

REGULATION OF LFA-1 (CD11a/CD18) FUNCTION

by

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ABSTRACT

In the immune system, the complex cellular interactions that are necessary for the effective surveillance of the body against pathogens, and for launching an appropriate immune response to eliminate these pathogens, are mediated by adhesion molecules. The proper function of the immune system relies on the strict regulation of adhesive interactions between immune cells. The leukocyte integrin LFA-1 and its major ligand ICAM-1 constitute one pair of adhesion molecules that play critical roles in immune responses. This thesis presents the results of experiments designed to elucidate the mechanisms regulating the binding of LFA-1 to ICAM-1. Murine recombinant soluble ICAM-1 was immobilized on polystyrene microspheres with a view to using the beads to probe the distribution of high-avidity LFA-1 on various cell lines. These microspheres bound specifically to high-avidity LFA-1 in a cytoskeleton-independent manner as treatment with cytochalasin B had no effect. Furthermore, the beads displayed a highly localized distribution on some cell types, whereas fluorescence staining indicated that LFA-1 was uniformly distributed on the cell surface. Thus, the cell surface distribution of high-avidity LFA-1 can be different from that of LFA-1 in general, and is potentially significant in regulating LFA-1-mediated adhesion.

The role of LFA-1 cytoplasmic domains in binding to ICAM-1 and in post-adhesion events was also investigated. Various truncated forms of LFA-1 α (CD11a) and β (CD18) chains were generated by PCR and co-transfected into murine fibroblast TNR-2 cells in various combinations. The transfected fibroblasts were tested for their ability to adhere to ICAM-1 immobilized on plastic, and to spread out following this adhesion. The results indicated that both LFA-1 cytoplasmic domains are important for adhesion to ICAM-1, but that they play different roles. Furthermore, both cytoplasmic domains are required for post-receptor spreading.

Fluorescent staining of these cells indicated no significant variation in the distribution of LFA-1 on the cell surface.

As a further probe into the roles of LFA-1 cytoplasmic domains in regulating adhesion, we overexpressed chimeras consisting of the LFA-1 subunit cytoplasmic domains attached to the extracellular portion of murine CD4 in a B cell line as well as in a T cell line, and examined the effect on adhesion to ICAM-1 and fibronectin. The CD4/18 chimera drastically inhibited adhesion of both lines to ICAM-1 and fibronectin, whereas the CD4/11 chimera and a truncated form of CD4 lacking most of the cytoplasmic domain had no effect. Cell spreading after binding to ICAM-1 was also inhibited by the CD4/18 chimera but not the CD4/11 chimera. These results suggest that the CD18 cytoplasmic domain interacts with intracellular molecules that regulate not only LFA-1 but also other integrins.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
BCECF	2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein
BSA	bovine serum albumin
CAM	cell adhesion molecule
cAMP	cyclic adenosine 3',5'-monophosphate
CD	cluster of differentiation
cDNA	complementary DNA
CHO	chinese hamster ovary
CR	complement receptor
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified minimal essential medium
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetate
ELISA	enzyme-linked immunosorbent assay
ESL	E-selectin ligand
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
F _C R	F _C receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
fMLP	f-Met-Leu-Phe
g	gram
GPI	glycosyl-phosphatidylinositol
HBSS	Hanks' balanced salt solution
HEV	high endothelial venule
hr	hour
HSA	heat stable antigen

ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
ILK	integrin linked kinase
IMF	integrin modulating factor
kb	kilobase
kD	kilodalton
LAD	leukocyte adhesion deficiency
LFA	lymphocyte function-associated antigen
mAb	monoclonal antibody
Mac-1	macrophage antigen-1
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MIDAS	metal ion-dependent adhesion site
min	minute
MLR	mixed lymphocyte response
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NK	natural killer
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PECAM	platelet endothelial CAM
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear
PSGL	P-selectin glycoprotein ligand
PTK	protein tyrosine kinase
RGD	Arg-Gly-Asp
RGE	Arg-Gly-Glu

RPMI	Roswell Park Memorial Institute
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
sICAM	soluble ICAM
TCR	T cell receptor
TRITC	tetramethylrhodamine isothiocyanate
VCAM	vascular cell adhesion molecule
VLA	very late antigen
WT	wild type

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To my wife and #1 fan,

Cindy

CHAPTER 1

Introduction

1.1 ADHESION MOLECULES OF THE IMMUNE SYSTEM

The orderly interaction of cells with other cells or with their extracellular environment is mediated by membrane-bound glycoproteins called cell adhesion molecules (CAMs). To date, many different adhesion molecules have been identified and they are subdivided into various categories or families based on structural and functional characteristics. The four major families include the cadherins, selectins, integrins and immunoglobulin (Ig) superfamily members. Collectively, these molecules are responsible for wide-ranging physiological and developmental processes including wound healing, tissue organization and maintenance, leukocyte traffic, and vertebrate embryonic development. In addition, they are implicated in various pathologies such as inflammation, which results from excessive accumulation of leukocytes at sites of tissue injury, and metastasis, which may stem from abnormal control of adhesion (Hynes & Lander, 1992).

In the immune system, molecules from the selectin, integrin and Ig superfamilies (Table 1) function cooperatively to mediate the cell/cell and cell/extracellular matrix interactions that are necessary for effective immune surveillance and response to foreign invasion. The selectins (Lasky, 1992) derive their name from the presence of a common N-terminal lectin-like domain. These molecules are believed to be involved in leukocyte recirculation, as well as in the binding of leukocytes to endothelium at sites of inflammation (see below). Ig-superfamily members are expressed predominantly by lymphocytes, and are exemplified in structure by antibody molecules. They contain one or more Ig-like domains, and generally function in antigen recognition and cell/cell interactions (Williams & Barclay, 1988). The integrins comprise

Table 1. Selected members of the major CAM families in the immune system¹

	Distribution	Ligands ²
<u>Integrins</u>		
Leukocyte integrins: LFA-1	lymphocytes, monocytes, neutrophils	ICAM-1, -2, -3
Mac-1	monocytes, neutrophils	ICAM-1, iC3b, FG, factor X
p150/95	monocytes, neutrophils	iC3b, FG
$\alpha_D\beta_2$	certain macrophages and T cells	ICAM-1, -3
VLA-4	lymphocytes, macrophages	VCAM-1, FN
<u>Ig Superfamily</u>		
ICAM-1	lymphocytes, neutrophils, thymocytes, monocytes, dendritic cells, endothelium, keratinocytes, epithelium	LFA-1, Mac-1, CD43, FG
ICAM-2	endothelium, monocytes, dendritic cells, some lymphocytes	LFA-1
ICAM-3	lymphocytes, monocytes, granulocytes	LFA-1, $\alpha_D\beta_2$
<u>Selectins</u>		
L-selectin	lymphocytes, neutrophils, thymocytes, monocytes, eosinophils, basophils, NK cells	sialyl Lewis ^{x,a} , GlyCAM-1, CD34, MadCAM-1
E-selectin	endothelium	sialyl Lewis ^{x,a} , ESL-1
P-selectin	platelets, endothelium, megakaryocytes	sialyl Lewis ^{x,a} , PSGL-1

¹ Adapted from Springer (1994) and Carlos and Harlan (1994).

² GlyCAM, glycosylation-dependent cell adhesion molecule; MadCAM, mucosal addressin CAM; PSGL, P-selectin glycoprotein ligand; ESL, E-selectin ligand; FG, fibrinogen; FN, fibronectin

heterodimeric proteins consisting of non-covalently associated α and β chains (Hynes, 1992). To date, at least 20 distinct receptors have been identified based on the association of these chains. Barring a few exceptions, α chains associate with only one β chain, thus limiting the potential diversity of integrins. These molecules are subdivided into families based on the expression of various β chains. Perhaps the best characterized subfamilies are the β_1 , β_2 and β_3 integrin families. The β_1 and β_3 integrins generally recognize the tripeptide sequence Arg-Gly-Asp (RGD) in their ligands (Ruoslahti, 1996), and function primarily in interactions of cells with the extracellular matrix. The β_2 integrins, in contrast, predominantly mediate interactions between hematopoietic cells, and between leukocytes and endothelial cells. The β_2 integrins have been dubbed the "leukocyte integrins" as their expression is restricted to leukocytes. To date, four leukocyte integrins have been discovered, and this thesis will focus on one of these - the lymphocyte function-associated antigen-1 (LFA-1).

1.2 ROLE OF CAMs IN THE IMMUNE SYSTEM

1.2.1) CAMs and Immune Responses

The specificity of cellular immune responses is determined by T cell receptor (TCR) recognition of foreign antigen in the context of self MHC present on antigen presenting cells (APC) (Reinherz et al., 1984; Clevers et al., 1988). However, this interaction contributes little to the overall adhesive strength between T cells and their targets. For example, a mAb to the TCR inhibited CTL-mediated killing of antigen-bearing target cells, but did not hinder conjugate formation between the cells (de Vries et al., 1986). Thus, CAMs function in an accessory capacity to fulfill this function. Many CAMs, including LFA-1, were originally discovered

when monoclonal antibodies (mAb) generated against cell surface antigens were found to inhibit CTL-mediated killing (Davignon et al., 1981; Pierres et al., 1982; Dialynas et al., 1982; Hildreth et al., 1983). In fact, the use of mAb has been instrumental in revealing the degree to which LFA-1 participates in the normal function of the immune system. Thus, LFA-1 has been implicated in NK cell-mediated cytotoxicity and antibody-dependent cytotoxicity mediated by granulocytes and peripheral mononuclear cells (Miedema et al., 1984; Schmidt et al., 1985). Furthermore, mAb to LFA-1 inhibited LAK cell-mediated cytolysis (Nishimura et al., 1985), as well as the ingestion of *Staphylococcus* by neutrophils (Ross et al., 1985). Helper T cell functions also rely on CAMs. T cell proliferation in response to viruses, alloantigens, xenoantigens and mitogens is inhibited by mAb to LFA-1 (Davignon et al., 1981; Pierres et al., 1982; Krensky et al., 1983; Hildreth & August, 1985; Dougherty & Hogg, 1987), as are the production of IL-2 (Kaufman & Berke, 1983; Golde et al., 1985), T cell-mediated B cell activation, and antigen presentation to T cells (Keizer et al., 1985; Howard et al., 1986; Springer et al., 1987).

1.2.2) Leukocyte Recirculation and Transmigration

In the course of patrolling the body for the presence of foreign antigens, lymphocytes undergo a repetitive cycle of migration from the vasculature, crossing the endothelium, into tissues or lymph nodes, and back into the circulation. This process is referred to as recirculation, and it proceeds regardless of injury or inflammation. There is a degree of specificity in the process as distinct cell subsets tend to migrate preferentially to certain anatomic sites. Some leukocytes, such as neutrophils and monocytes, do not normally

recirculate but, rather, are recruited to sites of inflammation along with recirculating cells only when stimulated by inflammatory mediators.

Transendothelial migration occurs through a cascade of adhesive events involving several families of adhesion molecules (Bevilacqua, 1993; Carlos & Harlan, 1994). Four distinct steps have been defined in this adhesion cascade: rolling of leukocytes along the vascular endothelium, signalling from the endothelium which leads to the activation of integrins with concomitant initiation of tight adhesion to the endothelium, and finally, transmigration into the surrounding tissue (Springer, 1994; Dunon et al., 1996).

Leukocyte rolling achieves the dual task of slowing the leukocytes, which are travelling under the force of blood flow, and bringing them into close proximity with the endothelial cells where they are exposed to various signals that trigger the subsequent events in the adhesion cascade. The rolling process is mediated predominantly by selectins (von Andrian et al., 1991; Lawrence & Springer, 1991; Abassi et al., 1993; Ley et al., 1993; Lawrence & Springer, 1993), although other receptor/ligand pairs are also capable of acting in this capacity. Of note, one of these alternative receptors is the integrin $\alpha_4\beta_1$ which can mediate rolling on VCAM-1 (Alon et al., 1995). This integrin is also involved in the subsequent steps of recirculation upon activation. This is an interesting observation because it indicates that integrins may undergo several adhesive states. Endothelial cells express E- and P-selectins, which bind to leukocyte surface molecules PSGL-1 and, perhaps, ESL-1 (Alon et al., 1994; Steegmaier et al., 1995), and are critical in the rolling process as demonstrated by the massive reduction in rolling efficiency of leukocytes from knockout mice simultaneously deficient in both these proteins (Frenette et al., 1996). However, knockout of either selectin individually reduced rolling only in the absence of inflammation, suggesting that other molecules can compensate for these selectins (Johnson et

al., 1995; Mayadas et al., 1993). L-selectin, which is present on all leukocytes, is also involved in rolling through its binding to any of several endothelial ligands (Butcher & Picker, 1996). Knockout of this selectin resulted in impaired lymphocyte migration to lymph nodes, and no lymphocyte binding to high endothelial venules (HEV) (Arbones et al., 1994). Furthermore, leukocyte recruitment into inflammatory sites was hampered in L-selectin knockout mice (Tedder et al., 1995). Interestingly, efficient rolling requires that the cell surface distribution of leukocyte molecules involved in the process be restricted to the tips of leukocyte villi (Picker et al., 1991; von Andrian et al., 1995), thereby dramatically increasing the local concentration of tethering molecules. This distribution is probably effected via an interaction with the cytoskeleton (Pavalko et al., 1995; von Andrian et al., 1995).

A rolling leukocyte is stopped by virtue of a triggering step which serves to activate integrins and leads to stronger secondary adhesion. Triggering may occur via binding of soluble factors, especially chemokines, to G-protein-coupled receptors on leukocytes (Bargatze & Butcher, 1993; Laudanna et al., 1996). Alternatively, ligation of L-selectin on neutrophils may initiate a signalling pathway leading to the activation of Mac-1 (Simon et al., 1995; Crockett-Torabi et al., 1995). Whatever the mechanism, activation of integrins must occur within seconds of the initiation of leukocyte rolling.

Integrins involved in mediating firm secondary adhesion to endothelium include the leukocyte integrins LFA-1 and Mac-1, as well as $\alpha_4\beta_1$ and $\alpha_4\beta_7$ (von Andrian et al., 1991; Muller & Weigl, 1992; Springer, 1994; Imhof & Dunon, 1995). Mice deficient in LFA-1 due to knockout of the α_L subunit displayed reduced leukocyte numbers in selected tissues, demonstrating its importance in homing to these tissues (Schmits et al., 1996).

Transmigration across the endothelium is the final step in the adhesion cascade, and is a complex process involving several transient and regulated receptor/ligand interactions, including those of LFA-1 and Mac-1 with ICAM-1, and $\alpha_4\beta_1$ with VCAM-1 (Furie et al., 1991; Kavanaugh et al., 1991; Oppenheimer-Marks et al., 1991; Meerschaert & Furie, 1995). It appears that β_2 integrins are used for transmigration by neutrophils, whereas lymphocytes use $\alpha_4\beta_1$. Another important receptor/ligand pair involved in this process is that of $\alpha_v\beta_3$, which is found on most circulating cells of the immune system, and PECAM-1 (CD31), which is expressed by endothelium (Liao et al., 1995).

1.2.3) Leukocyte Adhesion Deficiency

The physiological importance of CAMs in the immune system is underscored by patients afflicted with a recessive inherited deficiency termed Leukocyte Adhesion Deficiency (LAD) type 1. LAD-1 is a disease that subjects patients to life-threatening microbial and fungal infections (Anderson & Springer, 1987), and in the absence of bone marrow transplantation, patients die at an early age. The disease is characterized by defective neutrophil mobility and phagocytosis, lack of pus formation, and by the absence of lymphocytes and granulocytes in infected lesions despite chronic leukocytosis. The cause of LAD-1 has been traced to a deficiency in leukocyte integrin expression (Springer et al., 1984; Arnaout et al., 1984) which results from the inability of leukocytes to express a functional β_2 chain (Lisowska-Grospierre et al., 1986; Marlin et al., 1986). Thus, the leukocyte integrin α chains, which are synthesized at normal levels and are competent for surface expression (Marlin et al., 1986), cannot associate with the β chains to form functional heterodimers. Transfection of functional β_2 cDNA into

cells derived from LAD-1 patients restores LFA-1 surface expression and function (Hibbs et al., 1990).

Several different β_2 mutations have been discovered that can result in LAD-1 patients displaying symptoms ranging from severe to moderate (Springer et al., 1984; Anderson et al., 1985; Kishimoto et al., 1987a). The severe phenotype is attributed to a complete lack of leukocyte integrin surface expression. This may result from a complete absence of β_2 precursor or to point mutations in the precursor that render the two subunits incapable of associating. Various other mutations result in the synthesis of unusually large or small β_2 precursors which do not associate efficiently with the α chains.

To date, no instance of individuals deficient in only one of the leukocyte integrins has been reported, suggesting that these mutations may be masked by a normal phenotype. However, an LFA-1 -deficient mouse was created by genetic knockout of the α_L chain (Schmits et al., 1996). Leukocytes from this mouse exhibited impaired homotypic aggregation, proliferation in MLR, and responses to mitogens *in vitro*. While mounting normal CTL responses to systemic infections, mutant mice did not reject immunogenic tumors. These data indicate that LFA-1 deficiency negatively impacts peripheral, but not systemic, immune responses.

More recently, a second type of LAD, termed LAD-2, has been characterized in patients exhibiting a congenital defect in neutrophil adhesion to endothelium and emigration into inflamed tissue. The cause of this defect has been traced to a generalized deficiency in the synthesis of fucosylated carbohydrates (Etzioni et al., 1992), among which is a major ligand for the selectins, sialyl Lewis X (sLe^X) (Polley et al., 1991; Berg et al., 1992; Foxall et al., 1992).

The reduction in neutrophil emigration in LAD-2 patients is the consequence of reduced neutrophil rolling rather than adhesion since leukocyte integrin-mediated binding to endothelium could be induced by exposure of the cells to phorbol ester (Phillips et al., 1995). In addition to reduced neutrophil motility and adhesion, the clinical manifestations of LAD-2 are similar to those observed in LAD-1 of moderate severity, and include recurrent bacterial infections and developmental defects (Etzioni et al., 1992).

1.3 LFA-1

1.3.1) Structure, Distribution and cDNA cloning

The structure of LFA-1 exemplifies that of integrins in general (Figure 1.1). It is comprised of a 180 kD α_L subunit (CD11a) non-covalently associated with a 95 kD β_2 subunit (CD18) (Kurzinger & Springer, 1982; Sanchez-Madrid et al., 1983). Both chains are initially synthesized as separate precursors of 170 kD and 87 kD respectively, and consist of N-glycoside high mannose carbohydrate groups linked to polypeptide backbones of approximately 130 kD and 72 kD (Sanchez-Madrid et al., 1983; Sastre et al., 1986; Miller & Springer, 1987). The two precursor chains associate within the Golgi apparatus before processing of the N-linked carbohydrates to a complex form, and subsequent transport to the cell surface (Sanchez-Madrid et al., 1983; Sastre et al., 1986; Springer et al., 1984).

