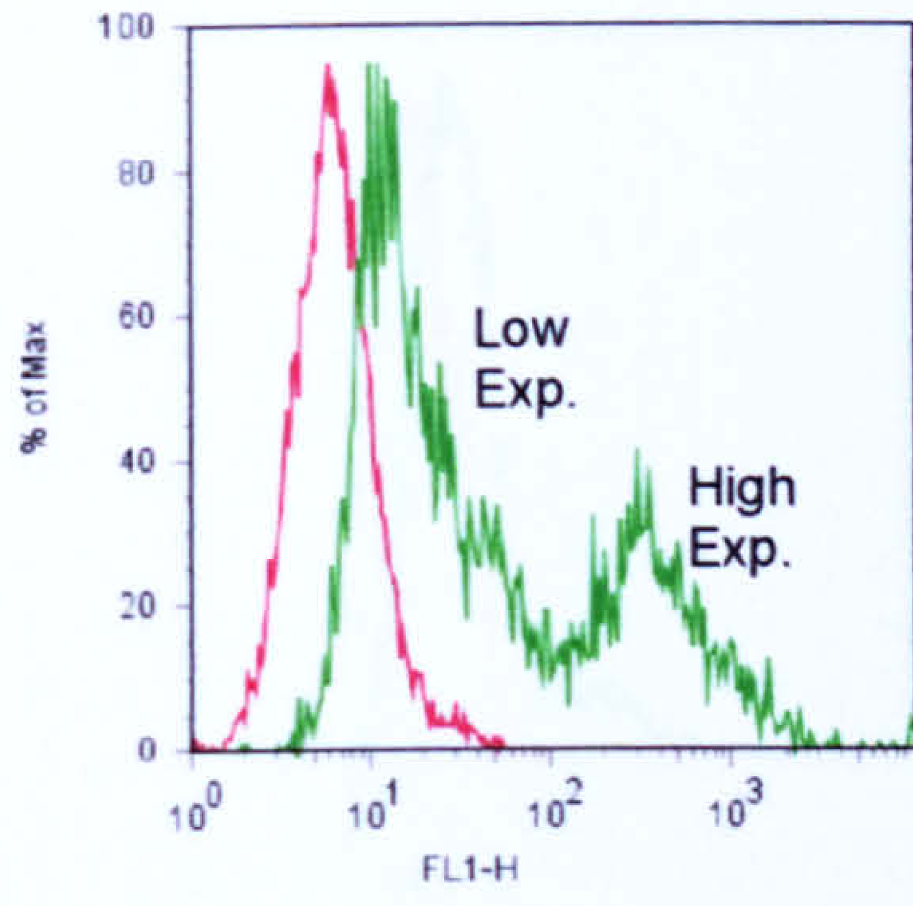
All cell types showed a histogram shift towards the right indicating an increase in fluorescence intensity due to positive cells. However, three cell populations were observed in NOK i.e. a negative, a low expressing and a high expressing one as shown by the two peaks in the histogram (Figure 2.7). This correlates with the immunocytochemistry results and suggests that three different cell populations may be present within NOK. Two cell populations were also seen for H357 cells by flow cytometry. However, the two peaks were not as distinct as NOK.

The histogram shift towards the right (increase in fluorescence intensity) was greatest for CAL27 and FaDu cells. However, the SCC4 cells showed the highest number of XCR1-positive cells (Figure 2.8).

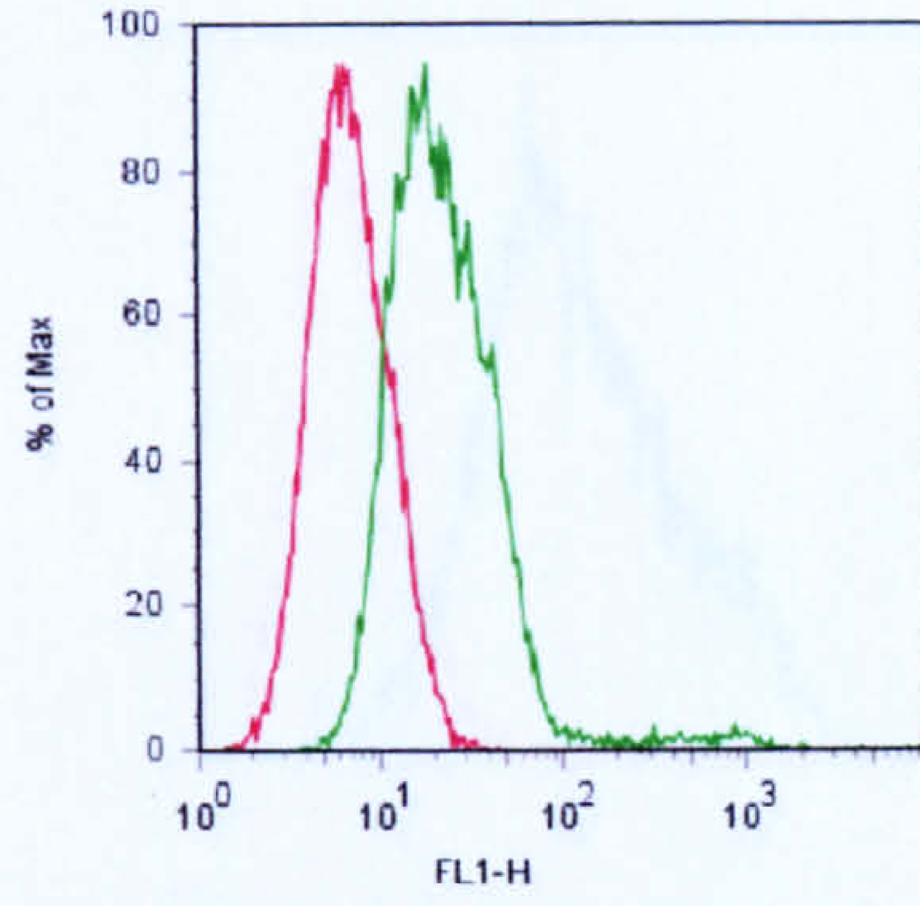


a) Isolated Neutrophils

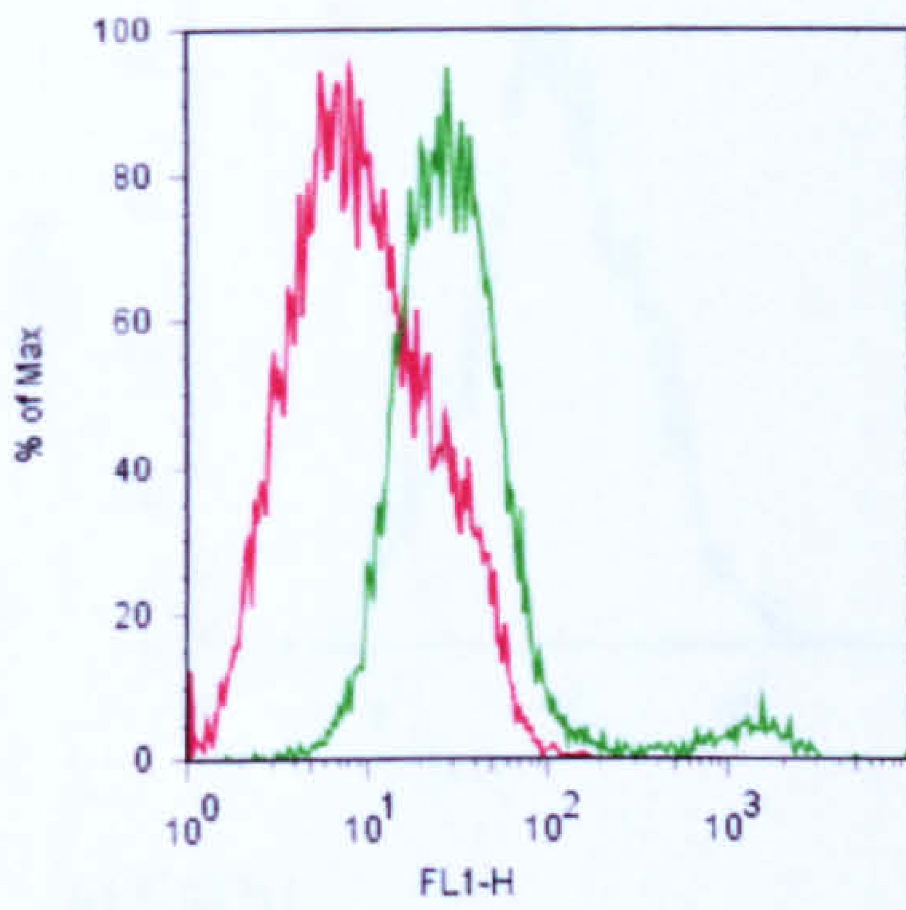
b) Jurkat



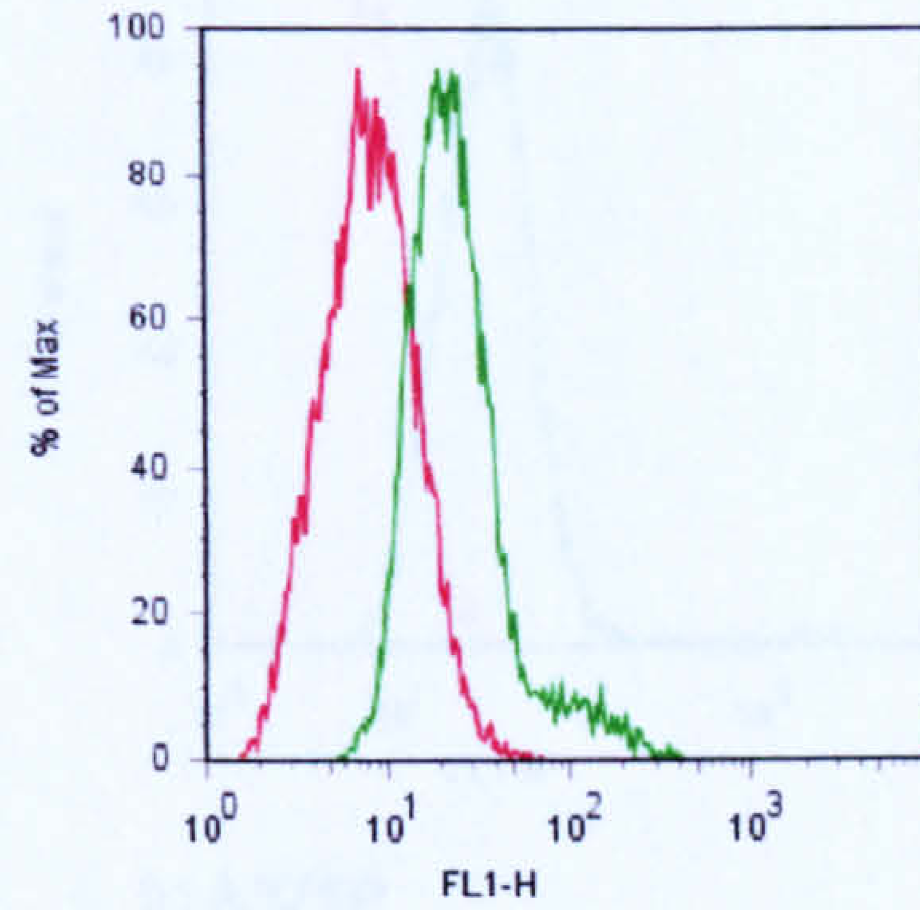
c) NOK



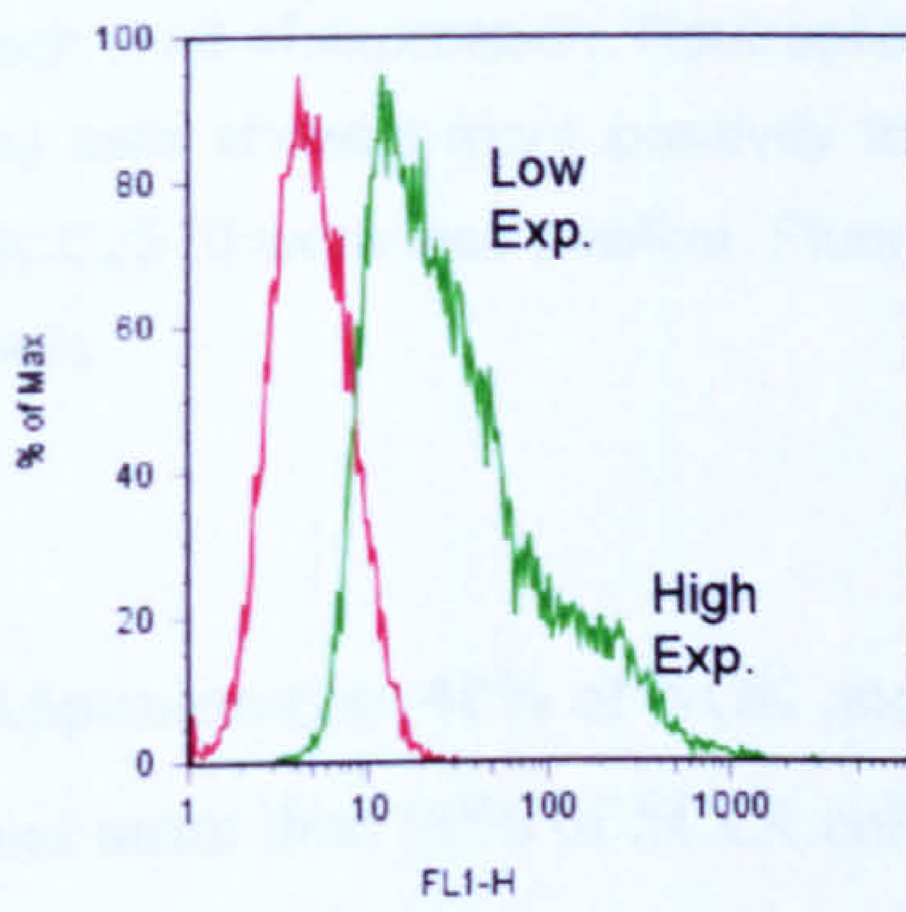
d) NSK



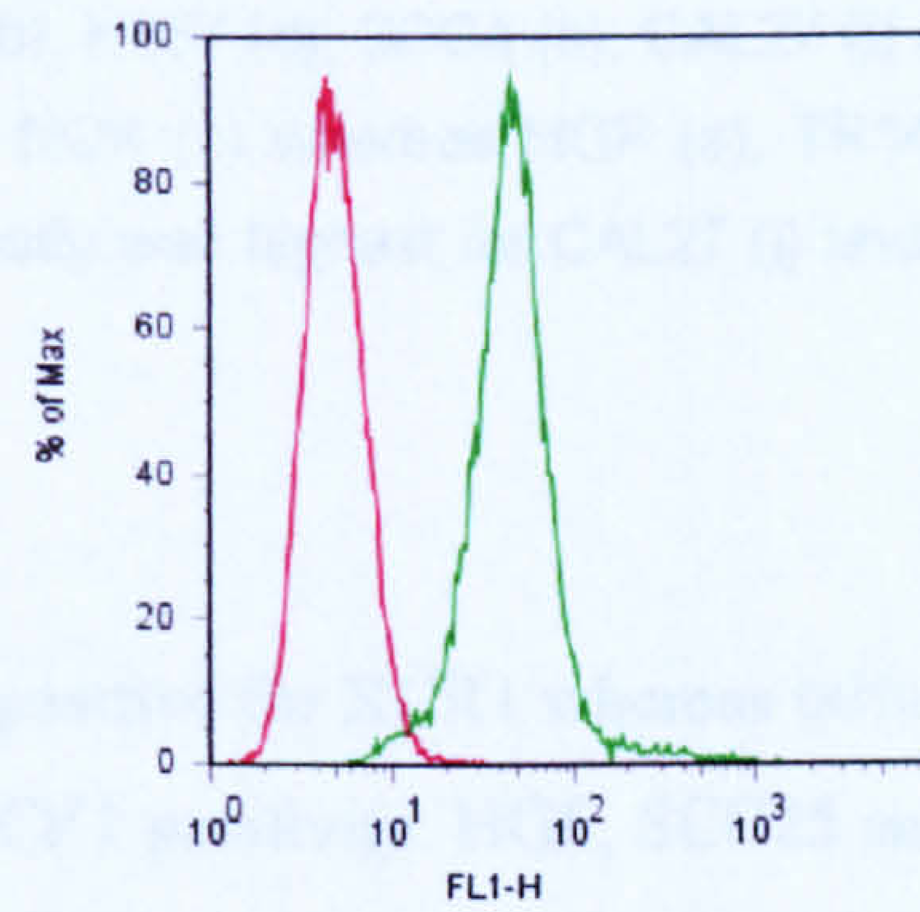
e) HGF



f) TR146



g) H357



h) SCC4

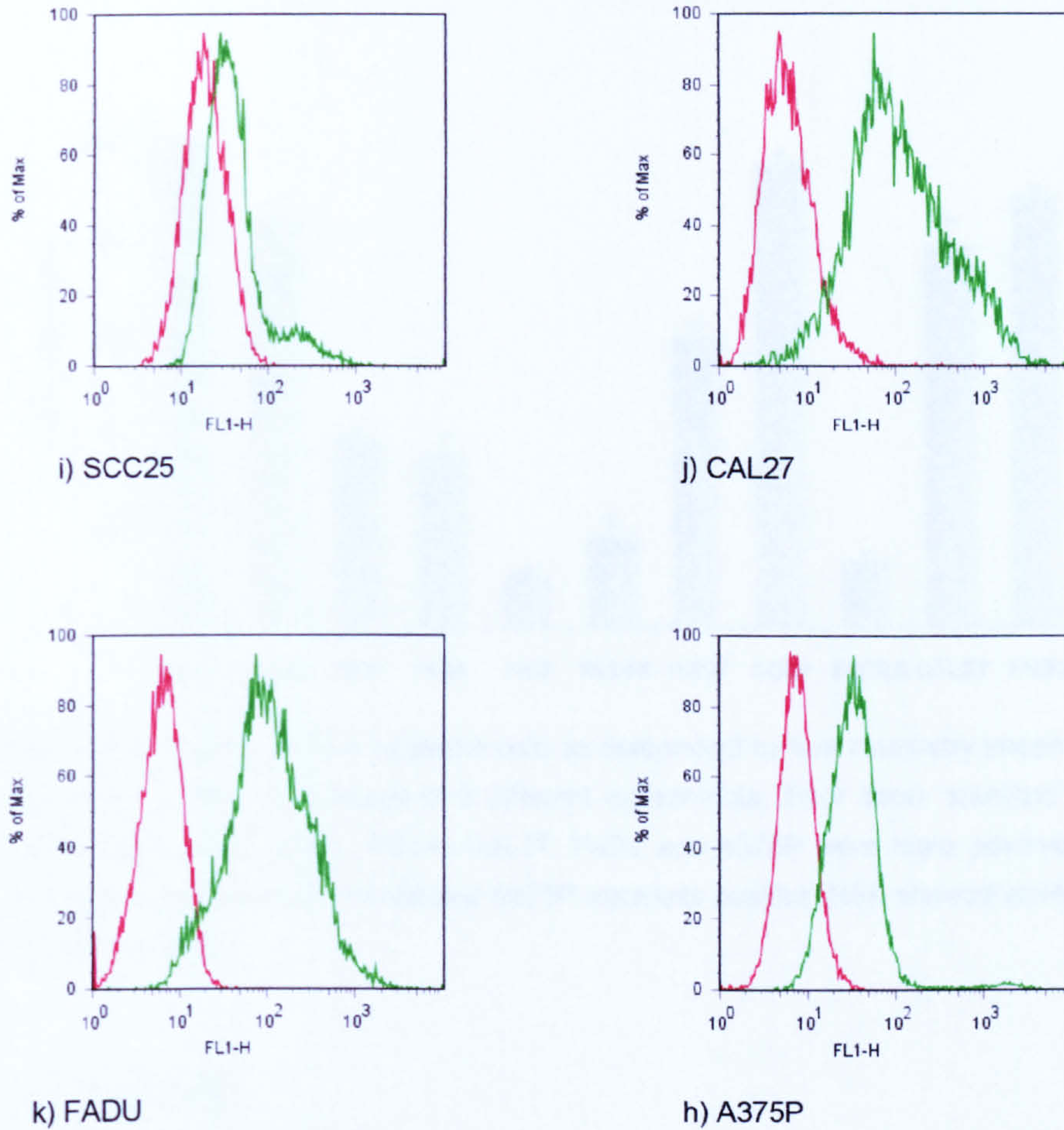


Figure 2.7. Representative histograms showing fluorescence intensity for XCR1 (Red-Negative Control; Green-XCR1). Where 'Low Exp' is low level of expression and 'High Exp' is high level of expression. Neutrophils (a), Jurkat (b), H357 (d), SCC4 (h), CAL27 (j) and FaDu (k) cells showed more positivity for XCR1 than NOK (c) whereas HGF (e), TR146 (f) and SCC25 (i) were less positive. Fluorescence intensity was highest for CAL27 (j) and FaDu (k) cells.

Approximately 40% of NOK and NSK were positive for XCR1 whereas 60% of H357 and more than 90% of SCC4 cells showed XCR1 positivity. HGF, SCC25 and TR146 showed the lowest percentage of XCR1-positive cells whereas isolated neutrophils were 100% positive and also showed high fluorescence intensity for XCR1 (Figure 2.7 and 2.8).

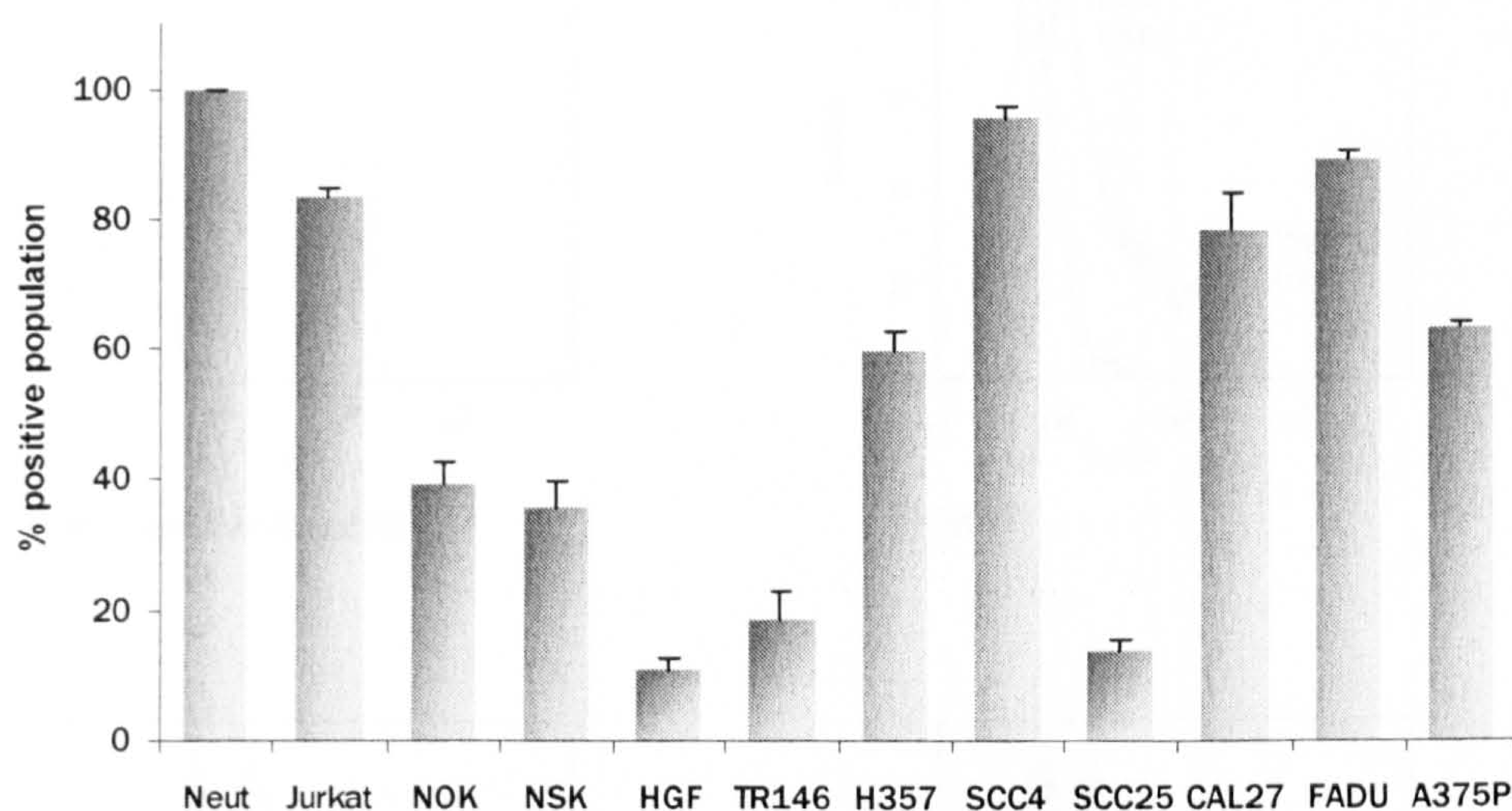


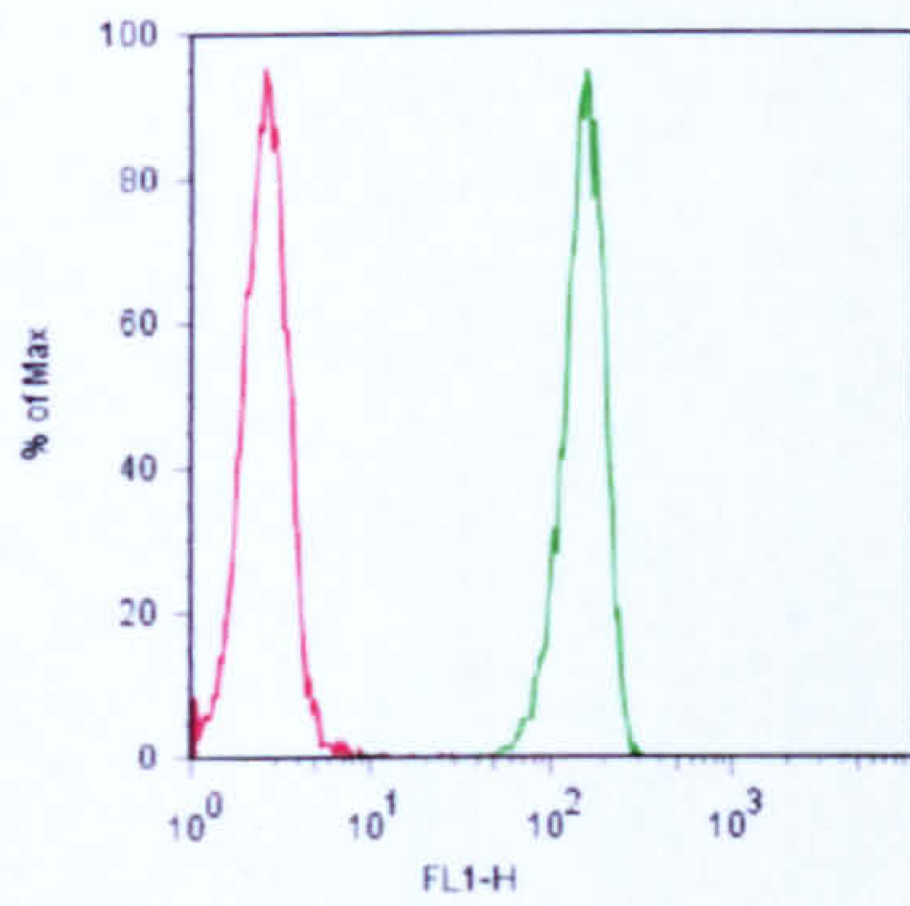
Figure 2.8. Number of XCR1-positive cells as determined by flow cytometry (mean % positive population ± SD) (n= average of 3 different experiments, Error bars= standard deviation). Neutrophils, Jurkat, H357, SCC4, CAL27, FaDu and A375P were more positive for XCR1 than NOK whereas HGF, TR146 and A375P were less positive. NSK showed similar % XCR1 positivity to NOK.

2.11.2.2 CXCR1

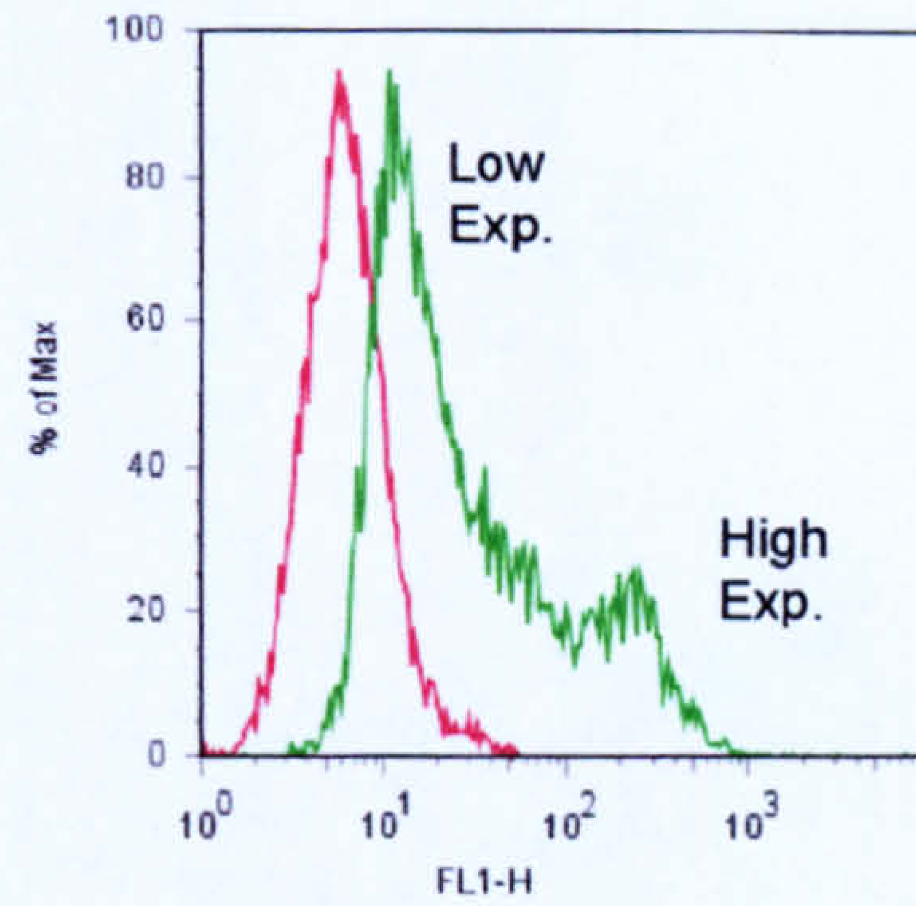
Both NOK and OCCL cells exhibited cell surface expression of CXCR1. Neutrophils and A375P cell line (positive controls) also showed CXCR1 expression in addition to HGF and NSK (Figure 2.9).

Histograms for all other cell types showed a shift towards the right and an increase in fluorescence intensity indicating positivity for CXCR1. Three distinct CXCR1 populations (i.e. negative, low expression and high expression) were observed in NOK, TR146, H357, SCC25 and FaDu cells indicated by an additional peak in the histogram (Figure 2.9).

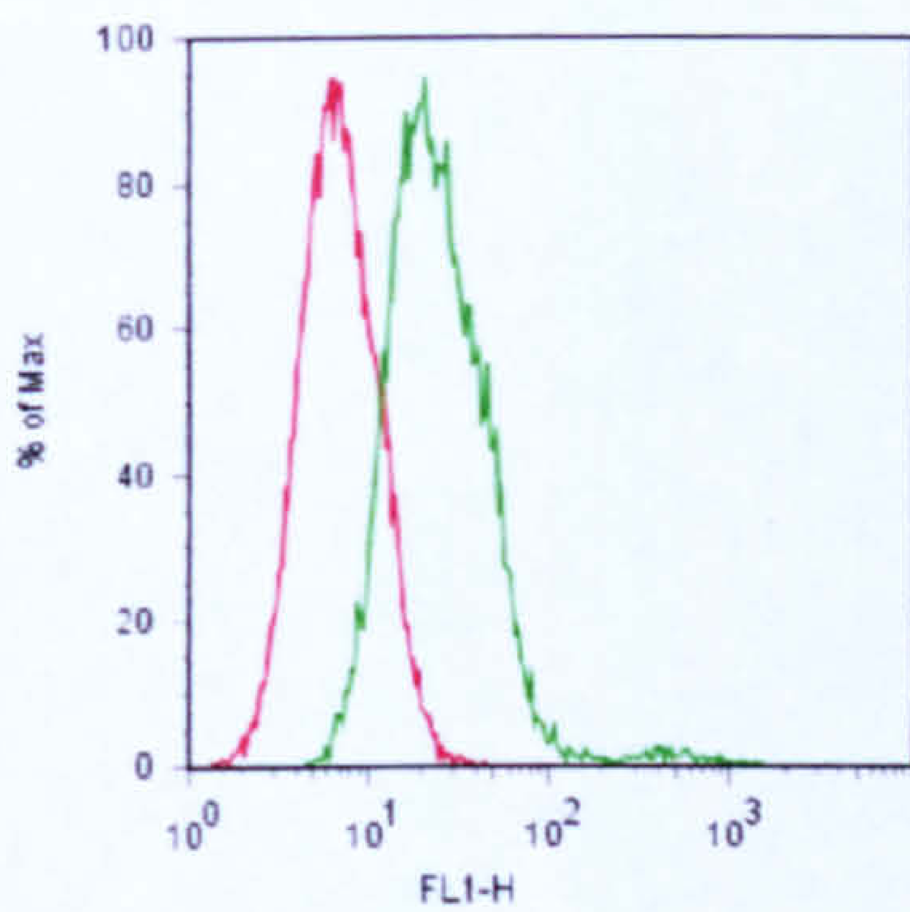
The percentage of CXCR1-positive cells was lowest for HGF, SCC25 and NOK and highest for SCC4, CAL27 and FaDu (Figure 2.10).



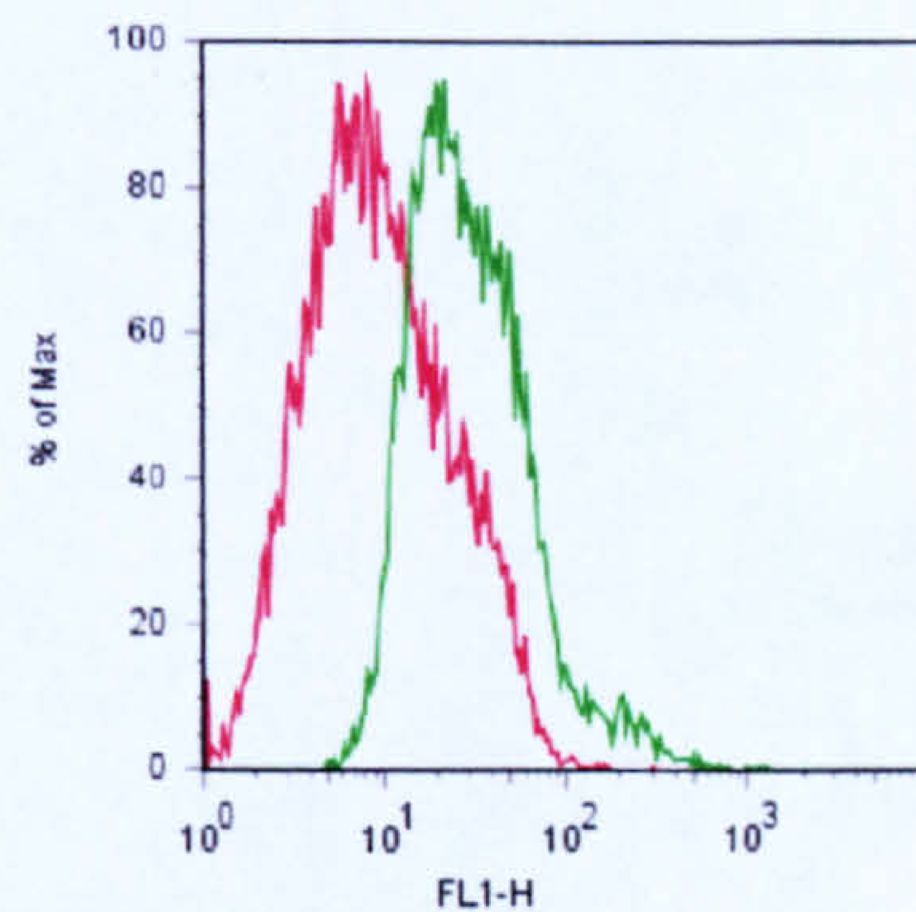
a) Isolated Neutrophils



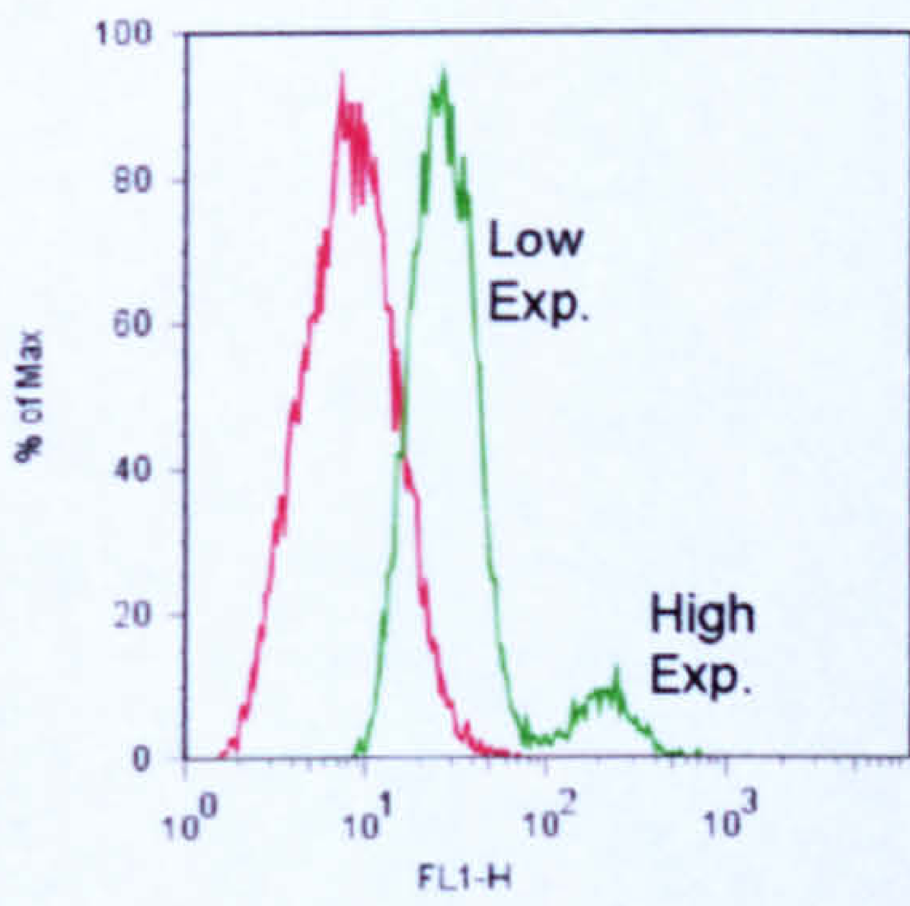
b) NOK



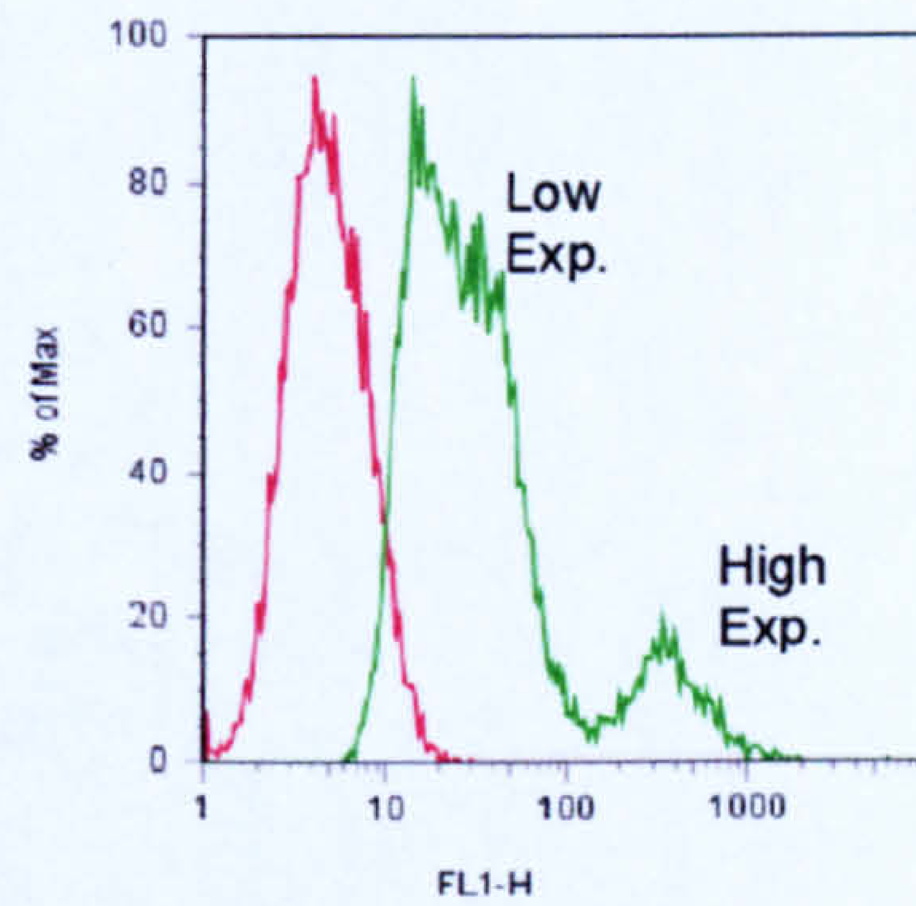
c) NSK



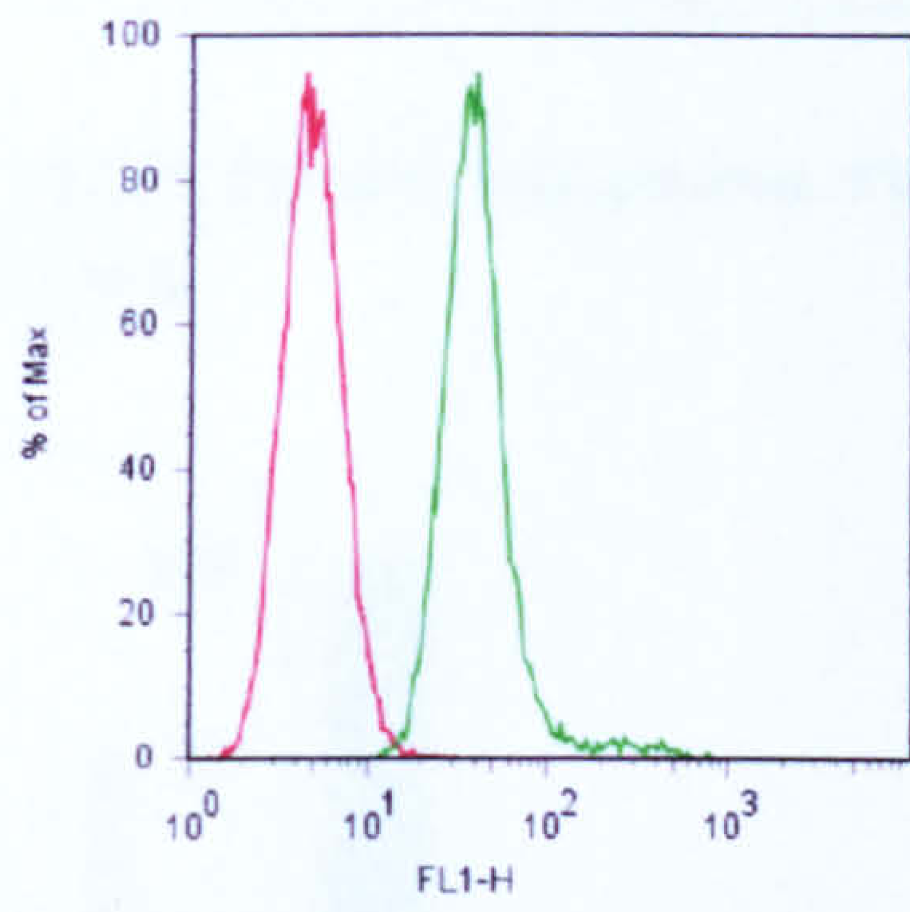
d) HGF



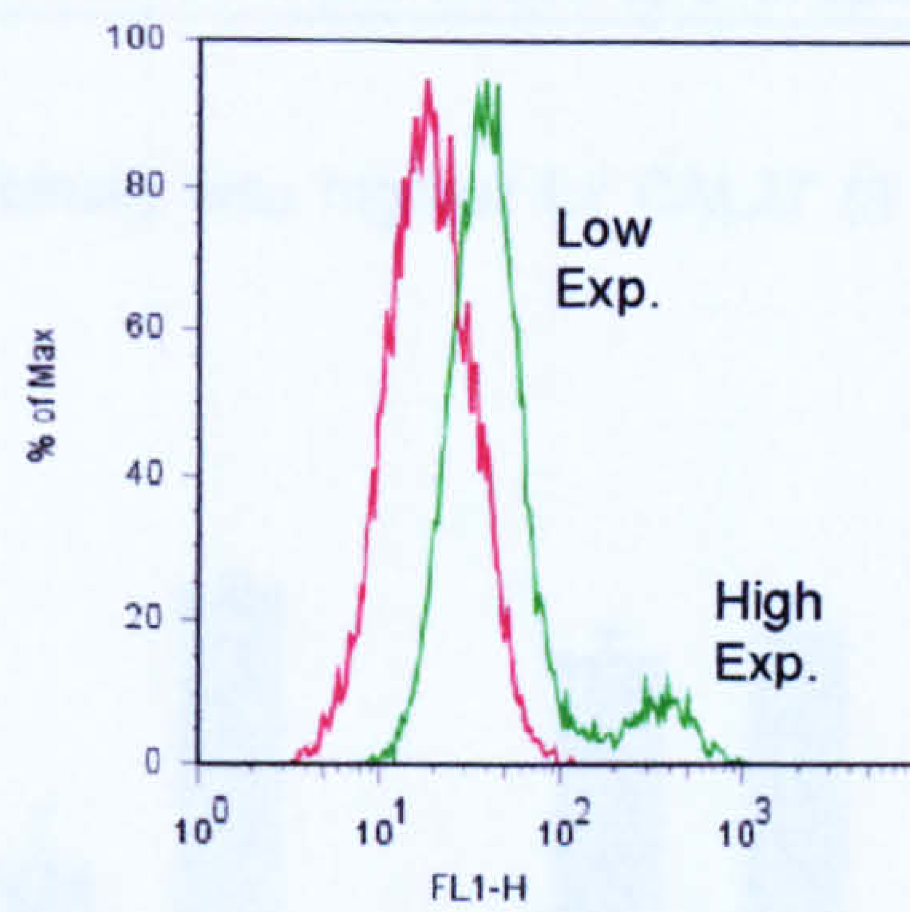
e) TR146



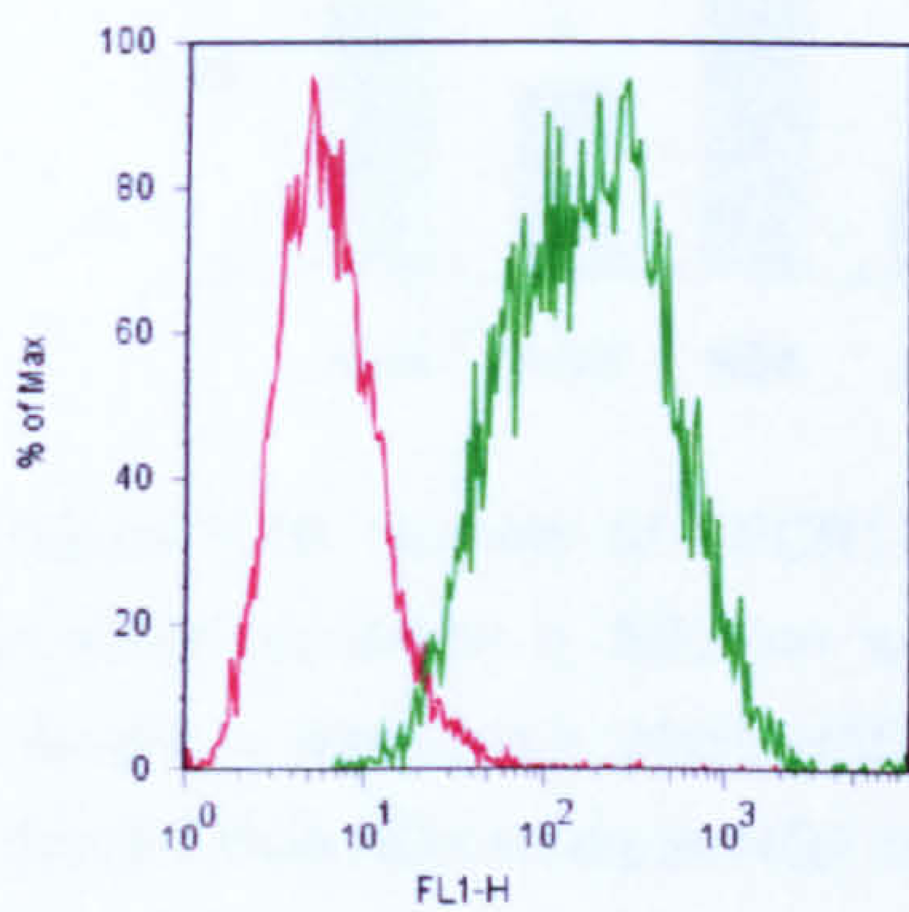
f) H357



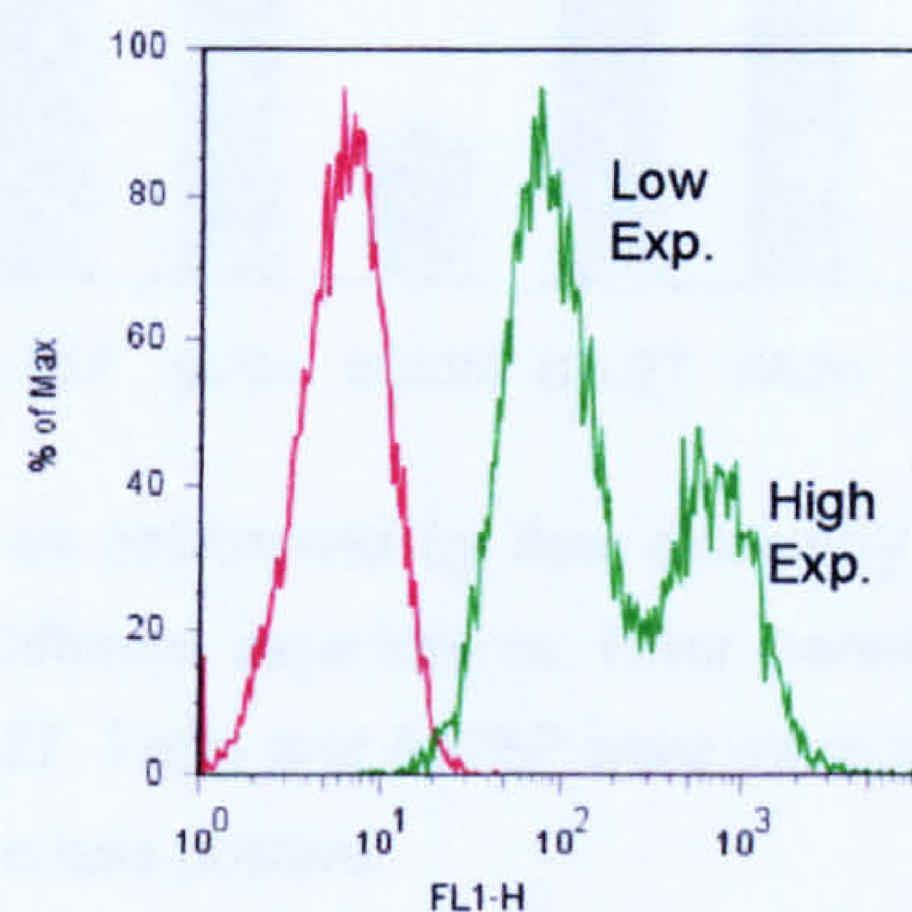
g) SCC4



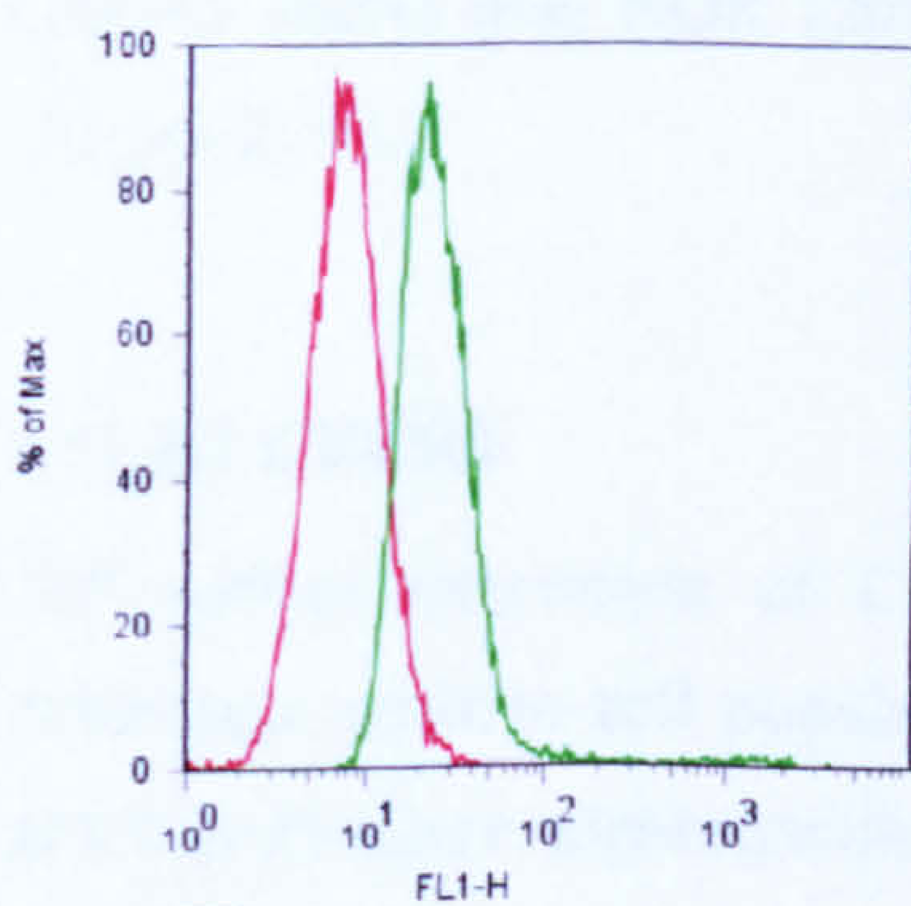
h) SCC25



i) CAL27



j) FADU



k) A375P

Figure 2.9. Representative histograms showing fluorescence intensity for CXCR1 (Red-Negative Control; Green-CXCR1). Where 'Low Exp' is low level of expression and 'High Exp' is high level of expression. Neutrophils (a), NSK (c), TR146 (e), H357 (f), SCC4 (g), CAL27 (i) and FaDu (j) cells showed more positivity for CXCR1 than NOK (b) whereas HGF (d) and

SCC25 (h) were less positive. Fluorescence intensity was highest for CAL27 (i) and FaDu cells (j).

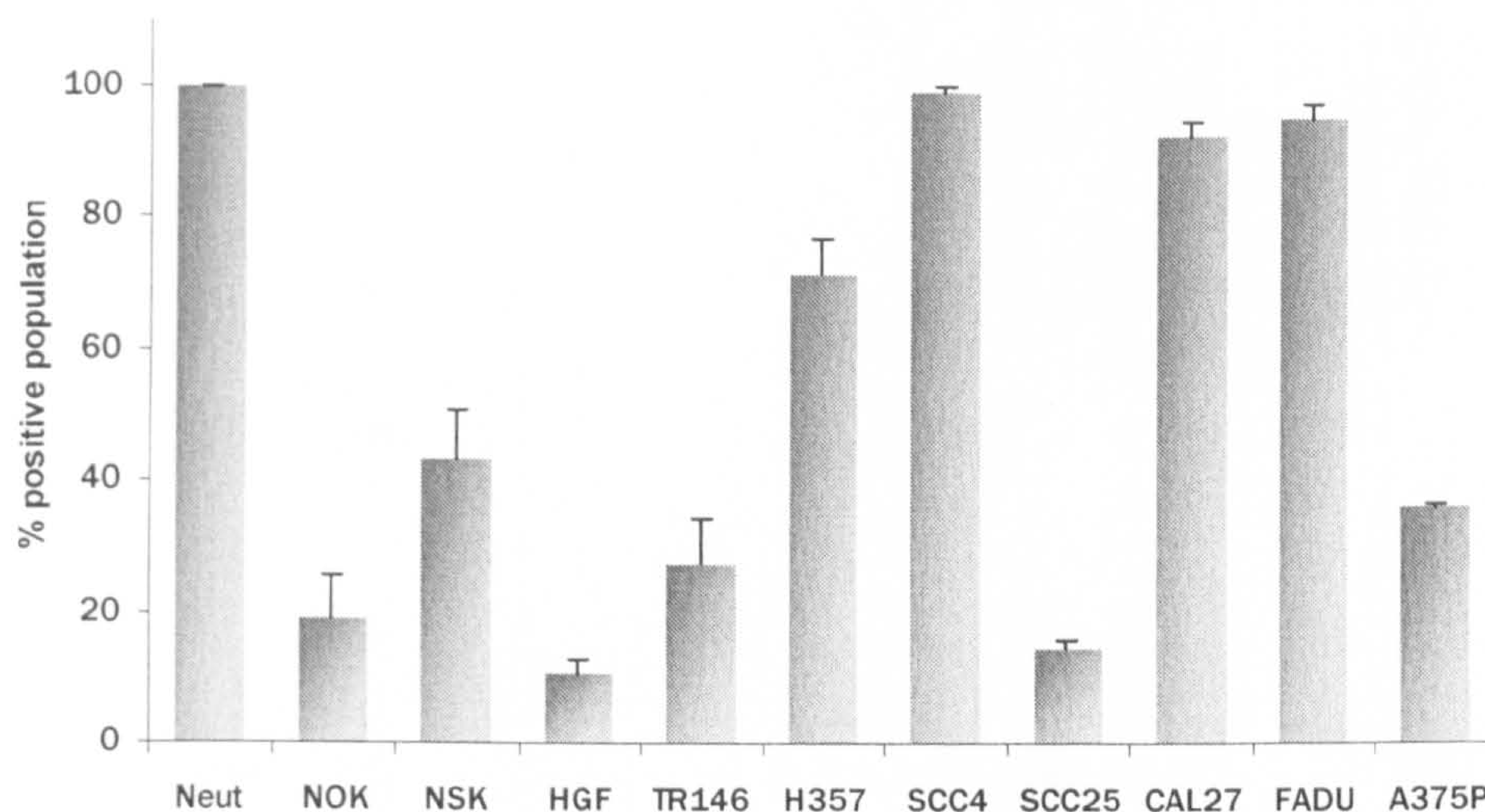


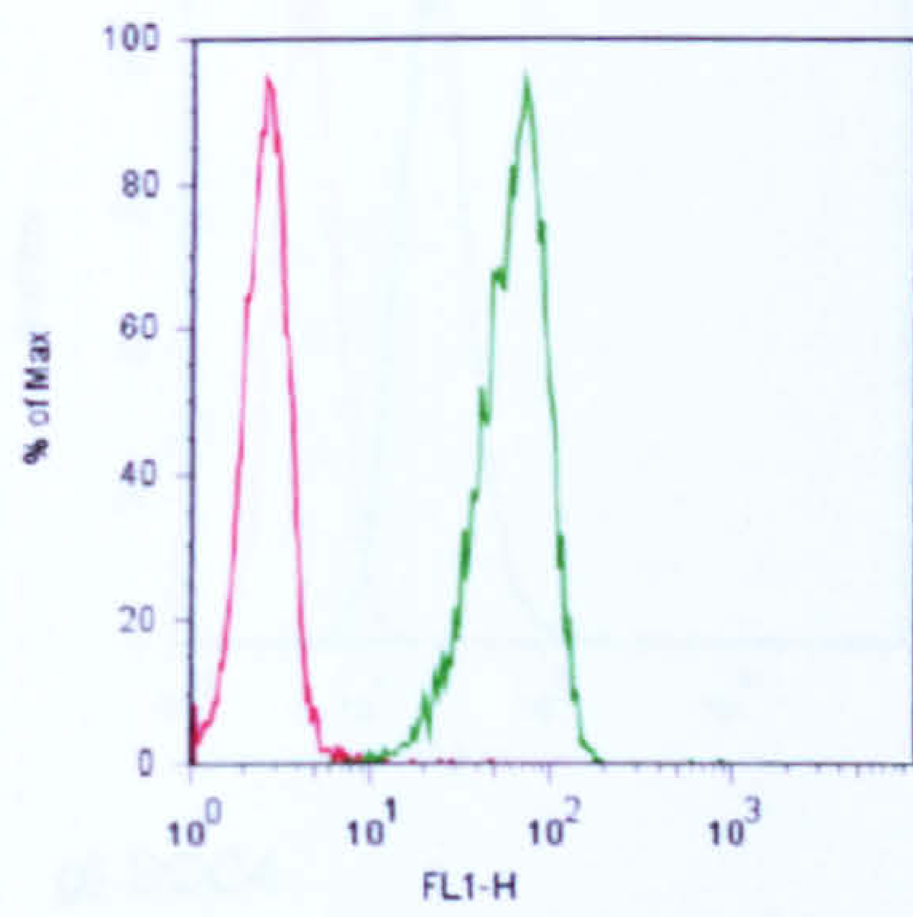
Figure 2.10. Number of CXCR1-positive cells as determined by flow cytometry (mean % positive population ± SD) (n= average of 3 different experiments, Error bars= standard deviation). Neutrophils, NSK, H357, SCC4, CAL27, FaDu and A375P were more positive for CXCR1 than NOK whereas HGF and A375P were less positive.

Neutrophils exhibited highest positivity for CXCR1 whereas between 80-100% of SCC4, CAL27 and FaDu were CXCR1-positive. More NSK were positive for CXCR1 (42%) than NOK (20%). HGF, SCC25 and TR146 were the least positive (Figure 2.10).

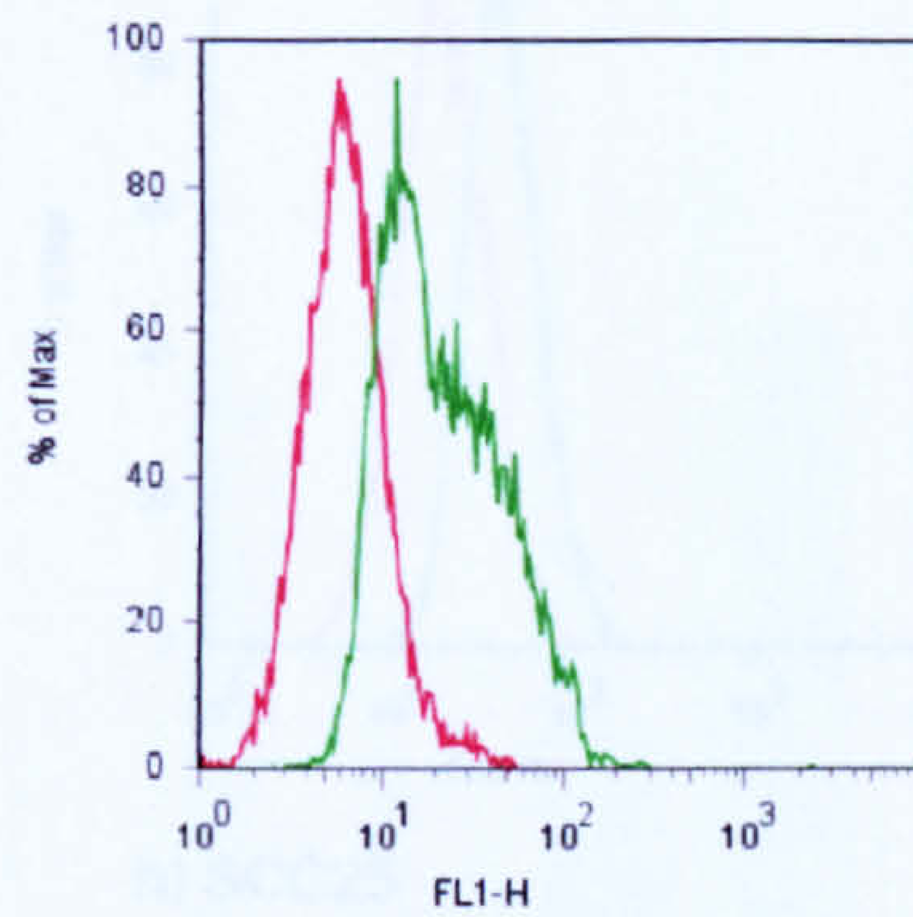
2.11.2.3 CXCR2

Cell surface expression of CXCR2 was detected on all cells other than HGF. Percentage positive cell population for CXCR2 was highest in isolated neutrophils, SCC4 and FaDu compared with all other cells (Figure 2.11).

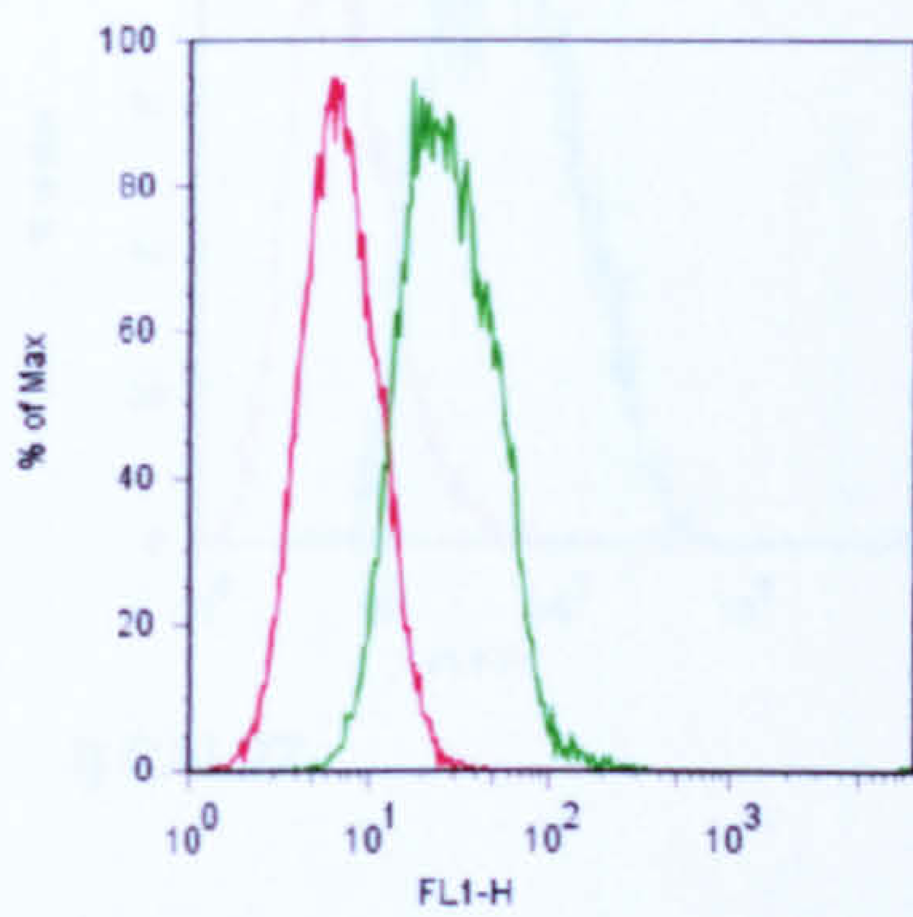
Expression was variable between different cell types and some cancer cells such as SCC25, TR146 and A375P were less positive than NOK. More NSK were positive for CXCR2 than NOK. The dual positive populations observed in NOK and H357 (for XCR1 and CXCR1) were not seen with CXCR2 (which is in agreement with the immunocytochemistry results) (Figure 2.11).



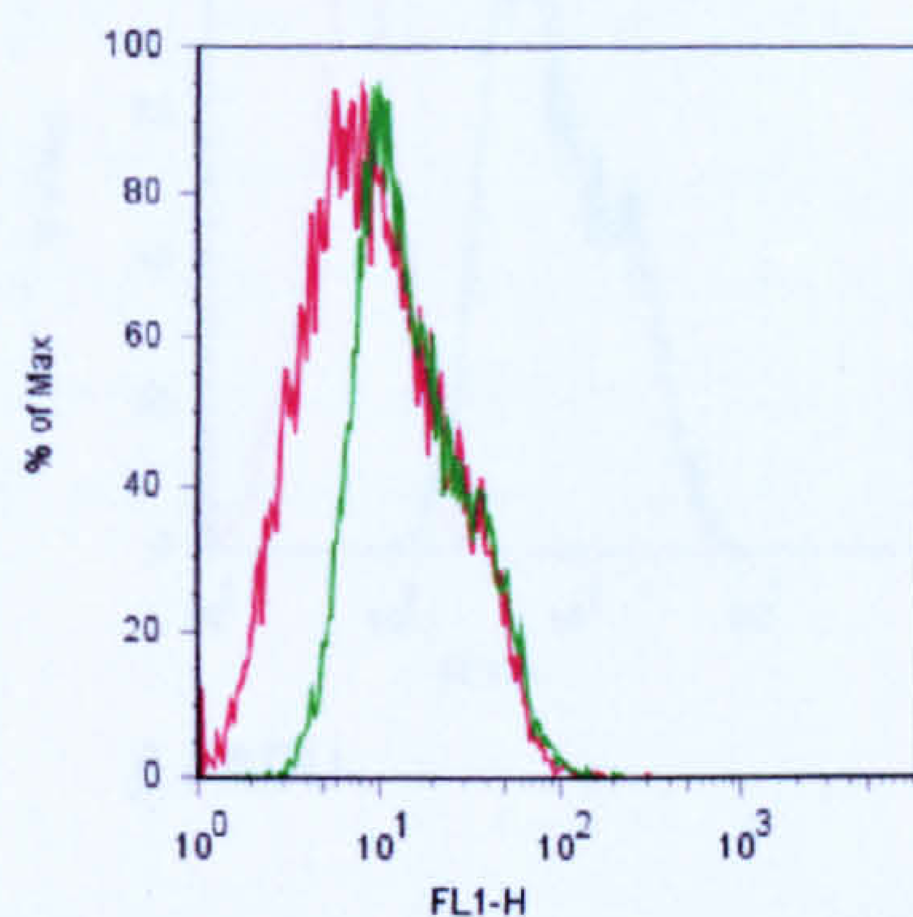
a) Isolated Neutrophils



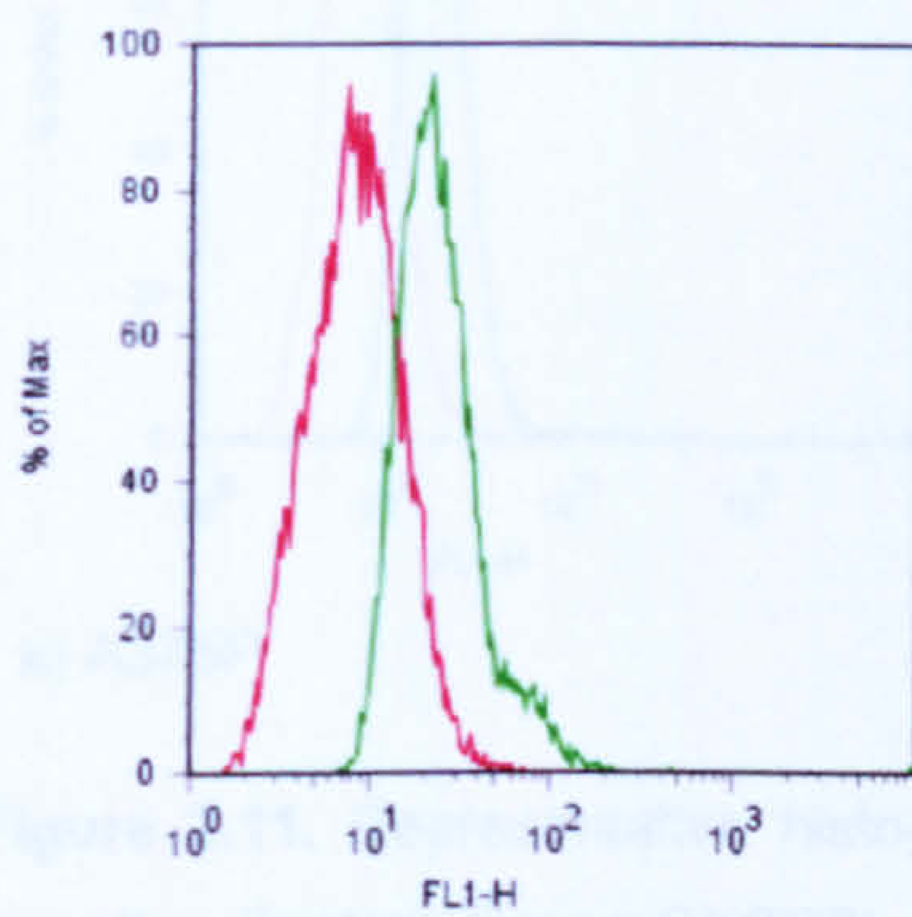
b) NOK



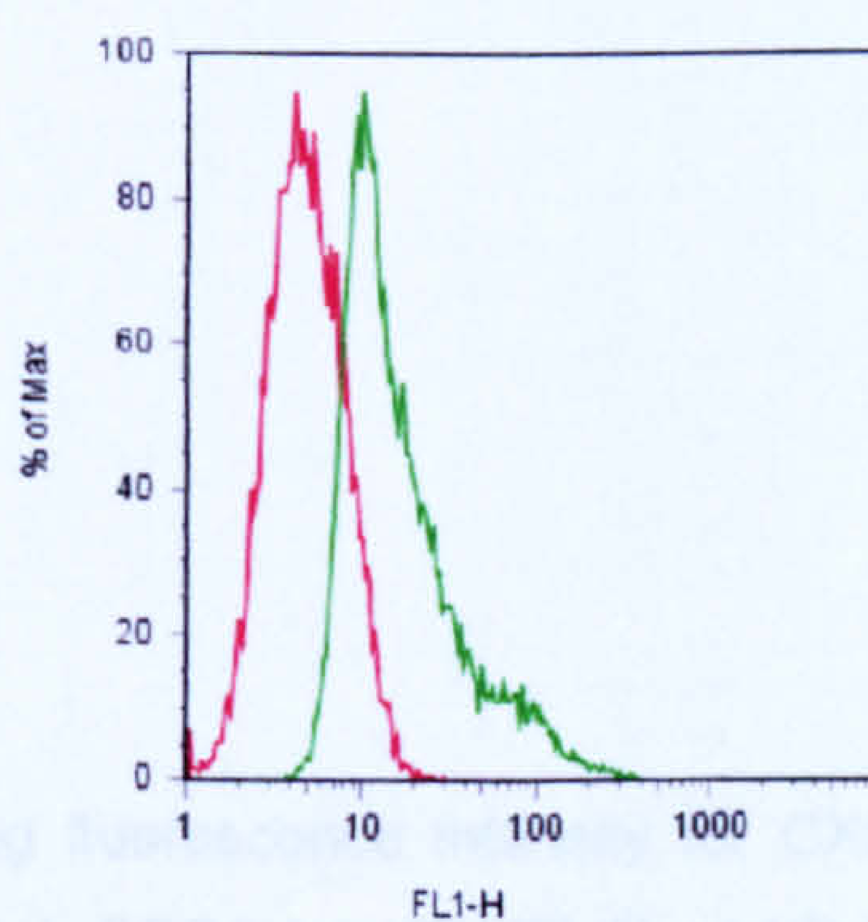
c) NSK



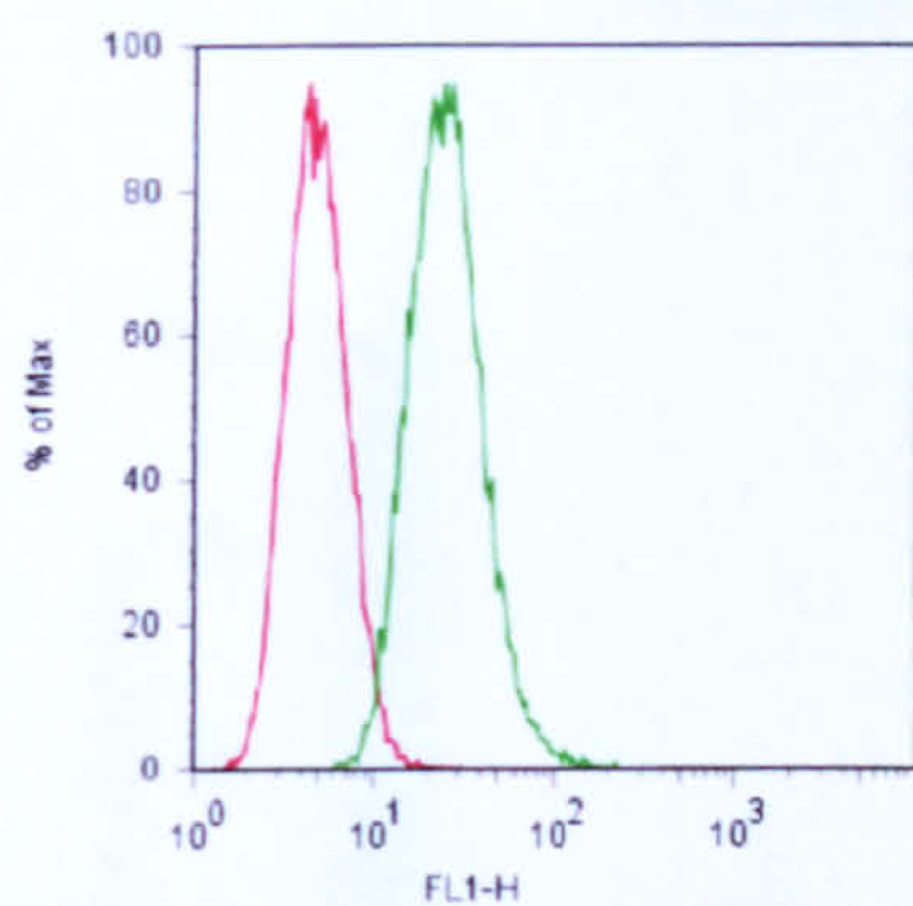
d) HGF



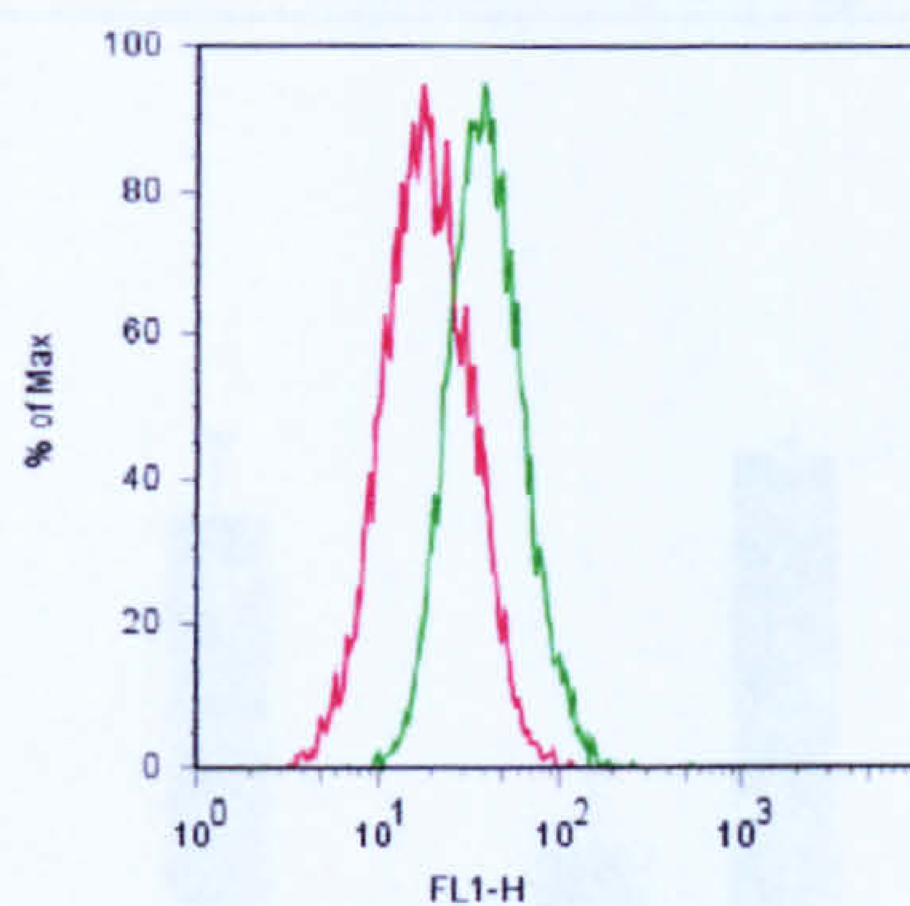
e) TR146



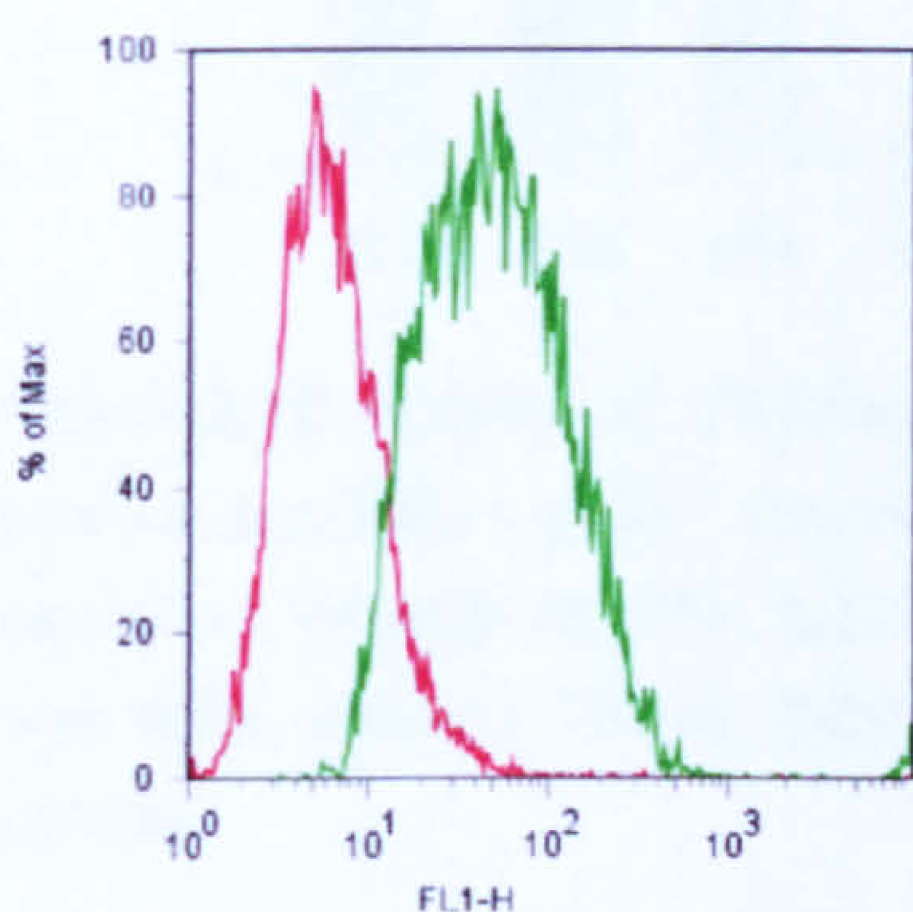
f) H357



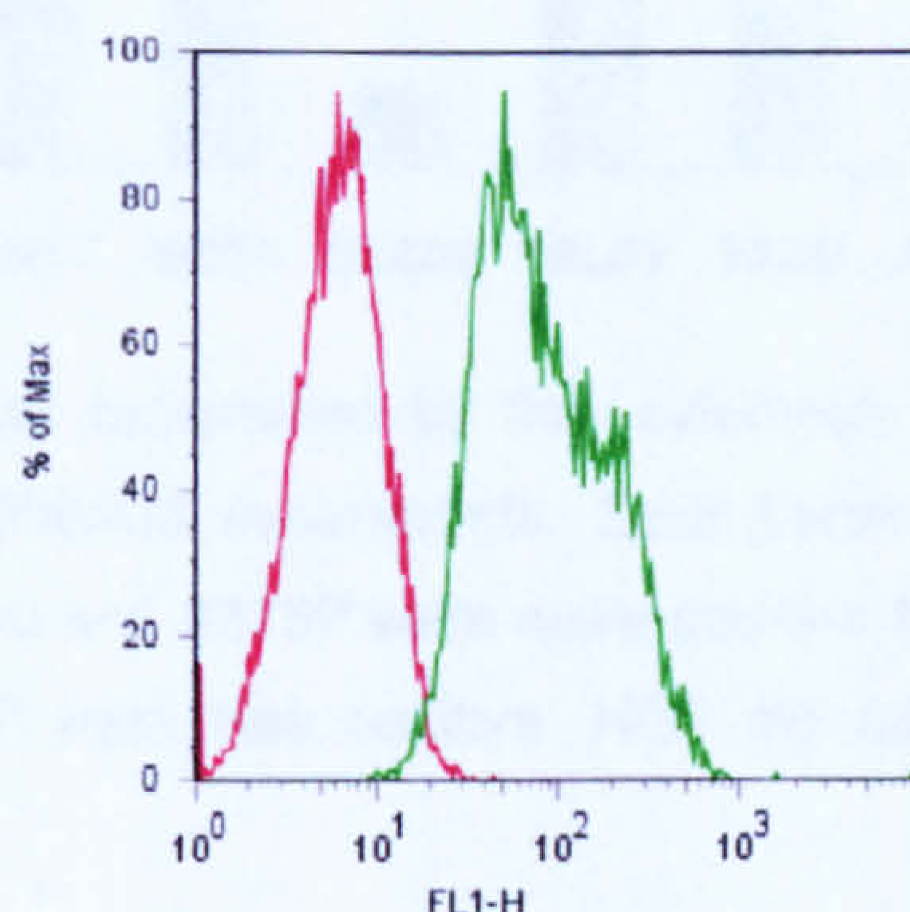
g) SCC4



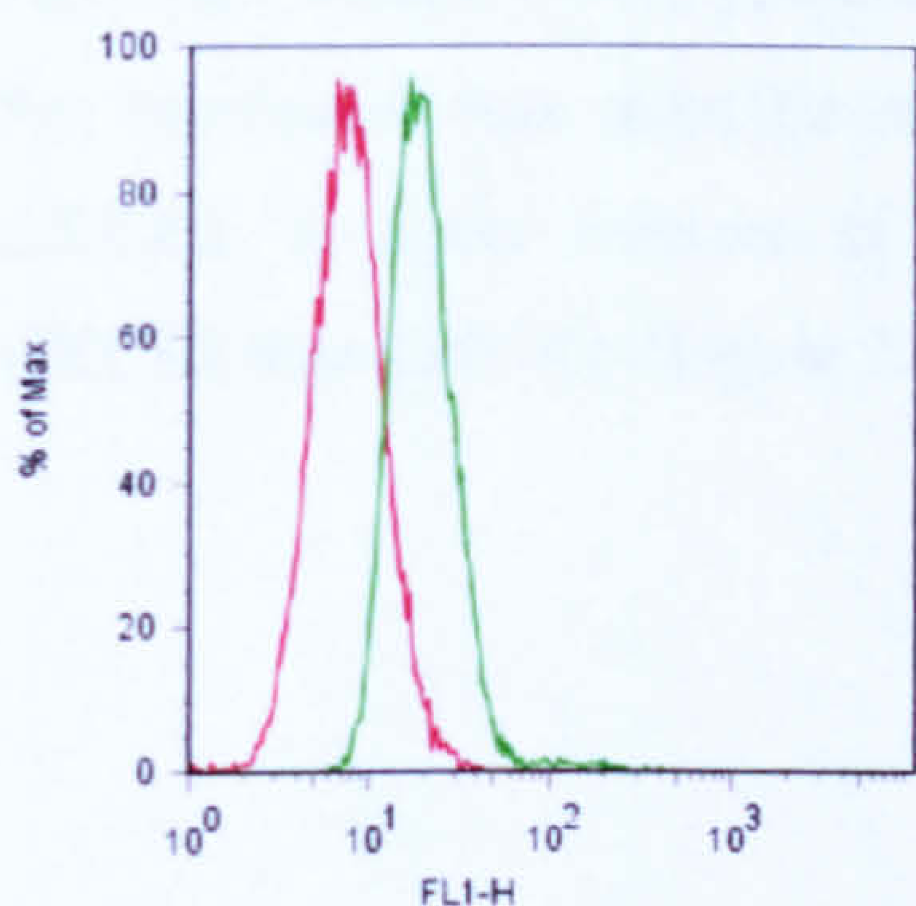
h) SCC25



i) CAL27



j) FADU



k) A375P

Figure 2.11. Representative histograms showing fluorescence intensity for CXCR2 (Red-Negative Control; Green-CXCR2). Neutrophils (a), SCC4 (g), CAL27 (i) and FaDu (j) cells showed more positivity for CXCR2 than NOK (b) whereas TR146 (e), SCC25 (h) and A375P (k) were less positive. Fluorescence intensity was highest for CAL27 (i) and FaDu (j) cells. HGF (d) did not show CXCR2 expression.

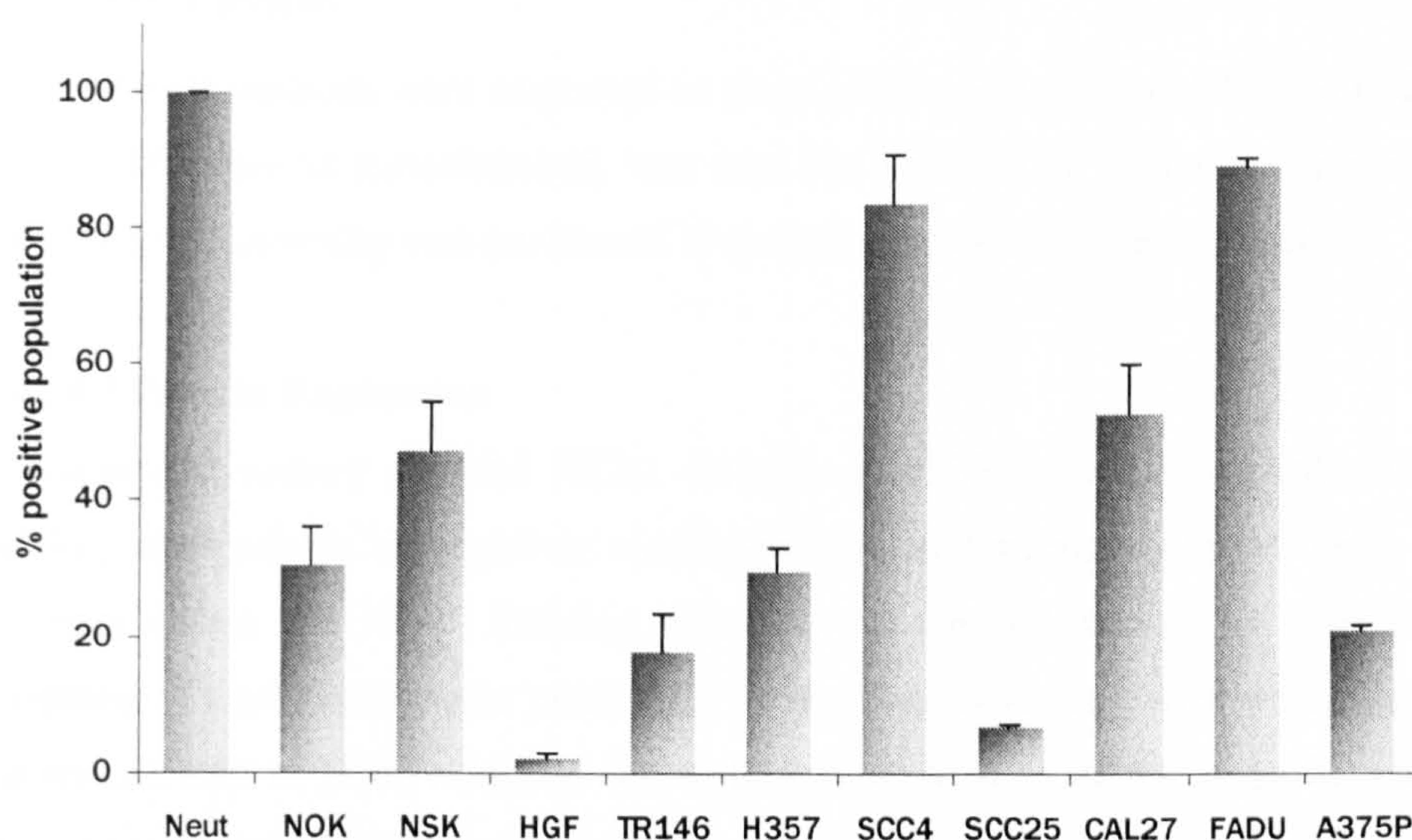


Figure 2.12. Number of CXCR2-positive cells as determined by flow cytometry (mean % positive population ± SD) (n= average of 3 different experiments, Error bars= standard deviation). Neutrophils, NSK, SCC4, CAL27, FaDu and A375P were more positive for CXCR2 than NOK whereas TR146, SCC25 and A375P were less positive. HGF did not express CXCR2.

Like CXCR1, more NSK were positive for CXCR2 (45%) than NOK (25%). However, more CXCR2 positive cells were observed in NOK and NSK than CXCR1. No expression was detected on HGF and SCC25 cells were the least positive for CXCR2. A fewer number of H357, CAL27 and A375P cells were positive for CXCR2 than CXCR1 (Figure 2.12).