LFA-1 is expressed exclusively on cells of hematopoietic origin. With the exception of some tissue macrophages (Kurzinger et al., 1981), all leukocytes, including thymocytes, neutrophils, monocytes and peripheral lymphocytes, express LFA-1 (Kurzinger et al., 1981; Krensky et al., 1983) as do leukocyte-derived cells such as macrophages and dendritic cells.

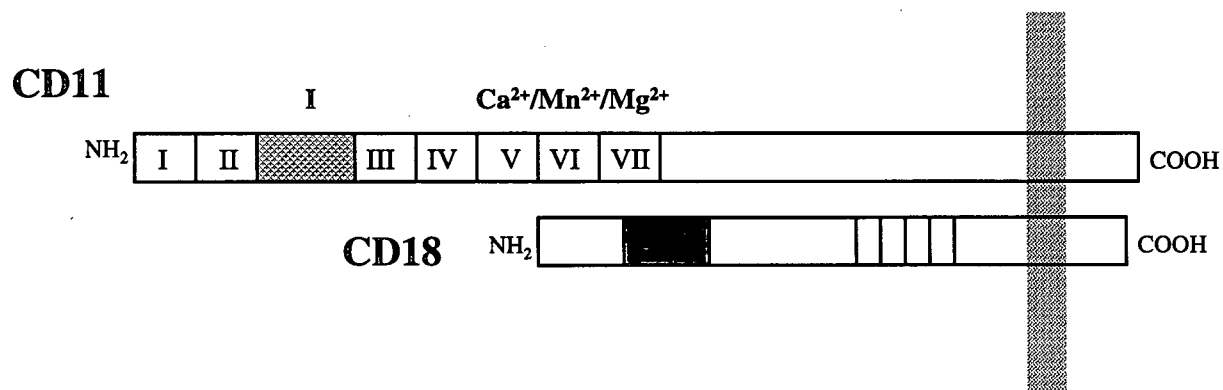


Figure 1.1. Schematic representation of LFA-1 structure. The α_L (CD11) chain contains 7 repeats postulated to fold into a β -propeller domain. The inserted I domain is essential for ligand binding, and contains a divalent cation binding site. Domains V-VII also contain consensus divalent cation binding sites. The α_L chain is non-covalently associated with the β_2 (CD18) chain. The four-fold repeat region of cysteine residue concentration is shown, as is another conserved region (shaded area) believed to be important in the maintenance of the heterodimer.

The cDNAs encoding both the human and murine α_L and β_2 subunits have been cloned and characterized (Kishimoto et al., 1987b; Law et al., 1987; Larson et al., 1989; Wilson et al., 1989; Kaufmann et al., 1991). Both chains are integral membrane proteins consisting of large extracellular domains, and relatively short transmembrane and cytoplasmic domains. Several putative N- glycosylation sites exist in the extracellular regions of both chains. All integrin α chains, including that of LFA-1, contain a series of seven repeats of approximately 60 amino acids at their N-termini (Corbi et al., 1987). These repeats are predicted to fold into a β -propeller domain as is seen for the G-protein β subunit (Springer, 1997; Huang & Springer, 1997; Huang et al., 1997). The three most C-terminal of these repeats contain motifs that share homology with the Ca^{2+} -binding EF-hand loop sequences of calmodulin, troponin C, and parvalbumin, and that are therefore believed to be divalent cation-binding sites (Corbi et al., 1987; Argraves et al., 1986; Poncz et al., 1987). In fact, the β -propeller model predicts that a putative Mg^{2+} ion binds to the upper face of the propeller, whereas the Ca^{2+} -binding motifs reside on the lower face (Springer, 1997). The presence of divalent cation binding sites correlates with the requirement for Mg^{2+} and, possibly, Ca^{2+} in integrin-mediated adhesion (Dransfield et al., 1992; Michishita et al., 1993; van Kooyk et al., 1994).

All integrin α subunits contain a conserved KXGFFKR motif in the plasma membrane-proximal region of the cytoplasmic domain. This sequence has been shown to bind the cytoplasmic chaperone calreticulin *in vitro* (Rojiani et al., 1991) and *in vivo* (Leung-Hagesteijn et al., 1994; Coppolino et al., 1995) and may be important in regulating adhesiveness of some integrins (O'Toole et al., 1994; Leung-Hagesteijn et al., 1994).

Another distinguishing feature of the α_L chain that is shared by several, but not all, integrins is the presence of a 200-amino acid segment in the extracellular domain called the I domain (for inserted/interactive domain). This domain is homologous to the A domains of von Willebrand factor, as well as to domains in the complement proteins C2 and factor B, and to cartilage matrix protein (Corbi et al., 1988; Arnaout et al., 1988; Larson et al., 1989), and has been implicated in the binding of ligand and divalent cations (Diamond et al., 1993; Landis et al., 1993; Michishita et al., 1993; Randi & Hogg, 1994; Bilsland et al., 1994; Zhou et al., 1994). Purified I domains from LFA-1, Mac-1 and VLA-2 were shown to directly bind to ligands ICAM-1, fibrinogen and collagen respectively (Randi & Hogg, 1994; Zhou et al., 1994; Kamata et al., 1994), and mutation of threonine or aspartate residues within the I domains of several integrins impairs adhesion by affecting cation binding (Michishita et al., 1993; Kern et al., 1994; Lee et al., 1995; Kamata et al., 1995). In addition, most anti-leukocyte integrin antibodies that have been demonstrated to inhibit interactions with ligand recognize epitopes in the I domain (Diamond et al., 1993; Landis et al., 1994; Champe et al., 1995; Binnerts et al., 1996), as do several integrin-activating antibodies (Randi & Hogg, 1994). Antibodies and mutational analysis have also yielded evidence that the ligand binding sites in the leukocyte integrins LFA-1 and Mac-1 overlap partially, but are distinct (Diamond et al., 1993; Landis et al., 1994; Zhang & Plow, 1996). The I domain is predicted to be tethered to the top of the β -propeller (Springer, 1997).

Recently, the crystal structures of the I domains from LFA-1 and Mac-1 have been determined (Lee et al., 1995; Qu & Leahy, 1995). These achievements were significant as they provided the first glimpse into the relationship between integrin structure and function. The structural features of both I domains were found to be very similar to each other, and revealed

the presence of a "metal ion-dependent adhesion site" (MIDAS) motif. A conserved sequence of five amino acid residues (DXSXS) contributes to the site, with two more residues contributing from discontinuous parts of the sequence. Mutation of these residues renders the integrins incapable of binding ligand (Michishita et al., 1993). MIDAS-like motifs have also been postulated to exist in conserved regions of integrin β chains, all of which contain the consensus DXSXS sequence (Lee et al., 1995). There is current speculation that an unstable ternary complex is formed between MIDAS domains on the integrin, divalent cation, and ligand, which results in the release of the cation and stable binding (D'Souza et al., 1994). Thus, divalent cation may play a role in integrin activation possibly by effecting conformational changes in the cation/ligand binding sites (see below).

The β_2 chain, and integrin β chains in general, is distinguished by its high cysteine content. The cysteines are particularly concentrated in a four-fold repeat region in the extracellular domain, and it is speculated that this region confers a rigid tertiary structure on the β_2 chain. Additional sequence conservation in the β chain exists in an area approximately 100 amino acids from the amino-terminus that is thought to be critical for the maintenance of the integrin heterodimer (Arnaout, 1990). Mutations in either of these regions result in leukocyte adhesion deficiency. The β_2 cytoplasmic domain has also been implicated in the regulation of LFA-1 function (see below).

1.3.2) Ligands

1.3.2.1) ICAM-1

The existence of a ligand for LFA-1 was first postulated when it was observed that LFA-1⁺ lymphocytes co-aggregated with LFA-1⁻ cells from patients afflicted with leukocyte adhesion deficiency upon stimulation with PMA. Human ICAM-1 was discovered using mAb against LFA-1⁻ cells that showed inhibition of this LFA-1-dependent homotypic aggregation (Rothlein et al., 1986). mAb inhibiting MLR led to the discovery of the murine ICAM-1 (Takei, 1985). ICAM-1 is a heavily-glycosylated single-chain glycoprotein of 80-115 kD. Both the human (Staunton et al., 1988) and murine (Horley et al., 1989; Siu et al., 1989) ICAM-1 cDNAs have been cloned and revealed that ICAM-1 is a member of the Ig superfamily by virtue of its five extracellular Ig-like domains. The human and murine proteins display 50% homology at the amino acid level, and 65% homology at the DNA level.

ICAM-1 is widely distributed on cells of both hematopoietic and nonhematopoietic origin, but its expression is low on the latter and on resting peripheral blood lymphocytes (Dustin et al., 1986). However, upon stimulation by phorbol esters, or a variety of inflammatory mediators, such as TNF α , IFN γ , and IL-1, the expression levels are rapidly upregulated, reaching maximal levels within 24 hours (Dustin et al., 1986; Rothlein et al., 1988; Dustin et al., 1988), which is typical of regulation at the RNA level. This situation contrasts that observed with LFA-1, which is constitutively expressed only on leukocytes (Kurzinger et al., 1981; Krensky et al., 1983). The increased level of ICAM-1 expression correlates with increased LFA-1-dependent adhesion, which has important ramifications for the regulation of inflammatory responses. For example, the upregulation of ICAM-1 expression on venule endothelium is

believed to facilitate the adhesion and subsequent transendothelial migration of leukocytes into inflammatory tissue, where appropriate effector functions can proceed (Springer, 1990; Dustin & Springer, 1991; Furie et al., 1991; Oppenheimer-Marks et al., 1991; Carlos & Harlan, 1994).

Antibody inhibition studies have further illustrated the importance of ICAM-1 (Springer, 1990; Dustin & Springer, 1991), largely reflecting the observed effects of anti-LFA-1 antibodies (see above). In addition, mice deficient in ICAM-1 expression have been recently generated, and exhibit prominent abnormalities in inflammatory responses and increased levels of circulating neutrophils and lymphocytes (Sligh et al., 1993; Xu et al., 1994). Neutrophil migration in response to chemical peritonitis was also impaired, and mice displayed delays in transplantation rejection. Despite these deficiencies, it is likely that other ICAMs can, at least partially, substitute for ICAM-1 deficiency.

In addition to binding to LFA-1, ICAM-1 has been shown to interact with another leukocyte integrin, Mac-1 (Smith et al., 1989; Diamond et al., 1991), CD43 (Rosenstein et al., 1991), fibrinogen (Bartfeld et al., 1993), hyaluronan (McCourt et al., 1994), and α -actinin (Carpen et al., 1992). Furthermore, ICAM-1 has been shown to be the major receptor for the rhinoviruses (Greve et al., 1989; Staunton et al., 1989b), and for Plasmodium-infected erythrocytes (Berendt et al., 1989). Thus, ICAM-1 is a multi-functional and versatile molecule.

1.3.2.2) ICAM-2

A second ligand for LFA-1 was postulated to exist when it was recognized that T cell adhesion to endothelial cells could be completely inhibited by mAb to LFA-1, but only partially inhibited by anti-ICAM-1 (Dustin & Springer, 1988). Furthermore, purified LFA-1 incorporated into membranes was shown to support adhesion of an ICAM-1⁺ T cell line (Dustin

& Springer, 1989). The new ligand was cloned by screening COS cells transfected with a cDNA expression library for their ability to bind to purified LFA-1 in the presence of anti-ICAM-1 mAb (Staunton et al., 1989a), and was designated ICAM-2. ICAM-2 is expressed mainly on leukocytes and endothelial cells as a 55 kD integral membrane protein containing two Ig-like domains that display the highest similarity (35% identity) with the two outermost domains of ICAM-1 (Staunton et al., 1989a). Since these outermost domains of ICAM-1 have been implicated in the binding of LFA-1 (Staunton et al., 1990), the sequence homology may extend to similarities in binding to LFA-1. In contrast to ICAM-1, ICAM-2 expression levels are several-fold higher on resting lymphocytes, but its expression is not affected by cytokine stimulation (Staunton et al., 1989a; de Fougerolles et al., 1991; Nortamo et al., 1991). Because of the small size of ICAM-2, questions have been raised as to the significance of the LFA-1:ICAM-2 interaction *in vivo*. ICAM-2 does not protrude from the cell surface to the same extent as ICAM-1 suggesting that closer contact between cells may be necessary, and that its interaction with LFA-1 may be weaker. However, extension of the ICAM-2 binding site by the insertion of 5 extra Ig-like domains did not enhance LFA-1-mediated adhesion (Damle et al., 1992a) suggesting that the strength of this interaction is determined by the structure of the binding site rather than by its distance from the cell surface. Alternatively, ICAM-1 has been suggested to occur as a dimer in the plasma membrane (Miller et al., 1995; Reilly et al., 1995) whereas ICAM-2 seems to exist as a monomer (Reilly et al., 1995), and this difference might explain the difference in the strength of adhesion to LFA-1. Although the LFA-1:ICAM-2 interaction may serve to supplement that of LFA-1 to ICAM-1 in nature (Springer, 1990), other studies have demonstrated that ICAM-2 can mediate effective interaction with LFA-1 in the absence of ICAM-1 (Carpenito et al., 1995). Based on its expression on endothelium, ICAM-2

is believed to be involved in lymphocyte recirculation (Dustin & Springer, 1991; Carlos & Harlan, 1994). Moreover, ICAM-2 was shown to transmit a costimulatory signal to CD4⁺ T cells when it was coimmobilized on plastic with mAb against TCR (Damle et al., 1992a,b), and one recent report (Helander et al., 1996) provides a glimpse into a potential involvement of ICAM-2 in leukocyte-target cell interactions. In this study, ICAM-2 was implicated as a recognition target for IL-2-activated NK cells.

The murine ICAM-2 was isolated from a lymphoma library using the human ICAM-2 as a probe (Xu et al., 1992), and from a mouse lung cDNA library using a PCR-based strategy (Carpenito et al., 1995). These data revealed a 60% identity of murine ICAM-2 with human ICAM-2 at the protein level.

1.3.2.3) ICAM-3

Evidence for a third counterreceptor for LFA-1 stemmed from the observation that certain cell lines aggregated in an LFA-1 -dependent, but ICAM-1 and -2 -independent manner (de Fougères et al., 1991; de Fougères & Springer, 1992). This observation eventually led to the identification and cloning of ICAM-3 (Fawcett et al., 1992; Vazeux et al., 1992; de Fougères et al., 1993). Like ICAM-1, ICAM-3 has 5 Ig-like extracellular domains, with the two outermost domains providing the LFA-1 binding sites (Fawcett et al., 1992). The Ig-like domains display high homology to those of ICAM-1 (52% identity) and ICAM-2 (37% identity) (de Fougères et al., 1993).

ICAM-3 is expressed constitutively at high levels on all leukocytes, and it is absent from endothelial cells (de Fougères & Springer, 1992; Acevedo et al., 1993; Staquet et al., 1995). ICAM-3 expression is not induced by exposure to cytokines or other agonists. The adhesion of

resting T lymphocytes to purified LFA-1 occurs predominantly through ICAM-3, suggesting that this molecule may play an important role in initiating immune responses (Fawcett et al., 1992; Vazeux et al., 1992; de Fougerolles et al., 1993; de Fougerolles et al., 1994). It was also demonstrated that purified ICAM-3, in addition to supporting adhesion through LFA-1, could provide costimulatory signals to resting T lymphocytes (de Fougerolles et al., 1994). Crosslinking experiments have also implicated ICAM-3 in T cell signal transduction (Juan et al., 1994). Finally, ICAM-3 was shown to regulate LFA-1:ICAM-1 - mediated T lymphoblast interaction (Arroyo et al., 1994). These data collectively suggest that ICAM-3 may function in a stimulatory capacity. Recently ICAM-3 was suggested to bind to a fourth leukocyte integrin CD11d/CD18, but the significance of this interaction is unknown (Van der Vieren et al., 1995).

The precise physiological significance of interactions with ICAM-2 and -3 is still uncertain, despite the data currently available. However, the different patterns in distribution and expression suggest that functional differences exist.

1.4 OTHER LEUKOCYTE INTEGRINS

1.4.1) Mac-1

Mac-1 displays a more limited distribution than does LFA-1. It is expressed primarily on myeloid and natural killer cells (Kishimoto et al., 1989), and the level of this expression depends upon the stage of differentiation, with mature granulocytes and macrophages expressing the highest levels (Miller et al., 1986; Hickstein et al., 1989a). The expression of Mac -1 is inducible upon exposure of cells to retinoic acid or phorbol esters (discussed below) (Miller et al., 1987; Bainton et al., 1987; Buyon et al., 1988; Hickstein et al., 1989b). mAb to Mac-1 have been shown to inhibit the binding of myeloid cells to iC3b, a component of complement, thus

defining Mac-1 as the complement receptor type-3 (CR3) (Beller et al., 1982). It has subsequently become apparent that Mac-1 can bind multiple ligands including ICAM-1 (Smith et al., 1989; Diamond et al., 1990), fibrinogen (Altieri et al., 1988; Wright et al., 1988), factor X (Altieri & Edgington, 1988), and heparin (Diamond et al., 1995). The adhesion and transmigration of myeloid cells to endothelium and epithelium has been attributed to Mac-1 (Lo et al., 1989; Smith et al., 1989; Anderson et al., 1990; Parkos et al., 1991) as has neutrophil homotypic aggregation and chemotaxis, and the binding and phagocytosis of opsonized particles (Wright et al., 1983; Anderson et al., 1986; Beller et al., 1982).

1.4.2) p150/95

The distribution of p150/95 ($\alpha_x\beta_2$; CD11c/CD18) generally resembles that of Mac-1, and the two integrins are often co-expressed on the surface of myeloid cells such as macrophages, monocytes and granulocytes (Miller et al., 1986; Hogg et al., 1986; Bilsland et al., 1994). p150/95 is also found on some activated lymphocytes (Keizer et al., 1987a; Postigo et al., 1991a). The similarity with Mac-1 is further extended by the existence of a partial overlap between ligands. This overlap has made the elucidation of the function of p150/95 more difficult. The first role to be discovered was as a receptor for iC3b (Micklem & Sim, 1985; Malhotra et al., 1986; Myones et al., 1988). Antibodies to p150/95 were shown to inhibit the binding of neutrophils to substrates (Anderson et al., 1986), and in one case, they inhibited conjugate formation between CTL and APC (Keizer et al., 1987a). A role for p150/95 in monocyte adhesion and chemotaxis was also suggested (Keizer et al., 1987b). Although several studies have demonstrated that p150/95 binds to iC3b, transfection of the human p150/95 α and β chains into COS or chinese hamster ovary (CHO) cells yields a receptor that cannot bind iC3b

(Diamond et al., 1993). However, COS cells transfected with a hybrid receptor consisting of the human α_X chain and a chicken β_2 chain can bind iC3b (Bilsland et al., 1994) suggesting that interactions between the two chains can regulate adhesiveness through p150/95.

Recently, an activating mAb to the CD11c subunit was shown to induce the binding of a rabbit T cell clone to ICAM-1, implicating the latter as a ligand for p150/95 (Blackford et al., 1996). The physiological significance of this interaction remains to be determined.

1.4.3) $\alpha_D\beta_2$

$\alpha_D\beta_2$ is the most recently discovered member of the leukocyte integrin family, and it exhibits a highly restricted tissue distribution (Danilenko et al., 1995; Van der Vieren et al., 1995). In dogs and humans, expression of $\alpha_D\beta_2$ is limited to macrophages in splenic red pulp, lymph node medullary regions, and bone marrow, as well as to a minor subpopulation of CD8⁺ T cells. The low expression of $\alpha_D\beta_2$ on peripheral blood monocytes and granulocytes distinguishes the tissue distribution of this molecule from those of Mac-1 and p150/95. Immunoprecipitation experiments revealed an α chain of approximately 155 kD that was antigenically distinct from the other leukocyte integrin α chains (Danilenko et al., 1995). The functional role of this molecule is, as yet, unknown. However, human $\alpha_D\beta_2$ has been shown to bind preferentially to ICAM-3 over ICAM-1, and the binding is unaffected by treatment with PMA or Mn^{2+} , both positive regulators of LFA-1 (Van der Vieren et al., 1995). Moreover, the cytoplasmic and I domain sequences of α_D differ markedly from those of other leukocyte integrins suggesting that $\alpha_D\beta_2$ plays a unique role in immune responses.

1.5 REGULATION OF LFA-1 FUNCTION

A significant feature of integrin-mediated adhesion is that the process is not passive, whereby receptors simply interact stereochemically with their counter-receptors. Rather, a high degree of order is observed in the regulated attachment and de-attachment of cells from their substrates. This stringent regulation is crucial to the functional effectiveness of the immune system. Leukocytes patrol the body in search of foreign antigens, and must both circulate as nonadherent cells in the blood and lymph, and migrate as adherent cells through tissues (Imhof & Dunon, 1995; Springer, 1995). Furthermore, they must adopt the adherent phenotype in order to congregate in lymphoid organs, cross endothelium to aggregate at sites of infection, and interact with antigen presenting cells. This thesis is concerned with the regulation of adhesion of LFA-1 to ICAM-1, and the following discussion will center primarily on this interaction. Where appropriate, information on the regulation of other integrins will be presented for comparison.

1.5.1) Inside-Out Signalling

The ability of LFA-1 to bind ICAM-1 was shown early on to be dependent on cellular metabolism, and on the activation state of the cell. Treatment of cells with sodium azide and 2-deoxy-D-glucose, thereby blocking the production of ATP, abrogates LFA-1-mediated adhesion of T cells to ICAM-1 (Marlin & Springer, 1987). Moreover, cooling cells to 4° C completely inhibited this interaction, establishing the temperature dependency of LFA-1-mediated adhesion. Finally, this adhesion was shown to require a functional cytoskeleton as cytochalasin B treatment, which disrupts microfilaments, inhibited adhesion (Rothlein & Springer, 1986).

In addition to metabolic considerations, LFA-1-mediated binding of T cells to ICAM-1 is dependent on the activation state of the cells. Stimulation of cells with phorbol esters (e.g. PMA), which are analogs of diacylglycerol and, therefore, directly stimulate the serine/threonine kinase PKC, or with mAb to CD3 results in an increase in LFA-1-mediated adhesion within minutes (Dustin & Springer, 1989). Anti-TCR mAb increases adhesion through β_1 integrins as well (Shimizu et al., 1990). PMA stimulation increases cell adhesion to ICAM-1 immobilized on plastic, but not the adhesion to purified LFA-1 immobilized on plastic, demonstrating that the increase in adhesion occurs as the result of a change in LFA-1 and not in ICAM-1. The increased adhesion is referred to as an increase in LFA-1 avidity to reflect the fact that binding is the result of multivalent interactions, with each receptor/ligand pair displaying its own affinity. Of note is the fact that PMA activation does not increase the amount of cell surface LFA-1, and protein synthesis is not required (Rothlein & Springer, 1986; Rothlein et al., 1986; Dustin & Springer, 1989; van Kooyk et al., 1989). The kinetics of the increase in adhesion following stimulation by PMA versus triggering via the TCR differ in that the latter results in a rapid but transient increase whereas the former yields a slower but sustained increase (Dustin & Springer, 1989). Regulation of adhesion by TCR is more physiologically relevant since it provides a link between antigen recognition and adhesion, and explains the general observation made earlier that recognition of cell-bound antigen by lymphocytes was an efficient means of increasing cell-cell junctional avidity (Kupfer & Singer, 1989). These data suggest that TCR-derived signals do not elicit increased adhesion simply by activating PKC. However, PKC stimulation may serve as part of the LFA-1 activation pathway upon triggering through the TCR. The TCR-derived increase in adhesion can be partially blocked by inhibition of PKC and accumulation of cAMP

(Dustin & Springer, 1989; Rovere et al., 1996), indicating that intracellular signals link TCR-engagement to the increase in LFA-1 avidity.

The increase in LFA-1 avidity observed upon treatment of cells with PMA or through TCR-derived signals may be achieved by other means. For example, several cell surface proteins have been shown to upregulate LFA-1-mediated adhesion upon crosslinking with specific mAb. These proteins include CD2 (van Kooyk et al., 1989), B cell surface Ig (Dang & Rock, 1991), CD44 (Koopman et al., 1990), MHC II (Mourad et al., 1990), and CD45 (Spertini et al., 1994; Bernard et al., 1994). Again, no change in LFA-1 surface expression is observed during the time course of the increase in adhesion. Crosslinking of these structures is associated with leukocyte activation and the concomitant production of second messengers and elevation of intracellular Ca^{++} levels due to phosphatidylinositol breakdown.

Activation-induced upregulation of adhesion is also observed for other integrins. The $\alpha_{\text{IIb}}\beta_3$ integrin on platelets does not mediate binding to fibrinogen unless activated by various inflammatory mediators released by injured tissue (Smyth et al., 1993). As is true for LFA-1, a qualitative rather than quantitative change is observed in the receptor upon binding of transfected CHO cells to fibrinogen (O'Toole et al., 1990). Similarly, the adhesiveness of several β_1 integrins for extracellular matrix proteins is increased upon crosslinking of CD2 or CD3 on lymphocytes (Shimizu et al., 1990), or upon pulsing T or B cells with antigen or phorbol ester (Chan et al., 1991a; Postigo et al., 1991b; Shaw et al., 1993; Tanaka et al., 1993).

The common thread in all these experiments was that intracellular signals could be transmitted to LFA-1 and other integrins and that these signals could influence the ability of the integrins to mediate adhesion extracellularly. The term 'inside-out' signalling is used to refer to this process. This signalling may actually stem from an interplay of several events which are

discussed below. Conceivably, three methods may be postulated to explain upregulation of integrin adhesiveness: the degree of cell surface expression of adhesion receptors and their cell-bound ligands, the distribution of these receptors on the cell surface, and the affinity of the individual receptor/ligand interactions.

1.5.1.1) Cell Surface Expression

The number of molecules available at the cell surface for interaction with ligand can affect the overall adhesiveness between cells and their targets. As mentioned above, ICAM-1 expression is dramatically induced on leukocytes and endothelium upon stimulation by various inflammatory mediators. The increase in expression provides the opportunity for greater contact with LFA-1 (Dustin et al., 1986; Clark et al., 1986; Dougherty et al., 1988; Wawryk et al., 1989). Furthermore, memory T cells are capable of enhanced binding under resting conditions (Shimizu et al., 1991), a fact attributed to the high basal levels of most adhesion receptors on the surface. The 5-fold increase in LFA-1 surface density on these cells versus that on naive cells (Pardi et al., 1989) likely increases the overall avidity of the interaction with ICAM-1.

As is the case for LFA-1, Mac-1 and p150/95 also require activation in order to bind their ligands. Stimulation of neutrophils, with a variety of agonists, leads to a rapid and transient upregulation of Mac-1-dependent binding to iC3b-coated particles (Wright & Meyer, 1986; Detmers et al., 1990; Anderson et al., 1986; Dana et al., 1986), as well as to fibrinogen (Wright et al., 1988) and ICAM-1 (Smith et al., 1989; Diamond et al., 1990). However, in addition to a qualitative change in this receptor enabling it to bind, there is an additional level of regulation in this system. Mac-1 is stored in intracellular granules in monocytes and polymorphonuclear cells (Todd et al., 1984; Miller et al., 1987; Bainton et al., 1987) and is

rapidly mobilized to the cell surface upon stimulation with various chemoattractants, such as fMLP, complement component C5a, and leukotriene B₄. However, it should be noted that a simple quantitative change in leukocyte adhesion molecule expression may not, in itself, be sufficient to maintain adhesion since not all of these molecules may be competent to bind ligand (Diamond & Springer, 1993). For example, the upregulation of Mac-1 surface expression upon stimulation of neutrophils was not required for homotypic aggregation (Buyon et al., 1988; Philips et al., 1988) or for their adhesion to endothelium (Vedder & Harlan, 1988), and temperature conditions that prevented the increase in expression did not affect adhesion through Mac-1 (Schleiffenbaum et al., 1989). The surface expression of LFA-1 is not upregulated upon cell activation through TCR or by PMA suggesting that this method of regulating adhesion is not utilized to effect immediate adhesion through LFA-1.

1.5.1.2) Affinity Modulation

Recent experiments have suggested that structural modifications in integrin extracellular domains may be the result of intracellular signals being transduced through the integrin and altering the conformation of the extracellular binding site, and, by extension, its affinity for ligand.

Affinity modulation is believed to be the primary means of regulating the aggregation of platelets through the platelet glycoprotein $\alpha_{IIb}\beta_3$ (O'Toole et al., 1990). Evidence for conformational changes comes largely from the existence of mAb that recognize epitopes present only on activated integrins. The appearance of these activation epitopes correlates with the kinetics of the observed increase in adhesion. Thus, $\alpha_{IIb}\beta_3$ adopts a high affinity state for the binding of soluble fibrinogen by activated platelets, which coincides with the ability of an

activation-specific reporter mAb such as PAC-1 to recognize it (O'Toole et al., 1990; Shattil et al., 1985). It should be noted, however, that inactivated platelets are capable of binding immobilized fibrinogen, demonstrating that the 'inactive' receptor is functional. Therefore, activation serves to convert the molecule to a different affinity state.

Similar data have been obtained for β_2 and β_1 integrins. At least two groups have identified mAb that discriminate between LFA-1 on resting cells and that on T cells activated by CD3 crosslinking, phorbol ester stimulation, or exposure to IL-2 (van Kooyk et al., 1991; Dransfield & Hogg, 1989). Furthermore, two antibodies recognizing active conformations of Mac-1 on activated neutrophils and monocytes have been isolated (Diamond & Springer, 1993).

In addition to reporter antibodies, other mAb have been identified that actually induce the adhesive state in integrins, presumably by stabilizing an active conformation. The implication is that integrins may naturally adopt different conformations until an active conformation is stabilized as a result of cell activation events. Examples of leukocyte integrin-activating mAb include KIM127, which recognizes the β_2 chain, and therefore promotes adhesion through LFA-1 and Mac-1 (Robinson et al., 1992), NKI-L16, which induces homotypic aggregation of lymphocyte lines (Keizer et al., 1988), and MEM-83 that promotes T cell binding to ICAM-1 (Landis et al., 1993) and recognizes the α_L chain. Similarly, several anti- β_1 -specific mAb have been shown to induce the adhesion of β_1 integrins to extracellular matrix components (Chan & Hemler, 1993; van de Wiel-van Kemenade et al., 1992; Kovach et al., 1992; Faull et al., 1993; Arroyo et al., 1993). It should be noted that the effect of antibody-induced integrin activation depends on the cellular environment of the integrin, as well as on the particular ligand studied. For example, LFA-1-transfected K562 cells were induced to bind to

ICAM-1 and to ICAM-3 upon treatment of the cells with KIM127, whereas treatment with another β_2 -specific mAb, KIM185, induced adhesion only to ICAM-1 (Ortlepp et al., 1995). In contrast, others have shown that KIM185 induced the adhesion of a T cell clone to all ICAMs (Binnerts et al., 1994). These data suggest that integrins can exist in different activation states.

Finally, a third type of mAb has been identified which purportedly recognizes epitopes exposed only on integrins that have ligand bound to them (Cabanas & Hogg, 1993). The significance of such secondary conformational changes is currently unknown, but they may be involved in stabilizing the integrin/ligand interaction, or in eliciting distinct effector functions (Diamond & Springer, 1994).

In addition to using antibodies to gauge the affinity state of the receptor, several studies have used soluble ligands to determine directly the affinity of various receptors. This has been demonstrated for $\alpha_{IIb}\beta_3$ binding to fibrinogen (O'Toole et al., 1990), Mac-1 binding to factor X and fibrinogen (Altieri & Edgington, 1988; Altieri et al., 1988), and for LFA-1 binding to soluble ICAM-1 (Lollo et al., 1993). In the latter study, the authors demonstrated a 200 fold increase in the affinity for ICAM-1 of a subpopulation of LFA-1 on activated T cells as compared to the LFA-1 on resting T cells.

Although the available evidence supports the notion of conformational changes regulating integrin adhesiveness, little is known about the mechanisms inducing these changes. Several factors may serve cooperatively to effect a conformational change, and these factors are discussed in the next few sections.

1.5.1.3) Phosphorylation

Phorbol esters have been shown to upregulate LFA-1-mediated adhesion (Rothlein & Springer, 1986). Since these compounds are known to activate PKC (Truneh et al., 1985), it was thought that downstream phosphorylation events exerted by PKC could contribute to this increase in adhesion. While LFA-1 α subunits are constitutively phosphorylated, the β chains become phosphorylated predominantly on serine residues upon treatment with phorbol ester (Hara & Fu, 1986; Chatila et al., 1989; Buyon et al., 1990; Pardi et al., 1992b; Valmu et al., 1991; Hibbs et al., 1991a). The time course of this phosphorylation parallels the observed change in LFA-1 avidity (Buyon et al., 1990; Valmu et al., 1991). Therefore, it was speculated that the phosphorylation of β_2 could influence the heterodimer directly to increase adhesion. However, it has subsequently been shown by mutational analysis that the phosphorylation step can be dissociated from the PMA-stimulated increase in adhesion as substitution of the major phosphorylation site in the β_2 cytoplasmic domain does not affect the binding of LFA-1 to ICAM-1 (Hibbs et al., 1991a) or that of Mac-1 to iC3b-coated erythrocytes (Rabb et al., 1993). It is possible that phosphorylation may influence the association of LFA-1 cytoplasmic domains with cytoskeletal elements (Pardi et al., 1992a,b; Pavalko & LaRoche, 1993; Stewart et al., 1996) or with other cytoplasmic regulatory factors that may promote adhesion by eliciting integrin clustering (Burn et al., 1988). Such clustering has been shown to occur with Mac-1 (Detmers et al., 1987). In any event, it is likely that a complex network of phosphorylation events, involving both the heterodimer and other substrates, is associated with increased avidity. For example, in one report, treatment of B cells with the protein kinase inhibitor staurosporine or with the serine/threonine phosphatase inhibitor okadaic acid led to an increase and decrease,

respectively, in LFA-1-mediated adhesion to ICAM-1 (Hedman & Lundgren, 1992). A role for tyrosine phosphorylation is suggested from experiments in which tyrosine kinase inhibitors result in reduced activation of adhesion through CD3 (Kanner et al., 1993).

1.5.1.4) Divalent Cations

The preceding discussion indicated a requirement for divalent cations for the function of LFA-1. Indirect evidence that metal ions actually bind integrins comes from the observation that certain sites on integrins can be recognized by mAb only in the presence of divalent cations (Dransfield & Hogg, 1989; Dransfield et al., 1992; van Kooyk et al., 1994; Diamond et al., 1993). The experimental use of divalent cations *in vitro* has also been demonstrated to directly convert LFA-1 and other integrins into their active forms (Dransfield & Hogg, 1989; Dransfield et al., 1992; Altieri, 1991; Kirchhofer et al., 1991). This effect may occur through a direct association of the cations with the ligand binding site or by altering the conformation of the molecule such that cryptic binding sites are exposed (Loftus et al., 1994). Thus, Mg^{2+} and Mn^{2+} have been shown to increase integrin adhesiveness for ligand (Gailit & Ruoslahti, 1988; Kirchhofer et al., 1990; Masumoto & Hemler, 1993). The binding of Mn^{2+} directly activates integrins, whereas Mg^{2+} acts in concert with additional stimuli. Ca^{2+} has been implicated in determining the surface distribution of LFA-1 (Detmers et al., 1987; van Kooyk et al., 1994; van Kooyk et al., 1996). The importance of divalent cations in integrin function is evident by the fact that mutation of amino acid residues involved in divalent cation binding abolishes ligand recognition (Michishita et al., 1993; Loftus et al., 1990).

Although changes in divalent cation concentrations have been demonstrated to affect the binding of integrins, as have different divalent cations, they are not likely to be of primary

significance for physiological regulation of integrin function since the local concentrations of divalent cations do not fluctuate drastically. Therefore, the binding of divalent cations to integrins may be of secondary importance when compared to cell activation-induced conformational changes in the integrin (Diamond & Springer, 1994).

1.5.1.5) Cytoplasmic Domains

Recent attention has focused on the integrin cytoplasmic domains and their roles in transmitting signals across the plasma membrane. The available evidence suggests that both the α (Chan et al., 1992b; Filardo & Cheresch, 1994; Kassner & Hemler, 1993; O'Toole et al., 1991; O'Toole et al., 1994) and β chains participate in this process (Chen et al., 1994; Hayashi et al., 1990; Hibbs et al., 1991b; Hughes et al., 1995; Lewis & Schwartz, 1995; Pasqualini & Hemler, 1994). Much of the data concerning the contribution of integrin cytoplasmic domains to adhesiveness comes from experiments utilizing truncated integrins. Unfortunately, the results of these experiments are, at times, difficult to reconcile with each other. Truncation of either cytoplasmic domain of Mac-1 results in an increase in binding of transfected COS cells to iC3b (Rabb et al., 1993). In contrast, partial truncation of the β_2 cytoplasmic domain was shown to abrogate LFA-1-mediated binding of B cells and COS cell transfectants to ICAM-1 (Hibbs et al., 1991b). In the latter case, the region of importance was narrowed down to the carboxy-terminal third of the β_2 cytoplasmic domain, and specifically, to a threonine triplet and a phenylalanine residue, all of which are conserved in β_1 , β_3 , and β_7 integrins (Hibbs et al., 1991a). Mutation of these residues abrogates the constitutive binding of COS cells transfected with human LFA-1 to ICAM-1. However, mutants retain the ability to respond to phorbol ester stimulation, indicating that these residues do not themselves explain the regulation of β_2 integrin adhesiveness.