2.12 DISCUSSION

Two different methods were employed to study protein expression of XCR1, CXCR1 and CXCR2. Immunocytochemistry was used for a subjective analysis of expression whereas flow cytometry was performed to quantify the cell surface expression.

i. XCR1 Protein Expression

Immunocytochemistry showed XCR1 staining in NOK and the intensity of this staining was variable as negative, weakly-positive and strongly-positive cells were observed within the NOK. Staining was seen on the cell surface as well as the cytoplasm. These results were confirmed by the flow cytometry data which showed that around 40% of NOK exhibited cell surface expression of XCR1. A similar pattern of staining to that of immunocytochemistry was noticed as the histograms consistently showed two distinct positive cell peaks suggesting that this finding is indeed real and there is a variable level of XCR1 expression within the same NOK population (Figure 2.7c). XCR1 expression was also detected on NSK which had a similar number of XCR1-positive cells as NOK but had lower fluorescence intensity (Figure 2.7d).

A more uniform pattern of expression was seen by immunocytochemistry and flow cytometry in HGF and XCR1 staining was seen on the cell surface and in the cytoplasm. This finding is comparable to that of Blaschke *et al.*, who showed XCR1 expression in cultured fibroblast-like synoviocytes (FLS) (Blaschke *et al.*, 2003). They utilised in situ hybridization to study XCR1 expression in FLS which showed staining on the cell surface as well as in the cytoplasm.

Staining for XCR1 was also observed in cancerous oral and melanoma cells and this staining appeared stronger in some cancer cell lines. For example SCC4 cells showed stronger staining intensity for XCR1 than other cells. Immunocytochemistry also suggested that three distinct cell populations may be present in H357 cells, a negative, a weakly positive and a strongly positive population whereas other tested cells (HGF, TR146, SCC4 and A375P) showed a uniform staining pattern. Cultured monolayers of cells were not permeabilised during the staining procedure. In spite of that, XCR1 staining was seen on the cell surface and in the cytoplasm in all cells. The immunocytochemical results were confirmed by flow cytometry for XCR1 which also

showed cell surface expression on the positive controls and all cancer cell lines. A higher number of SCC4, CAL27 and FaDu cells were positive for XCR1 whereas TR146 and SCC25 were less positive. The CAL27 and FaDu cells also showed the highest fluorescence intensity for XCR1 (as indicated by the histogram shift) suggesting that these cells express high levels of XCR1 (Figure 2.7j, 2.7k).

Interestingly, flow cytometry also showed two distinct XCR1-positive cell populations in H357 cells with one population expressing higher levels than the other (as shown by two peaks in the histogram) (Figure 2.7g). This is in harmony with the immunocytochemistry results and suggests that XCR1 expression in H357 cells is different to other cells as they exhibit a mixed population of XCR1-negative, -weakly positive and -strongly positive cells. Flow cytometry histograms from a recent paper on the role of chemokine receptors in nasopharyngeal carcinoma show the existence of distinct cell populations within nasopharyngeal carcinoma cell lines when tested for CXCR6 and CCR7 (Ou *et al.*, 2006). However, the authors did not comment or provide an explanation for this phenomenon. There is a possiblity that chemokine receptor expression may be correlated to the cell size or cell cycle with cells in a certain stage of the cell cycle expressing higher levels than others. However, whether this is so is outside the scope of this study and hence the significance of this mixed population expression pattern remains to be determined.

To summarize, no distinct difference in staining intensity was observed between NOK and OCCL by immunocytochemistry. Flow cytometry results showed that some oral cancer cell lines (H357, SCC4, CAL27, FaDu) had more XCR1-positive cells compared with NOK, NSK and HGF. However, the TR146 and SCC25 cell lines were even less positive for XCR1 than normal cells. There does not appear to be a consistent change in XCR1 expression in OCCL compared with NOK.

These results validate the preliminary micro-array data which suggested XCR1 mRNA is present in NOK and OCCL and confirm XCR1 protein expression on normal and cancerous oral epithelial cells for the first time. Similarly, the melanoma cell line A375P has not been shown to express XCR1. Expression of XCR1 on epithelial cells has not been reported to date. However, the significance of this expression on oral epithelial cells and fibroblasts remains to be elucidated.

ii. Protein expression of CXCR1

The positive control A375P cell line stained for CXCR1 and this is in agreement with the findings of Varney *et al.* (Varney *et al.*, 2003). CXCR1 staining was observed on the cell surface and in the cytoplasm of NOK by Immunocytochemistry. An interesting pattern of staining was noticed as there were three distinct cell groups negative, weakly-staining and strongly-staining cells for CXCR1. This is somewhat different to the findings of Sfakianakis *et al.* who carried out staining for CXCR1 on gingival keratinocytes and reported 100% positivity for CXCR1 (Sfakianakis, Barr, and Kreutzer 2002). Flow cytometry substantiated our results and showed a negative, a low expressing and a high expressing positive cell population. CXCR1 cell surface expression was also detected on NSK which has not been shown previously. More NSK cells (approximately 40%) were positive for CXCR1 than NOK (20%).

CXCR1 expression was also observed on HGF which has not been reported before. HGF exhibited a more uniform CXCR1 staining pattern than NOK and staining was seen on the cell surface and in the cytoplasm.

CXCR1 staining was also seen in the OCCL. Like NOK, the H357 cells also showed a mixed positive cell pattern and negative, weakly-positive and strongly-positive cells were seen. SCC4 cells showed stronger staining intensity than TR146 cells but overall, no observable difference in staining intensity was noticed between the normal and cancer cells. Flow cytometry confirmed the immunocytochemical results and CXCR1 expression was detected on the positive controls and all OCCL. Other than SCC25, all oral cancer cells had a higher number of CXCR1-positive cells than NOK and SCC4, CAL27 and FaDu cells had more positive cells than H357 and TR146. In addition H357, TR146, SCC25 and FaDu cells also showed two distinct CXCR1-positive populations (a low expressing and a high expressing one). Similar results for CXCR1 expression have been reported in colonic epithelial cell lines CaCo-2 and HT-29 which show two distinct cell populations (Sturm *et al.*, 2005). Watanabe *et al.* have previously used flow cytometry to study cell surface expression of CXCR1 on NA and HSC-4 oral cancer cell lines with HSC-4 cells showing higher fluorescence intensity but did not report high-expressing and low-expressing cell populations (Watanabe *et al.*, 2002). In addition, they did not quantify the percentage of CXCR1-positive cells or compare it to NOK.

Overall, the results suggest a greater proportion of oral cancer cells than NOK are positive for CXCR1.

iii. Protein expression of CXCR2

Membranous and cytoplasmic staining for CXCR2 was observed on the positive control (A375P cell line) and NOK which is consistent with existing literature (Varney *et al.*, 2003; Sfakianakis, Barr, & Kreutzer 2002). Sfakianakis *et al.* reported that 90% of gingival keratinocytes in their study expressed CXCR2. However, their results show very weak CXCR2 staining predominantly localized in the perinuclear area which differs from our pattern. The significance of this not clear but it may be the result of use of different antibodies. Alternatively it may reflect differences in the site of origin of the NOK within the oral cavity. Flow cytometry confirmed CXCR2 expression on NOK cell surface (to date flow cytometry has not been performed for CXCR2 on NOK). A higher proportion of NSK were positive for CXCR2 than CXCR1. Cell surface expression of CXCR2 has not been reported on NSK and HGF to date. No CXCR2 expression was observed on HGF by immunocytochemistry or flow cytometry.

CXCR2 staining by immunocytochemistry was also observed on the positive control and OCCL and this was corroborated by flow cytometry. No difference in staining intensity was noticed between normal and cancerous oral cells. The staining pattern for CXCR2 in H357 cells was different to CXCR1 as no distinct weakly staining and strongly staining cells were seen. However, the number of CXCR2-positive cells for all cancer cell lines was consistently lower than CXCR1. This is in agreement with the findings of Watanabe *et al.* as they reported lower expression of CXCR2 on NA and HSC-4 (OCCL) than CXCR1. No consistent difference in CXCR2 expression was noticed between normal and cancer cells in our study as some cancer cells (TR146, SCC25) were less positive than NOK.

All these finding confirm protein expression of XCR1, CXCR1 and CXCR2 on normal and cancerous oral epithelial cells. Therefore, the next step was to study the functionality of the receptors. NOK, H357 and SCC4 cells were chosen for all the functional assays. H357 cells show an intermediate number of positive cells (25-70%) whereas 90-100% of SCC4 cells are positive for XCR1, CXCR1 and CXCR2. NOK

were included to provide a comparison between normal and cancerous cells and they also have the least number of XCR1, CXCR1 and CXCR2 positive cells (20-40%) out of the three chosen cells.

CHAPTER 3

Signal Transduction

3.1 INTRODUCTION

It was shown in the previous chapter that XCR1, CXCR1 and CXCR2 are expressed on the surface of normal and malignant oral epithelial cells. The next step was to establish whether these receptors are functional on the surface of oral epithelial cells. For this purpose, four different parameters were studied: signal transduction, migration/chemotaxis, invasion and proliferation. NOK, H357 and SCC4 cells were chosen for all functional assays.

The ERK1/2 (extracellular signal-regulated kinases 1 and 2) signaling pathway was studied for signal transduction. ERK1 and 2 are also known as p44 and p42 MAP kinases and as both these proteins are highly homologous they are collectively referred to as ERK1/2. The constitutive ERK1/2 present intracellularly is called total ERK1/2 and is in an unphosphorylated form. Stimulation of the cascade results in phosphorylation of ERK1/2 leading to activation. This cascade plays a vital role in the regulation of cell differentiation, cell physiology and neuronal function (Giovannini 2006; Lu and Xu 2006; Meloche and Pouyssegur 2007; Yoon and Seger 2006). Abnormal ERK1/2 activity has been associated with a number of pathological conditions including autoimmune diseases and cancer (Roberts and Der 2007).

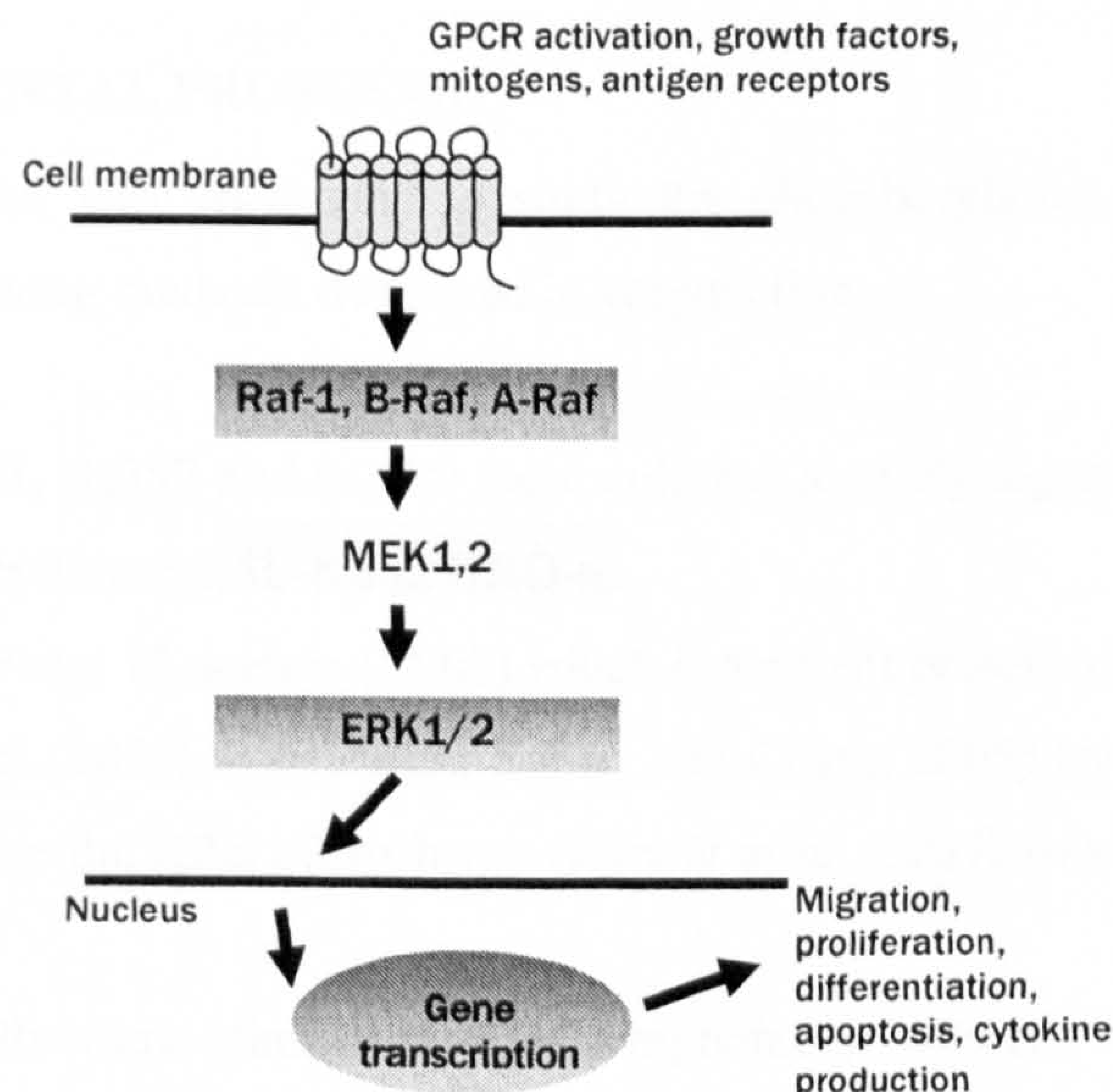


Figure 3.1. Overview of the ERK1/2 pathway. Stimulation of a cell surface receptor (by chemokines, growth factors etc.) activates downstream'Raf' followed by 'MEK' activation. MEK activates ERK1/2 which then acts on substrates within the nucleus leading to gene

transcription and facilitation of a range of actions including chemotaxis, proliferation and cell differentiation.

The ERK pathway is also involved in the signaling of chemokines including MIP-3α (Keates *et al.*, 2007) and SDF-1α (Barbieri *et al.*, 2006) in epithelial cells. Furthermore, a previous study has shown signal transduction in response to extracellular calcium through the ERK1/2 pathway in normal, pre-malignant and cancerous oral epithelial cells (Mukhopadhyay *et al.*, 2004). IL-8 and GRO-α have also been shown to signal through the ERK1/2 signaling cascade in Chinese hamster ovary (CHO) epithelial cells (Shyamala and Khoja 1998). However whether lymphotactin signals through the ERK1/2 pathway is not known.

3.2 AIM

The aim of this chapter was to establish whether the chemokine receptors XCR1, CXCR1 and CXCR2 mediate ERK1/2 signal transduction when exposed to their respective chemokines (lymphotactin and IL-8). CXCL1/GRO-α was included as it signals through CXCR2 only and not through CXCR1 (Ahuja and Murphy 1996).

3.3 EXPERIMENTAL PROTOCOL

A cell-based ELISA was performed to study the phosphorylation of intracellular ERK1/2. The following methods were used in conjunction:

- NOK and OCCL (H357 and SCC4) were cultured to study signal transduction in response to lymphotactin, IL-8 and GRO-α.
- Phorbol 12-myristat 13-acetate (PMA) which is a potent protein kinase C activator (Jorgensen *et al.*, 2005; Tepperman, Soper, and Chang 2005) and has also been shown to activate the ERK1/2 pathway (Hwang *et al.*, 2007) was used as positive control
- For XCR1, cells were stimulated with lymphotactin whereas for CXCR1 and CXCR2, IL-8 and GRO-α were used. GRO-α was used since it signals specifically through CXCR2 but not through any other receptor.
- The chemokine CCL25/TECK was used as an irrelevant chemokine control.

- Triplicate wells for each cell line and treatment were used in all assays and the ELISA was performed on three different occasions.

3.4 MATERIALS AND METHODS

3.4.1 CELL CULTURE

Cells were grown and maintained as described previously.

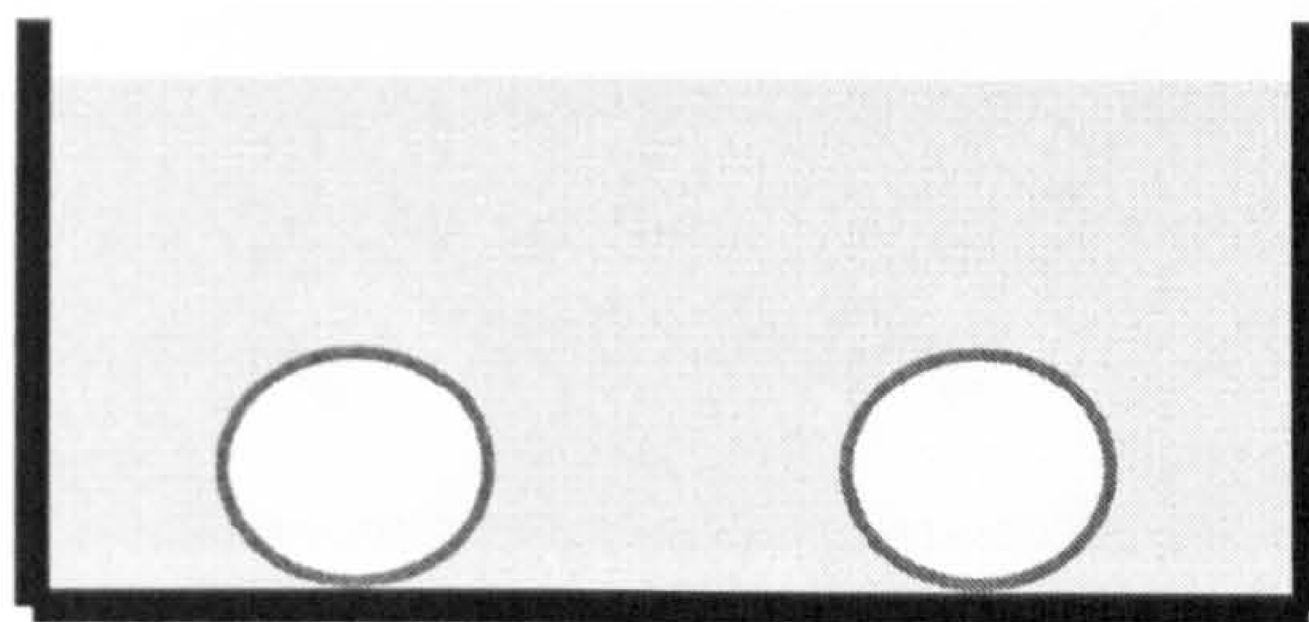
3.4.2 ERK1/2 ELISA

A fast activated cell-based ELISA (FACE) kit (Active Motif, Rixensart, Belgium) was used to study ERK1/2 phosphorylation. H357 cells were detached using cell dissociation buffer (Sigma) and seeded at a density of $6x10^3$/well in 96-well tissue culture plates (Corning, Lowell, MA, USA). Cells were grown to 80% confluence, washed with PBS and exposed to serum-free medium (Appendix 10.1.5) for 16h. Lymphotactin (100ng/ml) (R&D Systems), IL-8 (100ng/ml) (Sigma) and GRO-α (100ng/ml) (Peprotech, Rocky Hill, NJ, USA) were used to stimulate the cells for 10min before being fixed. TECK/CCL25 (Peprotech) was used as an irrelevant chemokine. Phorbol 12-myristat 13-acetate (PMA) (Sigma) was used as positive control (50ng/ml) whereas absence of ligands served as negative control.

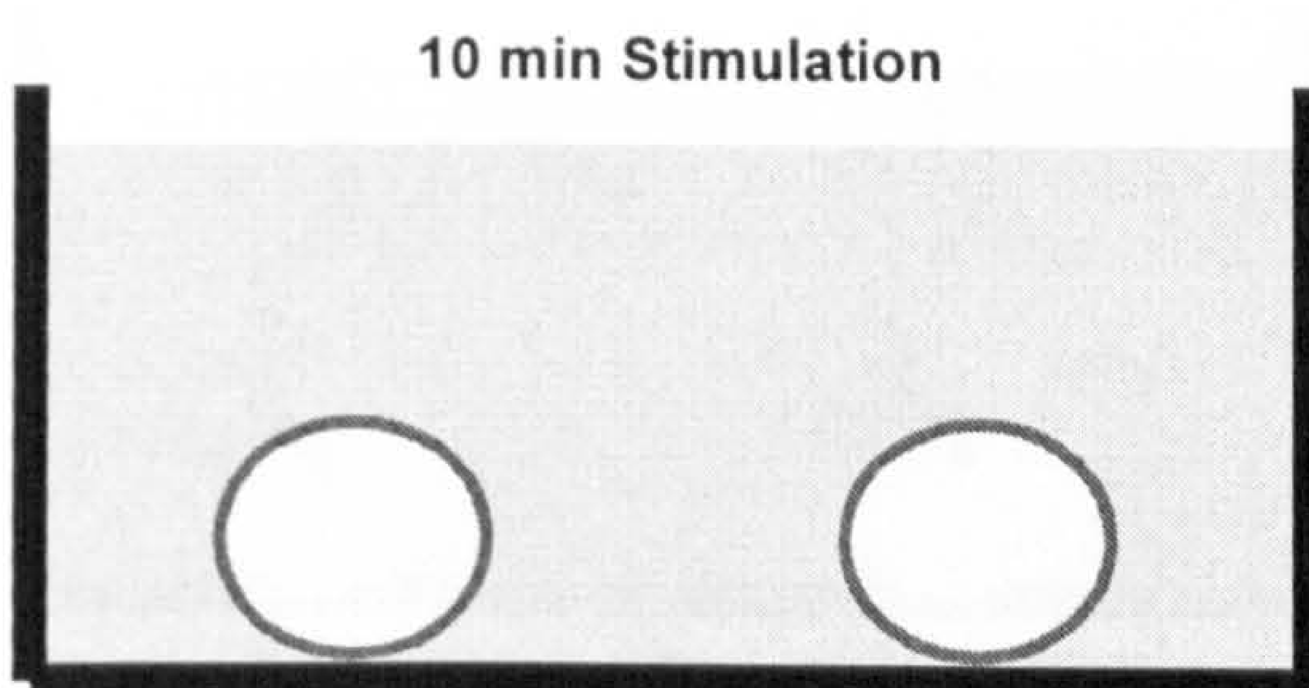
Cells were fixed with 2% paraformaldehyde for 20min at RT and washed three times with 100μl wash buffer (provided with kit) for 5min with gentle shaking. Wash buffer was removed and 100μl quenching buffer (provided with kit) was added for 20min at RT. After removal of quenching buffer, cells were washed two more times with wash buffer followed by the addition of antibody blocking buffer (provided with kit) for 1h. This was followed by the application of 40μl of diluted primary antibody for phosphorylated ERK1/2 and total ERK1/2 (in accordance with the manufacturer's instructions). The plate was sealed and cells incubated with primary antibody overnight at 4°C.

Primary antibody was removed the next day and cells washed three times as described previously. 100μl of secondary antibody was added (provided with kit), plate was sealed and incubated for 2h at RT with gentle shaking. Secondary antibody was

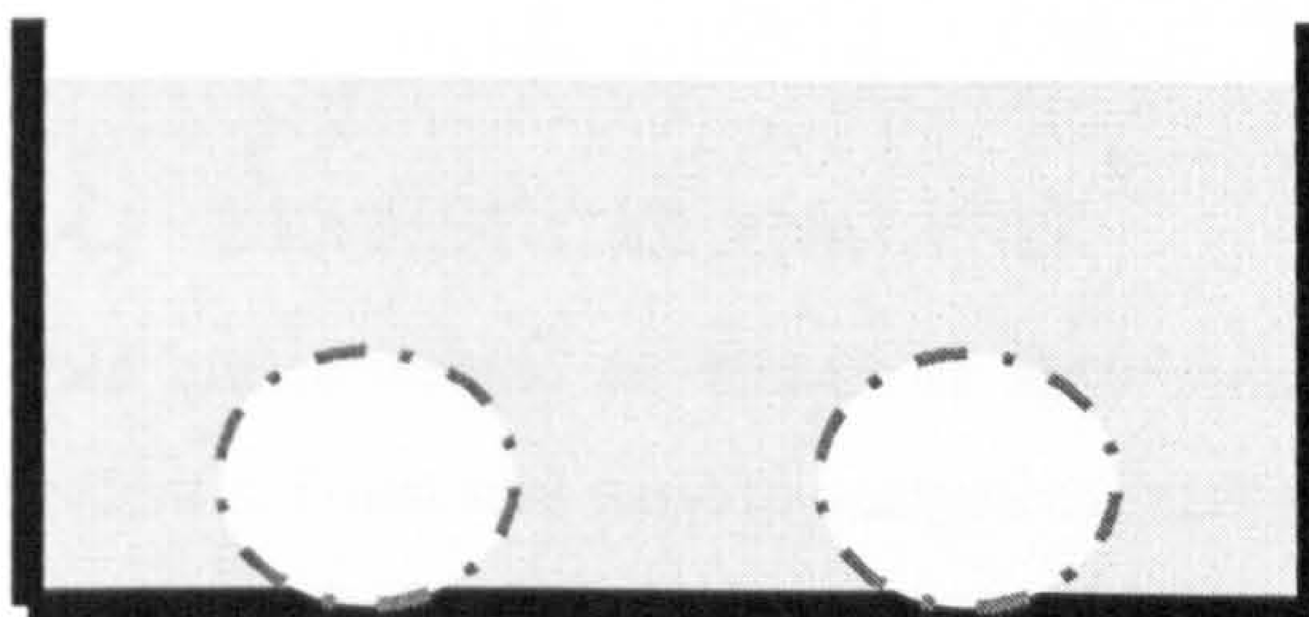
removed and cells washed followed by the addition of 100µl of developing solution (provided with kit) to each well. The plate was incubated for 10min at RT protected from direct light until the darkest staining wells were medium to dark blue. 100µl of stop solution (provided with kit) was added to stop the colour development which turned the blue colour to yellow. Absorbance was read on a spectrophotometer within 5min at 450nm.



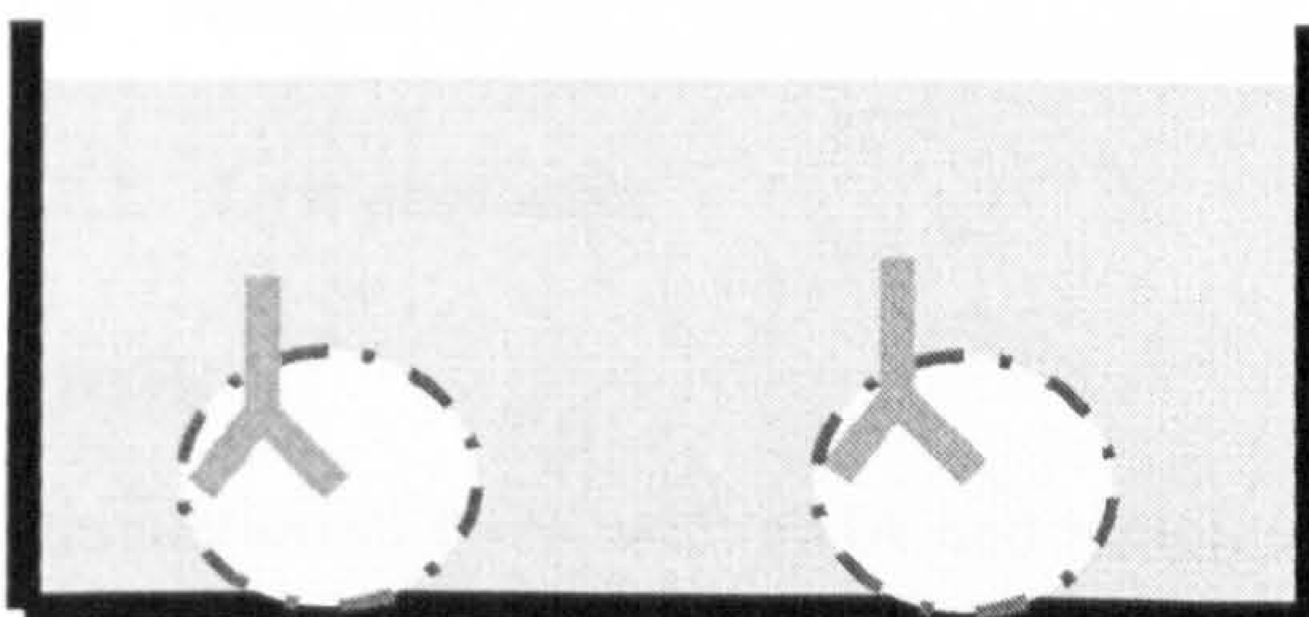
Cells seeded in 96-well tissue culture plates, allowed to adhere and serum-starved.



Cells stimulated with PMA, Lymphotactin, IL-8 and GRO-α for 10 min and immediately fixed using paraformadehyde.



Permeabilisation carried out to allow excess to the cell interior.



Primary antibody against phosphorylated and total ERK1/2 added overnight. Unbound antibody removed by washing.

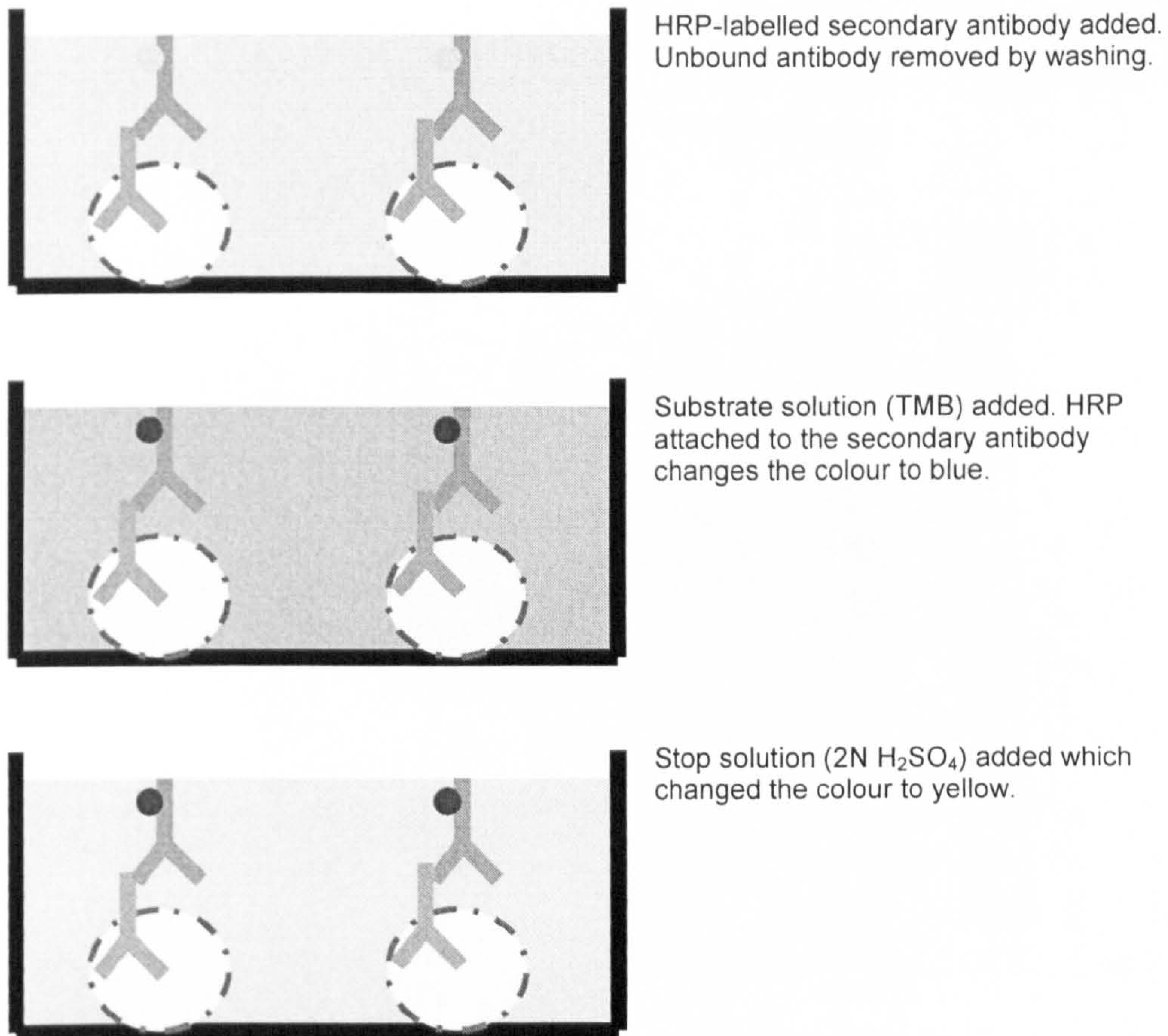


Figure 3.2. Overview of the ERK1/2 cell-based ELISA protocol.

3.4.3 STATISTICAL ANALYSIS

Data are presented as means of absorbencies +/- standard deviation (SD). Paired student's T-test was used to analyse the statistical significance of the results

3.5 RESULTS

3.5.1 Lymphotactin

i) NOK

Stimulation of NOK with PMA and lymphotactin resulted in a significant increase in intracellular ERK1/2 phosphorylation compared with unstimulated cells ($p<0.0001$ and $p<0.001$ respectively) (Figure 3.2). Lymphotactin increased ERK1/2

phosphorylation in a dose dependent manner in NOK. The irrelevant chemokine CCL25/TECK failed to elicit a response. No significant difference in total ERK1/2 was observed between any of the different treatments (Figure 3.2).

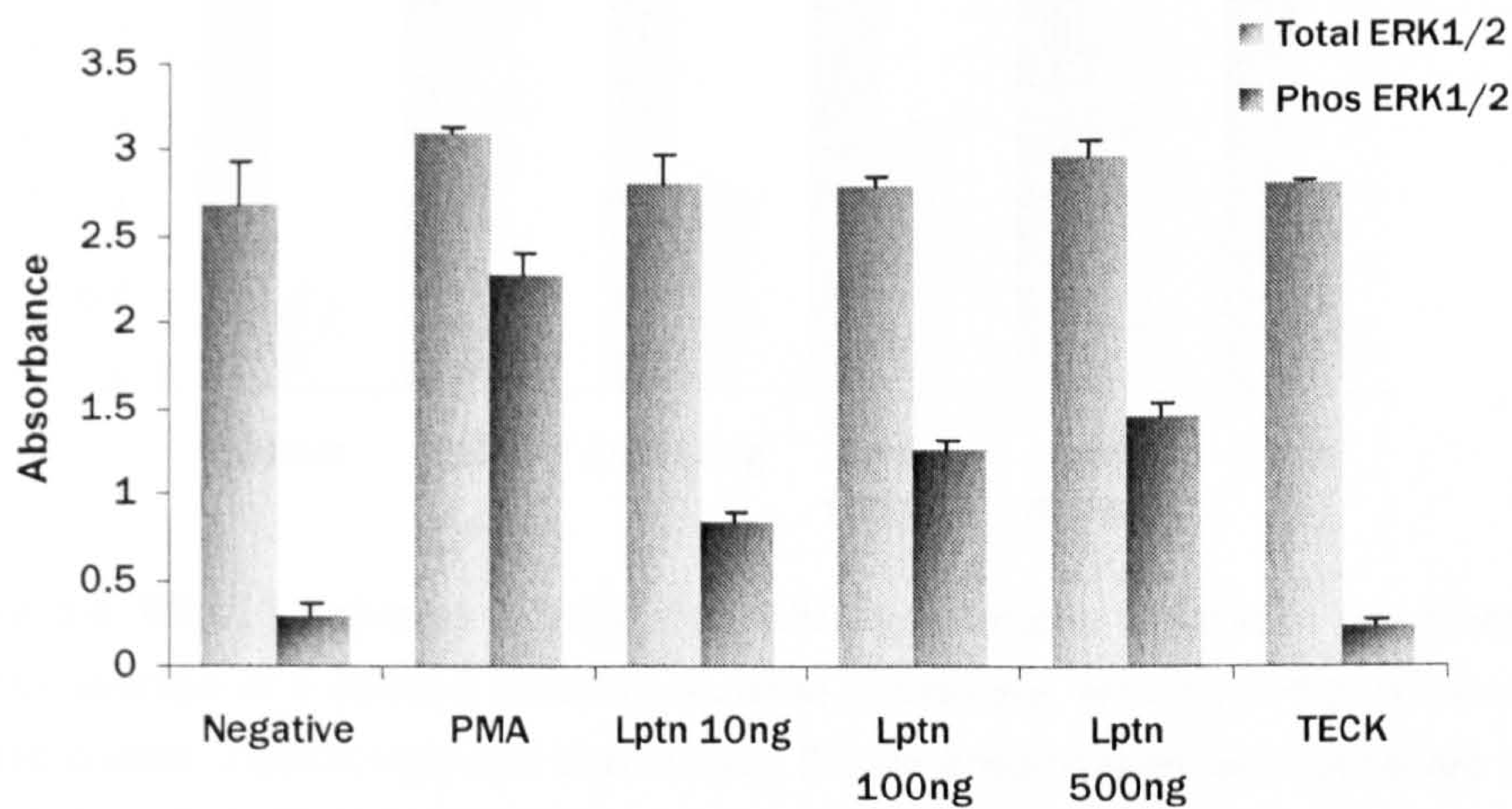


Figure 3.3. ERK1/2 activation in NOK in response to lymphotactin (mean absorbencies ± SD) (n= average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. Lymphotactin but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

ii) H357 and SCC4 cells

Stimulation of H357 and SCC4 cells with PMA (the positive control) caused a highly significant increase in ERK1/2 phosphorylation compared with unstimulated cells ($p<0.000001$ and $p<0.00001$ respectively) (Figure 3.3 and 3.4). Exposure to lymphotactin also caused a significant and dose-dependent increase in ERK1/2 phosphorylation in H357 and SCC4 cells compared with negative controls ($p<0.0001$ and $p<0.001$ respectively). However, this increase was less than the increase observed with PMA (Figure 3.3 and 3.4). TECK did not have any effect on ERK1/2 activation.

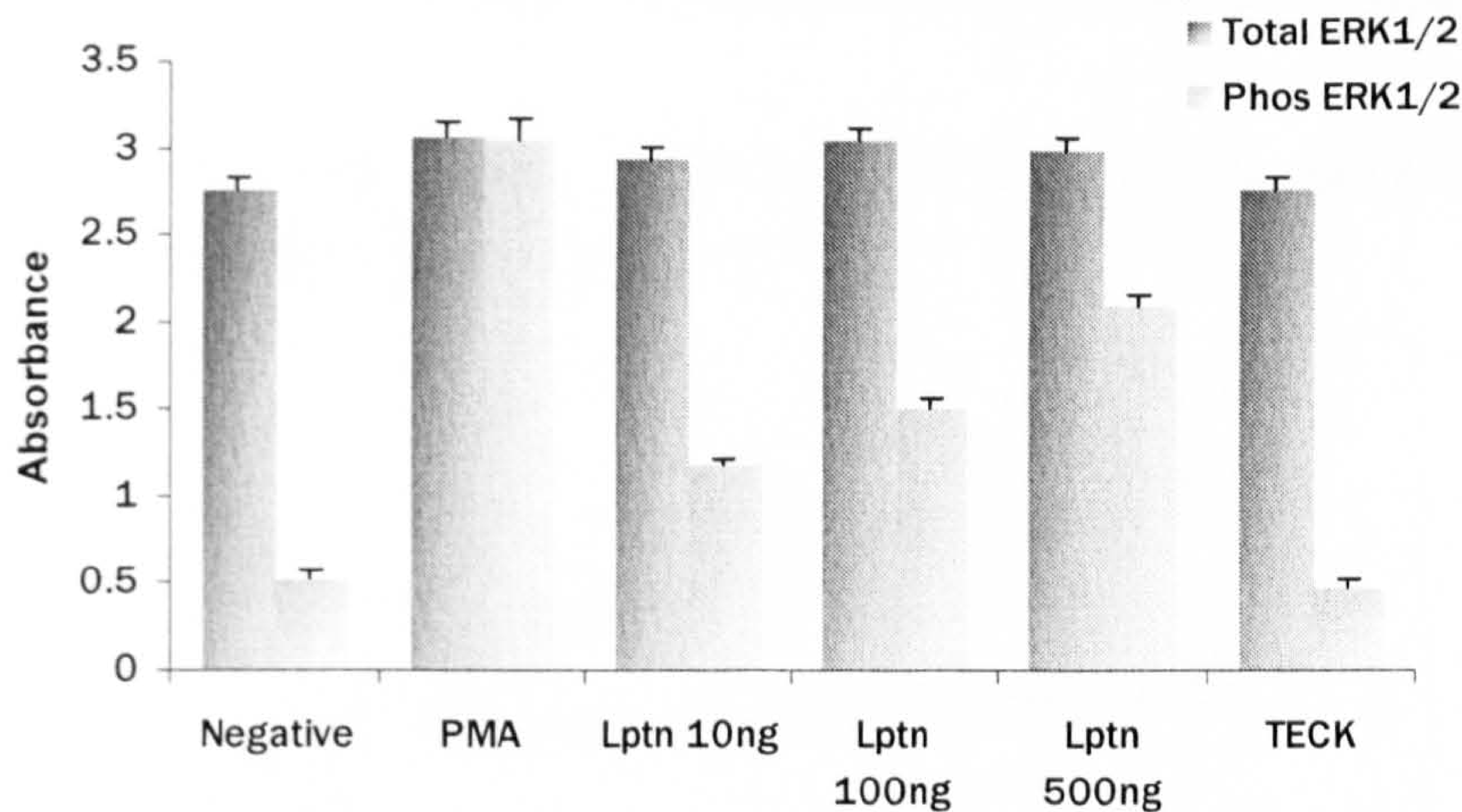


Figure 3.4. ERK1/2 activation in H357 cells after lymphotactin stimulation (absorbencies ± SD) (n= average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. Lymphotactin but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

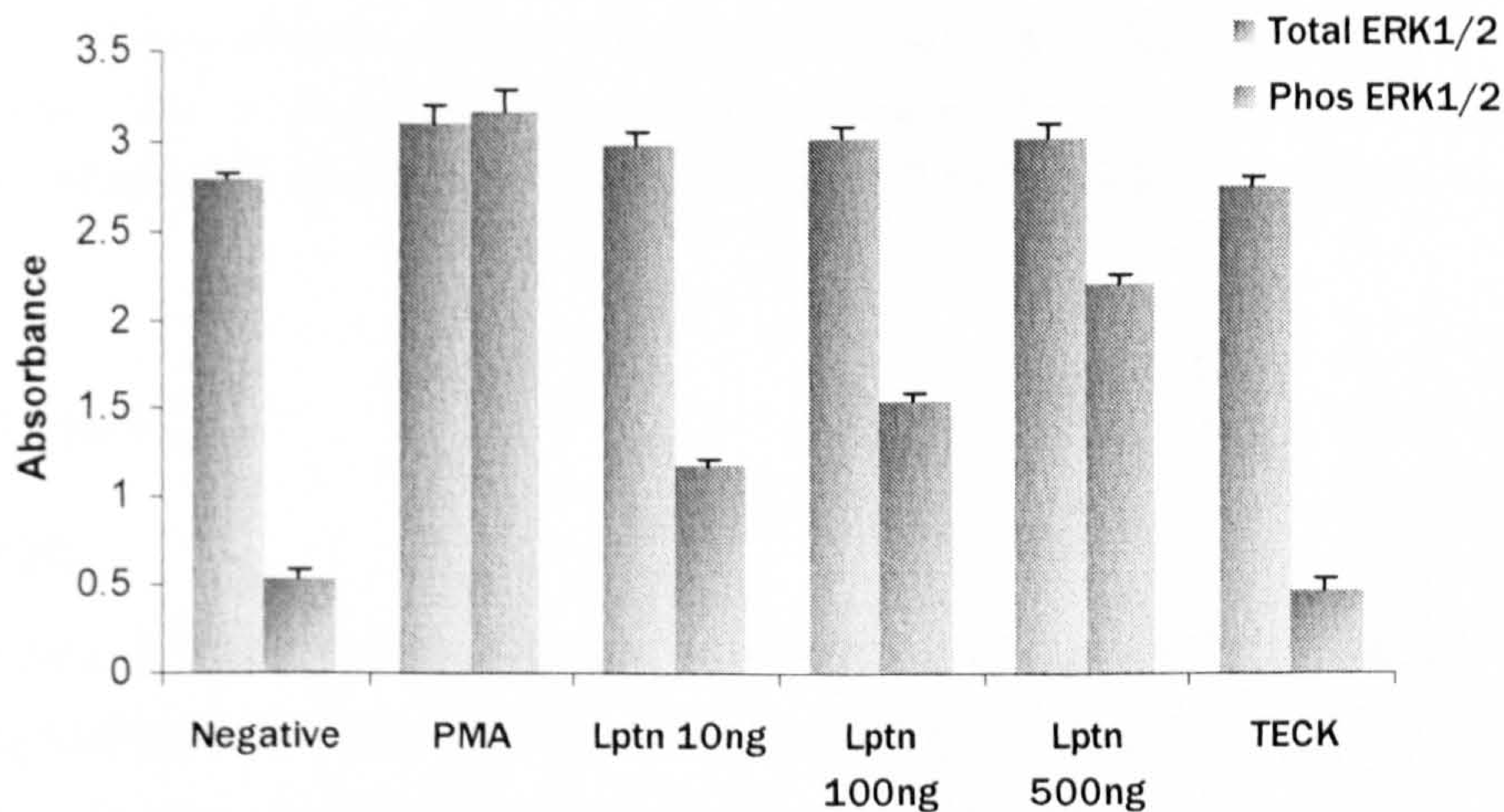


Figure 3.5. ERK1/2 activation in SCC4 cells after lymphotactin stimulation (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. Lymphotactin but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

iii) Comparison of lymphotactin-mediated ERK1/2 phosphorylation between NOK and OCCL

Phosphorylated ERK1/2 levels in H357 and SCC4 (OCCL) were significantly higher than NOK at all doses ($p<0.05$) (Figure 3.5).

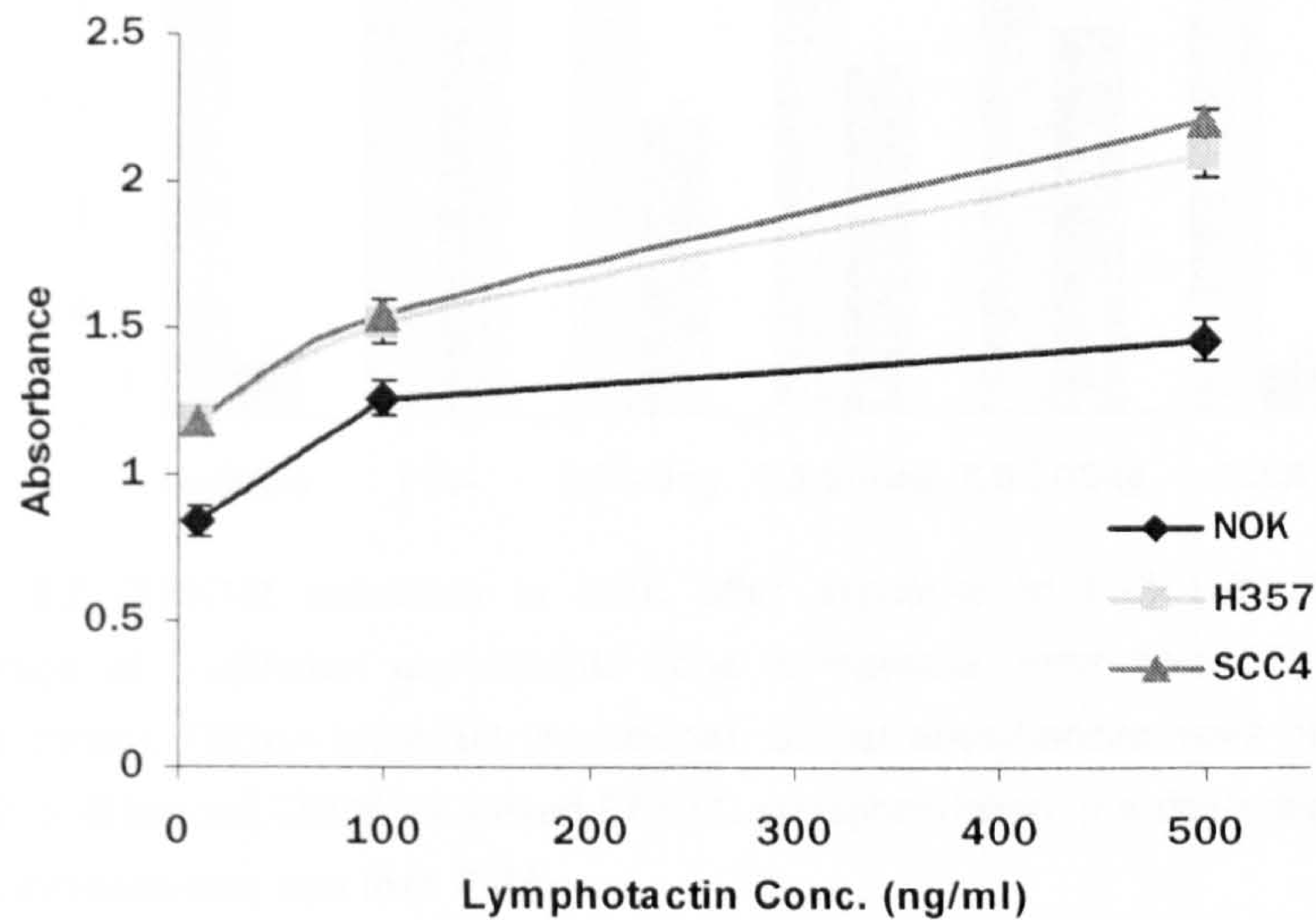


Figure 3.6. Comparison of lymphotactin mediated ERK1/2 phosphorylation in NOK, H357 and SCC4 cells (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). ERK1/2 phosphorylation was higher in the H357 and SCC4 cancer cells at all doses including baseline levels. No difference between H357 and SCC4 cells was observed.

3.5.2 IL-8

i) NOK

Exposure to PMA and IL-8 caused a significant increase in intracellular ERK1/2 phosphorylation in NOK compared with unstimulated controls ($p<0.0001$) and this effect was dose dependent. No effect was observed with the irrelevant chemokine CCL25/TECK (Figure 3.6).

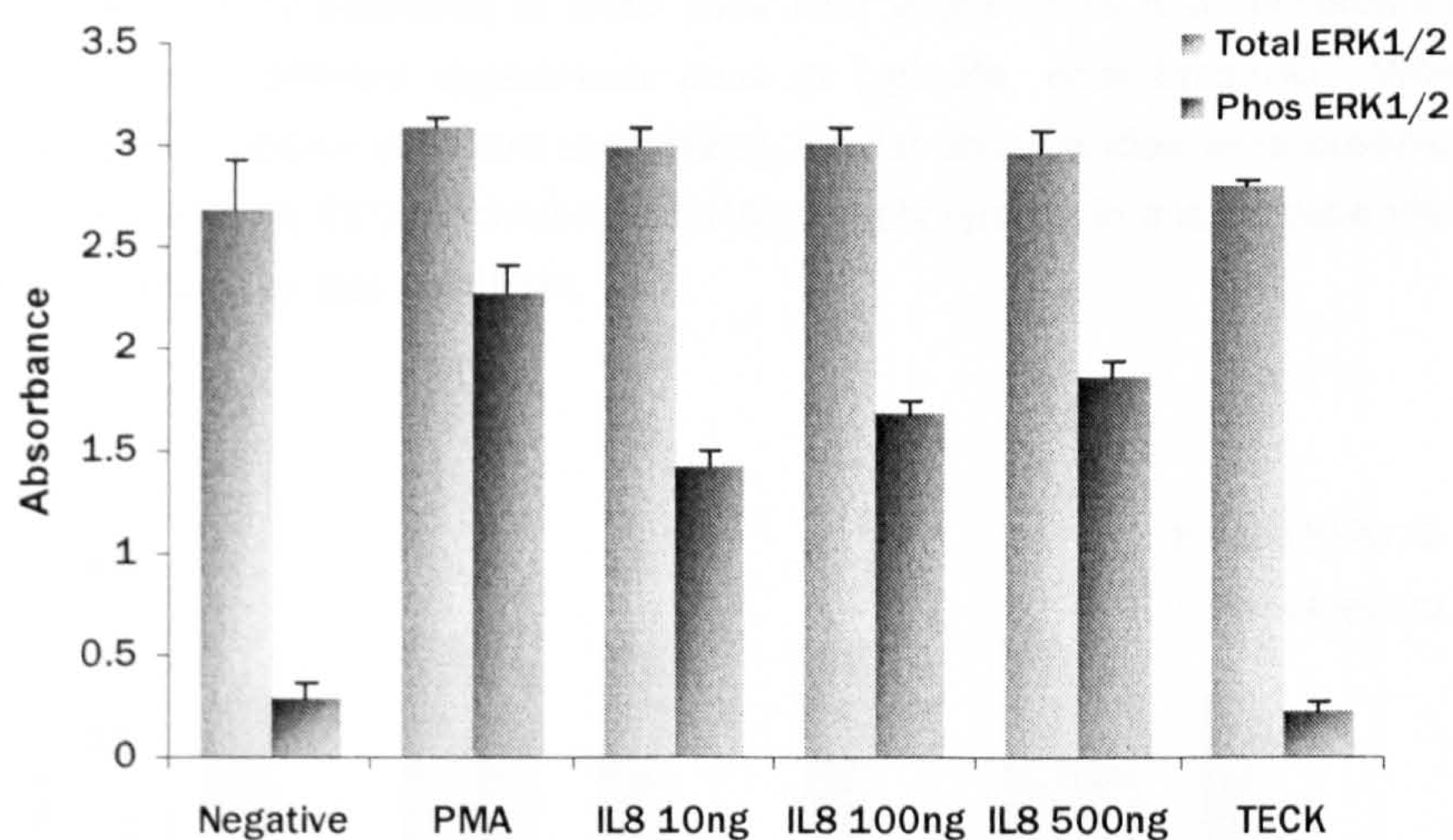


Figure 3.7. ERK1/2 activation in NOK after exposure to IL-8 (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. IL-8 but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

ii) H357 and SCC4 cells

A highly significant increase in ERK1/2 phosphorylation in H357 and SCC4 cells was observed after exposure to PMA compared with unstimulated cells ($p<0.000001$ and $p<0.00001$ respectively) (Figure 3.7 and 3.8). Stimulation of H357 and SCC4 cells with IL-8 but not TECK caused a significant increase in ERK1/2 phosphorylation compared with negative controls ($p<0.001$ and $p<0.00001$ respectively) in a dose dependent manner. However, this increase was not as high as observed with PMA.

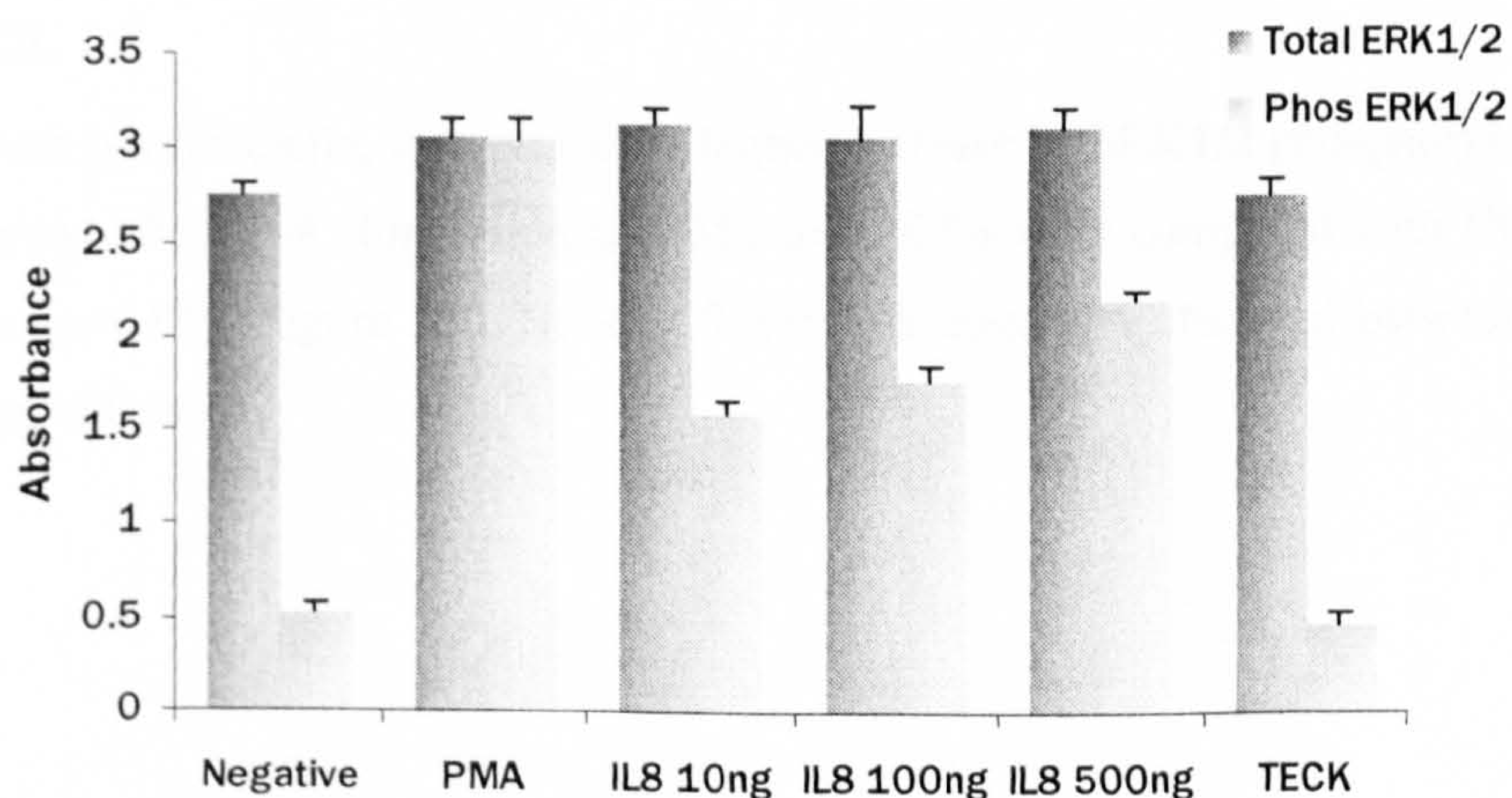


Figure 3.8. ERK1/2 activation in H357 cells after exposure to IL-8 (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. IL-8 but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

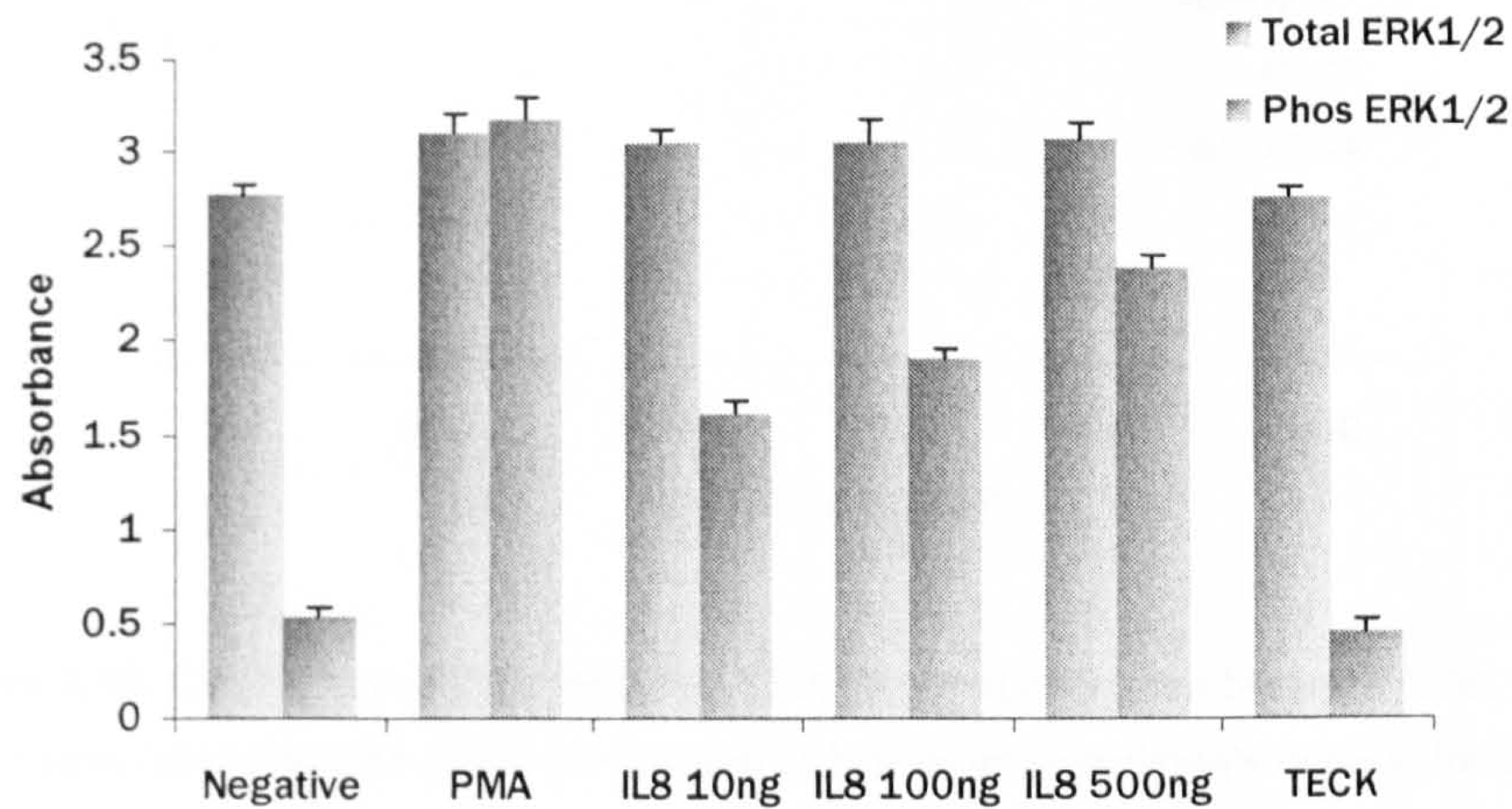


Figure 3.9. ERK1/2 activation in SCC4 cells after exposure to IL-8 (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. IL-8 but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

iii) Comparison of IL-8-mediated ERK1/2 phosphorylation between NOK and OCCL

As with lymphotactin, a significantly higher increase in ERK1/2 phosphorylation was observed after IL-8 stimulation of H357 and SCC4 cells compared with NOK at all doses ($p<0.05$) (Figure 3.9). No significant difference was observed between the two cancer cell lines.

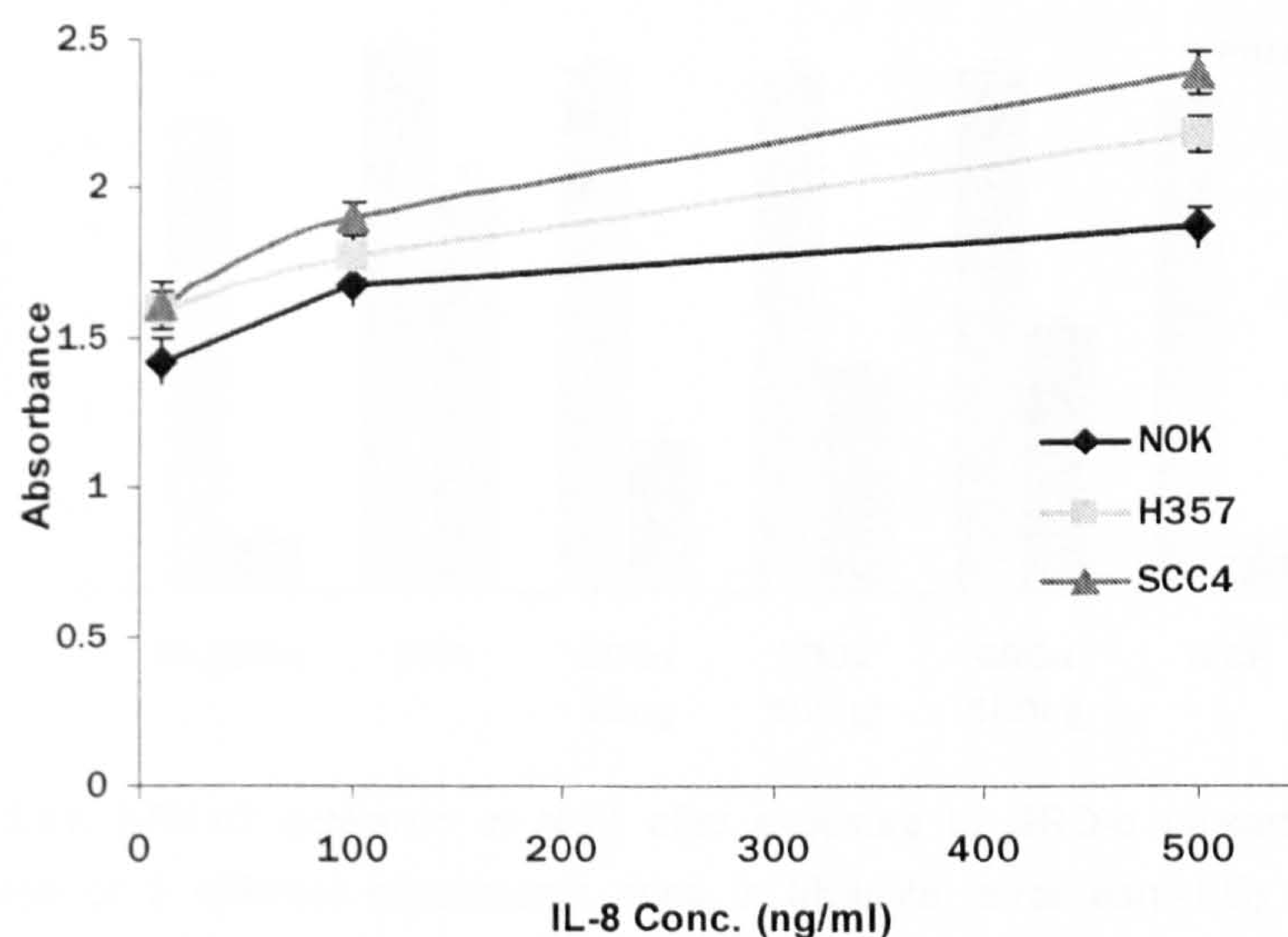


Figure 3.10. Comparison of IL-8 mediated ERK1/2 phosphorylation between NOK, H357 and SCC4 cells (absorbencies $\pm$ SD) (n=average of 3 different experiments done in triplicate, error bars=SD). ERK1/2 phosphorylation was higher in the H357 and SCC4 cancer cells at all doses including baseline levels. No difference between H357 and SCC4 cells was observed.

3.5.3 GRO-*α*

i) NOK

GRO-α facilitated a significant up-regulation of intracellular ERK1/2 phosphorylation in a dose dependent manner in NOK compared with unstimulated cells ($p<0.001$). The irrelevant chemokine CCL25/TECK did not cause any increase in phosphorylation. No difference in total ERK1/2 level between different treatments was observed (Figure 3.10).

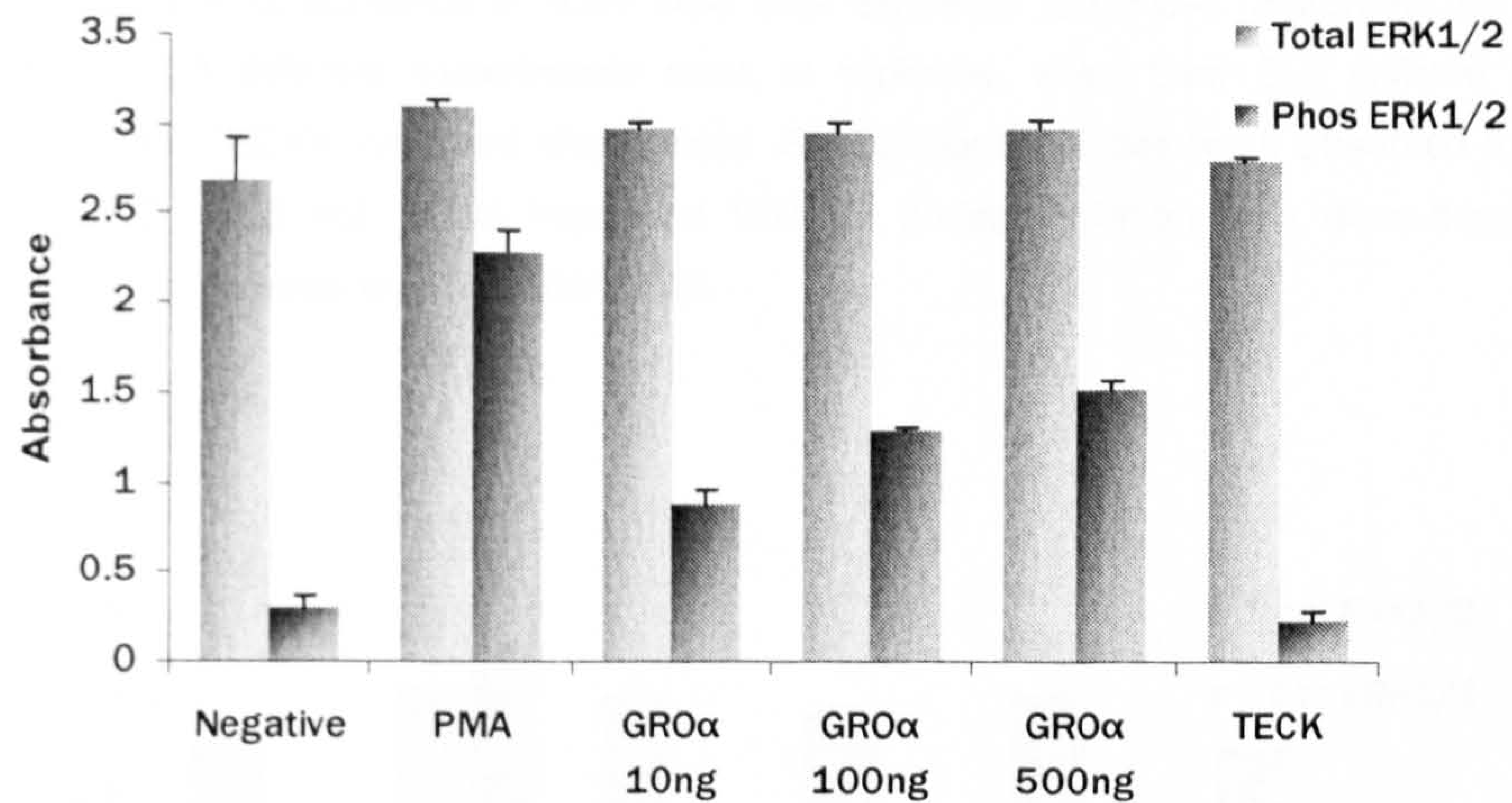


Figure 3.11. ERK1/2 activation in NOK after exposure to GRO-α (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. GRO-α but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

ii) H357 and SCC4 cells

Stimulation of H357 and SCC4 cells with PMA caused a highly significant increase in ERK1/2 phosphorylation compared with unstimulated controls ($p<0.000001$ and $p<0.00001$ respectively) (Figure 3.11 and 3.12). Exposure to GRO-α also significantly increased ERK1/2 phosphorylation compared with controls ($p<0.0001$ and $p<0.0001$ respectively) in a dose dependent manner but the levels of phosphorylated ERK1/2 were not as high as seen with PMA. No response was observed with TECK.

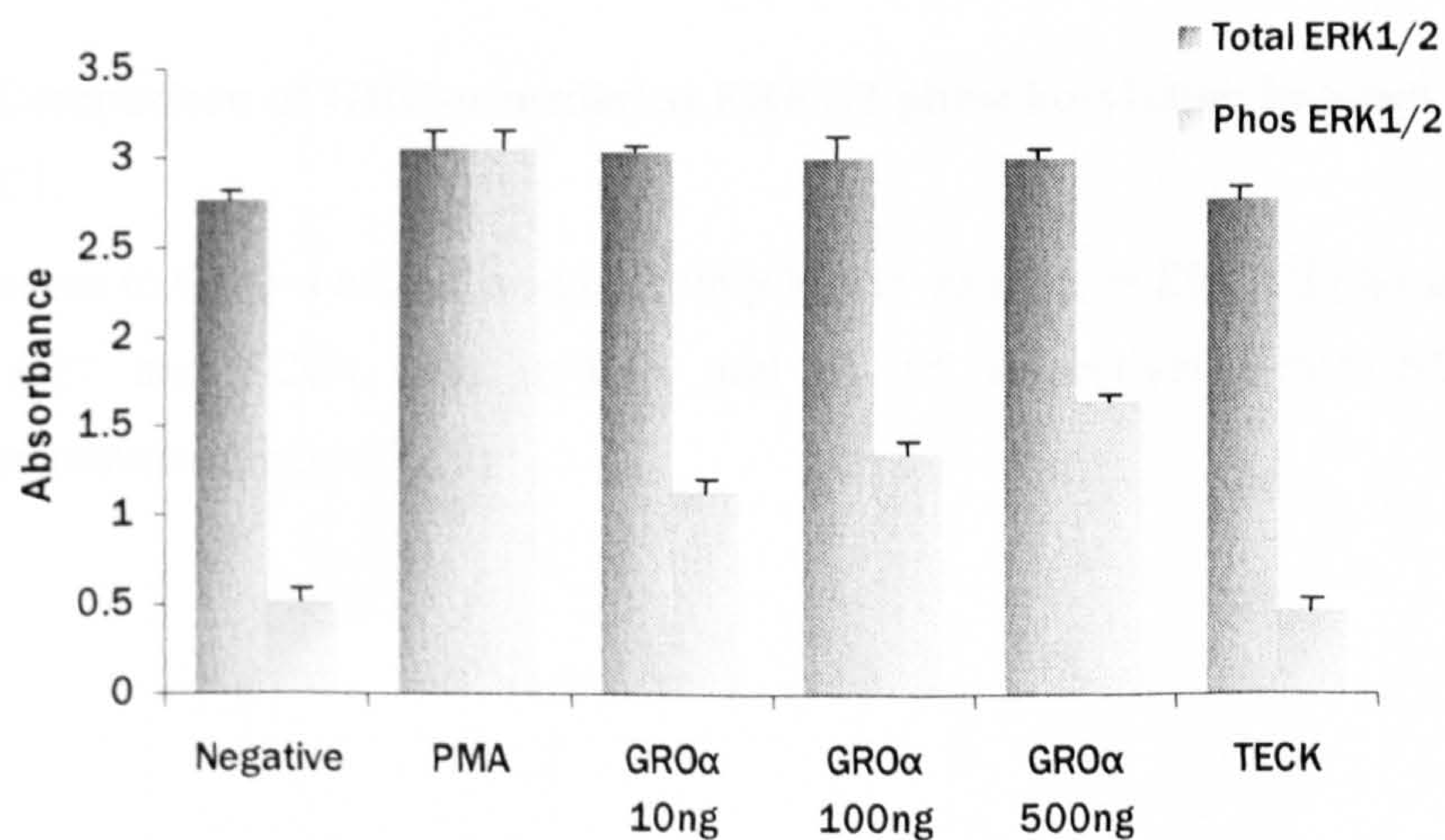


Figure 3.12. ERK1/2 activation in H357 cells after exposure to GRO-α (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. GRO-α but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

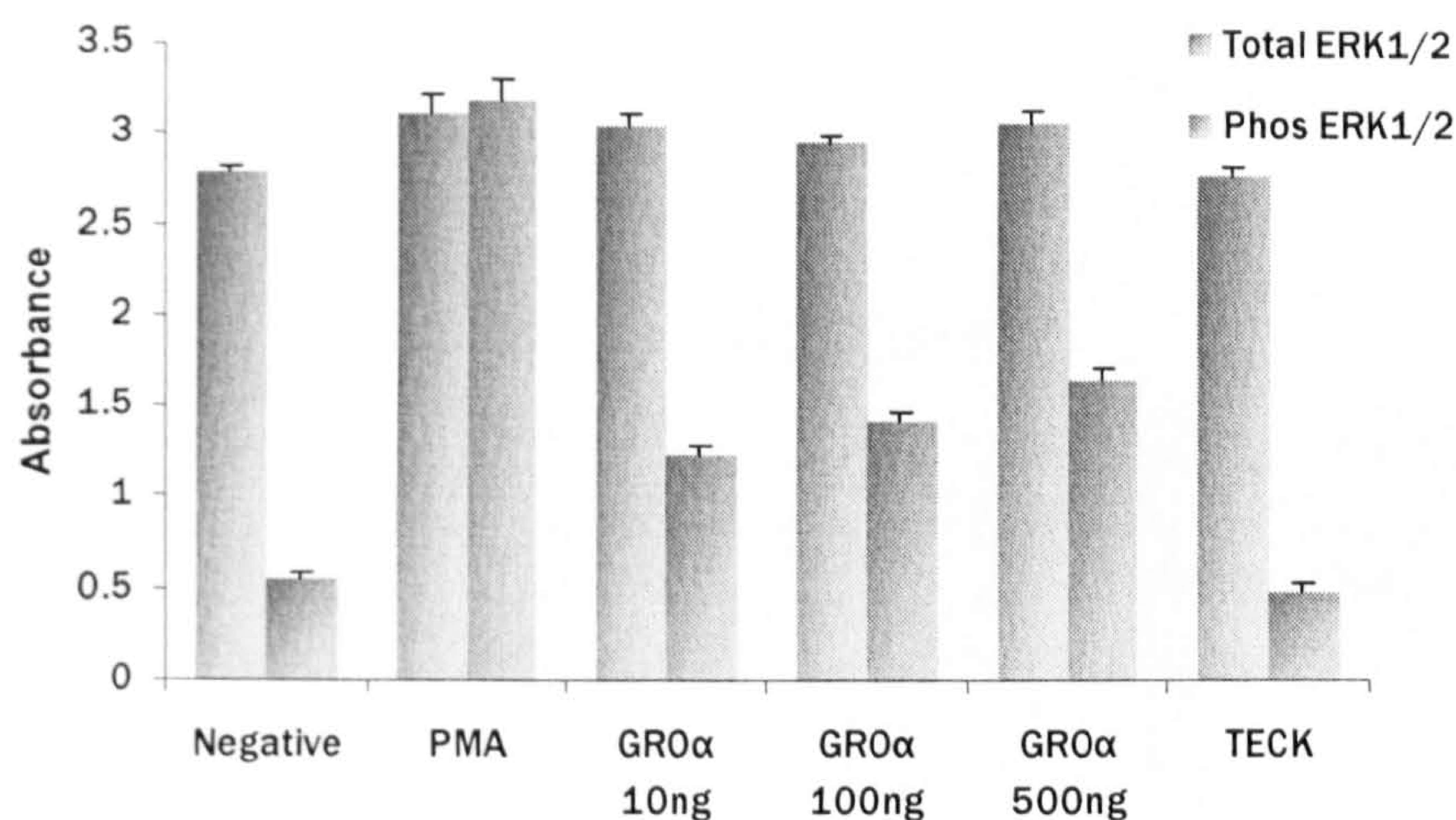


Figure 3.13. ERK1/2 activation in SCC4 cells after exposure to GRO-α (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD) (Where PMA= positive control, TECK= irrelevant chemokine). Similar absorbancies were observed for total ERK1/2. GRO-α but not TECK increased ERK1/2 phosphorylation in a dose-dependent manner but this increase was less than PMA.

iii) Comparison of GRO-α-mediated ERK1/2 phosphorylation between NOK and OCCL

Exposure to GRO-α caused a significantly higher increase in ERK1/2 phosphorylation in H357 and SCC4 cells ($p<0.05$ and $p<0.05$ respectively) than NOK at all concentrations (Figure 3.13).

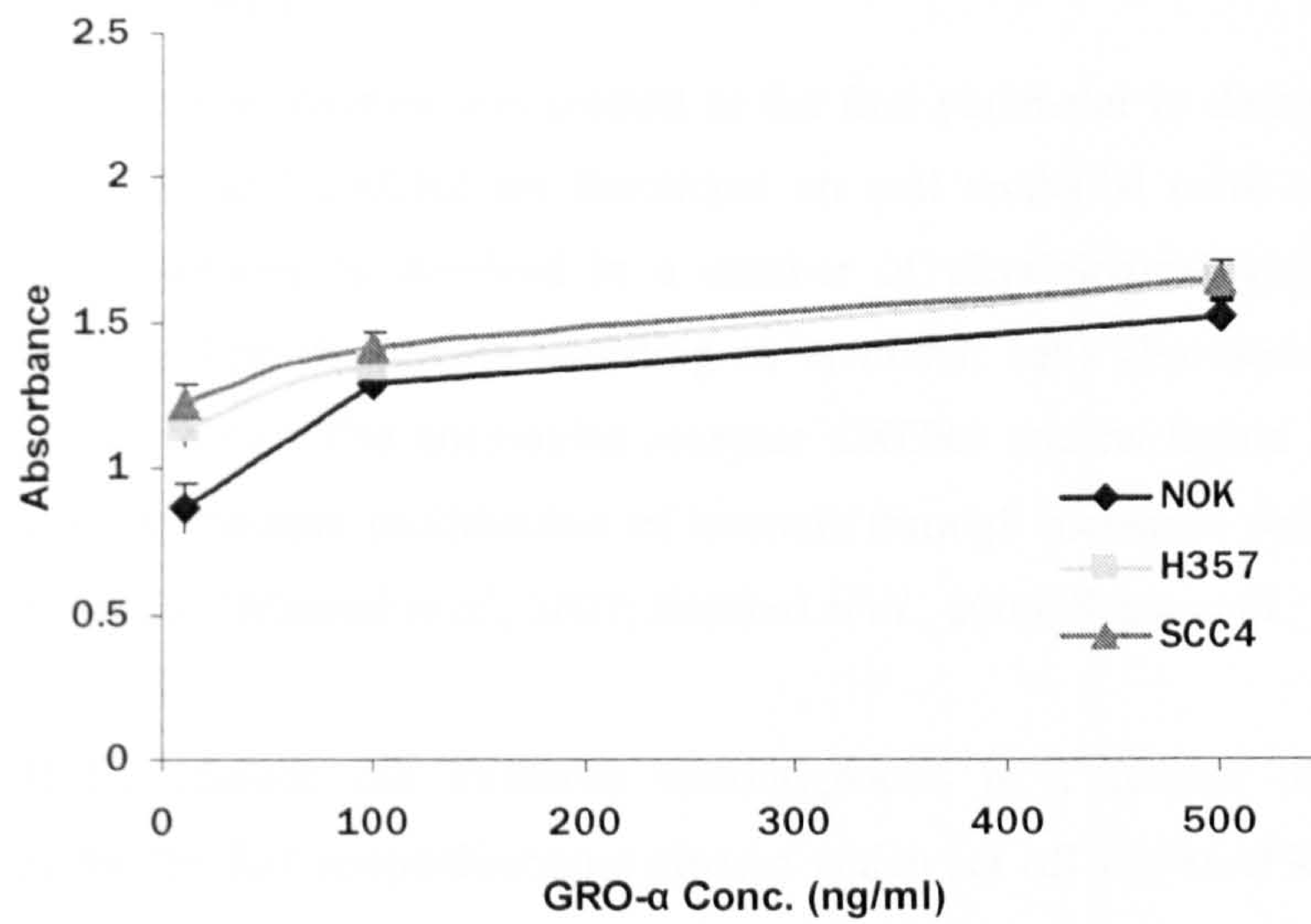


Figure 3.14. Comparison of GRO-α mediated ERK1/2 phosphorylation between NOK, H357 and SCC4 cells (absorbencies ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). ERK1/2 phosphorylation was higher in the H357 and SCC4 cancer cells at all doses including baseline levels. No difference between H357 and SCC4 cells was observed.

3.6 DISCUSSION

ERK1/2 signal transduction was studied as the first parameter to determine whether XCR1, CXCR1 and CXCR2 are functional on oral epithelial cells. As mentioned earlier, this pathway is involved in a number of physiological and pathological processes including chemokine signaling in epithelial cells (Barbieri *et al.*, 2006; Keates *et al.*, 2007). The chemokine receptor CXCR4 and its ligand SDF-1α have been shown to mediate proliferation of tumours through increased activation of the ERK1/2 cascade (Alsayed *et al.*, 2007; Barbieri *et al.*, 2006; Scala *et al.*, 2006).

The ERK1/2 cascade can influence tumourigenesis in a number of ways. It is regulated by the Raf serine/threonine kinases which set off the MAPK/ERK kinase (MEK) protein kinases, which then activate ERK1/2. Mutations of Raf in neoplasia suggest an important role for this pathway in oncogenesis (Davies *et al.*, 2002). In addition, the ERK1/2 pathway is a key downstream effector of Ras which is one of the most commonly mutated genes in cancer. Finally, Ras is a critical downstream effector of the epidermal growth factor receptor (EGFR), which is up-regulated in a range of cancers (Figure 3.13 and 3.14). Activation of ERK1/2 up-regulates expression of EGFR ligands, thus providing an autocrine growth mechanism to the tumour (Grandis and Sok 2004; Hynes and Lane 2005; Lynch 2004; Stephens *et al.*, 2004). Therefore, this pathway has been studied intensely with reference to cancer treatment.

i) XCR1

Exposure to lymphotactin significantly increased intracellular ERK1/2 phosphorylation in NOK and OCCL in a dose dependent manner. This strongly suggests that XCR1 is functional on the surface of NOK and OCCL since it is the only known receptor for lymphotactin. However, since XCR1 was not blocked in these experiments by use of an antibody it is possible that lymphotactin may mediate ERK1/2 phosphorylation through another receptor, as yet unidentified. CCL25/TECK which was used as an irrelevant chemokine did not cause a similar increase which suggests that the response was specific to lymphotactin.

Higher baseline levels of phosphorylated ERK were observed in OCCL compared

with NOK at all doses. As explained earlier, one possible explanation is that mutated Raf/Ras is present in the cancer cells and this may facilitate downstream activation of ERK1/2. Such a response has been documented in a range of cancers such as melanoma, lung and ovarian carcinoma (Mercer and Pritchard 2003; Rajagopalan *et al.*, 2002; Sieben *et al.*, 2004; Singer *et al.*, 2003; Vos *et al.*, 2003). Mutations of Ras have also been shown to play an important role in the pathogenesis of oral cancer (Milasin *et al.*, 1994; Sakata 1996). Another possible explanation is that EGFR which may be up-regulated in cancer, activates downstream Ras which in turn can influence the ERK1/2 pathway. Whether EGFR is up-regulated in the H357 and SCC4 cell lines is not known but aberrant expression of EGFR and its ligands have been documented in OSCC *in vivo* and EGFR and its ligands are up-regulated in head and neck malignancies and premalignant conditions compared to normal oral mucosa (Bankfalvi *et al.*, 2002; Rubin *et al.*, 1996; Rubin *et al.*, 1998; Rubin, Tweardy, and Melhem 1998; Rubin, Zeng, and Tweardy 1996; Srinivasan and Jewell 2001). Which of these possibilities explains the higher baseline levels of phosphorylated ERK in the cancer cell lines compared with NOK is not clear since nothing is known about the Raf/Ras mutations or EGFR expression in the cells used in our study.

To our knowledge, lymphotactin/XCR1 mediated activation of ERK1/2 signaling cascade either in lymphoid or non-lymphoid cells has not been shown to date. The significance of this signaling in oral epithelial cell regulation will be studied in the following chapters.

ii) CXCR1 and CXCR2

a) IL-8

Stimulation with IL-8 up-regulated ERK1/2 phosphorylation in a dose-dependent manner in both NOK and OCCL. No such response was seen with TECK/CCL25. This suggests that IL-8 acts through CXCR1 and/or CXCR2 on the surface of oral epithelial cells to increase ERK1/2. This is in agreement with existing literature as IL-8 signaling through ERK1/2 has been shown to induce growth, transcriptional activation and migration of endothelial cells in Kaposi's Sarcoma (Wang *et al.*, 2004). IL-8 mediated direct activation of ERK1/2 cascade in epithelial cells has also been shown previously. IL-8 facilitates phosphorylation of ERK1/2 through both CXCR1 and CXCR2 transfected in Chinese hamster ovary cells (CHO) (Shyamala and Khoja

1998). This phosphorylation is inhibited by the addition of pertussis toxin confirming the involvement of G-protein coupled receptors.

Higher baseline phosphorylated ERK1/2 levels were observed in OCCL compared with NOK. This maybe due to the autocrine growth loop of ERK, MEK and EGFR as discussed earlier but remains to be determined.

IL-8 mediated ERK1/2 signaling stimulates proliferation of non-small lung cancer cell lines A549 and NCI-H292 in a dose-dependent manner (Luppi *et al.*, 2006). Addition of an ERK1/2 inhibitor blocks this effect of IL-8. Interestingly, this IL-8 mediated proliferation is also blocked when an EGFR tyrosine kinase inhibitor or an anti-EGFR blocking antibody is used. This suggests that transactivation of EGFR can augment IL-8 facilitated ERK1/2 signaling and hence proliferation of cancer cells (Luppi *et al.*, 2006). We observed higher baseline levels of ERK in OCCL compared with normal cells which suggests higher constitutive ERK activity in OCCL.

b) GRO-α

Exposure of NOK and OCCL to GRO-α also caused a significant increase in ERK1/2 phosyphorylation and activation. The irrelevant chemokine TECK did not have any effect. This suggests that GRO-α acts on oral epithelial cells through CXCR2 which is its only known receptor.

GRO-α mediated ERK1/2 activation in epithelial cells has already been reported. Exposure to GRO-α leads to phosphorylation of ERK1/2 in CHO cells stably transfected with CXCR2 whereas addition of pertussis toxin blocks this response confirming the involvement of a G-protein coupled receptor (Shyamala and Khoja 1998). In addition, ERK1/2 activation by GRO-α has been shown in lymphoid cells. Airway smooth muscle cells release GRO-α which mediates neutrophil chemotaxis. Addition of PD-98059 (an ERK inhibitor) inhibits GRO-α secretion from airway smooth muscle cells suggesting that GRO-α release involves the ERK1/2 cascade (Issa *et al.*, 2006).

To summarise, stimulation of NOK and H357 and SCC4 oral cancer cell lines with IL-8 and GRO-α, causes a significant increase in intracellular ERK1/2

phosphorylation. This suggests that CXCR1 and CXCR2 mediate activation of ERK1/2 in oral epithelial cells when stimulated with their respective chemokines. Higher levels of phosphorylated ERK1/2 are observed in unstimulated and stimulated oral cancer cells than normal at all concentrations which suggests a potential role for ERK1/2 in the biology of oral cancer. However, more work needs to be done to establish the precise role of the ERK1/2 signaling cascade in this regard.

At present it is not clear whether the activation of ERK following IL-8 exposure is due to signaling through CXCR1 or CXCR2 or both. However it is clear that signaling through CXCR2 is able to activate ERK1/2 since exposure to GRO-α increases ERK1/2 and CXCR2 is its only known receptor.

CHAPTER 4

Role of XCR1, CXCR1 AND CXCR2 in Oral Epithelial Cell Migration and Invasion

4.1 INTRODUCTION

Previous chapters have shown that XCR1, CXCR1 and CXCR2 are expressed on normal and cancerous oral epithelial cells at mRNA and protein level and that they facilitate activation of the ERK1/2 signaling cascade when stimulated with their respective ligands. In order to determine whether XCR1, CXCR1 and CXCR2 are functional on oral epithelial cells, the effects of stimulation on cell migration and invasion were also investigated.

Cell migration and invasion are fundamental aspects of wound healing and tumour metastasis. A wide range of mechanisms appear to be involved in these processes ranging from alteration in expression and/or behaviour of cytokines, growth factors and adhesion molecules. A role for chemokines and their receptors in cell migration and invasion has been established in a range of cancers (Kim *et al.*, 2006; Kleeff *et al.*, 1999; Liang *et al.*, 2004; Moore 2001; Muller *et al.*, 2001; Scala *et al.*, 2006; Scotton *et al.*, 2001; Zhang *et al.*, 2007; Zlotnik 2006). To date, XCR1/lymphotactin interaction has not been shown to mediate migration or invasion of epithelial cells. IL-8 and GRO-α are better characterised than lymphotactin in that regard as they have previously been shown to facilitate epithelial cell migration (as discussed later in Section 4.6). However, very little is known about the individual roles of CXCR1 or CXCR2 in IL-8 or GRO-α mediated migration and invasion.

4.2 AIM

The aim of this chapter was to establish the role of the chemokine receptors XCR1, CXCR1 and CXCR2 in migration and invasion of oral epithelial cells.

4.3 EXPERIMENTAL PROTOCOL

- NOK, H357 and SCC4 cells were used in these assays.
- Migration assays were performed using Transwell® inserts in 24 well tissue culture plates to study the role of XCR1, CXCR1 and CXCR2 in chemotaxis of oral cells

towards lymphotactin, IL-8 and GRO-α.

- BD biocoat invasion chambers (coated with matrigel) were used to study the invasion of cells towards lymphotactin, IL-8 and GRO-α. 10% foetal bovine serum (FBS) was used as positive control in invasion studies.
- Blocking antibodies for XCR, CXCR1 and CXCR2 were also used in the assays to study the individual role of the receptors in migration and invasion
- CCL25/TECK was used as an irrelevant chemokine in all assays. CXCR1 was used as an irrelevant antibody in assays for XCR1/lymphotactin and GRO-α whereas XCR1 was used as an irrelevant antibody for IL-8 assays.
- Triplicate wells for each treatment and cell type were used in every migration and invasion assay. Assays were repeated three times on different occasions.

4.4 MATERIALS AND METHODS

4.4.1 CELL CULTURE

Cells were grown and maintained as described previously.