In contrast to the situation with the β_2 chain, truncation of the β_3 cytoplasmic domain does not affect the adhesiveness of $\alpha_{IIb}\beta_3$ to fibrinogen (O'Toole et al., 1991). Interestingly, however, a single point mutation in the β_3 cytoplasmic domain was seen to result in impaired $\alpha_{IIb}\beta_3$ activation (Chen et al., 1992) and deletion of a portion of the conserved membrane-proximal sequence KLLXXXXD results in constitutively active $\alpha_{IIb}\beta_3$ (Hughes et al., 1996). Truncation of the α_{IIb} cytoplasmic domain increased the binding affinity of $\alpha_{IIb}\beta_3$ for fibrinogen over that of the wild type molecule (O'Toole et al., 1991; Ylanne et al., 1993). Indeed, removal of just the conserved GFFKR sequence in the α chain led to $\alpha_{IIb}\beta_3$ being "locked" in a high affinity state (O'Toole et al., 1994). Finally, replacing the cytoplasmic domain of α_{IIb} with those from other integrin α subunits causes the chimeric integrin to behave differently from its wild type counterpart, further underscoring the importance of cytoplasmic domains in integrin regulation (O'Toole et al., 1991; O'Toole et al., 1994).

Studies testing the effect of β_1 integrin cytoplasmic domain truncation have yielded discordant data. Partial truncation of five carboxy-terminal amino acids in the β_1 cytoplasmic domain impairs adhesion and localization of integrin to focal contacts, whereas deletion of the entire cytoplasmic domain inhibits only the latter function (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990). Further complication derives from experiments showing that truncation of certain α chains has little or no effect on the binding of β_1 integrins to extracellular matrix proteins (Briesewitz et al., 1993; Bauer et al., 1993), whereas truncation of other α subunits abolishes the binding to ligand (Kassner & Hemler, 1993).

Because of these paradoxical results, it has been difficult to formulate a unifying model that explains the precise roles of cytoplasmic domains in integrin regulation. One possibility is

that intracellular regulatory elements interact with integrin cytoplasmic domains to modulate adhesiveness. Several examples of cytoskeletal proteins associating with integrins have been reported (see section 1.5.1.6). These proteins may function by altering the affinity of the integrin for ligand (O'Toole et al., 1991; O'Toole et al., 1994; Chen et al., 1992) and/or by influencing post-receptor binding events such as the cell surface distribution of integrin or cell spreading (Chan et al., 1992b; Ylanne et al., 1993; LaFlamme et al., 1992; Hayashi et al., 1990; Balzac et al., 1993; Reszka et al., 1992; van Kooyk et al., 1994; Peter & O'Toole, 1995).

In addition to cytoskeleton-mediated modulation, other regulatory elements may serve to modulate integrin function. Recently, a novel lipid, termed integrin modulating factor (IMF) -1 and believed to be a fatty acid or isoprenoid acid, was isolated. The lipid was postulated to control the avidity of Mac-1 and possibly LFA-1 on PMNs (Hermanowski-Vosatka et al., 1992). Stimulation of PMNS increases the level of IMF-1 which parallels a rise in Mac-1 avidity for purified iC3b. Moreover, IMF-1 purified from activated PMNs and added back to resting PMNs results in increased Mac-1-mediated binding. In another study, phosphatidic acid was shown to promote the binding of fibrinogen by $\alpha_{\text{IIb}}\beta_3$ (Smyth et al., 1992). The appeal of lipids as integrin regulatory molecules resides in the fact that cell activation leads to changes in the plasma membrane composition, which may promote the association of these lipids with integrin transmembrane domains.

Other recent reports have identified novel cytoplasmic regulatory proteins that interact with integrin β chains. These proteins, the integrin-linked kinase (ILK) (Hannigan et al., 1996), cytohesin-1 (Kolanus et al., 1996), and β_3 endonexin (Shattil et al., 1995) have been identified using the yeast two-hybrid system, and associate with β_1 , β_2 and β_3 cytoplasmic domains

respectively. Whereas the physiological significance of β_3 endonexin is unknown, ILK has been shown to modulate the adhesiveness of transfected epithelial cells to ECM (Hannigan et al., 1996), and cytohesin-1 was shown to have both positive and negative regulatory capabilities for the binding of Jurkat cells to ICAM-1 (Kolanus et al., 1996).

Finally, a tyrosine kinase, p125^{FAK} (FAK), is concentrated in focal contacts, which are sites of integrin (predominantly β_1) adhesion to ECM characterized by an accumulation of components of the actin-based cytoskeleton, and has been shown to interact with integrin β subunit peptides *in vitro* (Schaller et al., 1995). Its presence in focal contacts suggests that phosphorylation of components present at these contacts may be required to maintain their integrity. Furthermore, FAK may be a central player in integrin-mediated "outside-in" signalling (see section 1.5.2).

1.5.1.6) Cell Surface Distribution

Another potential mechanism of regulating integrin mediated adhesion is through modulation of the cell surface distribution of the molecules. Clustering of integrins or their ligands would serve to increase the local concentration of the receptors at the site of cell contact, thereby increasing the avidity of the interaction without necessarily modulating the affinity of the individual receptor:ligand interactions. A pertinent example of this comes from experiments testing the binding of CD2 to the transmembrane versus the GPI-linked forms of its ligand LFA-3 incorporated into artificial membranes (Chan et al., 1991b). This study showed dramatically improved adhesion of CD2 to the latter, presumably because the higher mobility of GPI-linked proteins within the membrane allows greater accumulation of receptor/ligand interaction at the site of contact. In other studies, phorbol ester treatment of neutrophils resulted in the

aggregation of Mac-1 in the absence of ligand (Detmers et al., 1987) and stimulation with fMLP or phorbol ester induced the clustering specifically of activated Mac-1 (Diamond & Springer, 1994). ICAM-1 was also demonstrated to be localized in uropods (Dougherty et al., 1988). Circumstantial evidence for the importance of clustering is provided by the inability of some leukemic T-cell lines that do not display clustering of LFA-1 to exhibit adhesion through LFA-1 in response to stimulation with phorbol ester or other agonists (van Kooyk et al., 1993). Finally, one recent report showed that the induction of a high-affinity state of a chimeric $\alpha_{\text{Ib}}\beta_3$ /LFA-1 integrin by deleting the GFFKR motif in the α_L subunit was not sufficient for stable adhesion - multimerization of the receptor was required (Peter & O'Toole, 1995).

Aggregation of integrins may be mediated by constitutive or induced interactions with cytoskeletal elements (Burn et al., 1988; Pavalko & LaRoche, 1993; Meijne et al., 1994; Peter & O'Toole, 1995; van Kooyk et al., 1994), and may be regulated by the rho family of small GTPases (Tominaga et al., 1993; Hotchin & Hall, 1995). Several lines of investigation have suggested that LFA-1 becomes physically associated with the actin-based cytoskeleton upon cell activation. Co-precipitation studies have revealed the association of LFA-1 β_2 subunits with the cytoskeletal proteins α -actinin (Pavalko & LaRoche, 1993) and filamin (Sharma et al., 1995). In *in vitro* binding experiments, α -actinin was shown to bind to a peptide corresponding to an 18 amino acid region in the N-terminal half of the β_2 cytoplasmic domain (Pavalko & LaRoche, 1993). This is consistent with what has been observed for β_1 integrins in fibroblasts (Horwitz et al., 1986; Otey et al., 1990). Filamin also bound to the N-terminal half of the β_2 cytoplasmic domain, but not to the α -actinin binding site, suggesting that these cytoskeletal proteins bind to distinct, but overlapping sites. Moreover, talin, another cytoskeletal protein, has been observed

to co-localize with LFA-1 at sites of cell-cell contact between helper T cells and B cells (Kupfer et al., 1990; Burn et al., 1988). In spite of this information, the mechanisms involved in the association of integrins with the cytoskeleton are incompletely understood.

Events following receptor binding to ligand probably lend a significant contribution to adhesion. For example, when VLA-5 on T cells is stimulated by an activating mAb, adhesion to immobilized fibronectin is increased via an increase in the affinity of the receptor. Phorbol ester treatment also increases adhesion, but through cytoskeletal changes which lead to cell spreading (Faull et al., 1994). Thus, it appears that two different mechanisms exist for regulating adhesion through this receptor.

1.5.2) Outside-In Signalling

In addition to their roles as cell adhesion molecules, integrins are purported to function in an "outside-in" signalling capacity. The activation of T cells by anti-CD3 antibody can be enhanced if LFA-1 is also crosslinked by mAb (Van Seventer et al., 1990; Wacholtz et al., 1989) or engaged by ICAM-1 immobilized on plastic (Kuhlman et al., 1991). Signalling induced by antibody-mediated crosslinking of LFA-1 leads to the phosphorylation of intracellular proteins on tyrosine residues (Kanner et al., 1993; Arroyo et al., 1994; Fuortes et al., 1994; Graham et al., 1994; Berton et al., 1994), as well as to an elevation of cytosolic calcium concentration and phosphatidylinositol turnover (Pardi et al., 1989). Ultimately, these signals can affect various cellular functions including apoptosis, cytotoxicity, production of cytokines, and antigen presentation (Moy & Brian, 1992; Koopman et al., 1994). The integrins VLA-4 and VLA-5 have also been implicated in providing co-stimulatory signals to T cells (Dustin & Springer, 1991; Shimizu & Shaw, 1991). The effect of signalling through these molecules is dependent

on the cell type involved. Signalling through LFA-1 on T cells results in increased proliferation, whereas signalling through Mac-1 on neutrophils promotes exocytosis and phagosome activity (Jaconi et al., 1991). Another potential consequence of outside-in signalling may be to direct cellular differentiation. The progression of murine fetal thymocytes from the CD4⁻CD8⁻ phenotype to the CD4⁺CD8⁺ phenotype can be inhibited by antibodies to LFA-1 or ICAM-1 (Fine & Kruisbeek, 1991).

Recent information suggests that integrins may be involved in cis interaction with other proteins in the plane of the membrane. For example, the β_1 subunit has been shown to associate with CD9 (Masselis-Smith & Shaw, 1994; Rubinstein et al., 1994; Berditchevski et al., 1996), and Mac-1 associates with Fc receptor (FcR; CD16) (Stockl et al., 1995). A topic of recent interest is whether these associations could be responsible for outside-in signalling through integrins. Evidence for this is suggested in one study in which the phosphorylation of cytoskeletal protein paxillin by stimulation of FcR was shown to require the presence of Mac-1 (Graham et al., 1994). Alternatively, clustering of integrins into focal adhesion plaques may serve to appose putative PTKs associated with the integrin cytoplasmic domains (Kolanus et al., 1993), and may thus initiate a signalling cascade. The structure and function of focal contacts has been the subject of intense research effort in recent years. Integrins in focal contacts bind to ECM-derived ligands such as laminin, collagen or fibronectin, and interact directly or indirectly with cytoskeletal proteins, such as α -actinin, talin, vinculin, paxillin, and tensin, via their cytoplasmic domains (Dedhar & Hannigan, 1996; Yamada & Geiger, 1997). The accumulation of cytoskeletal proteins may provide a scaffold for the association of other regulatory or signalling molecules such as the PTK FAK. In a recent report, FAK, phosphorylated as a result of integrin engagement by fibronectin, was linked to the ras signal transduction pathway via

Grb-2 and SOS proteins (Schlaepfer et al., 1994). Another consequence of integrin engagement and clustering is the activation of mitogen-activated protein kinase (MAPK), which may occur in a ras-dependent or -independent process, and which may lead to altered gene expression (Dedhar & Hannigan, 1996). Thus, the large aggregates of proteins in adhesion plaques may serve to provide high local concentrations of structural and effector molecules that facilitate the activation of signalling pathways and the organization of adhesive sites. The functions of the components present in adhesion complexes may be regulated by the state of aggregation of the receptors, and by conformational changes induced by ligand occupancy (Yamada & Geiger, 1997). It should be noted that LFA-1 has not been reported to localize in focal adhesion plaques except in one case (Peter & O'Toole, 1995) where the authors used a chimeric integrin consisting of the extracellular and transmembrane regions of $\alpha_{\text{IIb}}\beta_3$ and the cytoplasmic domains of LFA-1. Therefore, it is possible that the observed results may not be applicable to native LFA-1.

1.6 THESIS OBJECTIVES

This introduction describes the current state of knowledge pertaining to the regulation of LFA-1-mediated adhesion. At the start of this thesis work, the function of LFA-1 had been well characterized, but the complexity of its regulation had only begun to be appreciated. It was well established that a qualitative change in LFA-1 upon cell activation led to increased adhesion through this receptor, but little was known about the precise mechanisms involved in the conversion of LFA-1 from an inactive form to an active one. I set out to investigate this problem by testing the hypotheses that the distribution of LFA-1 at the cell surface plays an important role in adhesion, and that the cytoplasmic domains are involved in the process of

inside-out signalling. To test the first hypothesis, polystyrene microspheres, coated with recombinant sICAM-1, were used to probe the distribution of 'activated' LFA-1 on several cell types. The results of these experiments form the subject of chapter 3. Chapter 4 describes experiments designed to elucidate the roles of the LFA-1 cytoplasmic domains in adhesion to ICAM-1. Various mutant forms of LFA-1 were constructed and transfected into fibroblasts. The ability of the transfectants to bind sICAM-1 immobilized on plastic was subsequently tested. In chapter 5, the role of LFA-1 cytoplasmic domains in regulating adhesion was further tested by overexpressing isolated cytoplasmic domains in lymphocytes, and determining the effect on adhesion to ICAM-1.

CHAPTER 2

Materials and Methods

2.1) ANIMALS

BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were maintained in the Joint Animal Facility of the B.C. Cancer Research Center.

2.2) ANTIBODIES

Anti-murine ICAM-1 mAb YN1/1.7 has been described (Takei, 1985; Horley et al., 1989). Rat hybridomas producing anti- α_L (ATCC TIB213:FD441.8), anti- β_2 (ATCC HB226:2E6), and anti-CD4 (ATCC TIB207:GK1.5), and the mouse hybridoma producing anti-rat IgK (ATCC TIB169:RG11/39.4) were obtained from the American Type Culture Collection (Rockville, MD). FD441.8 was purified from the hybridoma supernatant using an Avid-AL column (BioProbe International) as per the manufacturer's instructions. 2E6 was grown in Protein Free Hybridoma Medium (Canadian Life Technologies, Burlington, ON) and was purified by precipitation from the culture supernatant with 50% saturated ammonium sulfate followed by dialysis against saline. FITC-conjugated anti-murine CD4 was purchased from Boehringer Mannheim (Laval, PQ) and rabbit anti-rat IgG - tetramethylrhodamine isothiocyanate (TRITC) was purchased from Sigma Chemical Co. (St. Louis, MO).

2.3) CELL CULTURE

The murine T cell hybridoma line T28 (Falo et al., 1986), (a gift from Dr. K. Rock, Dana Farber Cancer Institute, Boston, MA), P388D1 monocytic cells (Koren et al., 1975), (obtained from Dr. N. Reiner, Department of Medicine, University of British Columbia), the B cell line A20A8, a subline of A20 (Kim et al., 1979) (obtained from Dr. R. W. McMaster, Department of Medical Genetics, University of British Columbia, Canada), the T cell line EL-4 (Takei, 1983),

and the fibroblastoid L cell line (Sanford et al., 1948) were all maintained in Dulbecco's modified essential medium (DMEM) supplemented with 5% fetal calf serum (FCS). The murine fibroblast line TNR-2, an N-ras-transformed NIH3T3 line, was provided by Dr. R. Kay (Terry Fox Laboratory, Vancouver, B.C.) and was maintained in DMEM + 10% FCS. Splenic T cells from BALB/c mice were isolated by passage of splenocytes over a nylon wool column essentially as described previously (Julius et al., 1973). Briefly, 1 g of dried nylon wool (Polysciences Inc., Warrington, PA) was packed into a 12 ml disposable syringe, and autoclaved. Columns were equilibrated for 1 hr at 37° C in RPMI 1640 + 5% FCS. Single cell suspensions of spleen cells were loaded onto the column and incubated at 37° C for 1 hr. Non-adherent cells were eluted with pre-warmed RPMI + 5% FCS, and contaminating red blood cells (RBC) were lysed with Tris-NH₄Cl. This procedure has been shown to yield a population of resting T cells that is greater than 90% pure (Julius et al., 1973).

2.4) GENERATION OF RECOMBINANT cDNA's

The cDNA clones encoding murine α_L (CD11a) (obtained from Dr. T. A. Springer, Center for Blood Research, Boston, MA) and β_2 (CD18) (obtained from the American Type Culture Collection) were subcloned into pBluescript (Stratagene, La Jolla, CA). Truncations of the cytoplasmic domains of both chains were generated using polymerase chain reactions (PCR) in which the transmembrane and portions of the extracellular domains were amplified, cut with restriction endonucleases, and re-ligated with the remaining portions of the extracellular domains. All PCR reactions described herein were performed according to the following protocol: 94° C, 30 sec; 55° C, 30 sec; 72° C, 90 sec. The primers used to amplify the α_L

subunit were 5'-CCACACTAGAGGCCTTGG-3' and 5'-CTAGTAGAGCGCCAGGAAAAT-3'. The fragment was digested with BamHI and XbaI and ligated into the cognate sites of the original cDNA in pBluescript. Similarly, a β_2 PCR fragment was amplified using the primers 5'-TGAATGCACGGCTGGTAGA-3' and 5'-CTACCAGATGACCAGGAGGAG-3', and was subcloned into the NdeI/BamHI sites of the original plasmid in pBluescript. The resulting constructs, as well as the wild-type α_L cDNA, were then excised with XhoI/NotI and subcloned into the expression vector pBCMGSNeo (Karasuyama et al., 1990). In addition, the wild-type β_2 and α_L subunits were subcloned into pRC6 (Kay & Humphries, 1991) at the SmaI site. To generate chimeric cDNA clones encoding glycosyl-phosphatidylinositol (GPI)-anchored LFA-1, a DNA fragment encoding the GPI-anchoring signal derived from the heat stable antigen (HSA) cDNA (Kay et al., 1990) was generated by PCR using the oligonucleotides 5'-CAGGATCCGGGGTGGCAGCTCCCTGCAG-3' and 5'-TTCTAGAGATATGGTAACAGCCAATT-3' as primers. The PCR product was subcloned into BamHI and XbaI sites of pBluescript to generate the plasmid pGPIBST. A 360 bp fragment encoding a portion of the CD11a extracellular domain proximal to the transmembrane region was generated by PCR using 5'-GCGAATTCCCACACTAGAGGCCTTGG-3' and 5'-GAATGATCAACATCTCCTTCTCGTGGATCA-3' as primers. The fragment was digested with EcoRI and BclI and ligated with pGPIBST digested with EcoRI and BamHI. A 510 bp DNA fragment encoding a portion of the CD11a extracellular domain fused with the GPI-anchoring signal was isolated from this plasmid by digestion with BamHI and NotI. The full length CD11a cDNA in pBluescript was digested with BamHI and Not I and ligated with the 510 bp CD11a/GPI fragment. The cDNA clone encoding GPI-anchored murine CD18 was generated in a similar fashion. A DNA fragment encoding a portion of the CD18 extracellular

domain was generated by PCR using 5'-GGAATTCTGAATGCACGGCTGGTAGA-3' and 5'-TTGGATCCCCTTCACACTCTAGACT-3' as primers. The PCR product was digested with EcoRI and BamHI and ligated with pGPIBST. The resultant clone as well as the CD18 cDNA in pBluescript were digested with NdeI and NotI and ligated together at these sites. The DNA clones in pBluescript encoding GPI-anchored CD11a and CD18 were digested with XhoI and NotI and subcloned into the mammalian expression vector pBCMGSNeo (Karasuyama et al., 1990). All constructs were verified by nucleotide sequencing.

For the generation of LFA-1 chimeras, the transmembrane and cytoplasmic domains of murine CD11a and CD18 were amplified by PCR, as was the extracellular domain of murine CD4. The sequences of the oligonucleotides were 5'-GAATTCAATGTGGCTGCCATCGTAG-3' and 5'-GGATCCCATGCTCTAGCTTTCAGCA-3' for CD18, 5'-ATCGATTCCCAACCAACAAGAGCTCA-3' and 5'-GAATTCTGTCTGGTTCACCCCTCTGGA-3' for CD4, and 5'-GGATCCATGCTTCACGTGTACGTGC-3' and 5'-TTCCTGGGCTGTCAGTGTATCA-3' for CD11a. The CD18 and CD11a PCR fragments were subcloned into pBluescript at EcoRI/BamHI and BamHI/NotI sites respectively, and the CD4 fragment was ligated into SstI/EcoRI sites of pUC18. A HindIII/EcoRI fragment from the latter was then ligated upstream from the CD18 transmembrane and cytoplasmic domains in pBluescript that had been digested with HindIII/EcoRI. This procedure resulted in a chimera containing two extra amino acids at the junction of the CD4 and CD18 domains. Similarly, a XhoI/EcoRI fragment consisting of the extracellular portion of CD4 was liberated from the CD4/CD18 construct and was inserted upstream from the CD11a transmembrane and cytoplasmic domains in pBluescript, yielding a chimera with 8 extra amino acids at the junction of the two domains. As a control, a truncated

CD4 lacking all but four amino acids in the cytoplasmic domain, and thus incapable of interacting with the leukocyte specific tyrosine kinase p56^{lck} (Turner et al., 1990), was also generated by PCR using the primers 5'-GATTCCCAACCAACAAGAGCTCA-3' and 5'-GAATTCTTAGTGCCGGCACCTGACACAGCAG-3'. The fidelity of the reading frames for all these constructs was ascertained by nucleotide sequencing. All cDNA constructs were subcloned into the mammalian expression vector pBCMGSneo using XhoI and NotI sites.

2.5) TRANSFECTION AND ISOLATION OF CELL LINES

2.5.1) LFA-1⁺ L cells.

Murine fibroblast L cells were transfected using the calcium precipitation method with cDNA encoding murine CD11a and CD18 in pRC6, a derivative of pAX114 (Kay & Humphries, 1991). The transfected L cells were selected in DMEM + 5% FCS containing 0.25 mg/ml hygromycin B (Calbiochem, La Jolla, CA), and cells expressing LFA-1 on the surface were sorted on a FACStar flow cytometer (Becton-Dickinson & Co., Lincoln Park, NJ). Sorted cells were cloned by plating at low density and picking isolated colonies. FACS analysis of transfected L cells was performed by incubating cells with FD441.8 hybridoma supernatant for 30 min at 4° C, followed by a 1/100 dilution of FITC-conjugated secondary mAb RG11/39.4 in Hanks' balanced salt solution (HBSS) + 5% FCS for 30 min at 4° C. Dead cells were gated out using the dye 7-Aminoactinomycin D. Experiments were performed using either the bulk population of sorted cells, or one of the LFA-1⁺ clones.

2.5.2) LFA-1 cytoplasmic domain truncations

The murine ICAM-1 cDNA (Horley et al., 1989) was subcloned into pBCMGSNeo and electroporated into T28 cells which do not express endogenous ICAM-1. An ICAM-1⁺ T28 line was established by flow cytometric cell sorting using anti-ICAM-1 mAb (YN1/1.7). TNR-2 cells were transfected with 3 µg each of wild-type or mutant α_L and β_2 cDNA in various combinations using the lipofectin procedure (Canadian Life Technologies) according to the manufacturer's instructions. The transfected cells were selected with 0.3 mg/ml G418 (GIBCO-BRL, Grand Island, NY) and, for cells expressing wild-type β_2 , with 0.25 mg/ml hygromycin B (Calbiochem). Cells expressing LFA-1 were isolated by a series of panning and cell sorting procedures using anti- β_2 and anti- α_L mAb, and were subsequently maintained in DMEM +10% FCS + 0.2 mg/ml G418 and hygromycin B (where appropriate) with periodic panning. FACS analysis of TNR-2 transfectants was performed by incubating cells with FITC-conjugated anti- β_2 (5 µg/ml) for 30 min at 4° C. For LFA-1-GPI transfectants, the cells were further incubated with a saturating concentration of phycoerythrin-conjugated streptavidin for 30 min at 4° C. ICAM-1⁺ T28 cells were stained as described above with YN1/1.7 mAb conjugated to FITC for 30 min at 4° C. Stained cells were analyzed using a FACScan flow cytometer (Becton-Dickinson & Co.).

2.5.3) LFA-1 chimeras

A20A8 and EL-4 cells were transfected with 10 µg of vector alone or of CD4 Δ CY, CD4/18 or CD4/11 chimeric cDNA in pBCMGSneo by electroporation. The transfectants were selected and subsequently maintained in DMEM + 5% FCS containing 0.3 mg/ml (EL-4) or 0.5 mg/ml (A20A8) G418. Cells expressing the chimeras were isolated by flow cytometric cell

sorting using anti-CD4 mAb conjugated to FITC. Subsequent FACS analysis of the transfected lines was performed using anti-CD4-FITC and 2E6-FITC at 5 µg/ml for 15 min at 4° C.

2.6) PI-PLC TREATMENT AND ISOLATION OF SOLUBLE LFA-1

Phosphatidylinositol-specific phospholipase C (PI-PLC) was a gift from Dr. W. Jefferies (Biotechnology Laboratory, University of British Columbia). A Centricon-30 concentrator (Amicon, Beverly, MA) was used to concentrate the PI-PLC and remove low molecular weight contaminants. TNR-2 cells expressing GPI-anchored LFA-1 were resuspended in HBSS at a concentration of $2-4 \times 10^6$ /ml and PI-PLC (approximately 1 unit) was added. The cells were incubated at 37° C for 1 hour and analyzed by flow cytometry. For the immunoprecipitation of soluble LFA-1 generated by PI-PLC treatment, cells were surface-biotinylated as described in section 2.7 prior to the treatment with PI-PLC. For the isolation of soluble LFA-1 on a larger scale, approximately 8×10^7 TNR-2 cells expressing GPI-anchored LFA-1 were treated with PI-PLC as described above. Cells were removed by centrifugation at 12,000 X g for 30 min, and the supernatant was recovered and incubated with control rat antibody-coupled beads for 1 hour at 4° C. The unbound fraction was recovered and incubated with anti- α_L antibody-coupled beads for 1 hour at 4° C. The beads were then washed with 10 mM Tris-HCl (pH 7.4) containing 0.15 M NaCl and 2 mM MgCl₂. LFA-1 was eluted from the beads with 0.1M Na₂CO₃ (pH 11) containing 0.15 M NaCl and 2 mM MgCl₂, and the eluate was immediately neutralized with a predetermined amount of 1M NaH₂PO₄. The eluate was concentrated using a Filtron concentrator (FILTRON Technology Corporation).

2.7) IMMUNOPRECIPITATION AND WESTERN BLOT

Cell surface biotinylation was carried out using sulfo-N-hydroxy succinimide biotin (NHS-LC-biotin, Pierce Chemical Co., Rockford, IL) essentially as described (Food et al., 1994). EL-4 cells (4×10^6) and TNR-2 cells ($2-4 \times 10^6$) were washed in 5 ml HBSS followed by 5 ml biotin labelling buffer (HBSS minus phosphates), and were suspended at 5×10^6 /ml in labelling buffer containing 0.5 mg/ml NHS-LC-biotin. The biotinylated cells were washed three times with serum-free DMEM and lysed with 1 ml of 1% Triton X-100 in HBSS containing 2% bovine serum albumin (BSA). The cell lysates were cleared by microcentrifugation for 10 min. The cleared TNR-2 cell lysates (0.5 ml aliquots) were mixed with 15 μ l of Affigel-10 (BioRad Laboratories, Mississauga, ON) beads coupled with anti- α_L or anti- β_2 (2 mg antibody per ml beads), and incubated at 4° C for 2-3 hours with continuous mixing. EL-4 lysate supernatants were pre-cleared with 30 μ l RG11/39.4-complexed Affigel-10 beads for 90 min at room temperature, re-centrifuged, and then incubated with 20 μ l of GK1.5 or FD441.8 culture supernatant for 60 min at 4° C, followed by RG11/39.4-Affigel-10 for a further 60 min with continuous inversion. Following the immunoprecipitation, the beads were washed four times with 1% Triton X-100 in HBSS, before eluting the bound proteins with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 4% SDS. The eluted proteins were separated on a 10% SDS-PAGE gel, electro-blotted onto an Immobilon-P membrane (Millipore, Bedford, MA), and visualized by enzyme chemiluminescence using reagents obtained from Amersham (Oakville, ON) or Pierce Chemical Co.

2.8) PREPARATION OF sICAM-1-COATED MICROSPHERES

The production and purification of recombinant sICAM-1 has been described (Welder et al., 1993). Briefly, the cDNA encoding murine ICAM-1 (Horley et al., 1989) was digested with Sca I to remove the transmembrane and cytoplasmic domains, subcloned into the expression vector pBCMGSneo (Karasuyama et al., 1990), and transfected into NS-1 (Kohler et al., 1976) cells. sICAM-1 was purified from the culture supernatant of one of the cloned transfectants by antibody affinity chromatography. SDS-PAGE analysis of the proteins eluted from the column showed a single band of approximately 90 kD. Polystyrene microspheres (Seradyn, Indianapolis, IN), 2 μm in diameter, were washed of adsorbed surfactant and low molecular weight contaminants in an Amicon 8010 stirred ultrafiltration cell (Amicon, Beverly, MA) using a 300 000 molecular weight cutoff membrane. The microspheres were coated with sICAM-1 by co-incubation in either 0.1M sodium carbonate buffer (pH 9.5) or 0.1M sodium bicarbonate buffer (pH 8.0) for 1 hour at room temperature. Unbound sICAM-1 was washed away, and microspheres were treated with 0.5 mg/ml BSA to block non-specific binding sites. BSA-coated microspheres were prepared following the same protocol, and were used in control experiments. The density of sICAM-1 obtained on the microspheres using this protocol was approximately 6000 ICAM-1 sites/ μm^2 as determined in an ELISA assay measuring the degree to which sICAM-1-coated microspheres inhibit the binding of anti-ICAM-1 mAb to ICAM-1 immobilized on plastic (Welder et al., 1993).

2.9) BINDING OF MICROSPHERES TO CELLS

For the binding of microspheres to splenic T cells, resting or pre-activated (with 50 ng/ml phorbol 12-myristate 13-acetate; PMA (Sigma), dissolved in dimethyl sulfoxide; DMSO)

cells (3×10^6 in RPMI 1640 + 5% FCS) were co-incubated with microspheres (55 - 85 beads/cell) at 37° C for 45 min in microtitre wells in a total volume of 100 μ l. T28 cells were treated identically except that 2×10^6 cells were used. Where appropriate, splenic T cells were pre-incubated with 20 μ M cytochalasin B (Sigma) for 25 min at 37° C, PMA was added, and the cells were incubated for a further 20 min before co-incubation with microspheres. The mixtures were then layered onto a percoll solution (density = 1.1176 - 1.1217) and centrifuged at 100 X g for 5 min at room temperature to separate unbound microspheres. The pellets were gently resuspended, and aliquots were transferred into microtitre wells and photographed on an inverted microscope using a 40 X objective. This protocol is represented diagrammatically in Figure 2.1a.

For T28 cells, quantitation of the pattern of microsphere binding was performed blind in three independent tests. Approximately 100 cells were scored per test. Binding was deemed localized if several (at least 5) microspheres bound to a single site on the cell surface comprising not greater than one half of the circular cell's perimeter. In the case of A20A8 and P388 cells, percoll separation of unbound beads could not be performed due to the high degree of aggregation seen upon activation of these cells. Therefore, an alternate procedure was performed. Flat-bottom microtitre wells (Nunc, Denmark) were coated with 2% gelatin in HBSS at 37° C for 2 hours. After washing with HBSS, the gelatin layer was treated with 0.5% glutaraldehyde in HBSS for 15 min at room temperature, and washed with excess HBSS to remove free glutaraldehyde. The cells, stimulated with 50 ng/ml PMA where appropriate, were dispensed into the wells, centrifuged briefly and incubated at room temperature for 15 min to allow covalent attachment of the cells to the microtitre wells. The wells were then flooded with excess 1% gelatin/HBSS to saturate free glutaraldehyde binding sites. The cells were incubated

a

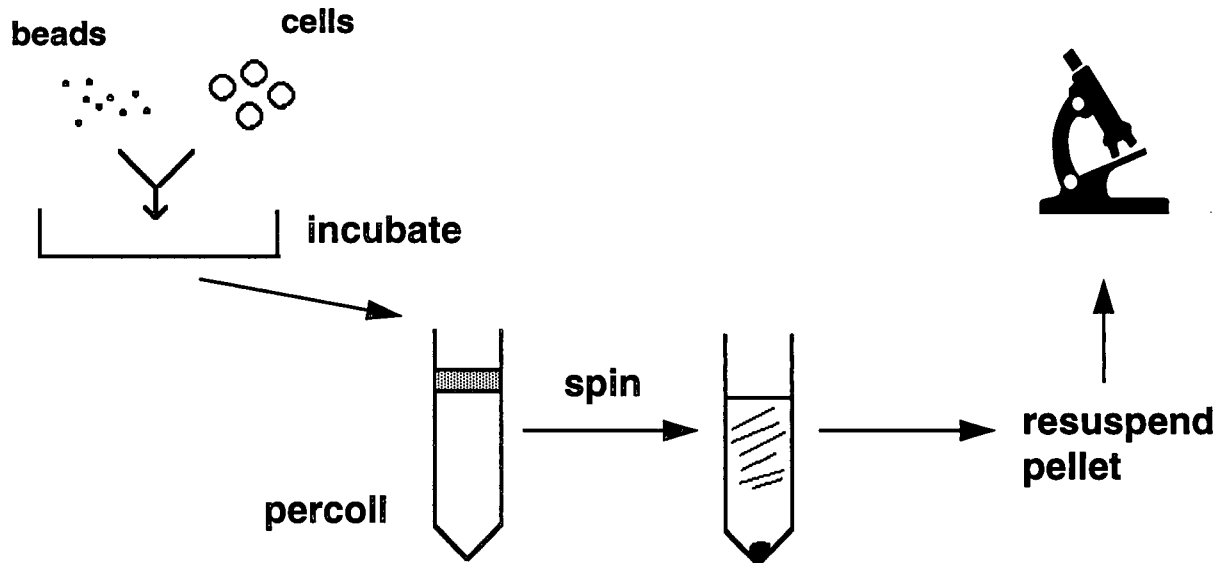
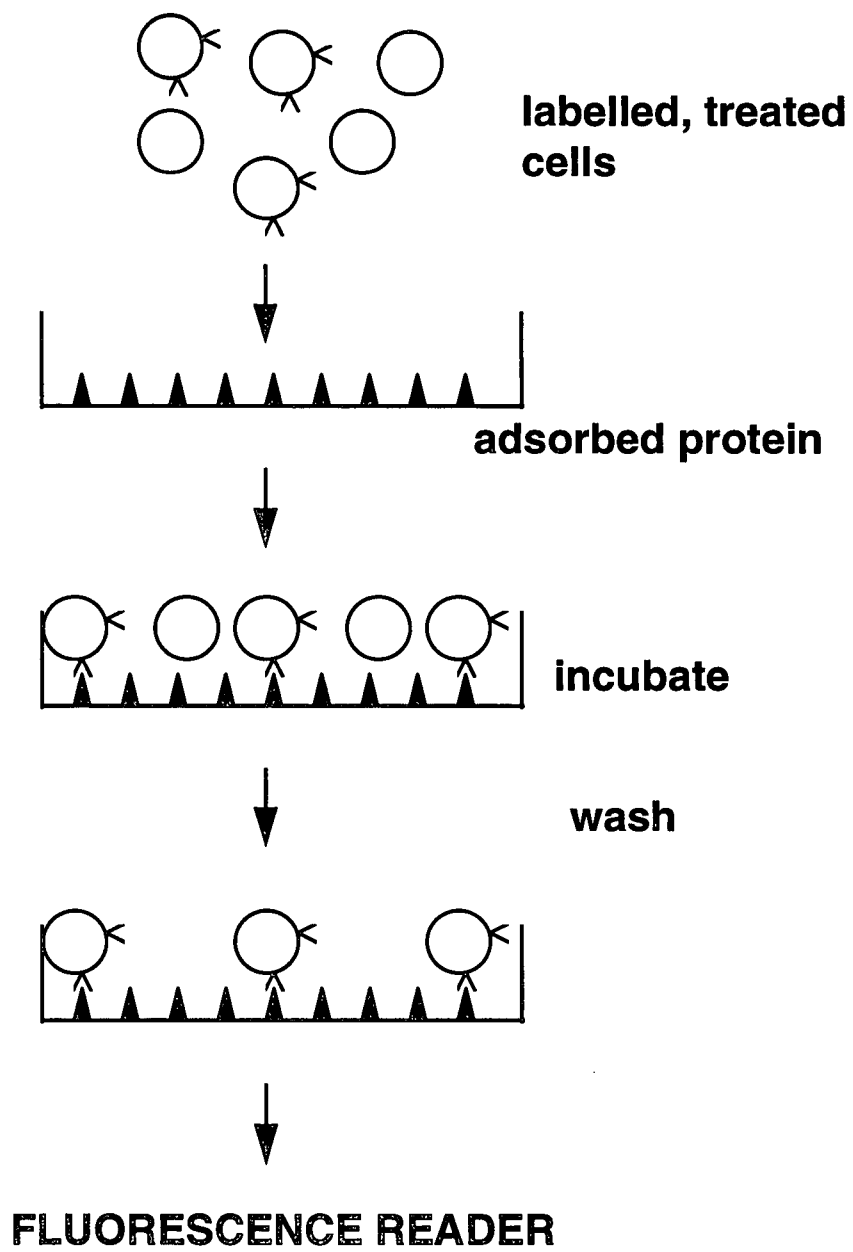


Figure 2.1. Protocols of adhesion assays used in this thesis. **(a)** Microsphere adhesion assay. Cells of interest were treated as required, and were incubated with sICAM-1-coated microspheres as described in Section 2.9. Unbound microspheres were separated from cell-bound microspheres by layering the mixture on a 25% percoll solution, and centrifuging at low speed. Unbound beads were aspirated away, and the pellet was gently resuspended in buffer and fixed with glutaraldehyde before photographing. **(b)** Plate adhesion assay. Cells of interest were treated as required, and introduced into microtitre wells previously coated with protein (e.g. sICAM-1). The cells were allowed to settle onto the protein coat, and plates were incubated at 37° C. Washing was performed using pre-warmed medium, and manually flicking the plates. Quantitation of cells bound was performed by measuring the reduction of MTT or the fluorescence of metabolically labelled cells (see section 2.10).

b



with microspheres for 20 min at 37° C. Unbound microspheres were washed away with pre-warmed 1% gelatin/HBSS, and the cells were photographed as described above.