4.4.2 MIGRATION ASSAY

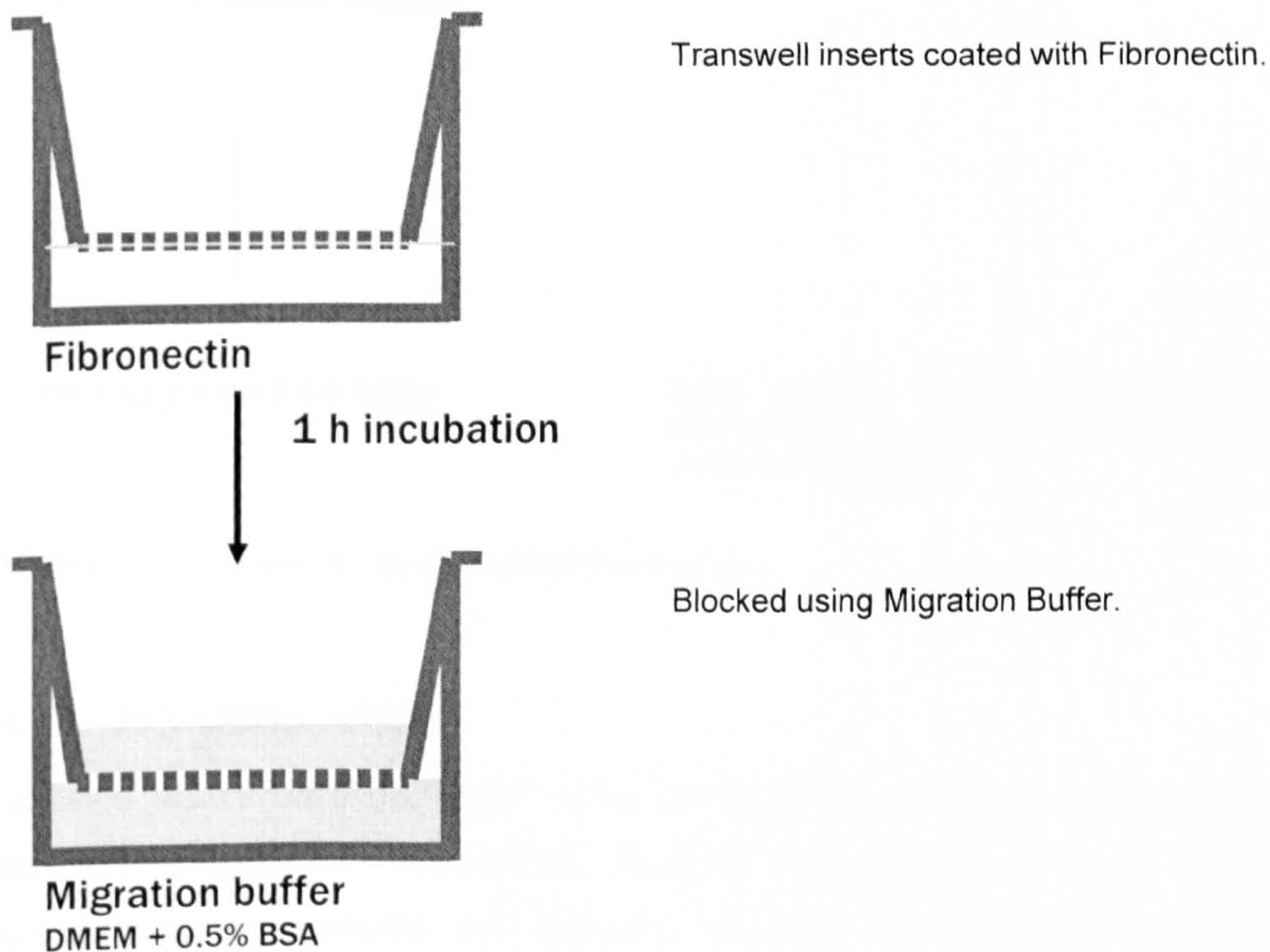
Chemotaxis assays were performed using Transwell® insert polycarbonate membranes (8μm pore size) (Corning, Lowell, MA, USA) in 24-well tissue culture plates. The undersides of the inserts were coated with fibronectin (Sigma)(10μg/ml) via a 1h incubation in a CO_2 incubator. The inserts were blocked with migration buffer (DMEM:F12, 3:1 with 0.5% BSA; filter sterilized) by another 1h incubation.

Cells were detached from T75cm^2 tissue culture flasks using a non-enzymatic cell dissociation solution (Sigma) and re-suspended in migration buffer. Some cells were incubated with XCR1 (Lifespan), CXCR1 and CXCR2 antibodies (R&D Systems) (20μg/ml) or an irrelevant antibody for 30min at RT before being used in the assay. The same concentration of antibodies was used as for immunocytochemistry and flow cytometry for consistency. Also, the product details suggested these concentrations to be

optimal for receptor neutralisation. $1x10^5$ cells were seeded in the top compartment whereas lymphotactin (R&D Systems), IL-8 (Sigma) and GRO-α (Peprotech) (100ng/ml) were added to the lower chamber. The control wells received no lymphotactin or an irrelevant chemokine CCL25/TECK (Peprotech) (100ng/ml).

Cells were allowed to migrate for 4h in a CO_2 incubator. The inserts were fixed in 10% formalin for 10min and stained in 0.5% crystal violet for 10min at RT. The inserts were rinsed briefly in water and non-migratory cells on the upper membrane surface removed with a cotton swab. The membranes were cut and mounted on slides using DPX. Migrated cells in five random fields were counted using a light microscope for each membrane and mean number of cells per assay calculated. All assays were performed in triplicate. Data was expressed as migration index.

$$\text{Migration Index} = \frac{\text{Number of cells migrating to fibronectin}}{\text{Number of cells migrating to test substance}} \times 100$$



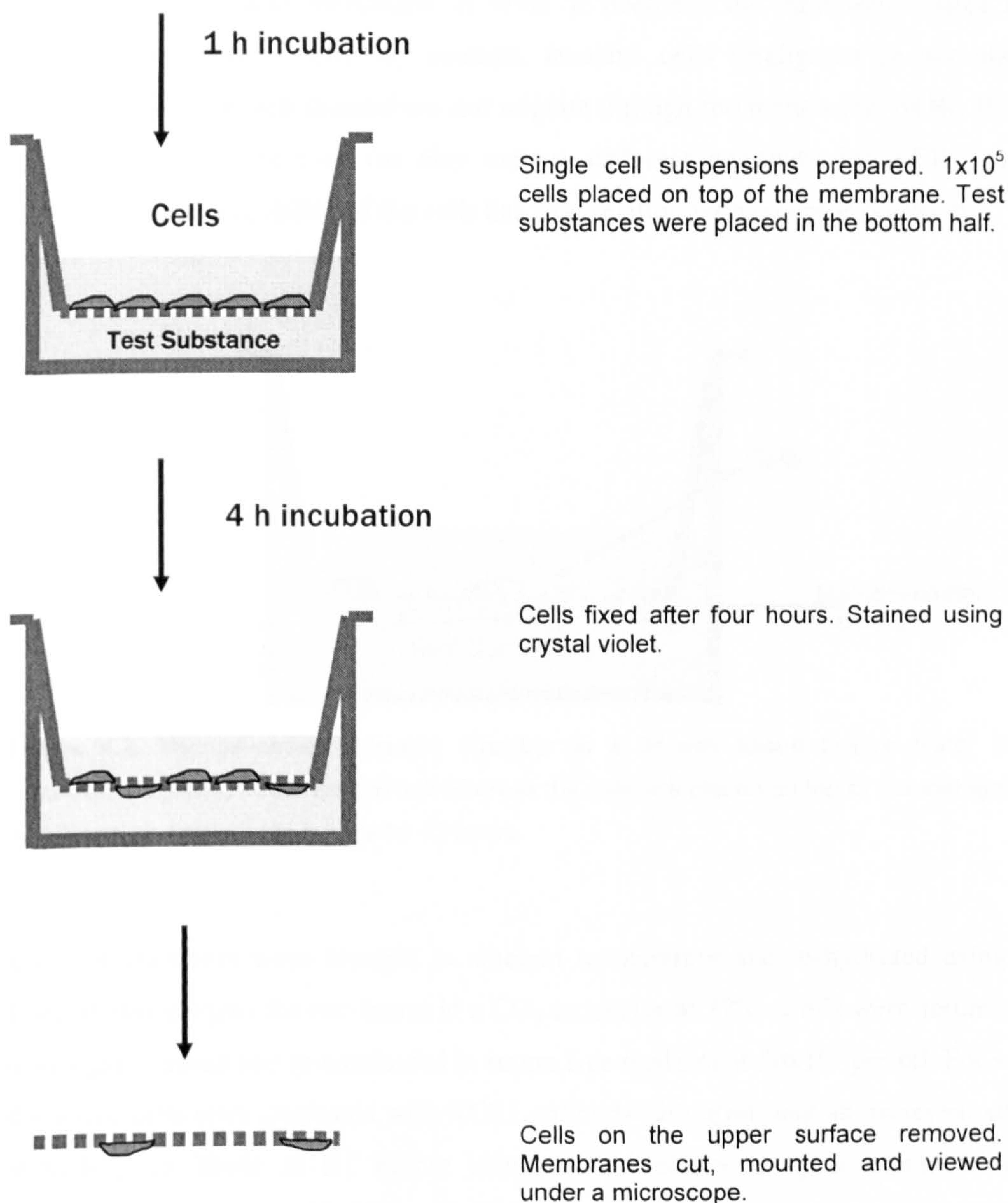


Figure 4.1. Overview of migration/chemotaxis assay.

4.4.3 INVASION ASSAY

Invasion assays were performed using Becton Dickinson biocoat invasion chambers (matrigel-coated) (BD Biosciences, Bedford, MA, USA) to study the effect of lymphotactin on epithelial cell invasion. The layer of matrigel matrix serves as a

reconstituted basement membrane *in vitro*. It occludes the membrane pores thereby blocking non-invasive cells. By contrast, invasive cells (malignant or non-malignant cells) are able to detach themselves and migrate through the membrane. NOK, H357 and SCC4 cell lines were used (as they express different positivity for XCR1) to study whether invasive capability of the cells has a correlation with receptor positivity.

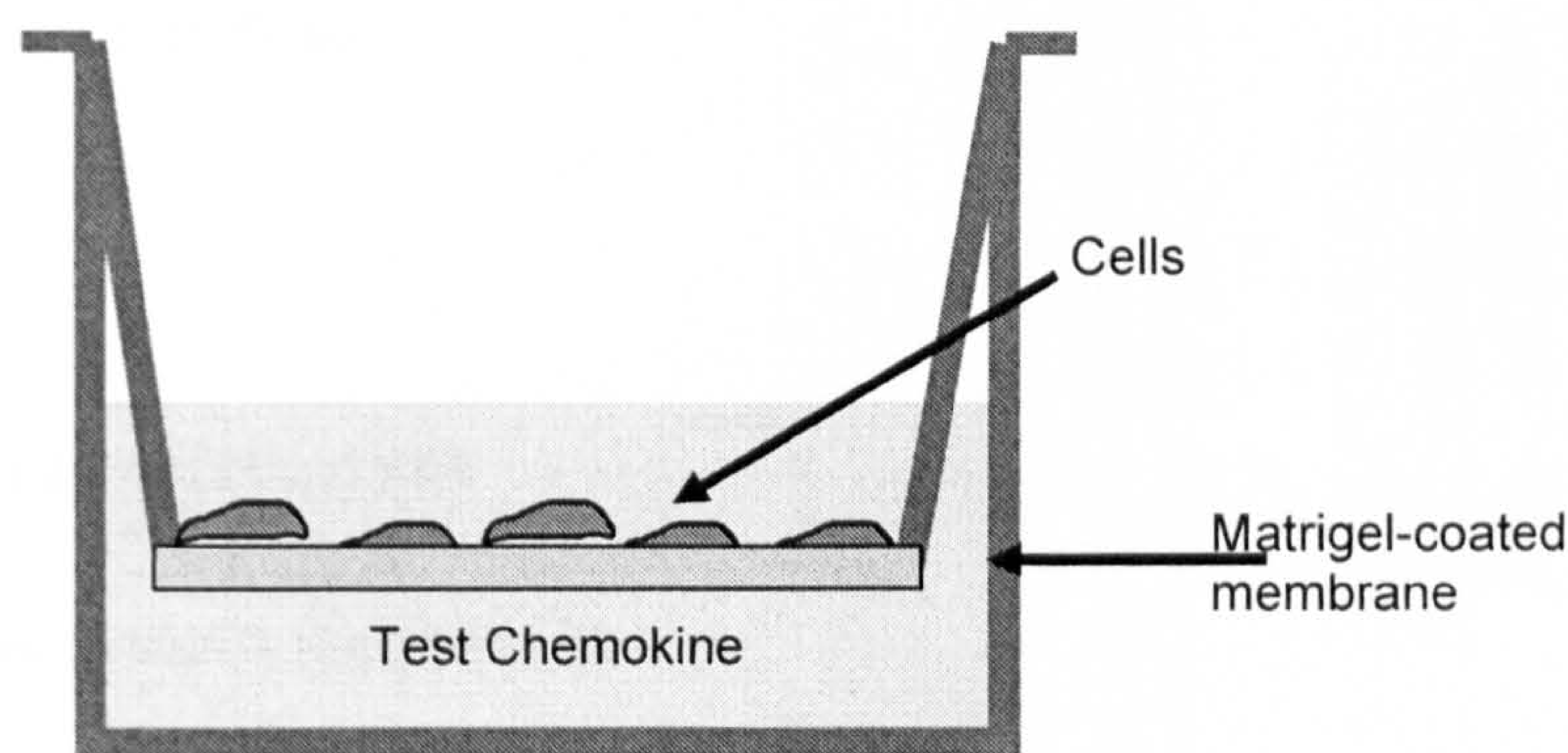


Figure 4.2. Matrigel-coated invasion chamber in a 24-well tissue culture plate. The test chemokine is placed in the bottom half whereas the cells are placed on top of the matrigel-coated membrane and allowed to invade for 22 hours.

Invasion chambers were brought to ambient temperature and re-hydrated using warm DMEM (Invitrogen) for two hours in a CO_2 incubator at 37°C. Cells were serum-starved overnight, washed and re-suspended in serum free medium at $50x10^3$ per ml. For some of the wells, cells were incubated with XCR1 antibody (20µg/ml) and an irrelevant antibody (CXCR1) for 30min at RT before being used. Medium in upper compartment was removed and replaced with 500µl of cell suspension. Lymphotactin (R&D Systems), IL-8 (Sigma), GRO-α (Peprotech) or an irrelevant chemokine TECK (Peprotech)(100ng/ml) were added to the bottom half and 10% serum was used as positive control. The invasion chambers were incubated for 20h, fixed in formalin and stained using 0.5% crystal violet. Chambers were washed and remaining non-invasive cells on the upper surface were wiped off using a cotton swab. The matrigel membranes were cut and mounted on slides using DPX. Number of invasive cells in five random fields per membrane was counted using a light microscope and mean number of cells per assay calculated. All assays were

performed in triplicate and performed three times. Data was expressed as mean number of invasive cells per assay.

4.4.4 STATISTICAL ANALYSIS

Data are presented as means +/- SD. Paired student's T test was used to determine the significance of the results obtained from the migration and invasion assays. A p-value of <0.05 was considered significant.

4.5 RESULTS

4.5.1 XCR1 / LYMPHOTACTIN

4.5.1.1 Migration towards lymphotactin

i) NOK

Addition of lymphotactin resulted in a significant increase (30%) in migration of NOK compared with fibronectin alone ($p<0.05$). No increase in migration was observed with the irrelevant chemokine TECK. Pre-incubation of cells with XCR1 antibody caused a significant decrease in lymphotactin-mediated migration ($p<0.05$) but did not reduce it to the control level. The irrelevant antibody CXCR1 failed to have any effect. (Figure 4.3).

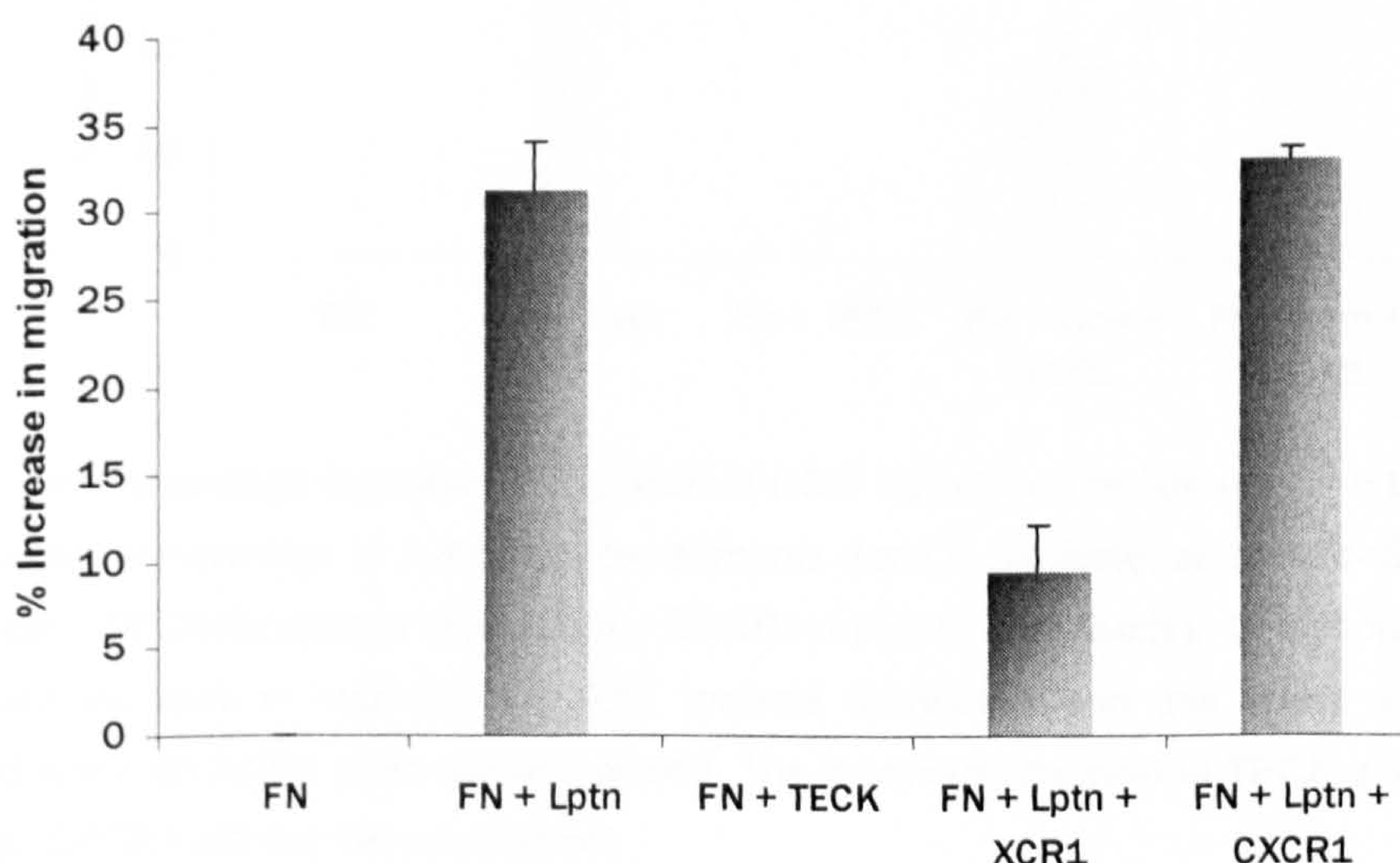


Figure 4.3. Percentage increase in migration of NOK towards fibronectin in the presence of lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (Where FN-fibronectin, TECK=irrelevant chemokine, CXCR1=irrelevant antibody). Lymphotactin caused a significant increase in migration of NOK towards fibronectin and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

ii) H357 and SCC4

A similar response was observed in H357 and SCC4 cells as their migration increased significantly (46.3% and 56.3% respectively) towards lymphotactin compared with fibronectin alone ($p<0.0001$ and $p<0.0001$ respectively) (Figure 4.4 and 4.5). Addition of XCR1 antibody resulted in a significant reduction in migration in H357 and SCC4 cells ($p<0.001$ and $p<0.001$ respectively) but migration was not reduced to control levels. The irrelevant controls TECK or CXCR1 did not have any effect.

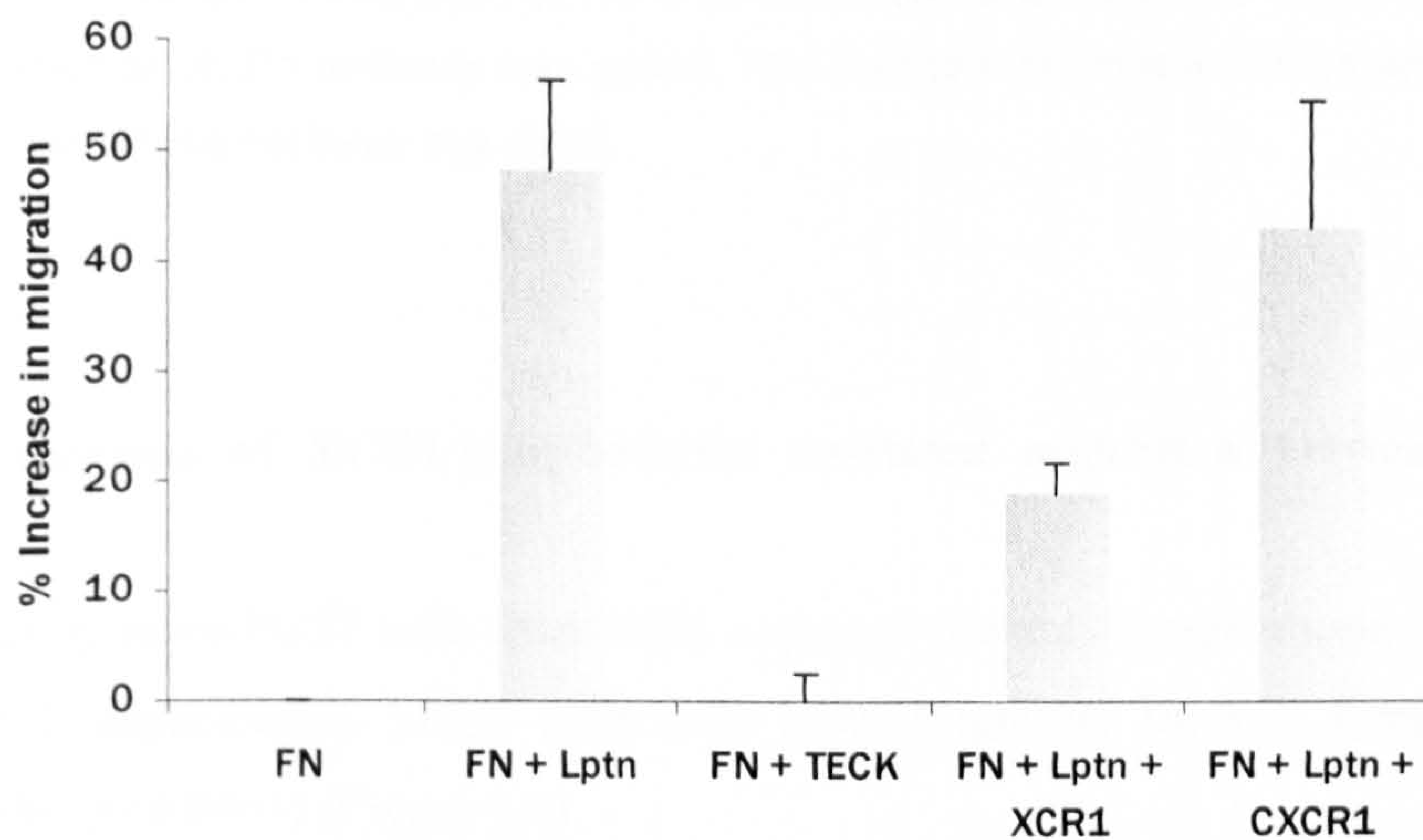


Figure 4.4. Percentage increase in migration of H357 cells towards fibronectin in the presence of lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (where FN-fibronectin, TECK=irrelevant chemokine, CXCR1=irrelevant antibody). Lymphotactin caused a significant increase in migration of NOK towards fibronectin and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

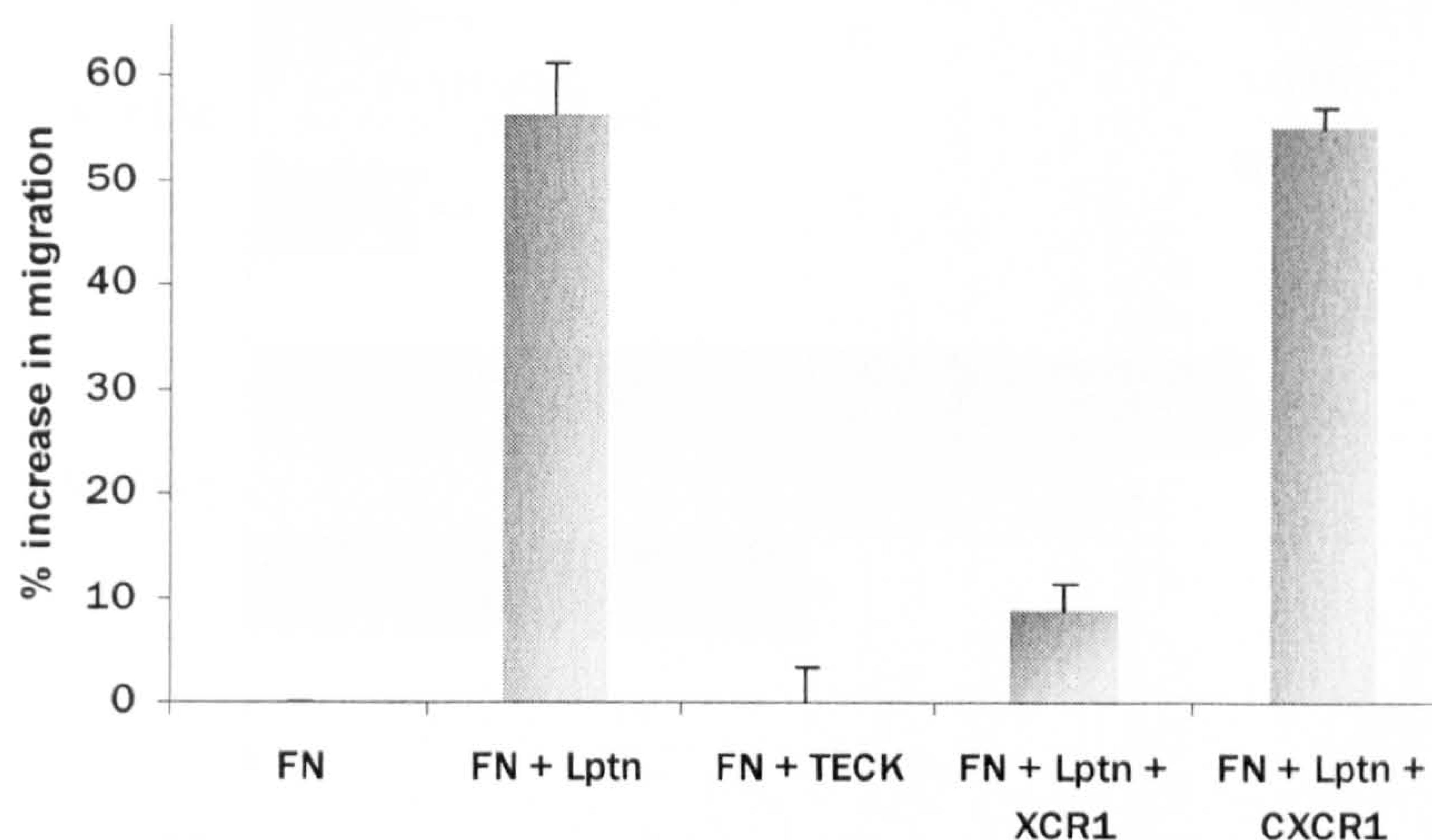


Figure 4.5. Percentage increase in migration of SCC4 cells towards fibronectin in the presence of lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (where FN- fibronectin, TECK=irrelevant chemokine, CXCR1=irrelevant antibody). Lymphotactin caused a significant increase in migration of NOK towards fibronectin and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

iii) Comparison of XCR1/lymphotactin mediated migration between NOK and OCCL

Significantly more H357 cells than NOK migrated towards lymphotactin ($p<0.0001$ and $p<0.00001$ respectively). SCC4 cells were more migratory towards lymphotactin than H357 cells ($p<0.0001$) (Figure 4.6).

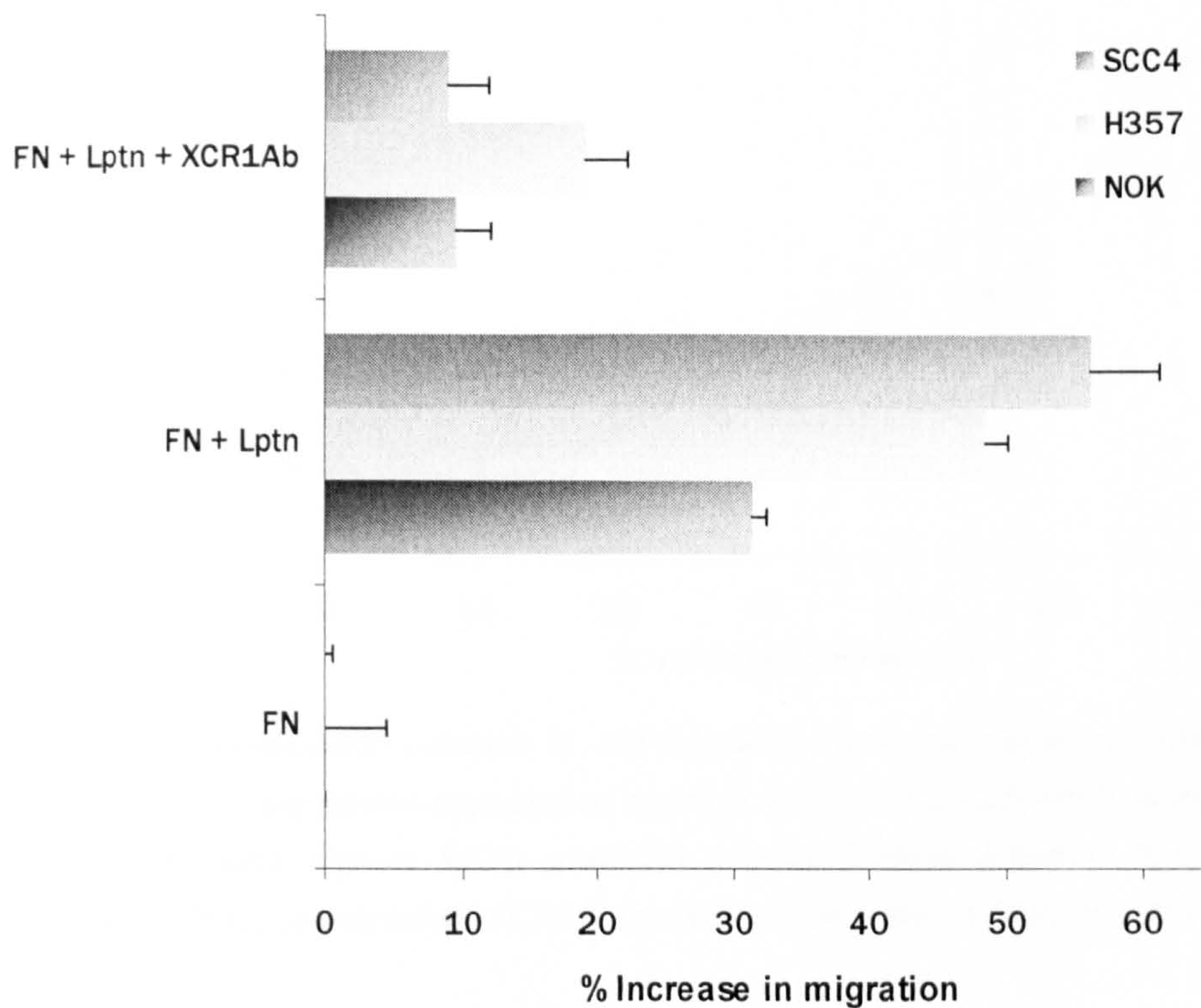


Figure 4.6. Comparison of percentage increase in migration of NOK, H357 and SCC4 cells in the presence of lymphotactin + fibronectin compared with fibronectin alone (n=average of 3 different experiments done in triplicate, error bars=SD). SCC4 cells were the most migratory towards lymphotactin, followed by H357 whereas NOK were the least migratory.

An increase of 56.3% in lymphotactin mediated migration of SCC4 cells was seen compared with 48.3% in H357 cells. This correlates well with the expression data as a higher number of SCC4 express XCR1 (>90%) than H357 cells (60%). The percentage increase in migration was lowest for NOK (31.3%) which are the least XCR1-positive of the three tested cells. The relationship between receptor expression and the migratory response of NOK, H357 and SCC4 cells is shown in Figure 4.7.

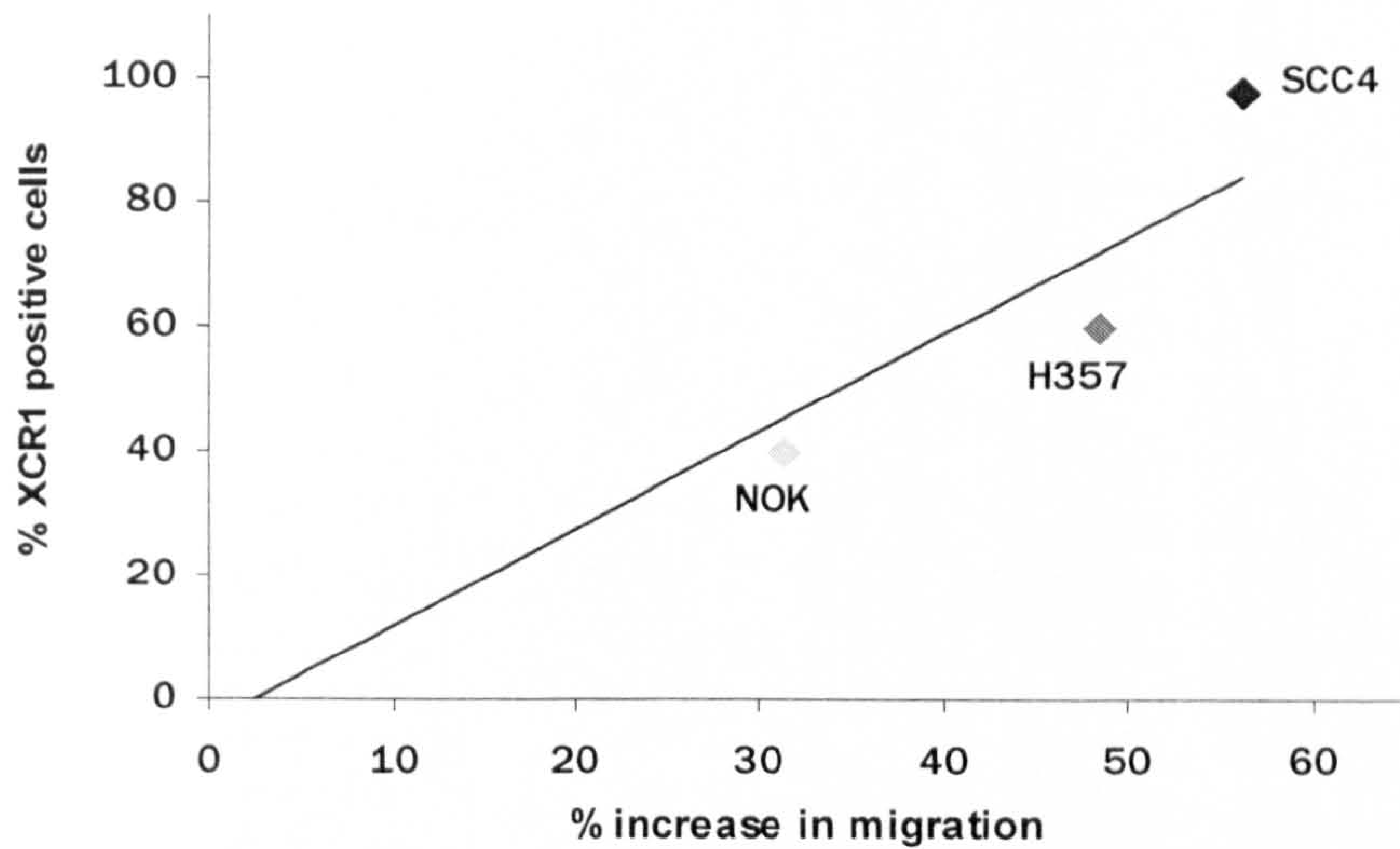


Figure 4.7. Relationship between % XCR1-positive cells and migration towards lymphotactin. Extent of XCR1 expression appeared to correlate with the migratory ability towards lymphotactin. SCC4 cells (with highest XCR1 positivity) showed highest migration towards lymphotactin followed by H357 (intermediate XCR1 expression). NOK were the least migratory.

4.5.1.2 Invasion towards lymphotactin

i) NOK

The number of NOK that invaded towards lymphotactin was significantly lower than the positive control FBS ($p<0.00001$) but was significantly higher than the background ($p<0.0001$). Very little invasion was observed in negative control or in response to TECK (Figure 4.8).

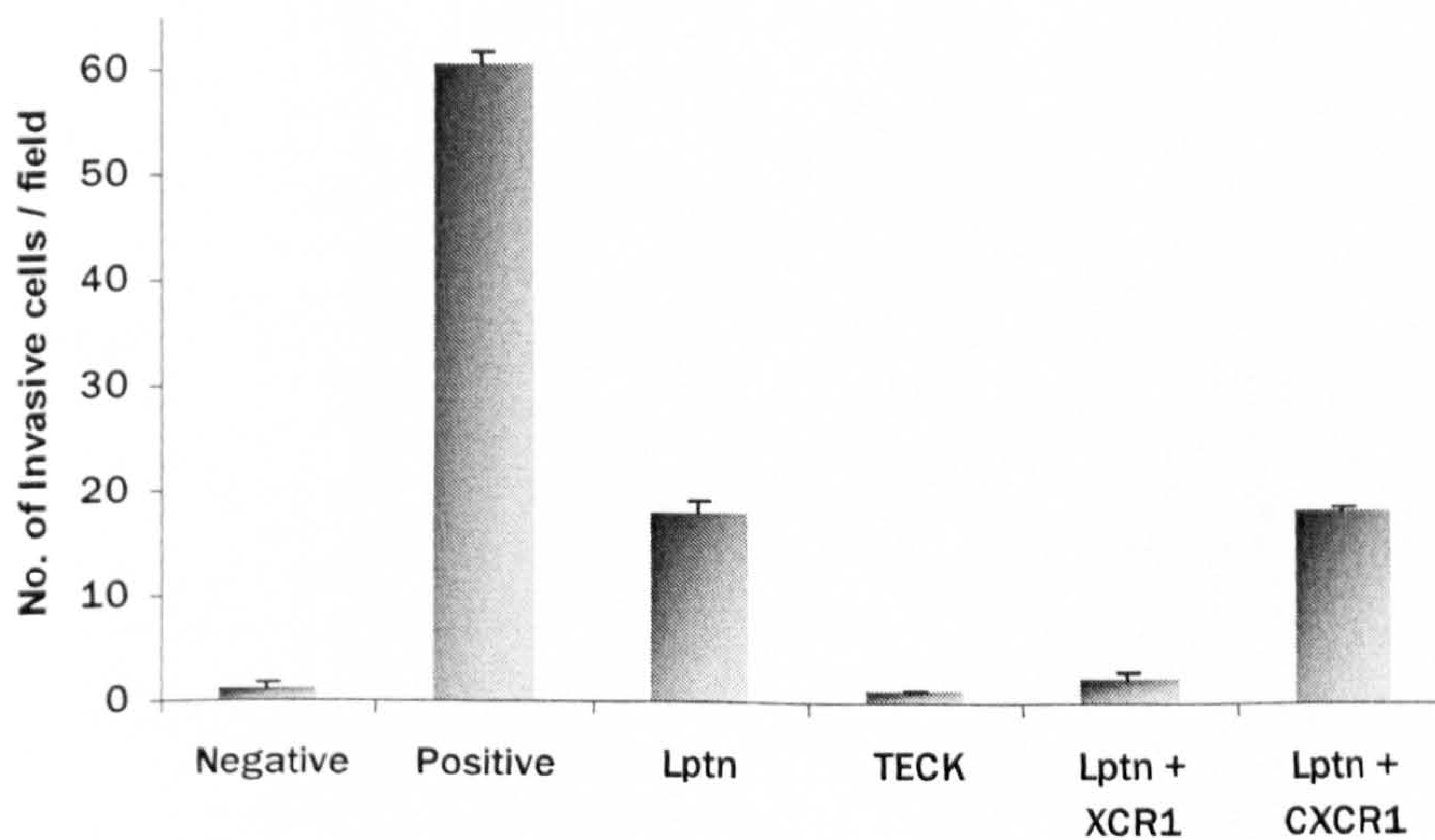


Figure 4.8. Invasion of NOK towards lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, CXCR1= irrelevant antibody). Lymphotactin caused a significant increase in invasion of NOK and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

Administration of XCR1 blocking antibody almost completely abolished this lymphotactin-mediated invasion ($p<0.0001$) but did not reduce it to control levels. No effect was observed with the irrelevant control antibody CXCR1 (Figure 4.8).

ii) H357 and SCC4 cells

Invasion of H357 and SCC4 cells was also significantly enhanced in the presence of FBS and lymphotactin ($p<0.0001$ and $p<0.0001$ respectively) compared with the background (Figure 4.9 and 4.10). Pre-incubation of H357 and SCC4 cells with XCR1 antibody resulted in a significant reduction in invasion ($p<0.00001$ and $p<0.0001$ respectively) but did not reduce it to the level of background. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not exert any influence on invasion.

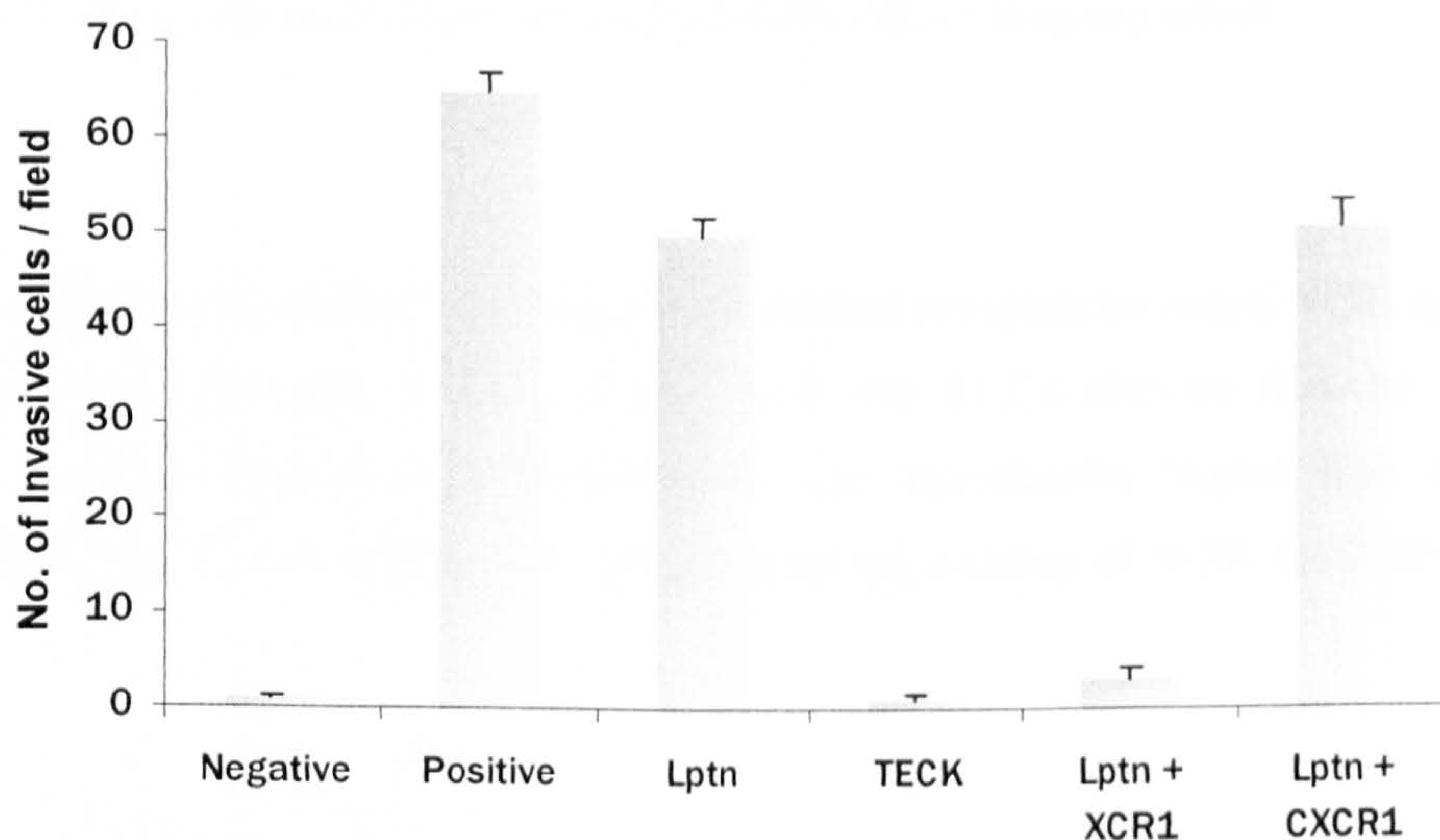


Figure 4.9. Invasion of H357 cells towards lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine,

CXCR1= irrelevant antibody). Lymphotactin caused a significant increase in invasion of NOK and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

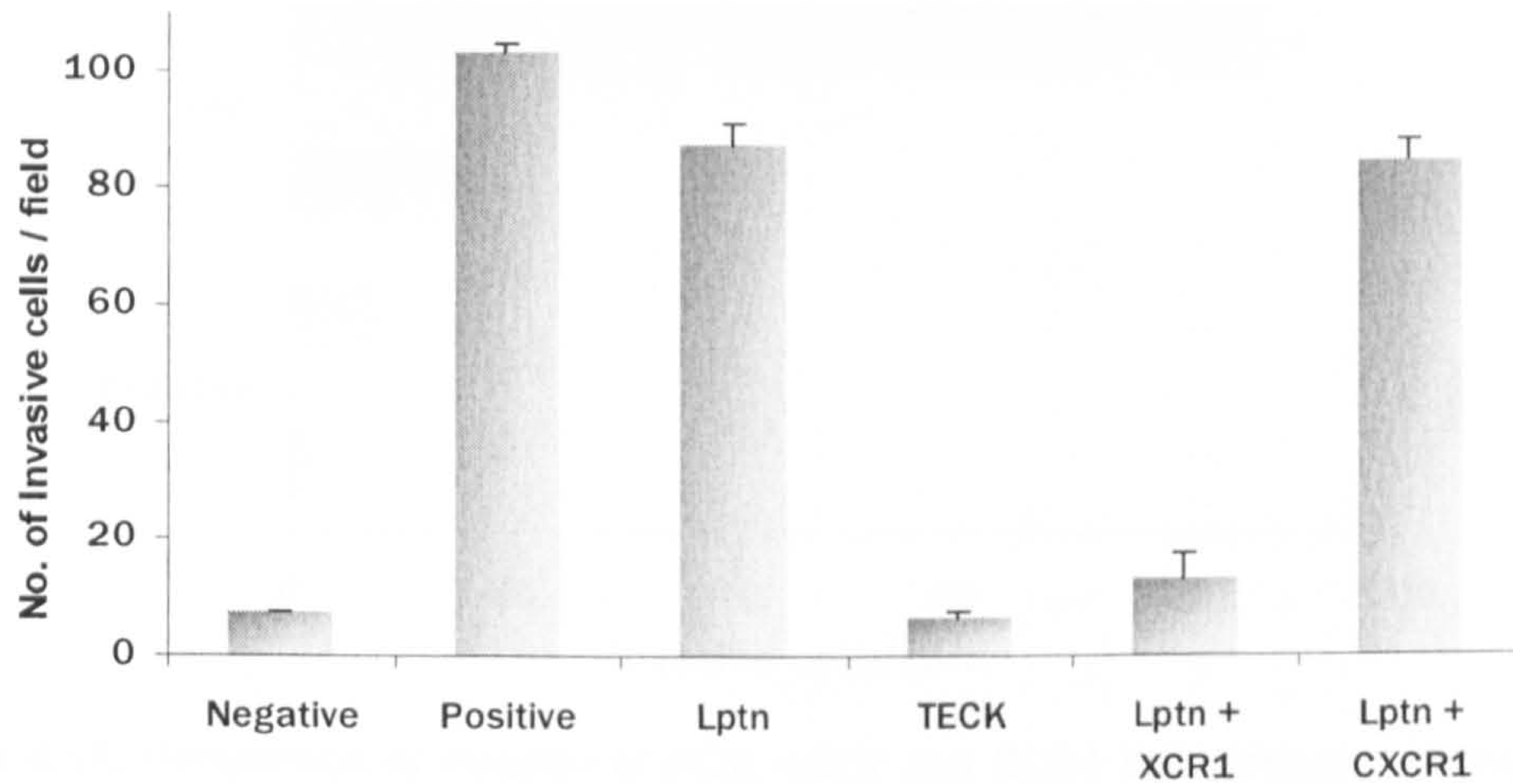


Figure 4.10. Invasion of SCC4 cells towards lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, CXCR1= irrelevant antibody). Lymphotactin caused a significant increase in invasion of NOK and this effect was significantly reduced when an XCR1 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

iii) Comparison of XCR1/lymphotactin mediated invasion between NOK and OCCL

Comparison of invasion between NOK, H357 and SCC4 showed that the number of SCC4 cells invading towards lymphotactin was significantly higher than H357 cells ($p<0.0001$) which was significantly greater than the number of NOK ($p<0.0001$) (Figure 4.11).

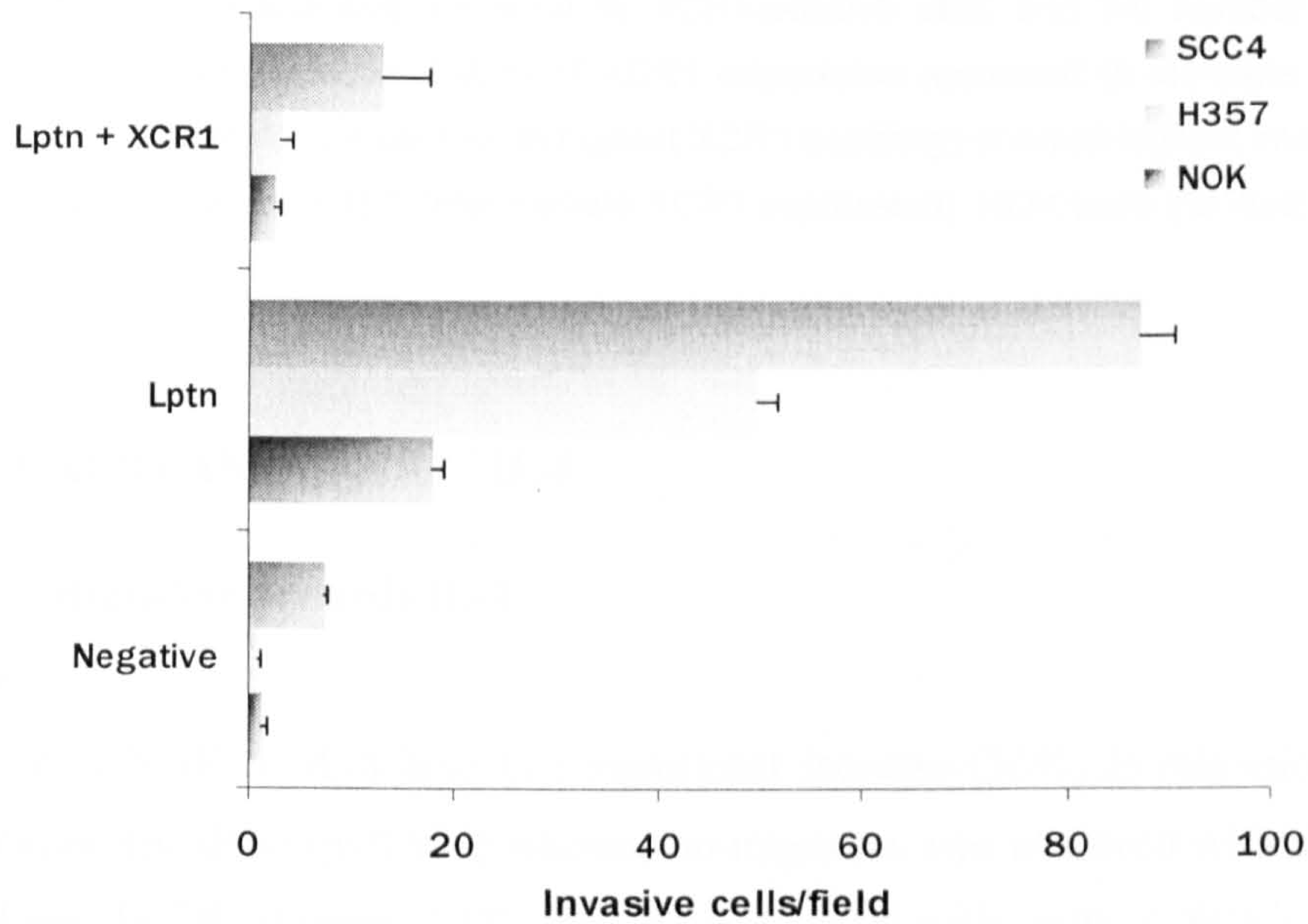


Figure 4.11. Comparison of invasion of NOK, H357 and SCC4 cells towards lymphotactin n= average of 3 different experiments done in triplicate, error bars=SD). Invasion towards lymphotactin was highest for SCC4 cells followed by H357 whereas NOK were the least invasive.

This is in agreement with XCR1 expression data as more than 90% of SCC4 cells are positive for XCR1 (as shown by flow cytometry) whereas fewer H357 cells express XCR1. The NOK are the least positive for XCR1 and the least invasive towards lymphotactin (Figure 4.12).

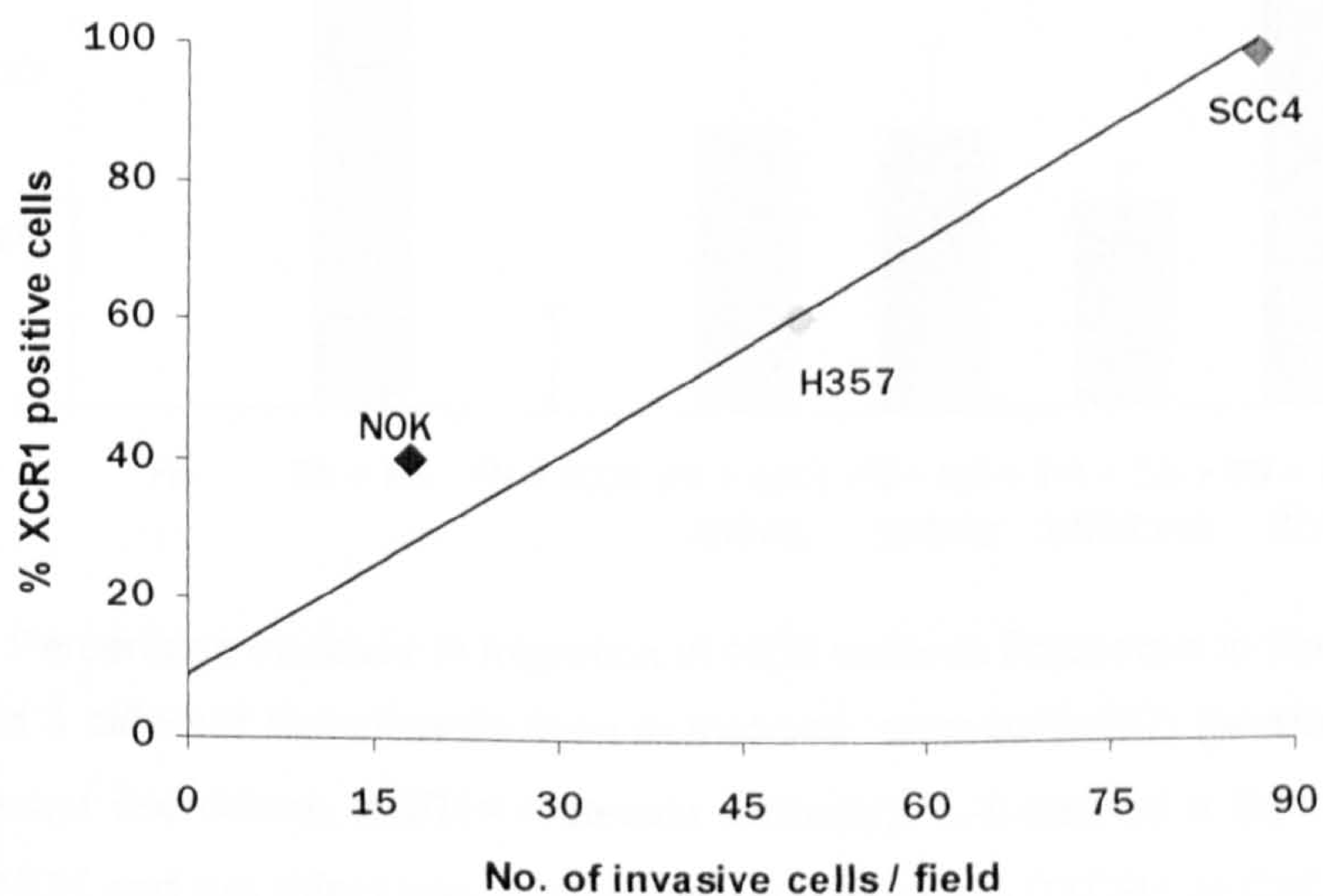


Figure 4.12. The relationship between % XCR1-positive cells and the number of cells that invaded towards lymphotactin. Extent of XCR1 expression appeared to correlate with invasion towards lymphotactin. SCC4 cells (with highest XCR1 positivity) showed highest invasion towards lymphotactin followed by H357 (intermediate XCR1 expression). NOK were the least invasive.

4.5.2 CXCR1 AND CXCR2 / IL-8

4.5.2.1 Migration towards IL-8

i) NOK

Exposure of NOK to IL-8 lead to a significant increase (36%) in migration compared with fibronectin alone ($p<0.005$) whereas no migration was observed with the irrelevant chemokine TECK (Figure 4.13). Pre-incubation of cells with CXCR1 or CXCR2 antibody individually significantly decreased migration ($p<0.005$) while the irrelevant antibody XCR1 failed to do so. However, when both CXCR1 and CXCR2 antibodies were used in combination, no further decrease was observed compared with CXCR1 or CXCR2 on their own.

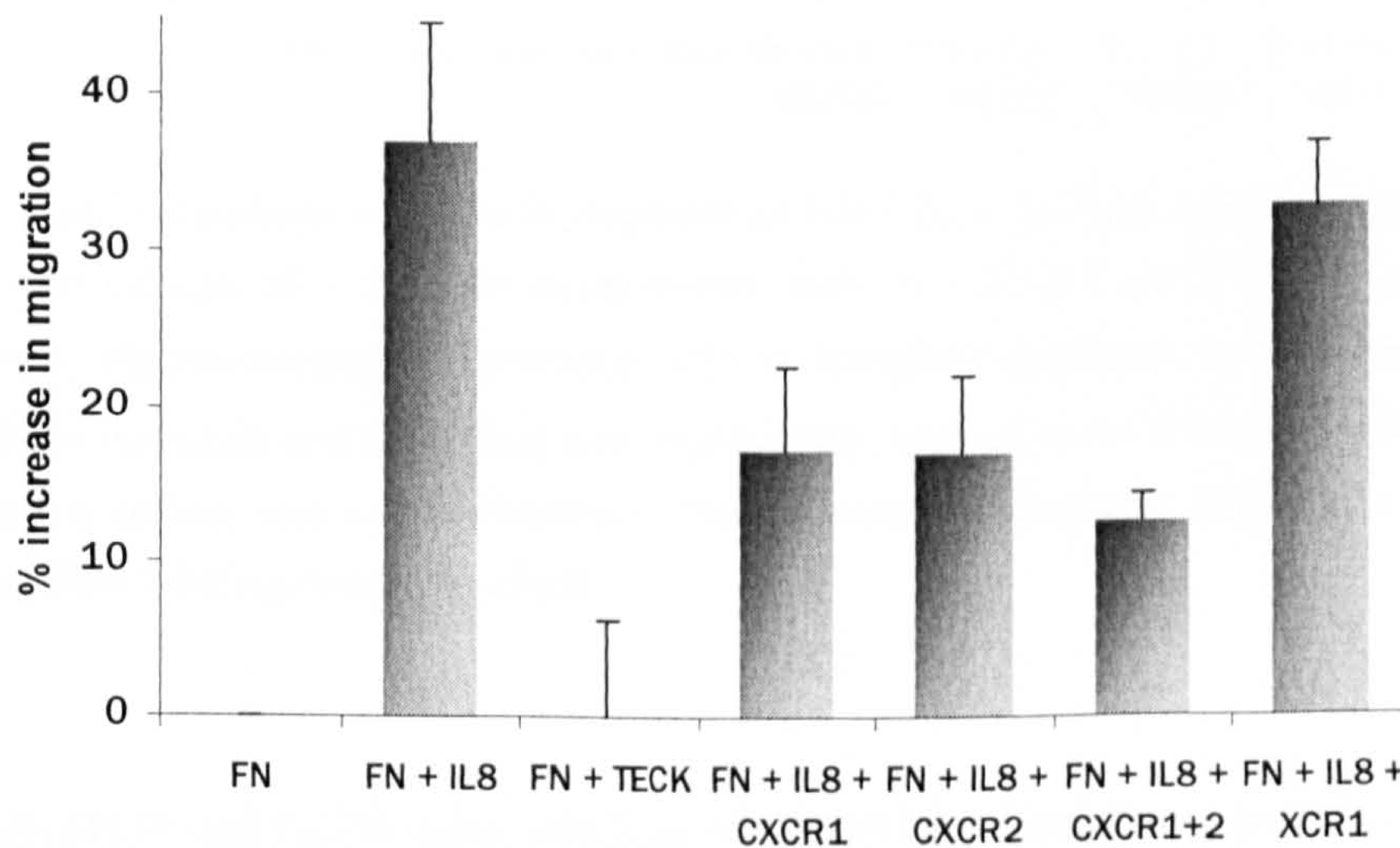


Figure 4.13. Percentage increase in migration of NOK towards fibronectin in the presence of IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where FN= fibronectin, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in migration of NOK and this effect was significantly reduced when CXCR1 or CXCR2 antibody was

added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

ii) H357 and SCC4 cells

Migration of H357 and SCC4 cells also increased significantly (47.2% and 74.2% respectively) in the presence of IL-8 compared with fibronectin alone ($p<0.000001$ and $p<0.001$ respectively) (Figure 4.14 and 4.15). As with NOK, the irrelevant chemokine TECK failed to increase migration.

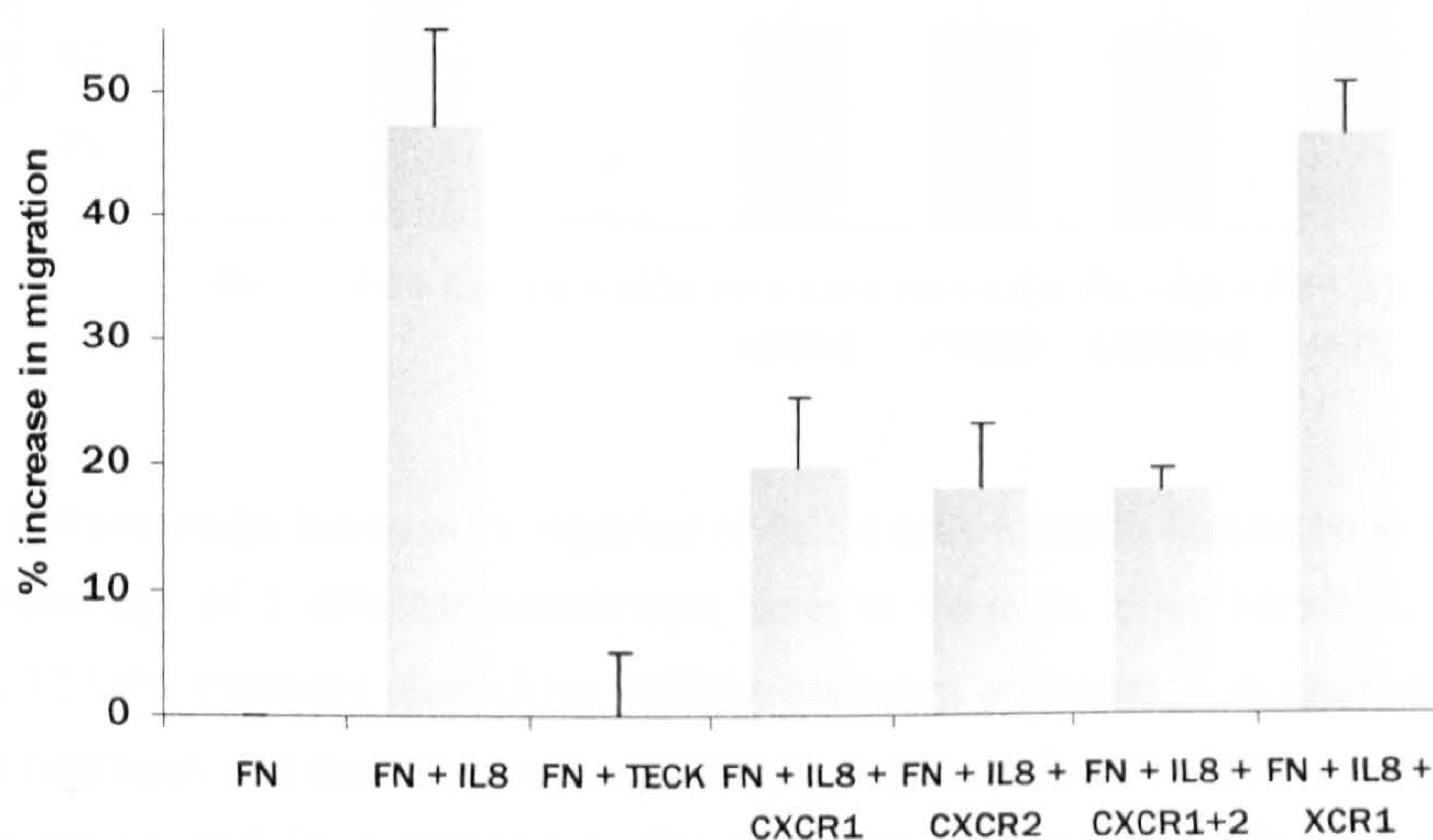


Figure 4.14. Percentage increase in migration of H357 cells towards fibronectin in the presence of IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where FN= fibronectin, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in migration and this effect was significantly reduced when CXCR1 or CXCR2 antibody was added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

For both H357 and SCC4 cells, addition of CXCR1 ($p<0.0001$ and $p<0.001$ respectively) or CXCR2 antibody ($p<0.0001$ and $p<0.0001$ respectively) facilitated a significant decrease in migration but did not reduce it to the control levels. Pre-incubation with CXCR1 and CXCR2 antibodies in combination did not cause any additional reduction in

migration compared to the individual antibodies. The irrelevant antibody XCR1 had no effect on migration (Figure 4.14 and 4.15).

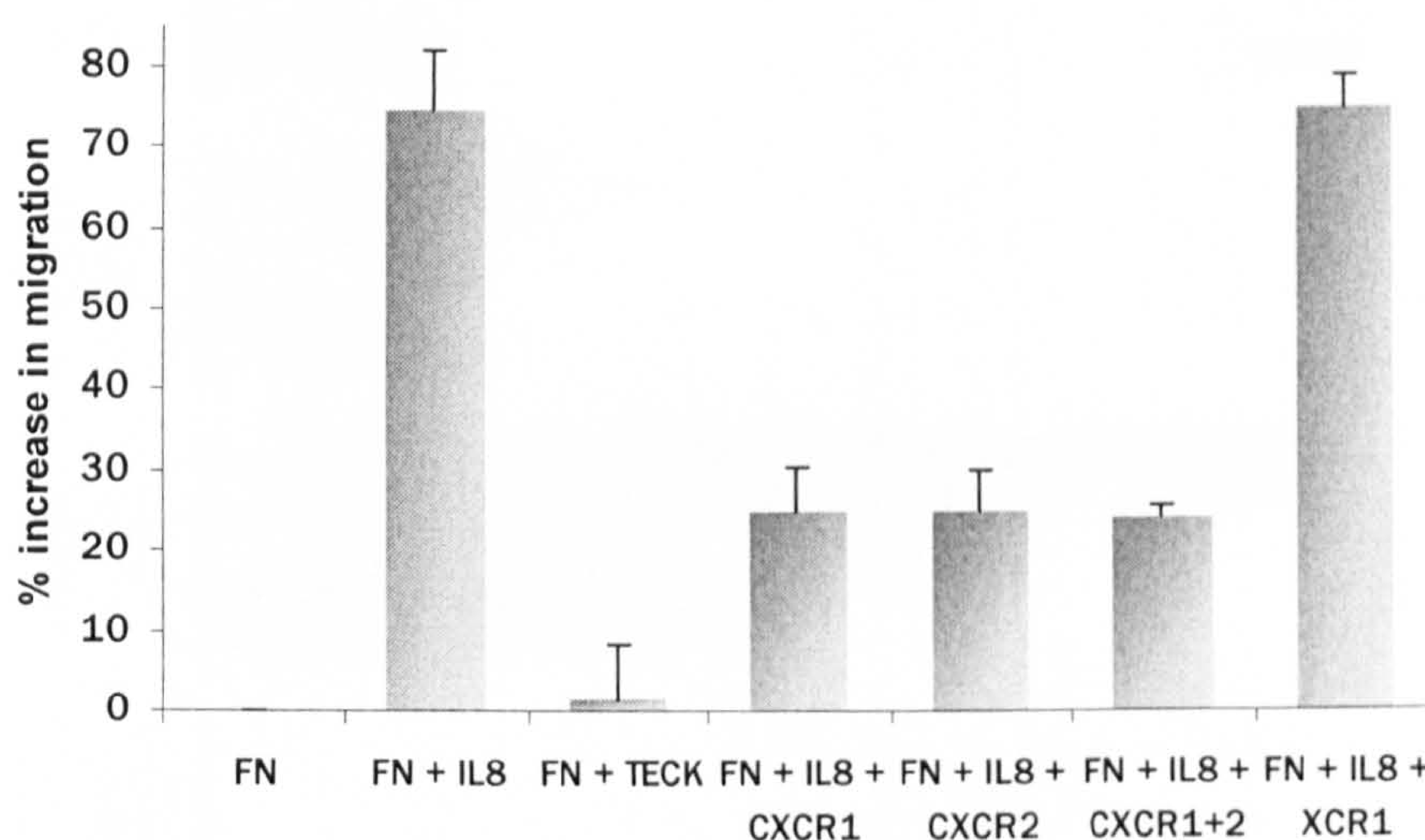


Figure 4.15. Percentage increase in migration of SCC4 cells towards fibronectin in the presence of IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where FN= fibronectin, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in migration and this effect was significantly reduced when CXCR1 or CXCR2 antibody was added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

iii) Comparison of IL-8 mediated migration between NOK and OCCL

As for lymphotactin, the number of SCC4 cells that migrated towards IL-8 was significantly higher than the number of H357 cells ($p<0.000001$). Similarly significantly more H357 cells than NOK migrated towards IL-8 ($p<0.01$) (Figure 4.16).

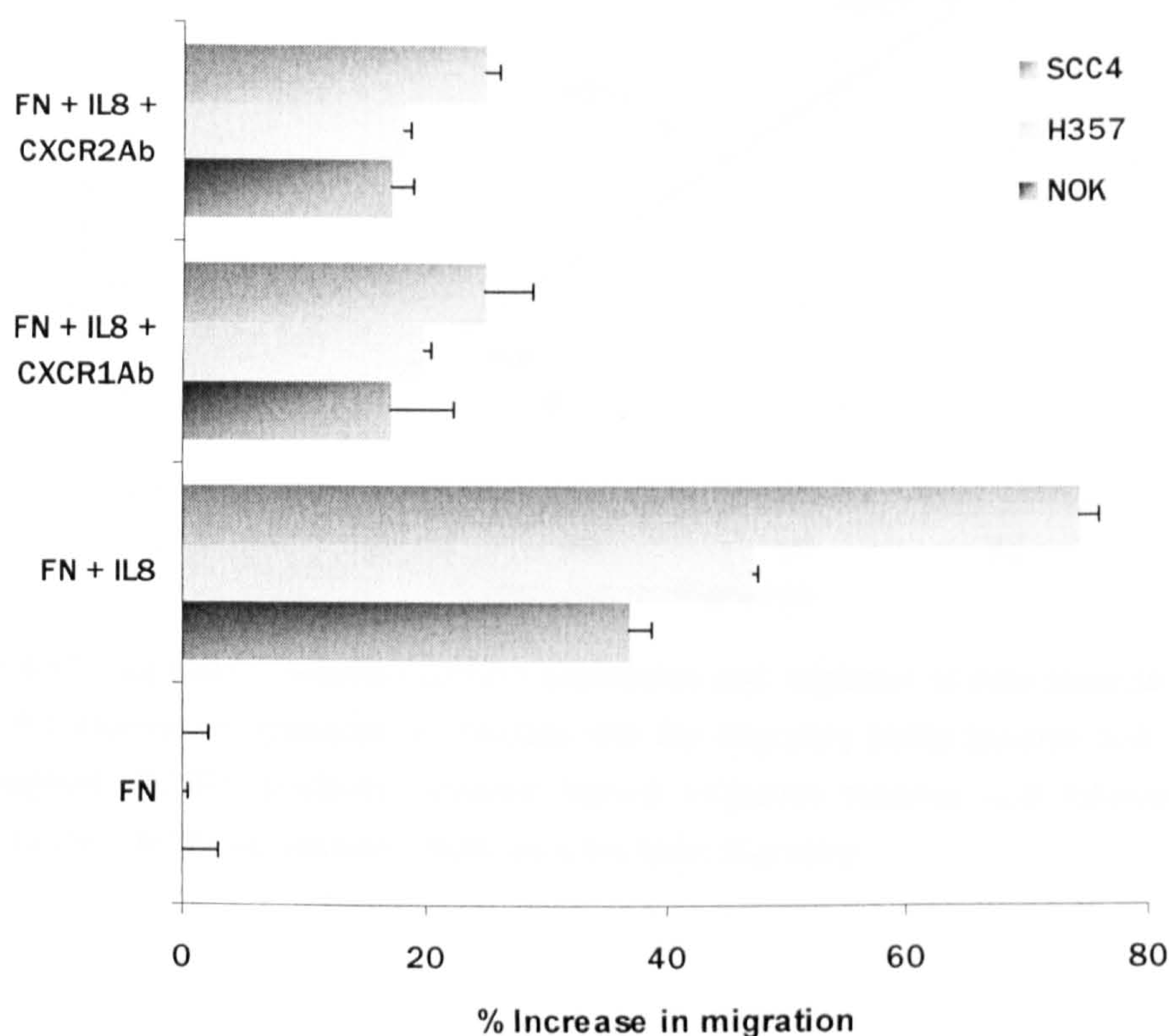


Figure 4.16. Comparison of percentage increase in migration of NOK and OCCL towards IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD). Migration of cells was significantly increased in the presence of IL-8 compared with fibronectin alone. SCC4 cells showed the highest migration followed by H357 cells while NOK were the least migratory. Addition of CXCR1 or CXCR1 antibody (alone and in combination) significantly reduced this migration.

The relationship between the percentage of CXCR1 and CXCR2 positive cells and the percentage increase in migration is shown in Figures 4.17 and 4.18 respectively. Different patterns were observed for CXCR1 and CXCR2 as CXCR1 expression showed a more direct relationship with percentage expression of migrating cells than CXCR2.

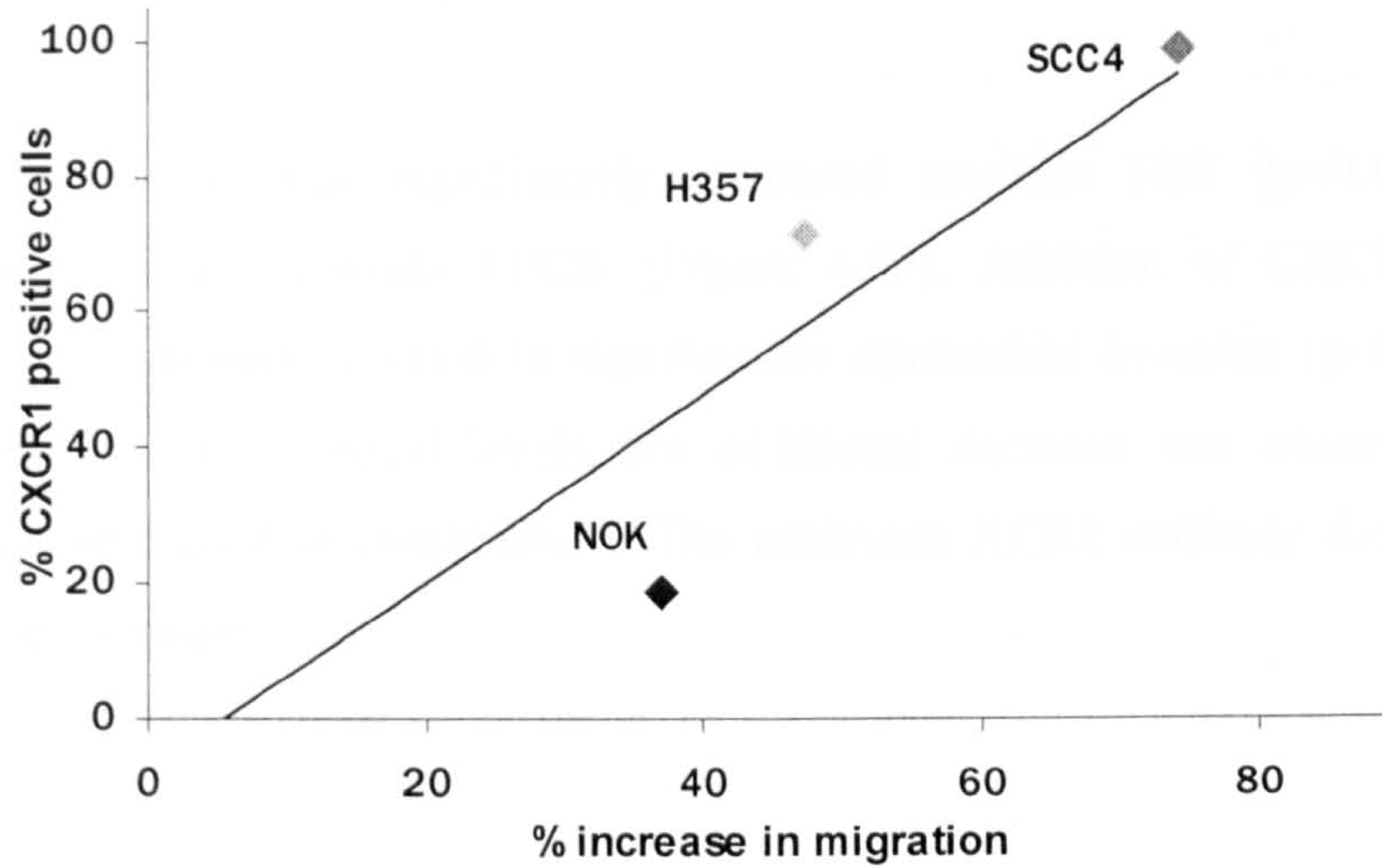


Figure 4.17. Correlation between CXCR1 expression and migration of cells towards IL-8. Extent of CXCR1 expression appeared to correlate with the migratory ability towards IL-8. SCC4 cells (with highest CXCR1 positivity) showed highest migration towards IL-8 followed by H357 (intermediate CXCR1 expression). NOK were the least migratory.

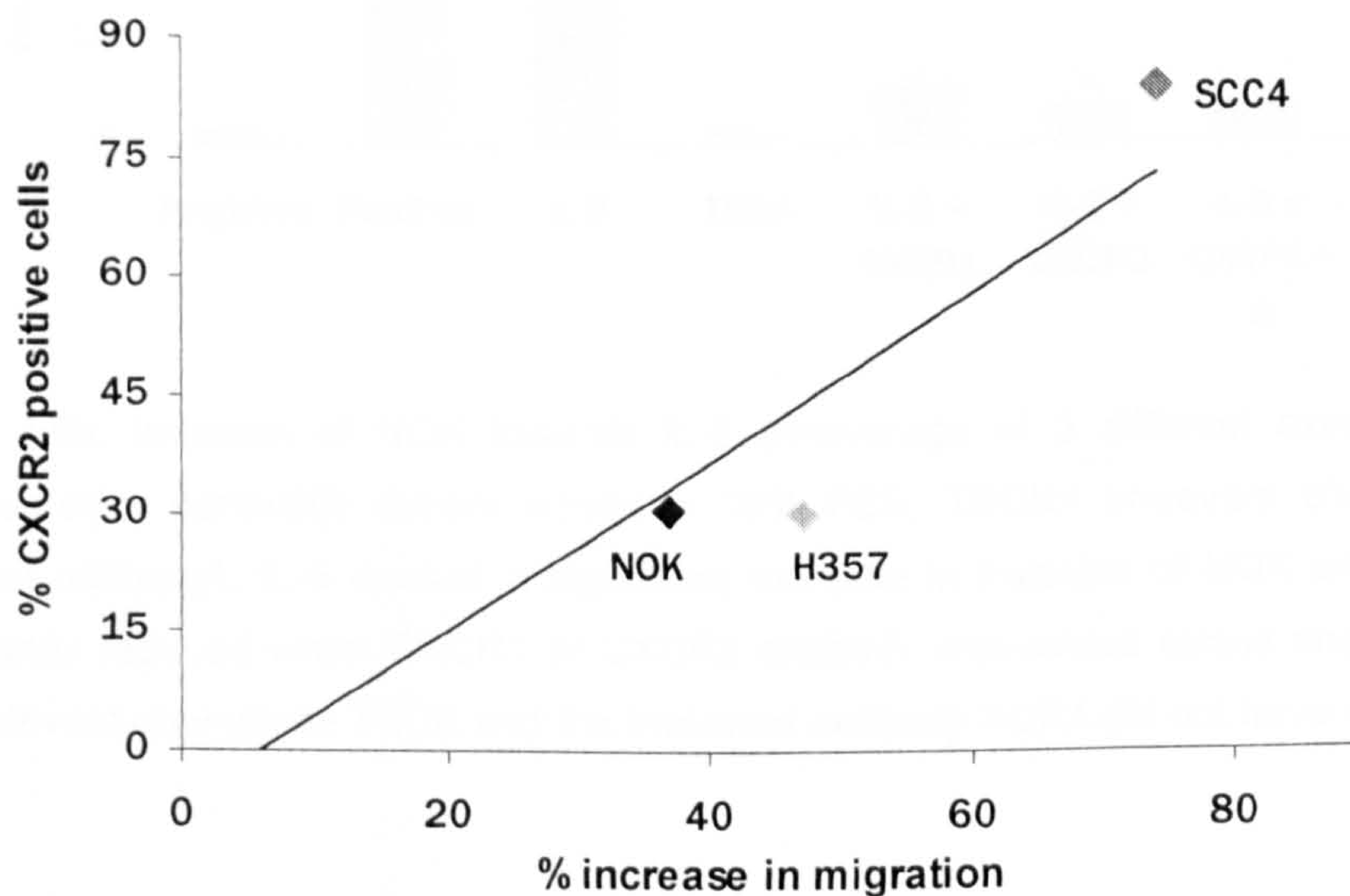


Figure 4.18. Correlation between CXCR2 expression and migration of cells towards IL-8. Extent of CXCR2 expression did not correlate very well with the migratory ability towards IL-8 (compared with CXCR1). SCC4 cells (with highest CXCR2 positivity) showed highest migration towards IL-8. H357 and SCC4 cells had similar CXCR2 positivity but greater migration was seen in H357 cells compared with NOK.

4.5.2.2 Invasion towards IL-8

i) NOK

Invasion of NOK was significantly increased towards FBS ($p<0.001$) and IL-8 ($p<0.0001$) but not towards TECK (Figure 4.19). Addition of CXCR1 or CXCR2 antibody on their own resulted in significantly diminished invasion ($p<0.0005$) but did not reduce it to the control levels. No additional decrease was observed when both antibodies were used in combination. The irrelevant XCR1 antibody did not cause any decrease in invasion.

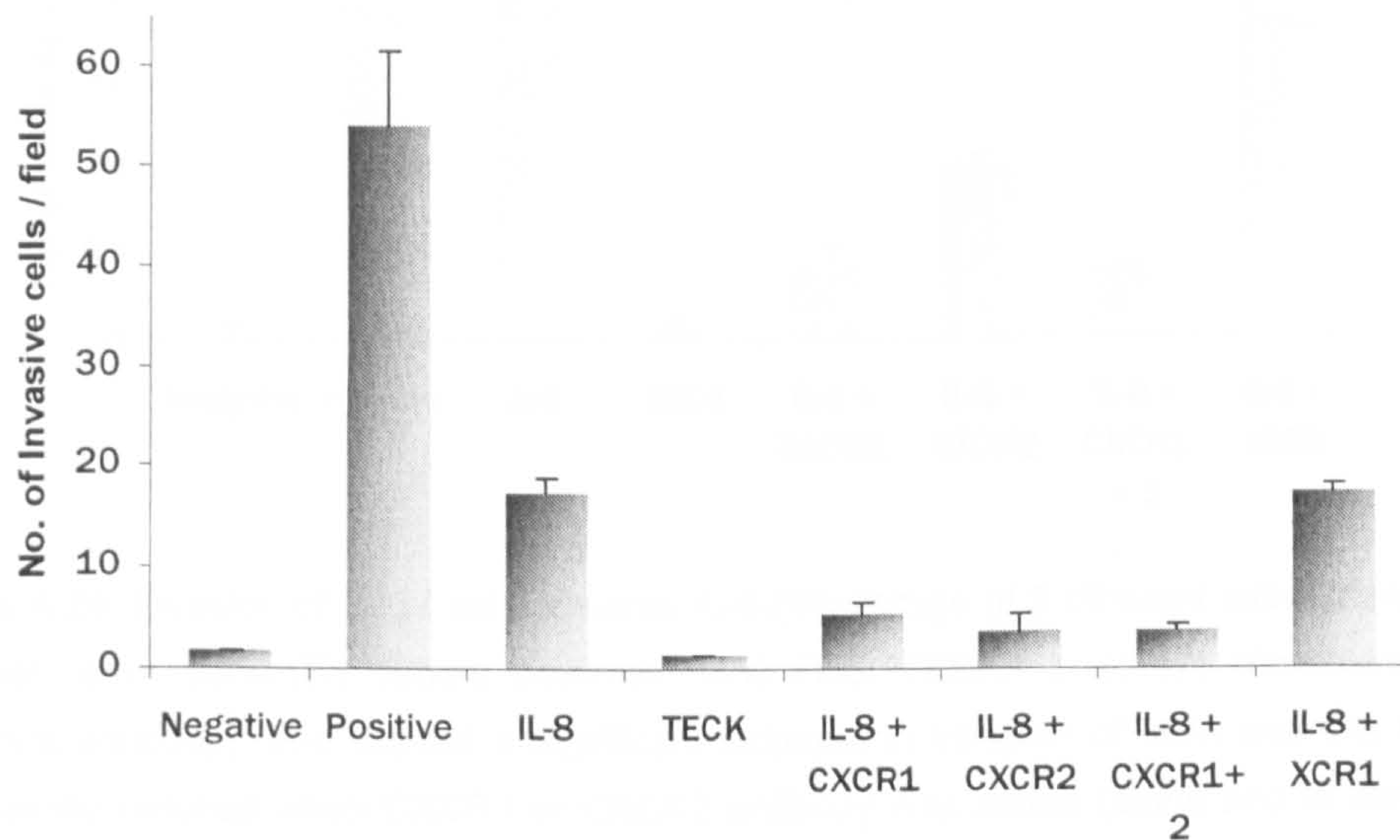


Figure 4.19. Invasion of NOK towards IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR1 or CXCR2 antibody was added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

ii) H357 and SCC4 cells

A similar response was observed in H357 and SCC4 cells. Exposure of H357 and SCC4 cells to FBS ($p<0.00001$) and IL-8 ($p<0.0000001$ and $p<0.0001$) significantly enhanced invasion compared with negative controls (Figures 4.20 and 4.21). In both H357 and SCC4 cells administration of CXCR1 ($p<0.00001$ and $p<0.01$ respectively) or CXCR2

(p<0.00001 and p<0.01 respectively) blocking antibodies individually or in combination (p<0.000001 and p<0.0001 respectively) significantly reduced invasion but did not reduce it to control levels. The reduction in invasion of H357 with CXCR1 antibody was significantly higher than CXCR2 (p<0.01) whereas no such effect was observed in the SCC4 cells (Figure 4.20). The irrelevant chemokine TECK or the irrelevant antibody XCR1 failed to elicit any response.

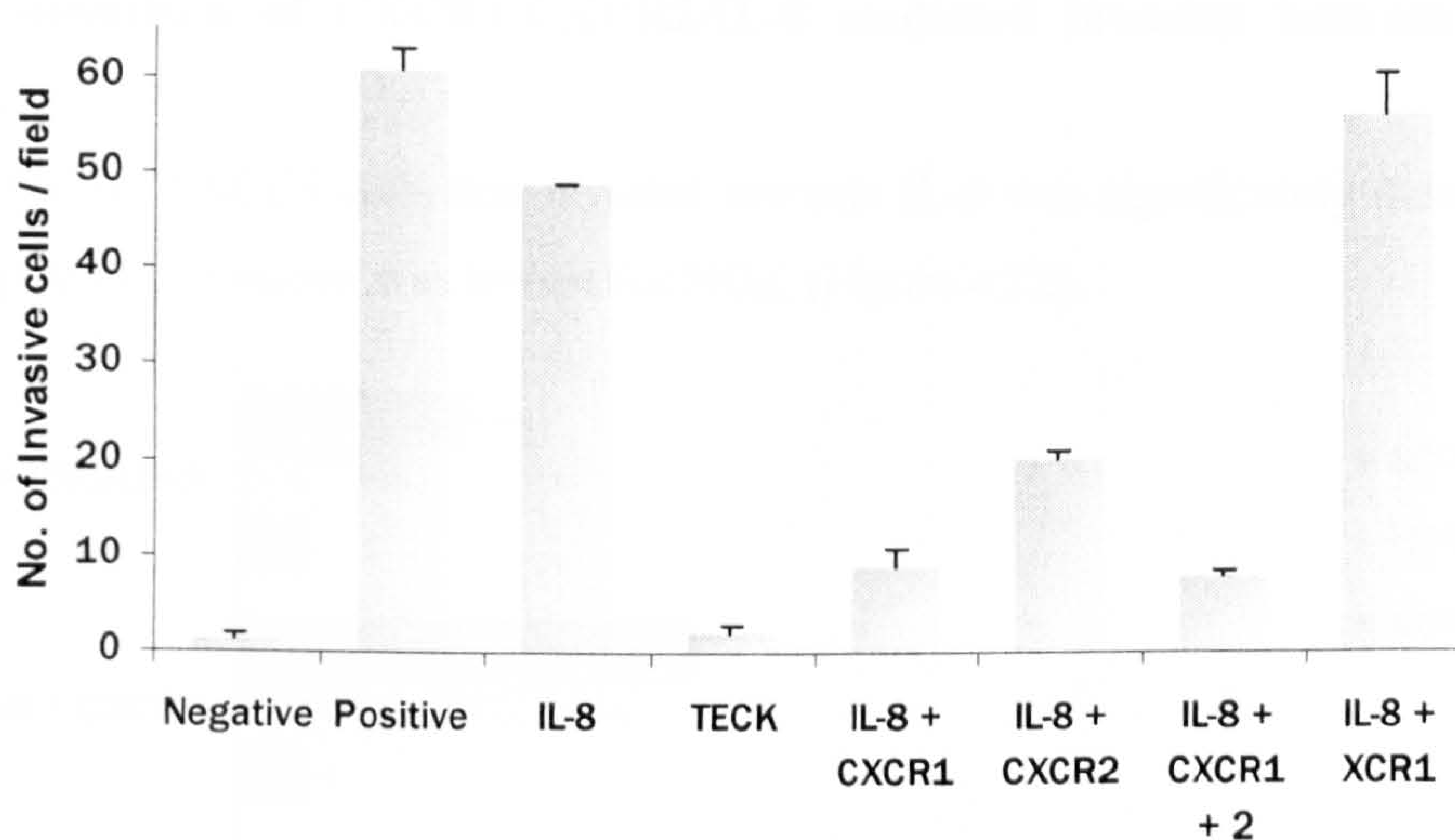


Figure 4.20. Invasion of H357 cells towards IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR1 or CXCR2 antibody was added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

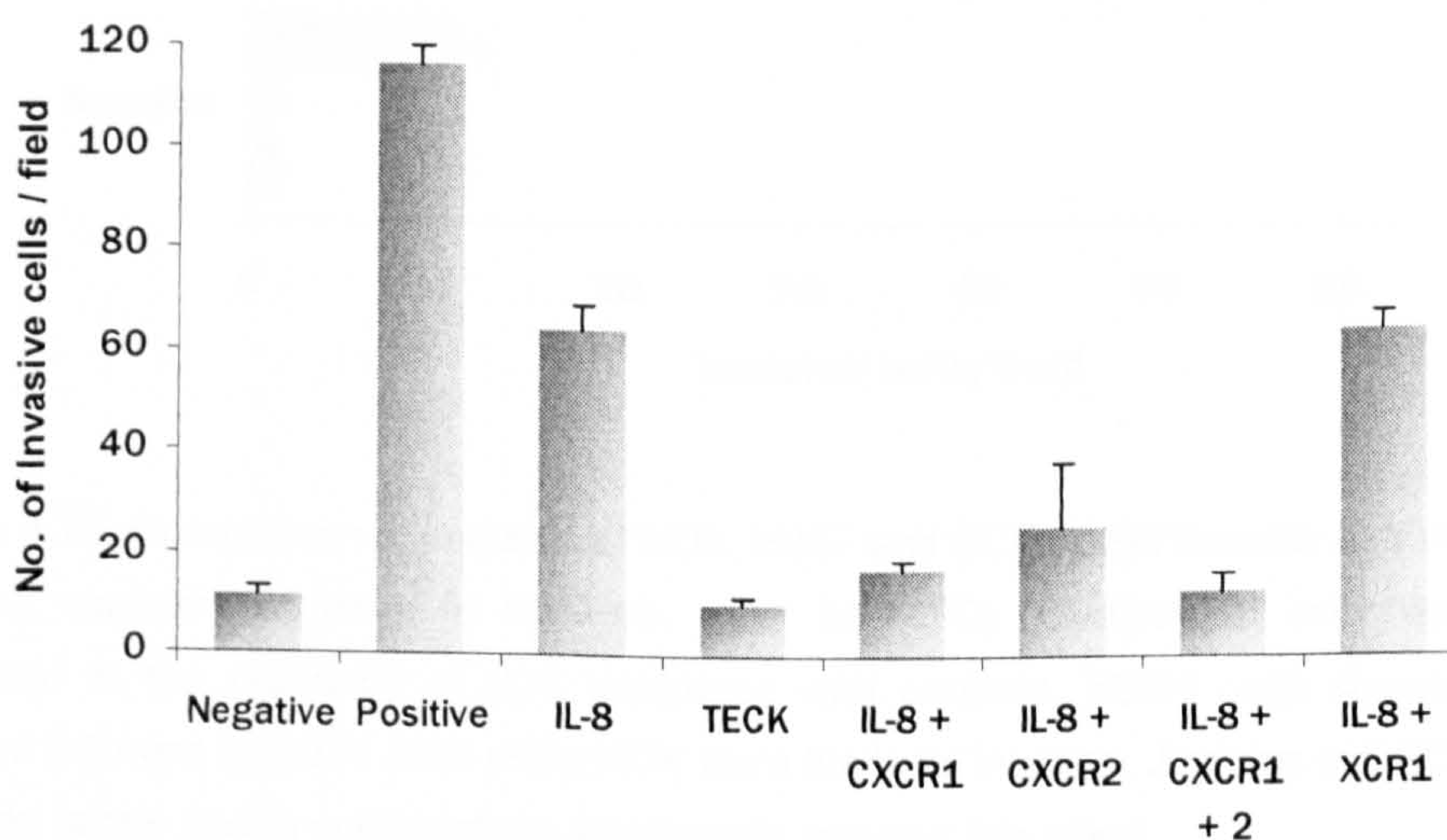


Figure 4.21. Invasion of SCC4 cells towards IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, XCR1= irrelevant antibody). IL-8 caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR1 or CXCR2 antibody was added (alone and in combination). The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any effect.

iii) Comparison of CXCR1-CXCR2/IL-8 mediated invasion between NOK and OCCL

The number of SCC4 cells that invaded towards IL-8 was significantly more than H357 cells ($p<0.01$). Invasion was lowest for NOK (Figure 4.22).