For the binding of microspheres to L cells, approximately 4×10^7 microspheres in 10 μ l of DMEM + 5% FCS were dispensed into flat-bottom microculture wells which had been seeded with $1-5 \times 10^4$ L cells 1-2 days previously. The wells were incubated at 37° C for 25 min and then washed 10-15 times with DMEM + 5% FCS pre-warmed to 37° C. The cells were subsequently fixed in 0.5% glutaraldehyde in phosphate buffered saline (PBS), stained in Giemsa, and photographed as described above. The degree of localized microsphere binding was scored in three independent tests performed blind (approximately 100 cells counted per test). Binding was scored as localized if several (more than 5) microspheres bound to a single site on the cell surface.

2.10) CELL ADHESION AND SPREADING ASSAYS

The general protocol used in performing adhesion assays to proteins immobilized in microtitre wells is illustrated in Figure 2.1b. Single cell suspensions of L cells were prepared by treating the cells in monolayer cultures with 10 mM EDTA in PBS at 37° C for 10 min, followed by vigorous pipetting. Flat-bottom microtitre wells (Nunc, Denmark) were coated with sICAM-1 (50 - 100 μ g/ml in 0.1M sodium carbonate buffer, pH 9.5, or in 0.1M sodium bicarbonate buffer, pH 8.0) for 60 min at room temperature, and unbound sICAM-1 was removed by washing with PBS. LFA-1⁺ cells in RPMI + 5% FCS were dispensed into the wells, centrifuged at 10 X g for 2 min, and incubated at 37° C for 5 - 7 min. Unbound cells were washed away from the wells by gently filling the wells with 150 μ l aliquots of pre-warmed medium and manually flicking the plate of its contents. The wells were washed several times,

and the remaining bound cells were quantitated in an assay measuring the reduction of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Mosmann, 1983) and using a standard curve generated from known numbers of cells.

Single cell suspensions of TNR-2 cells expressing LFA-1 truncations were prepared by treating the cells with 5 mM EDTA in HBSS for 10 min at 37° C. Where appropriate, the cells were treated with 20 μ M cytochalasin D for 30 min before stimulation with 50 ng/ml PMA for a further 30 min. A20A8 and EL-4 cells expressing LFA-1 chimeras were similarly stimulated with PMA. All cells were labeled with 1 μ g/ml Calcein-AM or 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF-AM, Molecular Probes, Eugene, OR) in HBSS for 10 min, washed with serum-free HBSS, and resuspended in HBSS containing 2% FCS. The adhesion of labelled cells to ELISA wells coated with 30 μ g/ml of sICAM-1 was assayed as described above, except that HBSS + 2% FCS was used as the assay buffer. In addition, the adhesion of A20A8 transfectants to microtitre wells coated with 30 μ g/ml whole human fibronectin (Sigma) was assayed. Cell adhesion to immobilized proteins was quantitated using a Cytofluor 2300 fluorescence microplate reader (Millipore), and was expressed as the percentage of input cells remaining in the wells after washing away unbound cells. Soluble LFA-1 was also immobilized in microwells, and the adhesion of ICAM-1⁺ T28 cells to the coated wells was tested in the same way as described for the adhesion of LFA-1⁺ TNR-2 cells to ICAM-1.

For the assay of cell spreading following cell binding to immobilized sICAM-1, adhesion assays were performed as described above, with the exception that cells were not labelled with metabolic dye. After washing to remove unbound cells, the remaining cells were incubated at 37° C for a further 7-8 min (TNR-2) or 20 min (A20A8), and were subsequently fixed in 0.5%

glutaraldehyde in HBSS. The degree of cell spreading was visualized on an inverted microscope using a 20X or a 40X objective. The irregular shape of EL-4 cells made them unsuitable for the analysis of spreading.

2.11) FLUORESCENCE MICROSCOPY

LFA-1⁺ L cells and TNR-2 transfectants were grown on glass coverslips, washed in PBS, and either fixed for 15-20 min with 1% paraformaldehyde at room temperature, or left unfixed. The cells were incubated with anti-LFA-1 (FD441.8) culture supernatant for 30 min followed by rabbit anti-rat IgG-TRITC secondary antibody for 30 min, washed, and mounted on glass slides in 30% glycerol in PBS. Antibody staining was performed at room temperature for fixed cells, and at 37° C for unfixed cells containing bound microspheres. In other experiments, LFA-1⁺ L cells, grown on coverslips, were incubated with sICAM-1-coated microspheres for 20 min at 37° C. After washing away unbound microspheres, cells were stained with rat anti-LFA-1 mAb for 20 min at 37° C, followed by rabbit anti-rat IgG-TRITC for 20 min at 37° C, and mounted on microscope slides. T28 cells containing bound microspheres (see above) were immobilized on glass slides via poly-L-lysine (100 mg/ml) (Sigma) for 5 min at 37° C, and then fixed with 1% paraformaldehyde in PBS. As a control, T28 cells in suspension were stripped of bound microspheres by incubation at 4° C in PBS containing 0.1% sodium azide and 1mM EDTA before fixation. In addition, untreated T28 cells were immobilized on glass slides prior to fixation with paraformaldehyde. All cells were incubated with rat anti-LFA-1 antibody for 30 min at room temperature, followed by either rabbit anti-rat IgG-TRITC, or biotinylated goat anti-rat Ig, and avidin-TRITC. Fluorescently stained cells were photographed on a Zeiss

epifluorescence microscope at 40X magnification using Kodak Tri-X 400 black and white print film.

CHAPTER 3

Cell surface distribution of high-avidity LFA-1 detected by sICAM-1-coated microspheres

The results presented in this chapter were published in the following manuscript:

AM Pyszniak, CA Welder and F Takei (1994) Cell Surface Distribution of High-Avidity LFA-1
Detected by Soluble ICAM-1-Coated Microspheres. J Immunol 152:5241.

3.1) INTRODUCTION

The importance of the interaction between LFA-1 and ICAM-1 in the normal function of the immune system has been extensively documented (Springer et al., 1987; Springer, 1990). This interaction is critically regulated by cellular activation, which induces a qualitative rather than quantitative change in LFA-1, thereby influencing its ability to bind ICAM-1. In T cells, this is exemplified by a rapid increase in LFA-1-mediated adhesion to ICAM-1 immobilized on plastic upon stimulation with phorbol esters or TCR cross-linking (Dustin & Springer, 1989). Antibodies that bind to LFA-1 on activated, but not on resting cells have been produced (Keizer et al., 1988; Dransfield & Hogg, 1989). These antibodies appear to recognize epitopes on LFA-1 which become exposed upon cell activation, suggesting that LFA-1 may undergo a conformational change from a low- to high-avidity form. However, precise mechanisms by which cell activation results in the conversion of LFA-1 to a high-avidity form, and the nature of the putative conformational change remain to be elucidated.

Previous studies in this laboratory have demonstrated that monovalent sICAM-1 is unable to bind efficiently to LFA-1⁺ cells unless it is first rendered multivalent by coupling to polystyrene microspheres (Welder et al., 1993). These results illustrate the importance of multivalency in the LFA-1:ICAM-1 interaction. Multivalency may be achieved physiologically by a redistribution of the cell surface molecules involved in cell:cell interaction. ICAM-1 has been previously shown to exhibit a localized distribution on the cell surface (Dougherty et al., 1988; Dustin et al., 1992). In contrast, LFA-1 has been reported to be localized at the site of cell contact on some cells (Kupfer & Singer, 1989) but not on others (Dougherty et al., 1988; Dustin et al., 1992). Distribution may be regulated through the association of LFA-1 with the

cytoskeleton via the cytoskeletal protein talin (Burn et al., 1988; Kupfer et al., 1990). It is of interest that an inhibitor of actin polymerization, cytochalasin B, abrogates cell adhesion mediated by LFA-1 (Marlin & Springer, 1987), suggesting that the putative association with the cytoskeleton may be important in the regulation of LFA-1 function. Whether this association influences the conformation of LFA-1, or its distribution on the cell surface, is currently unknown.

In this chapter, results are presented demonstrating that polystyrene microspheres, coated with sICAM-1 (Welder et al., 1993), specifically bind to activated LFA-1, and that cytochalasin B has no effect on this binding. Furthermore, the cell surface distribution of high-avidity LFA-1, as detected by the binding of the microspheres, is highly localized on some types of LFA-1⁺ cells, whereas LFA-1 in general, as determined by anti-LFA-1 antibody binding, remains evenly distributed on the surfaces of these cells. These results suggest that the distribution of high-avidity LFA-1 may, in some instances, differ from that of low-avidity LFA-1, and that sICAM-1-coated microspheres may be utilized in identifying high-avidity LFA-1.

3.2) RESULTS

3.2.1) Binding of sICAM-1-coated microspheres to high-avidity LFA-1

Uniform polystyrene microspheres were coated with recombinant murine sICAM-1 by physical adsorption as described in Materials and Methods. The binding of sICAM-1-coated microspheres to activated and non-activated splenic T cells was first examined. These cells constitutively express LFA-1, but they do not adhere to sICAM-1 immobilized on microtitre wells without prior activation (Welder et al., 1993). To test the binding of sICAM-1-coated

microspheres to splenic T cells, microspheres and cells were mixed in suspension, incubated for 45 minutes at 37° C, then separated by centrifugation over a solution of percoll (density 1.1176 - 1.1217). Approximately 50% of splenic T cells were recovered using this procedure. The binding of sICAM-1-coated microspheres was strictly dependent on prior cell activation (Fig. 3.1). T cells stimulated for 20 min with 50 ng/ml PMA readily bound sICAM-1-coated microspheres and aggregated into complexes of cells and microspheres (Fig. 3.1a), whereas few microspheres bound to unactivated T cells (Fig. 3.1b). However, not all PMA-stimulated cells participated in aggregate formation (Fig. 3.1a). The reason for this is unclear. A few isolated cells bound small numbers of microspheres whereas others bound none. This observation may reflect different activation states of the cells in this population. The binding of the microspheres was almost completely inhibited in the presence of mAb to LFA-1, demonstrating the specificity of this effect (Fig. 3.1c). The binding of BSA-coated microspheres to PMA-activated T cells was negligible, further confirming the specificity (Fig. 3.1d). These data collectively indicate that sICAM-1-coated microspheres bind exclusively to high-avidity LFA-1 present on activated cells. Surprisingly, pre-treatment of splenic T cells with 20 μ M cytochalasin B had no appreciable effect on the pattern of sICAM-1-coated microsphere binding following stimulation with PMA (Fig. 3.1e). Identical treatment with cytochalasin B almost completely inhibited the adhesion of PMA-stimulated cells to sICAM-1 immobilized on microtitre wells (Fig. 3.2). This observation implies that the cytoskeleton may not be directly involved in the initial interaction between LFA-1 and ICAM-1, or in the conversion of LFA-1 from a low- to high-avidity form.

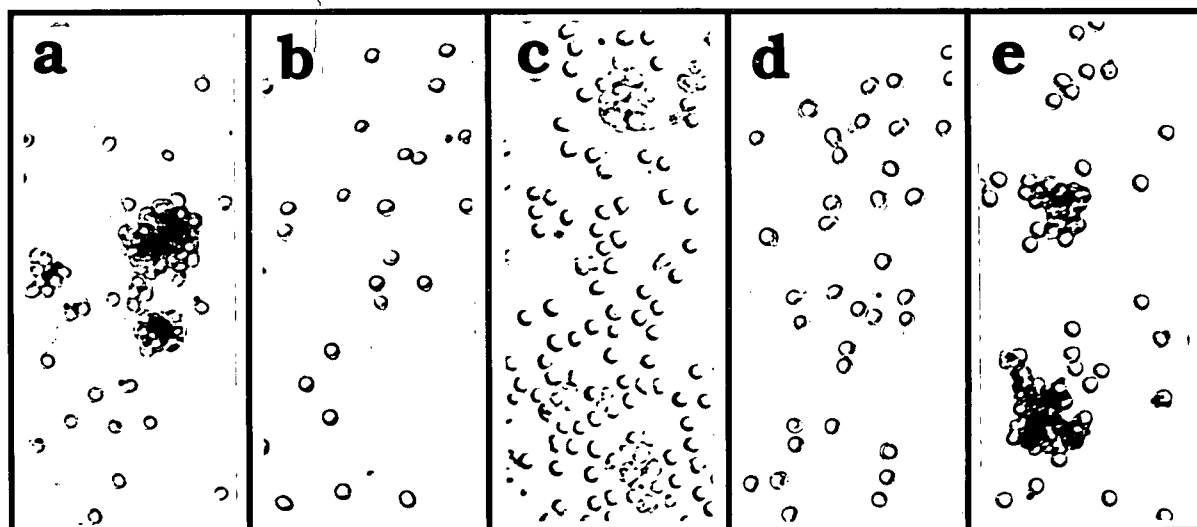


Figure 3.1. Binding of sICAM-1-coated microspheres (2 μ m diameter) to nylon wool-purified splenic T cells. (a) sICAM-1-coated microspheres binding to PMA-stimulated T cells. After separation of unbound microspheres by centrifugation through percoll, approximately 50% of input cells were recovered, of which 30% (exclusive of cells in large aggregates) exhibited detectable numbers of bound microspheres. (b) sICAM-1-coated microspheres binding to unstimulated T cells. (c) sICAM-1-coated microspheres binding to PMA-stimulated cells in the presence of anti-LFA-1 mAb. (d) BSA-coated microspheres binding to PMA-stimulated T cells. (e) sICAM-1-coated microspheres binding to PMA-stimulated cells after incubation with 20 μ M cytochalasin B. Of the recovered cells, approximately 20% bound microspheres.

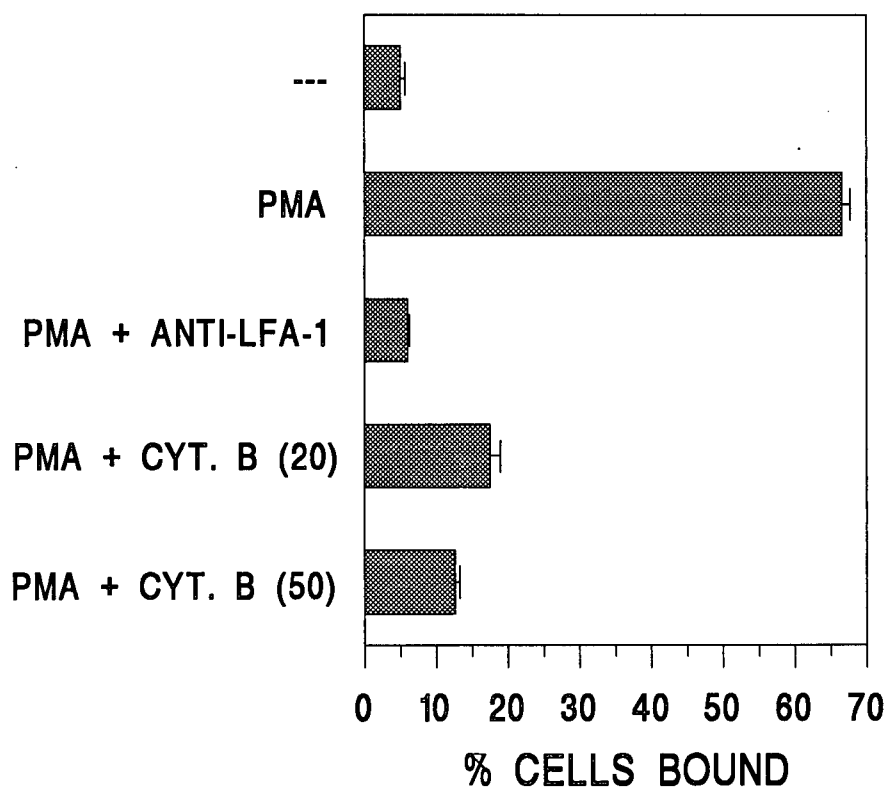


Figure 3.2. Adhesion of nylon wool-purified splenic T cells to sICAM-1 immobilized on polystyrene microtitre wells. The adhesion of PMA-stimulated T cells to immobilized sICAM-1 in the presence and absence of mAb to LFA-1 is depicted. The effect on adhesion of treatment with 20 μ M or 50 μ M cytochalasin B prior to stimulation with PMA is also shown.

3.2.2) Binding of sICAM-1-coated microspheres to other leukocytes

The murine T cell hybridoma line T28 exhibited enhanced binding of sICAM-1-coated microspheres following PMA stimulation (Fig. 3.3a). Close examination of the pattern of binding revealed that the microspheres, and, by extension, high-avidity LFA-1, were non-randomly distributed on the surfaces of some of these cells, often being restricted to a single region (Figs 3.3a and 3.3b, left frames). Data from three independent blind analyses of the microsphere distribution revealed that $46 \pm 8\%$ (mean \pm SEM) of T28 cells exhibited localization of bound microspheres. The observed localization was not due to binding of aggregated microspheres, since the latter were in a monodispersed form when used in these experiments (Fig. 3.3a, inset). To test whether all the LFA-1 molecules on these cells were localized at these regions, we attempted to determine the pattern of binding of microspheres coated with a mAb to LFA-1 (FD441.8). However, interpretation of the results was hampered by the fact that most of the cells binding these microspheres formed large aggregates, whereas relatively few singular cells bound any microspheres (Fig. 3.3b, center frame). Therefore, we performed an alternative procedure in which the cells were resuspended at 4° C in buffer containing azide and EDTA to detach the bound microspheres, before being rapidly fixed in paraformaldehyde. Fixed cells were then stained with anti-LFA-1 mAb followed by biotinylated secondary antibody and avidin-TRITC. This procedure fluorescently stained the entire cell surface uniformly, indicating that the overall distribution of LFA-1 on these cells remained even (Fig. 3.3b, right frame). We have also examined the pattern of fluorescent staining on cells to which bound microspheres were fixed with paraformaldehyde, and have observed that cells exhibiting localization of bound microspheres (Fig. 3.3c, left frame) are often fluorescently

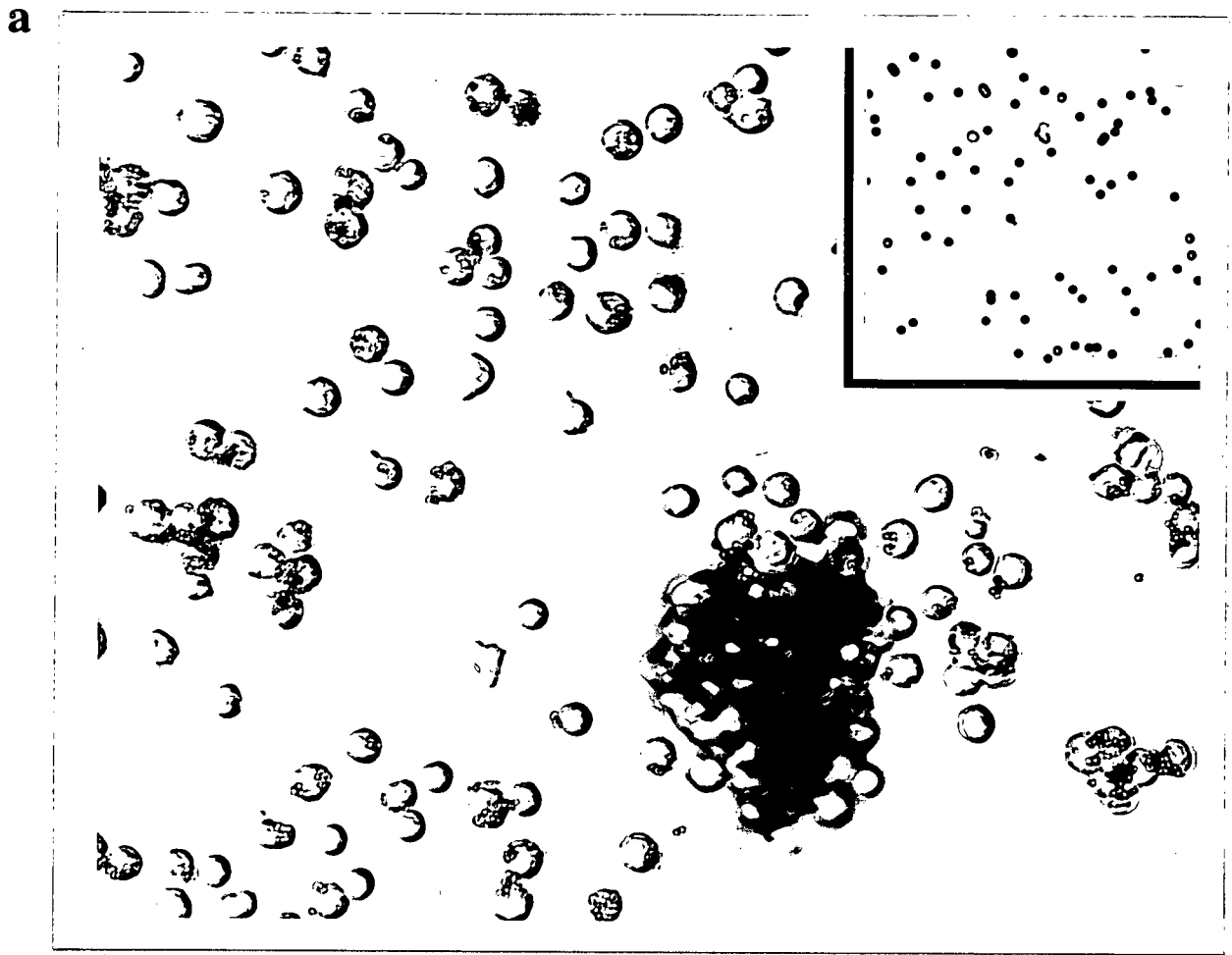
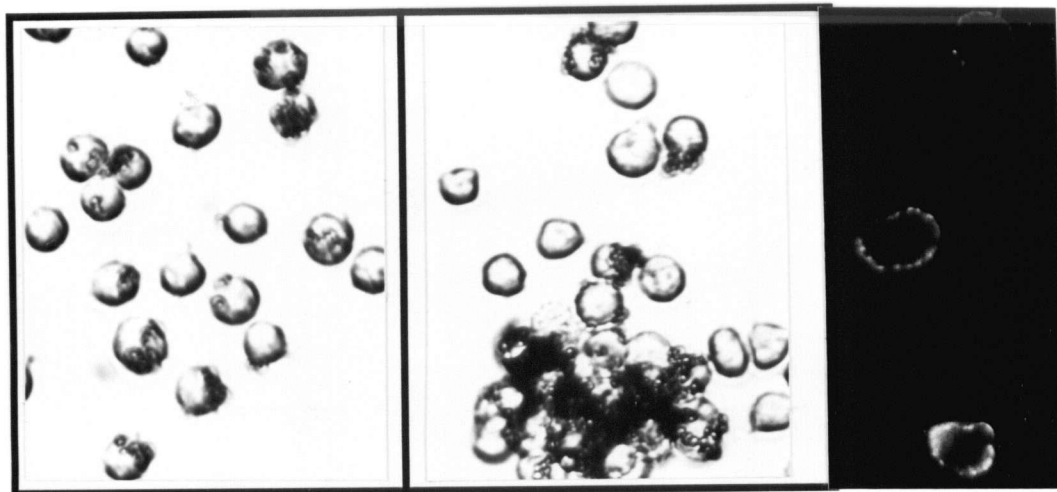
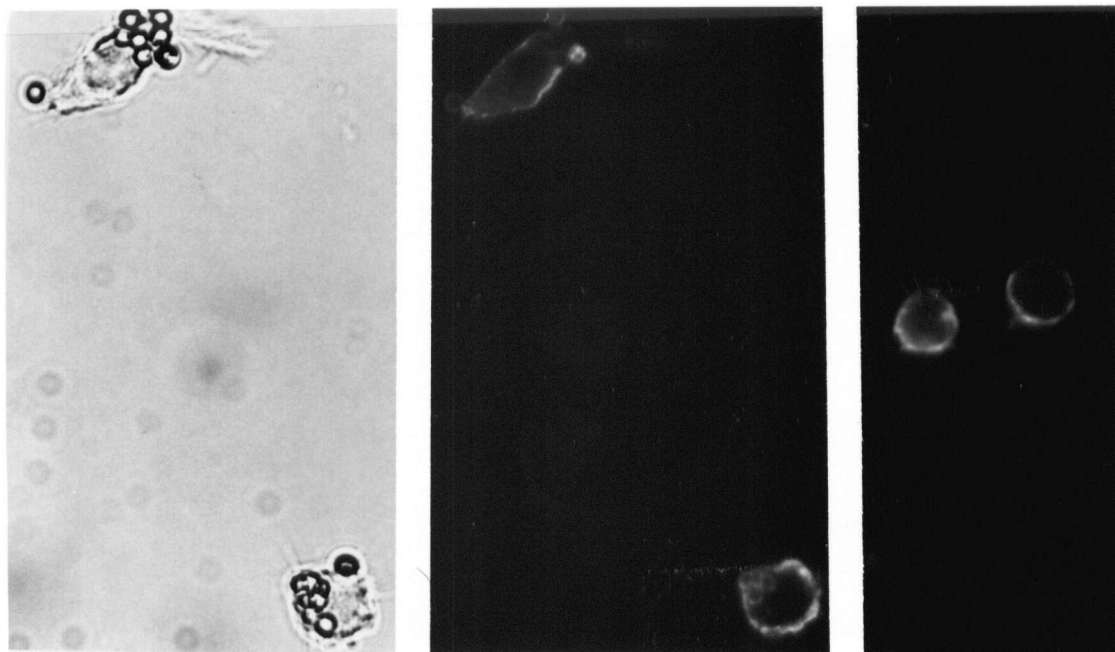


Figure 3.3. Binding of sICAM-1-coated microspheres (2 μm diameter) to T28 cells. (a) Low magnification view of sICAM-1-coated microspheres binding to PMA-stimulated T28 cells. Approximately 60% of the cells contained bound microspheres, excluding cells in large aggregates. Recovery of cells after centrifugation through percoll was 65% of the input number of cells. *Inset.* High-magnification view of the sICAM-1-coated microsphere preparation (diluted 1/25) used in these binding experiments. (b) *Left.* Higher magnification view of sICAM-1-coated microspheres binding to T28 cells. *Center.* Anti-LFA-1 mAb-coated microspheres binding to T28 cells. *Right.* Fluorescent staining of LFA-1 on T28 cells following detachment of sICAM-1-coated microspheres, and fixation in paraformaldehyde. (c) T28 cells exhibiting localization of bound sICAM-1-coated microspheres (left frame) were stained with anti-LFA-1 mAb followed by TRITC-conjugated secondary antibody as described in chapter 2 (center frame). Fluorescent stain is evident along the entire cell periphery. *Right frame.* Anti-LFA-1 fluorescent staining of T28 cells fixed in paraformaldehyde.

b



c



labelled along their entire perimeter (Fig. 3.3c, middle frame). An identical staining pattern was also observed on T28 cells not treated with microspheres (Fig. 3.3c, right frame). These results suggested that high-avidity LFA-1 is localized on some T28 cells whereas LFA-1 in general (mostly low-avidity) is randomly distributed on the cell surface.

In an effort to determine the general applicability of these results, we investigated the binding of microspheres to other types of leukocytes including a B cell line (A20A8), and a monocytic cell line (P388D1). These cell lines express both LFA-1 and ICAM-1 and form large cell aggregates upon PMA treatment, making it difficult to determine the binding of microspheres by the same method used for splenic T cells and T28 cells. Therefore, these leukocytes were immobilized on microtitre wells (see Section 2.9) and the binding of microspheres to the immobilized cells was determined. Both A20A8 and P388D1 cells treated with PMA specifically bound sICAM-1-coated microspheres (Fig. 3.4a,b, left frames), and this binding was inhibited by anti-LFA-1 mAb (center frames). The localization of sICAM-1-coated microspheres seen on T28 cells was not evident to the same degree on these cells although some cells clearly exhibit such binding (Fig. 3.4a,b, left frames). Anti-LFA-1 mAb-coated beads bound to these cells in a qualitatively indistinguishable fashion with some cells exhibiting apparent localized binding and others showing a more random distribution of microspheres (Fig. 3.4a,b, right frames). Therefore, high-avidity LFA-1, as detected by the binding of sICAM-1-coated microspheres, seems to be localized on some leukocytes, but not on others.

3.2.3) Binding of sICAM-1-coated microspheres to LFA-1⁺ L cells

To gain further insight into the possible localized distribution of high-avidity LFA-1, we investigated the distribution of sICAM-1-coated microspheres on murine LFA-1-transfected L

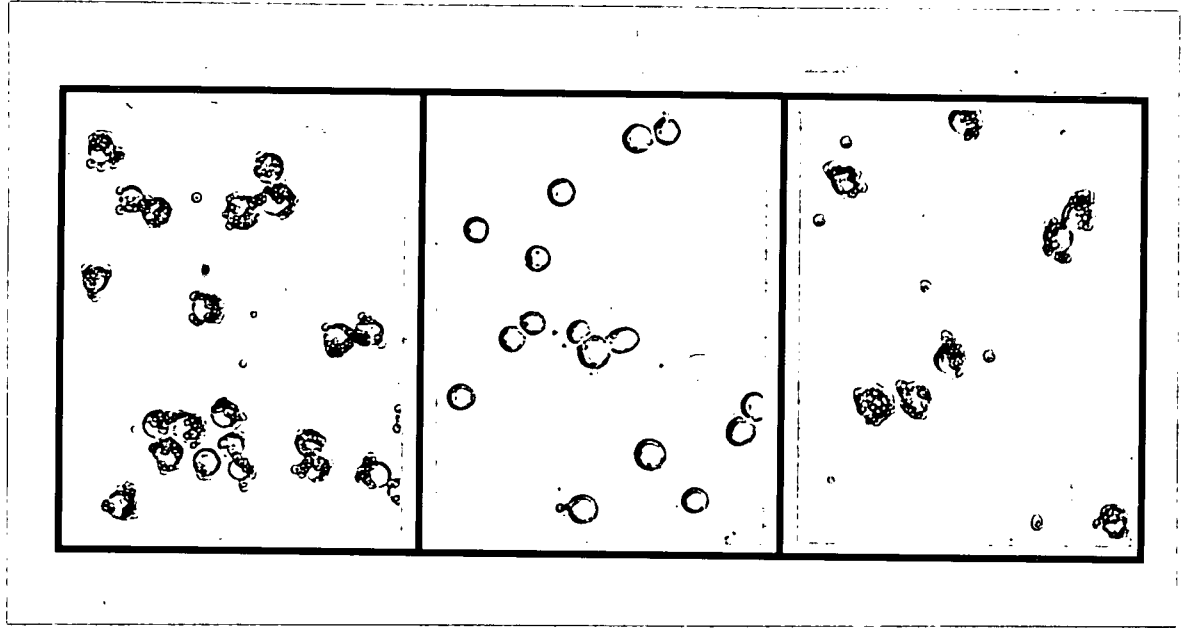
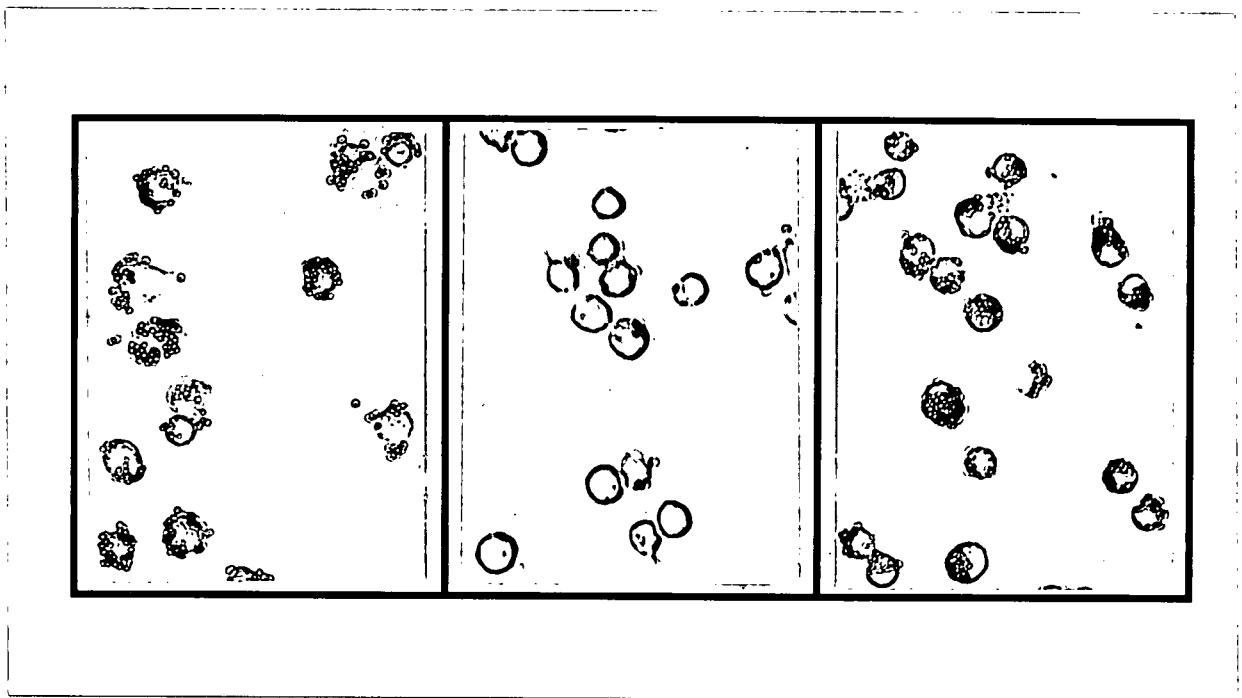
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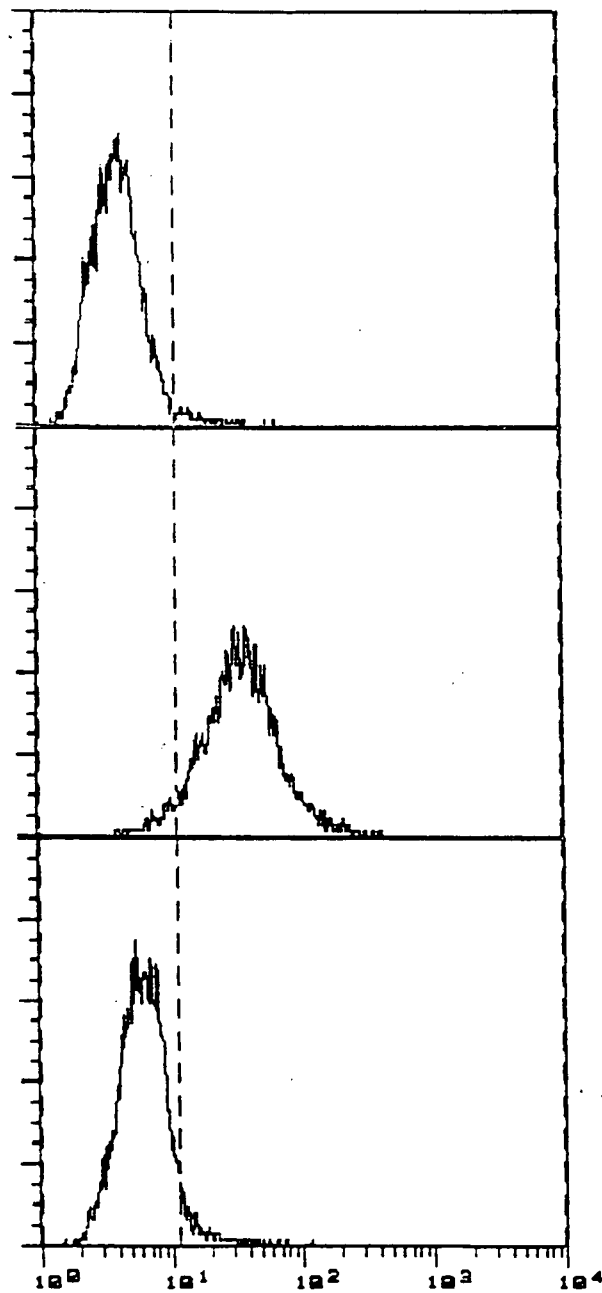
Figure 3.4. Binding of microspheres (2 μm diameter) to (a) A20A8 cells, and (b) P388D1 cells. *Left frames.* sICAM-1-coated microspheres. *Center frames.* Binding of sICAM-1-coated microspheres in the presence of anti-LFA-1 mAb. *Right frames.* Anti-LFA-1 mAb-coated microspheres.

cells. The cDNA encoding murine CD11a and CD18 were subcloned into the mammalian expression vector pRC6, and co-transfected into murine L cell fibroblasts. LFA-1⁺ L cell lines were established by two rounds of FACS sorting. Analysis of the resulting bulk population of LFA-1⁺ L cells revealed that approximately 95% of the cells expressed LFA-1 on their surfaces (Fig. 3.5a). To determine the state of LFA-1 on these cells, an adhesion assay, measuring their ability to bind to sICAM-1 immobilized on microtitre wells, was performed. As a control, T28 cells were also tested. T28 cells exhibited a low basal level of binding to immobilized sICAM-1 that was markedly upregulated by prior treatment with PMA (Fig. 3.5b). In contrast, LFA-1⁺ L cells, with or without prior treatment with PMA, bound equally well to sICAM-1-coated wells, and this adhesion was inhibited by mAb to CD11a. Since PMA treatment did not enhance the adhesiveness of these cells, we conclude that at least some of the LFA-1 molecules on these cells exist in a constitutively avid state.