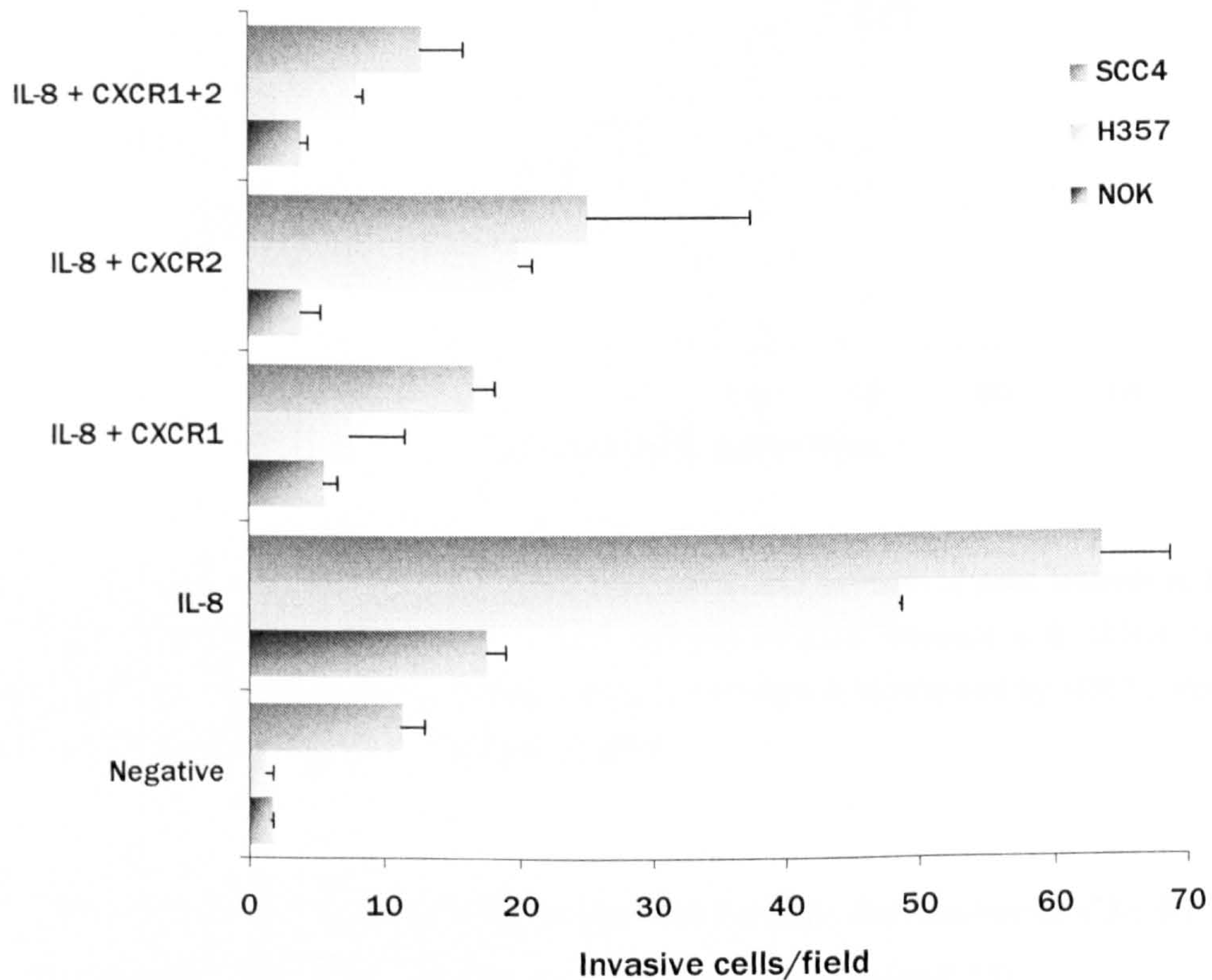


Figure 4.22. Comparison of invasion of NOK, H357 and SCC4 cells towards IL-8 (n=average of 3 different experiments done in triplicate, error bars=SD). Invasion of cells was significantly increased in the presence of IL-8 compared with controls. SCC4 cells showed the highest invasion followed by H357 cells while NOK were the least invasive. Addition of CXCR1 or CXCR1 antibody (alone and in combination) significantly reduced this effect.

This is in agreement with CXCR1 and CXCR2 expression data. More SCC4 cells are positive for CXCR1 and CXCR2 compared with NOK and H357 cells and showed the highest number of cells that invaded towards IL-8.

The correlation between CXCR1 and CXCR2 expression and the number of cells that invaded towards IL-8 is shown in Figure 4.23 and 4.24.

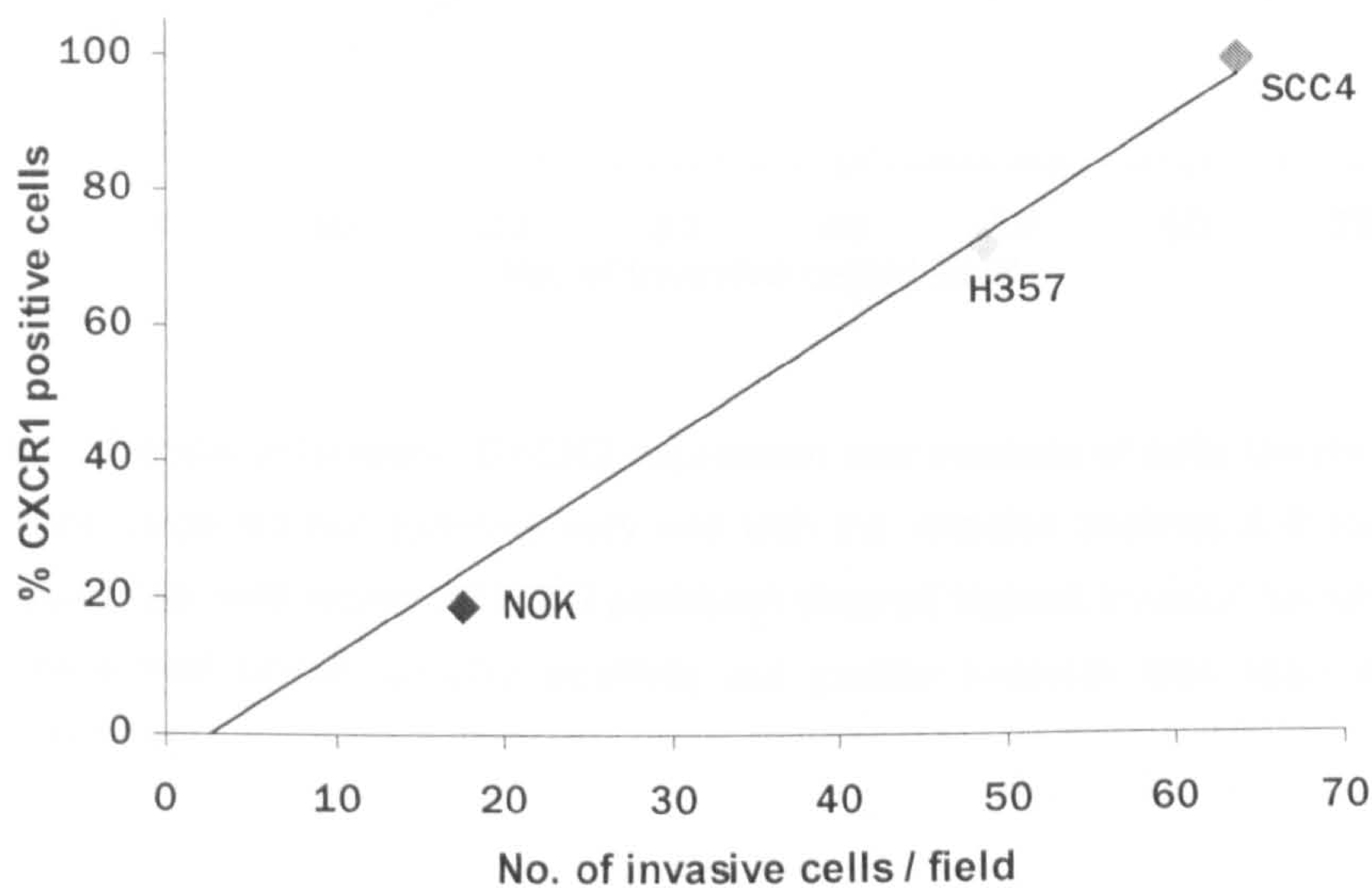


Figure 4.23. Relationship between CXCR1 expression and invasion of cells towards IL-8. Extent of CXCR1 expression appeared to correlate with the invasion towards IL-8. SCC4 cells (with highest CXCR1 positivity) showed highest invasion towards IL-8 followed by H357 (intermediate CXCR1 expression). NOK were the least invasive.

For CXCR1, a direct relationship was observed between the number of CXCR1 positive cells and the number of cells that invaded towards IL-8 (Figure 4.25).

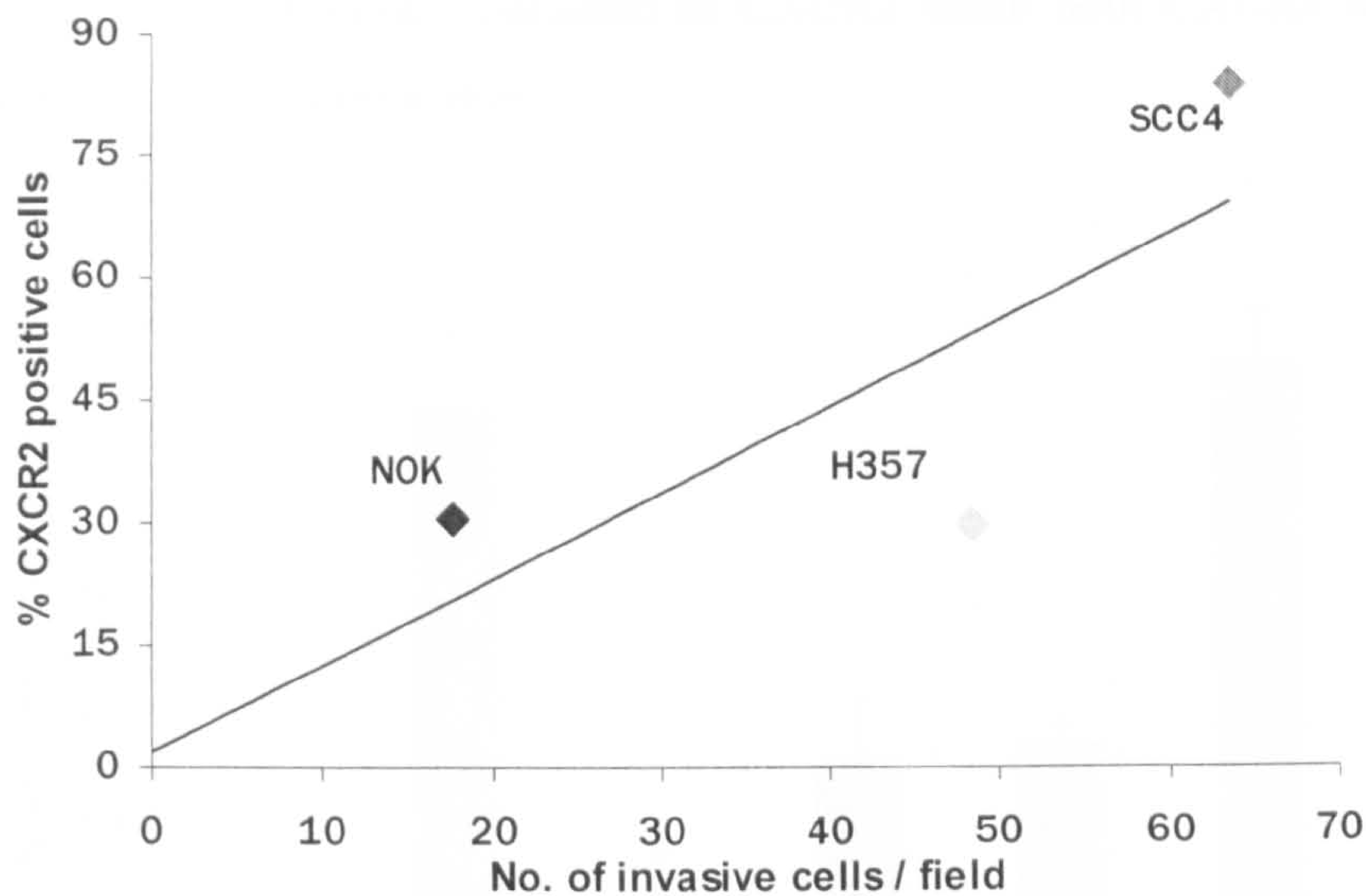


Figure 4.24. Relationship between CXCR2 expression and invasion of cells towards IL-8. Extent of CXCR2 expression did not correlate very well with the invasion towards IL-8 (compared with CXCR1). SCC4 cells (with highest CXCR2 positivity) showed highest invasion towards IL-8. H357 and SCC4 cells had similar CXCR2 positivity but greater invasion was seen in H357 cells compared with NOK.

The results were very different for CXCR2. NOK and H357 cells have a similar percentage of CXCR2-positive cells however more H357 cells than NOK invaded towards IL-8 (Figure 4.24).

4.5.3 CXCR2 / GRO-α

4.5.3.1 Migration towards GRO-α

i) NOK

Migration of NOK increased significantly by 45% in the presence of GRO-α compared with fibronectin alone ($p<0.00001$). Pre-incubation of cells with CXCR2 antibody caused a significant decrease in this migration ($p<0.001$) but did not reduce it to control levels. TECK and the irrelevant antibody CXCR1 failed to elicit any response (Figure 4.25). No

further decrease was observed compared to CXCR2 when both CXCR1 and CXCR2 antibodies were used in combination.

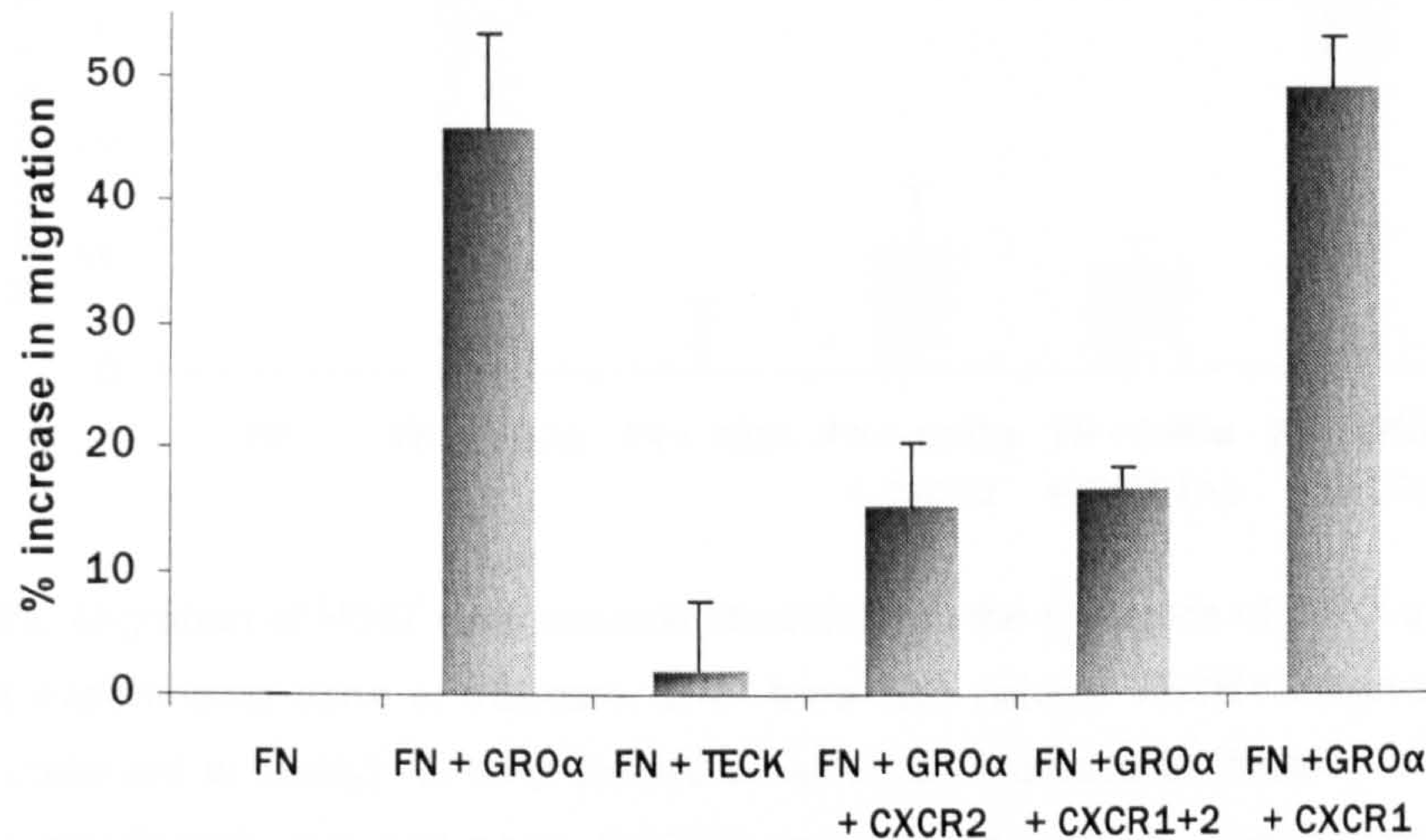


Figure 4.25. Percentage increase in migration of NOK towards fibronectin in the presence of GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

ii) H357 and SCC4 cells

Migration of H357 and SCC4 cells increased significantly (by 45% and 59.3% respectively) in the presence of GRO-α compared with fibronectin alone ($p<0.01$ and $p<0.0001$ respectively) (Figure 4.26 and 4.27). Pre-incubation with CXCR2 but not CXCR1 antibody significantly reduced migration of H357 and SCC4 cells towards GRO-α ($p<0.05$ and $p<0.01$ respectively) but failed to reduce it to control levels. No further reduction in migration compared to CXCR2 was observed when both CXCR1 and CXCR2 antibodies were used in combination. No migration was seen in response to TECK.

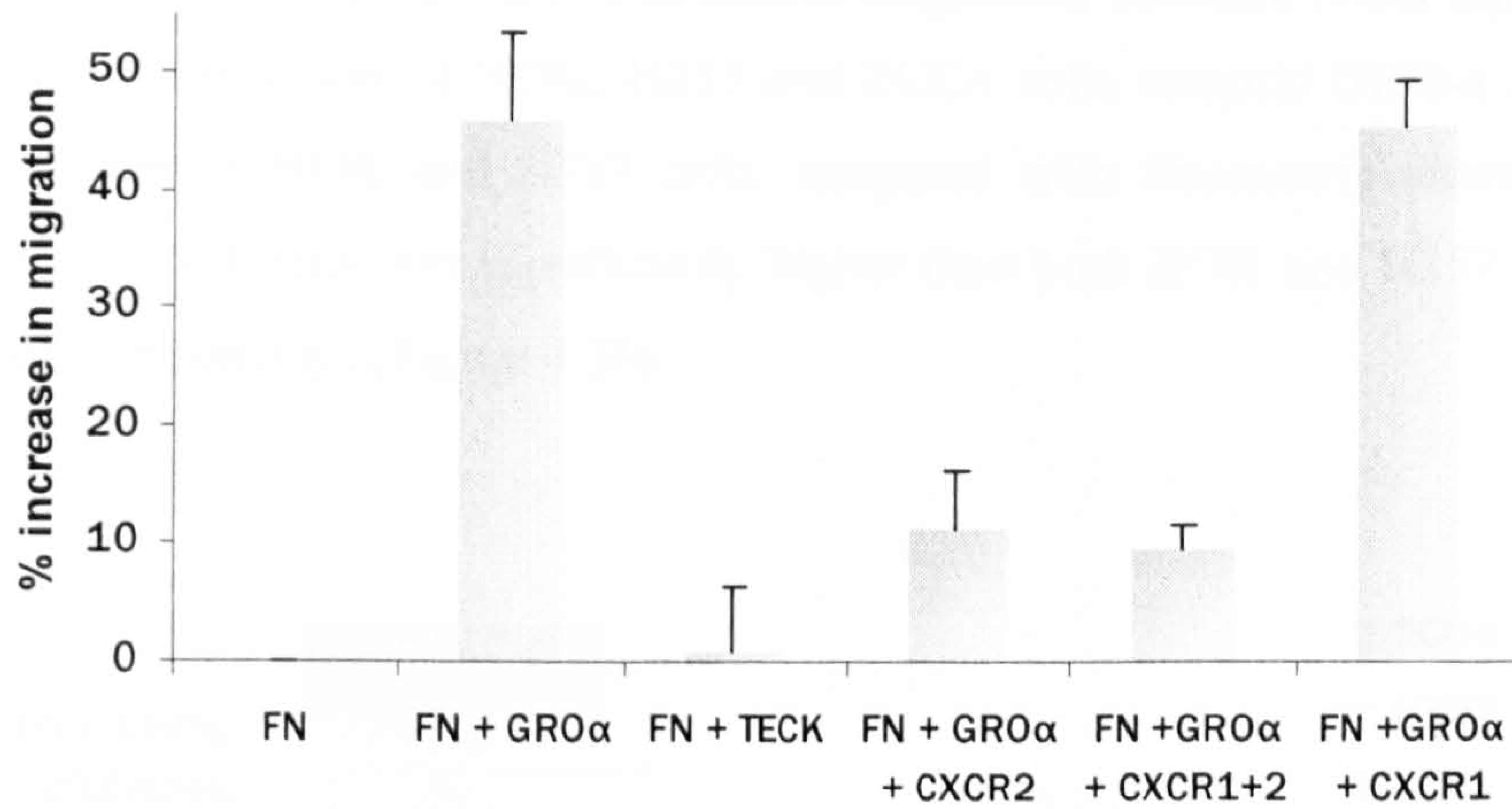


Figure 4.26. Migration of H357 cells towards fibronectin in the presence of GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

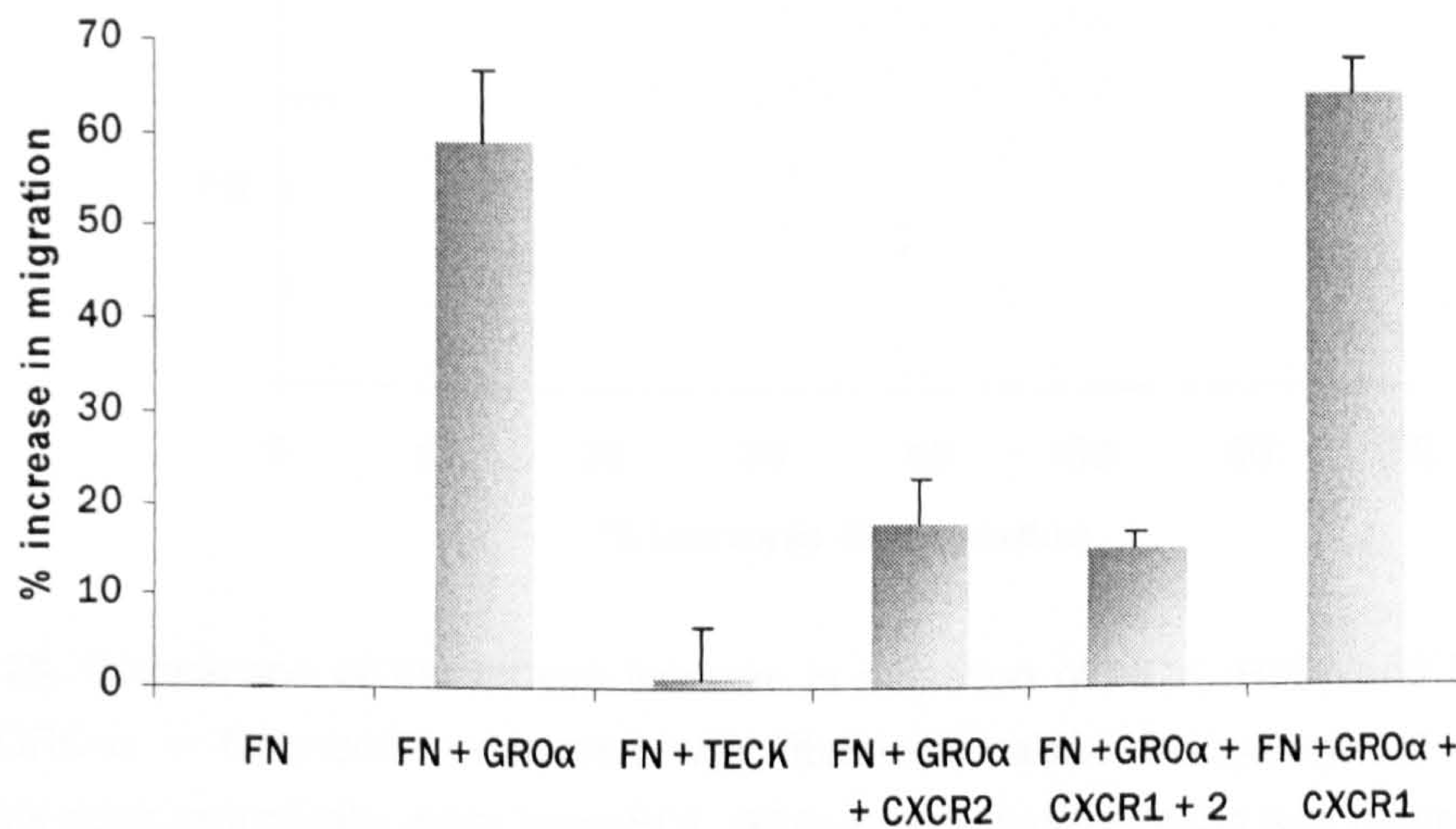


Figure 4.27. Migration of SCC4 cells towards fibronectin in the presence of GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

iii) Comparison of CXCR2/GRO-α mediated migration between NOK and OCCL

Comparison of migration of NOK, H357 and SCC4 cells towards GRO-α revealed no difference between NOK and H357 cells compared with fibronectin alone. However, migration of SCC4 cells was significantly higher than both NOK and H357 ($p<0.00001$ and $p<0.01$ respectively) (Figure 4.28).

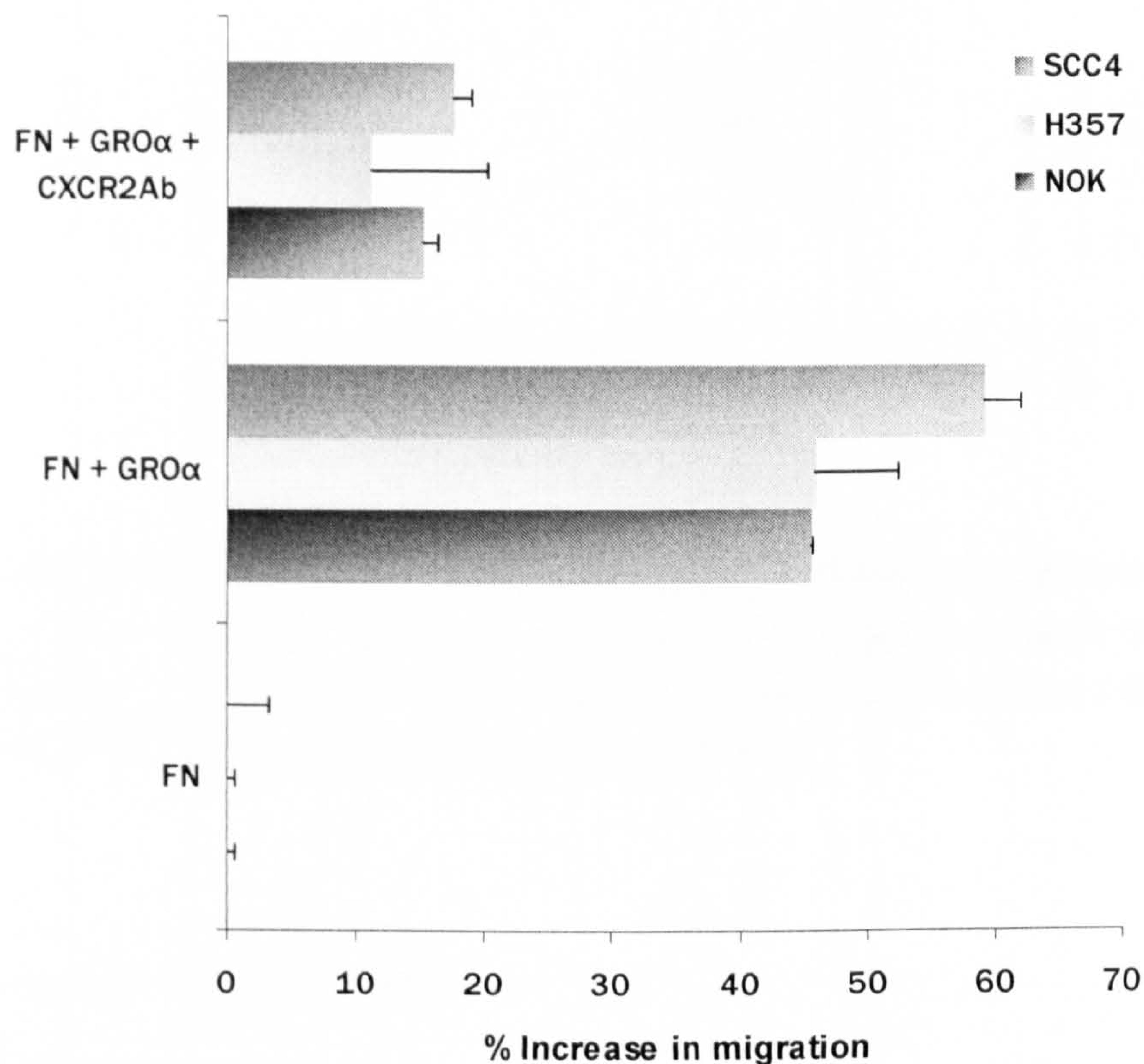


Figure 4.28. Comparison of percentage increase in migration of NOK, H357 and SCC4 cells towards GRO-α + fibronectin compared with fibronectin alone (n=average of 3 different experiments done in triplicate, error bars=SD). GRO-α mediated migration was highest in SCC4 cells whereas NOK and H357 migrated to a similar extent. Addition of CXCR2 antibody significantly reduced GRO-α mediated migration.

The relationship between percentage of CXCR2 positive cells and the percentage increase in migration is shown in Figure 4.29. NOK and H357 showed almost identical CXCR2 expression and migration whereas more SCC4 cells were CXCR2 positive and their

migration was greater.

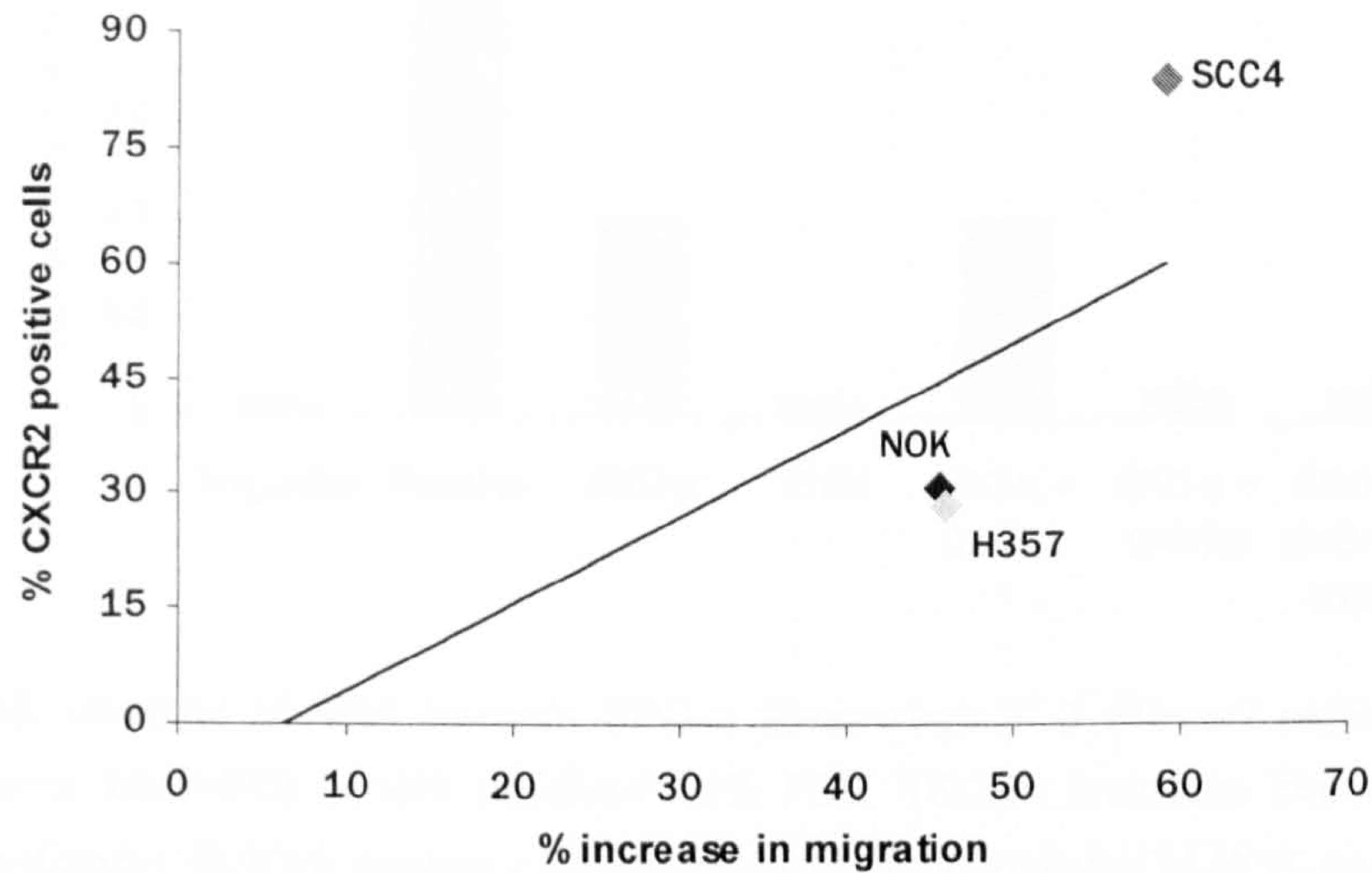


Figure 4.29. Relationship between CXCR2 expression and migration of cells towards GRO-α. Extent of CXCR2 expression appeared to correlate with the migration towards GRO-α. SCC4 cells (with highest CXCR2 positivity) showed highest migration towards GRO-α. H357 and NOK had similar CXCR2 expression and migrated to a similar extent.

4.5.3.2 Invasion towards GRO-α

i) NOK

Invasion of NOK was significantly enhanced in the presence of FBS and GRO-α compared with negative controls ($p<0.000001$). Addition of CXCR2 but not CXCR1 antibody significantly decreased invasion towards GRO-α ($p<0.00001$) but did not reduce it to control levels (Figure 4.30).

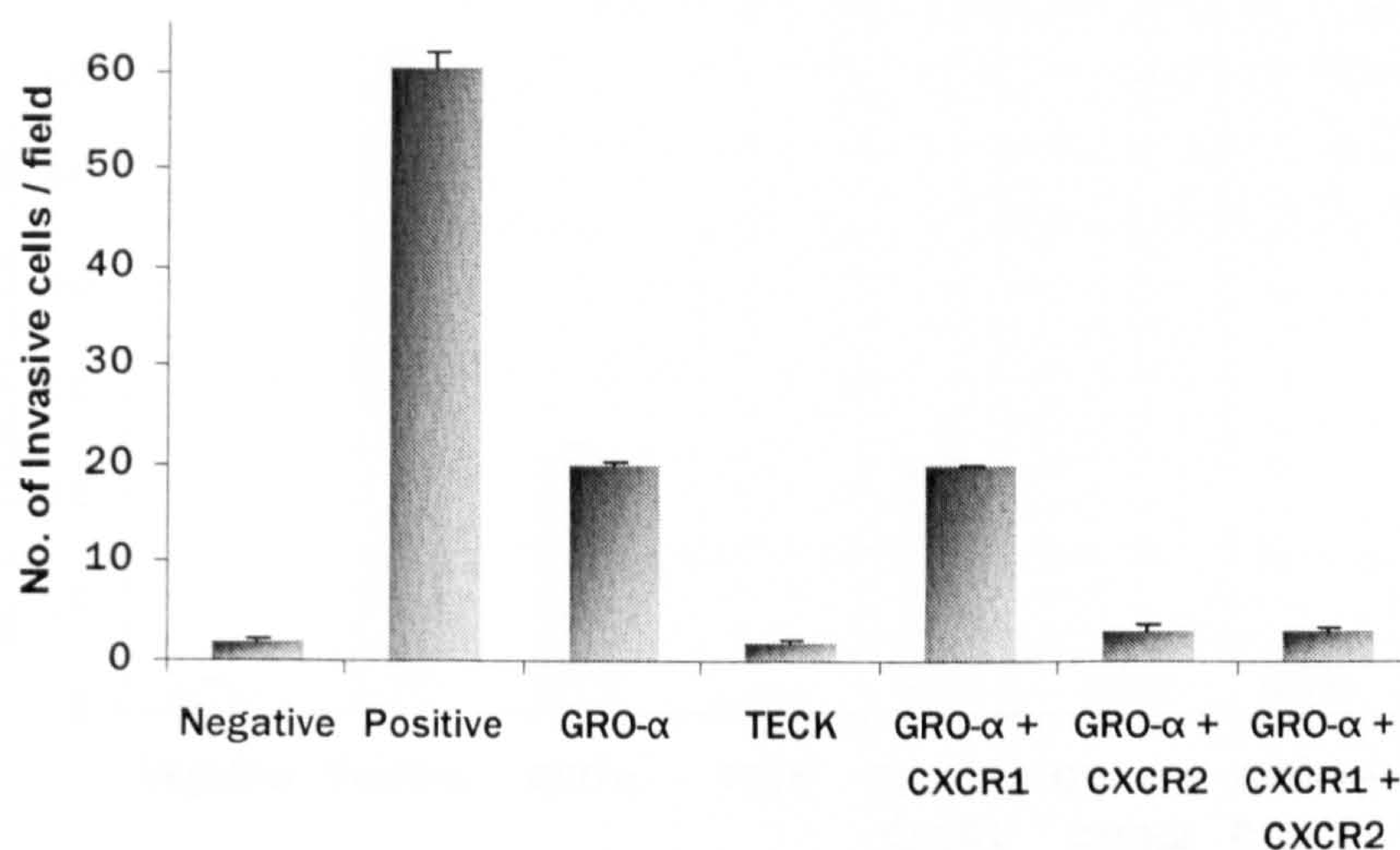


Figure 4.30. Invasion of NOK towards GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

Combination of CXCR1 and CXCR2 antibodies did not cause further reduction in invasion compared with CXCR2. No invasion was seen in response to TECK (Figure 4.30).

ii) H357 and SCC4 cells

FBS and GRO-α also caused a significant increase in invasion of H357 ($p<0.0000001$ and $p<0.00001$ respectively) and SCC4 cells ($p<0.00001$ and $p<0.0001$ respectively) compared with negative controls whereas TECK failed to do so (Figure 4.31 and 4.32). Addition of CXCR2 antibody decreased GRO-α mediated invasion significantly in both H357 and SCC4 cells ($p<0.0001$ and $p<0.0001$ respectively) but did not reduce it to control levels.

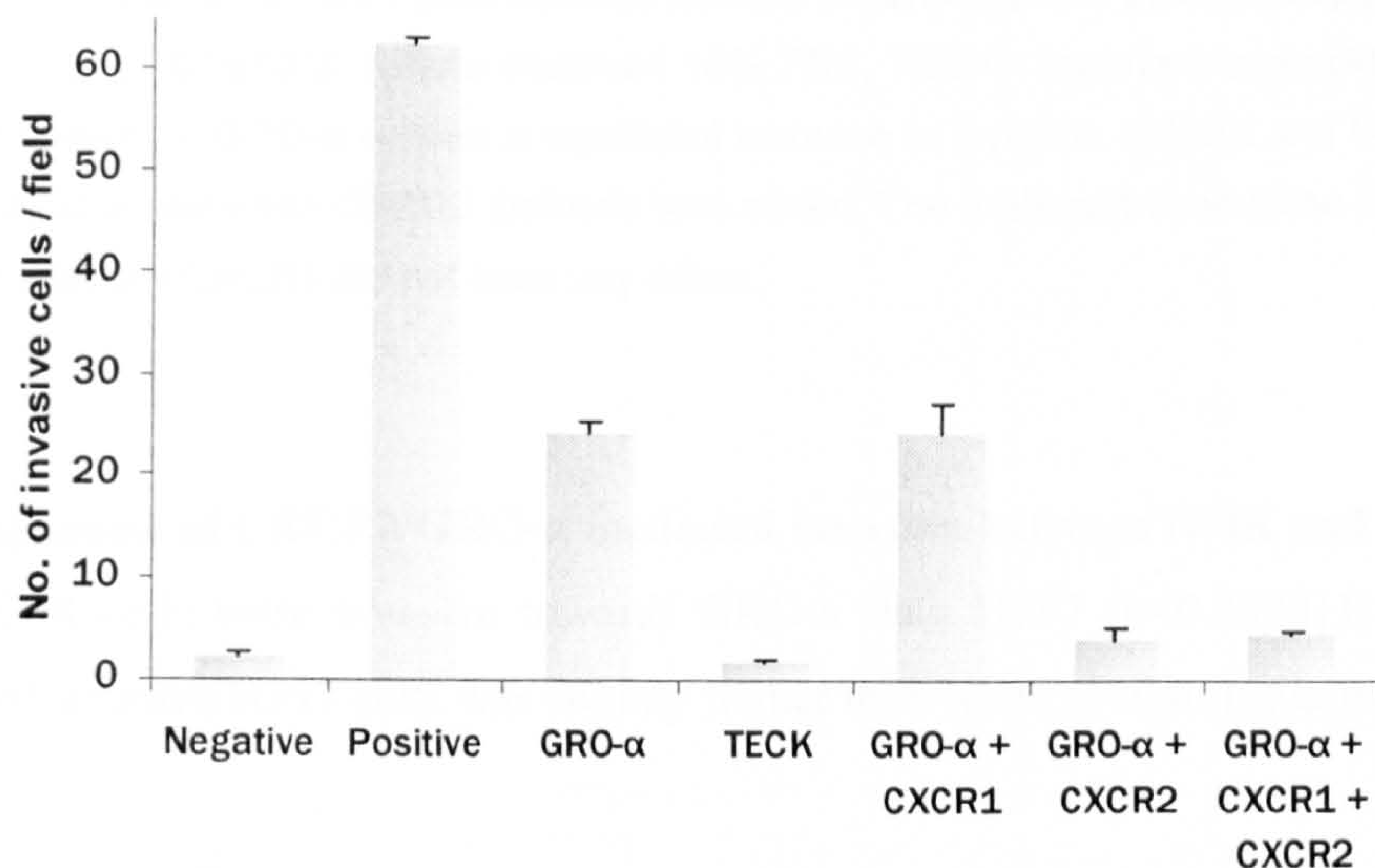


Figure 4.31. Invasion of H357 cells towards GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

Use of CXCR1 and CXCR2 antibodies in combination did not cause any further reduction in invasion compared with CXCR2. No decrease in invasion was seen with the irrelevant CXCR1 antibody (Figure 4.31 and 4.32).

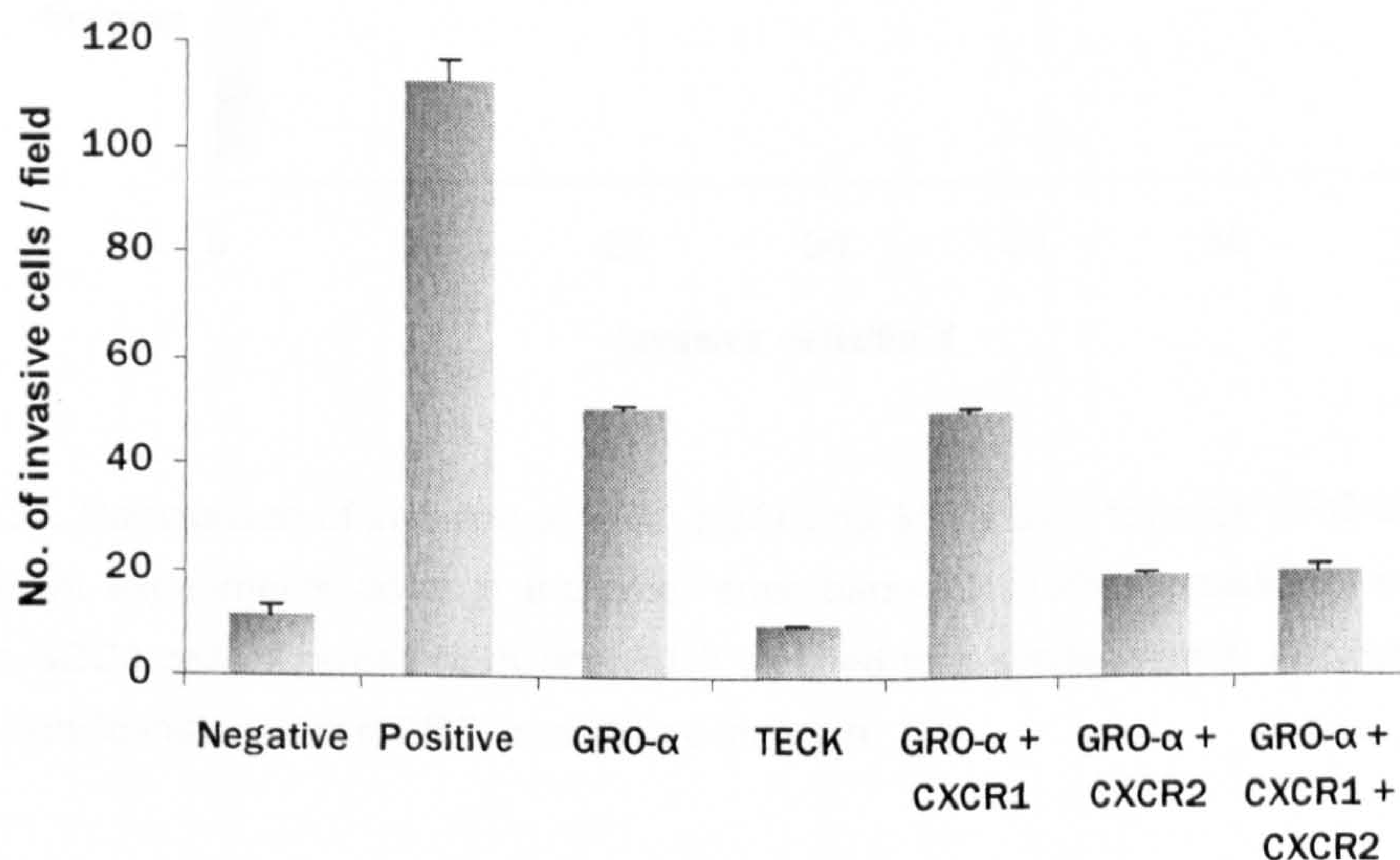


Figure 4.32. Invasion of SCC4 cells towards GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD) (where positive= 10% FBS, TECK= irrelevant chemokine, CXCR1= irrelevant antibody). GRO-α caused a significant increase in invasion of NOK and this effect was significantly reduced when CXCR2 antibody was added. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect.

iii) Comparison of CXCR2/GRO-α mediated invasion between NOK and OCCL

More SCC4 cells were invasive towards GRO-α than H357 ($p<0.00001$) whereas the number of invasive H357 cells was slightly higher than NOK ($p<0.01$) (Figure 4.33).

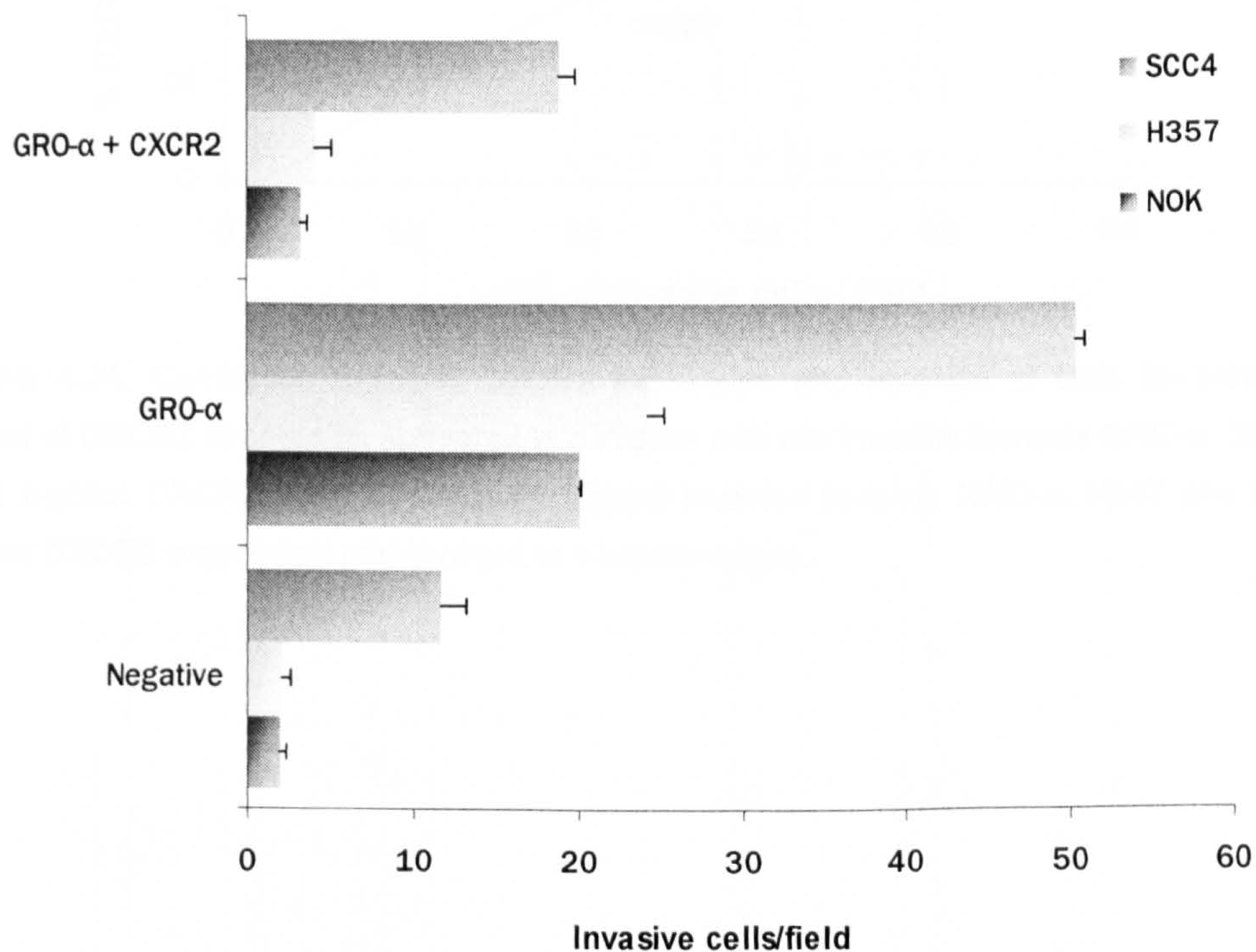


Figure 4.33. Comparison of invasion of NOK, H357 and SCC4 cells towards GRO-α (n=average of 3 different experiments done in triplicate, error bars=SD). GRO-α mediated invasion was highest in SCC4 cells whereas NOK and H357 invaded to a similar extent. Addition of CXCR2 antibody significantly reduced GRO-α mediated invasion.

NOK and H357 cells exhibited identical CXCR2 expression but more H357 cells invaded towards GRO-α than NOK. The relationship between percentage of CXCR2 positive cells and number of invasive cells towards GRO-α is shown in Figure 4.34.

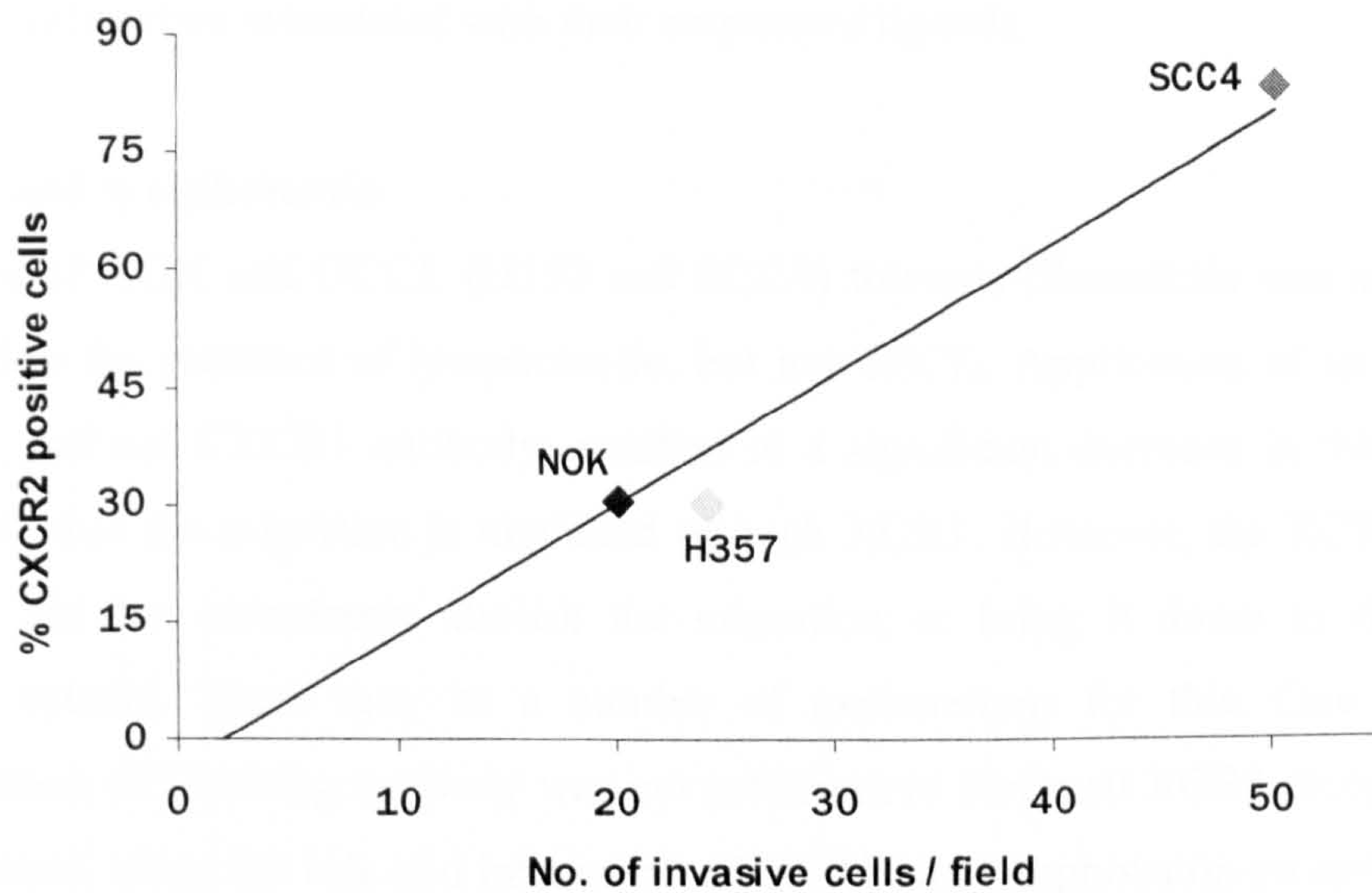


Figure 4.34. Correlation between CXCR2 expression and invasion of cells towards GRO-α. Extent of CXCR2 expression appeared to correlate with the invasion towards GRO-α. SCC4 cells (with highest CXCR2 positivity) showed highest invasion towards GRO-α. H357 and NOK had similar CXCR2 expression and invaded to a similar extent.

4.6 DISCUSSION

Migration and invasion assays were performed to study the role of XCR1, CXCR1 and CXCR2 in oral epithelial cell chemotaxis and invasion. The results suggest that XCR1, CXCR1 and CXCR2 may mediate migration and invasion of normal and cancerous oral epithelial cells when stimulated with their respective ligands.

i) XCR1 and lymphotactin

Migration of NOK and OCCL (H357 and SCC4) towards fibronectin was significantly enhanced in the presence of lymphotactin, but not TECK. Application of an anti-XCR1 antibody, but not CXCR1 antibody, resulted in a significant decrease in this migration suggesting that the migration is mediated through XCR1. However, the XCR1 blocking antibody did not completely abolish the migration or bring it down to the level of negative control. There may be a number of explanations for this. One is that the concentration of blocking antibody was not sufficient to block all XCR1 receptors on the cells. Second, since the role and interaction of XCR1 and lymphotactin on epithelial cells are not yet fully characterized, it is a possibility that lymphotactin may be signaling through a receptor not yet identified. However, migration of cells towards lymphotactin appeared proportional to the number of XCR1 expressing cells in the different cell populations used. Significantly more SCC4 cells (>90% XCR1 positive) migrated towards lymphotactin compared with H357 (60% XCR1 positive) whereas NOK (30% XCR1 positive) were the least migratory. This shows that a relationship exists between the number of XCR1 positive cells and the migratory capability towards lymphotactin and further suggests that lymphotactin mediates its effects through XCR1.

Similar results were obtained for invasion assays. Exposure to lymphotactin caused a significant and specific increase in invasion of H357 and SCC4 cells, since no response to TECK was seen. Invasion of a few NOK towards lymphotactin was also observed. Administration of an XCR1 antibody significantly reduced this invasion to a much greater extent than migration but still failed to bring it back to control levels. No response was seen to the irrelevant CXCR1 antibody or TECK. Like migration, a direct relationship between the number of XCR1 positive cells and the number of invasive cells

was observed as significantly more SCC4 cells invaded towards lymphotactin than H357 followed by NOK. Keratinocytes are known to migrate and invade *in vivo* during wound healing and a previous study has shown invasion of normal human keratinocytes *in vitro* (Livant *et al.*, 2000). However, this group did not describe the location of origin of the keratinocytes used in their study.

To summarize, these results show that XCR1 is functional on the surface of oral epithelial cells and these cells respond to lymphotactin by migrating and invading. The significance of lymphotactin's role in oral epithelial cell migration and invasion and its potential implications will be discussed later in Chapter 9.

ii) CXCR1, CXCR2 and IL-8

Migration of NOK and OCCL was significantly enhanced in the presence of IL-8. Addition of anti-CXCR1 or -CXCR2 antibody on its own reduced migration to a similar extent but no further decrease was observed when a combination of both antibodies was used. These findings are in agreement with those of Watanabe *et al.*, who studied the migration of oral cancer cell lines NA and HSC-4 towards IL-8 and reported a significant increase in migration of cells in the presence of IL-8 (Watanabe *et al.*, 2002). This group also used CXCR1 and CXCR2 blocking antibodies in their migration assays and reported that almost an identical reduction was observed with CXCR1 or CXCR2 antibody and only partial inhibition of migration could be achieved. Our results suggest that migration in response to IL-8 is mediated through both CXCR1 and CXCR2.

Comparison of the three cell types indicated a direct relationship between overall number of receptor positive cells and the chemotactic response. More SCC4 cells than H357 migrated towards IL-8 whereas NOK were the least migratory. A direct relationship between the percentage positive CXCR1 population and the percentage increase in migration of NOK, H357 and SCC4 cells was observed. However, the same was not true for CXCR2. Although approximately 30% of NOK and H357 are positive for CXCR2,

the percentage increase in migration was higher for H357 cells (45%) than NOK (35%). This may be explained by Figure 4.35.

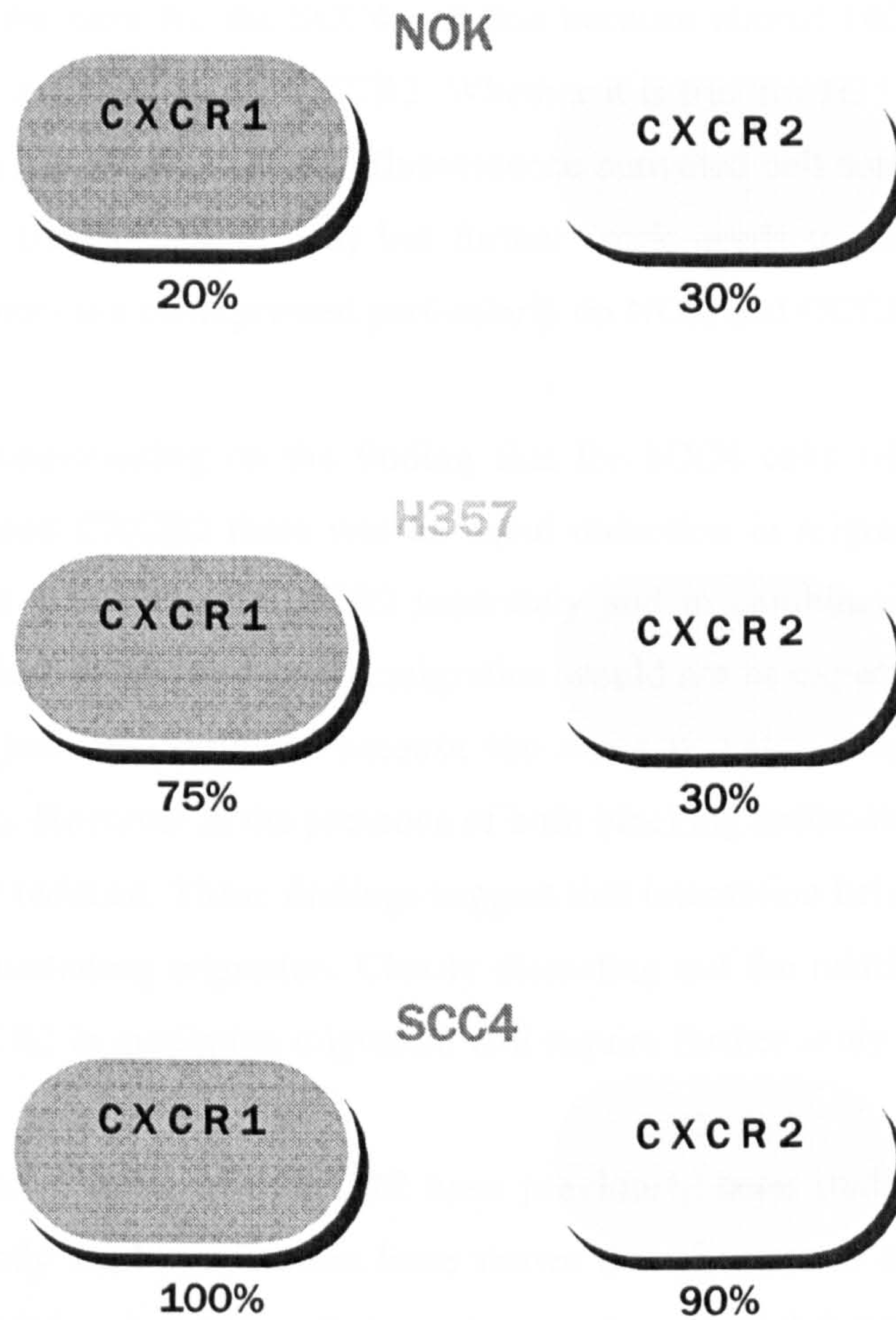


Figure 4.35. Percentage positivity for CXCR1 and CXCR2 in NOK, H357 and SCC4 cells. 20% of NOK are positive for CXCR1 and 30% for CXCR2. For H357 cells, 75% and 30% are positive for CXCR1 and CXCR2 respectively. Whereas for SCC4 cells, 100% and 90% are positive for CXCR1 and CXCR2 respectively.

Since more H357 cells are positive for CXCR1 than NOK (75% compared with 20% for NOK) CXCR1 might play a more important role than CXCR2 in these experiments as more CXCR1 receptors will be available to mediate the effects of IL-8.

As mentioned earlier, no further reduction in migration was observed when CXCR1 and CXCR2 antibodies were used in combination with any of the cells. This suggests that CXCR1 and CXCR2 may be co-expressed on the same cells rather than on separate cells. This is certainly the case for the SCC4 cell line because almost 100% are positive for CXCR1 and 90% are positive for CXCR2. Whether it is true for H357 and NOK cells is not clear. Preliminary FACS analysis (fluorescence activated cell sorting) suggested this may be the case (results not shown) but further work needs to be done to establish whether the receptors are co-expressed particularly on NOK and OCCL.

It is also worth commenting on the finding that for SCC4 cells which almost all co-express CXCR1 and CXCR2 there was an equal reduction in migration in response to antibodies against CXCR1 and CXCR2 separately and in combination. If migration is induced through both receptors equally, migration would not be expected to be reduced in the presence of just one antibody because the other receptor would be available to mediate migration. However in the presence of both blocking antibodies migration should have been greatly reduced. These findings suggest that interaction between receptors may be important in mediating migration. Clearly dissecting out the relative contributions of CXCR1 and CXCR2 in mediating migration will require further study.

Individual roles of CXCR1 and CXCR2 have previously been studied in IL-8 induced migration. Antibody inhibition studies have shown that chemotaxis of epithelial cells in colon carcinoma and endothelial cells in melanoma is mediated through CXCR1 (Bates, DeLeo, III, and Mercurio 2004; Ramjeesingh, Leung, and Siu 2003) as CXCR1 blocking antibody caused a much greater reduction in migration than CXCR2. However, higher CXCR1 expression was exhibited by cells in both cases after stimulation. This suggests that the greater reduction in migration with CXCR1 antibody may only be due to greater expression of CXCR1 compared with CXCR2. In contrast, migration of human vein umbilical endothelial cells (HUVEC) and human dermal microvascular endothelial cells (HMEC) towards IL-8 is mediated through CXCR2 (Li *et al.*, 2005). This group did not study the cell surface expression of CXCR1 or CXCR2 on HMECs and therefore it is possible that more HMECs are positive for CXCR2 than CXCR1. Colonic epithelial cell

lines CaCo-2 and HT-29 show very weak expression of CXCR2 compared with CXCR1 and their migration in chemotaxis/wound assays is mediated through CXCR1 (Sturm *et al.*, 2005). They also reported that addition of a CXCR1 antibody significantly reduced migration towards IL-8 but even extensive doses of CXCR1 do not block the migration completely. This is similar to our findings as addition of both antibodies individually or in combination failed to reduce migration to the control level. It is also possible that IL-8 may have the potential to act through other pathways.

IL-8 also stimulated invasion of NOK, H357 and SCC4 cells. Invasion was decreased after addition of CXCR1 or CXCR2 antibody individually and in combination. This suggests that like migration, invasion may be mediated through both receptors. A direct relationship between the percentage positive CXCR1 cells and number of invasive cells was seen in NOK, H357 and SCC4 cells. The same was not true for CXCR2. Even though similar proportions of NOK and H357 are positive for CXCR2 (30%) a much higher number of H357 cells than NOK invaded towards IL-8. As discussed above this may be explained by the much higher percentage of CXCR1-positive than CXCR2-postive H357 cells.

A different effect of the blocking antibodies on invasion of H357 cells compared with the SCC4 cells and NOK was seen. Interestingly a greater reduction in invasion occurred with antibodies against CXCR1 than CXCR2 and addition of both antibodies in combination significantly reduced invasion compared with CXCR2 but not CXCR1. This may be explained by the fact that more H357 cells are positive for CXCR1 than CXCR2 (see above). However such an effect was not seen with antibodies against CXCR1 and CXCR2 on the migration of H357 cells. Clearly the difference in the percentage of CXCR1 and CXCR2 positive cells is not the only explanation. A further difference between the effects of blocking antibodies on migration and invasion was observed as antibodies were more effective in invasion than migration assays and reduced invasion almost to control levels. One possible explanation for this could be the design of the two experiments. The migration assays were less sensitive than invasion assays as a large number of cells migrated towards fibronectin even in the absence of the chemokines. In

invasion assays, fibronectin was not employed and cells (serum starved overnight) were allowed to invade only towards the chemokines for 22 hours. Therefore the improved blocking efficiency in invasion assays may just be due to greater sensitivity of the invasion assays.

A role for IL-8 in cancer cell migration and invasion has previously been reported by a number of groups. IL-8 facilitates chemotaxis and invasion in a number of cancer cells including ovarian (So *et al.*, 2004), breast (Kim *et al.*, 2005; Yao *et al.*, 2007; Youngs *et al.*, 1997) colon (Li, Varney, and Singh 2001), gastric (Kitadai *et al.*, 2000) pancreatic (Kuwada *et al.*, 2003) and hepatocellular cancer (Kubo *et al.*, 2005). All these findings suggest that both CXCR1 and CXCR2 can facilitate migration or invasion in response to IL-8. The possible implications of the interaction between CXCR1, CXCR2 and IL-8 in oral epithelial cell behaviour will be discussed later.

iii) CXCR2 and GRO-α

GRO-α was used for comparison with IL-8 in migration and invasion assays as it only signals through CXCR2 and not CXCR1. Migration and invasion of cells was significantly enhanced in the presence of GRO-α. Use of an anti-CXCR2 antibody but not CXCR1 antibody resulted in a significant decrease in migration. When antibodies against CXCR1 and CXCR2 were used in combination no further decrease in migration was observed compared with CXCR2 which is in agreement with the literature as GRO-α signals through CXCR2 and not through CXCR1. A role for GRO-α in epithelial cell migration and invasion has been shown in uveal melanoma (Woodward *et al.*, 2002), colon carcinoma (Li, Varney, and Singh 2004) and breast cancer cell lines (Li and Sidell 2005; Youngs *et al.*, 1997). However, the role of GRO-α in oral epithelial migration or invasion has not been reported to date. The above mentioned findings suggest that GRO-α can facilitate migration and invasion of oral epithelial cells and its effects are mediated principally through CXCR2.

To summarize, these results show that IL-8 is able to stimulate migration and invasion of both normal and malignant epithelial cells and these effects appear to be mediated by

both CXCR1 and CXCR2. The relative contribution of these receptors appears to depend on the proportion of receptor positive cells and differs between cell types. The response of the SCC4 and H357 cell lines to IL-8 is greater than that of NOK and this also appears to relate to the percentage of positive cells. There is a suggestion that interaction between receptors may be important at least in the migration and invasion of SCC4 cells.

GRO-α also facilitates chemotaxis and invasion of normal and cancerous oral epithelial cells. Higher migration and invasion are observed in OCCL compared with NOK and GRO-α appears to mediate these effects through CXCR2.

The implications of these findings in the regulation of oral epithelial cell behaviour *in vivo* will be discussed in detail in Chapter 9.

CHAPTER 5

Role of XCR1, CXCR1 and CXCR2 in Oral Epithelial Cell Proliferation

5.1 INTRODUCTION

Previous chapters have shown that in addition to facilitating cell migration and invasion, lymphotactin, IL-8 and GRO-α can also activate the ERK1/2 signaling cascade in oral epithelial cells and this may be mediated through their respective receptors XCR1, CXCR1 and CXCR2. The ERK1/2 pathway plays an important role in a range of physiological and pathological processes. A strong association between ERK1/2 activation and proliferation of cells has also been reported (Barbieri *et al.*, 2006; Luppi *et al.*, 2006). Therefore, the next parameter to establish functional expression of XCR1, CXCR1 and CXCR2 was to study their role in oral epithelial cell proliferation.

5.2 AIM

The aim of this chapter was to establish the role of the chemokine receptors XCR1, CXCR1 and CXCR2 (and their ligands) in the proliferation of normal and malignant oral epithelial cells.

5.3 EXPERIMENTAL PROTOCOL

- NOK and OCCL (H357 and SCC4) were cultured in 96 well tissue culture plates to study proliferation in response to lymphotactin, IL-8 and GRO-α.
- Proliferation assays were performed using CellTitre 96® aqueous one solution cell proliferation reagent (Promega).
- Negative controls did not receive any of the chemokines whereas TECK/CCL25 was used as an irrelevant control chemokine.
- CXCR1 was used as an irrelevant antibody for lymphotactin and GRO-α whereas XCR1 was used for IL-8.
- Triplicate wells for each treatment and cell type were used in every experiment. All proliferation assays were performed three times.

5.4 MATERIALS AND METHODS

5.4.1 CELL CULTURE

Cells were grown and maintained as described previously.

5.4.2 PROLIFERATION ASSAY

The CellTitre 96® aqueous one solution cell proliferation reagent (Promega) was used to study proliferation. It contains the MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethophenyl)-2- (4-sulfophenyl)- 2H- tetrazolium. MTS is bio-reduced by cells into a coloured formazan product by NADPH or NADH produced by enzymes in metabolically active cells thus providing an indirect account of the number of cells.

Cells were detached using cell dissociation buffer (Sigma) and seeded at a density of $2x10^3$/well in 96 well plates (Corning). Cells were allowed to adhere overnight, medium was aspirated and cells washed with PBS. Lymphotactin (R&D Systems), IL-8 (Sigma) and GRO-α (Peprotech) were diluted to a concentration of 100ng/ml in serum-free medium (Appendix 10.1.5) and cells were incubated for 72h. In addition to the ligands, antibodies against XCR1, CXCR1 and CXCR2 were also added to some wells to study the individual role of these receptors in lymphotactin-, IL-8- and GRO-α-mediated cell proliferation. For receptor blocking, cells were incubated with antibodies against XCR1, CXCR1 and CXCR2 (20μg/ml) in serum-free medium at 37°Cfor at least 1h before addition of the chemokines. Absence of ligands served as the negative control whereas keratinocyte growth medium (KGM) (Appendix 10.1) was used as the positive control. All treatments were performed in triplicate wells.

After 72 hours, 20μl of MTS was added to each well and incubated in a CO_2 incubator for 30-60 min. Absorbance was recorded at 492nm using a spectrophotometer and plotted against the concentration of the chemokines. A standard curve for each assay determined the number of cells.

5.4.3 STATISTICAL ANALYSIS

Averages and standard deviations were calculated from the obtained values of all samples and paired Student's T-test was used to analyze the significance of cell proliferation in response to lymphotactin, IL-8 and GRO-α compared with negative control. Data was expressed as increase in cell number relative to unstimulated cells. A p-value of less than 0.05 was considered significant.

5.5 RESULTS

5.5.1 Role of XCR1/lymphotactin in proliferation

i) NOK

Keratinocyte growth medium (KGM) (used as positive control) caused a highly significant increase in proliferation of NOK ($p<0.0001$). Stimulation of NOK with lymphotactin but not TECK caused a significant increase in proliferation compared with the negative control ($p<0.01$). Addition of XCR1 antibody significantly diminished this lymphotactin-mediated proliferation ($p<0.01$) but failed to bring it back to the negative control level. No reduction in proliferation was seen with the irrelevant antibody CXCR1 (Figure 5.1).

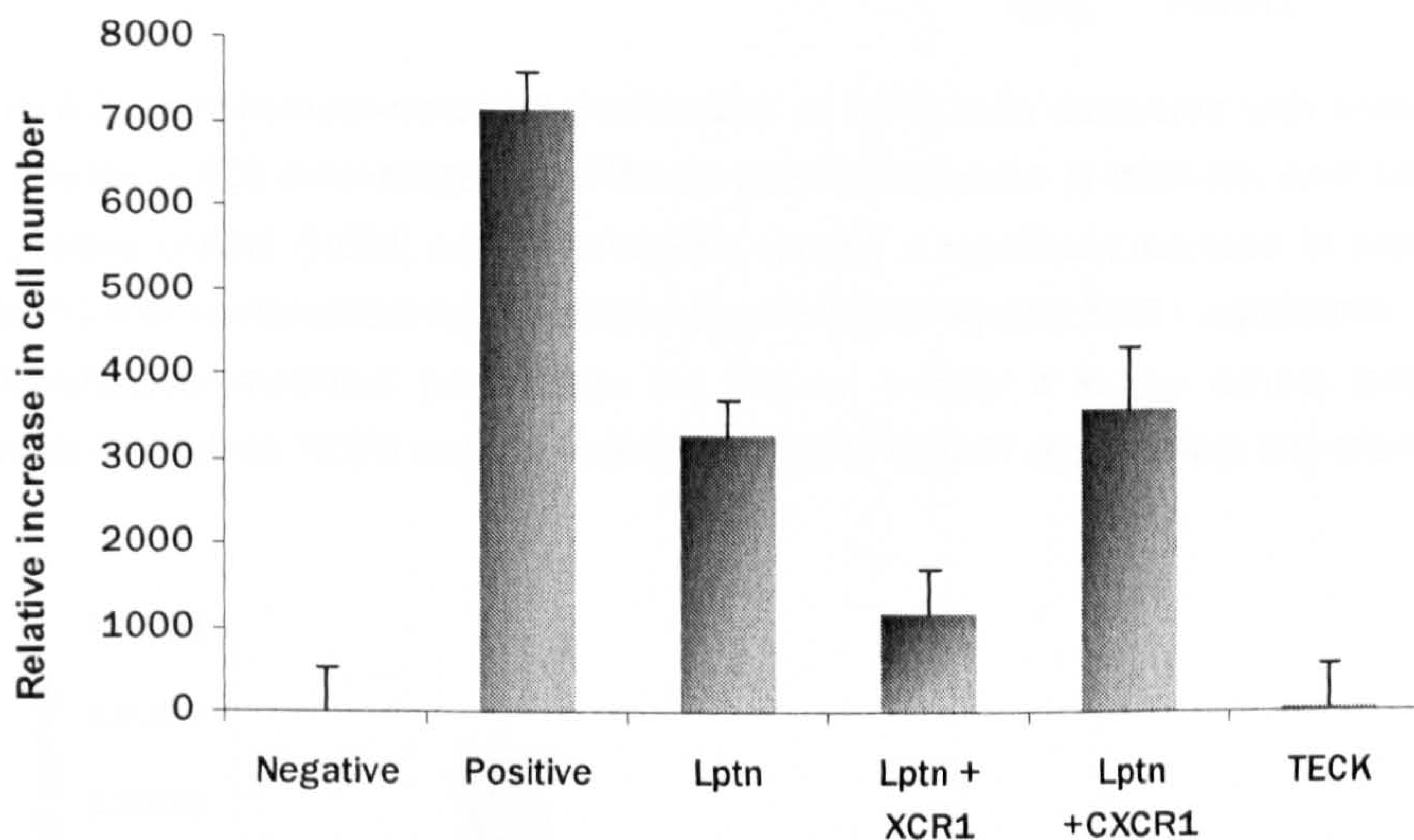


Figure 5.1. Lymphotactin-mediated proliferation of NOK compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and lymphotactin caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against XCR1 significantly reduced the lymphotactin-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

ii) H357 and SCC4 cells

Exposure to KGM significantly increased proliferation of OCCL (H357 and SCC4 cells) ($p<0.001$ and $p<0.001$ respectively). Lymphotactin also increased proliferation of H357 and SCC4 cells ($p<0.001$ and $p<0.001$ respectively) whereas addition of XCR1 antibody significantly reduced lymphotactin mediated proliferation ($p<0.001$

and $p<0.0001$ respectively). The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any effect (Figure 5.2 and 5.3).

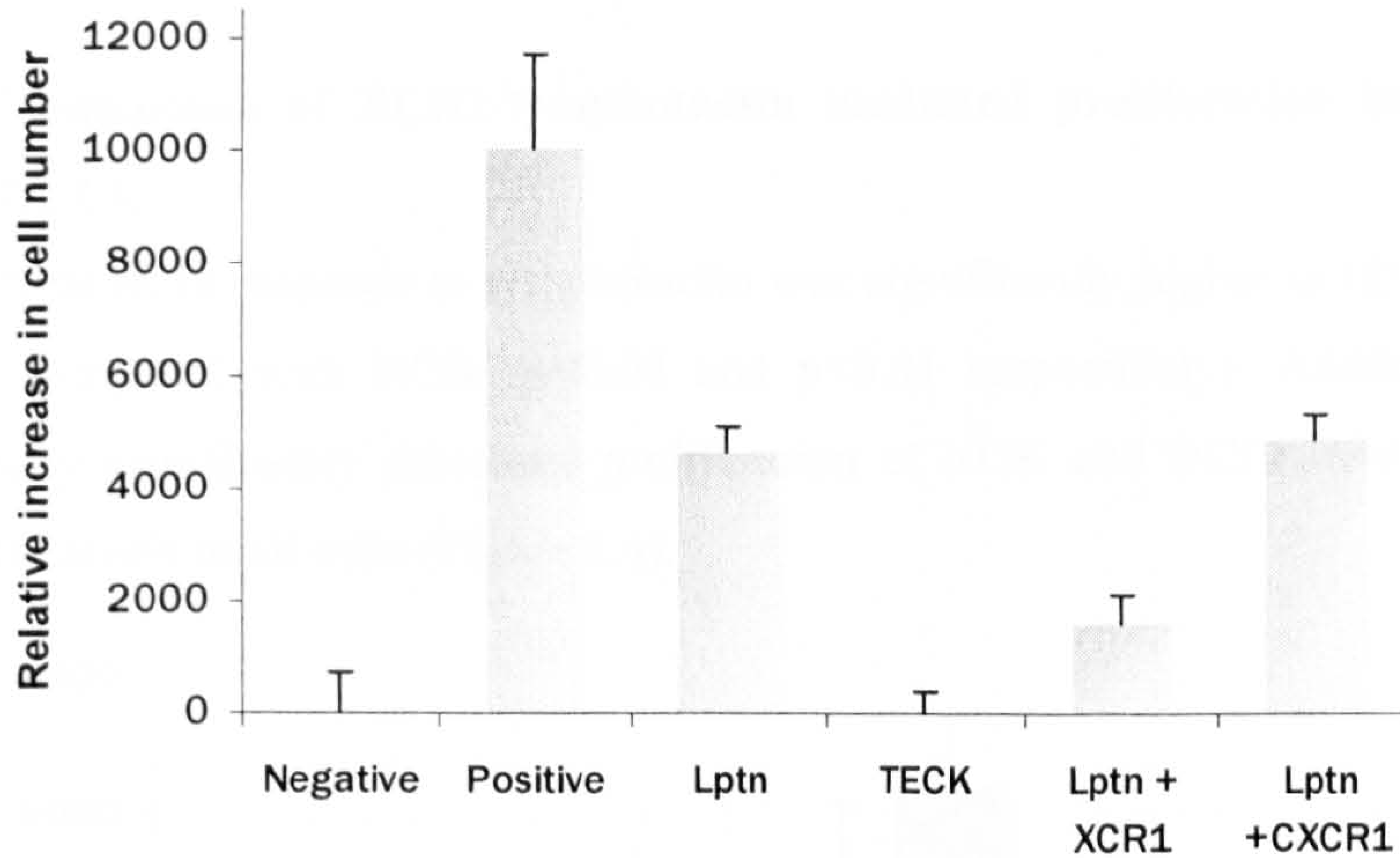


Figure 5.2. Lymphotactin-mediated proliferation of H357 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and lymphotactin caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against XCR1 significantly reduced the lymphotactin-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

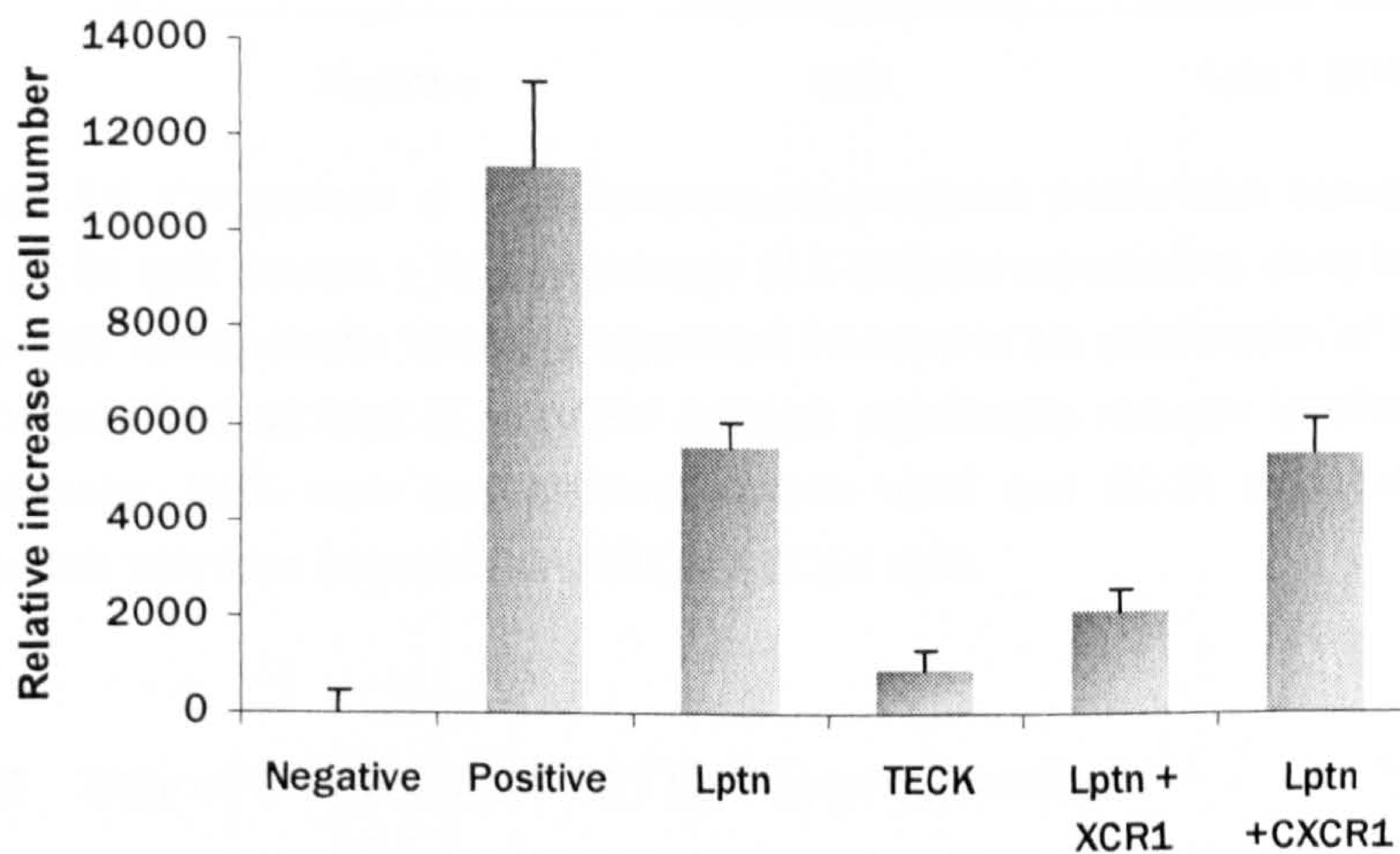


Figure 5.3. Lymphotactin-mediated proliferation of SCC4 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and lymphotactin caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against XCR1 significantly reduced

the lymphotactin-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

iii) Comparison of XCR1/lymphotactin mediated proliferation between NOK and OCCL

Proliferation in response to lymphotactin was significantly higher in H357 and SCC4 cells compared with NOK ($p<0.05$ and $p<0.01$ respectively). Addition of XCR1 antibody significantly decreased proliferation of NOK and OCCL and reduced it to similar levels in all cells (Figure 5.4).

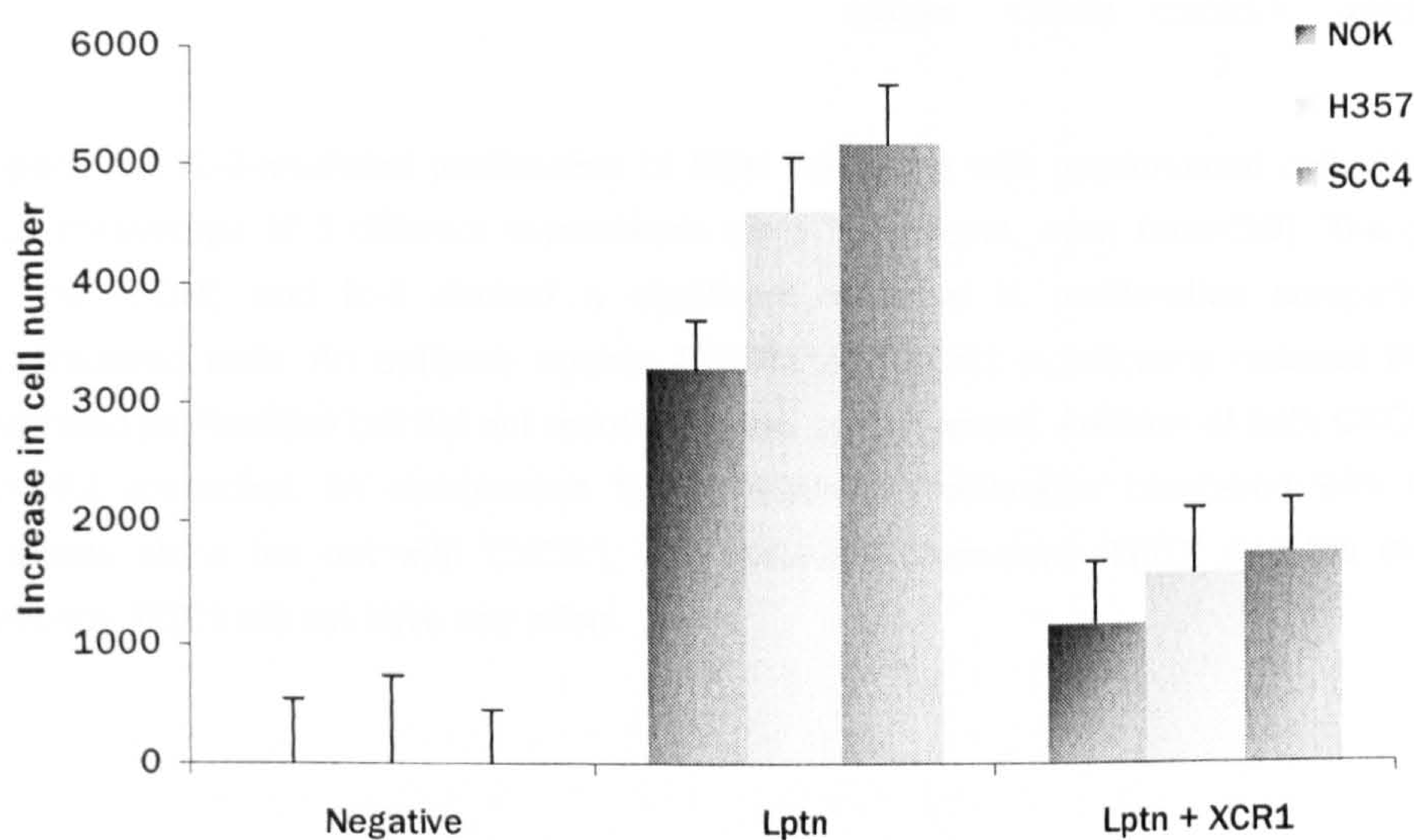


Figure 5.4. Comparison of XCR1/lymphotactin-mediated proliferation between NOK, H357 and SCC4 cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). Lymphotactin caused a significant increase in the proliferation of NOK, H357 and SCC4 cells and addition of an XCR1 antibody significantly reduced lymphotactin-mediated proliferation. NOK were less proliferative than H357 and SCC4 cells but no significant difference was seen between the H357 and SCC4 cells.

5.5.2 Role of CXCR1, CXCR2 / IL-8 in proliferation

i) NOK

Proliferation of NOK was significantly increased after stimulation with KGM and IL-8 ($p<0.0001$ and $p<0.001$ respectively). Addition of CXCR1 or CXCR2 antibody significantly decreased proliferation ($p<0.01$ and $p<0.01$ respectively) but did not reduce it to the control level (Figure 5.5).

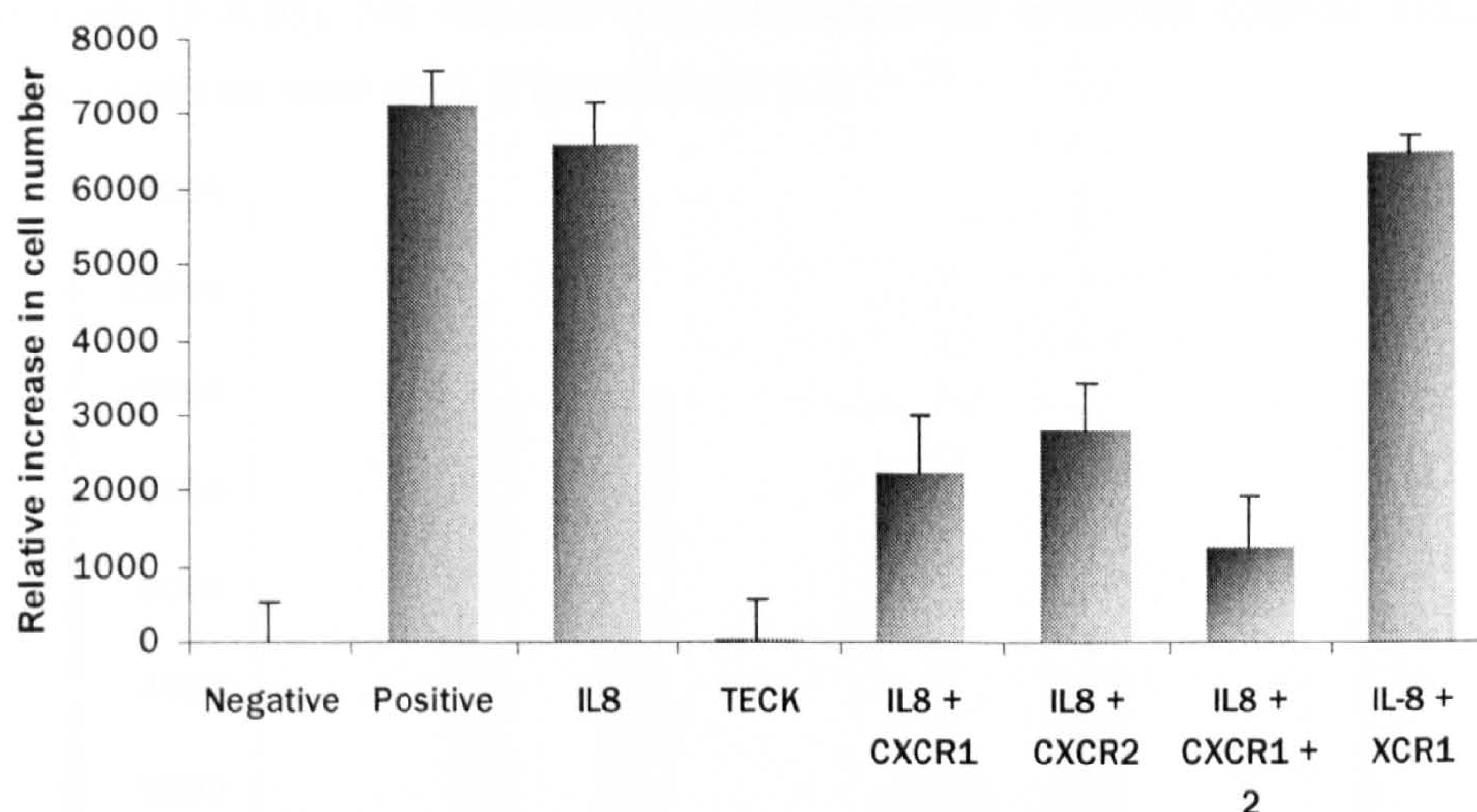


Figure 5.5. IL-8-mediated proliferation of NOK compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and IL-8 caused a significant increase in proliferation compared with unstimulated cells. An antibody against CXCR1 or CXCR2 significantly reduced the IL-8-mediated proliferation but did not reduce it to the control levels. Addition of both CXCR1 and CXCR2 antibodies on combination further reduced proliferation compared with CXCR2 antibody alone but not with CXCR1. The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any affect.

When both CXCR1 and CXCR2 antibodies were used in combination, a further decrease in NOK proliferation was observed compared with CXCR2 alone ($p<0.05$) but not with CXCR1. The irrelevant chemokine TECK and CXCR1 antibody did not have any influence on proliferation (Figure 5.5).

ii) H357 and SCC4 cells

KGM and IL-8 significantly increased proliferation of H357 ($p<0.001$ and $p<0.01$ respectively) and SCC4 cells ($p<0.001$ and $p<0.0001$ respectively). A significant decrease in IL-8 mediated proliferation of H357 and SCC4 cells was noticed when either CXCR1 or CXCR2 antibody was added ($p<0.05$ and $p<0.001$ respectively). When both antibodies were used in combination, proliferation of H357 and SCC4 cells was decreased further compared with CXCR2 alone, but not with CXCR1

antibody ($p<0.05$). No response was seen when the irrelevant controls TECK or XCR1 antibody were used. (Figure 5.6 and 5.7)

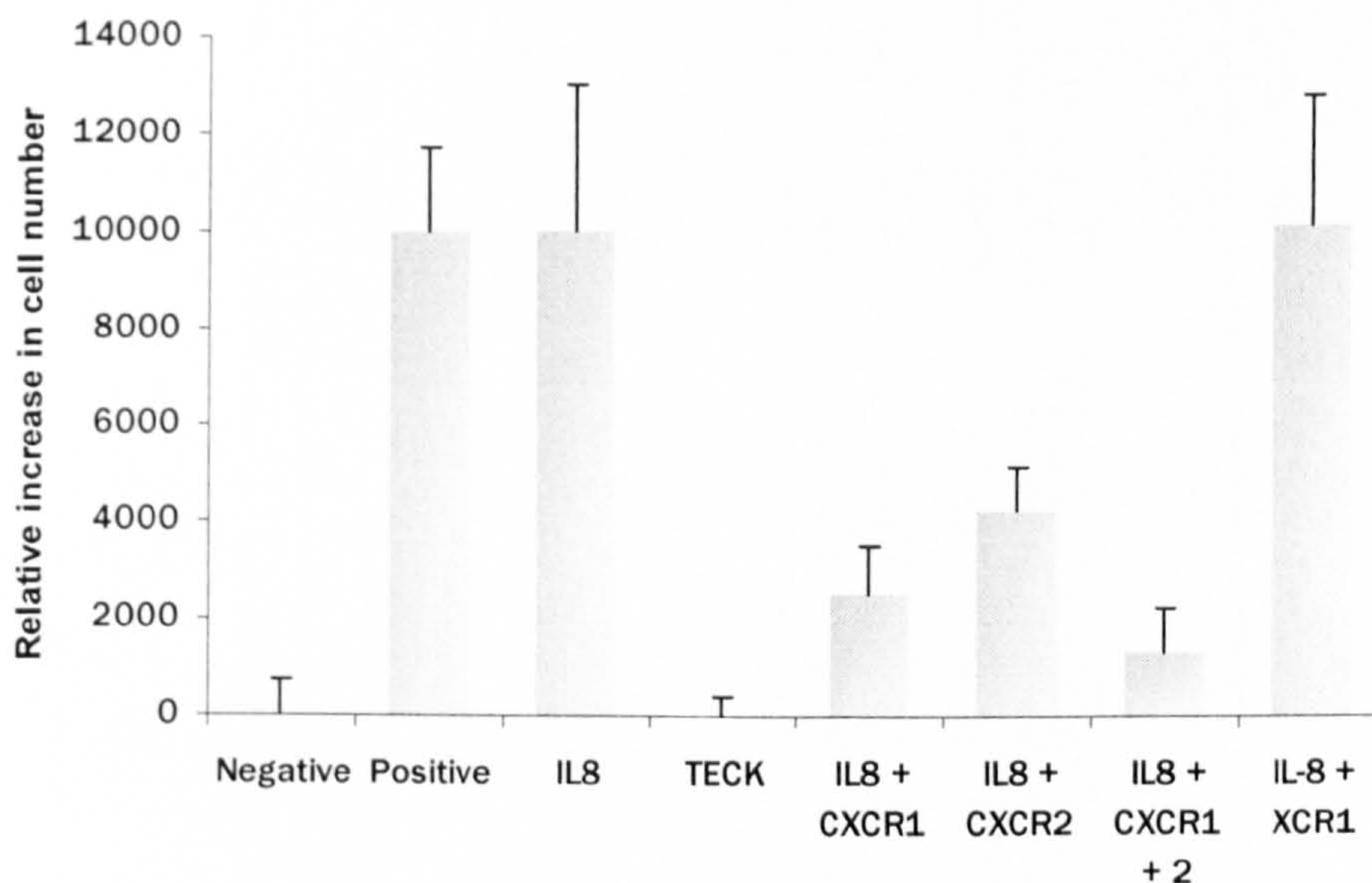


Figure 5.6. IL-8-mediated proliferation of H357 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and IL-8 caused a significant increase in proliferation compared with unstimulated cells. An antibody against CXCR1 or CXCR2 significantly reduced the IL-8-mediated proliferation but did not reduce it to the control levels. Addition of both CXCR1 and CXCR2 antibodies on combination further reduced proliferation compared with CXCR2 antibody alone but not with CXCR1. The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any affect.

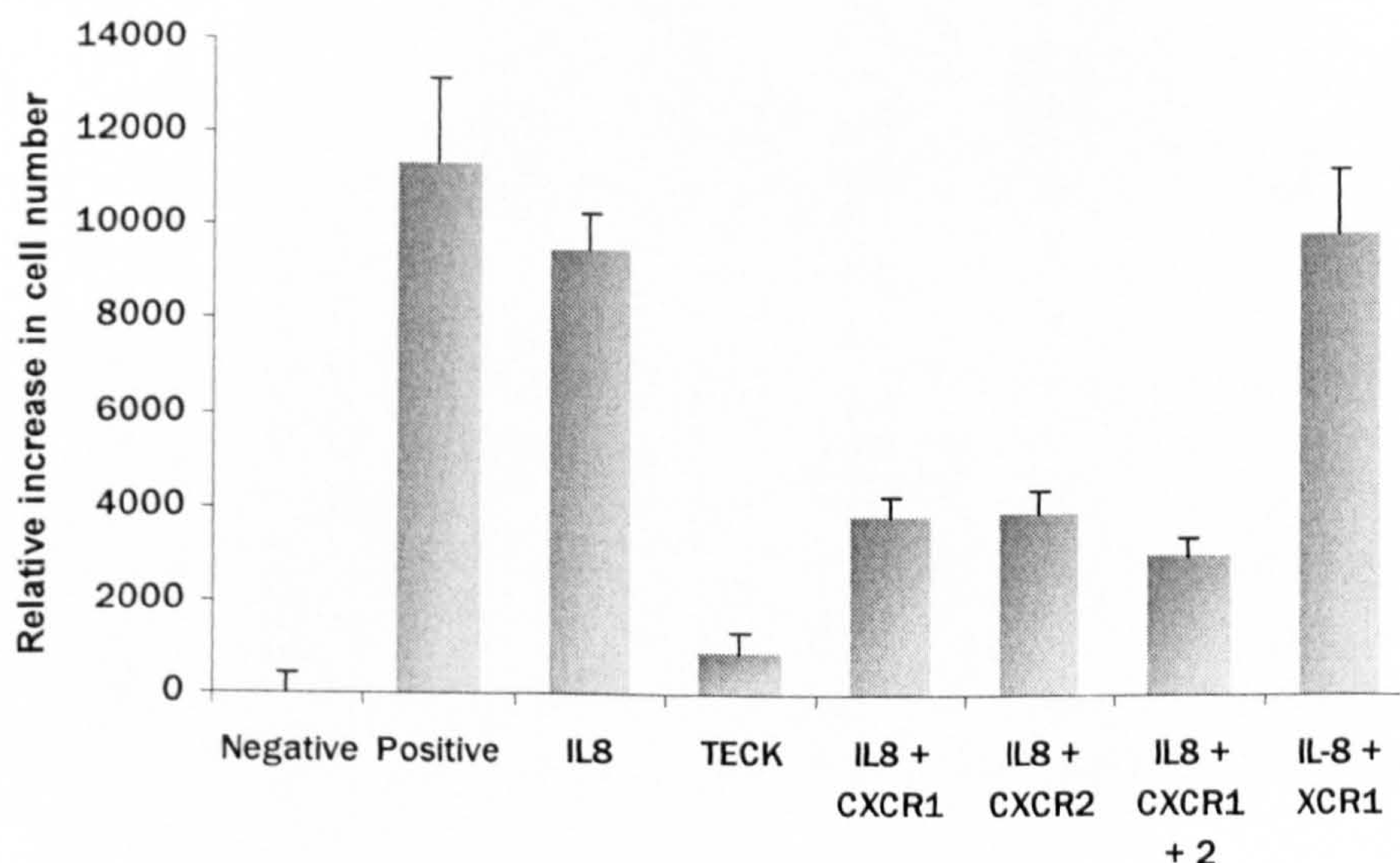


Figure 5.7. IL-8-mediated proliferation of SCC4 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and IL-8 caused a significant increase in proliferation compared with unstimulated cells. An antibody against CXCR1 or CXCR2 significantly reduced the IL-8-mediated proliferation but did not reduce it to the control levels. Addition of both CXCR1 and CXCR2 antibodies on combination further reduced proliferation compared with CXCR2 antibody alone but not with CXCR1. The irrelevant chemokine TECK and the irrelevant antibody XCR1 did not have any affect.

iii) Comparison of CXCR1-CXCR2/IL-8 mediated proliferation between NOK and OCCL

Comparison of NOK and OCCL showed that IL-8 facilitated a significantly higher increase in proliferation of H357 and SCC4 cells compared with NOK ($p<0.05$ and $p<0.01$ respectively) (Figure 5.8).

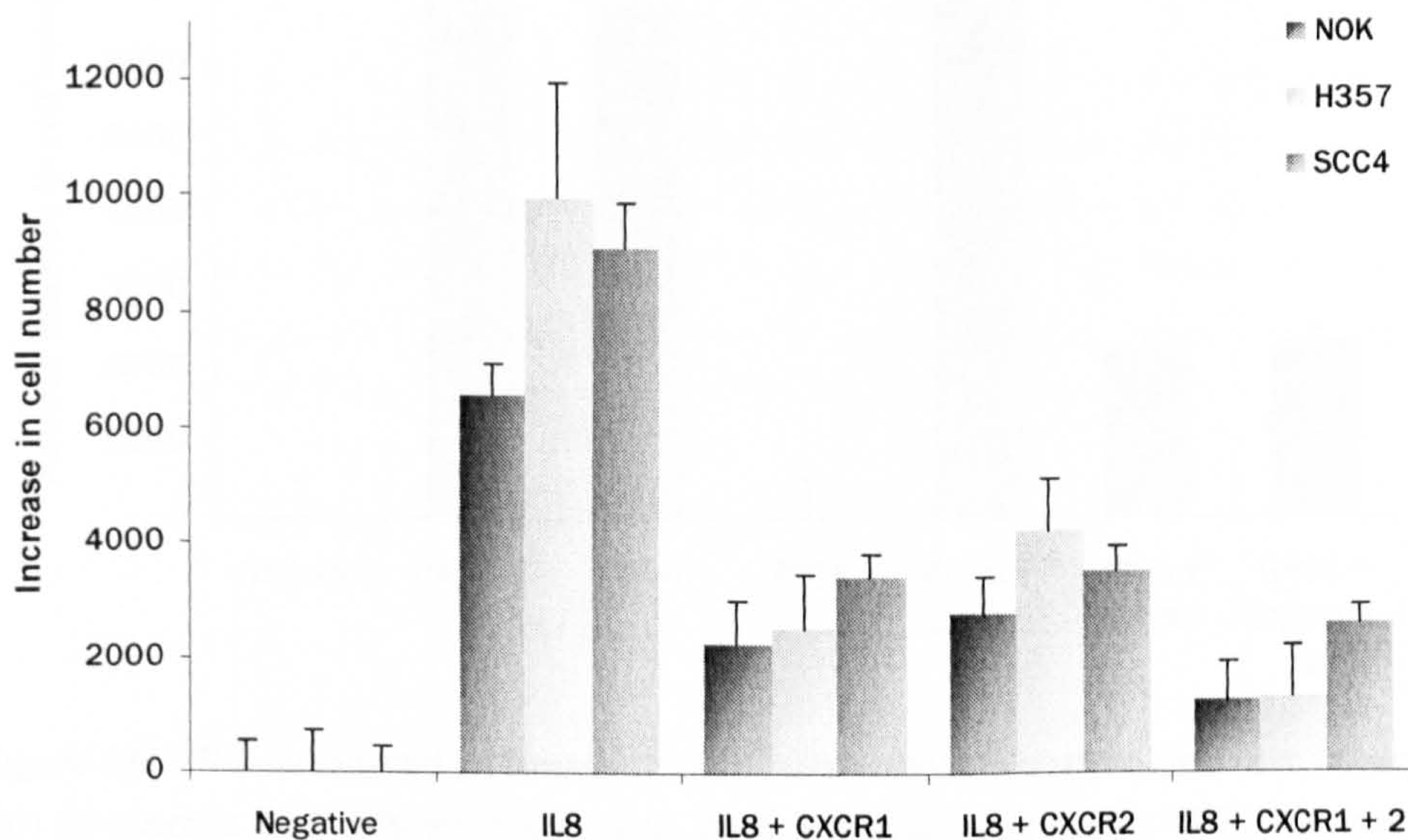


Figure 5.8. Comparison of CXCR1, CXCR2 / IL-8 mediated proliferation (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). IL-8 caused a significant increase in the proliferation of NOK, H357 and SCC4 cells and addition of a CXCR1 or CXCR2 antibody significantly reduced IL-8-mediated proliferation. When both CXCR1 and CXCR2 antibodies were used a further reduction in proliferation was seen compared with CXCR2 but not with CXCR1 antibody. NOK were less proliferative than H357 and SCC4 cells but no significant difference was seen between the H357 and SCC4 cells.

A significant reduction in this proliferation was observed when CXCR1 or CXCR2 antibodies were used individually or in combination (Figure 5.8).

5.5.3 Role of CXCR2 / GRO-α in proliferation

i) NOK

A significant increase in the number of NOK was seen after addition of KGM and GRO-α indicating that it facilitates proliferation of these cells ($p<0.0001$). TECK failed to induce such a response. Addition of CXCR2 antibody significantly decreased proliferation ($p<0.001$) but did not reduce it to the control level. CXCR1 antibody on its own or in combination with CXCR2 did not reduce proliferation (Figure 5.9).

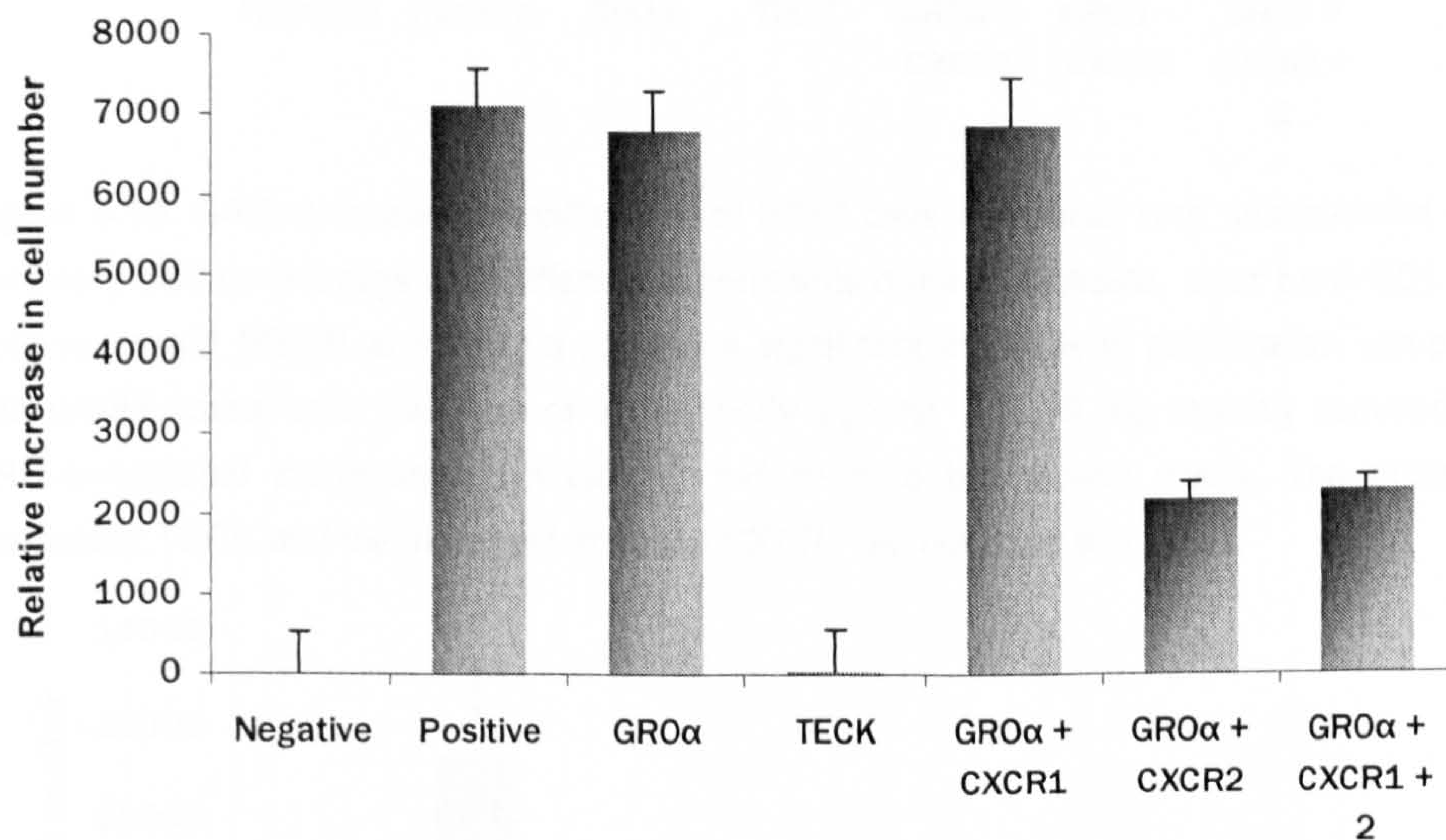


Figure 5.9. GRO-α mediated proliferation of NOK compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and GRO-α caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against CXCR2 significantly reduced the GRO-α-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

ii) H357 and SCC4 cells

H357 and SCC4 cells also showed a significant increase in proliferation after stimulation with KGM ($p<0.001$ and $p<0.001$ respectively) and GRO-α ($p<0.01$ and $p<0.0001$ respectively) compared with negative control. TECK failed to induce

proliferation. The proliferation of H357 and SCC4 cells was significantly reduced when anti-CXCR2 antibody was added ($p<0.001$ and $p<0.001$). No reduction in proliferation was observed with the CXCR1 antibody (Figure 5.10 and 5.11).

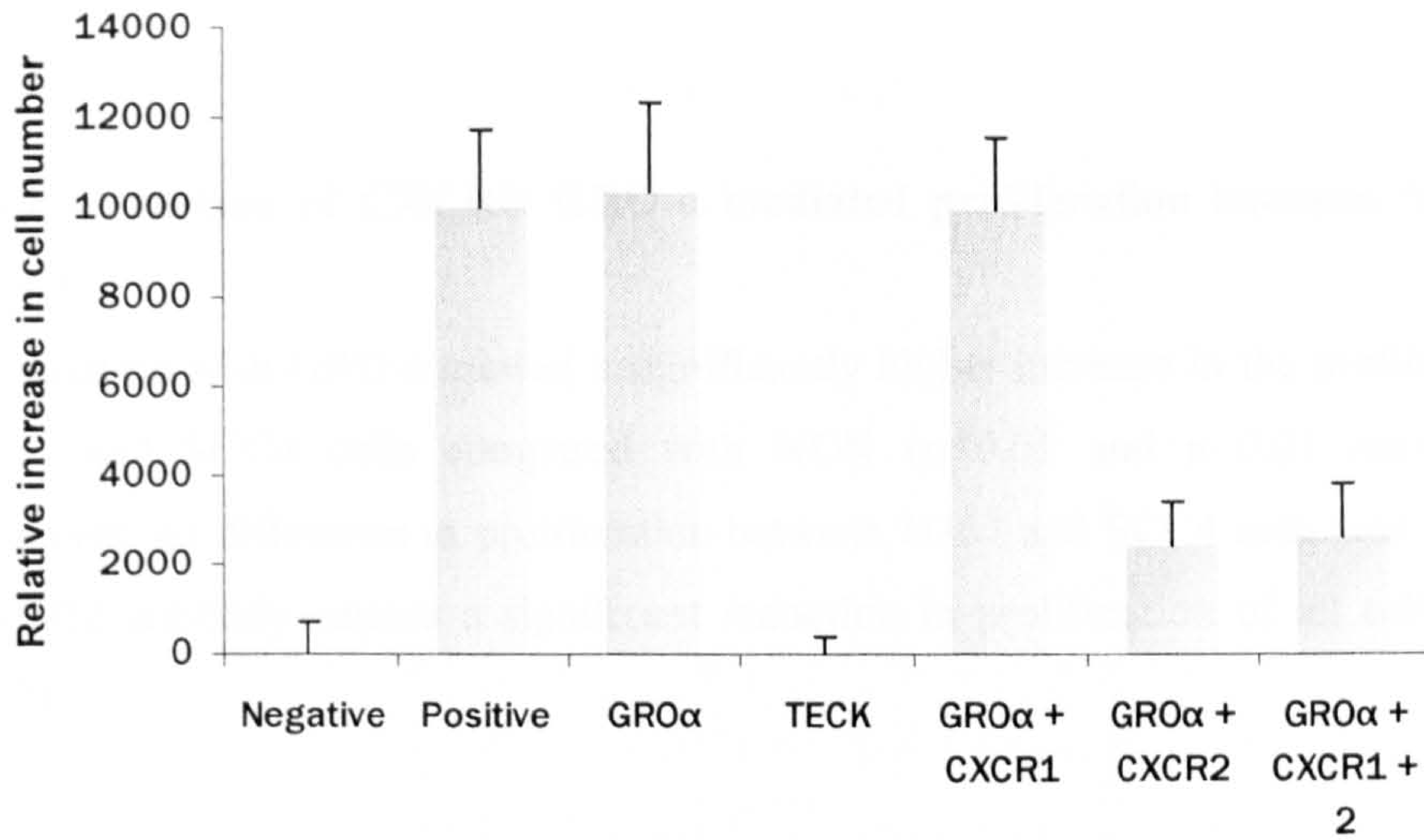


Figure 5.10. GRO-α mediated proliferation of H357 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The positive control (KGM) and GRO-α caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against CXCR2 significantly reduced the GRO-α-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

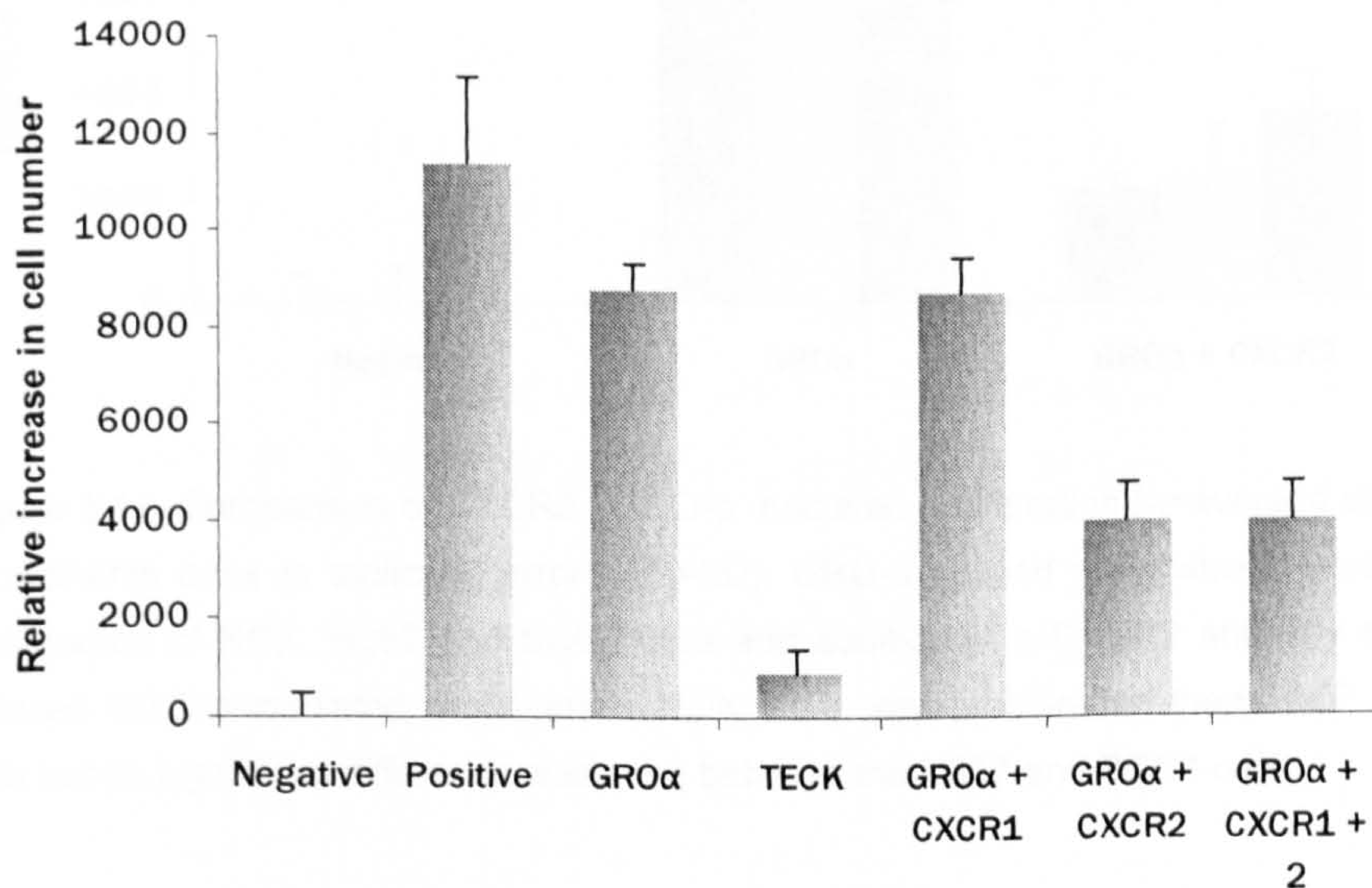


Figure 5.11. GRO-α mediated proliferation of SCC4 cells compared with unstimulated cells (means ± SD) (n=average of 3 different experiments done in triplicate, error bars=SD). The

positive control (KGM) and GRO-α caused a significant increase in proliferation compared with unstimulated cells. Addition of an antibody against CXCR2 significantly reduced the GRO-α-mediated proliferation but did not reduce it to the control levels. The irrelevant chemokine TECK and the irrelevant antibody CXCR1 did not have any affect.

iii) Comparison of CXCR2/ GRO-α mediated proliferation between NOK and OCCL

Stimulation with GRO-α caused a significantly higher increase in the proliferation of H357 and SCC4 cells compared with NOK ($p<0.05$ and $p<0.01$ respectively). However, no difference in proliferation between H357 and SCC4 cells was observed. CXCR2 antibody caused a significant reduction in proliferation of all cells (Figure 5.12).

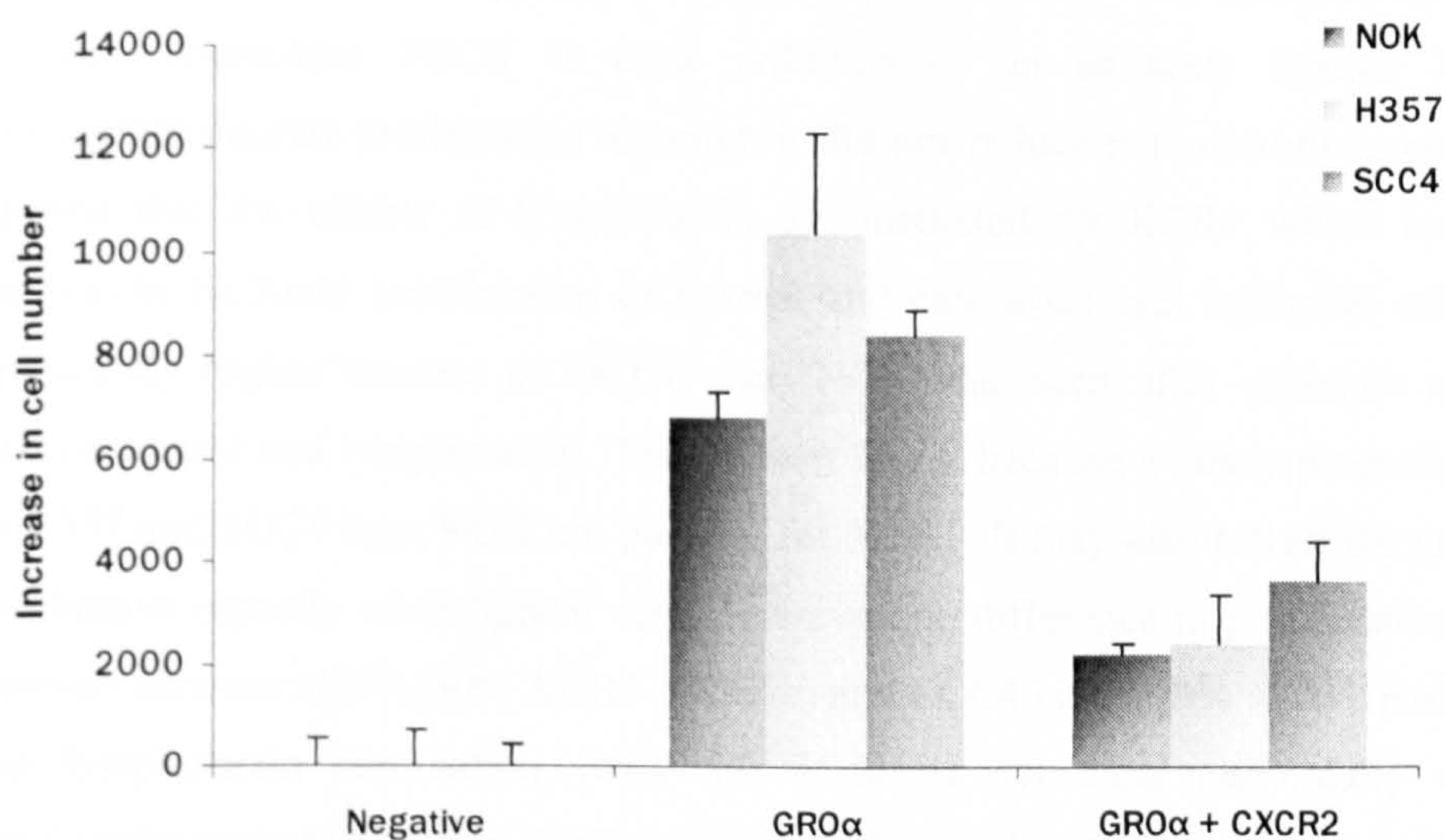


Figure 5.12. Comparison of CXCR2 / GRO-α mediated proliferation (n=average of 3 different experiments done in triplicate, error bars=SD). GRO-α caused a significant increase in the proliferation of NOK, H357 and SCC4 cells and addition of a CXCR2 antibody significantly reduced GRO-α-mediated proliferation. NOK were less proliferative than H357 and SCC4 cells but no significant difference was seen between the H357 and SCC4 cells.

5.6 DISCUSSION

The aim of this chapter was to establish the role, if any of XCR1, CXCR1 and CXCR2 in the proliferation of oral epithelial cells. Exposure to lymphotactin, IL-8 and GRO-α increased proliferation of normal and cancerous cells and this proliferation was significantly decreased after addition of antibodies against their respective receptors. This suggests that XCR1, CXCR1 and CXCR2 mediate proliferation of oral epithelial cells when exposed to their respective ligands.

i) XCR1 and lymphotactin

Lymphotactin has only been reported to stimulate proliferation of CD8+ T lymphocytes to date (Cerdan *et al.*, 2004). The findings from this chapter suggest that lymphotactin can also act as a proliferative agent and trigger the growth of oral epithelial cells. This effect appears specific since no such effect is observed when an irrelevant chemokine TECK is used. Addition of an antibody against XCR1 significantly inhibits proliferation although it did not reduce it to control level. This suggests that the effects of lymphotactin are mediated by XCR1 which has the potential to facilitate proliferation of normal and cancerous oral epithelial cells. A significantly higher number of OCCL than NOK was seen after exposure to the positive control and lymphotactin. This is most likely because a larger proportion of the H357 and SCC4 than NOK are positive for XCR1. It may also reflect the greater proliferative capacity of malignant cells. However, no difference in proliferation was observed between H357 (60% XCR1 positive) and SCC4 cells (99% XCR1 positive) after lymphotactin stimulation. Why this is so is not clear particularly when significantly more SCC4 than H357 migrate and invade in response to lymphotactin. It may be that XCR1 is not as important in proliferation as in migration or invasion.

Although this chapter suggests that lymphotactin mediates both normal and malignant epithelial cell proliferation through XCR1 *in vitro* the significance of these findings *in vivo* is not clear.

ii) CXCR1, CXCR2 and IL-8

Exposure to IL-8 also caused a significant increase in proliferation of normal as well

as cancerous oral cells. However, the proliferative response was greater in OCCL than NOK. Administration of CXCR1 and CXCR2 antibodies individually resulted in a significant reduction in proliferation but failed to reduce it to control levels. This suggests that proliferation is mediated through both CXCR1 and CXCR2. Interestingly a further reduction in proliferation was observed when both CXCR1 and CXCR2 antibodies were used in combination but was only significant compared with CXCR2 and not with CXCR1. This reduction occurred with NOK and both H357 and SCC4 cell lines. These results suggest that for all cells CXCR1 may be more important in mediating the effects of proliferation than CXCR2. This is particularly true of NOK which are only 20% positive for CXCR1 but 30% positive for CXCR2. Although more SCC4 cells are positive for CXCR1 than H357 cells (75% and 90% respectively) no difference in proliferation was observed between the two cell lines. Why this is so is not clear but it may be that the cells were proliferating maximally in the assay conditions and no further increases were possible.

The role of IL-8 in proliferation of oral epithelial cells has also been studied previously. In contrast to our results, Watanabe *et al.*, reported no increase in oral cancer cell proliferation after stimulation with IL-8 (Watanabe *et al.*, 2002). A possible explanation may be that the two oral cancer cell lines (NA and HSC-4) used in their study are different to the ones in ours and there may be heterogeneity in response. An indirect association between IL-8 expression and proliferation in periodontitis has been shown as basal keratinocytes expressing IL-8 *in vivo* are more positive for PCNA/cyclin (a proliferation marker) compared with controls (Jarnbring, Gustafsson, and Klinge 2000) suggesting that IL-8 may facilitate proliferation of oral epithelial cells. However, this group did not study the expression of CXCR1 and CXCR2 on oral epithelial cells and also did not show any direct influence of IL-8 on cell proliferation.

A role for IL-8 in epithelial cell proliferation has been reported by a number of groups. IL-8 induces proliferation of a range of cancer cells *in vitro* including prostate (Araki *et al.*, 2007; Murphy *et al.*, 2005), melanoma (Lazar-Molnar *et al.*, 2000; Varney *et al.*, 2003), lung (Luppi *et al.*, 2006; Zhu *et al.*, 2004), colon (Li, Varney, and Singh 2001), breast (Yao *et al.*, 2007), hepatocellular (Akiba *et al.*, 2001) and epidermoid cancer cells (Metzner *et al.*, 1999). However, other groups have reported

contrasting results as IL-8 does not induce *in vitro* proliferation in hepatocellular (Kubo *et al.*, 2005), pancreatic cancer cells (Kuwada *et al.*, 2003) and intestinal epithelial cells (Sturm *et al.*, 2005). Individual roles of CXCR1 and CXCR2 in IL-8 mediated proliferation have also been studied. Murphy *et al.*, reported that IL-8 induced proliferation in prostate cancer is mediated through CXCR1 and CXCR2 as addition of either antibody leads to significant decrease in proliferation (Murphy *et al.*, 2005). Varney *et al.*, reported similar findings for malignant melanoma (Varney *et al.*, 2003). However, Akari *et al.*, showed that proliferation in response to IL-8 in prostate cancer is mediated through CXCR1 and not CXCR2 (Araki *et al.*, 2007). On the other hand, Metzner *et al.*, showed that IL-8 mediated proliferation in epidermoid carcinoma is mediated through CXCR2 only (Metzner *et al.*, 1999). These differences between our findings and other groups could be explained by the use of different CXCR1 and CXCR2 antibodies in these studies which might have affected the results. Another possibility could be that IL-8 exerts differential effects on different cells and that its signaling through CXCR1 or CXCR2 depends upon the expression of the receptors as some cells are more positive for CXCR1 than CXCR2 and vice versa.

iii) CXCR2 and GRO-α

Addition of GRO-α also significantly increased proliferation of NOK and OCCL. As observed with IL-8, H357 and SCC4 cells showed more proliferation than NOK. 90% of SCC4 cells are positive for CXCR2 compared to 30% for H357. However, no significant difference between the proliferation of H357 and SCC4 cells was observed. This suggests that the cancer cells may possess an intrinsic ability to proliferate more compared to normal cells and their proliferation may be influenced by additional factors. To date, a role for GRO-α in oral epithelial cell proliferation has not been reported. However, it has been shown to mediate cell proliferation in malignant melanoma (Lazar-Molnar *et al.*, 2000), colon carcinoma (Li, Varney, and Singh 2004), epidermoid (Metzner *et al.*, 1999) and oesophageal carcinoma (Wang *et al.*, 2006). GRO-α also stimulates proliferation of skin keratinocytes and plays an important role in wound healing (Rennekampff *et al.*, 1997; Steude, Kulke, and Christophers 2002).

In summary these findings show that IL-8 stimulates proliferation of both NOK and OCCL and CXCR1 may be more important than CXCR2 in mediating these effects.

However CXCR2 is important in mediating the effects of GRO-α on the proliferation of both NOK and OCCL. The proliferation of NOK to both IL-8 and GRO-α is less than the response of the OCCL.

Proliferation is a hall mark of a wide range of physiological and pathological processes (including epithelial cell turnover, wound healing and cancer) and the significance of these findings in these physiological and pathological scenarios will be discussed in Chapter 9.

Although the results on CXCR1 and CXCR2 were interesting it was not feasible to carry on studying all three receptors due to time constraints. Since XCR1 expression on epithelial cells has never been shown before, the results obtained for XCR1 and its ligand lymphotactin are potentially exciting. Therefore, it was decided to focus the study on further exploration of the role of XCR1 and lymphotactin in the regulation on oral epithelial cell behaviour.

CHAPTER 6

In vivo Expression of XCR1 and Lymphotactin

6.1 INTRODUCTION

The results from chapters 2-5 have not only shown that XCR1 is expressed on oral epithelial cells *in vitro* but that it is functional and mediates proliferation, migration and invasion. However, the significance of these findings *in vivo* is not known. For that reason, the next step was to study whether XCR1 is expressed *in vivo* in normal and diseased oral mucosa and furthermore to determine the source of lymphotactin, if any in oral mucosa.

In vivo expression of XCR1 on non-lymphoid cells has been reported only on fibroblast-like synoviocytes in rheumatoid synovium (Blaschke *et al.*, 2003) and recently in melanocytic lesions (Seidl *et al.*, 2007). On the other hand, Lymphotactin expression *in vivo* has only been shown in infiltrating lymphocytes, dendritic cells and mononuclear cells in rheumatoid arthritis and Crohn's disease (Blaschke *et al.*, 2003; Middel *et al.*, 2001) and in mouse epidermal intraepithelial lymphocytes (IEL) (Boismenu *et al.*, 1996). Whether it is produced in the oral mucosa is not known.

6.2 AIM

The aim of this chapter was to study and establish the *in vivo* expression of XCR1 and the source of lymphotactin in normal and diseased oral mucosa.

6.3 EXPERIMENTAL PROTOCOL

- Expression of XCR1 and lymphotactin in normal oral epithelium was subjectively compared with expression in oral cancer. Expression was also studied in dysplasia, lichen planus and ulcers to observe if the presence or absence of an inflammatory infiltrate had any influence on expression. Paraffin-embedded tissue blocks of normal oral mucosa, OSCC, dysplasia, lichen planus and non-specific ulceration were obtained from the departmental tissue archive (Department of Oral Pathology, University of Sheffield) and 4μm serial sections were cut.

- An immunoperoxidase technique was employed. A Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) was used in accordance with the manufacturer's instructions for this purpose.

- Staining was performed at least three times on each sample on three different occasions.

6.4 MATERIALS AND METHODS

6.4.1 IMMUNOHISTOCHEMISTRY

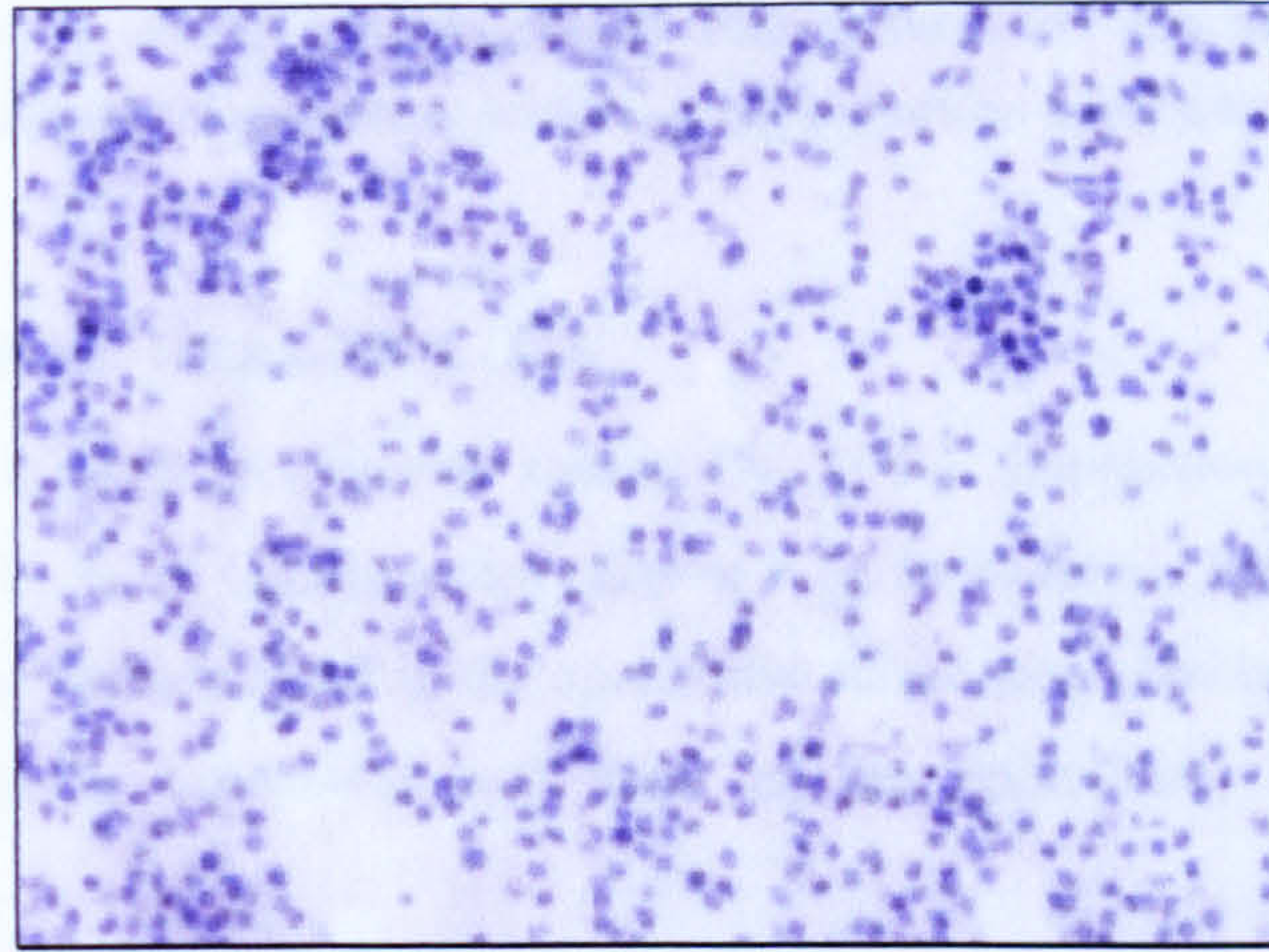
Serial sections were cut from three cases of normal oral mucosa, oral lichen planus, dysplasia, eight cases of OSCC and two cases of non-specific ulceration. Mononuclear cells were isolated from blood, fixed, clotted, embedded in paraffin and used as positive control (Appendix 10.3 and 10.4). 2% hydrogen peroxide in methanol was used to block endogenous peroxidase. For XCR1, antigen retrieval was performed by heating the tissue sections in citrate buffer (pH 6.0) in a microwave for 8min whereas sections were heated for 12min for lymphotactin. Polyclonal anti-human XCR1 antibody (Lifespan) (1:500 dilution) and polyclonal anti-human lymphotactin antibody (Peprotech) (1:25 dilution) were added overnight at 4°C. Rabbit serum served as negative control for XCR1 whereas for lymphotactin antibody was pre-absorbed with recombinant lymphotactin (R&D Systems) before application. A Vector NovaRed substrate kit (Vector Laboratories) was used to visualize peroxidase activity resulting in a red-brown positive reaction. Sections were counterstained with haematoxylin, dehydrated and mounted in DPX.

At least three serial sections for each sample were stained on different occasions.

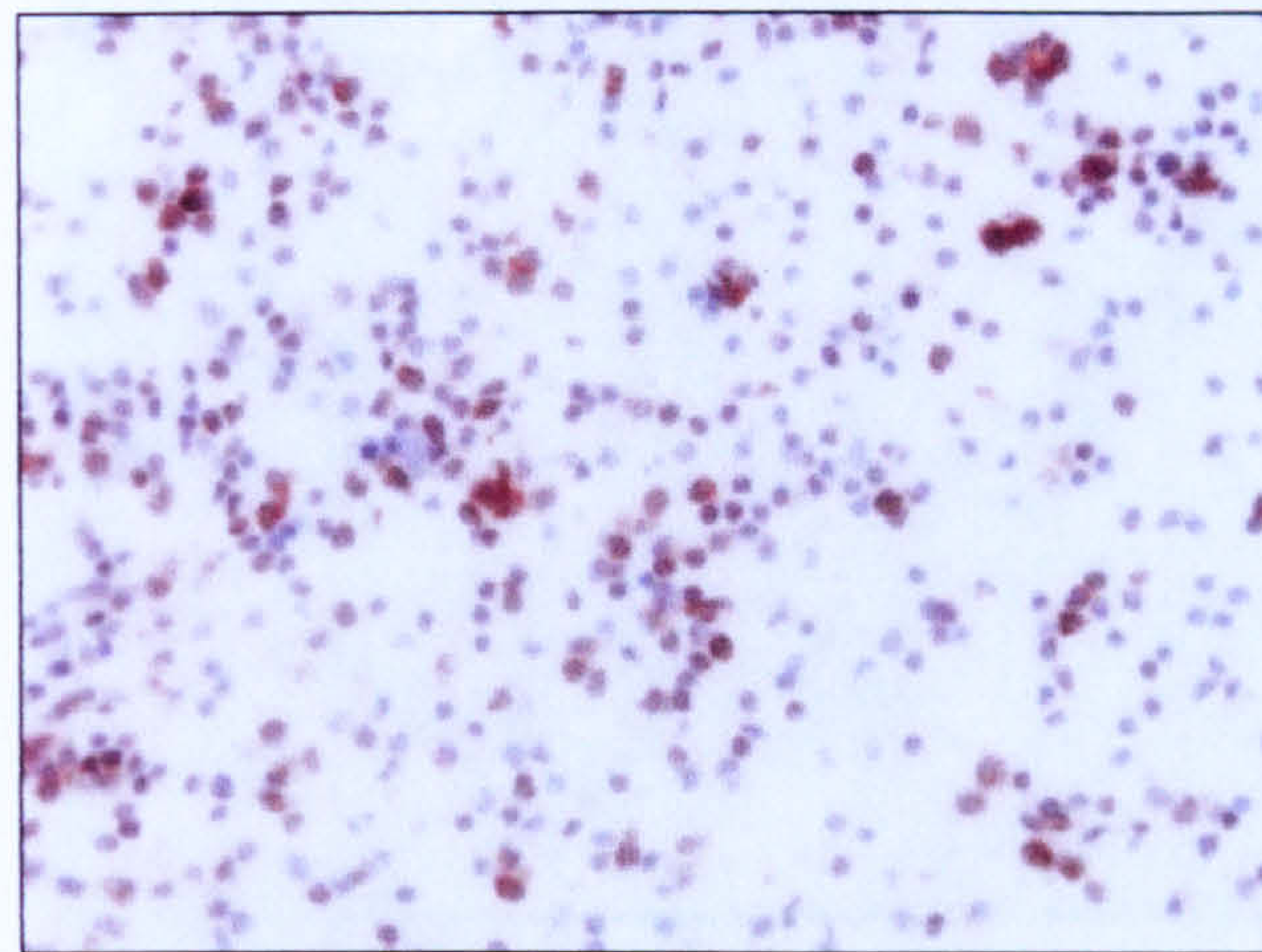
6.5 RESULTS

6.5.1 ISOLATED MONONUCLEAR CELLS

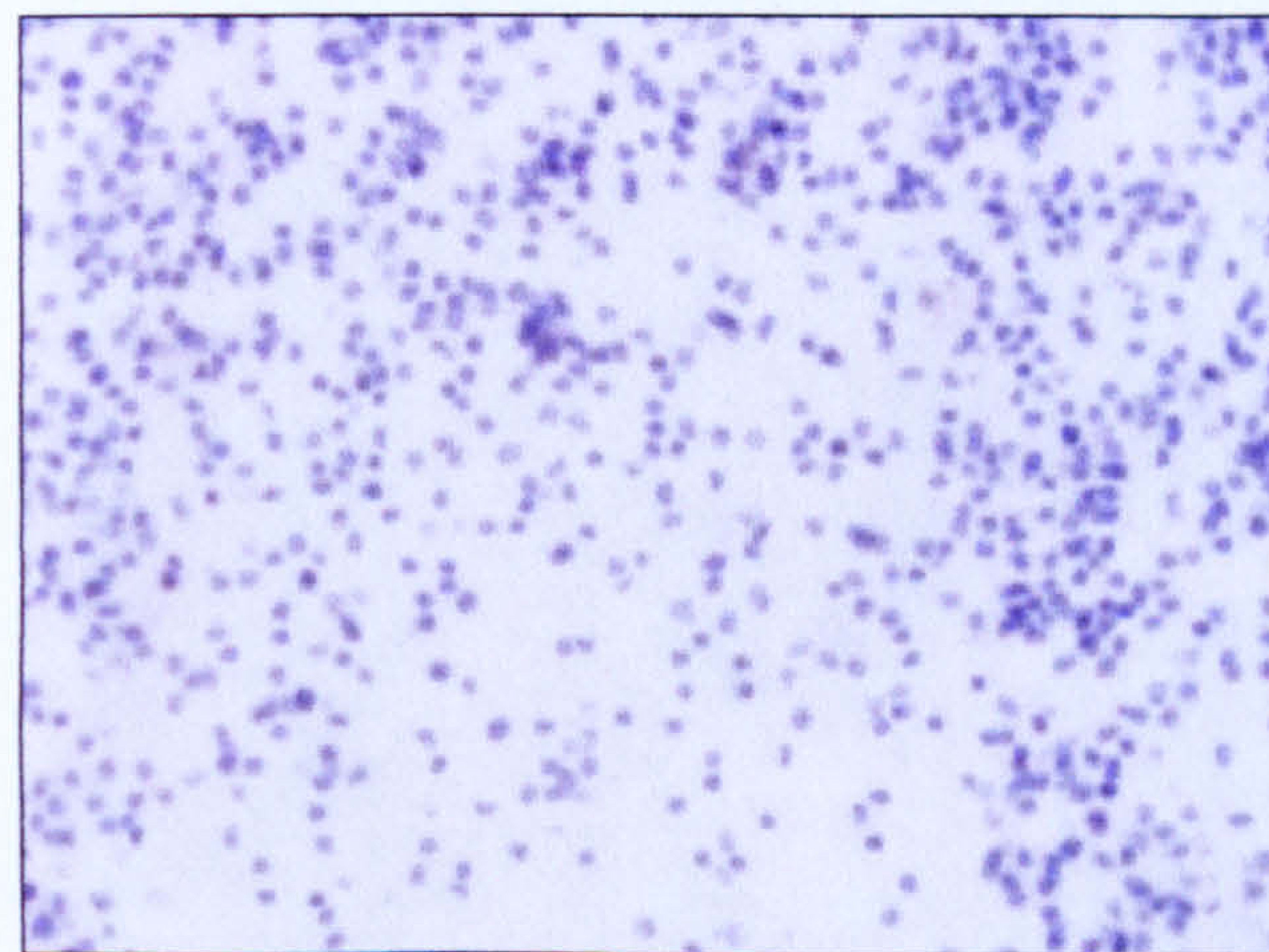
Strong expression of XCR1 was seen in stimulated mononuclear cells. Staining was observed in lymphocytes and neutrophils but not in monocytes. Expression was observed on the cell surface as well as in the cytoplasm. No expression of lymphotactin was observed (Figure 6.1).



a) Negative control



b) XCR1



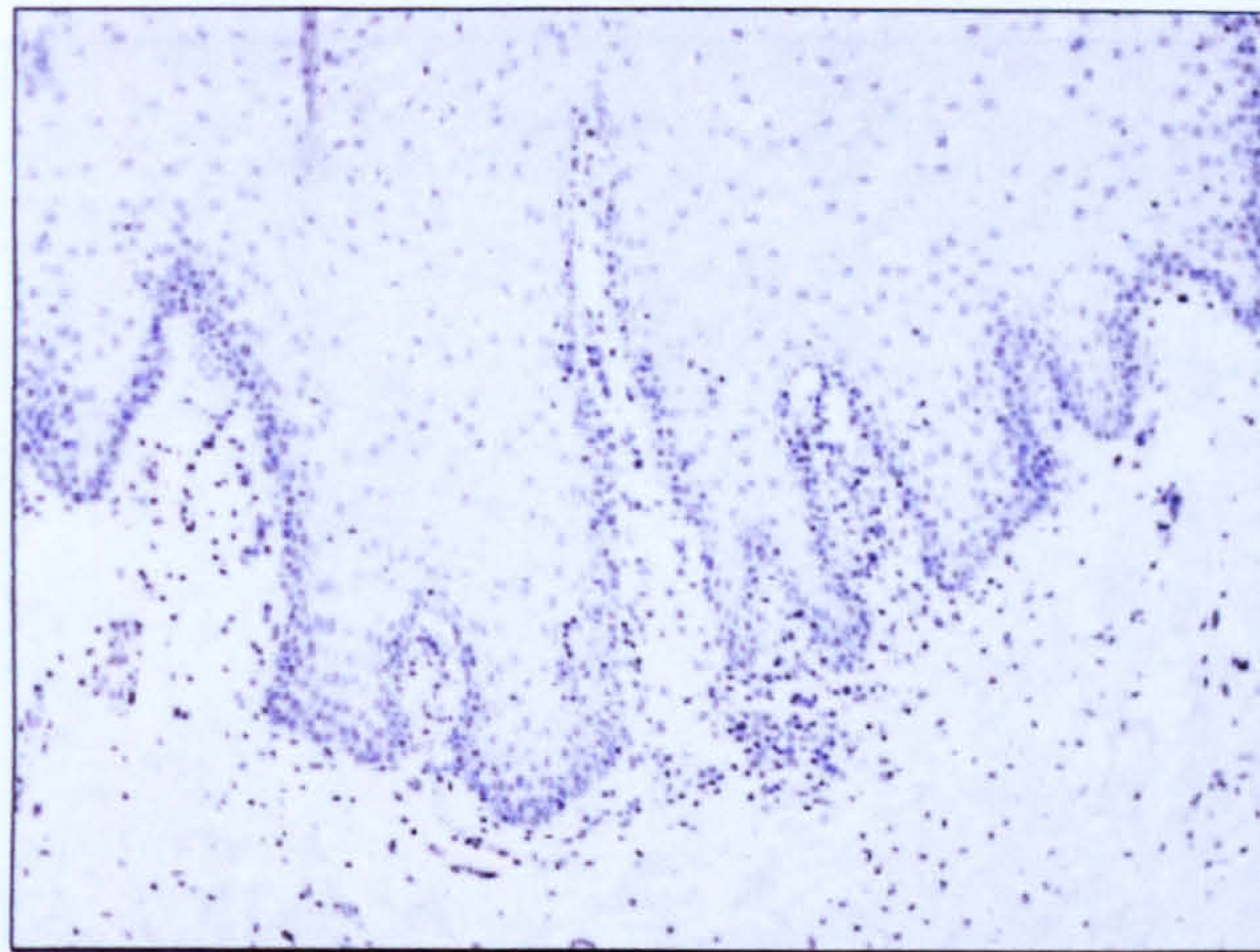
c) Lymphotactin

Figure 6.1. Immunohistochemical expression of XCR1 and lymphotactin in isolated mononuclear cells (x60 magnification) (Representative sample). No staining was seen in

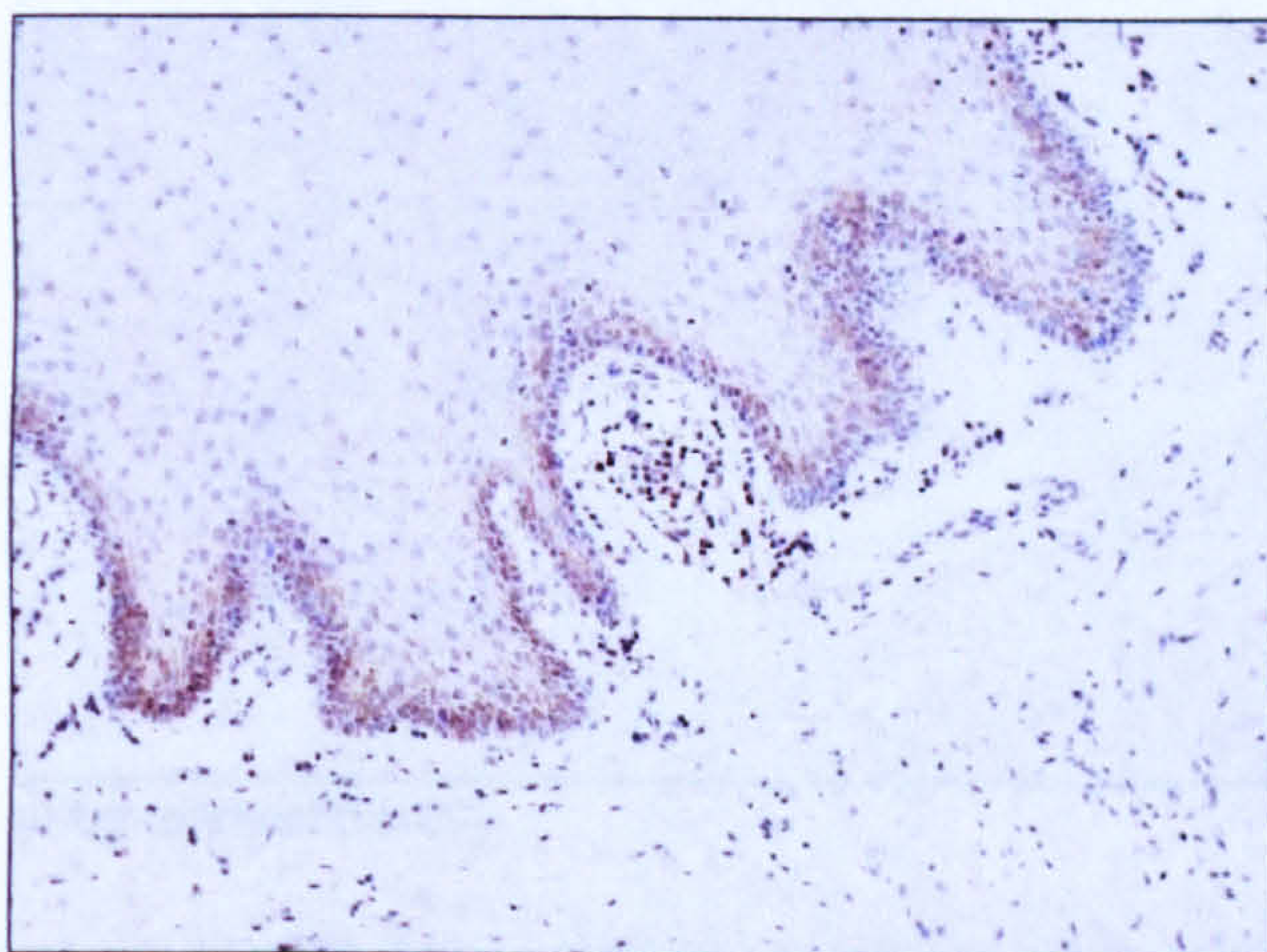
negative control (a) and for lymphotactin (c). Staining for XCR1 was seen on neutrophils but not on monocytes (b). Staining was performed three times on every sample.

6.5.2 NORMAL ORAL MUCOSA

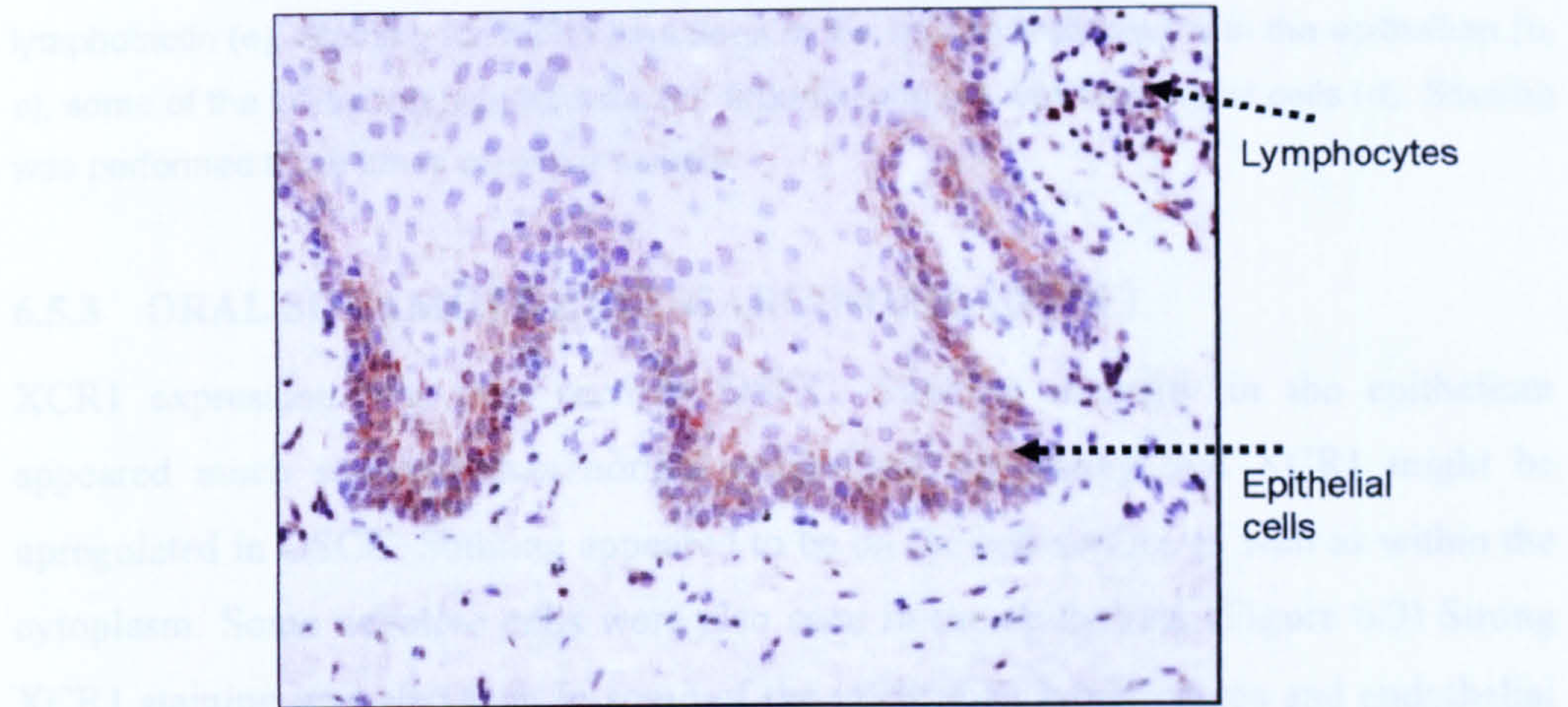
Normal oral mucosa consistently stained for XCR1. Expression was predominantly observed in the basal keratinocytes (Figure 6.2). In addition, fibroblasts, endothelial cells and some lymphocytes in the connective tissue also showed XCR1 expression. No lymphotactin expression was seen in the epithelium, IEL or infiltrating lymphocytes.



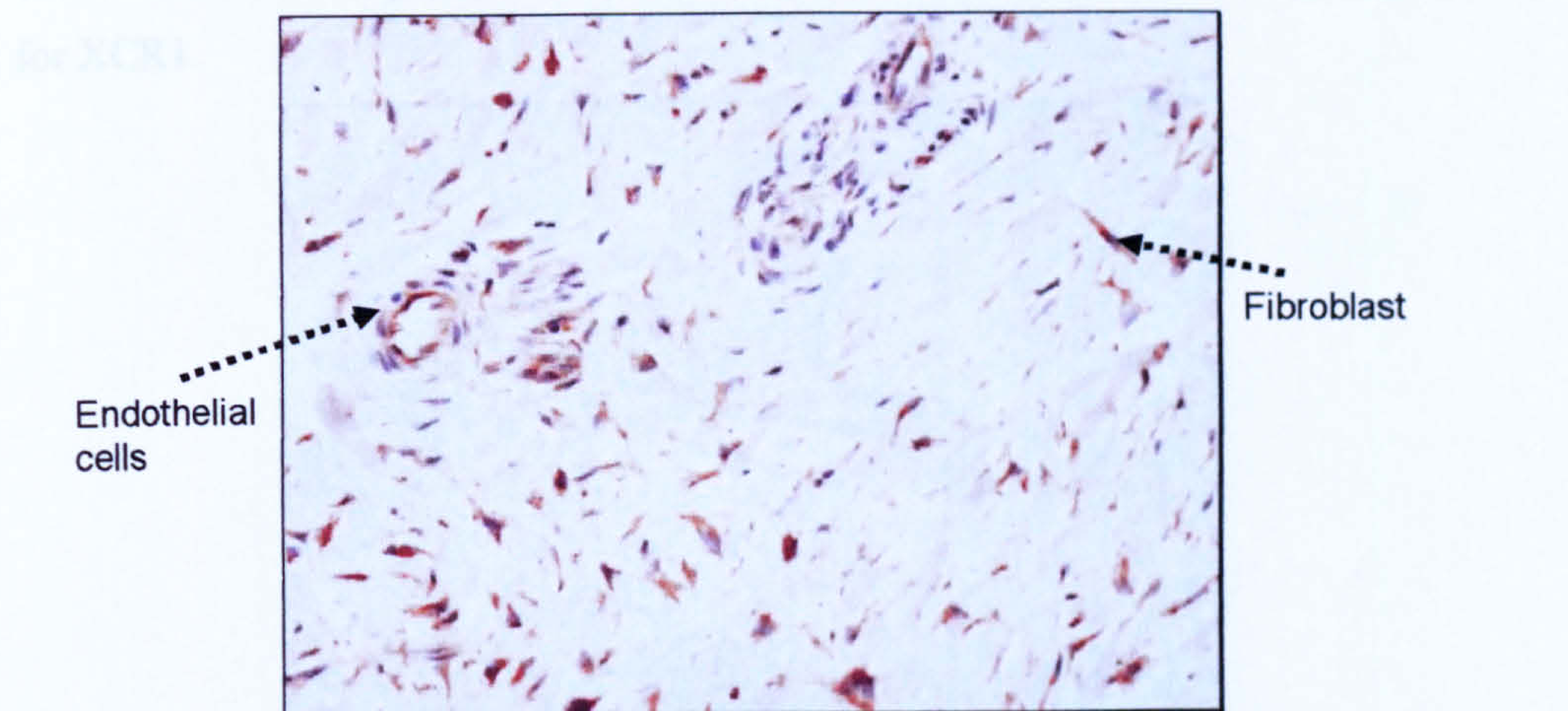
a) Negative control (x20)



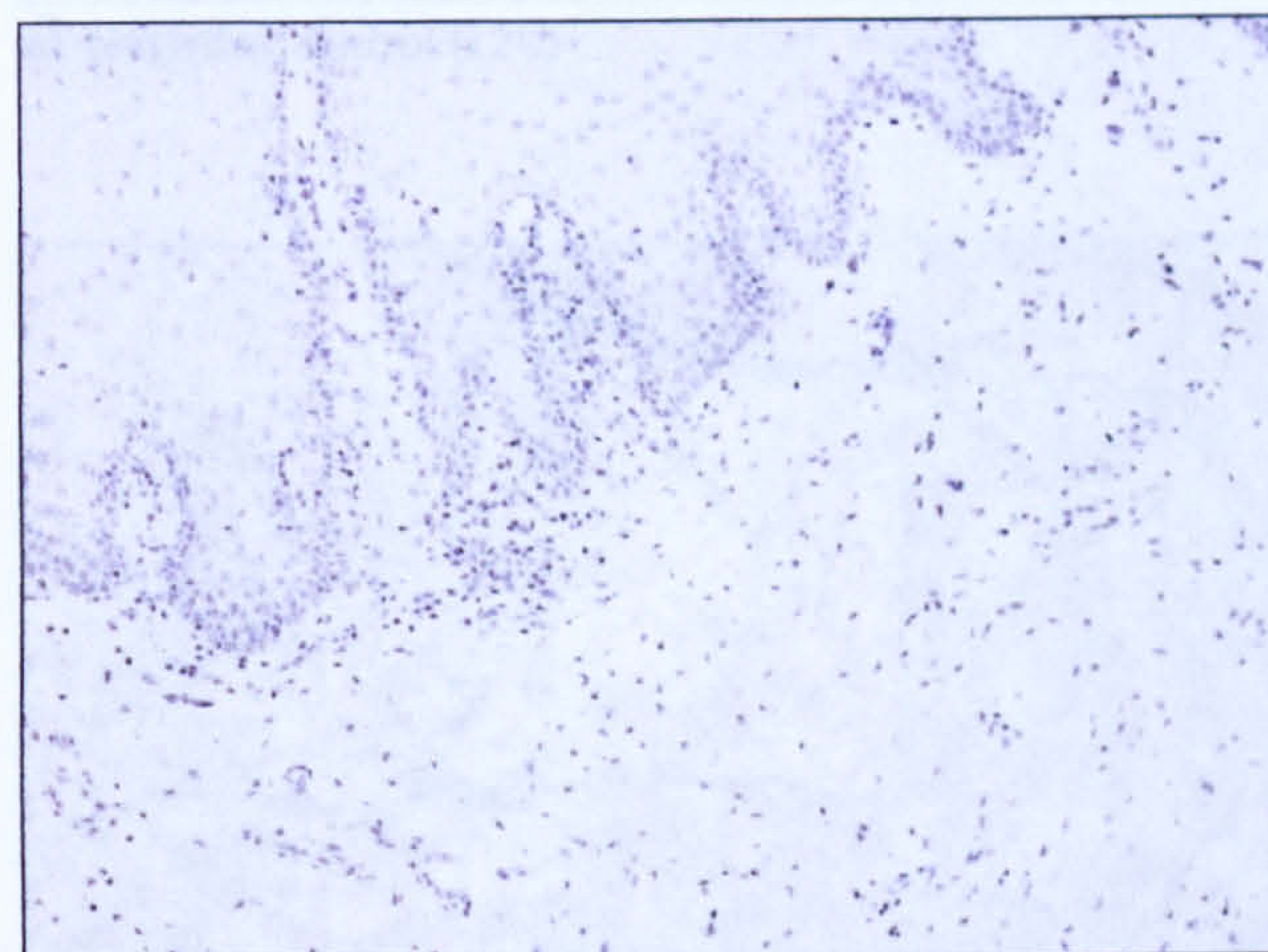
b) XCR1 (x20)



c) XCR1 (x40)



d) XCR1 (x40)



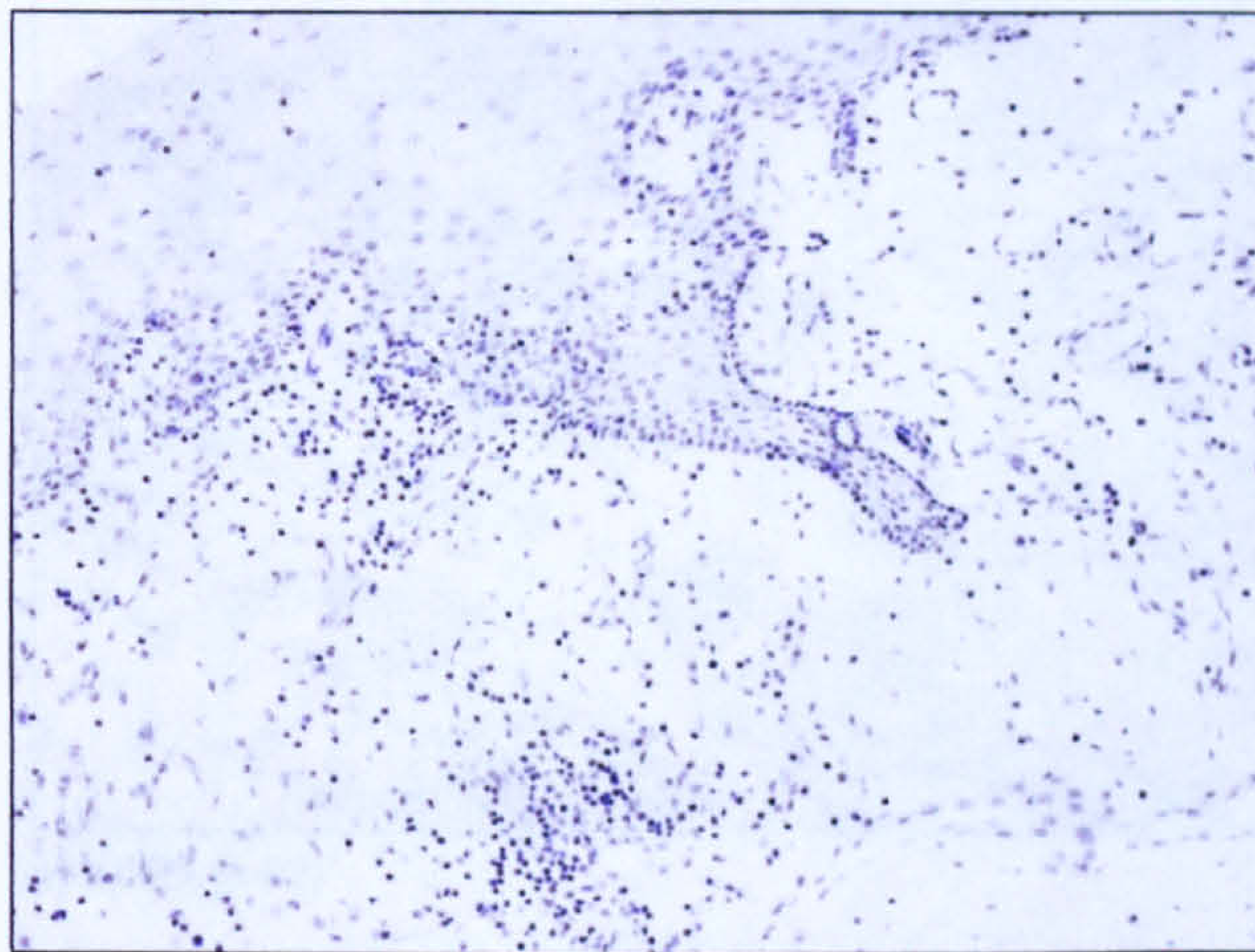
e) Lymphotactin (x20)

Figure 6.2. Normal oral mucosa- Immunohistochemical expression of XCR1 and lymphotactin (Representative sample). No staining was observed in the negative control (a) or for

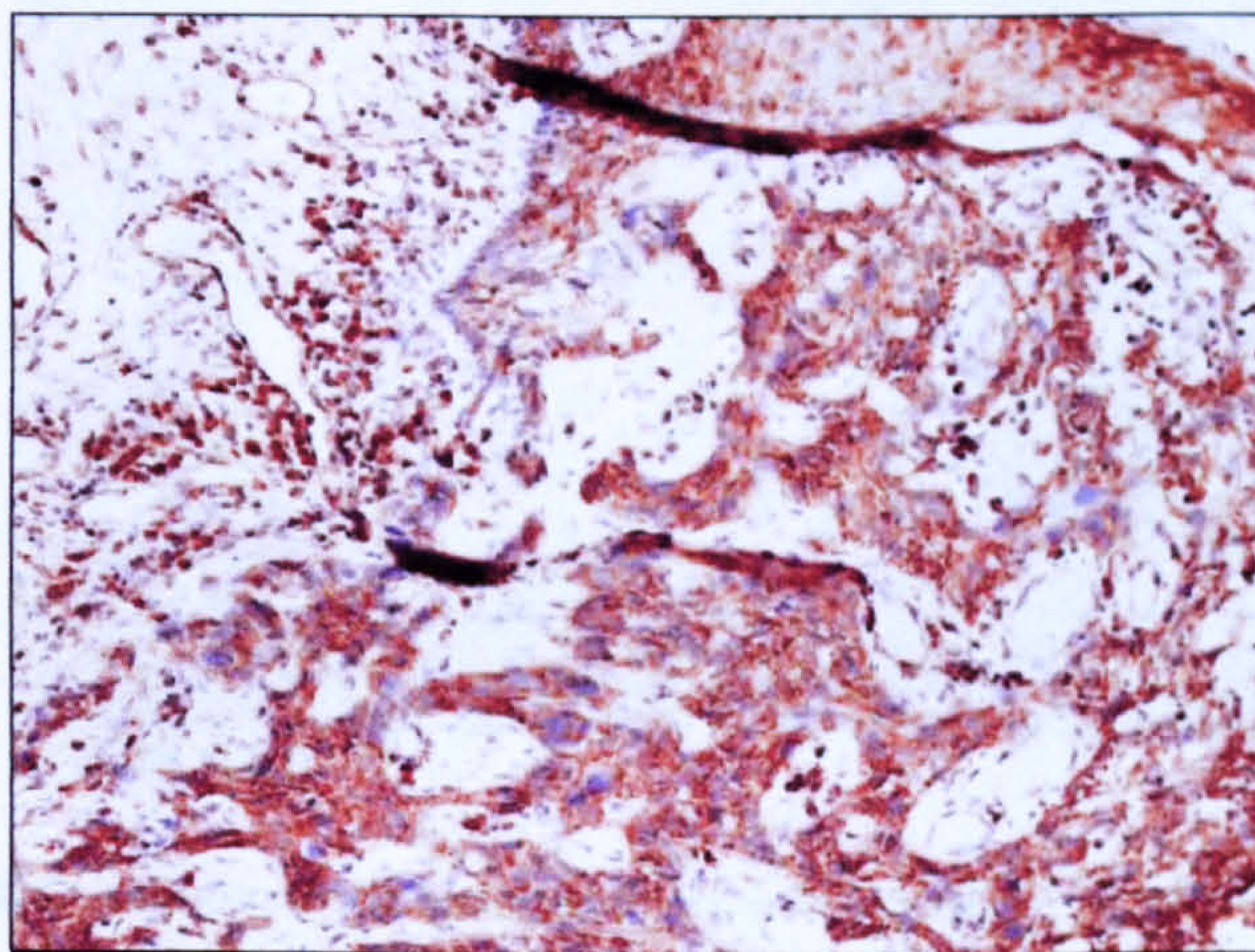
lymphotactin (e). Staining for XCR1 was seen in the basal keratinocytes in the epithelium (b, c), some of the infiltrating lymphocytes (c), firboroblasts (d) and endothelial cells (d). Staining was performed three times on every sample.

6.5.3 ORAL SQUAMOUS CELL CARCINOMA (OSCC)

XCR1 expression was also seen in OSCC. Staining intensity in the epithelium appeared much stronger than normal epithelium suggesting that XCR1 might be upregulated in OSCC. Staining appeared to be on the cell surface as well as within the cytoplasm. Some negative cells were also seen in the epithelium (Figure 6.3) Strong XCR1 staining was also seen in some of the infiltrating lymphocytes and endothelial cells. All the invading tumour cells and cells in tumour islands showed strong staining for XCR1.



a) Negative control (x20)



b) XCR1 (x20)

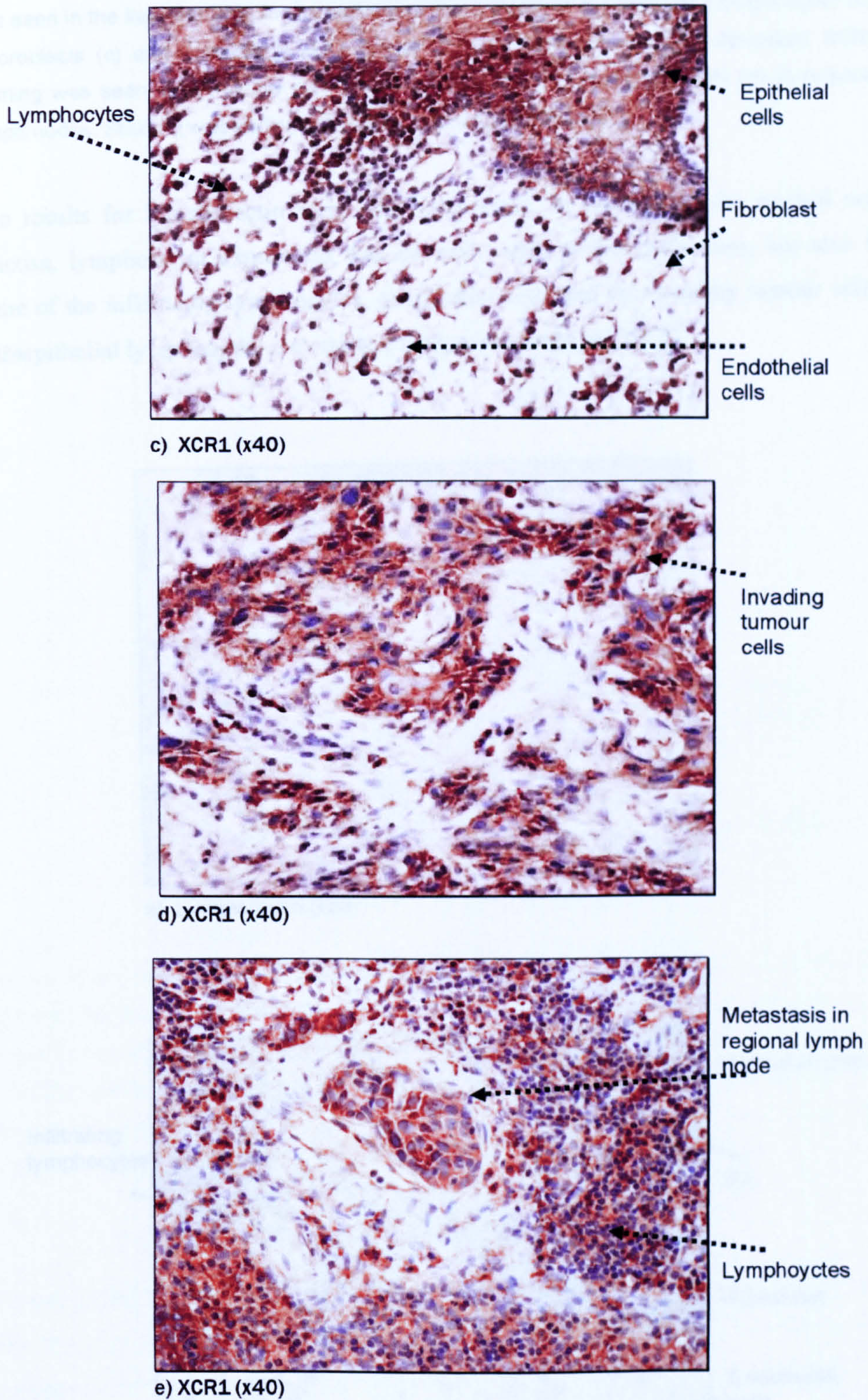
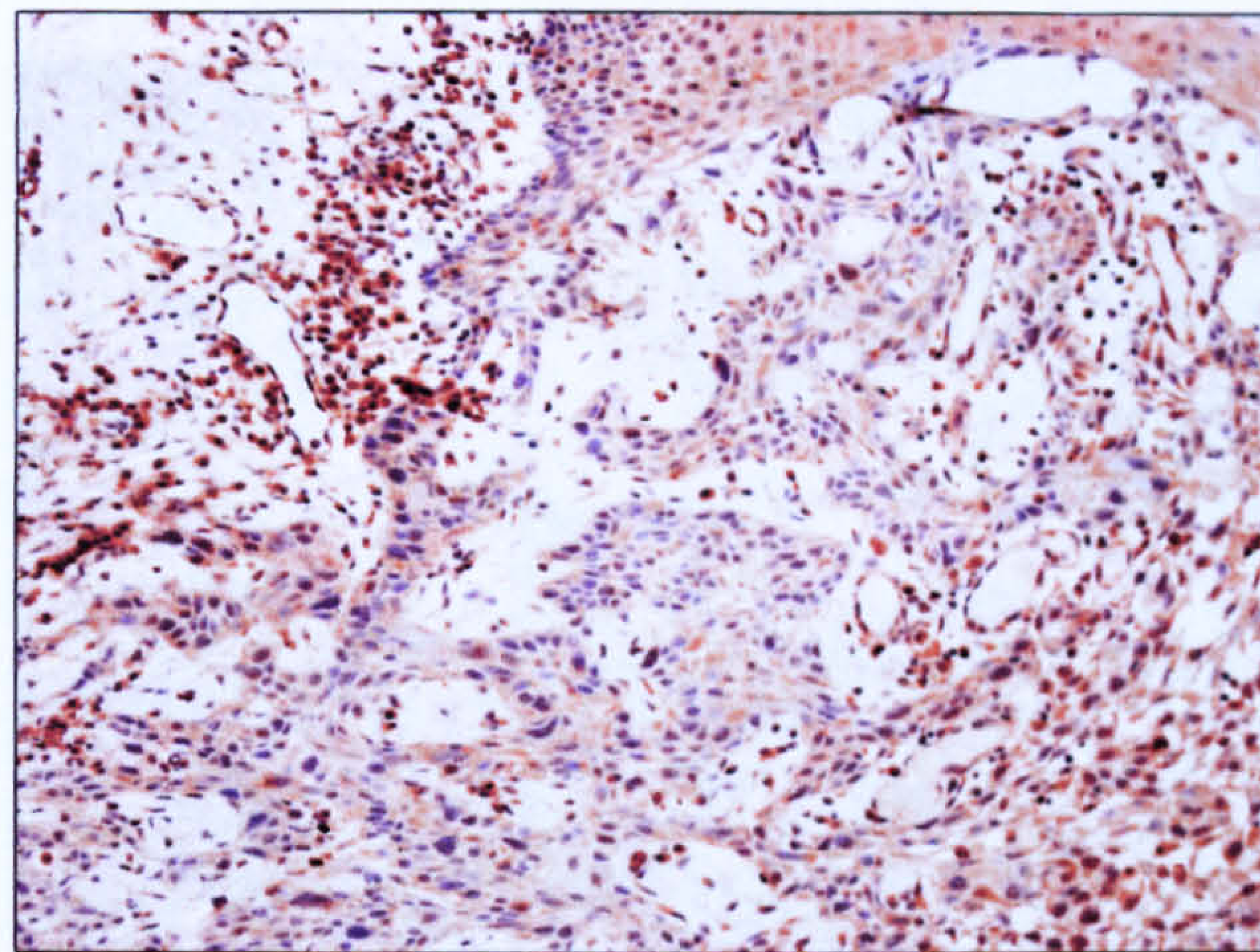


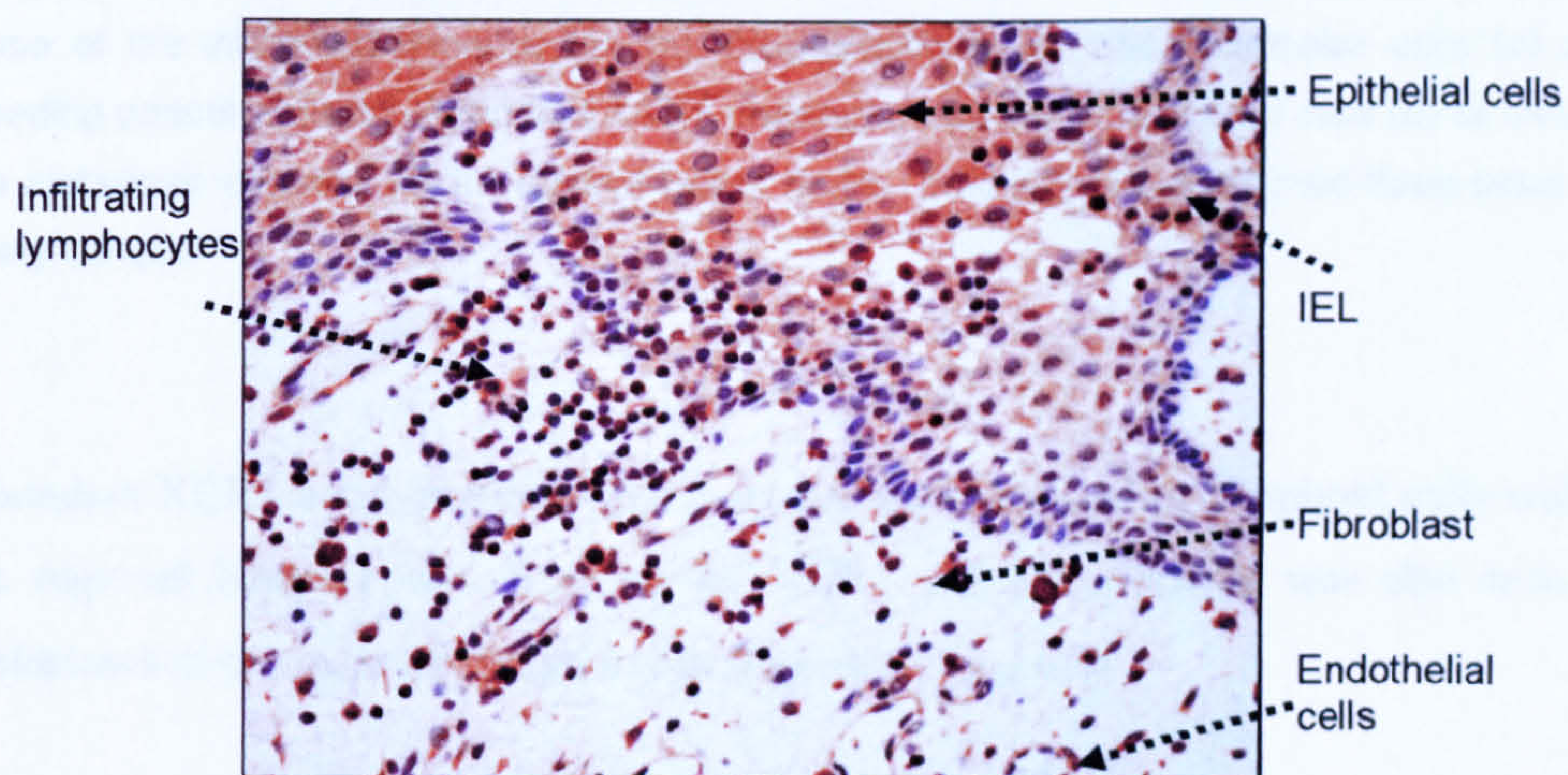
Figure 6.3. OSCC- Immunohistochemical expression of XCR1 (Representative sample). No staining was observed in the negative control (a) or for lymphotactin (e). Staining for XCR1

was seen in the keratinocytes in the epithelium (b, c), some of the infiltrating lymphocytes (c), firboroblasts (c) and endothelial cells (c) and invading tumour cells (d). Abundant XCR1 staining was seen on lymphoid cells (e) as well as the metastatic deposits (e) in regional lymph nodes. Staining was performed three times on every sample.

The results for lymphotactin were very interesting. In contrast to the normal oral mucosa, lymphotactin expression was not only seen in the epithelium, but also in some of the infiltrating lymphocytes, endothelial cells and the invading tumour cells. Intraepithelial lymphocytes also stained for lymphotactin in OSCC.



a) Lymphotactin (x20)



b) Lymphotactin (x40)

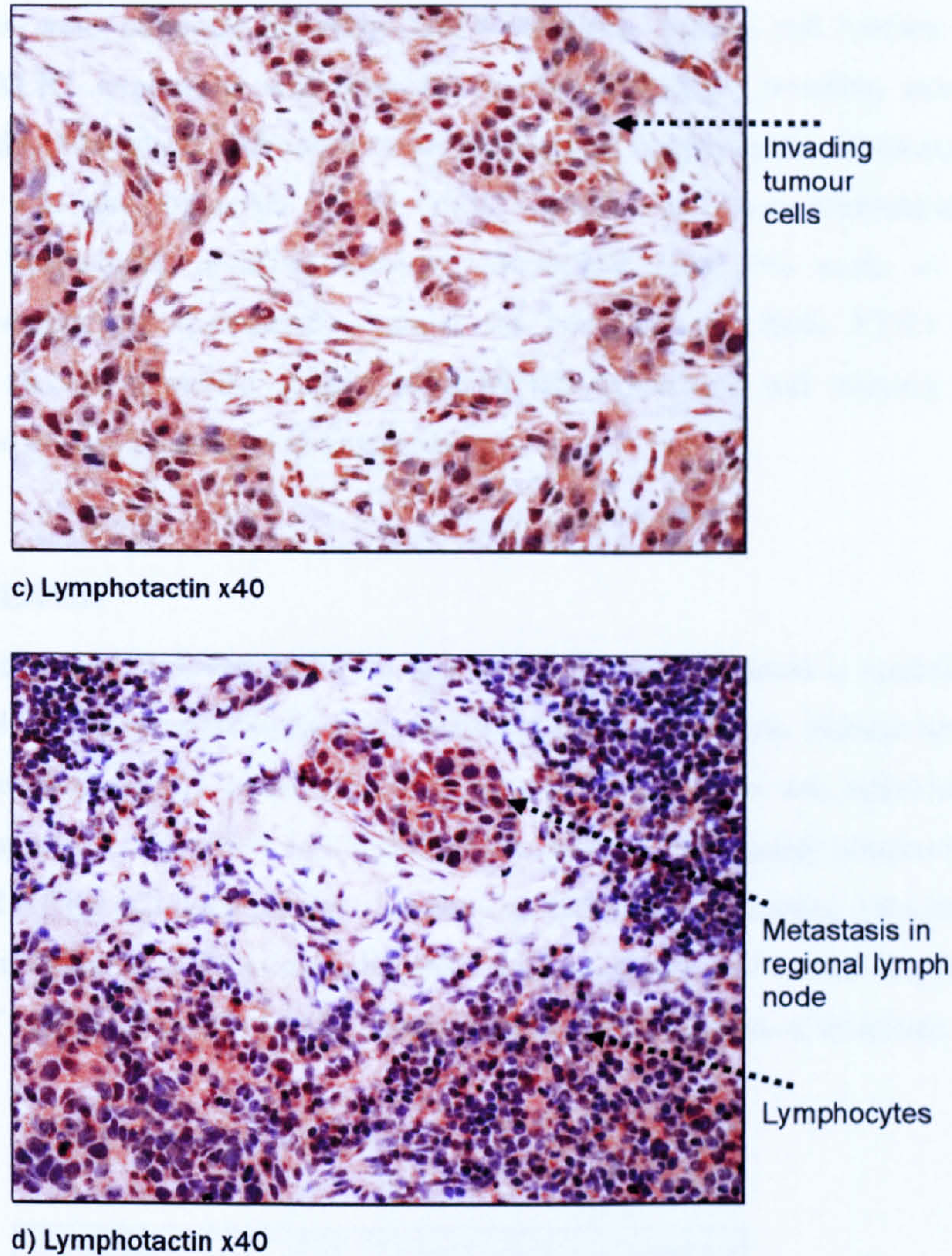


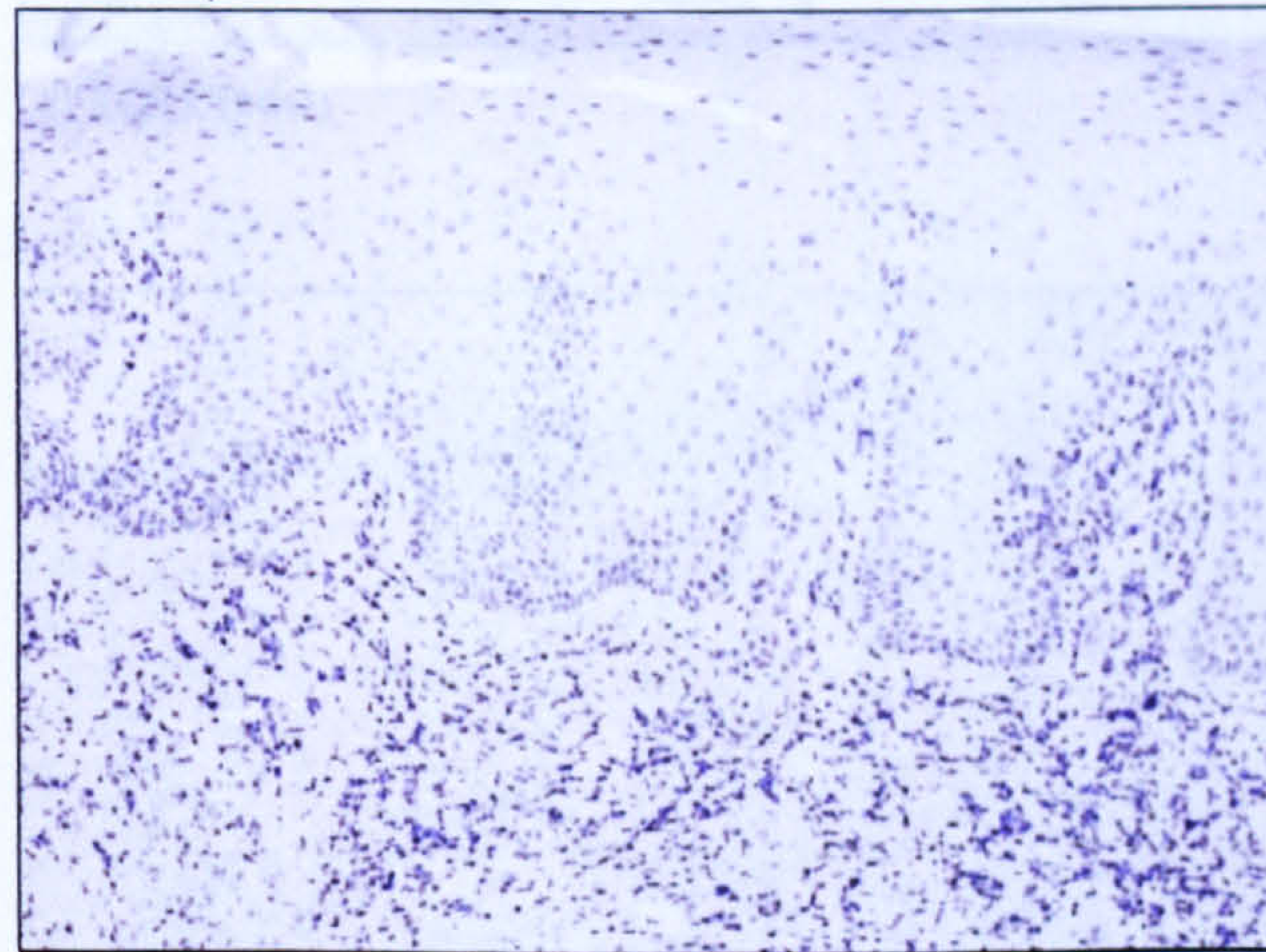
Figure 6.4. OSCC- Immunohistochemical expression of lymphotactin (Representative sample). Staining for lymphotactin was seen in the keratinocytes in the epithelium (a, b), some of the infiltrating lymphocytes (b), firboroblasts (b, c) and endothelial cells (b) and invading tumour cells (c). Abundant XCR1 staining was seen on lymphoid cells (d) as well as the metastatic deposits (d) in regional lymph nodes. Staining was performed three times on every sample.

Abundant XCR1 and lymphotactin expression was observed in lymphoid cells within the regional lymph nodes. Staining for XCR1 and lymphotactin was also seen in metastases in the regional lymph nodes (Figure 6.3 and 6.4).

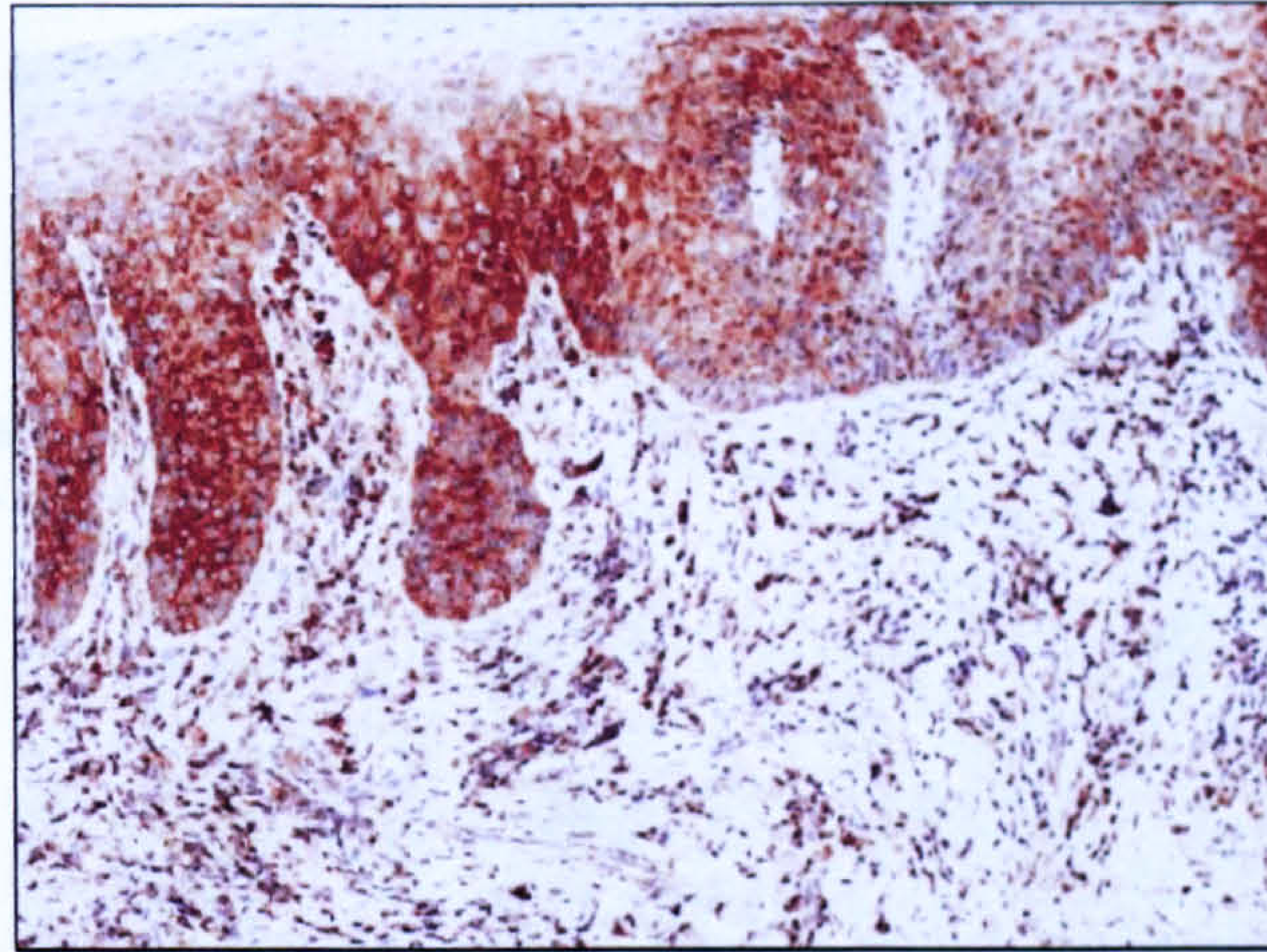
Similar results were obtained for other OSCC samples. Strong cell surface and cytoplasmic XCR1 expression was observed in the epithelium, invading tumour, endothelial cells, fibroblasts and some of the infiltrating lymphocytes. Staining for lymphotactin was also observed in all OSSC samples and was predominantly cytoplasmic. Epithelium, invading tumour, endothelial cells and some of the infiltrating lymphocytes consistently stained for lymphotactin. Both XCR1 and lymphotactin showed a similar pattern of epithelial expression and staining was localized to the lower two third of the epithelium.

6.5.4 DYSPLASIA

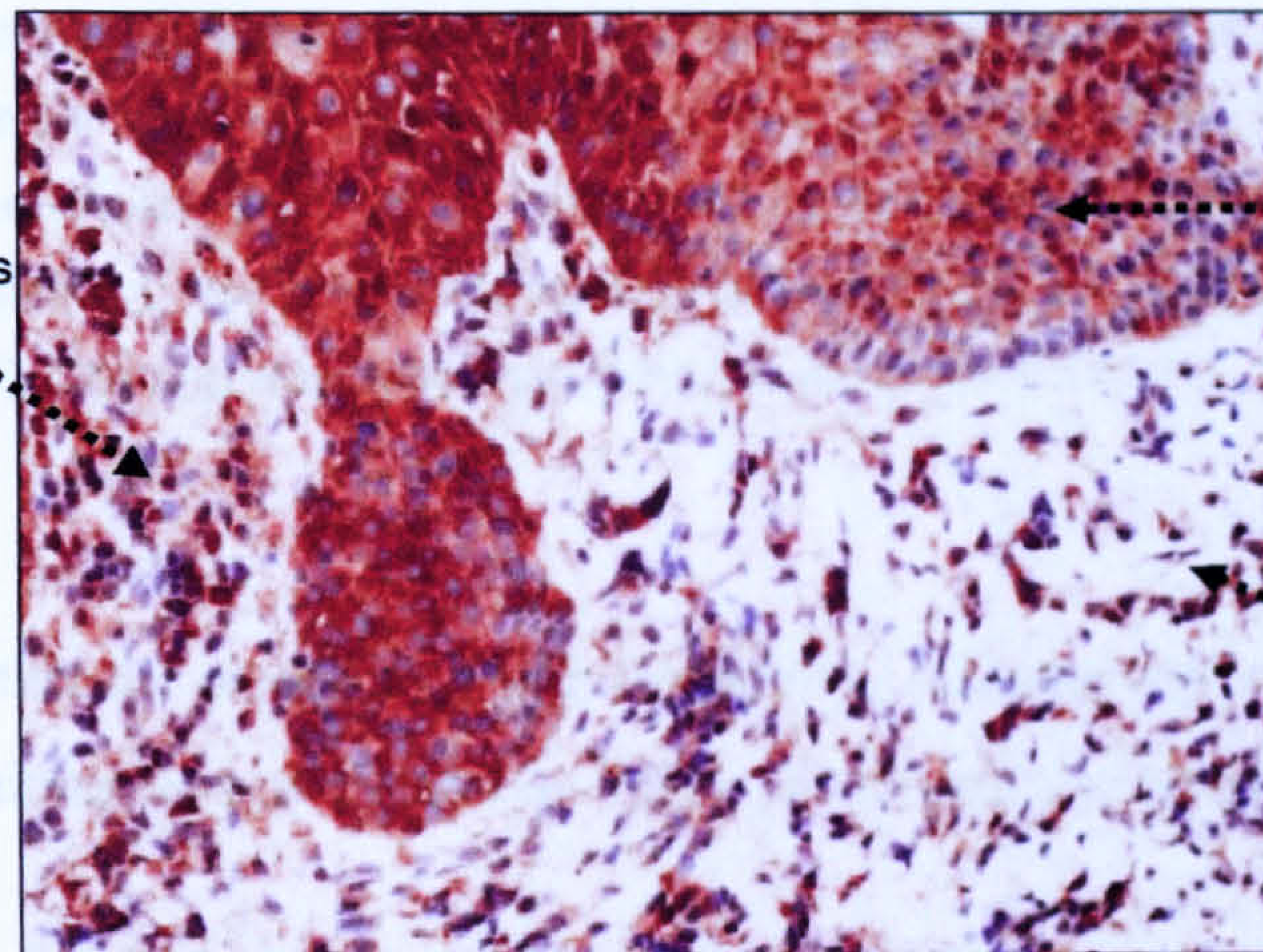
To study whether up-regulation of XCR1 and lymphotactin expression is specific to OSCC, a number of other conditions were studied including dysplasia. Similar results to OSCC were obtained and expression of XCR1 and lymphotactin was consistently noticed in dysplasia in the lower two thirds of the mucosa. Infiltrating lymphocytes also expressed XCR1 and lymphotactin. No obvious difference in staining intensity of XCR1 and lymphotactin was observed between mild, moderate and severe dysplasia. The pattern of staining was also similar between the different degrees of dysplasia.



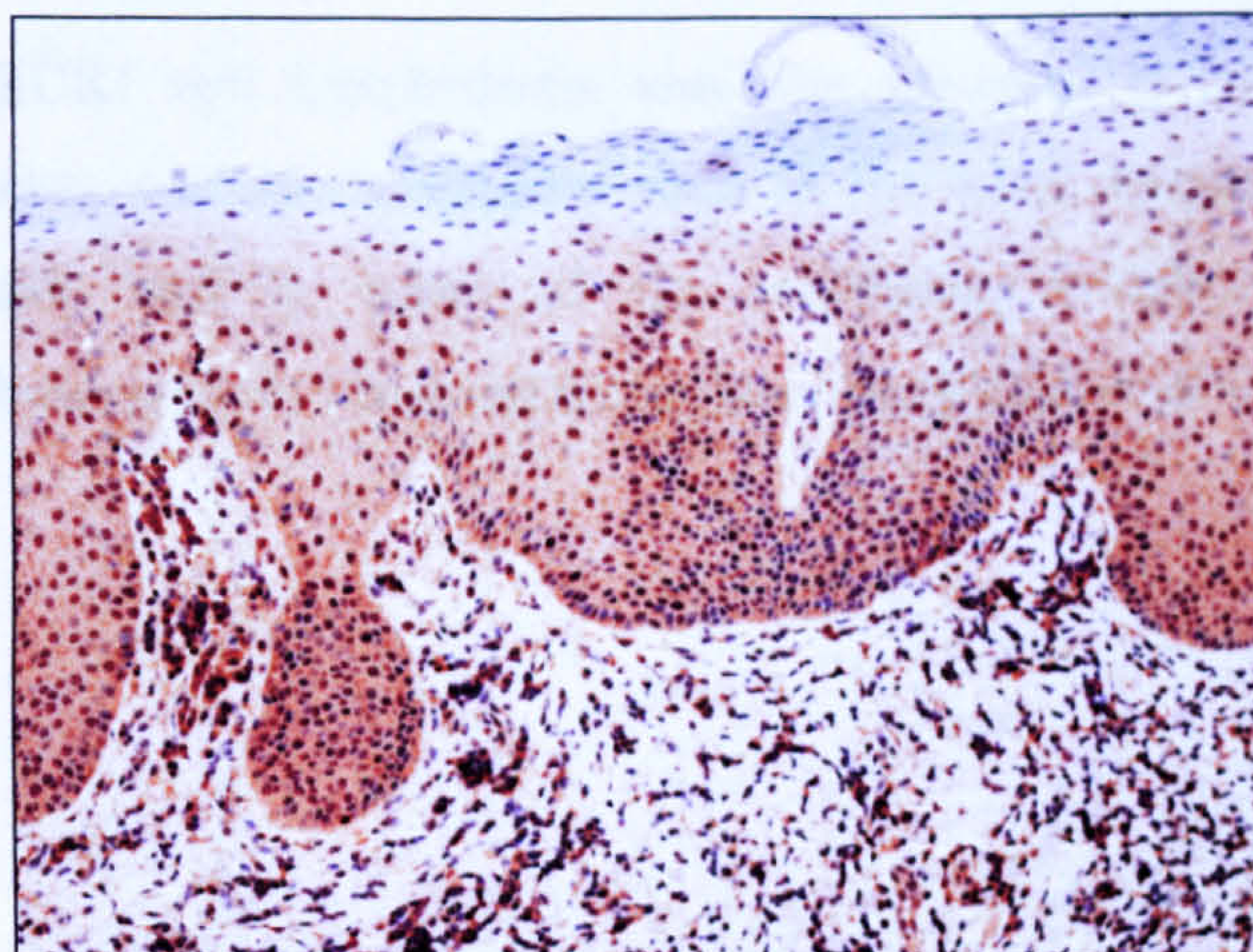
a) Negative control (x20)



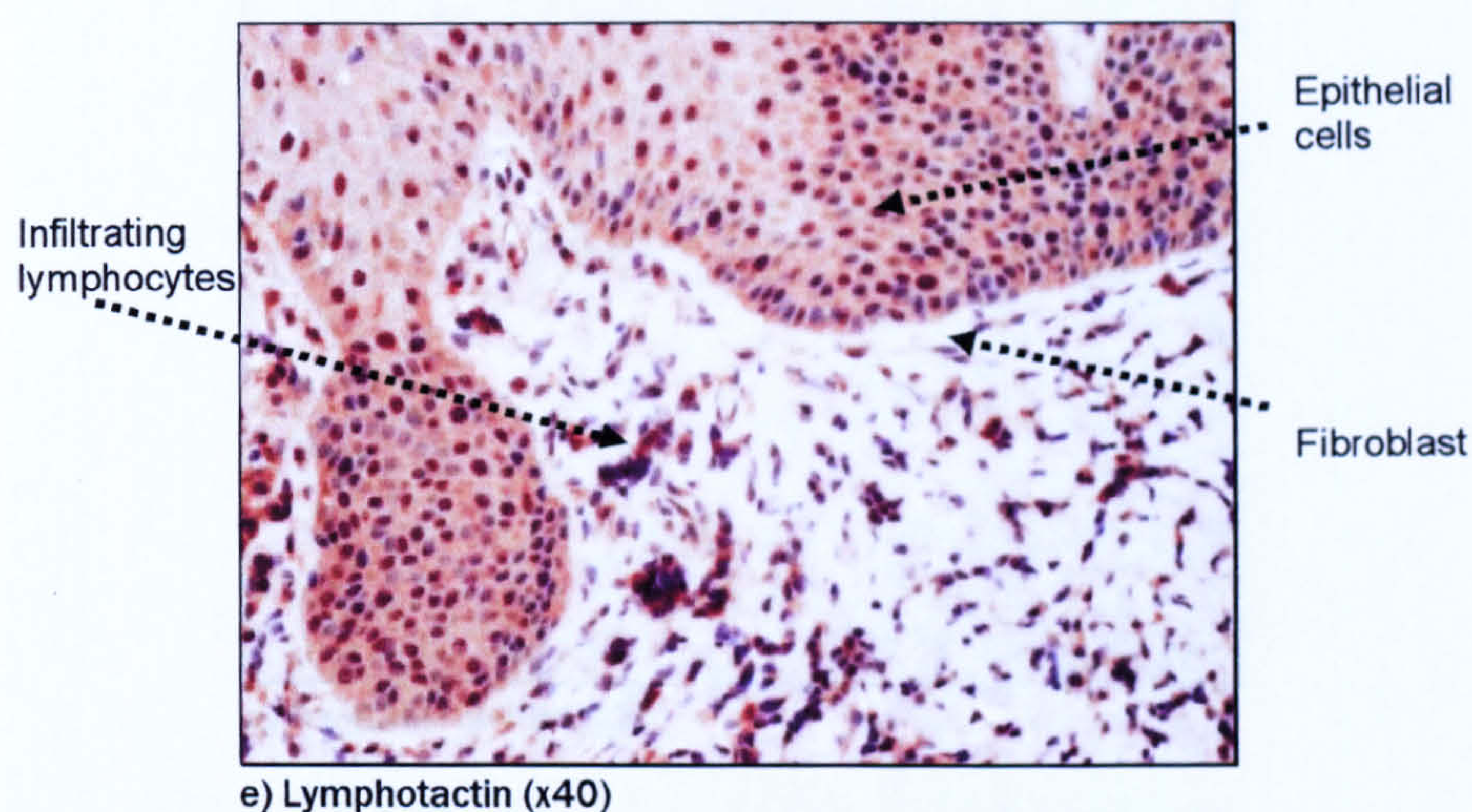
b) XCR1 (x20)



c) XCR1 (x40)



d) Lymphotactin (x20)



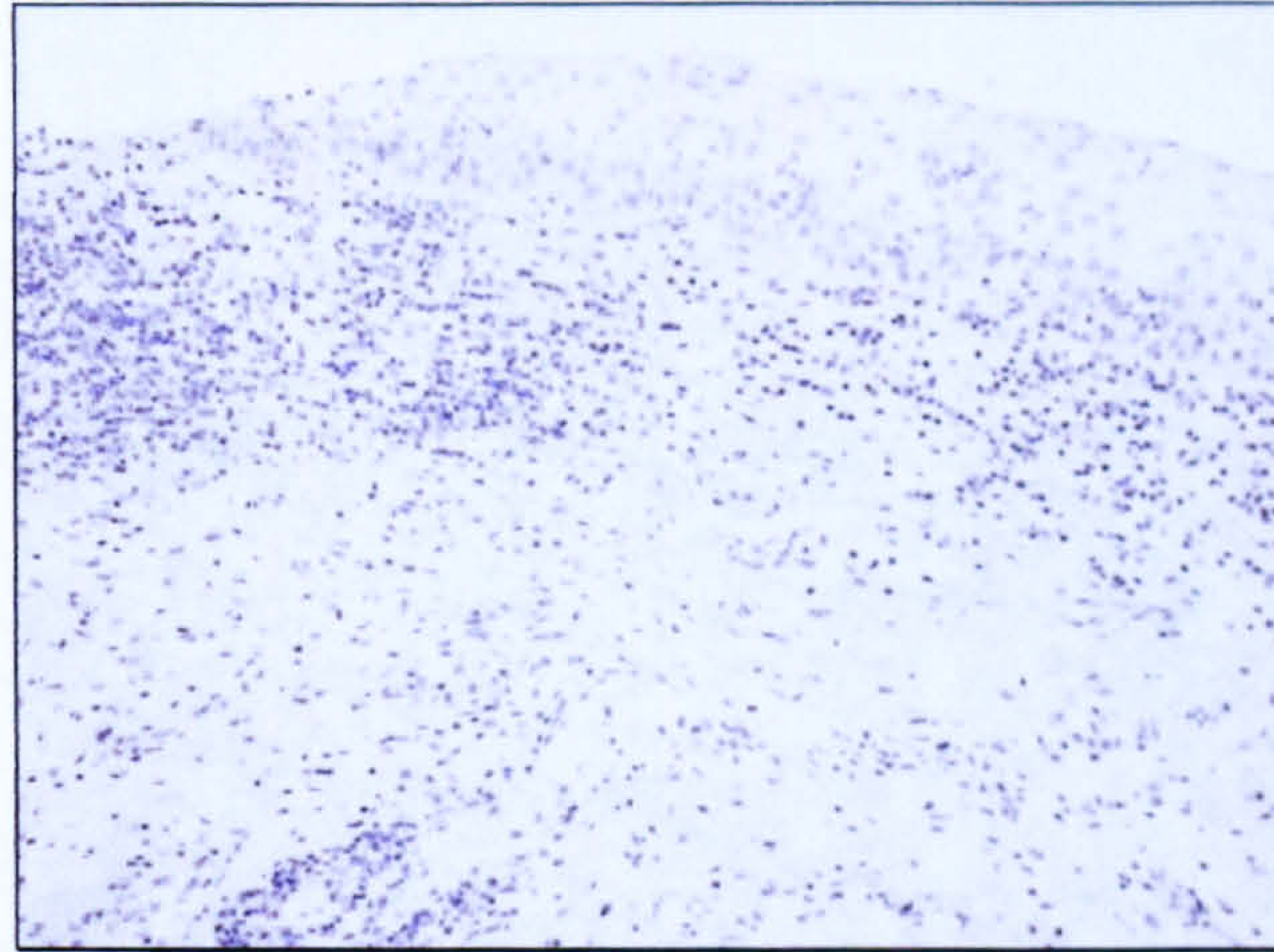
e) Lymphotactin (x40)

Figure 6.5. Severe dysplasia- Immunohistochemical expression of XCR1 and lymphotactin (Representative sample). No staining was seen in the negative control (a). Staining for XCR1 was seen on epithelial cells (b, c), infiltrating lymphocytes (b, c) and fibroblasts (b, c). Lymphotactin staining was also observed on epithelial cells (d, e), lymphocytes (d, e) and fibroblasts (d, e). Staining was performed three times on every sample.

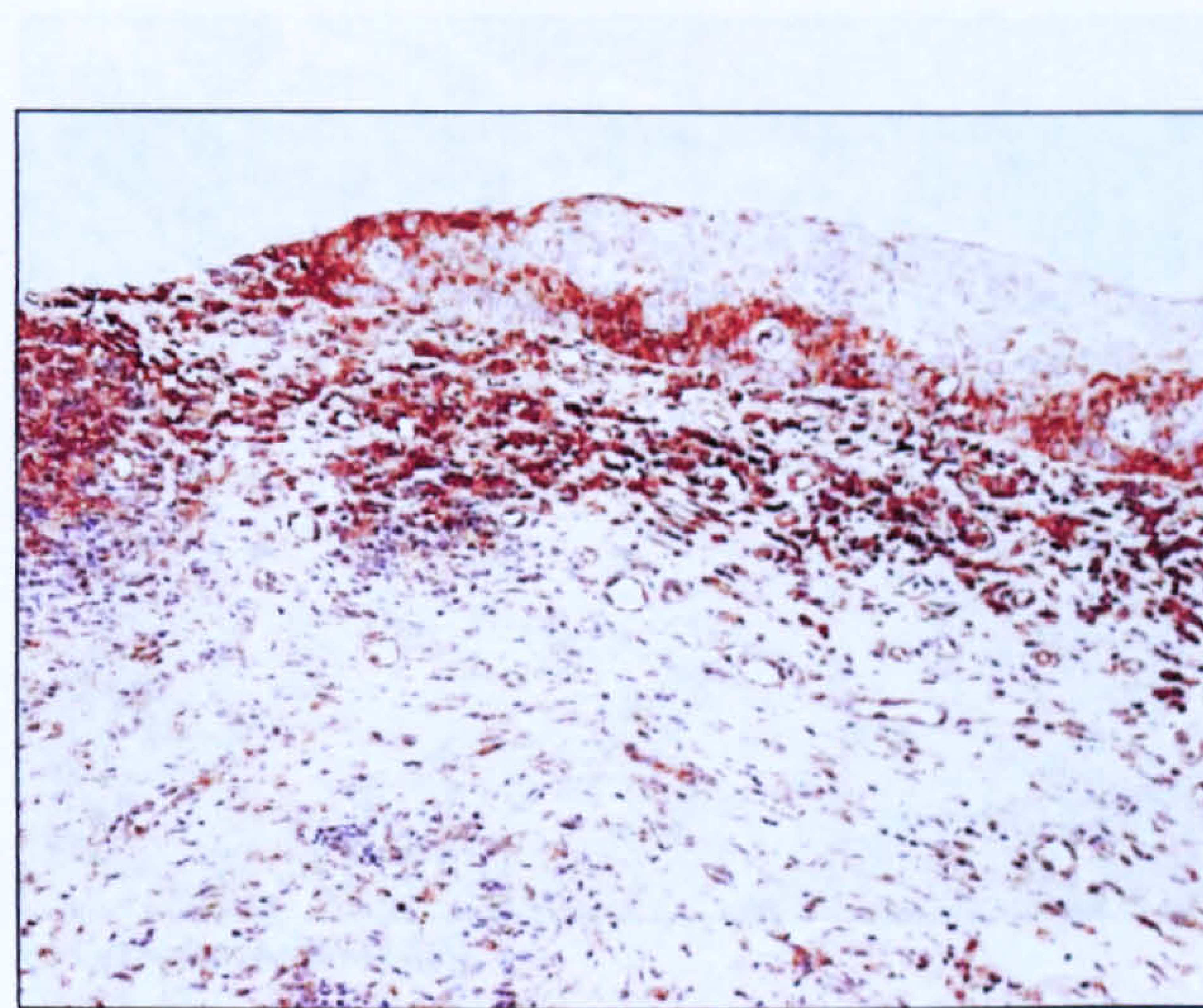
To study whether the presence of an inflammatory infiltrate was correlated to this up-regulation, staining was performed on lichen planus and non-specific ulceration.

6.5.5 LICHEN PLANUS

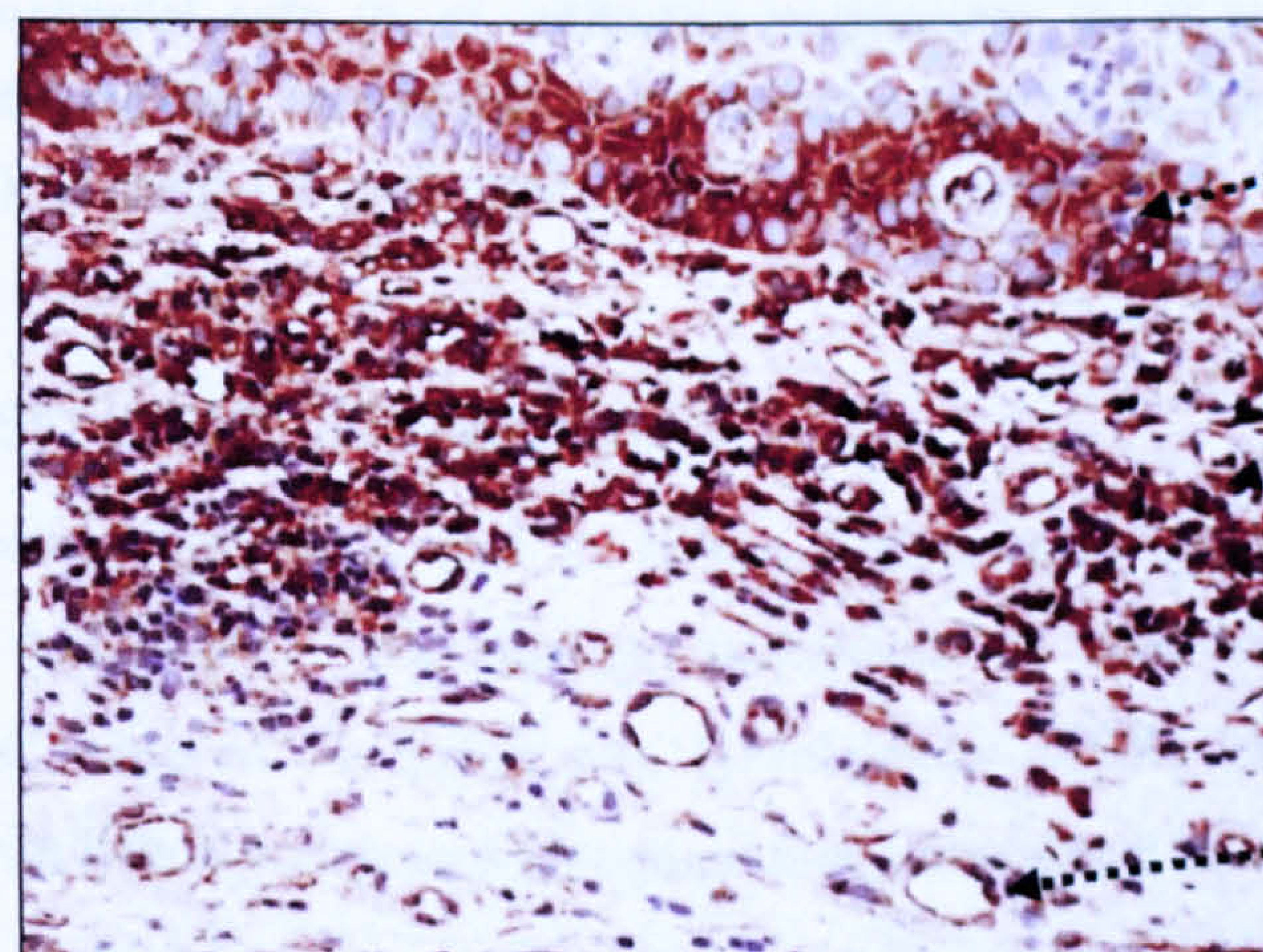
Staining for XCR1 and lymphotactin was also observed in lichen planus. Strong XCR1 expression was predominantly seen in the basal keratinocytes, endothelial cells, fibroblasts and in some of the infiltrating lymphocytes. Staining for lymphotactin however was observed throughout the thickness of the epithelium.



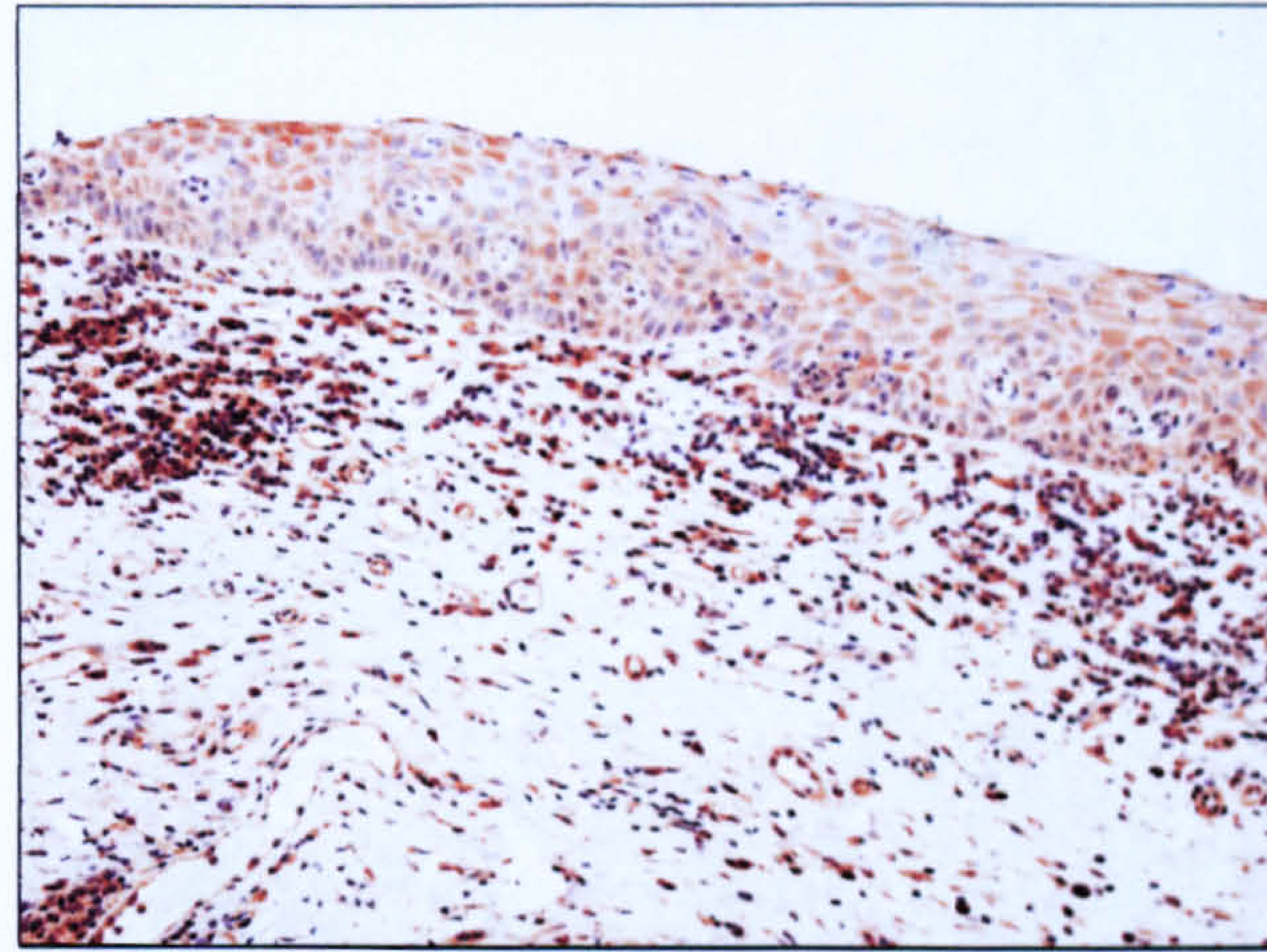
a) Negative control (x20)



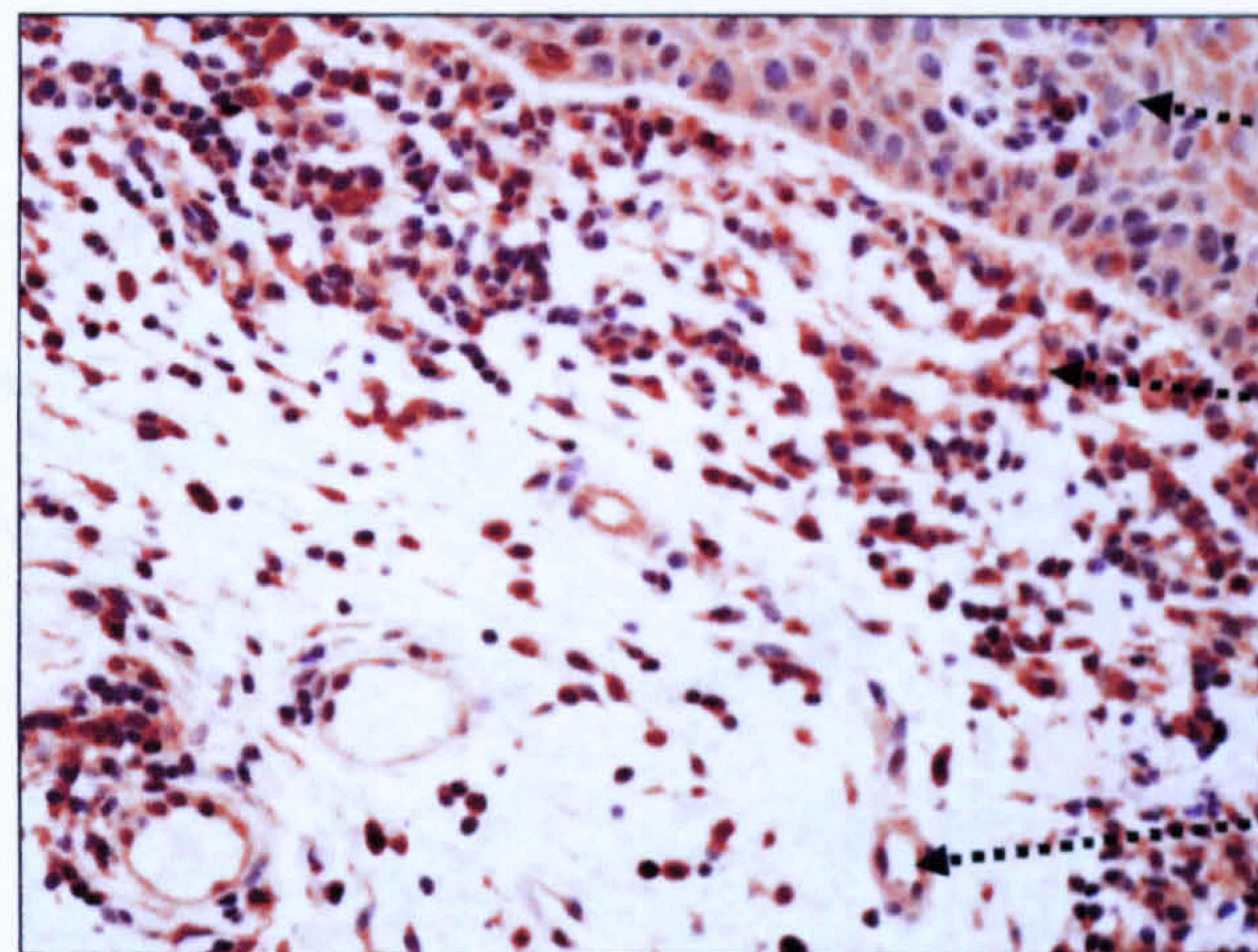
b) XCR1 (x20)



c) XCR1 (x40)



d) Lymphotactin (x20)



e) Lymphotactin (x40)

Figure 6.6. Lichen planus- Immunohistochemical expression of XCR1 and lymphotactin (Representative sample). No staining was seen in the negative control (a). Staining for XCR1 was seen on epithelial cells (b, c), infiltrating lymphocytes (b, c) and fibroblasts (b, c). Lymphotactin staining was also observed on epithelial cells (d, e), lymphocytes (d, e) and fibroblasts (d, e). Staining was performed three times on every sample.

6.5.6 NON-SPECIFIC ULCERATION

Ulceration showed similar results to lichen planus and staining for XCR1 and lymphotactin was seen in the adjacent epithelium as well as endothelial cells, fibroblasts and some of the infiltrating lymphocytes. XCR1 expression appeared much stronger in lichen planus and ulceration than normal oral mucosa.

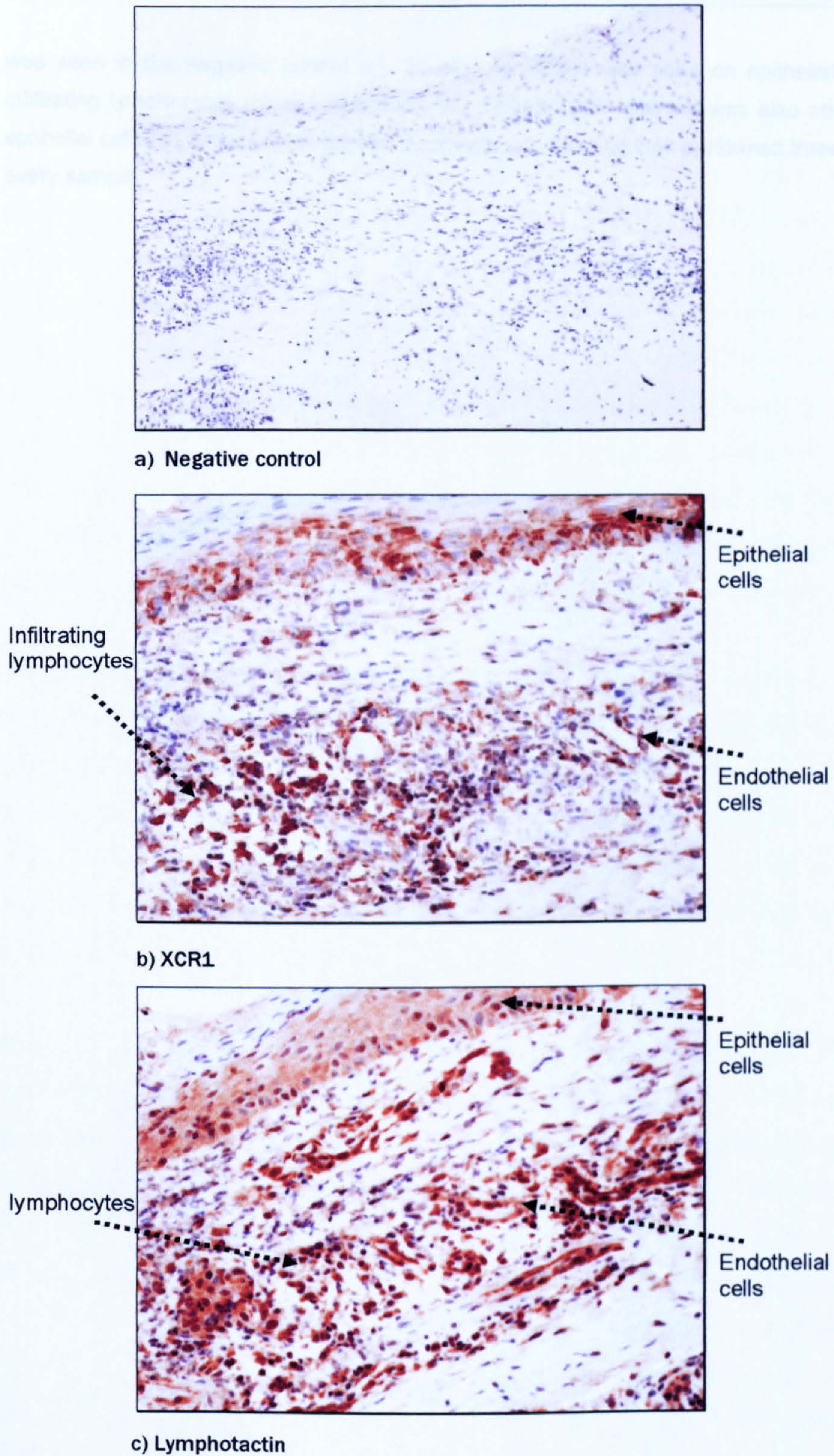


Figure 6.7. Adjacent epithelium in non-specific ulceration- Immunohistochemical expression of XCR1 and lymphotactin (Original magnification x 40) (Representative sample). No staining

was seen in the negative control (a). Staining for XCR1 was seen on epithelial cells (b), infiltrating lymphocytes (b) and fibroblasts (b). Lymphotactin staining was also observed on epithelial cells (c), lymphocytes (c) and fibroblasts (c). Staining was performed three times on every sample.

6.6 DISCUSSION

The immunohistochemistry results showed that XCR1 is expressed *in vivo* in normal oral epithelium and appears to be up-regulated in cancer. This corroborates our previous *in vitro* results which also showed an up-regulation of XCR1 expression in cancerous oral epithelial cells compared with normal keratinocytes. However, this up-regulation does not appear to be specific to OSCC and was also seen in other diseases such as dysplasia, lichen planus and ulceration. A similar pattern of XCR1 staining was observed in all samples and staining was predominantly localized to the lower two third of the mucosa. In addition to the epithelium, XCR1 expression was also observed on other cell types including lymphocytes, fibroblasts and endothelial cells. No obvious difference in XCR1 staining intensity was noticed between OSCC, dysplasia, lichen planus or ulceration. However this was not studied in depth.

XCR1 staining in the lower two third of the mucosa in some conditions (such as dysplasia) suggests that XCR1 expression might be correlated to proliferation as the basal cell layer is continuously renewing itself and maturing cells are moving upwards in the mucosa. In support of this is our finding that lymphotactin stimulates epithelial cell proliferation through XCR1 *in vitro*. However, this potential correlation between XCR1 expression and oral epithelial cell proliferation/maturation needs to be studied in more detail to be established.

Stronger staining for XCR1 was also seen in infiltrating lymphocytes in OSCC compared with normal tissue. In addition many more lymphocytes in OSCC appeared to express XCR1 than in normal epithelium but this was only a subjective assessment. This apparent up-regulation of XCR1 in lymphoid and epithelial cells may have a number of implications. Increased XCR1 expression by epithelial cells may allow them to respond to lymphotactin by migrating, invading and proliferating. The increased recruitment of XCR1 expressing lymphocytes suggests they may have been attracted by lymphotactin and these lymphocytes may exert anti-tumour effects or produce pro-inflammatory cytokines which may or may not regulate the expression of XCR1 and lymphotactin and ultimately play a role in the biology of the disease. Therefore it is important to establish the source of lymphotactin if any in the oral epithelium.

Very different results were obtained for lymphotactin. No lymphotactin expression was seen in the normal oral mucosa. Intraepithelial lymphocytes (IEL), infiltrating lymphocytes and epithelial cells did not stain for lymphotactin. In contrast to that, lymphotactin expression was observed in diseased oral mucosa which was unexpected. Lymphotactin staining was not only seen in the IEL and infiltrating lymphocytes but surprisingly also in the epithelium. Boismenu *et al.* have shown lymphotactin expression in activated IEL in mouse epidermis (Boismenu *et al.*, 1996). However, our results suggest that there may be multiple potential sources for lymphotactin in the oral epithelium. It appears that IEL in oral mucosa only express lymphotactin in conditions like inflammation, dysplasia and cancer and not in normal mucosa suggesting that some sort of trigger is required for lymphotactin expression. However the nature of this trigger is not known. In addition, staining for lymphotactin on infiltrating lymphocytes in diseased oral mucosa suggests that they are producing lymphotactin by a similar mechanism. And last but not the least; lymphotactin expression by oral epithelial cells suggests that lymphotactin might have the potential to act back on the epithelial cells through an autocrine mechanism.

All these *in vivo* findings confirm previous *in vitro* results and demonstrate expression of lymphotactin outside the lymphoid system for the first time. The different patterns of *in vivo* expression of XCR1 and lymphotactin between normal and diseased oral mucosa suggests an important role for them in oral epithelial cell regulation. It is possible that this increased receptor/ligand expression and interaction may act back on the oral epithelial cells in an autocrine manner and facilitate pathological cell migration, invasion and proliferation. Therefore, it was decided to study lymphotactin expression in oral epithelial cells (constitutive and in response to stimulation) *in vitro* to back up the immunohistochemistry results and to find out what triggers lymphotactin release from these cells.

CHAPTER 7

In vitro Expression of Lymphotactin in Oral Epithelial Cells

7.1 INTRODUCTION

Immunohistochemistry results showed *in vivo* expression of XCR1 in normal, inflammatory and cancerous oral epithelium whereas lymphotactin expression was only seen in inflammation and cancer and not in normal mucosa. XCR1 expression was in agreement with previous *in vitro* results. However, lymphotactin expression was surprising since its *in vivo* expression has not been shown outside the immune system. Therefore, it was important to establish the expression of lymphotactin *in vitro* on normal and cancerous oral epithelial cells to back up the *in vivo* results.

7.2 AIM

The aims of this chapter were to study whether lymphotactin is present in NOK and OCCL *in vitro* and if so, what might regulate its production.

7.3 EXPERIMENTAL PROTOCOL

1. RT-PCR

- RT-PCR was used to study mRNA expression of lymphotactin in NOK, HGF, OCCL (H357, TR146, SCC4, SCC25, CAL27 and FADU) and A375P cells.
- Neutrophil RNA was used as positive control.
- RNA was isolated from the cultured cells to be used for RT-PCR. Isolated RNA was quantified and its quality checked using gel electrophoresis and spectrophotometry.
- Sense and anti-sense primers for lymphotactin were designed.
- RNA samples were DNase-treated to remove genomic DNA contamination.
- RT-PCR was performed three times on different occasions for all cell types.

2. Immunofluorescence

- Immunofluorescence was performed on cultured monolayers of cells to study intracellular lymphotactin expression. Staining was carried out on three different occasions for each cell type.

3. Flow Cytometry

- Flow cytometry was performed on cultured cells (three times) to detect intracellular expression of lymphotactin.

4. ELISA

- An ELISA was performed to study the production of lymphotactin by oral epithelial cells in culture.
- Cells were stimulated with PMA, TNF-α and LPS as they have previously been shown to stimulate the release of chemokines from epithelial cells (von Asmuth *et al.*, 1994). Unstimulated cell supernatants were used as negative controls.
- Triplicate wells for each treatment and cell type were used in every experiment. Assay was performed three times.

7.4 MATERIALS AND METHODS

7.4.1 CELL CULTURE

Cells were grown and maintained as described previously.

7.4.2 RNA ISOLATION

Total RNA was isolated from the cultured cells, DNase-treated and quantified as described previously in Section 2.4.2.

7.4.3 RT-PCR

7.4.3.1 Primer Design

The following primer sequences were used:

F/Sense: CCTCCTTGGCATCTGCTCTC
R/Anti-sense: ATTGGTCGATTGCTGGGTTC

7.4.3.2 RT-PCR Reaction

The RT reaction was performed as previously described in section 2.4.4. The PCR mix was prepared and 24μl of this mix was moved to PCR tubes. 1μl of cDNA was

added to each tube and samples were placed in the PCR machine. The following cycle was used:

- 94 °C for 1 min (Denaturation)
- 60 °C for 2 min (Annealing)
- 72 °C for 3 min (Extention)

After 35 cycles of the above mentioned program, the following steps were carried out:

- 72 °C for 10 min (Final extention step)
- 4 °C forever (cooling cycle)

7.4.3.3 Gel for PCR Products

A 1% agarose gel was used to separate and visualize the PCR products.

7.4.4 IMMUNOFLUORESCENCE

Protein expression was subjectively assessed by performing immunofluorescence on cultured cell monolayers. Cells were grown on chamber slides as mentioned earlier and fixed/permeabilised with 2% paraformaldehyde containing 0.1% Triton-X100 (Sigma) for 10min. Fixative was removed and slides washed in PBS. After PBS removal, rabbit anti-human lymphotactin antibody (20μg/ml) (Peprotech) was added for one hour. Negative controls received pre-absorbed antibody with recombinant lymphotactin. Excess antibody was washed off and FITC-conjugated secondary antibody was applied for 30min. Slides were washed again and mounted in vectashield mounting medium containing DAPI (Vector Labs).

7.4.5 FLOW CYTOMETRY

IntraPrep ™ (fixation and permeabilisation) kit (Beckman Coulter, Fullerton, CA, USA) was used for intracytoplasmic staining. The kit contained 2 reagents, reagent 1 (which is formaldehyde/fixation agent) and reagent 2 (which is saponine/permeability agent).

Single cell suspensions were prepared as previously described and washed in staining buffer (PBS+2%FBS+1%BSA) by centrifugation (400xg, 5min, 4°C). The

supernatants were discarded and cells re-suspended ($1x10^6$cells/ml) in serum-free DMEM and stimulated with PMA (50ng/ml) (Peprotech) for two hours in a CO_2 incubator. PMA was used as it is a potent protein kinase C activator and has been shown to facilitate lymphotactin release from lymphocytes (Blaschke *et al.*, 2003). Cells were washed and resuspended in staining buffer. 50µl of sample was added to a new tube with 100µl of reagent 1 (formaldehyde), vortexed vigorously to avoid clumping and incubated at RT for 15min. Samples were washed using staining buffer as mentioned previously and supernatant was aspirated. 100µl of reagent 2 (saponine) was added to each tube and allowed to diffuse naturally into the cell pellet by incubating at RT for 5min. Primary antibody for lymphotactin was added (20µg/ml), mixed by vortexing and incubated for 30min at 4°C. Rabbit serum served as the negative control.

Samples were washed in 1ml staining buffer (400xg, 5 min, 4°C), and re-suspended. Swine anti-rabbit FITC-conjugated antibody (Dako) was added (10µl/million cells), samples were vortexed and incubated for 30min at 4°C in the dark. Samples were washed again and re-suspended in staining buffer. A FACSCalibur (Becton Dickinson) was used to acquire the data whereas analysis was performed using FlowJo software (Tree Star). Staining was considered positive when the percentage of positive cells of lymphotactin antibody was higher than the negative controls.

7.4.6 ELISA

Lymphotactin levels in culture supernatants were determined by an ELISA using lymphotactin duo set (R&D Systems) in accordance with the manufacturer's instructions. All assays were performed in triplicate and a standard curve for each assay determined the number of cells.

Lymphotactin capture antibody (360µg/ml of mouse anti-human lymphotactin antibody) was diluted to the working concentration in PBS and a 96-well microplate was coated with 100µl per well of the diluted capture antibody. The plate was sealed and incubated overnight at 4°C. The next day, the capture antibody was removed and each well washed three times with wash buffer (0.05% Tween20 in PBS). Remaining wash buffer was removed by aspirating and inverting the plate and blotting it against

clean paper towels. The plate was blocked by adding 300µl of reagent diluent (1% BSA in PBS-filtered with 2% normal goat serum) to each well and incubated at RT for two hours. The aspiration/wash was repeated as described earlier.

100µl of sample or standards were added per well. The plate was covered with an adhesive strip and incubated for two hours at RT. Samples were removed and the aspiration/wash step was repeated. This was followed by the addition of 100µl of detection antibody (36µg/ml of biotinylated goat anti-human lymphotactin antibody diluted in reagent diluent) to each well. The plate was covered with a new adhesive strip and incubated for two hours at RT. After two hours, detection antibody was removed and plate was washed three times with wash buffer. 100µl of working dilution of streptavidin-HRP was then added to each well. The plate was covered and incubated for 20min at RT in the dark. After three more washes with wash buffer, 100µl of substrate solution (TMB) (BD) was added to each well for 20min at RT in the dark till the strongest colouring wells were medium to dark blue. 50µl of stop solution (2N H_2SO_4) was added to each well to stop colour development. The optical density of each well was measured immediately, using a microplate reader set to 450nm.

7.5 RESULTS

7.5.1 RT-PCR

Lymphotactin mRNA was seen in NOK, HGF and OCCL (H357, SCC4, TR146, CAL27 and FADU), the positive control and the melanoma cell line A375P. Only SCC25 cells did not contain lymphotactin mRNA (Figure 7.1).

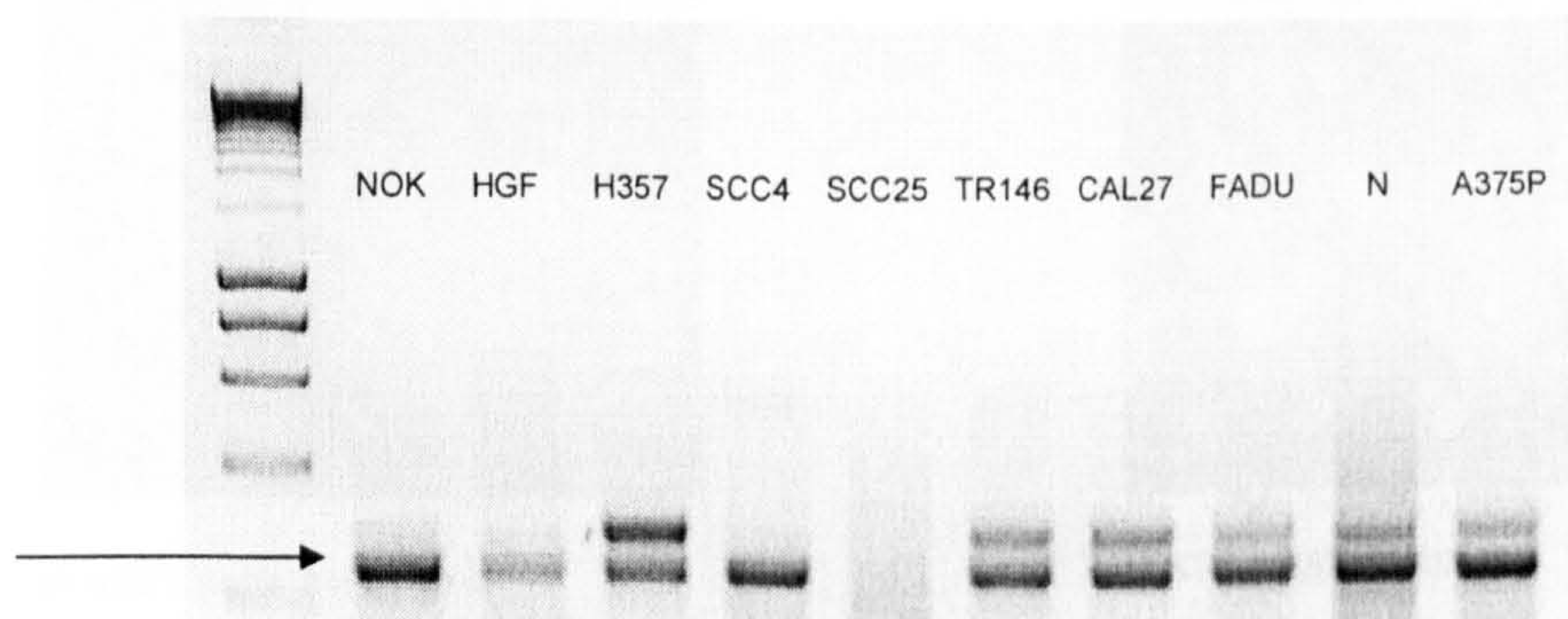


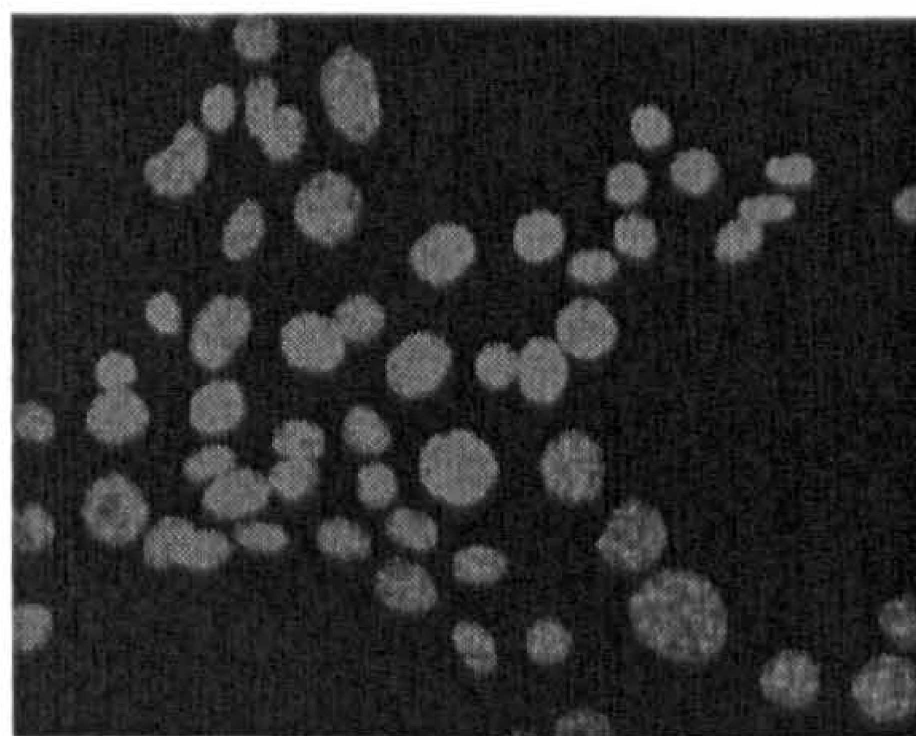
Figure 7.1. mRNA expression of lymphotactin (Representative picture). Strong bands were

observed for NOK, H357, SCC4, TR146, CAL27, FaDu, Neutrophils and A375P. HFG showed weak band intensity whereas SCC25 were negative for lymphotactin mRNA.

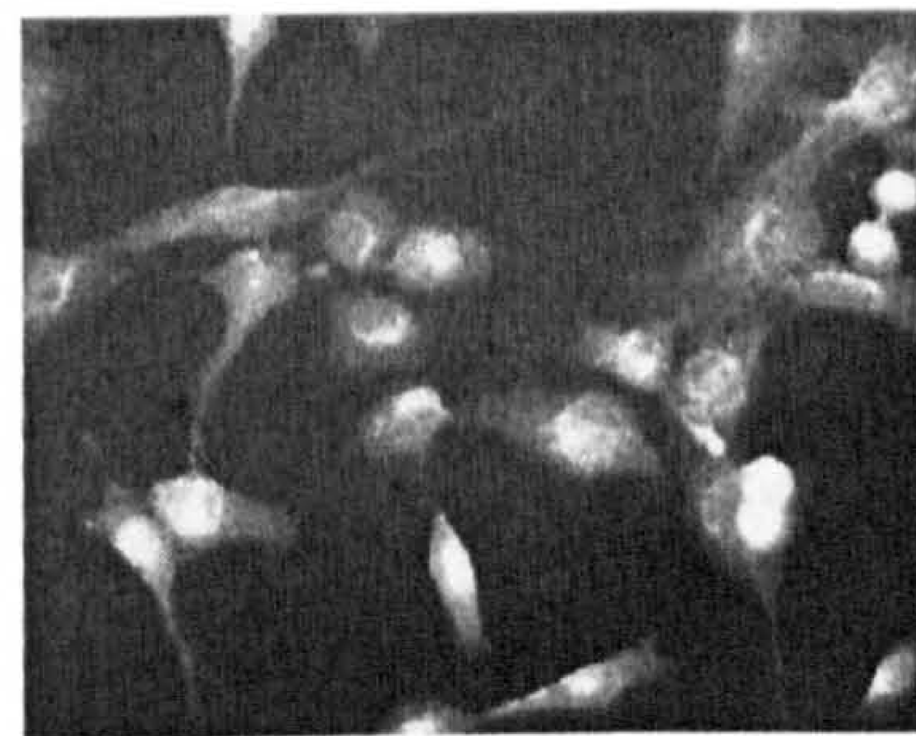
Multiple bands were observed in some cell types. All bands were gel extracted, transformed into *E. Coli* and mini-prepped. DNA sequencing was carried out which confirmed the lower bands as lymphotactin (Appendix 10.5).

7.5.2 IMMUNOFLUORESCENCE

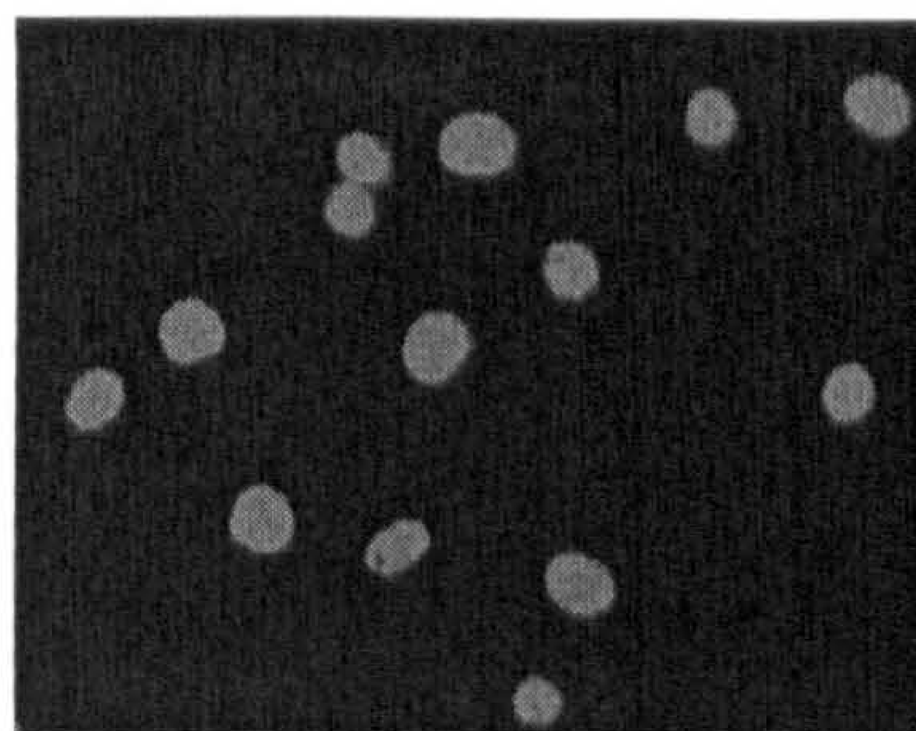
NOK, H357 and SCC4 cells were studied for intracellular expression of lymphotactin. Expression was seen in all tested cells whereas no expression was seen in the negative controls. Lymphotactin expression was predominantly cytoplasmic but some nuclear staining was also observed particularly in NOK and H357 cells. Staining intensity seemed slightly stronger in H357 and SCC4 cells compared with NOK (Figure 7.2). Some negative cells were also observed in NOK and OCCL.



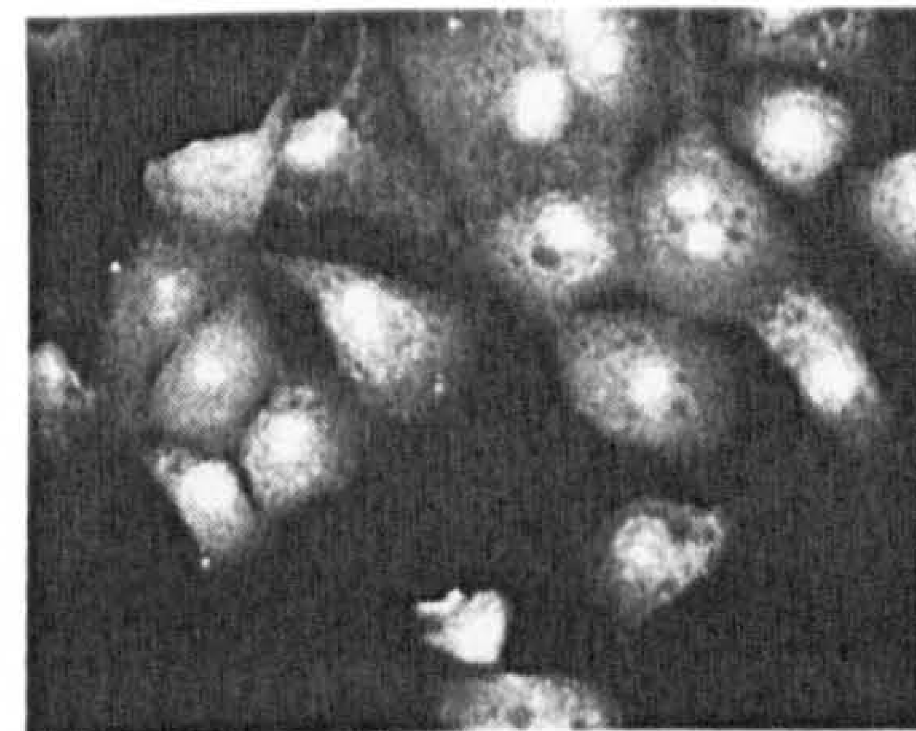
a) NOK negative control



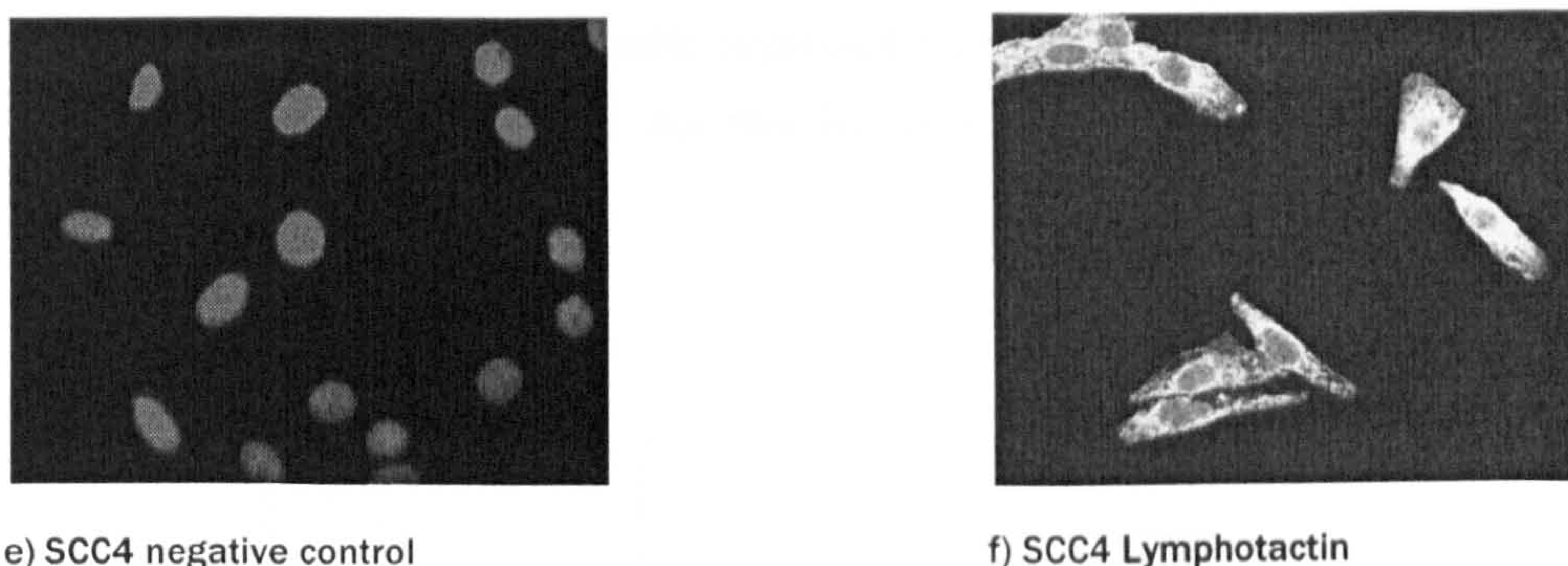
b) NOK Lymphotactin



c) H357 negative control



d) H357 Lymphotactin



e) SCC4 negative control

f) SCC4 Lymphotactin

Figure 7.2. Representative picture showing intracellular expression of lymphotactin in NOK, H357 and SCC4 cells as shown by immunofluorescence (Original magnification x 60 - antibody concentration 20μg/ml). No staining was observed in the negative controls (a, c, e). Intracellular staining for lymphotactin was seen in NOK (b), H357 (d) and SCC4 (e) cells.

7.5.3 FLOW CYTOMETRY

i) Jurkat

Constitutive intracellular expression of lymphotactin was observed intracellularly in the Jurkat cells as shown by a distinct shift towards the right in the histogram. Stimulation of the cells with PMA failed to cause a significant increase in intracellular lymphotactin (Figure 7.3).

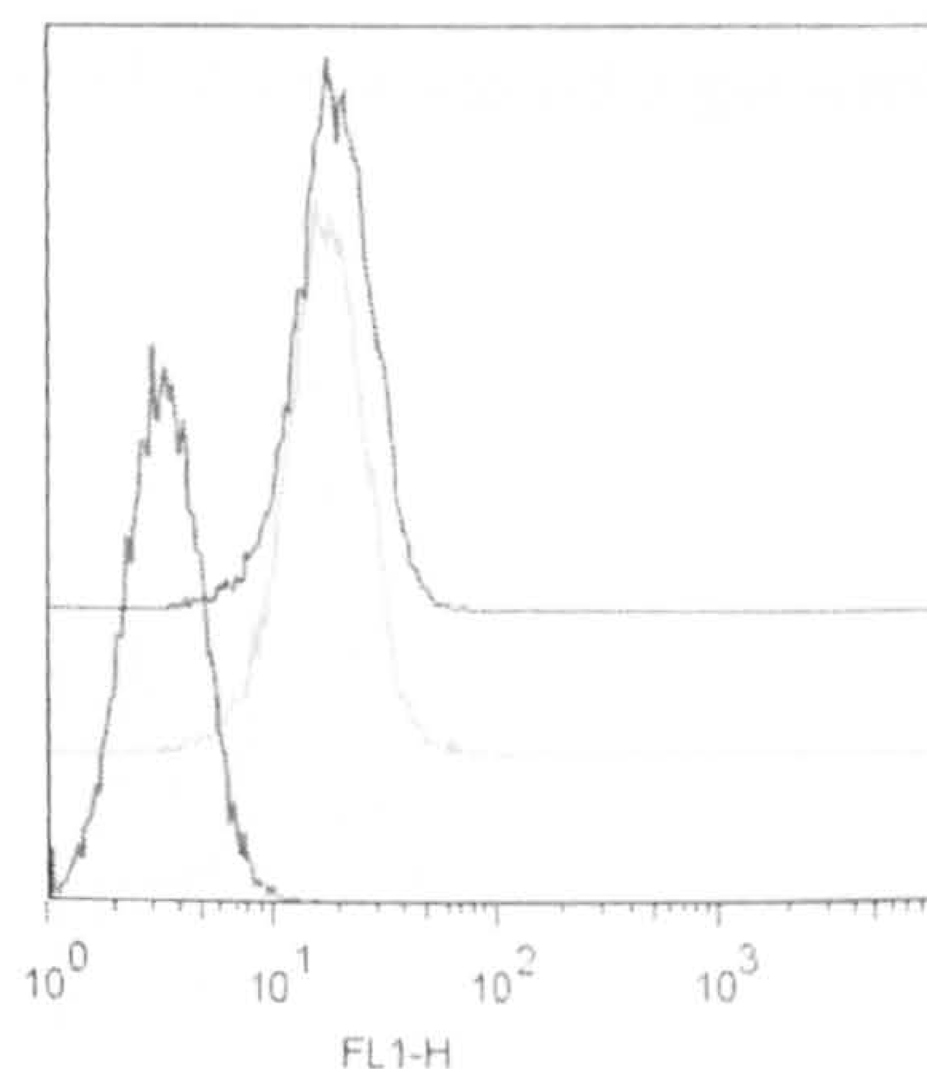


Figure 7.3. Representative histogram showing fluorescence intensity for lymphotactin expression in Jurkat cells. Red- Negative Control, Green- Unstimulated cells, Blue- Cells stimulated with PMA. Experiment was performed three times.

91.4% of unstimulated Jurkat cells expressed lymphotactin. Stimulation with PMA increased expression to 93.6% but this increase was not significant compared to unstimulated cells (Figure 7.4).

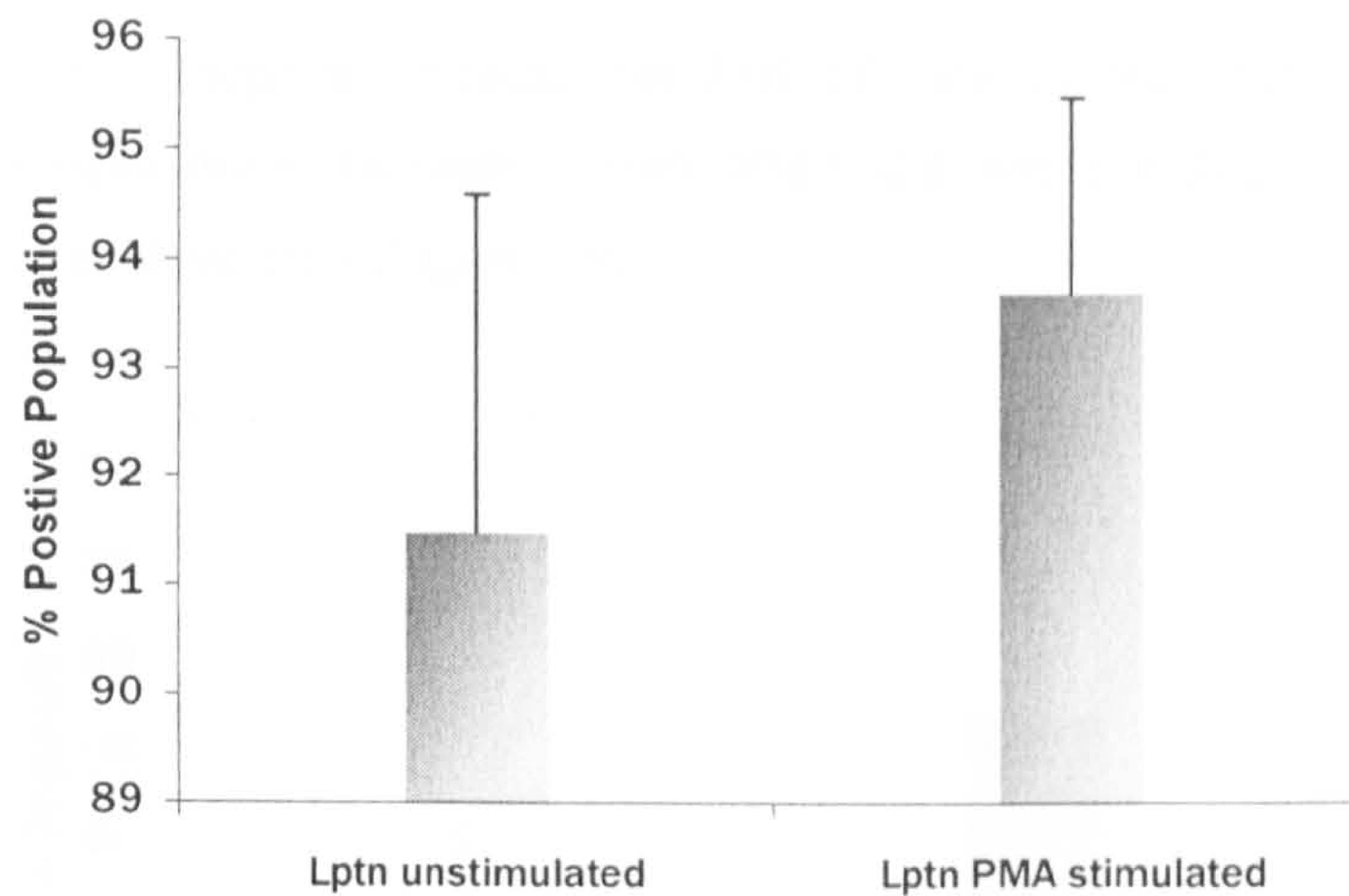


Figure 7.4. Percentage of Jurkat cells expressing lymphotactin (n=average of 3 different experiments, error bars=SD). Constitutive intracellular staining was seen in unstimulated cells. 2 hour stimulation with PMA caused a slight increase compared with unstimulated cells but was not significant.

ii) NOK

Lymphotactin was also detected in unstimulated NOK. This expression was slightly increased after exposure to PMA but was not significant (Figure 7.5).

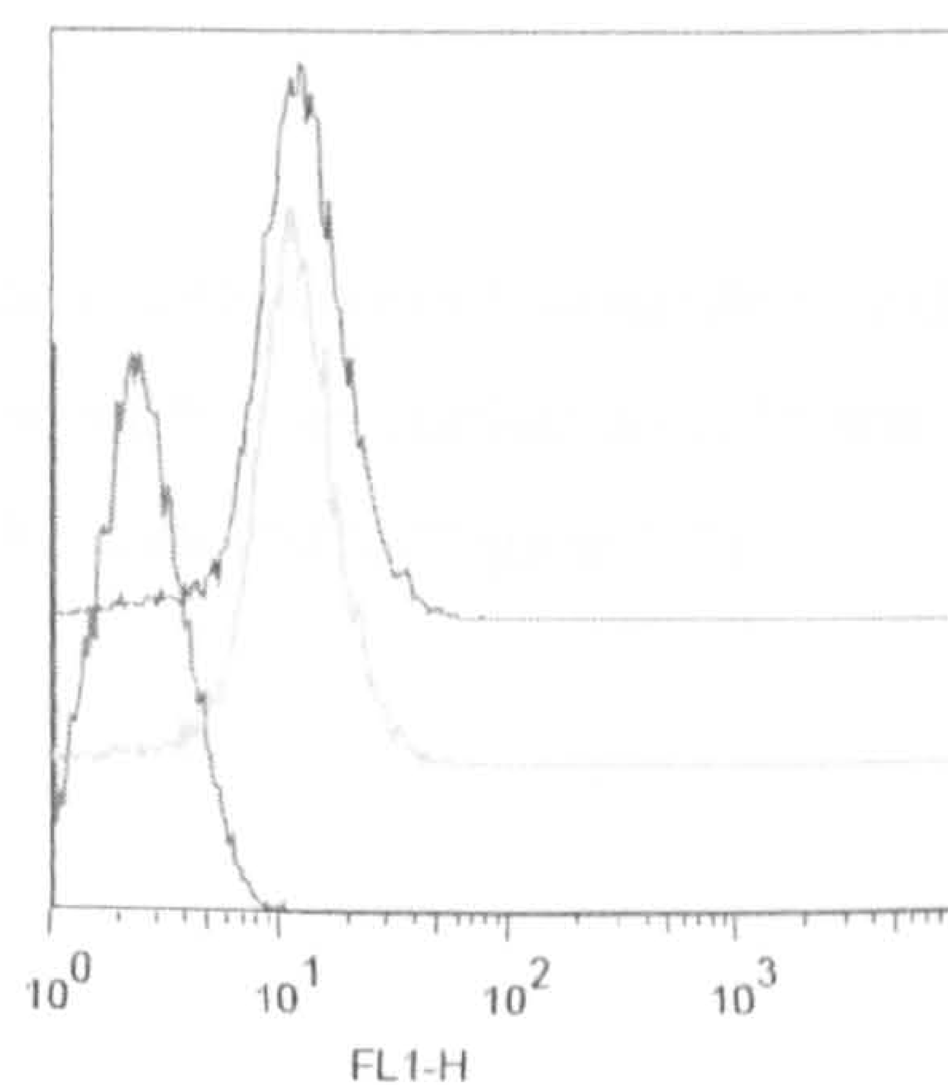


Figure 7.5. Representative histogram showing fluorescence intensity for lymphotactin

expression in NOK. Red- Negative Control, Green- Unstimulated cells, Blue- Cells stimulated with PMA. Experiment was performed three times.

Analysis of the histogram revealed that 53% of unstimulated NOK constitutively expressed lymphotactin. Incubation with PMA did not significantly change the percentage of positive cells (Figure 7.6).

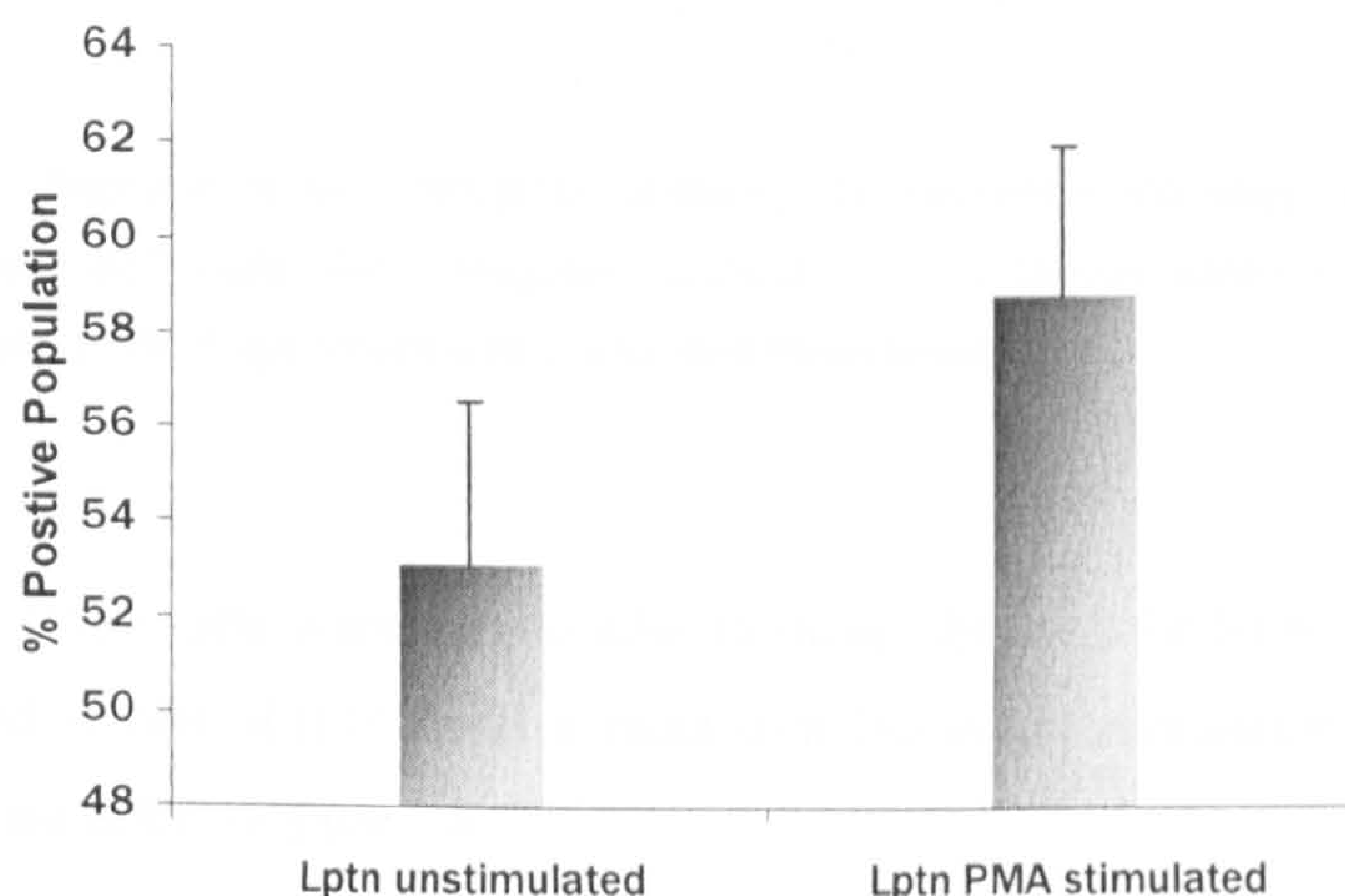


Figure 7.6. Percentage of NOK expressing lymphotactin (n=average of 3 different experiments, error bars=SD). Constitutive intracellular staining was seen in unstimulated cells. 2 hour stimulation with PMA caused a slight increase compared with unstimulated cells but was not significant.

iii) H357

Unstimulated H357 cells also showed consistent expression of lymphotactin (as shown by the histogram shift). No further shift in the histogram was observed after stimulation of H357 cells with PMA (Figure 7.7).

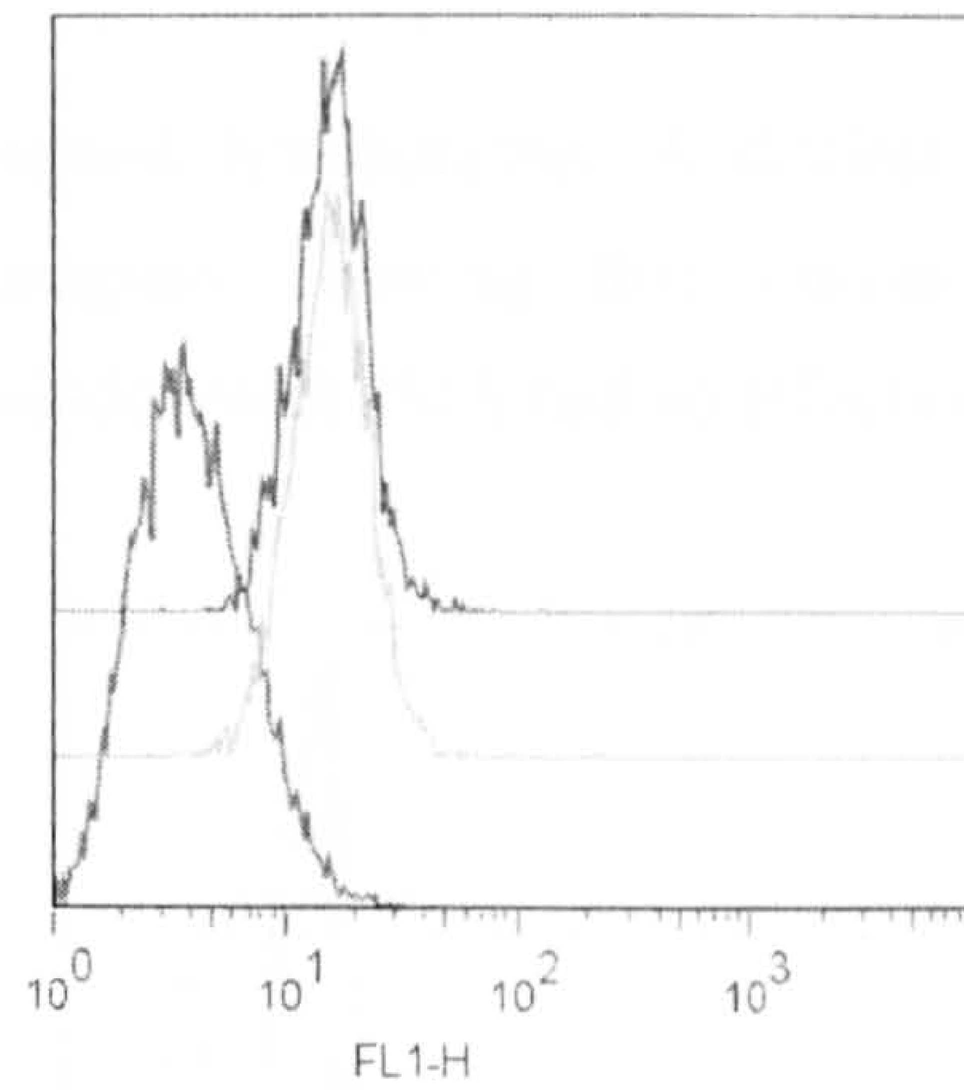


Figure 7.7. Representative histogram showing fluorescence intensity for lymphotactin expression in H357 cells. Red- Negative Control, Green- Unstimulated cells, Blue- Cells stimulated with PMA. Experiment was performed three times.

Results for H357 cells were very similar to those obtained for NOK. Lymphotactin was detected in 49% of H357 cells without stimulation and stimulation with PMA has not significant effect (Figure 7.8).

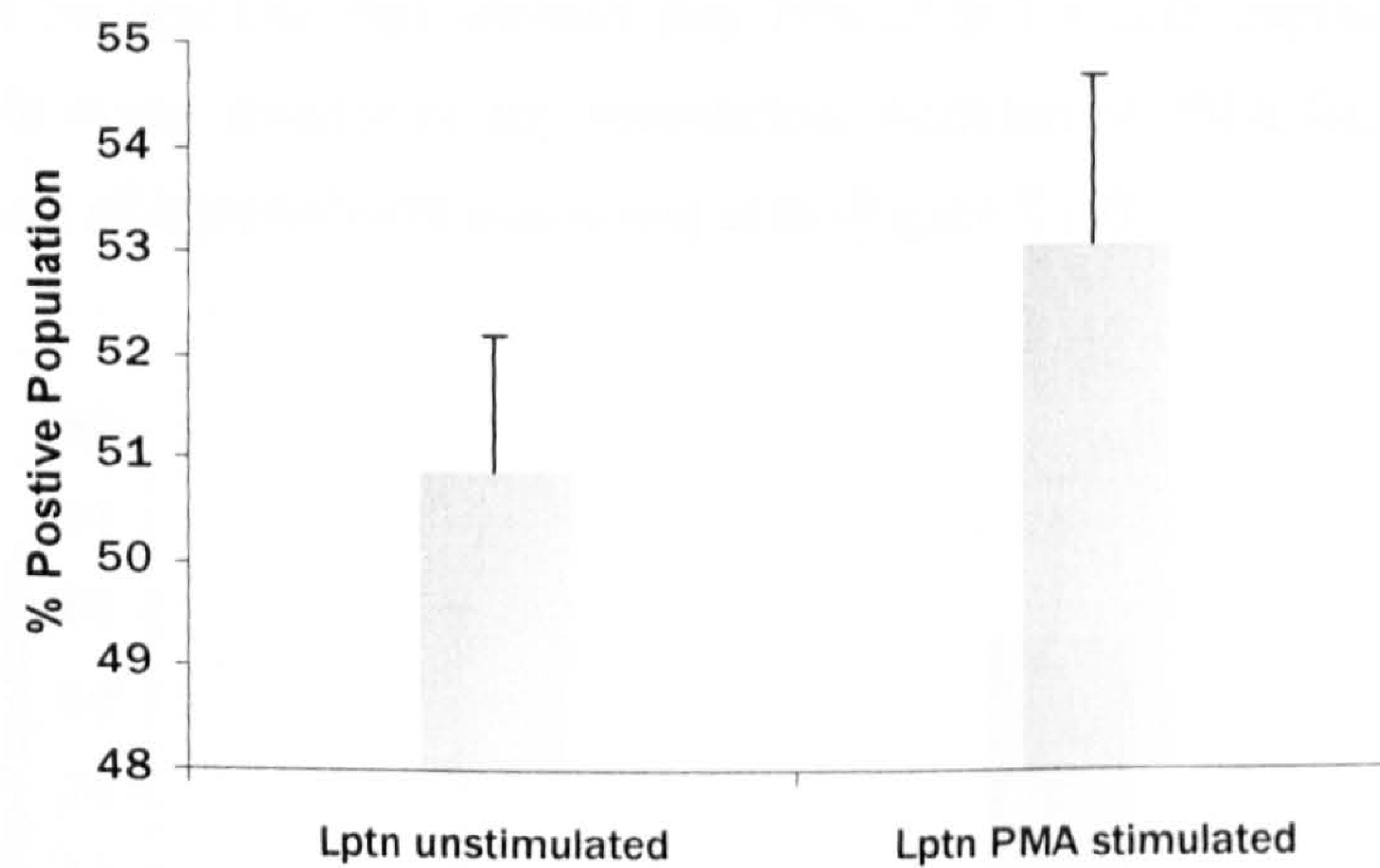


Figure 7.8. Percentage of H357 cells expressing lymphotactin (n=average of 3 different experiments, error bars=SD). Constitutive intracellular staining was seen in unstimulated cells. 2 hour stimulation with PMA caused a slight increase compared with unstimulated cells but was not significant.

iv) SCC4

SCC4 cells also expressed lymphotactin. A distinct shift towards the right was observed in the histogram showing that unstimulated SCC4 cells express lymphotactin and stimulation with PMA had no effect (Figure 7.9).

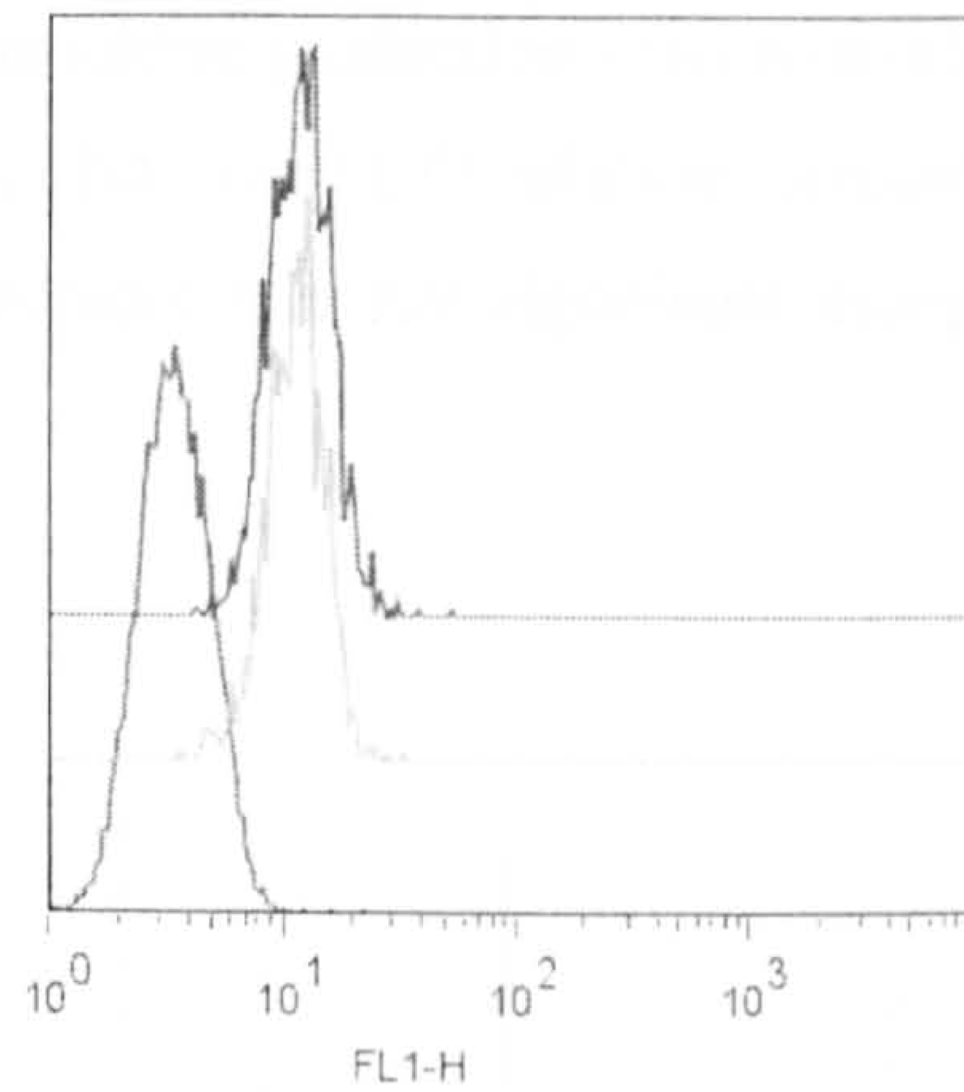


Figure 7.9. Representative histogram showing fluorescence intensity for lymphotactin expression in SCC4 cells. Red- Negative Control, Green- Unstimulated cells, Blue- Cells stimulated with PMA. Experiment was performed three times.

Analysis of the acquired data showed that 75% of SCC4 cells express intracellular lymphotactin in the absence of any stimulation. Addition of PMA failed to increase the percentage of lymphotactin expressing cells (Figure 7.10).

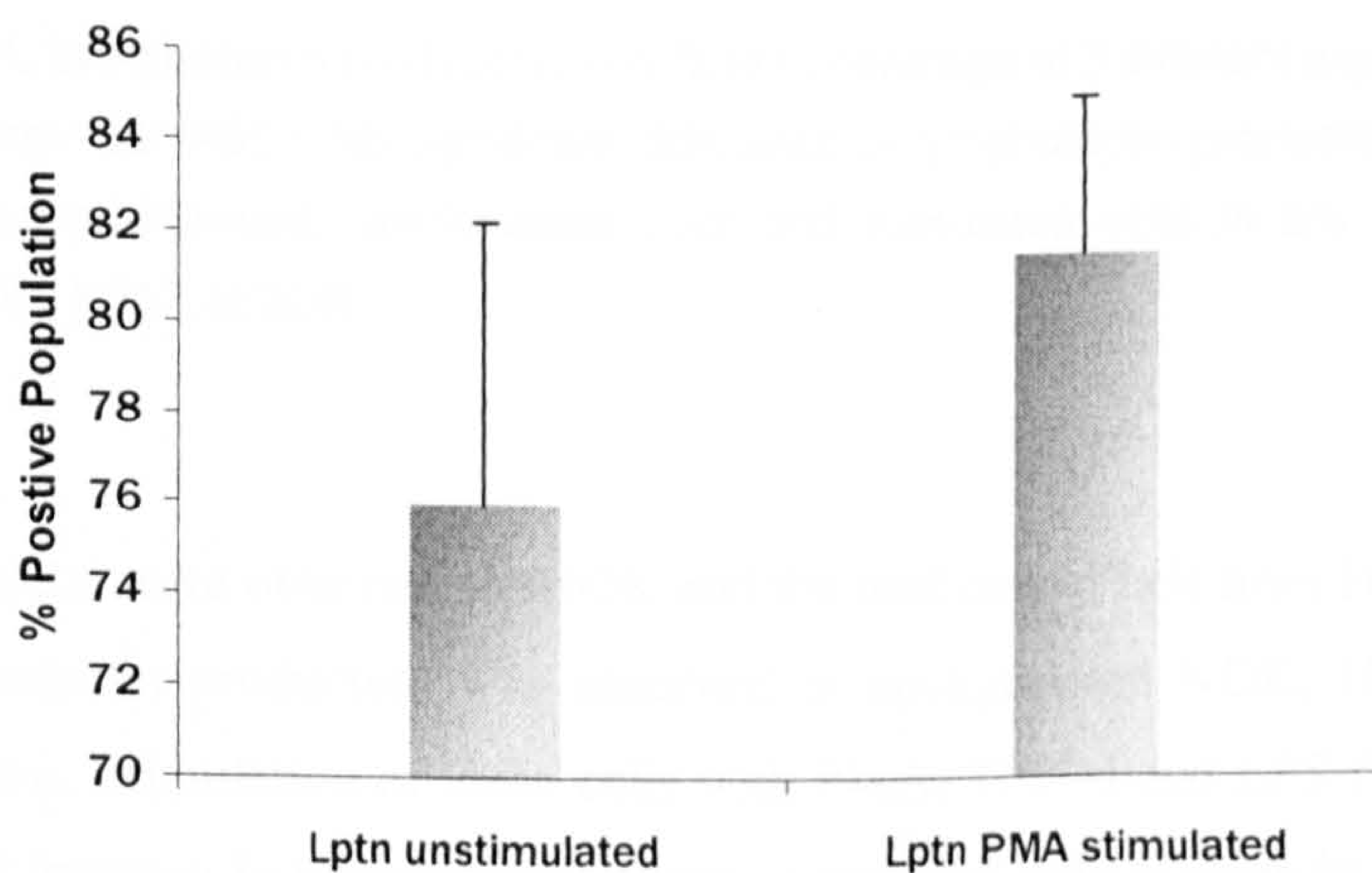


Figure 7.10. Percentage of SCC4 cells expressing lymphotactin (n=average of 3 different experiments, error bars=SD). Constitutive intracellular staining was seen in unstimulated

cells. 2 hour stimulation with PMA caused a slight increase compared with unstimulated cells but was not significant.

7.5.4 ELISA

Cells were stimulated with PMA, TNF-α and LPS as they have previously been shown to stimulate chemokine production (von Asmuth *et al.*, 1994). Stimulation of Jurkat cells with PMA, TNF-α and LPS slightly increased lymphotactin production in culture however the increase was not significant compared with unstimulated cells (Figure 7.11).

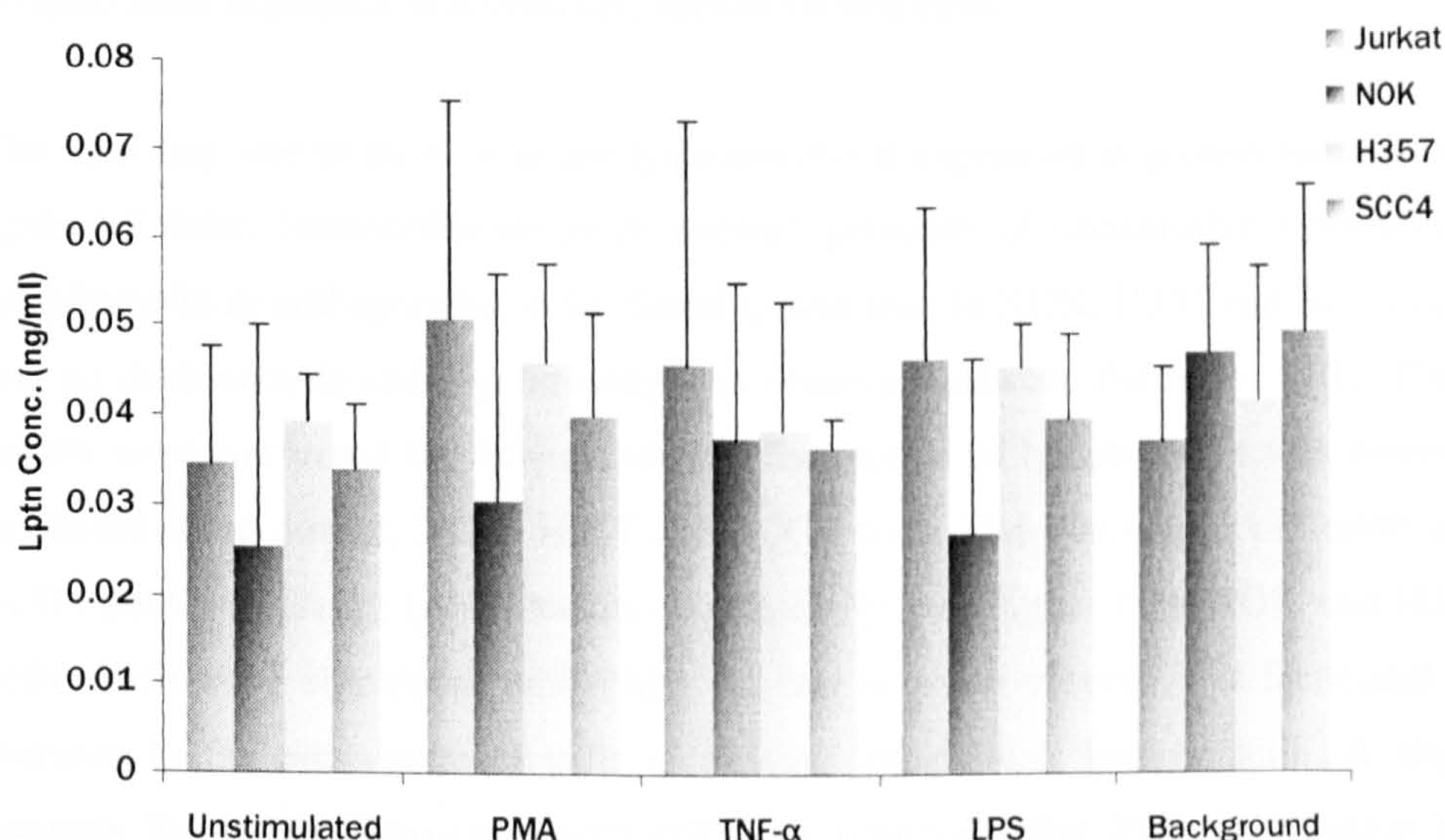


Figure 7.11. Lymphotactin production in culture (n=average of 3 different experiments done in triplicate, error bars=SD). No significant difference in lymphotactin production was observed between the background, unstimulated cells and stimulated cells in any of the cell lines (Jurkat, NOK, H357, SCC4).

Similar results were obtained for NOK and the oral cancer cell lines H357 and SCC4. No lymphotactin production was observed in unstimulated NOK, H357 and SCC4 cells *in vitro*. Stimulation of these cells with PMA, TNF-α and LPS failed to cause a significant increase in lymphotactin release compared with unstimulated cells (Figure 7.11).

7.6 DISCUSSION

After discovering *in vivo* expression of lymphotactin in inflammatory and cancerous oral mucosa, expression of lymphotactin was studied *in vitro* on normal and cancerous oral epithelial cells.

A band for lymphotactin mRNA was seen in neutrophil RNA as well as NOK and HGF. RT-PCR showed multiple bands but gel extraction, transformation and DNA sequencing confirmed the presence of lymphotactin mRNA. A similar band was observed in the OCCL (H357, SCC4, TR146, CAL27 and FADU) whereas SCC25 cells were negative for lymphotactin. This confirmed that the message for lymphotactin is present in normal and cancerous oral cells.

The next step was to study whether lymphotactin is expressed at protein level in oral epithelial cells. Immunofluorescence showed presence of constitutive intracellular lymphotactin in oral epithelial cells. Staining was seen in NOK, H357 and SCC4 cells and no difference in staining intensity was observed between the tested cells. These results were confirmed by flow cytometry. Expression of lymphotactin was detected in unstimulated Jurkat, NOK, H357 and SCC4 cells. The percentage of Jurkat and SCC4 cells expressing lymphotactin constitutively was higher than NOK and H357 cells. Cells were stimulated with PMA for two hours to observe if it facilitated an increase in the percentage of cells expressing intracellular lymphotactin. A slight increase in lymphotactin-expressing cells was observed after PMA stimulation but was not significant compared with controls.

An ELISA was used to study whether oral epithelial produce lymphotactin in culture. No lymphotactin secretion in cell supernatants was detected in unstimulated Jurkat, NOK, H357 or SCC4 cells. Cells were exposed to a range of stimulants including PMA, TNF-α and LPS to study whether they trigger lymphotactin secretion from cells in culture. However, no lymphotactin production was detected in any of the cells even after stimulation. This suggests that some other stimulant might be required to trigger lymphotactin production in culture. To date, lymphotactin secretion in culture has only been shown in primary isolated lymphotcytes. Stievano *et al.*, reported that CD8+ αβ+ T cells lacking expression of CD5 on cell surface are the major source of

lymphotactin in peripheral blood. Stimulation with PMA significantly increases lymphotactin production in culture (Stievano *et al.*, 2003). Lymphotactin production has been shown by CD4+ and CD8+ T cells only after T cell-receptor (TCR)-dependent stimulation using staphylococcus superantigens (TSST-1 and SEB) and not after treatment by inflammatory cytokines TNF-α, IL-1βor LPS (Tikhonov *et al.*, 2001). This is in accordance with our results as TNF-α and LPS failed to facilitate an increase in lymphotactin release from NOK and OCCL. Since TCR activation is mandatory for staphylococcus superantigen mediated T lymphocytes activation, this suggests that lymphotactin production can be induced by T cell-receptor signals. A role for *Porphyromonas gingivalis* in lymphotactin production *in vitro* has also been reported. Stimulation of mouse peritoneal macrophages with live *P. gingivalis* resulted in lymphotactin secretion however *P. gingivalis* LPS or its fimbrial protein FimA failed to do so (Zhou *et al.*, 2005). This suggests that immune cells can detect live bacteria and their components in a different manner and this may influence the inflammatory response.

To conclude, lymphotactin mRNA is present in NOK and OCCL and the protein is constitutively expressed intracellularly. However, no lymphotactin was detected in cell supernatants even after stimulation of normal and cancerous oral cells with PMA, TNF-α and LPS. In previous chapter abundant *in vivo* expression of lymphotactin in diseased oral epithelial cells was observed. Even though the mRNA for lymphotactin was observed in normal as well as cancerous oral epithelial cells, no production in culture was observed even after stimulation. This may be explained by the complex tissue microenvironment *in vivo* where a wide range of stimulants are operational. It is possible that the stimulants we used do not influence lymphotactin production from oral epithelial cells and some other stimulant may be more important in this regard.

CHAPTER 8

Role of XCR1 and Lymphotactin Interaction in Oral Epithelial Cell Adhesion and MMP Production

8.1 INTRODUCTION

Previous chapters confirmed constitutive intracellular *in vitro* expression of lymphotactin in normal and malignant oral epithelial cells. However, *in vivo* expression of lymphotactin is observed only in diseased oral epithelium. XCR1 on the other hand is expressed *in vitro* and *in vivo* in normal and diseased mucosa however its expression appears to be up-regulated in inflammation and cancer. We have also shown that XCR1 is functional on the surface of oral epithelial cells and can mediate migration, invasion and proliferation.

Epithelial cell adhesion is a fundamental aspect of cell migration and invasion. Epithelial cells need to adhere to ECM components in order to move and increased ability to do so may be evident in wound healing (Santoro and Gaudino 2005) as well as in cancer (Kramer, Shen, and Zhou 2005). The interaction of chemokine receptors and chemokines also influences adhesion and cell migration in wound healing and cancer (Raja *et al.*, 2007). Interaction of CX3CR1 with fractalkine has been shown to facilitate adhesion of prostate cancer cells (Shulby *et al.*, 2004). However the role of XCR1/lymphotactin interaction in epithelial cells adhesion has not been studied to date.

MMP production also facilitates the migratory/invasive response. They are a family of enzymes that help the cells to break down the ECM and therefore facilitate physiological and pathological cell migration and invasion (Bjorklund and Koivunen 2005; Folgueras *et al.*, 2004; Stamenkovic 2003). The role of chemokines in MMP regulation is well documented in a range of cancers. Chemokine receptors such as CXCR4, CCR7, CCR1 facilitate an increase in MMP production and hence play an important role in the biology of the cancer (Redondo-Munoz *et al.*, 2007; Wu *et al.*, 2007; Yuecheng and Xiaoyan 2007b). A role of lymphotactin in MMP regulation has been shown as stimulation of synoviocytes in the rheumatoid synovium with lymphotactin results in down-regulation of MMP-2 production (Blaschke *et al.*, 2003). However, nothing is known about role of XCR1 and lymphotactin interaction in MMP production and regulation in epithelial cells.

8.2 AIM

The aim of this chapter was to establish the role of XCR1 and its ligand lymphotactin in adhesion of oral epithelial cells to ECM components and in MMP production by these cells *in vitro.*

8.3 EXPERIMENTAL PROTOCOL

- NOK, H357 and SCC4 cells were used in these assays.
- Adhesion assays were performed to study the role of XCR1 and lymphotactin in adhesion to fibronectin and collagen using cell titre cell proliferation reagent (Promega).
- Production of total MMP-2, -7 and -9 was studied in culture supernatants since they have been shown to be important in the biology of OSCC which is discussed in detail later (de Vicente *et al.*, 2005; de Vicente *et al.*, 2007; Kato *et al.*, 2005; Patel *et al.*, 2007; Vairaktaris *et al.*, 2007). In addition, oral epithelial have been shown to produce MMP-2, MMP-7 and MMP-9 in culture. For this purpose, Quantikine ELISA kits were used.
- Blocking antibody for XCR1 was used to study its role in cell adhesion and MMP production.
- CCL25/TECK was used as an irrelevant chemokine and CXCR1 as an irrelevant antibody.
- Triplicate wells for each treatment and cell type were used in every adhesion and MMP assays. Both experiments were performed three times on different occasions.

8.4 MATERIALS AND METHODS

8.4.1 CELL CULTURE

Cells were grown and maintained as described previously.

8.4.2 ADHESION ASSAY

Adhesion assays were performed using fibronectin and collagen (Sigma) in 96-well tissue culture plates (Corning). The plates were coated with fibronectin or collagen

(Sigma) (10µg/ml) via an overnight incubation at 4°C. Negative control wells were not coated. Non-specific adhesion was blocked by incubating the plate for one hour with PBS containing 1% BSA (filter sterilized) at 37°C in a CO_2 incubator.
Cells were non-enzymatically removed from tissue culture flasks using cell dissociation solution (Sigma) and re-suspended in serum free medium (Appendix 10.1.5). Some cells were incubated with XCR1 antibody (Lifespan) (20µg/ml) or an irrelevant antibody for at least 30min at 37°C. Cells were then stimulated with lymphotactin (R&D Systems) (10ng/ml, 100ng/ml and 500ng/ml) for two hours at 37°C with gentle agitation. Sterile PBS was used to wash the 96 well plate and aspirated completely. $4x10^4$ cells were seeded in each well and allowed to adhere for an hour at 37°C. Cells in the control wells were stimulated with an irrelevant chemokine CCL25/TECK (Peprotech) (100ng/ml).

After an hour medium was aspirated and unbound cells were washed off by washing twice in PBS. Fresh medium was added to all wells. 20µl of cell titre proliferation reagent (Promega) was added to each well. It contains the tetrazolium salt MTS which is cleaved by metabolically active cells and provides a way to quantify the number and percentage of adhered cells. Absorbance was recorded at 492nm using a microplate reader. A standard curve for each assay determined the number of cells and all assays were performed in triplicate. Data was expressed as percentage increase in cell adhesion.

8.4.3 MMP PRODUCTION *IN VITRO*

8.4.3.1 Treatments and sample collection

MMP production in culture was studied using Quantikine ELISA kits for MMP-2, -7 and -9 (R&D Systems). NOK, H357 and SCC4 cells ($1x10^5$ cells per well) were seeded in 6 well tissue culture plates and allowed to adhere overnight. Medium was aspirated and cells washed thoroughly with PBS to remove any traces of FBS. In some wells, cells were incubated with XCR1 blocking antibody or the irrelevant CXCR1 antibody for 60 min at 37°C in a CO_2 incubator. Afterwards, cells were exposed to serum free DMEM containing lymphotactin (R&D Systems)(100ng/ml), PMA (Sigma)(50ng/ml), TNF-α (Peprotech)(100ng/ml) and LPS

(Peprotech)(100ng/ml) or the irrelevant chemokine TECK (Peprotech)(100ng/ml) for 48 hours. Supernatants were collected, briefly centrifuged to remove any cellular debris, aliquoted and stored at -80°C.

8.4.3.2 Assay procedure

The assays were performed in accordance with the manufacturer's instructions. All reagents and samples were brought to RT before use and prepared as directed. Samples were diluted in assay diluent and added to the 96 well plate (pre-coated with capture antibody), the plate was sealed and incubated for 2 hours at RT on a horizontal orbital microplate shaker set at 500 rpm.

All samples were aspirated and the plate was washed with wash buffer (provided with the kit) three times using a squirt bottle. Remaining wash buffer was removed by aspirating and the plate was dried by inverting and blotting it against clean paper towels. 200µl of conjugated secondary antibody was added to each well. The plate was sealed with a new adhesive strip and incubated for 2 hours at RT on the shaker. After 2 hours the secondary antibody was removed and the plate was washed three times and dried as described previously. 200µl of substrate solution was added to each well and incubated for 30min at RT in the dark. This was followed by the addition of 50µl of stop solution to each well which changed the colour from blue to yellow. The optical density of each well was determined using a microplate reader set to 450nm. All treatments were performed in triplicate.

8.4.4 STATISTICAL ANALYSIS

Data are presented as means +/- SD. Paired student's T test was used to determine the significance of the results obtained from the adhesion and MMP assays. A p-value of ≤ 0.05 was considered significant.

8.5 RESULTS

8.5.1 ADHESION ASSAYS

8.5.1.1 Adhesion to fibronectin

i) NOK

Stimulation of NOK with lymphotactin resulted in a significant increase in adhesion to fibronectin ($p<0.001$) in a dose dependent manner whereas no such effect was observed with the irrelevant chemokine TECK (Figure 8.1). Addition of XCR1 blocking antibody significantly reduced adhesion at all doses ($p<0.05$) and brought it back to control level. The irrelevant control antibody CXCR1 on the other hand failed to reduce adhesion.

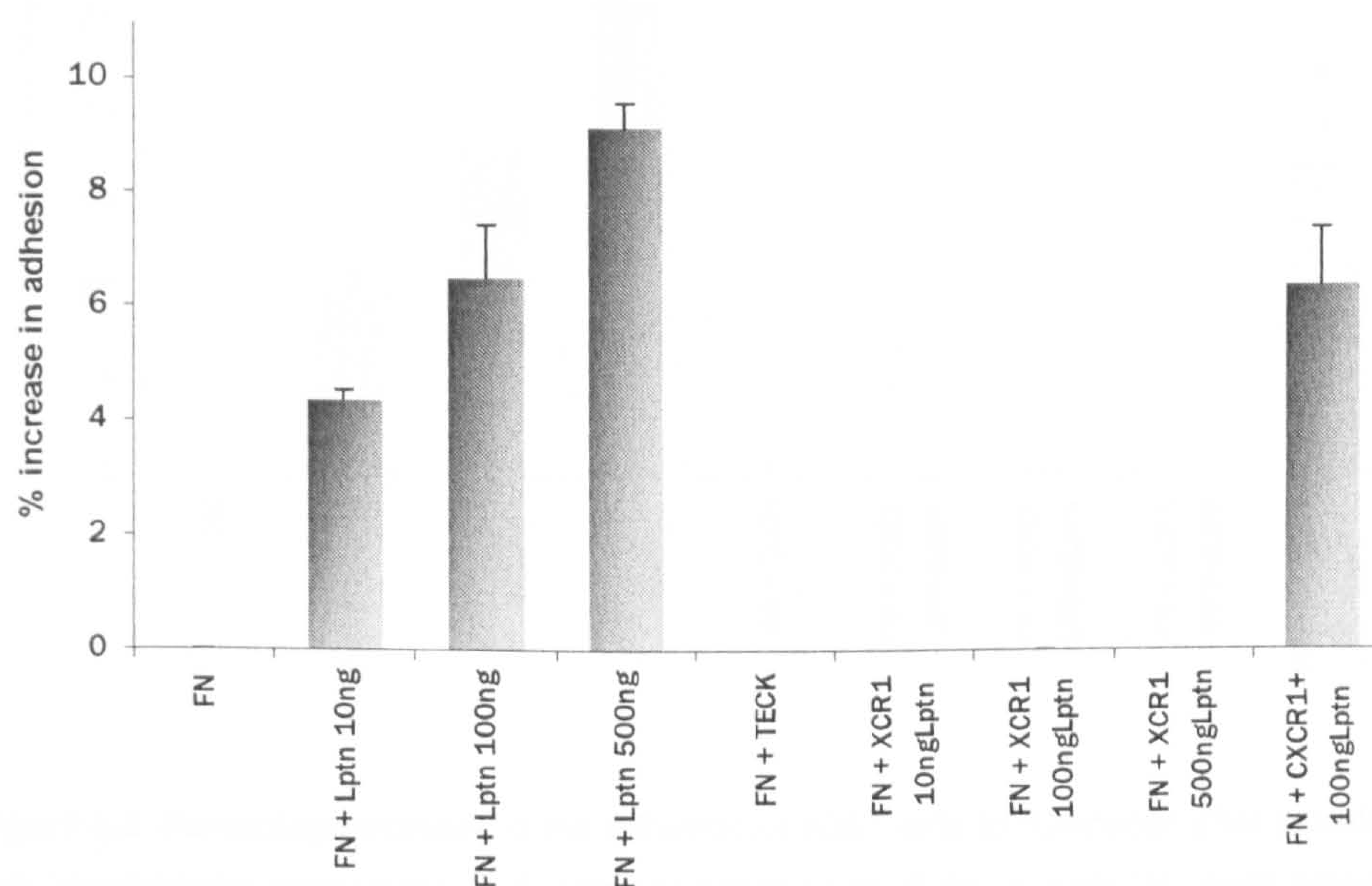


Figure 8.1. Percentage increase in the adhesion of NOK to fibronectin after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin increased adhesion to fibronectin in a dose dependent manner whereas no affect was seen with the irrelevant chemokine TECK. Preincubation with XCR1 antibody (and not CXCR1) blocked the increase in adhesion at all lymphotactin concentrations.

ii) H357 and SCC4

Adhesion of H357 and SCC4 cells to fibronectin also increased significantly after stimulation with lymphotactin in a dose dependent manner ($p<0.001$ and $p<0.0001$ respectively) (Figure 8.2 and 8.3). Pre-incubation of H357 and SCC4 cells with XCR1 antibody caused a significant reduction in adhesion at all lymphotactin doses ($p<0.001$ and $p<0.0001$ respectively) and reduced it to control levels. The irrelevant control chemokine and antibody TECK and CCL25 did not have any effect on adhesion.

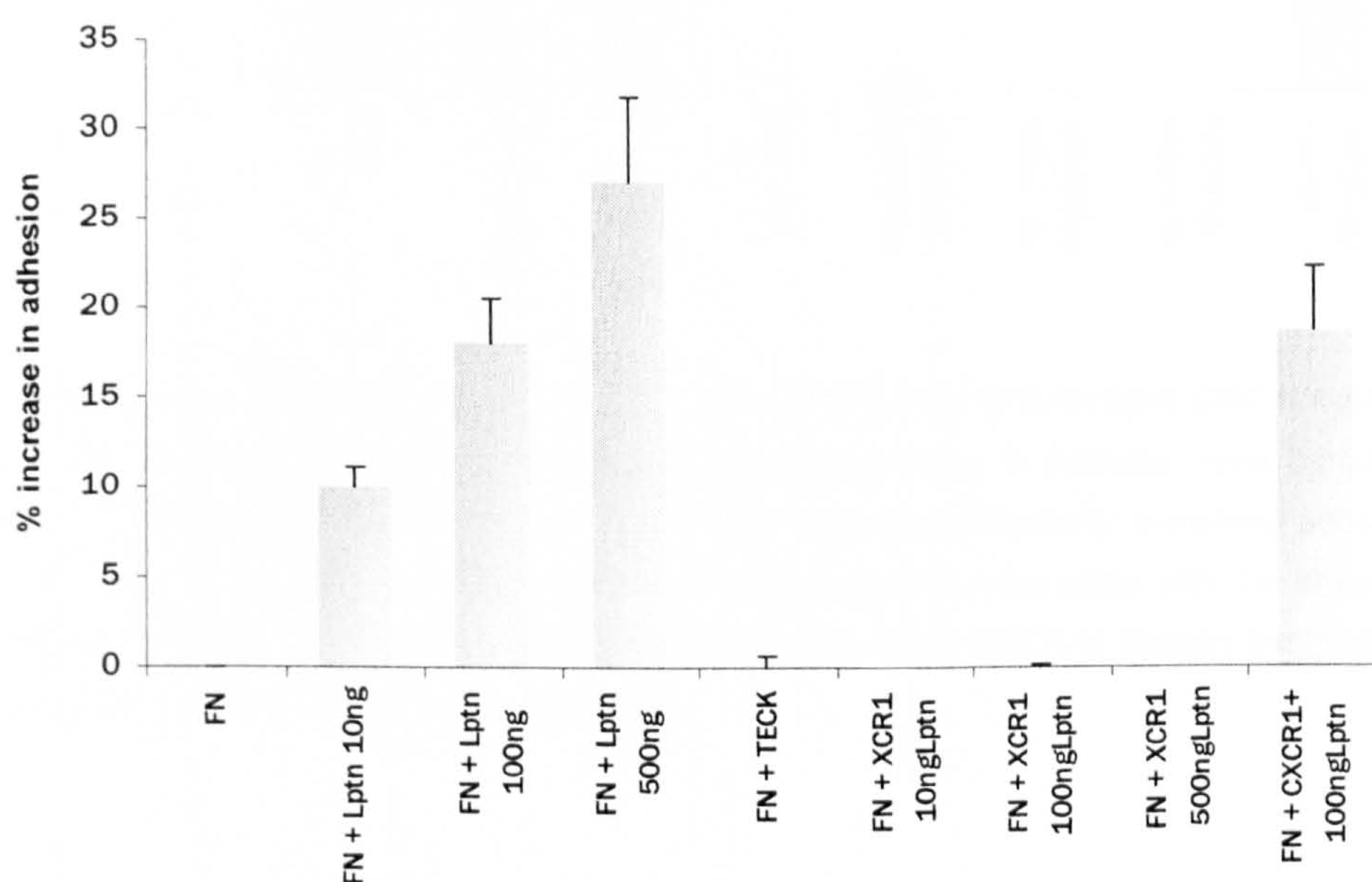


Figure 8.2. Percentage increase in the adhesion of H357 cells to fibronectin after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin increased adhesion to fibronectin in a dose dependent manner whereas no affect was seen with the irrelevant chemokine TECK. Preincubation with XCR1 antibody (and not CXCR1) blocked the increase in adhesion at all lymphotactin concentrations.

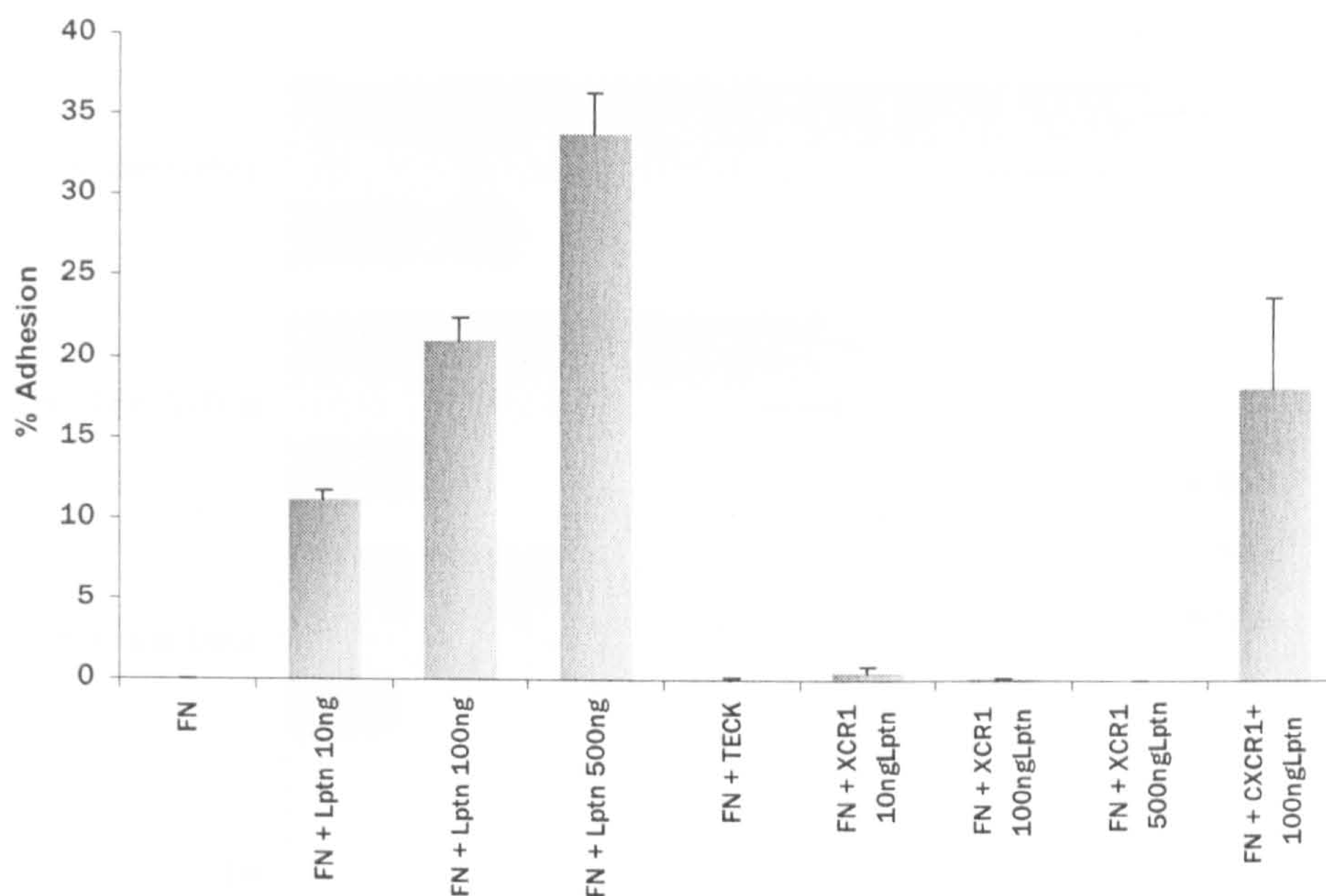


Figure 8.3. Percentage increase in the adhesion of SCC4 cells to fibronectin after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin increased adhesion to fibronectin in a dose dependent manner whereas no affect was seen with the irrelevant chemokine TECK. Preincubation with XCR1 antibody (and not CXCR1) blocked the increase in adhesion at all lymphotactin concentrations.

The NOK were the least adhesive to fibronectin of the tested cells. At three different doses of lymphotactin (10ng/ml, 100ng/ml and 500ng/ml) an increase in adhesion of 4.3%, 6.5% and 9.1% was observed respectively. Exposure to lymphotactin caused a significantly higher increase in adhesion of H357 cells than NOK to fibronectin (10%, 18.6% and 27.1%) ($p<0.01$). Similarly, significantly higher adhesion of SCC4 cells to fibronectin was seen compared with NOK after lymphotactin stimulation at all doses (11%, 20.9% and 33.6%) ($p<0.001$). No significant difference was observed between adhesion of H357 and SCC4 cells (Figure 8.4).

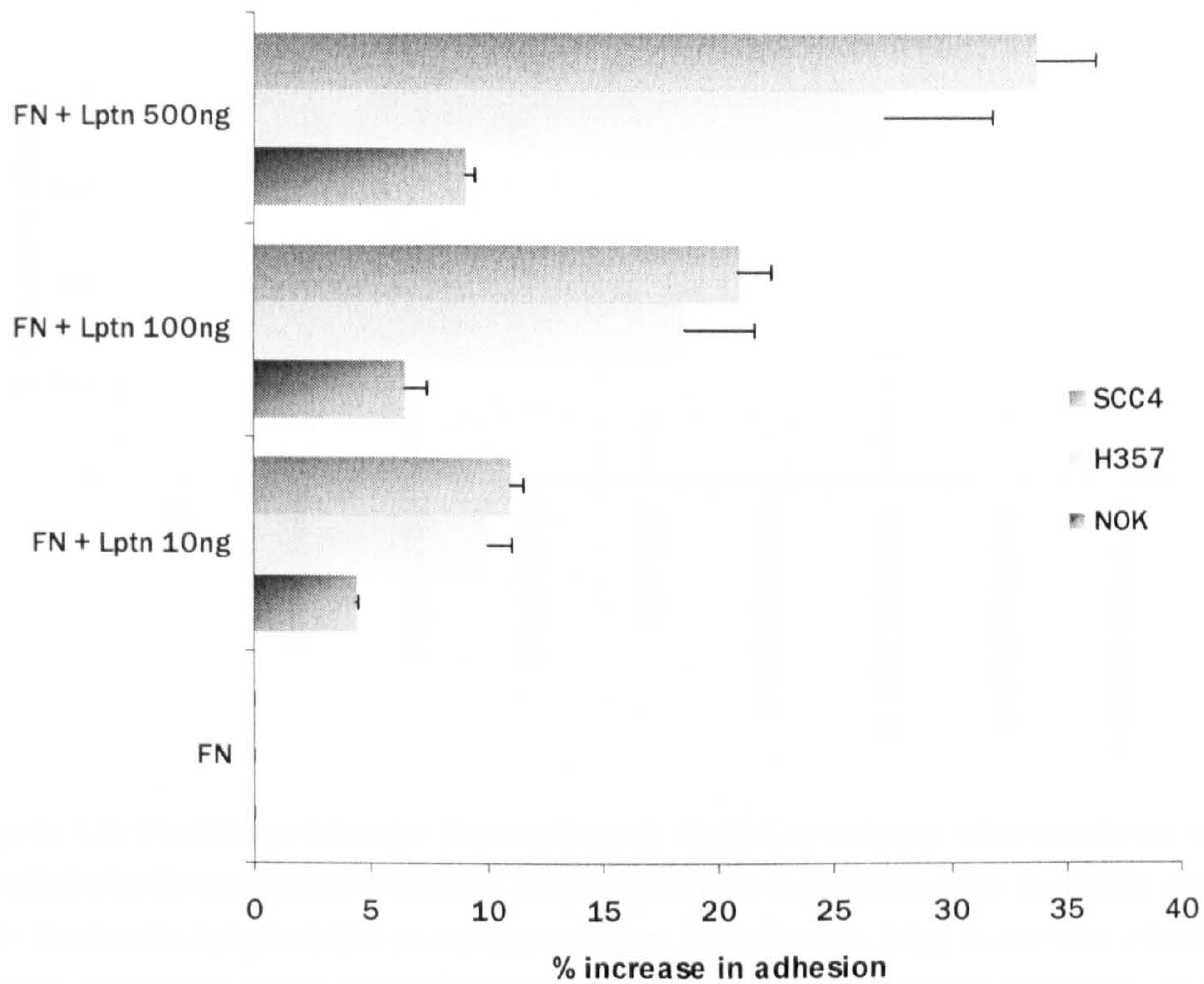


Figure 8.4. Comparison of percentage increase in adhesion of NOK, H357 and SCC4 cells to fibronectin after lymphotactin stimulation (n=average of 3 different experiments done in triplicate, error bars=SD). Lymphotactin increased adhesion to lymphotactin in a dose dependent manner. The H357 and SCC4 cells significantly higher adhesion than NOK.

8.5.1.2 Adhesion to Collagen

i) NOK

No increase in adhesion of NOK to collagen was observed after incubation with lymphotactin at any dose. No difference was observed between different treatments including addition of XCR1 antibody, TECK or CXCR1 antibody (Figure 8.5).

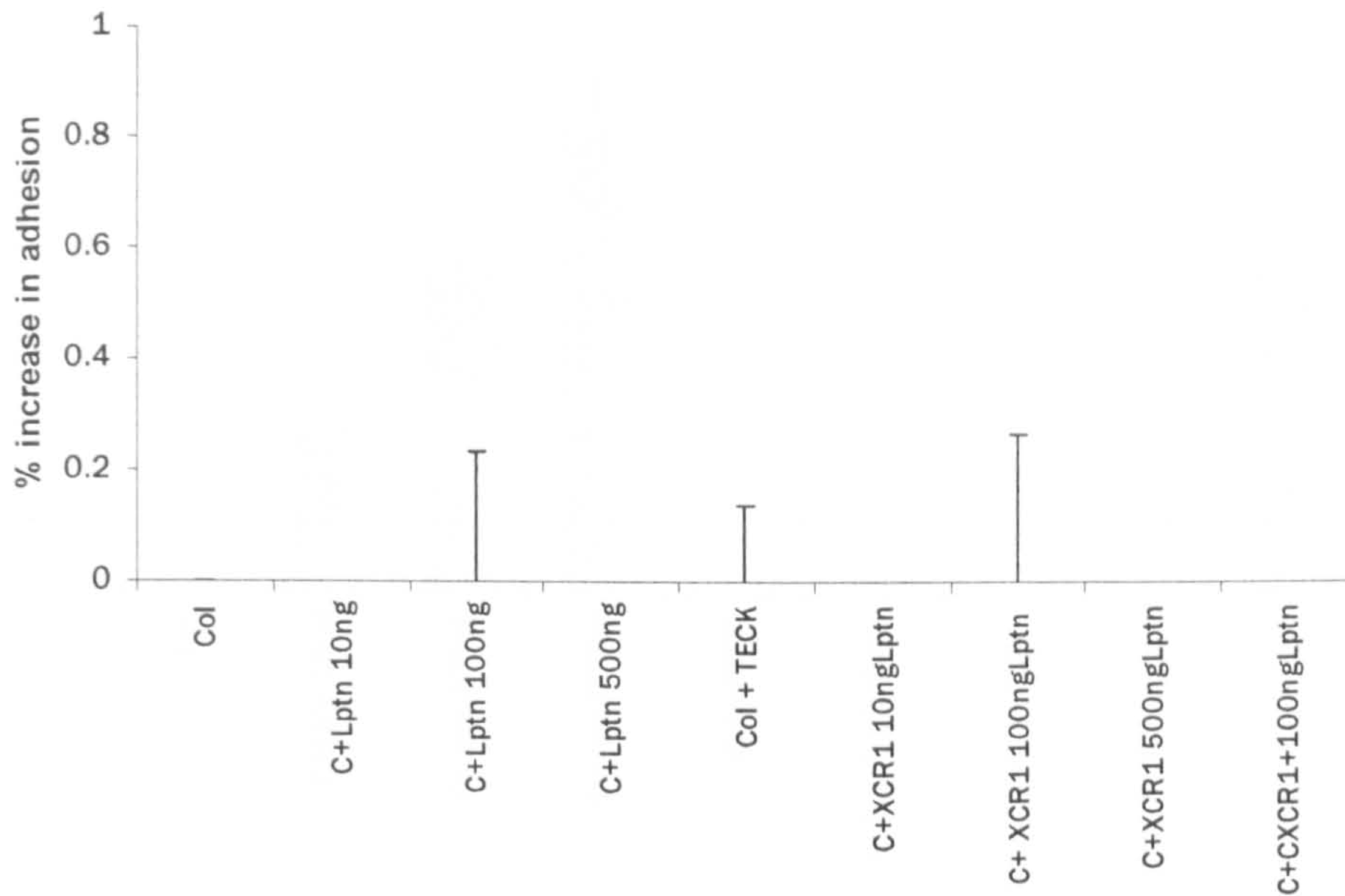


Figure 8.5. Percentage increase in the adhesion of NOK to collagen after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin failed to increase adhesion of NOK to collagen at any of the tested concentrations. Similarly no affect was seen with the irrelevant chemokine TECK, the XCR1 or the CXCR1 antibody.

ii) H357 and SCC4

Different results were obtained for the cancer cells. Adhesion of both H357 and SCC4 cells to collagen significantly increased after lymphotactin stimulation ($p<0.001$ and $p<0.001$ respectively) in a dose dependent manner (Figure 8.6 and 8.7). A significant decrease in this adhesion was observed after pre-incubation of H357 and SCC4 cells with XCR1 antibody ($p<0.001$ and $p<0.001$ respectively) and adhesion was reduced to control levels. The irrelevant chemokine TECK or the irrelevant antibody CXCR1 failed to elicit any response.

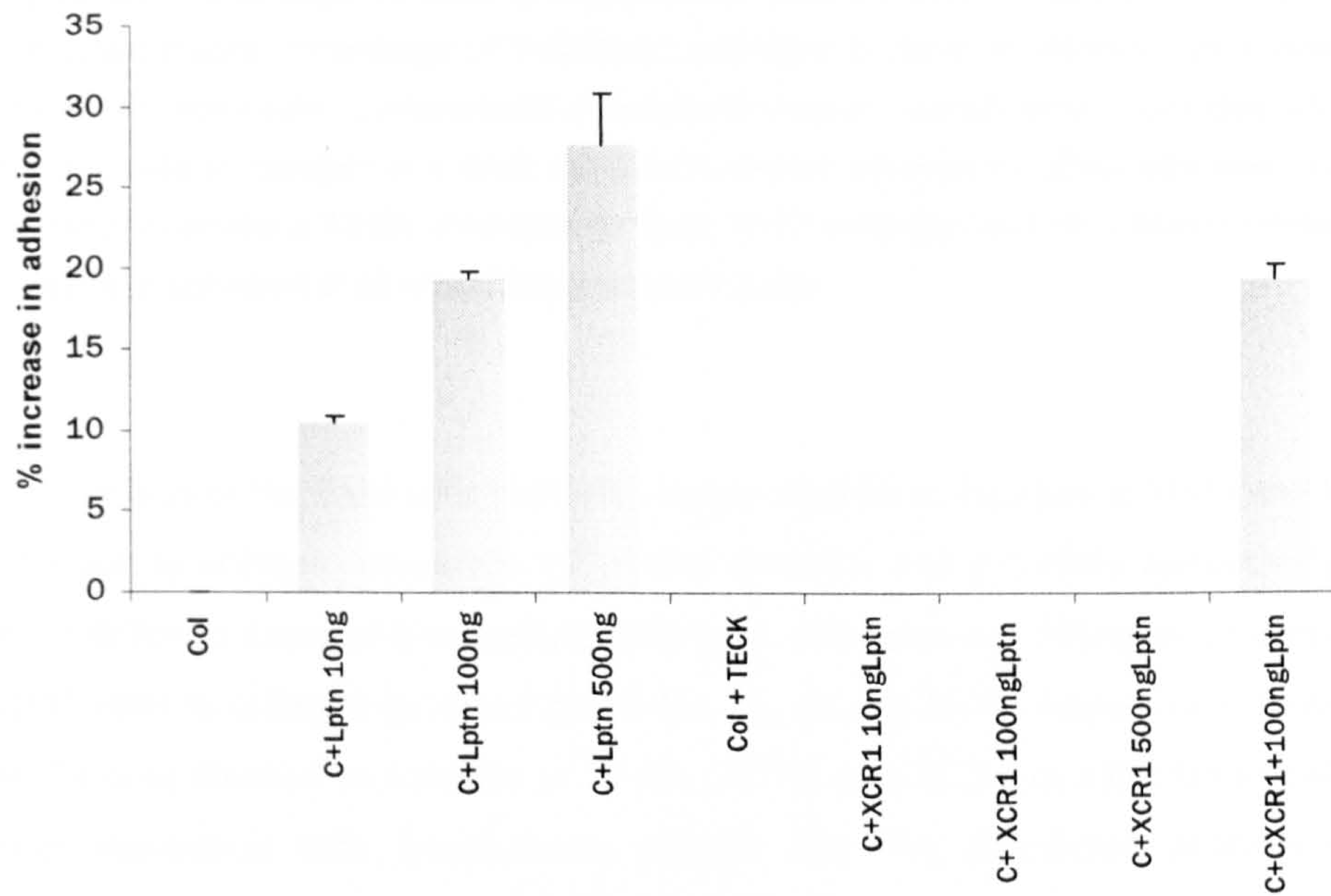


Figure 8.6. Percentage increase in the adhesion of H357 cells to collagen after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin increased adhesion of H357 cells to collagen in a dose dependent manner whereas no affect was seen with the irrelevant chemokine TECK. Preincubation with XCR1 antibody (and not CXCR1) blocked the increase in adhesion at all lymphotactin concentrations.

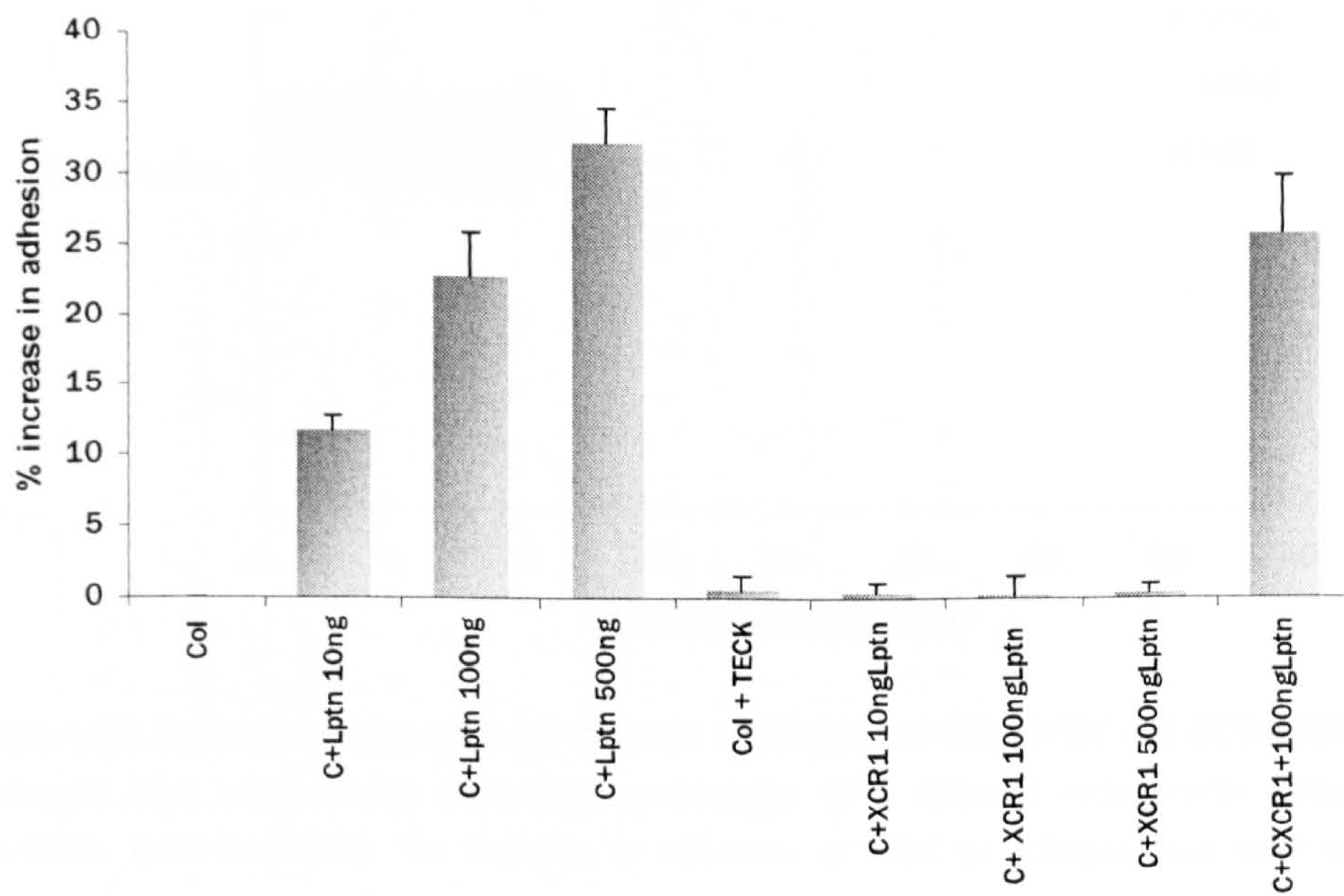


Figure 8.7. Percentage increase in the adhesion of SCC4 cells to collagen after stimulation with lymphotactin (n=average of 3 different experiments done in triplicate, error bars=SD) where FN= fibronectin, Lymphotactin concentration=ng/ml. Lymphotactin increased adhesion of H357 cells to collagen in a dose dependent manner whereas no affect was seen with the irrelevant chemokine TECK. Preincubation with XCR1 antibody (and not CXCR1) blocked the increase in adhesion at all lymphotactin concentrations.

Comparison of the three cells showed a highly significant increase in H357 and SCC4 adhesion to collagen compared with NOK ($p<0.001$ and $p<0.0001$ respectively). At three different doses of lymphotactin (10ng/ml, 100ng/ml and 500ng/ml), adhesion of H357 cells to collagen increased by 10.4%, 19.3% and 25.5% respectively. Similarly, SCC4 cells showed an increase of 11.6%, 22.7% and 32.2% in adhesion to collagen after incubation with lymphotactin (Figure 8.8). No significant difference was observed between adhesion of H357 and SCC4 cells.

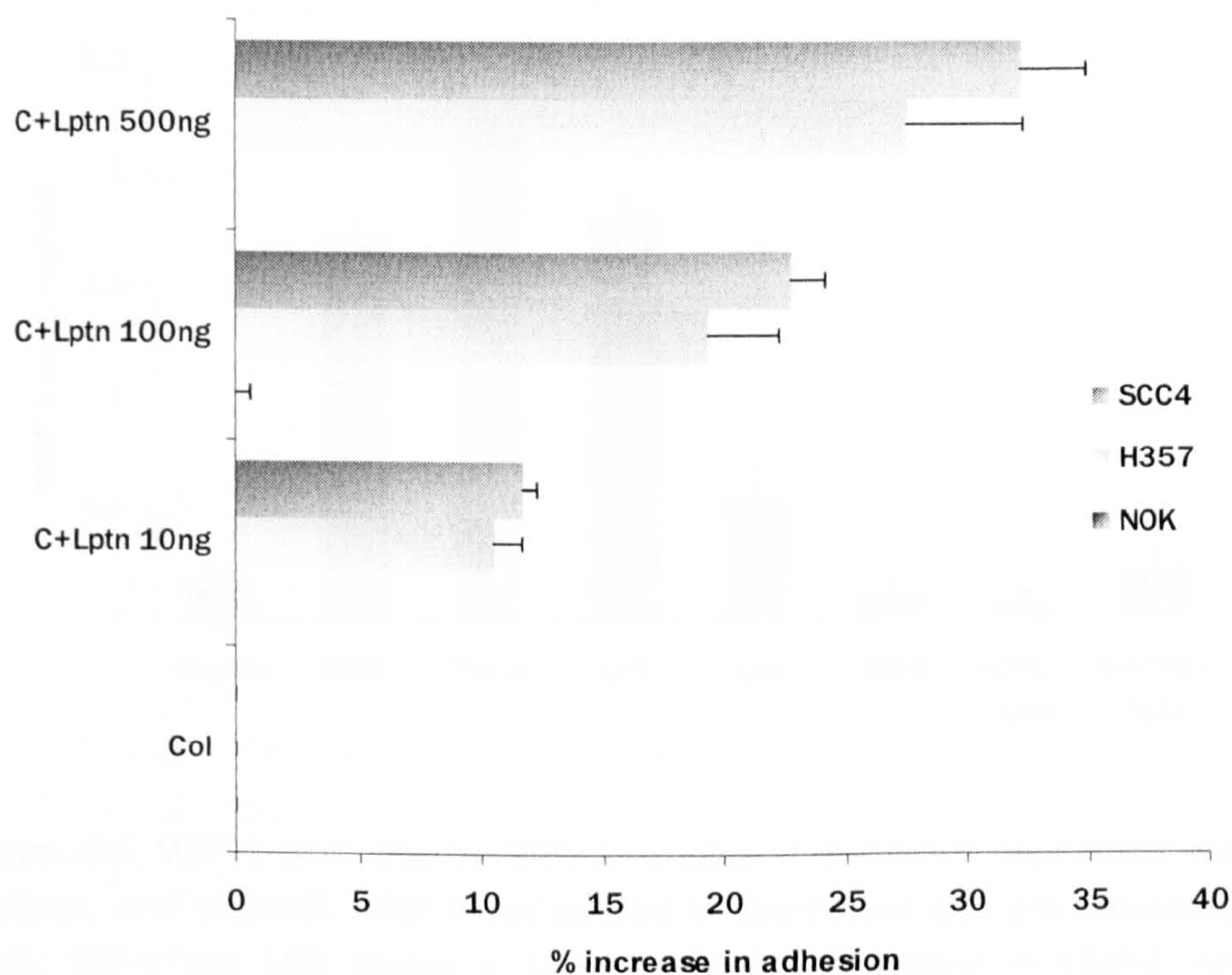


Figure 8.8. Comparison of percentage increase in adhesion of NOK, H357 and SCC4 cells to collagen after lymphotactin stimulation (n=average of 3 different experiments done in triplicate, error bars=SD). No increase in adhesion of NOK to collagen was seen after

lymphotactin stimulation. However, adhesion of H357 and SCC4 cells was significantly increased (compared with collagen alone) in a dose dependent manner.

8.5.2 MMP PRODUCTION

8.5.2.1 MMP-2

i) NOK

Constitutive MMP-2 expression was seen in NOK as unstimulated cells produced low levels. Incubation of NOK with PMA, TNF-α and LPS significantly increased MMP-2 production ($p<0.00001$) (Figure 8.9). A small yet significant increase in MMP-2 release was also seen after lymphotactin stimulation ($p<0.05$) and addition of XCR1 antibody significantly reduced MMP-2 expression ($p<0.01$). Addition of the irrelevant control chemokine TECK or the control antibody CXCR1 did not have any effect.

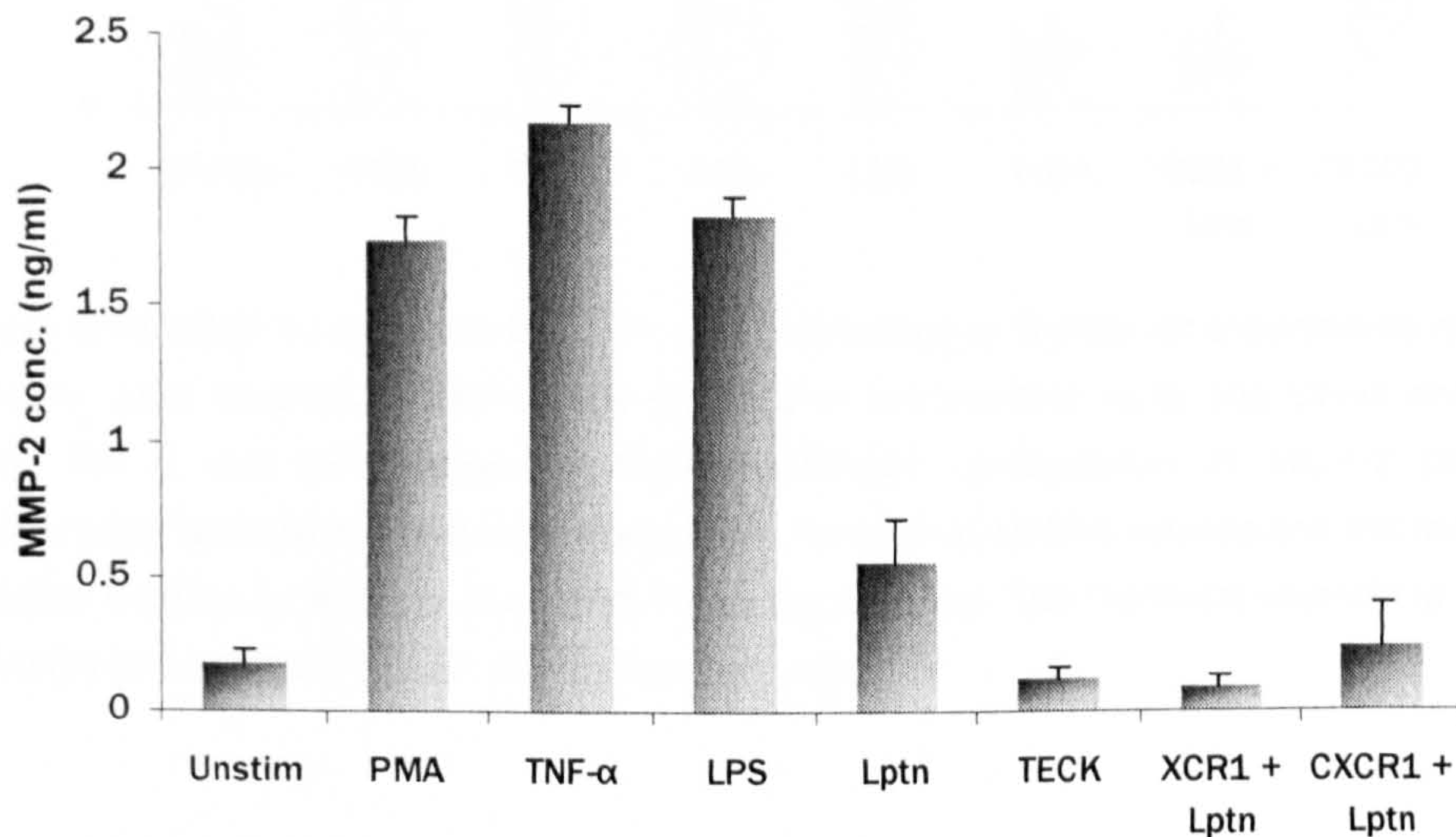


Figure 8.9. MMP-2 production by NOK (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-2 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-2 release. Lymphotactin also caused a small but significant increase in MMP-2 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

ii) H357 and SCC4 cells

Constitutive MMP-2 expression was also seen in supernatants of H357 and SCC4 cells. Stimulation with PMA, TNF-α and LPS caused a highly significant increase in MMP-2 release from H357 and SCC4 cells ($p<0.0001$ and $p<0.00001$ respectively) (Figure 8.10 and 8.11).

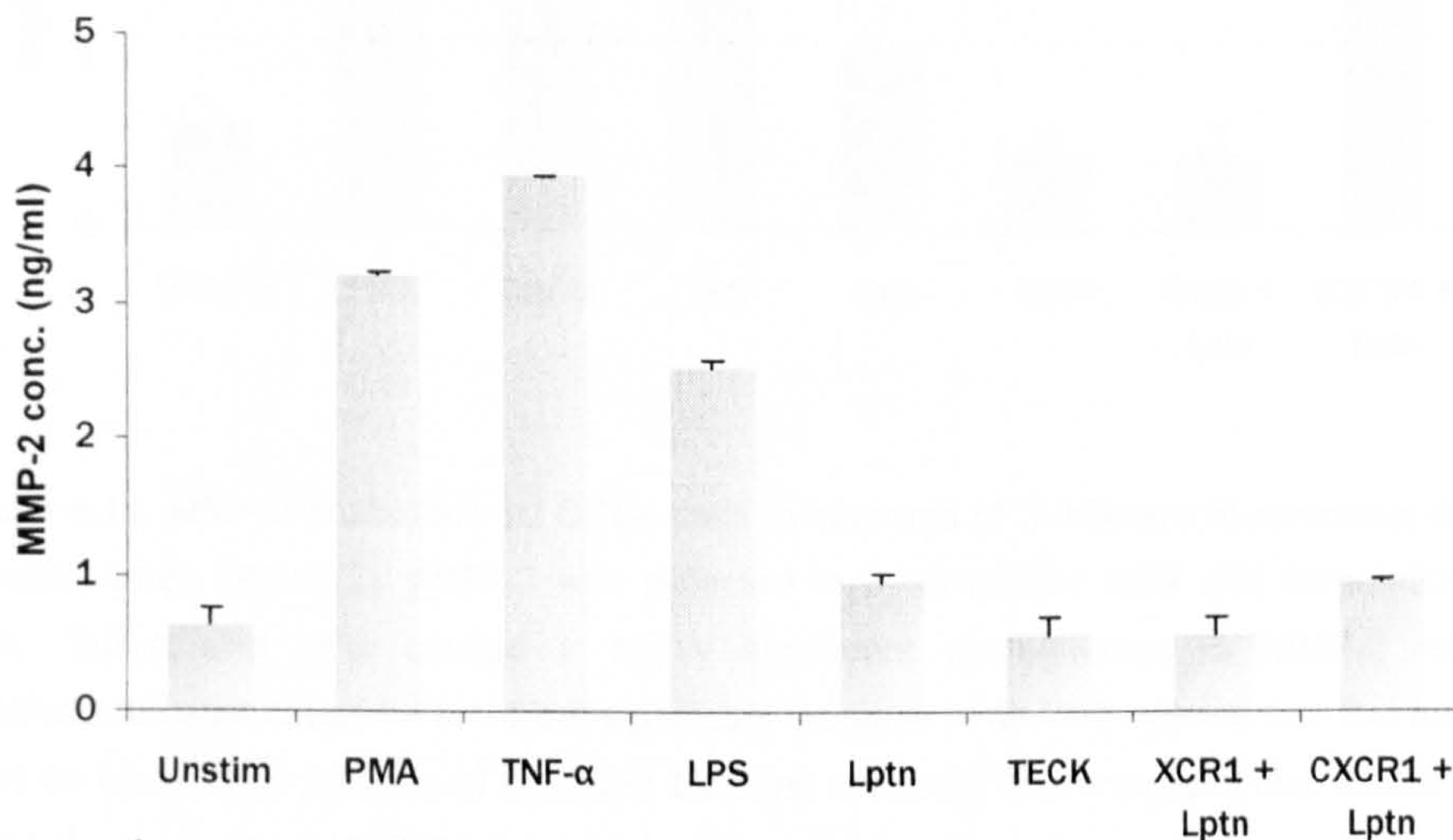


Figure 8.10. MMP-2 production by H357 cells (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-2 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-2 release. Lymphotactin also caused a small but significant increase in MMP-2 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

Addition of lymphotactin also caused a significant increase in MMP-2 production from H357 and SCC4 cells ($p<0.05$ and $p<0.001$ respectively) and administration of XCR1 blocking antibody reduced MMP-2 secretion to control levels. No response was observed when TECK or CXCR1 were used (Figure 8.10 and 8.11).

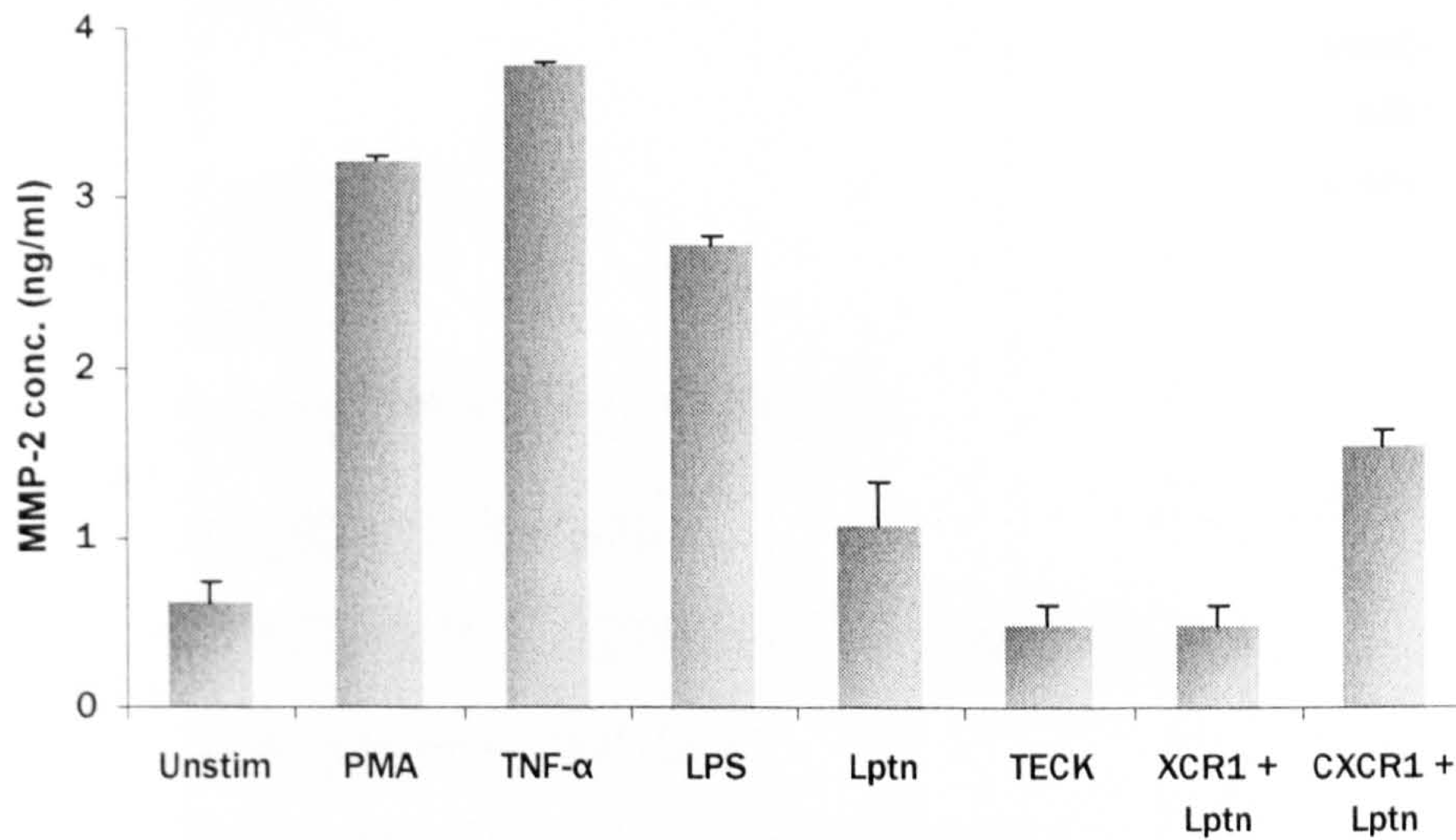


Figure 8.11. MMP-2 production by SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-2 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-2 release. Lymphotactin also caused a small but significant increase in MMP-2 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

Comparison of MMP-2 expression between NOK and OCCL revealed that the constitutive MMP-2 expression by H357 and SCC4 cells was significantly higher than NOK ($p<0.01$ and $p<0.01$ respectively) (Figure 8.12). Similarly addition of PMA, TNF-α, LPS and lymphotactin caused a higher increase in MMP-2 secretion from H357 ($p<0.00001$, $p<0.00001$, $p<0.001$ and $p<0.05$ respectively) and SCC4 cells ($p<0.00001$, $p<0.00001$, $p<0.00001$ and $p<0.0001$ respectively) than NOK. H357 and SCC4 cells also showed a significantly higher increase in MMP-2 release in response to lymphotactin stimulation than NOK ($p<0.05$ and $p<0.05$ respectively).

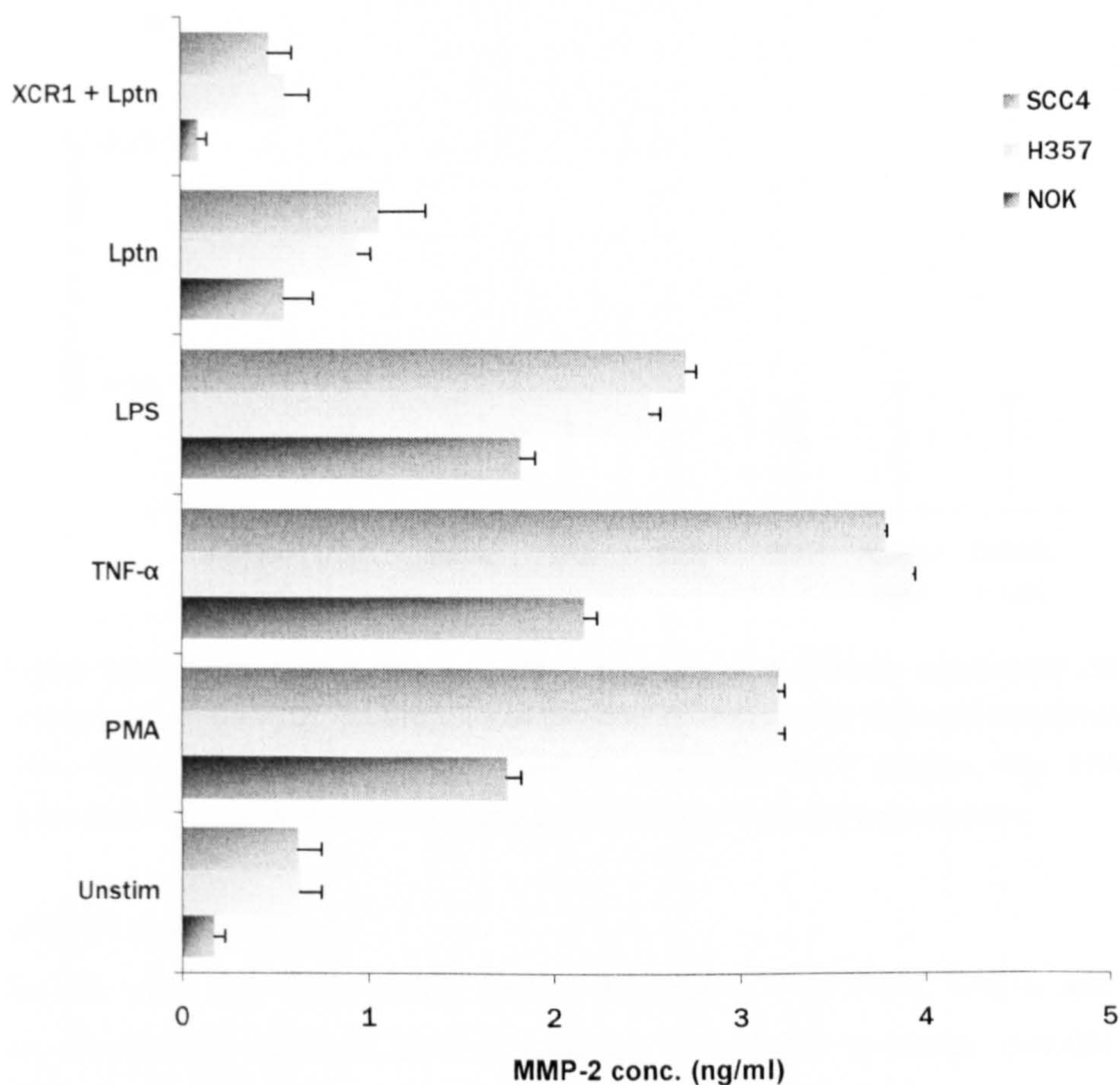


Figure 8.12. Comparison of MMP-2 production between NOK, H357 and SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). An increase in MMP-2 production was seen in response to PMA, TNF-α, LPS and lymphotactin. Addition of XCR1 antibody blocked the increase in response to lymphotactin. The H357 and SCC4 cells produced significantly higher amount of MMP-2 in culture compared with NOK.

8.5.2.2 MMP-7

i) NOK

No constitutive expression of MMP-7 was seen in the NOK supernatants. Addition of PMA, TNF-α or LPS failed to stimulate MMP-7 production. Similarly addition of lymphotactin, XCR1, TECK or CXCR1 did not have any effect (Figure 8.13).

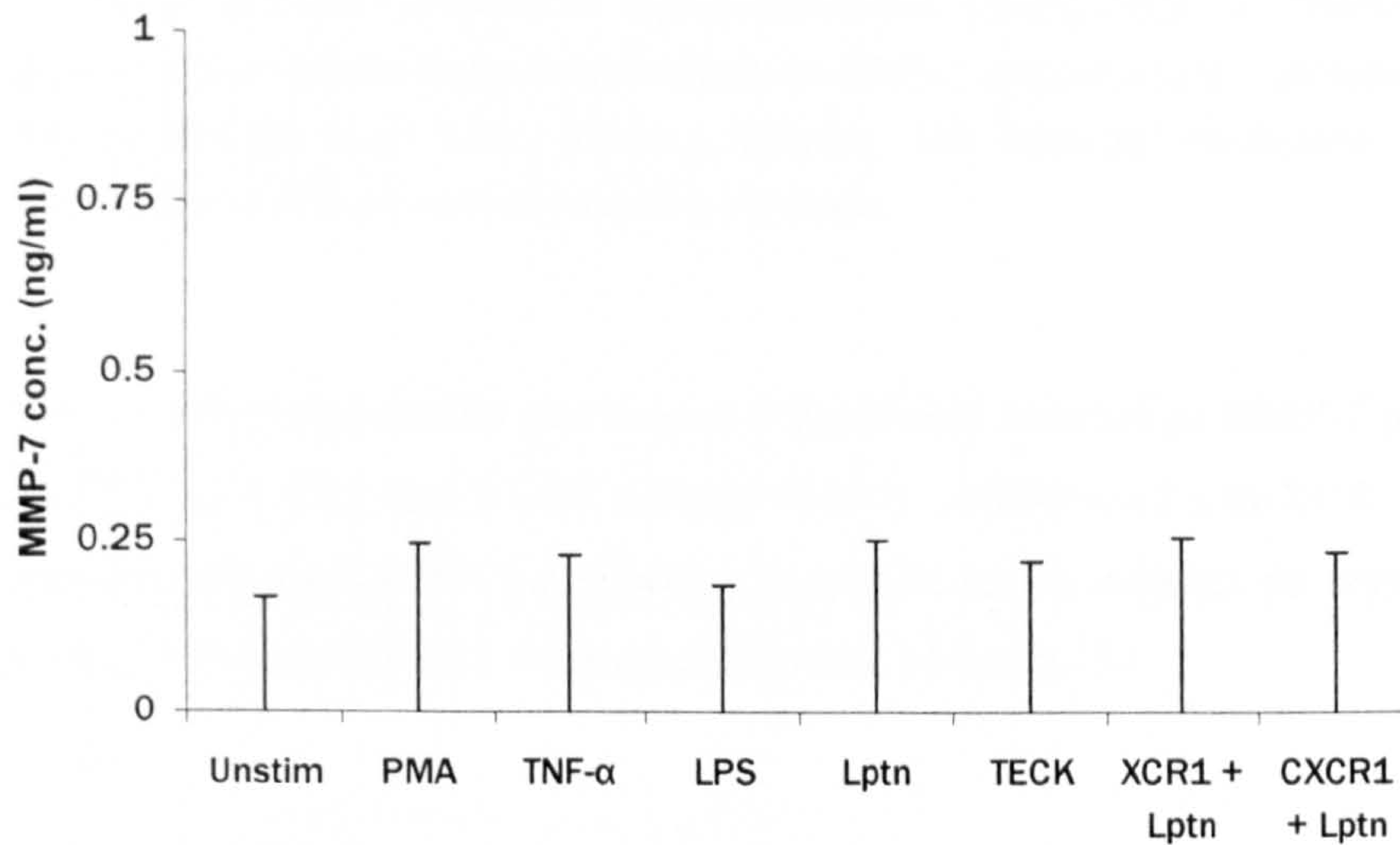


Figure 8.13. MMP-2 production by NOK (n=average of 3 different experiments done in triplicate, error bars=SD). No MMP-7 was detected in unstimulated NOK and stimulation with PMA, TNF-α, LPS or lymphotactin failed to stimulate MMP-7 release. The irrelevant chemokine TECK and irrelevant antibody CXCR1 also did not have any influence.

ii) H357 and SCC4 cells

Results were very different for OCCL. Stimulation with PMA, TNF-α and LPS significantly up-regulated MMP-7 secretion from H357 ($p<0.001$, $p<0.001$ and $p<0.0001$ respectively) and SCC4 cells ($p<0.0001$, $p<0.001$ and $p<0.0001$ respectively) compared with unstimulated cells (Figure 8.14 and 8.15).

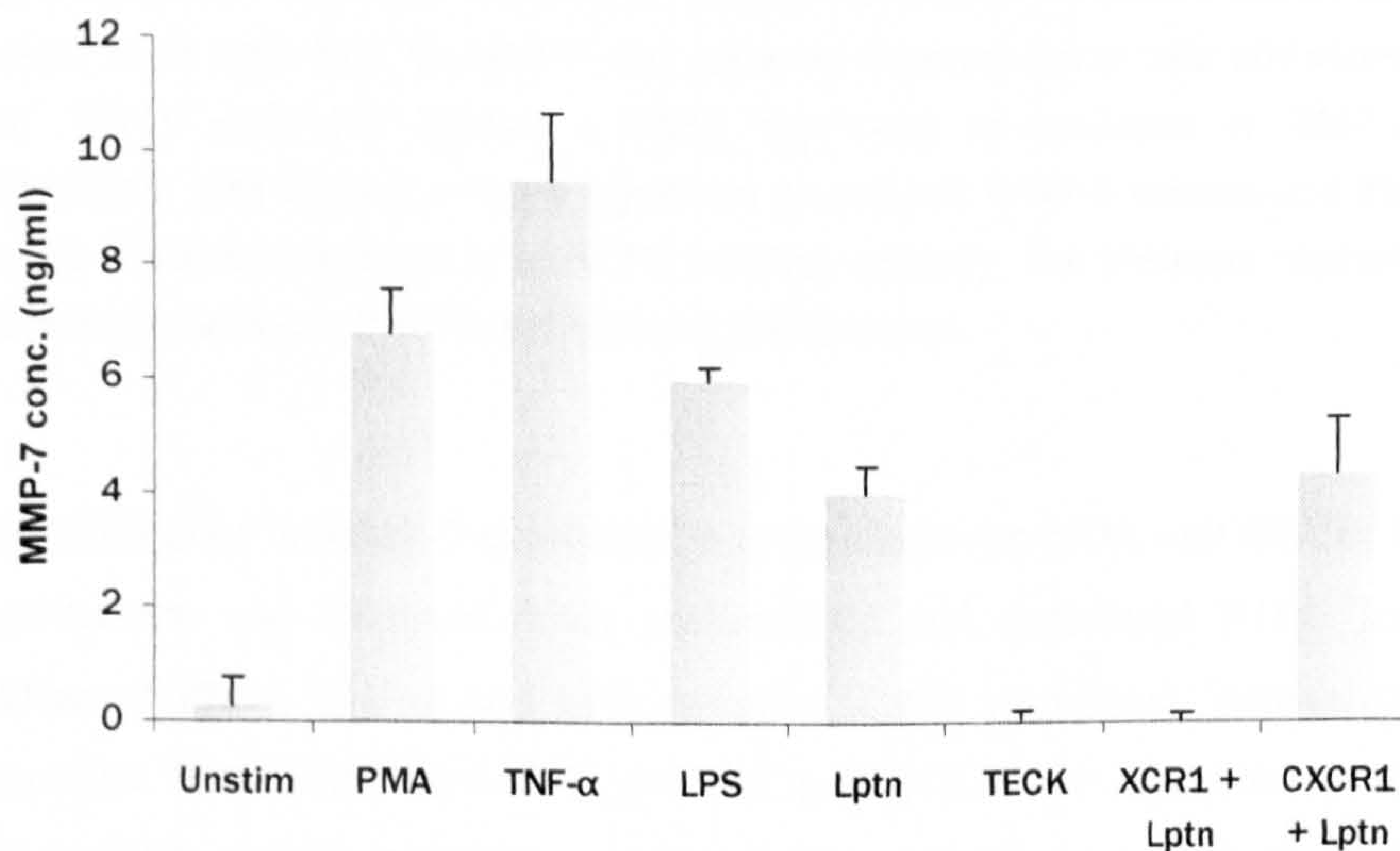


Figure 8.14. MMP-7 production by H357 cells (n=average of 3 different experiments done in triplicate, error bars=SD). No MMP-7 was detected in unstimulated cells and stimulation with

PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-7 release. Lymphotactin also caused a significant increase in MMP-7 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

Incubation with lymphotactin also caused a significant increase in MMP-7 production from H357 ($p<0.001$) and SCC4 cells ($p<0.001$). Addition of anti-XCR1 antibody significantly reduced MMP-7 production to control levels whereas no response was seen when TECK or CXCR1 were used (Figure 8.14 and 8.15).

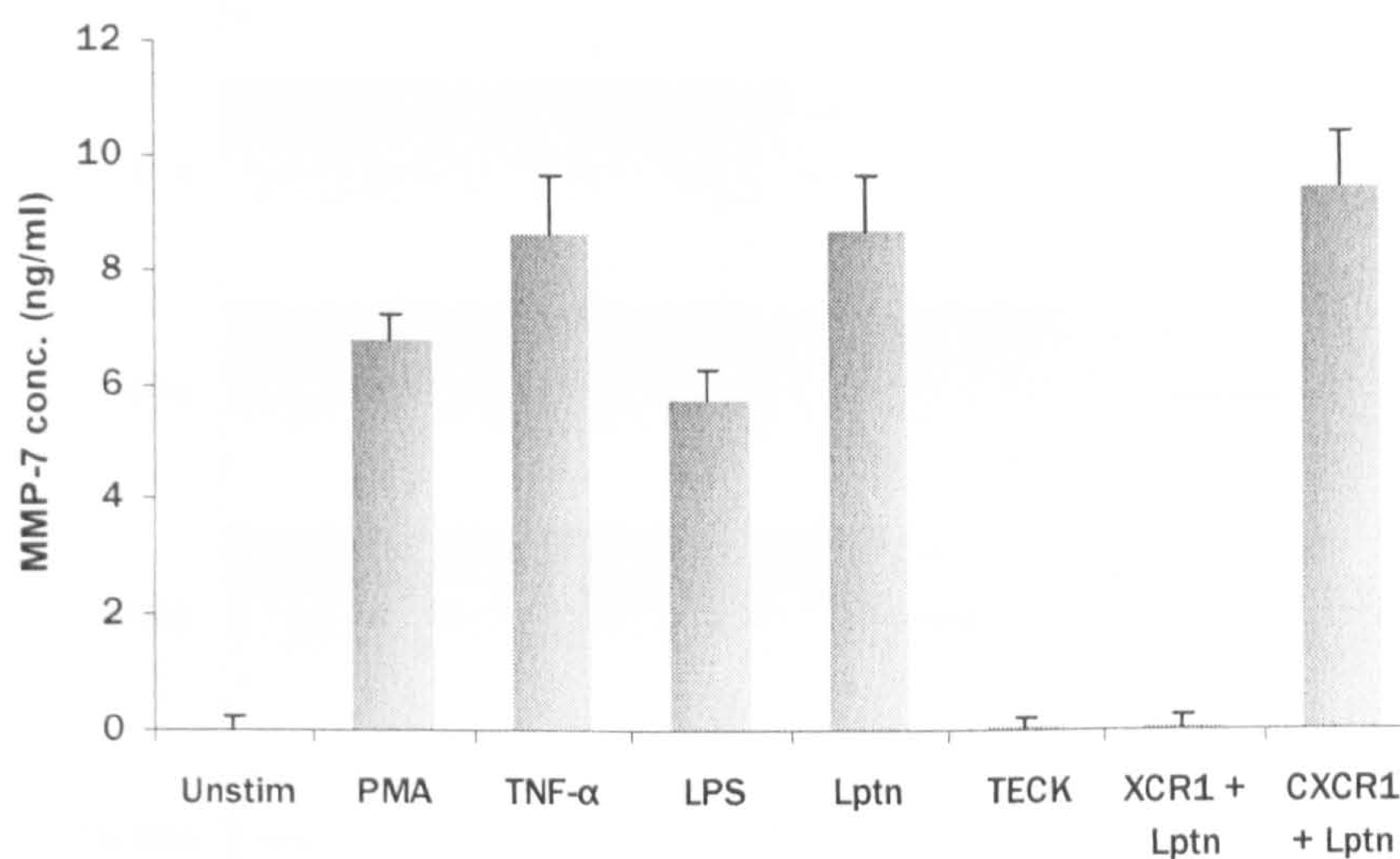


Figure 8.15. MMP-7 production by SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). No MMP-7 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-7 release. Lymphotactin also caused a highly significant increase in MMP-7 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

A big difference in MMP-7 expression was seen between NOK and OCCL. No MMP-7 production was observed from unstimulated and stimulated NOK. In contrast, addition of PMA, TNF-α and LPS caused a highly significant increase in MMP-7 production from H357 ($p<0.001$, $p<0.001$ and $p<0.00001$ respectively) and SCC4 cells ($p<0.01$, $p<0.01$, $p<0.01$ respectively) (Figure 8.16). Lymphotactin had no effect on MMP-7 release from NOK whereas H357 cells showed significantly higher MMP-

7 production than NOK ($p<0.001$). The SCC4 cells were the most responsive to lymphotactin and showed a highly significant increase in MMP-7 production compared to both NOK and H357 cells ($p<0.001$ and $p<0.001$ respectively) (Figure 8.16).

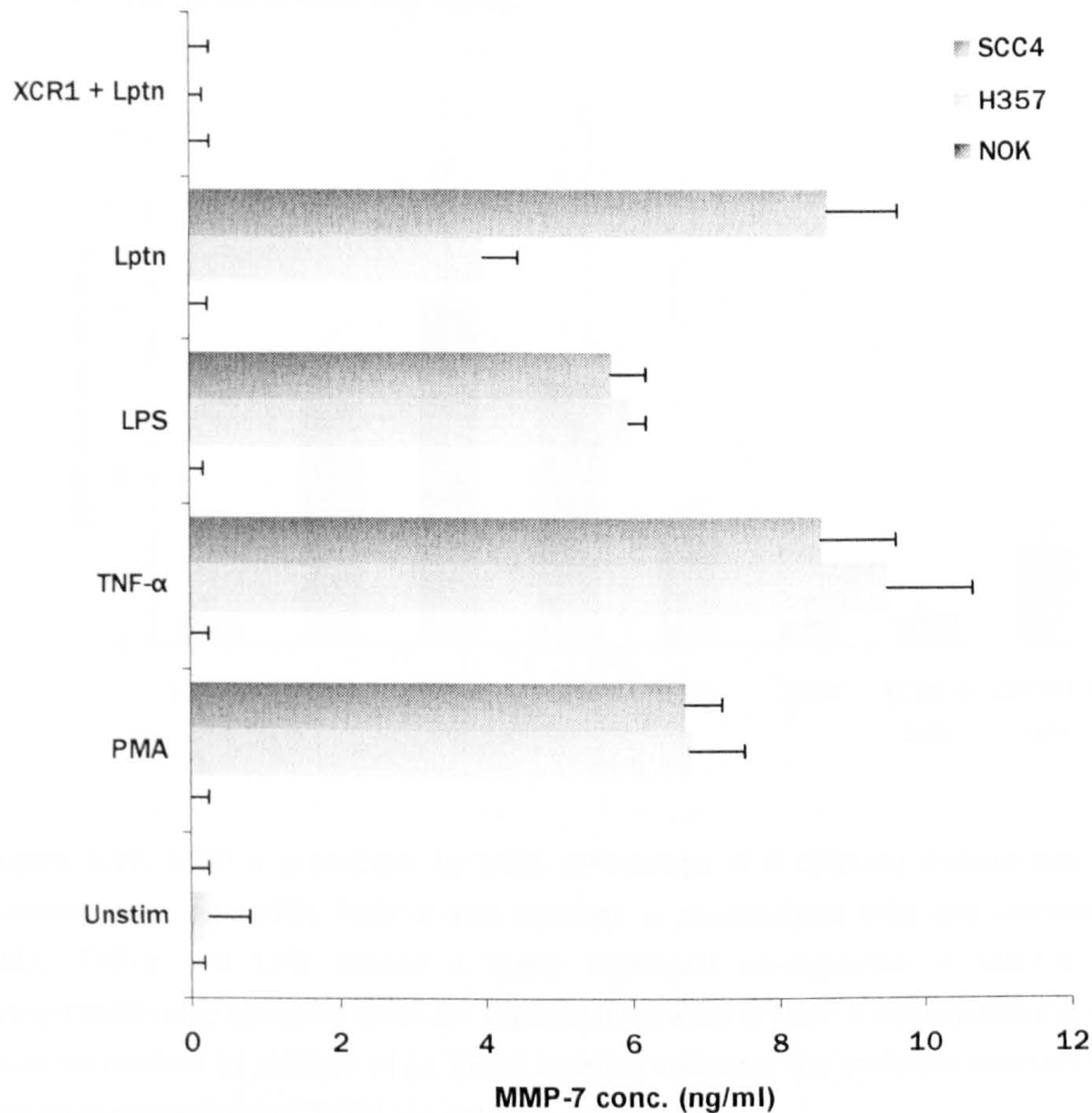


Figure 8.16. Comparison of MMP-7 production between NOK, H357 and SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). H357 and SCC4 cells showed an increase in MMP-7 production in response to PMA, TNF-α, LPS and lymphotactin. Addition of XCR1 antibody blocked the increase in response to lymphotactin. No MMP-7 production was detected in NOK supernatants.

8.5.2.3 MMP-9

i) NOK

Like MMP-2, constitutive expression of MMP-9 was detected in NOK culture supernatants. Incubation of NOK with PMA, TNF-α or LPS significantly increased

MMP-9 secretion compared with unstimulated cells ($p<0.01$, $p<0.05$ and $p<0.0001$ respectively) (Figure 8.17). Lymphotactin also caused a small but significant increase in MMP-9 release ($p<0.05$) and this effect was abolished by the addition of XCR1 blocking antibody ($p<0.05$). The irrelevant control chemokine and antibody (TECK and CXCR1) failed to have any effect.

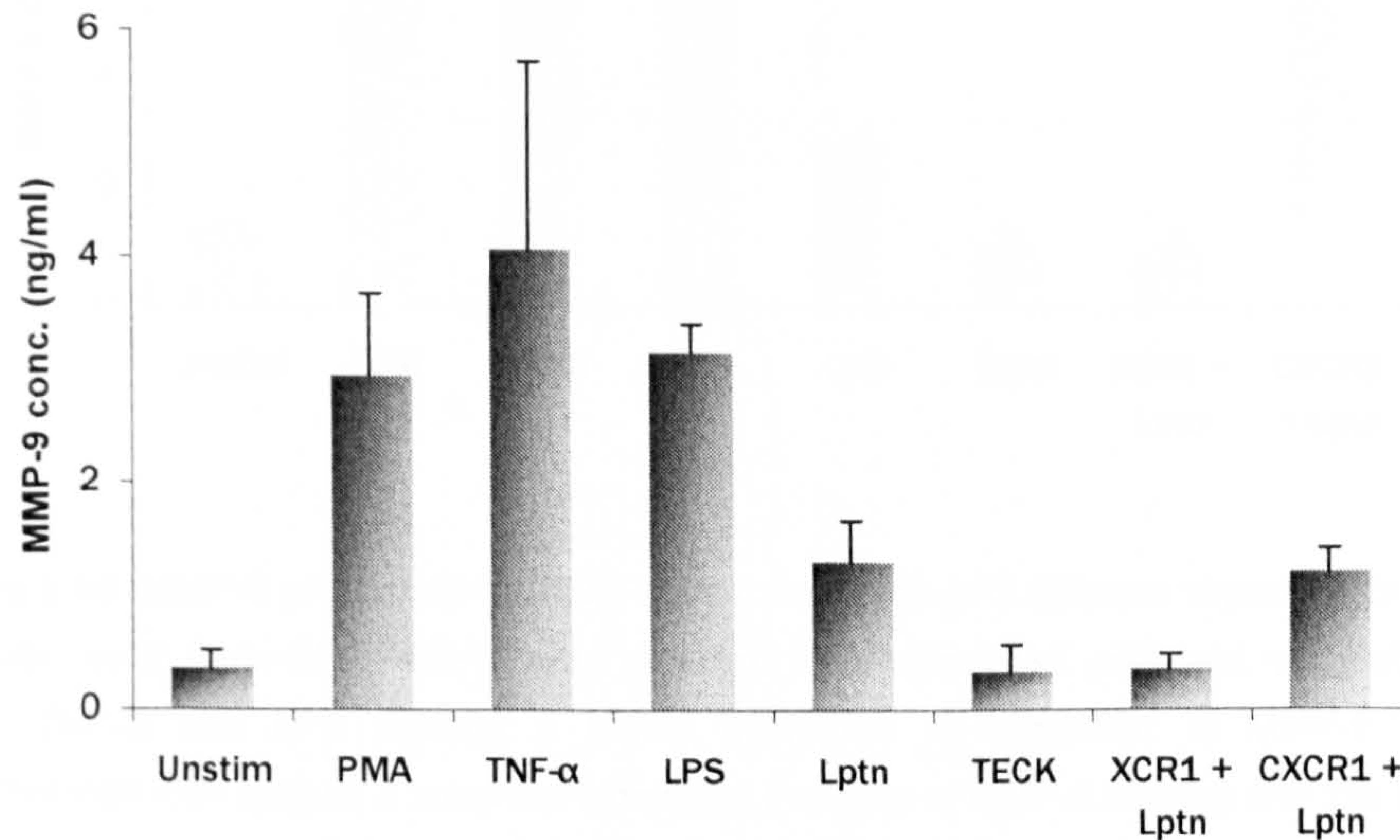


Figure 8.17. MMP-9 production by NOK (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-2 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-9 release. Lymphotactin also caused a small but significant increase in MMP-9 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

ii) H357 and SCC4 cells

Constitutive MMP-9 release was also detected in culture supernatants of H357 and SCC4 cells. Stimulation with PMA, TNF-α or LPS significantly increased MMP-9 secretion from H357 ($p<0.001$, $p<0.001$ and $p<0.01$ respectively) and SCC4 cells ($p<0.00001$, $p<0.0001$ and $p<0.01$ respectively) compared with unstimulated cells (Figure 8.18 and 8.19).

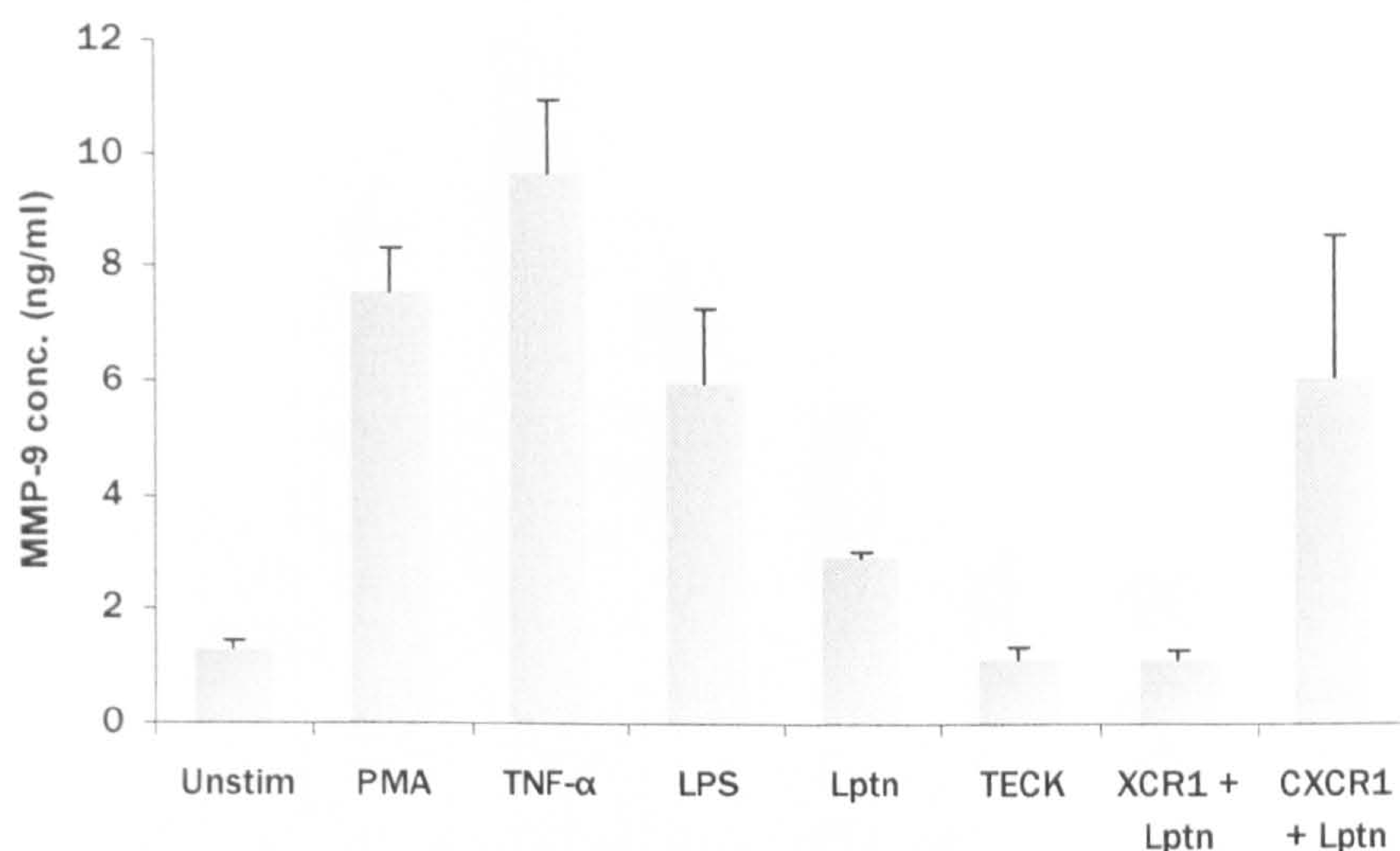


Figure 8.18. MMP-9 production by H357 cells (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-2 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-9 release. Lymphotactin also caused a small but significant increase in MMP-9 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

A significant increase in MMP-9 production was also seen after stimulation of H357 and SCC4 cells with lymphotactin ($p<0.001$ and $p<0.001$ respectively) but not with TECK. Preincubation of cells with an antibody against XCR1 caused a significant reduction in MMP-9 release ($p<0.0001$ and $p<0.0001$ respectively) and reduced it to control levels. The irrelevant CXCR1 antibody failed to reduce MMP-9 secretion (Figure 8.18 and 8.19).

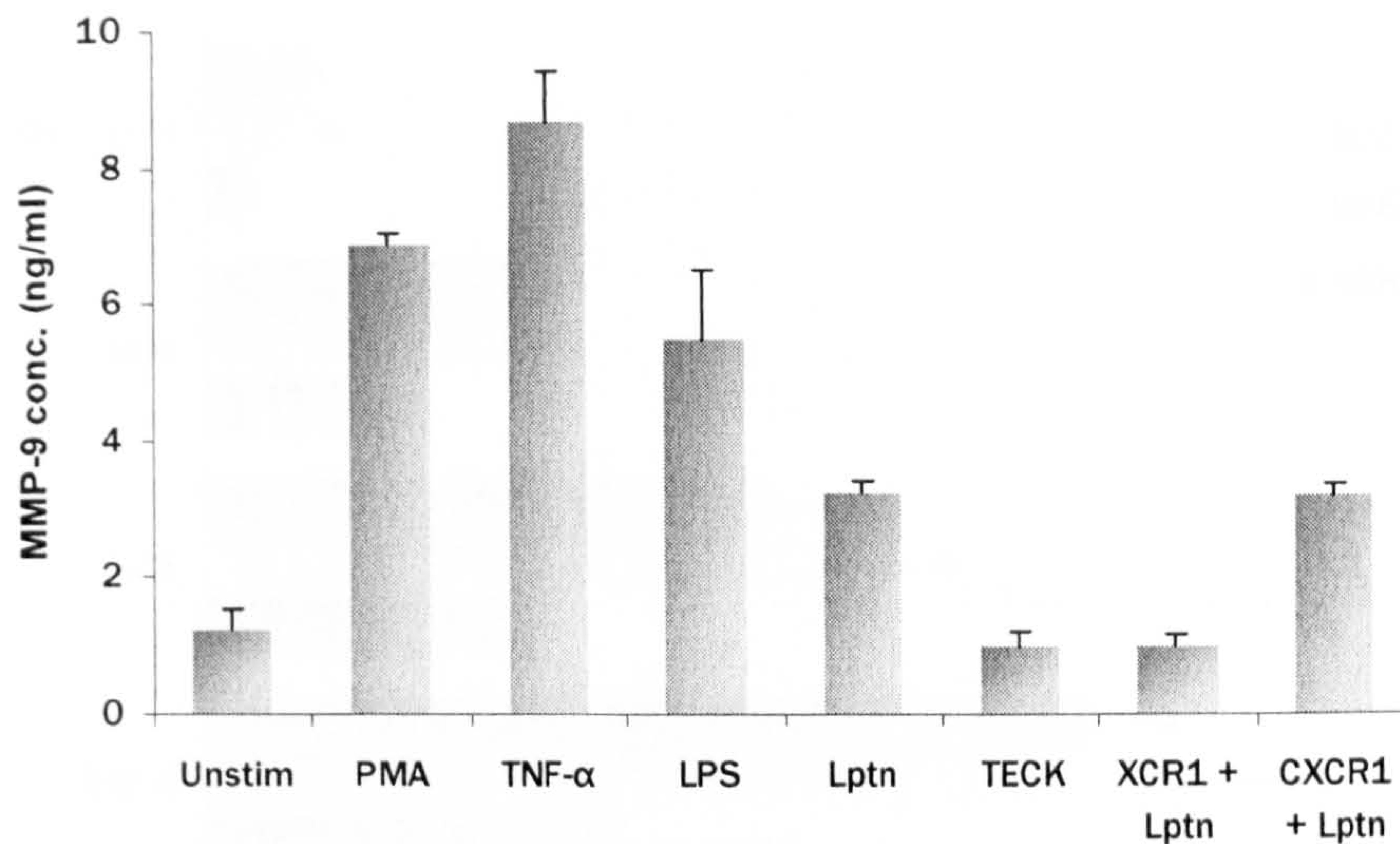


Figure 8.19. MMP-9 production by SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). MMP-9 was detected in unstimulated cells and stimulation with PMA, TNF-α and LPS caused a highly significant up-regulation in MMP-9 release. Lymphotactin also caused a small but significant increase in MMP-9 release and this increase could be blocked by addition of an XCR1 blocking antibody. The irrelevant chemokine TECK and irrelevant antibody CXCR1 did not have any influence.

Comparison of NOK and OCCL showed that the levels of constitutive MMP-9 released in culture from H357 and SCC4 cells were significantly higher than NOK ($p<0.01$ and $p<0.01$ respectively). Stimulation of H357 cells with PMA, TNF-α or LPS caused a significantly bigger increase in MMP-9 secretion compared with NOK ($p<0.01$, $p<0.05$ and $p<0.05$ respectively) (Figure 8.20). A similar response was observed in SCC4 cells as addition of PMA, TNF-α or LPS facilitated significantly higher levels of MMP-9 compared with NOK ($p<0.001$, $p<0.05$ and $p<0.05$ respectively). Exposure of H357 and SCC4 cells to lymphotactin also facilitated a bigger increase in MMP-9 production compared with NOK ($p<0.01$ and $p<0.001$ respectively). No significant difference in MMP-9 secretion was observed between H357 and SCC4 cells (Figure 8.20).

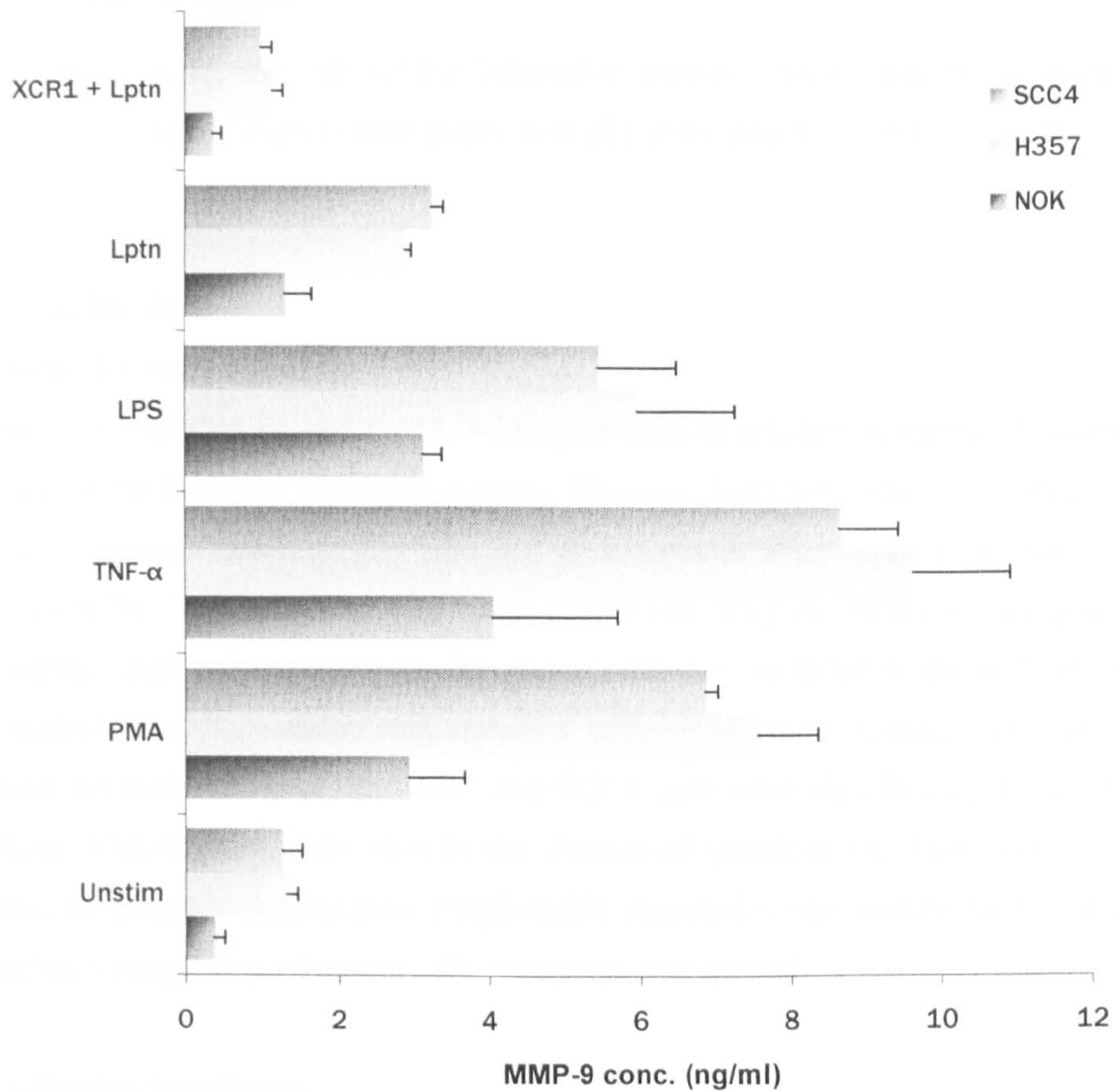


Figure 8.20. Comparison of MMP-9 production between NOK, H357 and SCC4 cells (n=average of 3 different experiments done in triplicate, error bars=SD). An increase in MMP-9 production was seen in response to PMA, TNF-α, LPS and lymphotactin. Addition of XCR1 antibody blocked the increase in response to lymphotactin. The H357 and SCC4 cells produced significantly higher amount of MMP-2 in culture compared with NOK.

8.6 DISCUSSION

In this chapter, the role of the interaction between XCR1 and lymphotactin was studied in adhesion and MMP production of normal and malignant oral epithelial cells *in vitro*.

1. ADHESION

Adhesion to fibronectin

Stimulation of NOK and OCCL with lymphotactin resulted in increased adhesion to fibronectin in a dose dependent manner. However, both the cancer cell lines H357 and SCC4 showed much greater adhesion to fibronectin after exposure to lymphotactin than NOK. This adhesion was completely blocked by an XCR1 blocking antibody whereas the irrelevant control chemokine and antibody failed to do so. These results suggest that lymphotactin may induce a stronger response in cancer cells compared with normal. However, the H357 and SCC4 cells were significantly more adhesive than NOK to fibronectin even in the absence of lymphotactin. Therefore it is likely that the greater adhesion after lymphotactin stimulation may just be due to the greater adhesive capability of cancer cells compared with normal.

Adhesion to collagen

The results were very different for adhesion to collagen. Addition of lymphotactin failed to increase adhesion of NOK to collagen at any dose. Similarly, no effect was observed after addition of XCR1 antibody, TECK or CXCR1 antibody. Contrasting results were obtained for OCCL as stimulation with lymphotactin caused a significant increase in H357 and SCC4 cell adhesion to collagen in a dose dependent manner. The increase in adhesion to collagen was completely abolished when XCR1 blocking antibody was added. These differential results between NOK and OCCL show that normal and cancer cells respond to lymphotactin in a different manner and that XCR1 may mediate an adhesive response in cancer cells and not in normal cells. It is also likely that XCR1-lymphotactin interaction is influencing expression of certain integrins or adhesion molecules on cancer cells that might have a different expression pattern compared with normal cells. Oral epithelial cells are known to bind to fibronectin through the integrins $\alpha5\beta1$ and $\alpha V\beta6$ (only in wound healing and cancer) whereas they utilise the $\alpha2\beta1$ integrin to bind to collagen (Poomsawat *et al.*, 2003;

Sugiyama *et al.*, 1993; Thomas *et al.*, 2001d; Thomas *et al.*, 2001c; Thomas *et al.*, 2001b; Thomas, Jones, and Speight 1997; Thomas and Speight 2001). It would be interesting to study the expression patterns of these integrins on NOK and OCCL and find out whether there is a difference in expression between normal and cancer cells. Another possibility is that lymphotactin may be altering the expression of these integrins in some manner (either affecting expression or avidity of these integrins). In addition, it is also possible that incubation of cells on collagen or fibronectin may affect XCR1 expression by upregulating or downregulating it.

Role of chemokines and their receptors in adhesion

A role of chemokines and their receptors in epithelial cell adhesion to ECM components has been shown. The chemokine receptor CXCR4 mediates tumour cell adhesion to ECM components. Stimulation of prostate cancer cell lines LNCaP and DU-145 with SDF-1α (ligand for CXCR4) significantly up-regulates adhesion to fibronectin, collagen and laminin by up-regulation of the integrins α5 and β3 and this effect is significantly reduced after addition of an anti-CXCR4 antibody (Engl *et al.*, 2006). A similar response is observed in small cell lung cancer (SCLC) cells as stimulation with SDF-1α in addition to CXCR4 activation upregulates α2, α4, α5 and β1 integrin expression (Hartmann *et al.*, 2005). In addition, binding of tumour cells to ECM can up-regulate expression of chemokine receptors and their ligands thus providing an autocrine mechanism of activation. Growth of pancreatic tumour cells on laminin-1 results in an up-regulation in CXCR4 expression (mediated by the α6β1 integrin) and IL-8 expression (mediated by the α6β1and α3β1 integrins) (Grzesiak *et al.*, 2007). Role of other chemokines and receptors in epithelial cell adhesion to ECM is not well characterized. CCL21 has been shown to increase adhesion of non-small cell lung carcinoma cell lines Lu-99 and A549 to endothelium. The chemokines fractalkine and CCL21 have been shown to increase adhesion of prostate and non-small cell lung cancer cells to the endothelium (Koizumi *et al.*, 2007; Shulby *et al.*, 2004). However, none of these studies compared the adhesion of normal cells to cancer cells.

Chemokines and their receptors in oral epithelial cell adhesion

The role of chemokines and their receptors in adhesion of oral epithelial cells to ECM components has not been shown to date. Recently a group has studied the expression

and role of the chemokine receptor CXCR4 in mucoepidermoid carcinoma of salivary glands. Gene knockdown of CXCR4 from highly metastatic mucoepidermoid carcinoma Mc3 cells results in significantly diminished adhesion to matrigel compared with their CXCR4-expressing counterparts (Wen *et al.*, 2007). However they did not study the effect of SDF-1α stimulation on these cells and also did not compare adhesion to normal keratinocytes.

Our findings show for the first time that chemokine-chemokine receptor interaction can facilitate adhesion of normal and malignant oral cells to ECM. Adhesion of both normal and cancer cells to fibronectin is increased after stimulation with lymphotactin whereas for collagen, lymphotactin stimulation only facilitates increased adhesion of cancer cells. This increased adhesion is mediated through XCR1 and may play a role in the increased migration and invasion in response to lymphotactin (as seen with our experiments in chapter 4). Due to time constraints, the effect of XCR1 and lymphotactin interaction on adhesion to other ECM components (such as laminin and tenascin) could not be studied. Similarly, the mechanism of adhesion and the integrins that are involved in the process were not studied. Since adhesion is a fundamental aspect of epithelial cells motility these results are very interesting and may have potential implications in oral epithelial cell migration and invasion in wound healing and cancer.

2. MMP PRODUCTION *IN VITRO*

Matrix metalloproteinases (MMPs) are a family of zinc and calcium-dependent proteases primarily involved in the breakdown of ECM. They play an important role in many physiological processes such as embryonic development, morphogenesis, reproduction and tissue remodeling (Bjorklund and Koivunen 2005; Stamenkovic 2003). MMPs are also implicated in many pathological processes such as arthritis (Xue *et al.*, 2007), cardiovascular disease (Sivakumar *et al.*, 2007) and cancer (Lynch and Matrisian 2002; Stamenkovic 2000).

a) MMP-2 and MMP-9

MMP-2 (gelatinase A) is primarily expressed in mesenchymal cells during development and tissue regeneration (Bai *et al.*, 2005; Xue, Le, and Jackson 2006). It

is highly expressed in stromal cells surrounding the invading front of metastasizing tumours and is associated with many connective tissue cells as well as neutrophils, macrophages and monocytes (Lynch and Matrisian 2002; Stamenkovic 2000). Together with MMP-9 (gelatinase B), it degrades type IV collagen (the major component of basement membranes) and gelatin. It can also degrade other types of collagens (V, VII and X) as well as elastin and fibronectin.

MMP-9 is also involved in inflammation (Xue *et al.*, 2007), tissue remodelling and wound healing (Puchelle *et al.*, 2006) in addition to tumour invasion and metastasis (Deryugina and Quigley 2006; Stamenkovic 2000). Substrates for MMP-9 include collagens type IV, V, VII, X and XI, denatured collagen type I (gelatin), fibrinogen, vitronectin, IL-1β and entactin, a molecule that bridges laminin and type IV collagen (Bjorklund and Koivunen 2005).

Expression and role of MMP-2 and MMP-9 in tumours

Expression of MMP-2 and MMP-9 has been shown in a wide range of tumours such as breast and prostate cancer (Incorvaia *et al.*, 2007), lung cancer (Drac *et al.*, 2007; Kopczynska *et al.*, 2007), pancreatic cancer (Bloomston *et al.*, 2002), melanoma (Hofmann *et al.*, 2000), bladder cancer (Kanayama 2001), hepatocellular carcinoma (Qin and Tang 2002), colorectal carcinoma (Mook, Frederiks, and Van Noorden 2004)and glioma (Nakada, Okada, and Yamashita 2003) where they facilitate ECM breakdown and migration, invasion and metastasis of tumour cells.

Expression of MMP-2 and MMP-9 in normal and malignant oral epithelium

Expression of MMP-2 and MMP-9 has also been shown in normal as well as malignant oral epithelial cells. MMP-2 and MMP-9 are highly expressed *in vivo* in OSCC patients (Guttman *et al.*, 2004) and expression is correlated to alcohol consumption and the grade of differentiation and is associated with poor survival rates (de Vicente *et al.*, 2005). In addition to *in vivo* tissue expression, MMP-2 and MMP-9 levels are also elevated in plasma compared with disease free individuals. Patients with lymph node metastases express significantly higher active MMP-2 and MMP-9 compared with patients without metastases (Patel *et al.*, 2005; Patel *et al.*, 2007). Constitutive *in vitro* expression of MMP-2 and MMP-9 is detected in culture supernatants of NOK as well as oral cancer cell lines KB and OC-2 (Tsai *et al.*, 2003).

However, the OCCL secrete significantly higher levels of MMP-2 and MMP-9 compared with NOK suggesting an important role for MMP-2 and MMP-9 in oral cancer biology.

Author / year (Reference)	**Association with clinicopathological parameters**
(Kusukawa *et al.*, 1993)	Lymph node metastasis and mode of invasion
(Kawamata *et al.*, 1997)	MMP2 expression related to lymph node metastasis and bone invasion
(Charous *et al.*, 1997)	Expression in tumour front- No difference between primary tumour and metastasis
(Ikebe *et al.*, 1999; Kurahara *et al.*, 1999)	Invasion and lymph node metastasis
(Hong *et al.*, 2000)	Lymph node metastasis (only MMP9)
(Imanishi *et al.*, 2000)	Associated with MT1-MMP and lymph node metastasis
(Tokumaru *et al.*, 2000)	Lymph node metastasis
(Riedel *et al.*, 2000a; Riedel *et al.*, 2000b)	Elevated serum levels associated with cancer stage, Worse survival (only MMP9).
(Yoshizaki *et al.*, 2001)	MMP-2 expression associated with poor prognosis.
(Yorioka *et al.*, 2002)	Lower MMP concentration associated with longer disease free survival
(Franchi *et al.*, 2002)	MMP9 expression associated with angiogenesis and p53 expression in OSCC.
(Robinson *et al.*, 2003)	Expression of MMP2 and MMP9 in OCCL associated with invasiveness *in vitro* and tumourigenesis *in vivo*.
(Katayama *et al.*, 2004)	Lymph node metastasis, Worse survival (only MMP9)
(Nagel *et al.*, 2004)	MMP2 expression associated with invasiveness of salivary gland tumours

Table 8.1. Summary of reported findings of MMP-2 and MMP-9 expression in OSCC.

Chemokines in MMP-2 and MMP-9 regulation

A role for chemokines and their receptors in MMP-2 and MMP-9 regulation is well documented. Interaction of CXCR4 and SDF-1α causes a significant increase in MMP-2 and MMP-9 production from ovarian cancer cells *in vitro* (Yuecheng and Xiaoyan 2007b). SDF-1α also stimulates MMP-9 release from lung cancer cell lines and silencing of the MMP-9 gene inhibits tumour cell invasion towards SDF-1α (Tang *et al.*, 2007a). However both these groups did not study the role of CXCR4 as they did not use blocking antibodies against the receptor. Knockdown of the chemokine receptor CCR1 from a hepatocellular carcinoma cell line HCCLM3 significantly inhibits MMP-2 production from the cells as well as reducing their invasive ability (Wu *et al.*, 2007) suggesting an important role for CCR1 in HCC biology. The role of chemokines and receptors in MMP regulation from oral epithelial cells is not very well characterized. Stimulation of OCCL with SDF-1α significantly up-regulates MMP-9 production in a dose dependent manner (Samara *et al.*, 2004). However they did not compare expression to NOK or study the role of CXCR4.

Chemokines have been shown to influence MMP production in other cancers. SDF-1α up-regulates MMP-9 expression in prostate cancer (Chinni *et al.*, 2006), colorectal cancer (Brand *et al.*, 2005), myeloma (Parmo-Cabanas *et al.*, 2006), HCC (Chu *et al.*, 2007), lung cancer (Tang *et al.*, 2007b) and ovarian cancer (Yuecheng and Xiaoyan 2007a). Treatment of pancreatic cancer cells with IL-8 significantly up-regulates MMP-2 expression (Kuwada *et al.*, 2003) whereas RANTES has a similar effect on breast cancer cells (Azenshtein *et al.*, 2002). As previously observed, none of these studies compared MMP expression between cancerous and normal cells or use a receptor blocking antibody.

Our findings

In agreement with the literature our results also showed constitutive MMP-2 and MMP-9 production in culture supernatants of NOK and OCCL. The level of constitutive MMP-2 and MMP-9 secretion in H357 and SCC4 cells was significantly higher than NOK. Stimulation of NOK, H357 and SCC4 cells with PMA, TNF-α or LPS resulted in a significant increase in MMP-2 and MMP-9 release and the secretion levels were higher in cancer cells than normal cells. Interestingly, exposure of normal and cancerous oral cells to lymphotactin also caused a small but significant increase in

MMP-2 and MMP-9 secretion and pre-incubation of cells with XCR1 blocking antibody reduced secretion to control levels (unstimulated cells). Lymphotactin facilitated a significantly bigger release in MMP-2 and MMP-9 production from H357 and SCC4 cancer cells than NOK. No difference between MMP-2 and MMP-9 expression could be detected between the two cancer cell lines H357 and SCC4 cells. These results suggest that lymphotactin can facilitate an increase in MMP-2 and MMP-9 concentration from oral keratinocytes. This effect is more pronounced in cancer cells than normal cells and is mediated through XCR1. Lymphotactin has been previously shown to down-regulate MMP-2 production from synoviocytes in culture which is different to our findings (Blaschke *et al.*, 2003). This may be explained by the difference in origin of the cells used in their study and ours. XCR1 expression between synoviocytes and oral epithelial cells may differ and it is possible that they respond differently when exposed to lymphotactin.

Expression of MMP-2 and MMP-9 by oral epithelial cells has previously been shown. Bennett *et al.* reported that a skin keratinocyte cell line UP and H357 oral cancer cells constitutively express both MMP-2 and MMP-9 which is in agreement with our findings (Bennett *et al.*, 2000). Stimulation of these cells with scatter factor significantly increases MMP-2 and MMP-9 activity and a blocking antibody against scatter factor inhibited this increase. However, they did not study expression in NOK. Robinson *et al.* reported that NOK and a range of OCCL (including H357 cells) constitutively express pro-MMP-2 and MMP-9 in culture (Robinson *et al.*, 2003). They also reported that pro-MMP-2 production from OCCL is generally higher than NOK whereas pro-MMP-9 levels are higher in NOK than OCCL. This could be explained by the pattern of integrin expression by these cells as a correlation between integrin expression and MMP-2 expression has been shown. Expression of the αvβ6 integrin has been shown to be directly proportional to MMP-9 expression (Thomas *et al.*, 2001a). Transfection of the αvβ6 integrin in the H357 cell line results in a significant increase in MMP-9 release from these cells. This increase in MMP-9 expression then facilitates migration of oral keratinocytes and their adhesion to fibronectin *in vitro*. The OCCL used in the study of Robinson *et al.* expressed very low levels of the αv subunit compared with NOK which may have influenced MMP-9 expression. The pattern of integrin expression is different between normal keratinocytes, in wound healing and in cancer. Therefore, it is possible that different

integrins may influence MMP expression in different conditions. Constitutive expression of MMP-2 and MMP-9 from normal and malignant oral epithelial cells has also been reported by Tsai *et al.* They found that NOK and OCCL (KB and OC2) constitutively produce MMP-2 and MMP-9 in culture and that the levels are significantly higher in OCCL compared with NOK which is in agreement with our findings (Tsai *et al.*, 2003).

b) MMP-7

Expression of MMP-7 (matrilysin) has been reported in epithelial cells of normal as well as diseased tissues (Hajj *et al.*, 2007; Kassim *et al.*, 2007; Kuivanen *et al.*, 2006). MMP-7 is capable of degrading a range of ECM proteins such as collagen IV, gelatin, laminin, aggrecan, entactin, elastin and versican. It can activate other proteinases such as urokinase plasminogen activator and pro-MMP-1, -2, -9, and can cleave additional substrates such as osteopontin (Ii *et al.*, 2006).

Expression and role of MMP-7 in tumours

MMP-7 is expressed in a variety of tumours including breast (Jiang *et al.*, 2005), colon (Adachi *et al.*, 2001), prostate (Hashimoto *et al.*, 1998), lung (Liu *et al.*, 2007), ovary (Sillanpaa *et al.*, 2006) and skin (Kawasaki *et al.*, 2007). MMP-7-mediated cleavage of Fas ligand protects tumour cells from chemotherapeutic drug cytotoxicity hence facilitating tumour growth and progression (Mitsiades *et al.*, 2001). MMP-7 expression has also been shown *in vivo* in endometrial cancers and the production levels directly correlate with lymph node metastasis (Shiomi and Okada 2003). *In vivo* expression has also been shown in colorectal and gastric carcinomas and a correlation is observed between staining intensity and vascular invasion and metastases (Aihara *et al.*, 2005; Kitoh *et al.*, 2004; Lee *et al.*, 2006; Zheng *et al.*, 2003). Liver metastases of colorectal carcinoma show up-regulation of MMP-7 mRNA and protein compared with the normal liver tissue. Strong *in vivo* expression of MMP-7 is observed localized in the tumour front and cytoplasm (Ogawa *et al.*, 2005; Pesta *et al.*, 2007). In addition, significantly higher levels of latent MMP-7 are detected in liver metastases samples compared with normal liver tissue but the active form of MMP-7 is only detected in the metastatic cells in the liver and not in the normal cells (Zeng *et al.*, 2002). This suggests an important role for active MMP-7 in metastasis of colorectal carcinomas to the liver. Transfection of the MMP-7 gene into

a breast cancer cell line MCF-7 results in production of increased levels of pro- and active MMP-7 and significantly increases invasion through matrigel compared with the untransfected cells (Wang, Reierstad, and Fishman 2006).

Expression of MMP-7 in oral cancer

A role for MMP-7 in the biology of oral cancer has also been reported (Birkedal-Hansen *et al.*, 2000; Impola *et al.*, 2004). Expression of MMP-7 is observed in oral tumours but not in normal tissue and is significantly correlated with lymph node metastasis and patient survival (de Vicente *et al.*, 2007). This suggests that MMP-7 can be used a prognostic indicator, particularly in patients with lymph node metastasis.

Chemokines in MMP-7 regulation

The role of chemokines in MMP-7 regulation is not very well characterized. To date, MMP-7 production in response to chemokine stimulation has been reported from malignant oral keratinocytes *in vitro*. Stimulation of two oral cancer cell lines NA and HSC-4 with IL-8 significantly up-regulates MMP-7 expression at mRNA level and its production in culture (Watanabe *et al.*, 2002). However, this group did not compare MMP-7 production from OCCL to that from NOK.

Our findings

Our experiment showed different results for MMP-7 compared with MMP-2 and MMP-9. No constitutive expression was observed in NOK or OCCL. Stimulation of NOK with PMA, TNF-α, LPS or lymphotactin failed to increase MMP-7 secretion in culture. However, in OCCL H357 and SCC4 a pronounced up-regulation in MMP-7 expression was observed after stimulation with PMA, TNF-α or LPS. In addition, stimulation of H357 and SCC4 cells with lymphotactin also caused a significant increase in MMP-7 release compared with unstimulated controls. Lymphotactin stimulation facilitated release of significantly very high levels of MMP-7 in SCC4 cells compared with H357 cells. Preincubation of cells with XCR1 antibody significantly reduced MMP-7 production to control levels. These results show that lymphotactin can stimulate production of MMP-7 from oral cancer cells but not from normal cells. In addition, SCC4 cells which are almost 100% positive for XCR1 secrete very high levels of MMP-7 after exposure to lymphotactin compared with

H357 cells which release a much smaller amount suggesting that MMP-7 production may have a correlation with number of XCR1 expressing cells.

These findings can have a number of implications. Up-regulation of MMP-2, MMP-7 and MMP-9 from NOK and OCCL after lymphotactin stimulation suggests that XCR1/lymphotactin interaction may be important in MMP production from oral epithelial cells. A large increase in MMP-7 production from SCC4 cells suggests a correlation between XCR1 expression and MMP-7 production and indicates that XCR1 and lymphotactin interaction may be more important in MMP-7 regulation than MMP-2 or MMP-9. However, the ELISA kits used in these experiments only measure total MMP and cannot distinguish between the pro and active forms of the enzymes.

Release of MMP-2, MMP-7 and MMP-9 is hallmark of wound healing, tumour progression, invasion and metastasis as increased expression allows cells to attach to and break down ECM thus allowing migration and invasion. As our previous results have shown abundant lymphotactin expression in diseased oral mucosa it is possible that XCR1 may play an important role in the biology of these processes by regulating MMP expression and thus influencing physiological epithelial cell migration and adhesion in wound healing or inflammation or by affecting tumour cell invasion and metastasis.

CHAPTER 9

Discussion and Conclusions

9.1 DISCUSSION

As explained in Chapter 1, the principal function of chemokines is chemoattraction, trafficking and localisation of lymphocytes. However, expression of chemokine receptors has been shown on epithelial cells where they mediate a range of effects (including cell migration and invasion) and influence the behaviour of cells. Chemokine receptors have been implicated in a wide range of cancers where they facilitate tumour invasion and metastasis to lymph nodes along the gradient of their respective chemokine.

Our preliminary microarray results suggested a difference in mRNA levels of the chemokine receptors XCR1 and CXCR1 between normal and cancerous oral epithelial cells. These results were exciting as XCR1 expression has never been shown on epithelial cells whereas the expression of CXCR1 on oral epithelial cells is not well characterised. XCR1 binds to the chemokine lymphotactin whereas CXCR1 (along with CXCR2) binds with the highest affinity to IL-8. To date, expression of XCR1 outside the lymphoid system has only been shown on fibroblast-like synoviocytes in rheumatoid synovium (Blaschke *et al.*, 2003) and very recently in melanocytic lesions (Seidl *et al.*, 2007). Therefore, the preliminary results were explored further and the aim of this project was to establish the expression of XCR1 and CXCR1 (and CXCR2 since it also binds to IL-8) on normal and malignant oral epithelial cells and to observe if they have a role in the regulation of oral epithelial cell behaviour.

9.1.1 CXCR1 and CXCR2

Our results show that CXCR1 and CXCR2 are expressed at mRNA and protein level by normal as well as cancerous oral epithelial cells. More cancer cells express the receptors than normal keratinocytes and show a greater response to IL-8 (acting through CXCR1 and CXCR2) in activation of the ERK1/2 signaling cascade, migration, invasion and proliferation *in vitro*.

The findings with NOK show that normal cells have the ability to respond to the ligands if provided with the apt stimulus. Activation of ERK1/2, increase in proliferation and

migration of NOK *in vitro* suggests a potential role for CXCR1 and CXCR2 *in vivo* in physiological epithelial cell turnover as well as in wound healing and inflammatory conditions. A role for IL-8 in wound healing has been shown in the skin (Engelhardt, *et al.*, 1998). Our finding that CXCR1 and CXCR2 are expressed by NOK suggests that IL-8 and GRO-α may play a role in wound healing in the oral cavity by stimulating proliferation and migration of epithelial cells. However whether IL-8 or GRO-α is expressed by oral epithelial cells or other cell types in oral wounds is unknown. Normal oral epithelial cells express CXCR1 and CXCR2 *in vivo* (Sfakianakis, Barr, and Kreutzer 2002) and expression is also observed in periodontitis. However, the results from this study are not very convincing and whether CXCR1 and CXCR2 expression is altered in oral wounds is not clear.

In general, a higher percentage of OCCL were positive for CXCR1 and CXCR2 than NOK and cancer cells showed a stronger response to stimulation with the ligands than normal cells. This difference in expression and behaviour between normal and cancer cells suggests a possible role for CXCR1 and CXCR2 in the biology of oral cancer. Expression of IL-8, CXCR1 and CXCR2 has previously been shown in oral cancer *in vitro* and *in vivo* (Cohen *et al.*, 1995; Richards *et al.*, 1997; Watanabe *et al.*, 2002). However, expression between normal and cancerous cells has not been compared before. In addition, the individual roles of CXCR1 and CXCR2 in IL-8 mediated effects in epithelial cell invasion and proliferation have never been reported. CXCR1 and CXCR2 have been shown to mediate OCCL migration in response to IL-8 in only one study (Watanabe *et al.*, 2002). However this group did not use both antibodies in combination to find out whether it caused any further decrease in migration compared to CXCR1 or CXCR2 alone. The results of this thesis suggest that CXCR1 and CXCR2 mediate both invasion and migration but that interaction between both receptors may be important in some cancer cell lines. Furthermore CXCR1 appears to play a more important role that CXCR2 in proliferation although both may mediate the effects of their respective ligands. Strong IL-8 expression is observed in OSCC and expression in OSCC primary cultures and cell lines is upregulated after stimulation with TNF-α and IL-1β both of which are expressed in tumours (Cohen *et al.*, 1995; Watanabe *et al.*, 2002). The abundant

expression of IL-8 in OSCC coupled with the fact that more oral cancer cells are positive for both IL-8 receptors and respond more than NOK to IL-8 (as indicated by our findings) suggests that CXCR1 and CXCR2 may play a role in the pathogenesis of oral cancer by facilitating tumour cell proliferation, migration, invasion and possibly metastasis.

9.1.1.1 CONCLUSIONS (CXCR1 and CXCR2)

Our findings show that CXCR1 and CXCR2 are expressed on normal and malignant oral epithelial cells and are functional as they facilitate signaling, migration, invasion and proliferation in response to IL-8 and GRO-α. While CXCR1 and CXCR2 appear to mediate both migration and invasion there is some evidence that CXCR1 may be more important in proliferation. These findings raise the possibility that CXCR1 and CXCR2 may regulate the behaviour of normal oral epithelial cells *in vivo* in physiological processes such as epithelial cell turnover as well as in wound healing. In addition, cancer cells appear more migratory, invasive and proliferative in response to the ligands than NOK and these findings suggest a potential role for CXCR1 and CXCR2 in the biology of oral cancer.

9.1.2 XCR1 AND LYMPHOTACTIN

XCR1 expression was observed at mRNA and protein level in NOK, HGF and OCCL. In general, a higher percentage of cancer cells were positive for XCR1 than NOK but exposure to lymphotactin facilitated *in vitro* signal transduction, migration, invasion and proliferation in both. The cancer cells however showed a greater quantitative response than NOK in all experiments. Differences between the responses of normal and cancer cells to lymphotactin were also observed. XCR1 mediated an increase in adhesion of both NOK and OCCL to fibronectin after stimulation with lymphotactin but for collagen, exposure to lymphotactin only increased adhesion of OCCL and not of NOK. The mechanisms underlying this increase is not clear but it is possible that lymphotactin may affect the expression or avidity of integrins involved in binding to fibronectin ($\alpha5\beta1$, $\alpha v\beta6$) and type I collagen ($\alpha1\beta1$, $\alpha2\beta1$). The difference in the response between NOK

and OCCL in adhesion to collagen may reflect differences in $\alpha1\beta1$, $\alpha2\beta1$ expression between the cells. Further work is required to investigate this in detail.

A difference in MMP production between normal and cancer cells was also noticed. XCR1/lymphotactin facilitated a small increase in MMP-2 and MMP-9 from NOK and OCCL. However, an increase in MMP-7 in response to lymphotactin was only seen in cancer cells and not in NOK. As observed with signaling, proliferation, migration and invasion the increase in adhesion and MMP-2 and MMP-9 was greater in OCCL than NOK.

Differences between normal and cancerous oral mucosa were also seen *in vivo*. XCR1 expression was seen in normal oral mucosa (NOM) but appeared much stronger in OSCC. Expression was not only seen on epithelial cells but also on infiltrating lymphocytes, fibroblasts and endothelial cells. In contrast no staining for lymphotactin was observed in normal mucosa (epithelium or IEL) but abundant lymphotactin expression was seen in OSCC on epithelial cells, infiltrating lymphocytes, IEL, fibroblasts and endothelial cells. In addition, strong XCR1 and lymphotactin expression was observed in regional lymph nodes. The invading tumour cells as well as the metastases in regional lymph nodes also exhibited strong expression of both XCR1 and lymphotactin. However, both NOK and OCCL constitutively expressed intracellular lymphotactin *in vitro* but failed to release it in culture. XCR1 and lymphotactin also appeared to be upregulated *in vivo* in dysplasia, lichen planus and non-specific ulceration suggesting that upregulation of XCR1 and lymphotactin expression is not restricted to OSCC but is also increased in inflammatory conditions.

All these findings suggest a role for XCR1 and lymphotactin in regulation of normal as well as cancer cells. Epithelial cells in NOM *in vivo* express XCR1 but do not stain for lymphotactin which raises a question about the function of XCR1 and the source of lymphotactin in healthy mucosa. Previously, IEL in murine epidermis have been shown to produce lymphotactin *in vivo* (Boismenu *et al.*, 1996) but we did not observe lymphotactin expression on IEL in NOM. However, only three cases of NOM were

studied and it is possible that the protocol or conditions of our immunocytochemical staining were not perfectly optimised. This may also be the explanation as to why lymphotactin was not found in the epithelial cells when it was present intracellularly in NOK *in vitro.*

Abundant lymphotactin and XCR1 expression was seen on epithelial cells in lichen planus and non-specific ulceration as well as on infiltrating lymphocytes which indicates that some sort of trigger is required to stimulate the production of lymphotactin. It is possible that the inflammatory infiltrate acts as a trigger by producing inflammatory mediators but it is also possible that the increase in lymphotactin production attracts the inflammatory infiltrate. Alternatively a combination of these may be operative. More work is required *in vivo* to study the relationship between the infiltrate and increased expression of lymphotactin and XCR1.

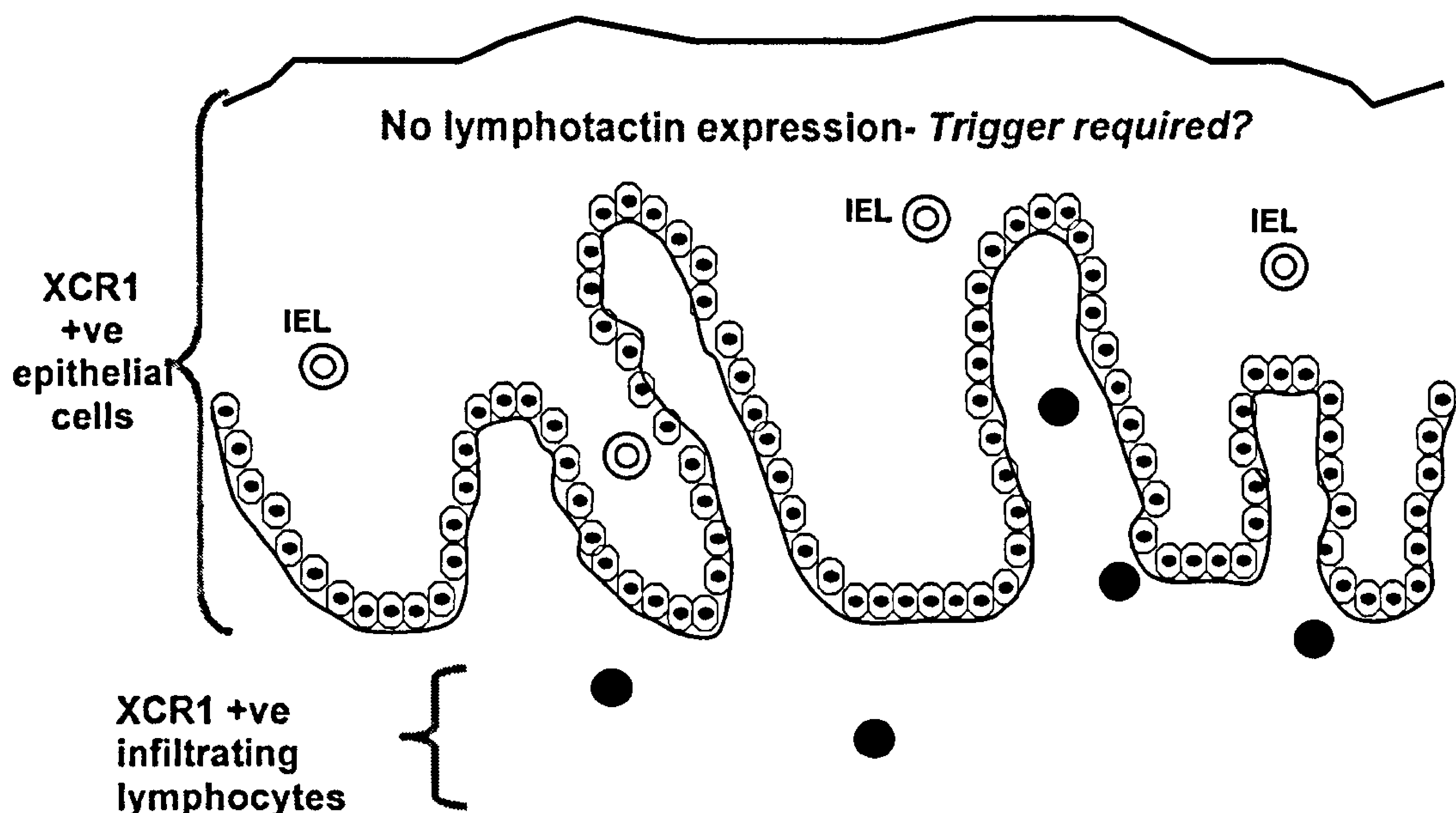


Figure 9.1. Suggested XCR1/lymphotactin expression in normal oral mucosa *in vivo*. XCR1 is expressed by normal oral epithelial cells as well as some of the infiltrating lymphocytes. Expression of XCR1 by epithelial cells may enable them to interact with the lymphotactin which may be produced by IEL thus influencing the behaviour of epithelial cells.

Since expression of XCR1 and lymphotactin has not been shown by epithelial cells before, nothing is known about the regulation of their expression. In addition to a possible role for inflammatory mediators, it possible is that lymphotactin itself may regulate expression of XCR1. However at this stage we do not know which mediator influences XCR1 or lymphotactin expression. Our *in vitro* studies were unable to show a role for PMA, TNF-α LPS in the release of lymphotactin from keratinocytes in culture. Recent reports have shown lymphotactin production from lymphocytes after stimulation with bacterial pathogens such as *Staphylococcus aureus* (Tikhonov *et al.*, 2001) and *Porphyromonas gingivalis* (Zhou *et al.*, 2005). Our preliminary results indicate that exposure of oral keratinocytes to *S. aureus* or *P. gingivalis* stimulates lymphotactin production from these cells (results not shown). However, more work needs to be done to establish the regulation of lymphotactin expression by epithelial cells.

The significance of the increased XCR1 and lymphotactin expression by epithelial cells and lymphocytes in lichen planus and non-specific ulceration is not clear. However expression in non-specific ulceration raises the possibility XCR1 and lymphotactin may play a role in wound healing by increasing proliferation and migration across the ulcer surface. These processes may be helped in part by the increases in MMP production and adhesion to fibronectin observed in this study. Another possible function of lymphotactin may be attraction of neutrophils and other inflammatory cells once the ulceration has occurred. In oral lichen planus destruction of the basal cells is associated a dense lymphocyte dominated infiltrate in the lamina propria. Lymphotactin may play a role in attracting this infiltrate but also may promote healing by stimulating proliferation.

Expression of XCR1 and lymphotactin is also detected in dysplasia and OSCC and appears to be upregulated compared with NOM. This difference in XCR1 and lymphotactin expression between NOM and OSCC suggests a potential role for XCR1 and lymphotactin interaction in the biology of OSCC. These findings provided an insight into the source of lymphotactin within the oral mucosa as expression was seen in IEL, infiltrating lymphocytes and in epithelial cells suggesting that there are a number of potential sources for lymphotactin within the oral mucosa in OSCC. The *in vivo*

expression of lymphotactin in abundance by lymphocytes and epithelial cells suggests that lymphotactin may facilitate tumour cell migration and invasion through increased adhesion and MMP production in addition to acting as strong proliferative influence. Thus lymphotactin may increase the aggressive behaviour of the tumour. However it is also possible that lymphotactin production by the epithelial cells and the tumour facilitates chemotaxis of cytotoxic T lymphocytes (with upregulated XCR1) which may act on the tumour and cause a reduction in its size and effects. These opposing potential effects combined with the possibility that the stimulated lymphocytic infiltrate releases other cytokines or growth factors which may play a role in lymphotactin or epithelial cell regulation .indicates the complexity of the scenario. Further work needs to be done to understand the precise role and regulation of XCR1 and lymphotactin in OSCC.

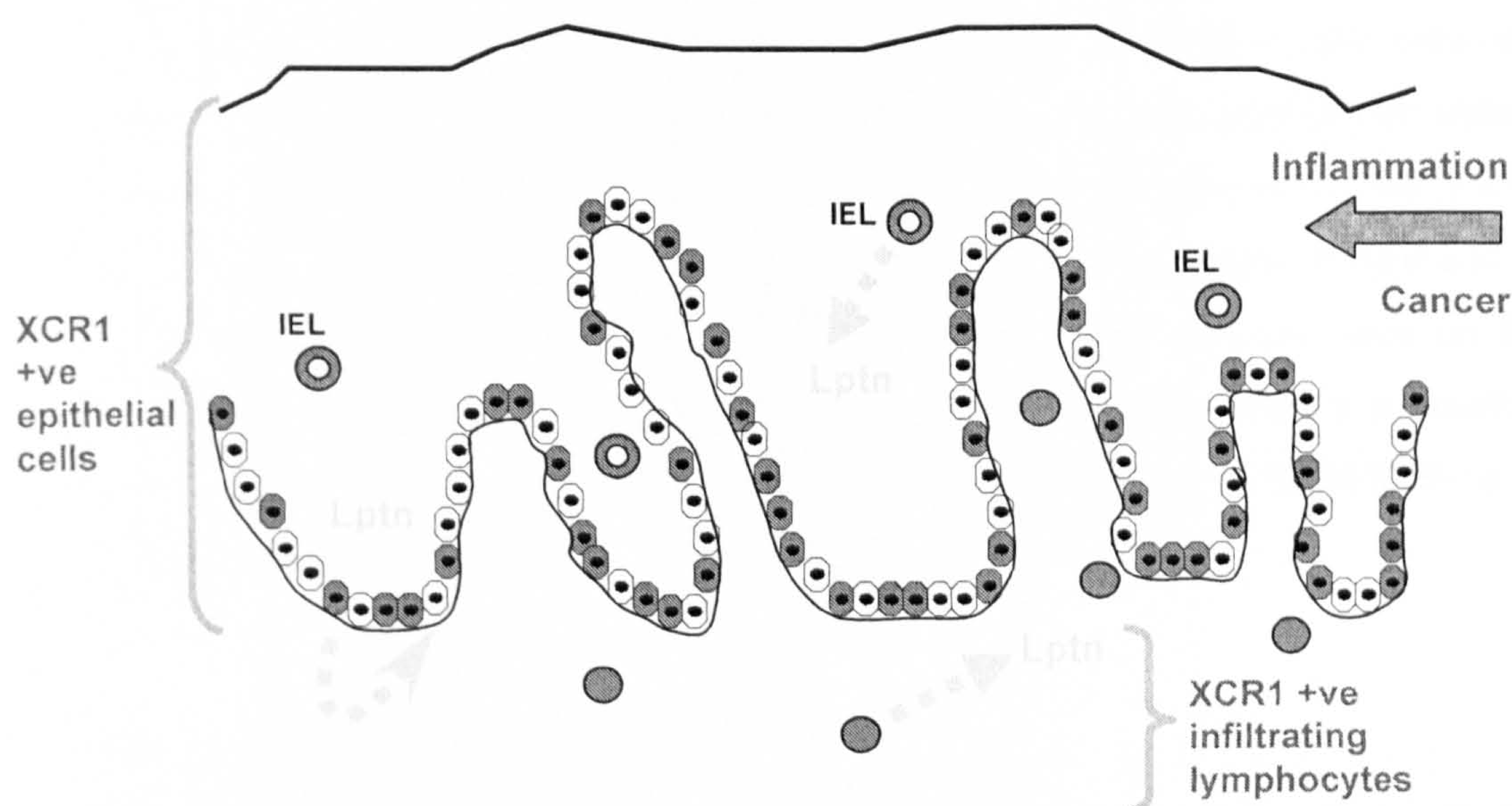


Figure 9.2. XCR1/lymphotactin expression in inflammation and OSCC *in vivo* showing possible sources of lymphotactin. In conditions like inflammation or cancer, Strong XCR1 expression is seen on the epithelial cells as well as the infiltrating lymphocytes. In addition, strong lymphotactin expression is also observed in these conditions and it appears to be produced by IEL, infiltrating lymphocytes and the epithelial cells thus providing multiple sources of lymphotactin within the oral epithelium.

As mentioned earlier the invading tumour cells as well as the metastatic deposits in the regional nodes exhibit strong XCR1 and lymphotactin expression and lymphotactin is produced in abundance in cervical lymph nodes. This may provide a gradient to tumour cells (with upregulated XCR1 expression) in the lymphatics allowing them to metastasise via increased adhesion, MMP production and invasion. The chemokine receptor CXCR4 and its ligand have been implicated in the metastasis of OSCC as increased CXCR4 expression is associated with increased tumour cell migration, invasion and metastasis (Almofti *et al.*, 2004; Ishikawa *et al.*, 2006; Muller *et al.*, 2006). Recent reports have shown autocrine chemokine/receptor signaling plays a more important role in distant OSCC metastases than paracrine signaling. Tumour cells capable of autocrine signaling (i.e. expressing CXCR4 as well as its ligand SDF-1α) show increased frequency and foci of metastasis in the regional lymph nodes compared with cells expressing only the receptor CXCR4 (thus having only paracrine activation). In addition, the cells co-expressing CXCR4/SDF-1α exhibit distant metastases to the lung whereas no distant metastases are seen with cells expressing CXCR4 on its own (Uchida *et al.*, 2007). Our results showed co-expression of XCR1 and lymphotactin in cancer cells *in vitro* and *in vivo* and strong expression of both the receptor and the ligand was also seen on the invading tumour cells and metastases in regional lymph nodes. Therefore, it is possible that XCR1/lymphotactin interaction may act in a similar manner to CXCR4/SDF-1α in OSCC metastasis.

9.1.2.1 CONCLUSIONS (XCR1)

Our results show widespread expression of XCR1 in normal and cancerous oral epithelial cells *in vitro* and *in vivo* and expression appears to be upregulated on cancer cells. Lymphotactin (the ligand for XCR1) is also expressed constitutively *in vitro* by NOK and OCCL however *in vivo* expression is only evident in inflammatory conditions and cancer. Findings from normal epithelial cells suggest that XCR1 may play a role in modulating the behaviour of these cells and therefore may have a role in physiological cell turnover as suggested by proliferation of NOK in response to lymphotactin. XCR1 may also mediate migration and invasion of normal epithelial cells in wound healing. Production of

lymphotactin from oral epithelial cells may facilitate chemotaxis of lymphocytes and neutrophils which may exert anti-inflammatory or pro-inflammatory effects. Up-regulation of XCR1 and lymphotactin on epithelial cells and lymphocytes in OSCC suggests a potential role in the pathogenesis of OSCC. Invading tumour cells and metastatic tumour deposits show strong expression of both XCR1 and lymphotactin and regional lymph nodes express lymphotactin and XCR1 in abundance which may provide a gradient for tumour cell invasion and metastasis. Together these findings suggest a potential role for XCR1/lymphotactin interaction in the pathogenesis of OSCC.

XCR1 and lymphotactin are widely expressed in the oral tissues *in vivo* and increases in both are seen in non-specific ulceration, oral lichen planus and oral malignancy. Both are also expressed by normal and malignant epithelial cells *in vitro* and lymphotactin mediates proliferation, migration, and invasion as well as increasing MMP production and cell adhesion. These findings raise the possibility that XCR1 and lymphotactin may play an important role in normal mucosa, inflammatory disease and oral cancer.

9.2 FUTURE WORK

Our findings have raised a number of questions that need to be addressed in detail to find out the precise role, regulation and mechanism of action of XCR1 and lymphotactin in the pathogenesis of OSCC.

1. Since XCR1 appears to be upregulated in OSCC compared with NOM, it would be interesting to study the reason for this increase in XCR1 expression and the regulation of its expression. It is possible that XCR1 expression is correlated to the cell cycle and could be studied using propidium idodide staining and flow cytometry. We could also study the effect of hypoxia on XCR1 expression as tumours have diminished oxygen supply and therefore the cells may be hypoxic. CXCR4 has been shown to be highly upregulated in response to hypoxia. We could also study whether lymphotactin has any role in the regulation of XCR1 expression.

2. Similarly it would be important to establish the regulation of lymphotactin expression and what triggers its production. A wide range of inflammatory mediators could be studied in that regard. As already mentioned, our preliminary results showed lymphotactin production from oral keratinocytes after exposure to *S. aureus* or *P. gingivalis* and this association between lymphotactin production and bacterial pathogens should be studied further.

3. The mechanism of XCR1 activation and signalling should also be studied. Since we have already studied the ERK1/2 pathway, we could analyse calcium mobilisation and actin polymerisation in oral epithelial cells in response to lymphotactin. Other signalling pathways such as FAK may also be studied in addition to the correlation between expression of XCR1 and other adhesion molecules (such as integrins).

4. Our understanding of the role of XCR1 and lymphotactin in oral epithelial cell migration and invasion could be improved by performing these assays using organotypic cultures or reconstructed oral mucosa models.

5. Similarly, knocking the XCR1 gene down in cancer cells using Si-RNA and repeating the functional assays (signaling, migration, invasion, proliferation, adhesion and MMP production) would lead to a better understanding of the role of XCR1 in cancer cell regulation. It would also be interesting to find out if knocking down the lymphotactin gene would have any effects on the functionality and expression of XCR1.

6. Since only the role of XCR1/lymphotactin interaction in adhesion of oral epithelial cells to fibronectin and type I collagen was studied it would be interesting to investigate adhesion to other ECM components such as laminin, tenascin and other types of collagen.

7. The association between XCR1/lymphotactin expression and OSCC pathogenesis should also be studied. This can be achieved by an in depth study of *in vivo* expression of XCR1 and lymphotactin and using more tissue samples, different grades and stages of OSCC and correlating it with clinicopathologic parameters such as lymph node

metastasis and patient survival. This could be backed up by *in vivo* studies on mice by studying whether increased XCR1 expression is related to increased tumourigenesis and whether knocking out XCR1 or lymphotactin genes makes any difference.

CHAPTER 10

Appendix

10.1 MEDIA FOR CELLS

10.1.1 Keratinocyte Growth Medium (KGM)

The following reagents were used to make up 400 ml of KGM.

- Dulbecco's modified eagle medium (DMEM) with sodium pyruvate and 1000 mg/ml glucose (Invitrogen): 270 ml.
- HAM F12 Nutrient mixture (Invitrogen): 90 ml.
- 10 % Foetal bovine serum (FBS), not heat activated (Invitrogen) : 40 ml.
- Epidermal growth factor (Sigma): 400µl (10 ng/ml)
- Hydrocortisone (Sigma): 4ml (400ng/ml)
- Transferrin (Sigma): 5µg/ml.
- Insulin (Sigma): 200µl (5µg/ml).
- Cholera Toxin (Sigma): 600µl (1×10^{-10}M)
- Penicillin/Streptomycin (Invitrogen): 4 ml (2.5 µg/ml)
- Fungizone (Invitrogen): 4 ml (2.5 µg/ml)
- Adenine (Sigma): 5ml (1.8×10^{-4} M).

10.1.2 Medium for HGF, TR146 and A375P cells

All utilize the same medium containing:

- DMEM (Invitrogen): 500ml
- Penicillin/Streptomycin (Invitrogen): 5ml (50µg/ml)
- Fungizone (Invitrogen): 5ml (2.5 µg/ml)
- 10 % FBS (Invitrogen): 50ml

10.1.3 Medium for SCC4 and SCC25

500ml of medium contained:

- DMEM:F12 (1:1 mix) with 15mM Hepes and L-Glutamine, (Invitrogen): 500ml
- Penicillin/Streptomycin (Invitrogen): 5ml (50µg/ml)
- Fungizone (Invitrogen): 5ml (2.5 µg/ml)

- Hydrocortisone: 4ml (400ng/ml).
- 10% FBS (Invitrogen): 50ml

10.1.4 Medium for CAL27 and FaDu cell lines

500ml of medium contained:

- RPMI-1640 with 25mM Hepes and L-Glutamine (Invitrogen): 500ml
- Penicillin/Streptomycin (Invitrogen): 5ml (50μg/ml)
- Fungizone (Invitrogen): 5ml (2.5μg/ml)
- 10 % FBS (Invitrogen): 50ml

10.1.5 Serum-free Media

Serum-free media for all cells (where required) were prepared using their respective medium (i.e. DMEM, RPMI or DMEM:F12) with penicillin and streptomycin but without FBS.

10.2 RNA ISOLATION, EXTRACTION AND ANALYSIS

10.2.1 Materials used for RNA isolation

- Chloroform (Sigma)
- RNase-free 0.1% DEPC water (Diethylpyrocarbonate) (Sigma)
- Isopropanol (Fluka Biochemika)
- Ethanol (Sigma)
- Heraeus Megafuge capable of > 10,000 x g
- RNase-free centrifuge tubes (2 ml) with secure closures

10.2.2 Materials used for RNA analysis

- Spectrophotometer (BioRad)
- Reagents and apparatus for preparation and electrophoresis of agarose gels.

10.2.3 Analysis of isolated RNA

10.2.3.1 Spectrophotometric analysis

The following values were obtained for some of the isolated RNA samples.

Serial No.	Sample Type	[RNA] µg/µl
1.	NOK	1.425 µg/µl
2.	HGF	0.864 µg/µl
3.	NSK	1.327 µg/µl
4.	TR146	0.628 µg/µl
5.	H357	1.236 µg/µl
6.	SCC4	1.924 µg/µl
7.	SCC25	2.469 µg/µl
8.	CAL27	1.576 µg/µl
9.	FaDu	1.628 µg/µl
10.	A375P	2.106 µg/µl

Table 7.1. Spectophotometric analysis of RNA samples (Representative values).

10.2.3.2 Gel electrophoresis

An example of the results of the gel electrophoresis of the isolated RNA samples is pictured below (Figure 10.1). This picture does not show every sample used.

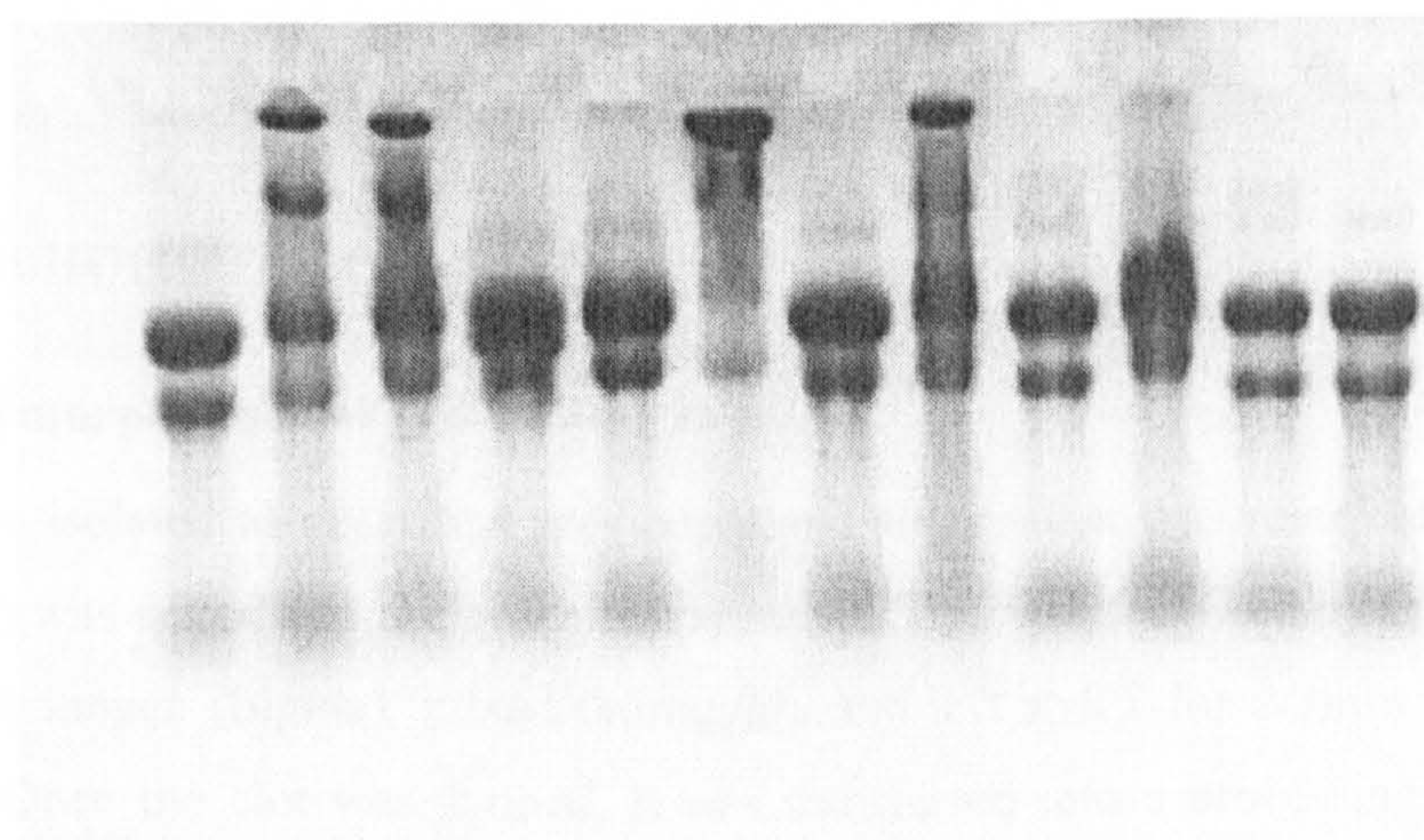


Figure 10.1. Sample result of RNA gel electrophoresis before DNase treatment. The 18S and

28S bands can be clearly observed in most of the samples. DNA contamination can be observed in some samples.

10.3 FLOW CYTOMETRY

10.3.1 Neutrophil Isolation

Human venous blood from healthy volunteers was obtained after informed consent (Division of Genomic Medicine, Royal Hallamshire Hospital, University of Sheffield) and was anti-coagulated with 0.1ml of 3.8%w/v sodium citrate solution per ml of blood. Blood was centrifuged at 400 x g for 20min and the upper serum layer removed. The remaining blood cells were diluted 50:50 with HBSS without Ca^{2+} or Mg^{2+} (Cambrex, East Rutherford, NJ, USA) overlaid onto lymphocyte separation medium (PAA, Pasching, Austria), and centrifuged at 400 x g for 40min. The mononuclear layer containing monocytes and lymphocytes was removed and washed once in HBSS. The polymorphonuclear leukocyte (PMNL)/erythrocyte layer containing the neutrophils was collected and PMNL isolated by hypertonic lysis. Erythrocytes were lysed by adding sterile water to the PMNL/erythrocyte phase for 30sec followed by an equal volume of 1.8% NaCl to bring the suspension to an isotonic state. Cells were centrifuged at 400 x g for 10min and the process repeated 3-4 times until all erythocytes had been lysed. Finally, PMNLs were washed in HBSS with 0.1% BSA and re-suspended to the required cell density.

10.4 IMMUNOHISTOCHEMISTRY

10.4.1 Polymorphonuclear cell clotting in fibrin

PMNL were isolated as described previously and supernatant was removed. 200μl FBS (Invitrogen) was added and the pellet resuspended. This was followed by the addition of 200μl of fibrinogen (Sigma), mixed thoroughly and left at RT for 2-3min to allow clot formation. Once the clot was formed, it was transferred into a processing cassette and fixed with 70% ethanol (Sigma) until processed.

10.5 GEL EXTRACTION AND DNA SEQUENCING

10.5.1 Gel Extraction

Gel extractions were carried out using a gel extraction kit (Qiagen, Crawley, UK). The DNA fragment was excised from the agarose gel using a sharp scalpel and weighed in a colourless tube. 3 volumes of buffer QG (provided with kit) were added to 1 volume of gel and incubated at 50°C for 10min (or until the gel had dissolved). To ensure gel dissolution, the tube was vortexed every 2-3min. The colour of the mixture turns yellow once the gel gets dissolved. 1 volume of isopropanol (Sigma) was added to the sample and mixed. The sample was then transferred to a QIAquick column in a 2ml collection tube (provided with kit) and centrifuged for 1min. The flow-through was discarded and the QIAcolumn was placed back in the same collection tube. 0.5ml of buffer QG was added to the QIAquick column and centrifuged for 1min to remove any remaining agarose. The column was then washed with 0.75ml of buffer PE (provided with kit) by centrifuging it for 1min. The flow-through was removed and the QIAquick column was centrifuged for an additional 1min at 17,900 x g (13000 rpm). The column was transferred to a clean 1.5ml microcentrifuge tube. 50µl of sterile water (Qiagen) was added to the center of the QIAquick membrane and centrifuged for 1min to elute the DNA.

10.5.2 Growth of bacterial culture

Top 10 *E. coli* cells (Promega, UK) were used for transformation. This strain carries the ampicillin gene; therefore bacterial growth was carried out in the presence of ampicillin (50mg/ml).

10.5.3 Transformation

For transformation, 100µl of cells per transformation were thawed on ice. 5µl of eluted DNA was added to each 100µl aliquot, mixed and incubated on ice for 30min. Cells were heat shocked by placing tubes in a water bath at 42°C for 30sec followed by chilling on ice for 2min. 900µl SOC medium (Invitrogen) was added to the cells and incubated at

37°C on a shaker for 1hr. Cells were plated out on LB agar plates containing ampicillin (50mg/ml) and incubated at 37°C overnight.

The next day, single colonies were picked from the agar plates with a pipette tip and transferred to a tube containing 2ml LB broth and ampicillin and incubated on a shaker at 37°C overnight. After 24hrs pipette tips were removed from the tubes. Medium was centrifuged at 2500-3000rpm for 10min after which a mini-prep kit (Qiagen) was used to isolate DNA using 1.5ml of sample.

10.5.4 Mini Prep

Bacterial pellet was resuspended in 250μl in buffer P1 (provided with kit) and transferred to a micro-centrifuge tube. This was followed by the addition of 200μl of buffer P2 (provided with kit) and thoroughly mixing was carried out by inverting the tube. 350μl of buffer N3 (provided with kit) added, mixed immediately by inverting the tube and centrifuged for 10min at 13000 rpm. Supernatant was added to a QIAprep spin column (Qiagen) and centrifuged for 30-60sec. The flow-through was drained and the spin column was washed by a 30-60sec centrifugation with 0.75ml buffer PE (provided with kit). The flow-through was removed and the spin column was centrifuged again to remove residual wash buffer. The QIAprep column was then moved to a clean 1.5ml micro-centrifuge tube. DNA elution was achieved by adding 50μl of water to the centre of the spin column, allowed to stand for 1min and then centrifuged for 1min. The eluted DNA was collected, its concentration using a spectrophotometer and stored in the freezer.

10.5.5 Restriction digests

8μl of DNA was digested with 0.5μl HindIII (Promega) and 0.5μl NotI (Promega) enzymes (mixed with 1μl buffer) and incubated at 37°C for 4hr. The samples were run on a 1% agarose gel (Figure 10.2).

10.5.6 Gel

The samples were loaded on the gel and run at 100 volts for 20-30min. The gel showed bands at 7000bp (representing plasmid/vector) and around 300bp (representing lymphotactin). The gel photograph was taken by Gene Snap software (Syngene).

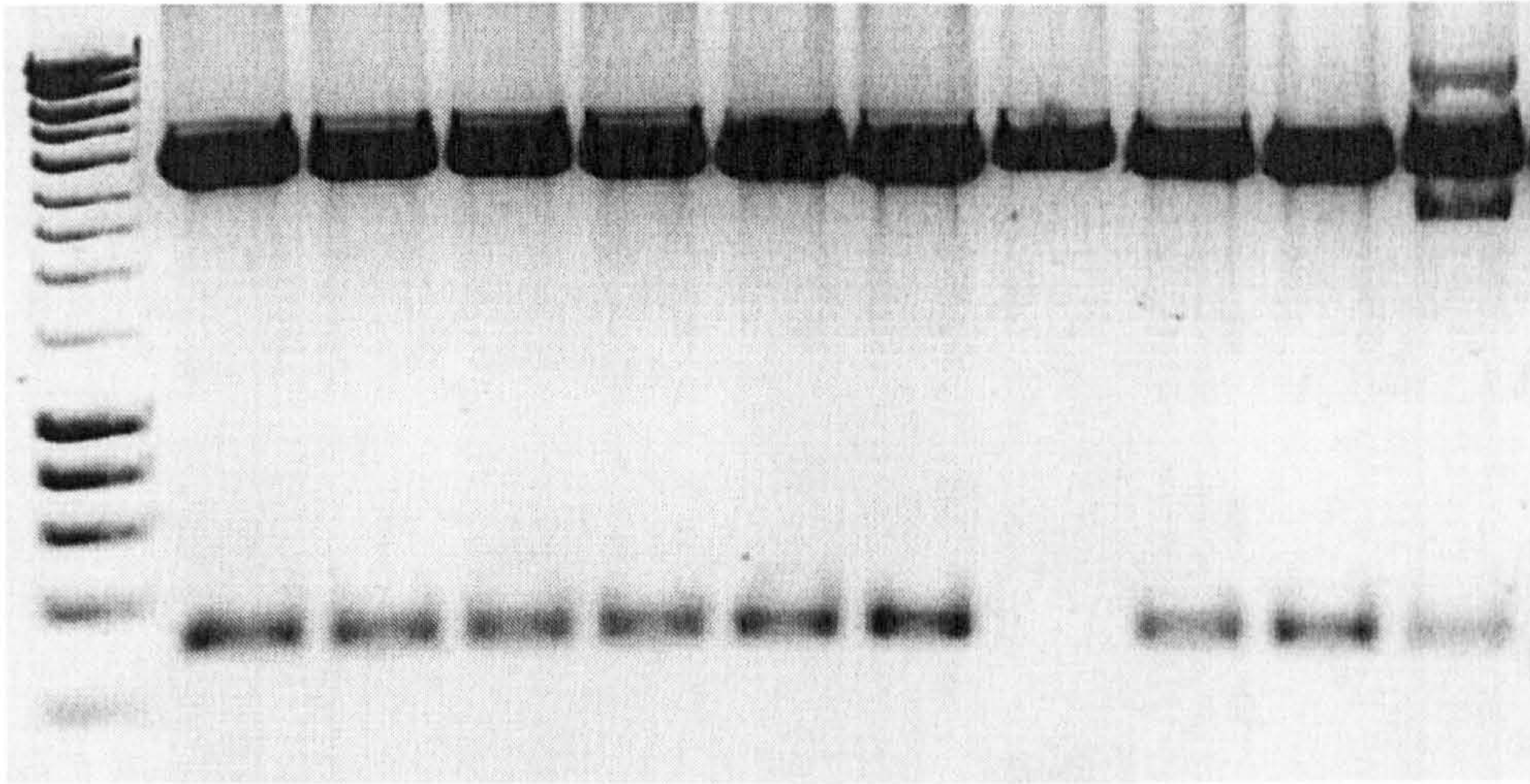


Figure 7.2. Gel electrophoresis showing plasmid/vector and lymphotactin insert.

The samples were sent for sequencing to the Genetic Core Facility, Division of Genomic Medicine, University of Sheffield, UK. Sequences were visualized using the FinchTV software (Geospiza Incorporated, Seattle, WA, USA) and confirmed using BLAST (http://www.ncbi.nlm.nih.gov/blast) {Altschul, 1990 69 /id} (Figure 10.3).

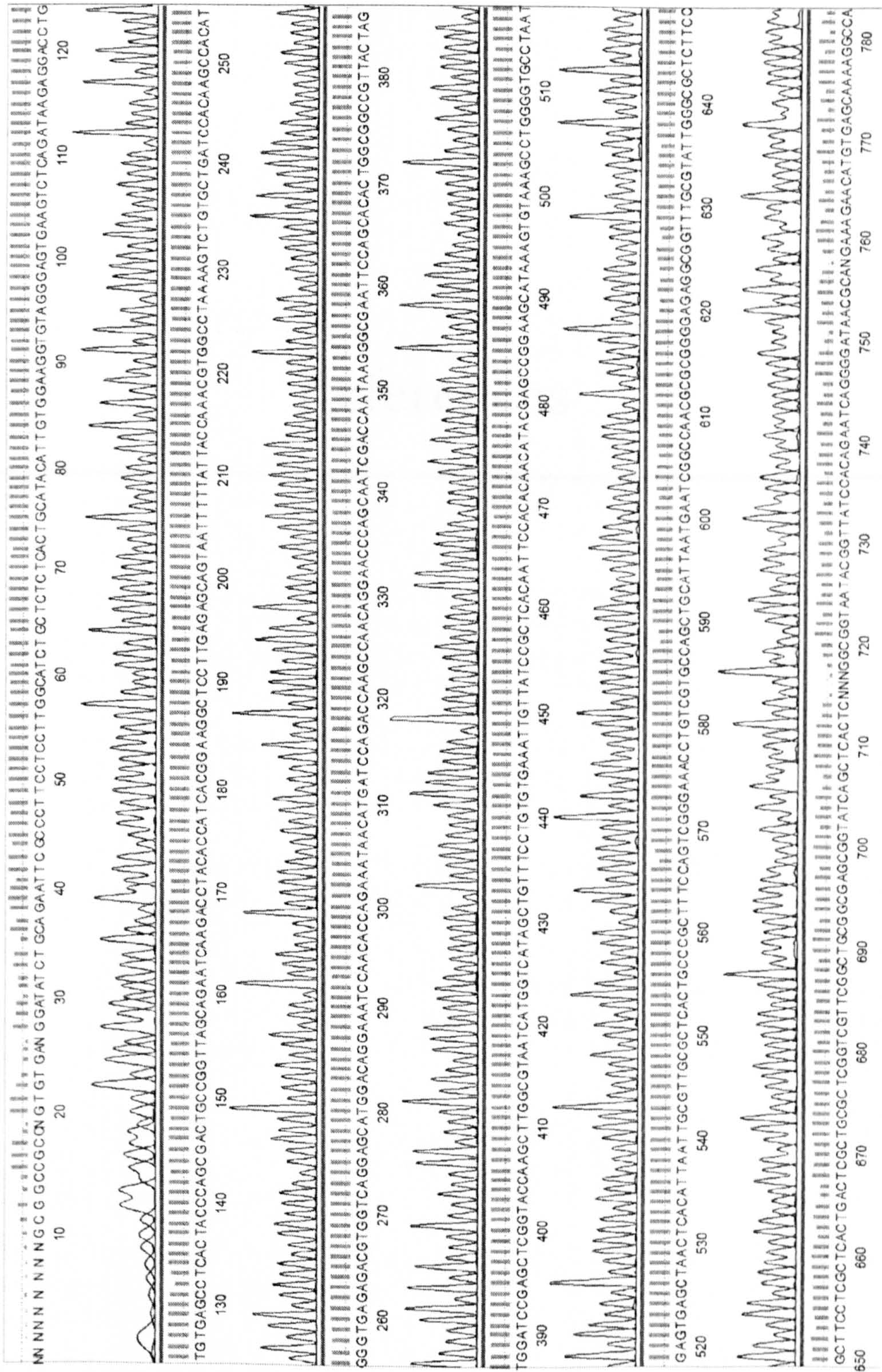


Figure 10.3. Representative lymphotactin sequence (from gel extraction) as observed with FinchTV software.

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