As expected from the cell adhesion assay, LFA-1⁺ L cells grown in monolayers bound sICAM-1-coated microspheres in the absence of activation with PMA (Fig. 3.6a). This binding was inhibited with mAb to LFA-1 (not shown), and BSA-coated microspheres failed to adhere to these cells (Fig. 3.6b). No sICAM-1-coated microspheres bound to untransfected L cells (Fig. 3.6c). Strikingly, the distribution of microspheres on the surfaces of many LFA-1⁺ L cells is not uniform (Fig. 3.6a). This is most easily observed with elongated L cells on which the microspheres often appear at the tips. In three independent tests performed blind, counts of approximately 100 cells per experiment revealed that $42 \pm 2\%$ (mean \pm SEM) of L cells exhibited localized binding of sICAM-1-coated microspheres. This effect is not due to capping

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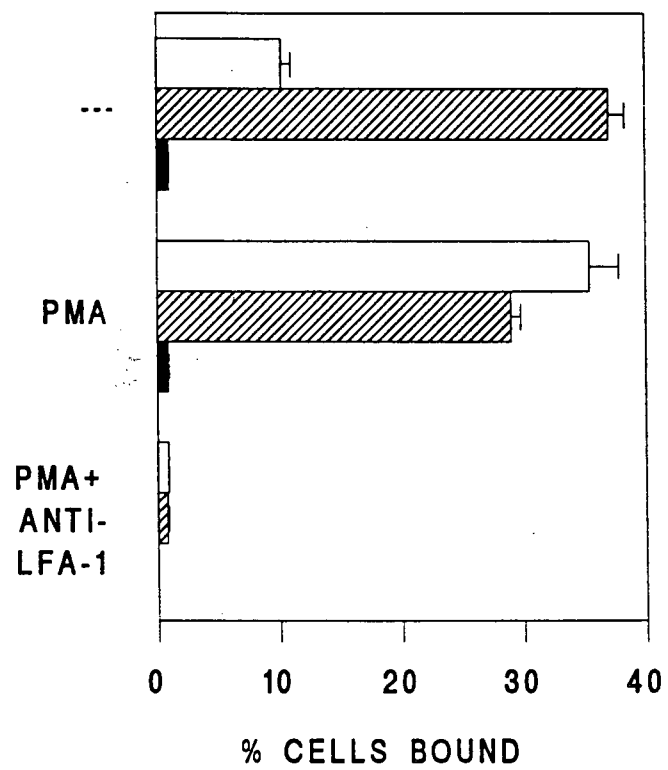
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Figure 3.5. Characterization of LFA-1-transfected L cells. (a) FACS analysis of LFA-1-transfected L cells. Transfected L cells were stained with either rat anti-ICAM-1 (top) or rat anti-LFA-1 (middle) mAb followed by FITC-conjugated anti-rat Ig. Anti-LFA-1 staining of untransfected L cells is also shown (bottom). (b) Adhesion of LFA-1-transfected L cells to sICAM-1 immobilized on polystyrene microtitre wells. The binding to immobilized sICAM-1 of the bulk population of LFA-1⁺ L cells obtained after two rounds of FACS sorting is shown. Clear bars, T28 cells; hatched bars, LFA-1⁺ L cells; solid bars, untransfected L cells. Each data point represents the mean of duplicate experiments.

b



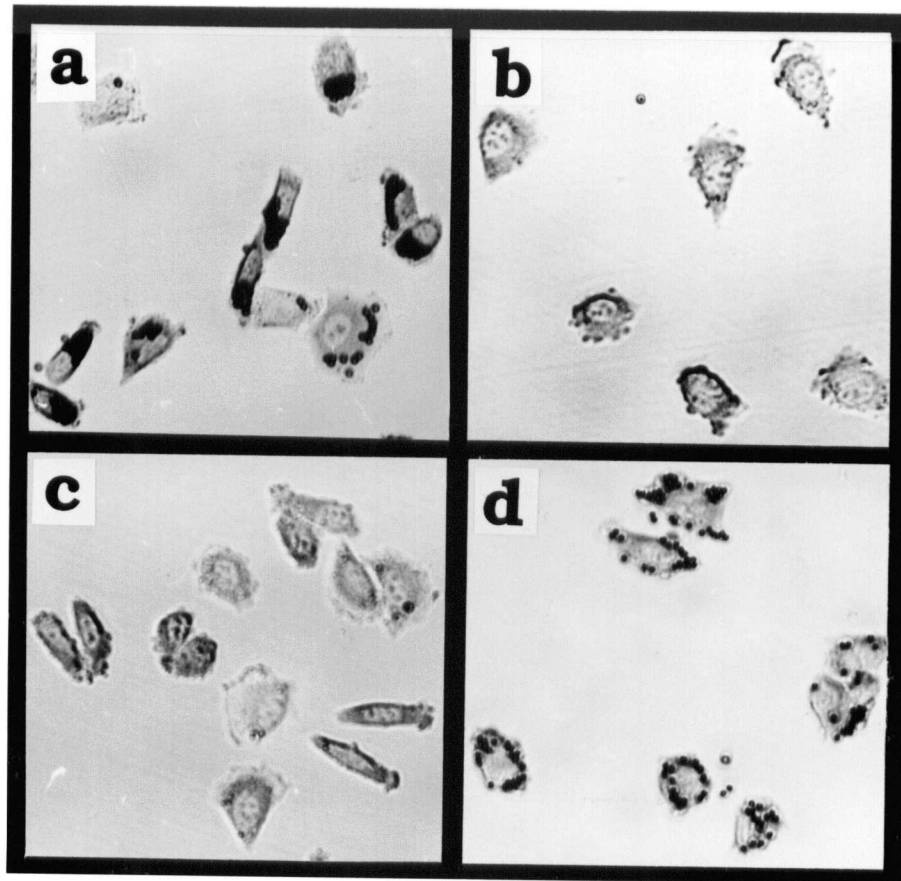


Figure 3.6. Binding of sICAM-1-coated microspheres (2 μm diameter) to LFA-1-transfected L cells. (a) Binding of sICAM-1-coated microspheres to LFA-1⁺ L cells, (b) binding of BSA-coated microspheres to LFA-1⁺ L cells, (c) binding of sICAM-1-coated microspheres to untransfected L cells, (d) binding of anti-LFA-1 antibody-coated microspheres to LFA-1⁺ L cells.

of the LFA-1 molecules as a result of the microspheres binding because microspheres coated with an antibody to LFA-1 labelled the L cell surface more uniformly (Fig. 3.6d). In this case, only $10 \pm 3\%$ of the L cells exhibited localized binding. The different labelling patterns of LFA-1⁺ L cells obtained with sICAM-1-coated microspheres versus those coated with anti-LFA-1 suggested that the distribution of high-avidity LFA-1 may be different from that of the low-avidity form, as was apparent in T28 cells. To test this possibility, we labelled monolayers of LFA-1⁺ L cells with sICAM-1-coated microspheres and subsequently stained them with anti-LFA-1 mAb, followed by secondary antibody conjugated to TRITC. Cells exhibiting a restricted distribution of microspheres (Fig. 3.7a) were often labelled along their entire perimeter by fluorescent antibody (Fig. 3.7b), indicating that LFA-1 molecules in general, remain uniformly distributed on the cell surface. Fluorescent label was also clearly visualized at the outer perimeter of LFA-1⁺ L cells not labelled with sICAM-1-coated microspheres (Fig. 3.7c). No such labelling was seen when the primary anti-LFA-1 mAb was omitted, demonstrating the specific nature of the labelling (not shown). The fluorescent staining of LFA-1⁺ L cells with anti-LFA-1 antibody often had a patchy appearance on these cells, implying that the LFA-1 molecules may be clustered on the cell surface. To determine whether this clustering was the result of antibody-mediated cross-linking of LFA-1 molecules on the cell surface, we examined the pattern of fluorescence on cells that had been fixed in paraformaldehyde prior to staining. The patchy appearance of the fluorescent label was observed on fixed cells as well (Fig. 3.7d).

3.3) DISCUSSION

The main conclusions from the data presented in this chapter are (1) sICAM-1-coated microspheres specifically bind to high-avidity, but not to low-avidity, LFA-1, (2) the

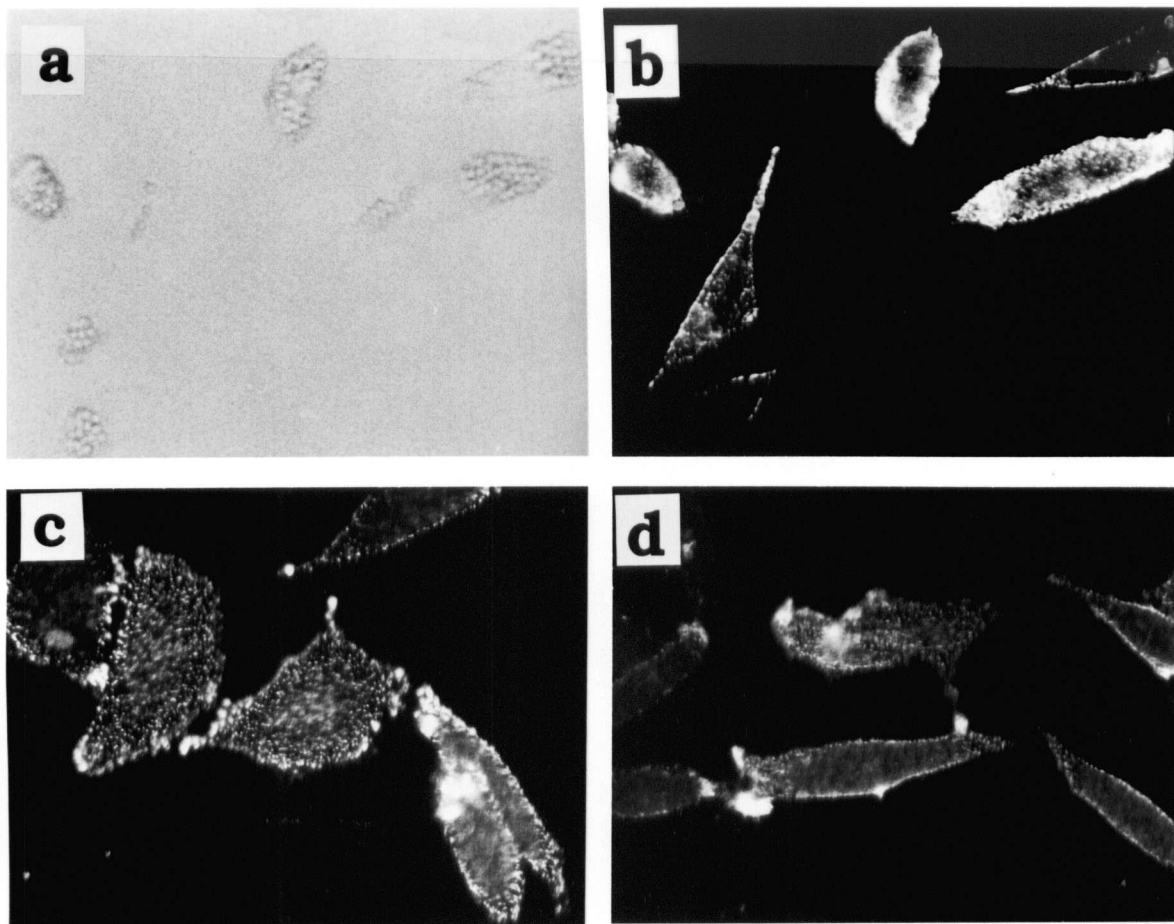


Figure 3.7. Fluorescent staining of LFA-1 on LFA-1-transfected L cells. (a) Bright-field image of sICAM-1-coated microspheres adhering to LFA-1⁺ L cells, (b) fluorescent staining of cells in (a) using rat anti-LFA-1 mAb followed by TRITC-conjugated anti-rat Ig, (c) anti-LFA-1 fluorescent staining of LFA-1⁺ L cells with no microspheres bound, (d) anti-LFA-1 fluorescent staining of LFA-1⁺ L cells fixed in paraformaldehyde. *beads = 2 μm diameter*

cytoskeletal inhibitor cytochalasin B does not affect the binding of sICAM-1-coated microspheres to LFA-1, (3) high-avidity LFA-1, as determined by the binding of sICAM-1-coated microspheres, is highly localized on some cells, whereas LFA-1 in general (high- and low-avidity forms) is evenly distributed on the cell surface, and (4) LFA-1-transfected L cells constitutively express high-avidity LFA-1 which is highly localized. Although most leukocytes constitutively express LFA-1, they do not adhere to purified ICAM-1 immobilized on solid surfaces unless they are pre-activated. Thus, cell activation seems to induce a qualitative change in LFA-1, converting low-avidity LFA-1 on resting cells to a high-avidity form. The exact mechanisms by which LFA-1 avidity for ICAM-1 is regulated by cell activation are unknown. However, antibodies recognizing LFA-1 on activated, but not on resting, cells have been produced (Keizer et al., 1988; Dransfield & Hogg, 1989) suggesting that LFA-1 may undergo a conformational change upon cell activation. The microspheres coated with recombinant sICAM-1 used in this study also bind only to activated cells as freshly isolated splenic T cells did not bind the microspheres unless they were first treated with PMA. Furthermore, the binding of sICAM-1-coated microspheres to these cells was efficiently inhibited by anti-LFA-1 antibody. Similarly, the murine T cell hybridoma T28 bound the microspheres upon activation whereas non-activated T28 cells bound significantly fewer microspheres. These data closely correlate with cell adhesion assay results in which the adhesion of these cells to sICAM-1 immobilized on microculture wells was quantitated (Welder et al., 1993). The results also indicate that sICAM-1-coated microspheres represent a useful tool in identifying high-avidity LFA-1. Furthermore, the microspheres add a functional dimension to the recognition of high-avidity LFA-1 since the latter is identified on the basis of its ability to bind ICAM-1 rather than solely on the presence of a particular activation epitope.

One important difference between the conventional cell adhesion assays, in which cell binding to ICAM-1 immobilized on microtitre wells is measured, and the present method utilizing sICAM-1-coated microspheres, is the sensitivity to cytochalasin B treatment. As previously reported (Marlin & Springer, 1987), cytochalasin B almost completely inhibited the adhesion of PMA-activated T cells to immobilized ICAM-1 in a conventional cell adhesion assay. In contrast, cytochalasin B did not affect the binding of sICAM-1-coated microspheres to identically treated cells. This result may be explained by the differences in the two protocols. In the conventional plate adhesion assays, cells are subjected to a washing process to remove non-adherent cells, which is likely to create some shear force. On the other hand, in the method using sICAM-1-coated microspheres, unbound microspheres and cells are separated by sedimentation through a percoll solution, which is unlikely to generate the same degree of shear force. The fact that sICAM-1-coated microspheres bind to cytochalasin B-treated splenic T cells indicates that the molecular interaction between high-avidity LFA-1 and ICAM-1 is not affected by cytochalasin B and that it does not inhibit the conversion of low-avidity LFA-1 into the high-avidity form. Therefore, the role of the cytoskeleton in LFA-1:ICAM-1-mediated cell adhesion may be to stabilize adhering cells to make them resistant to shear force. Similar experiments using T28 cells and L cells did not give meaningful results, because T28 cells are resistant to cytochalasin B, whereas L cells were lost during the washing procedure to remove unbound microspheres (data not shown).

In addition to determining whether given cells express high-avidity LFA-1, sICAM-1-coated microspheres enable us to visualize the distribution of high-avidity LFA-1 on the cell surface. The binding of the microspheres to T28 cells often appeared highly localized, whereas staining of LFA-1 in general showed an even distribution, suggesting that low- and high-avidity

LFA-1 may be unequally distributed on these cells. However, these results were not reproduced on A20A8 or P388D1 cells. This apparent discrepancy may be explained by considering that both cell types express ICAM-1 on their surfaces, and that upon activation, substantial aggregation of these cells is observed. This aggregation may be reflective of a redistribution of high-avidity LFA-1 to multiple sites on the cell surface following PMA stimulation. Activated T28 cells, in contrast, express little ICAM-1 on their surfaces, and aggregate to only a limited extent. Therefore, localization may be more readily observed on these cells. Alternatively, the observed localization of sICAM-1-coated microspheres on T28 cells may be a result of capping of high-avidity LFA-1. We consider this to be improbable since the same degree of localized microsphere binding was not observed with A20A8 and P388D1 cells.

To investigate the issue of distribution further, we generated LFA-1⁺ fibroblast L cells by transfecting cDNA encoding murine CD11a and CD18. Because L cells have a typical fibroblastic morphology, the distribution of the microspheres bound to the cell surface is more easily visualized. The binding of the microspheres to LFA-1⁺ L cells is often highly localized, although not all cells show this pattern of binding. The results with LFA-1-transfected L cells are consistent with those with T28 cells, confirming that the binding of sICAM-1-coated microspheres is not random but often highly localized. It is of interest that no such localization of LFA-1 on these cells is observed by fluorescence staining with anti-CD11a antibody. Moreover, fluorescent staining of LFA-1⁺ L cells that have the microspheres already bound to the cell surface has shown that LFA-1 is present in areas where no microspheres are bound. Because the anti-CD11a antibody used for the fluorescence staining cannot differentiate between low- and high-avidity forms of LFA-1, these results indicate that only high-avidity LFA-1 often

localizes at certain spots on the cell surface whereas low-avidity LFA-1 is more evenly distributed.

The localized binding of the microspheres is likely not due to capping of LFA-1 as induced by the binding of the microspheres because anti-LFA-1 antibody-coated microspheres, which should also bind and cross-link LFA-1, did not show similar localized binding, but rather a random distribution. These results indicate that the binding of sICAM-1-coated microspheres does not induce capping of LFA-1 on transfected L cells grown as adherent cells, and that the observed localization of sICAM-1-coated microspheres is likely due to true localization of high-avidity LFA-1 on these cells.

The difference in distribution of high- versus low-avidity LFA-1 on the cell surface may explain the inconsistency in the literature regarding the distribution of LFA-1. Kupfer and Singer (1989) reported that LFA-1 is localized at the site of cell contact using helper T cell lines, whereas Dustin et al. observed an even distribution of LFA-1 on different human lymphoid cell lines (Dustin et al., 1992). Using various murine lymphoid cell lines treated with PMA and forming cell aggregates, we have not been able to observe any localization of LFA-1 as determined by fluorescent staining with anti-CD11a antibody. However, if the majority of LFA-1 molecules on a given cell are in a high-avidity form, they are likely to be localized, and such localization should be detected by fluorescent staining using anti-LFA-1 antibodies that are not specific for high-avidity LFA-1. On the other hand, if only a small subpopulation of the LFA-1 molecules are in a high-avidity form, localized distribution of LFA-1 would not be detected using these antibodies.

It is of interest that LFA-1-transfected L cells adhere to sICAM-1-coated microtitre wells without prior activation, and that PMA does not enhance the cell adhesion. Similarly, sICAM-

sICAM-1-coated microspheres bind to the transfected L cells without PMA treatment. Therefore, the transfected L cells seem to constitutively express high-avidity LFA-1. This is consistent with the previous report on human LFA-1-transfected COS cells, which also constitutively express high-avidity LFA-1 (Larson et al., 1990). It should be noted, however, that not all the LFA-1 molecules on the transfected L cells seem to be in a high-avidity form. As discussed above, only high-avidity LFA-1 on L cells seems to have a localized distribution on the cell surface, but the same cells also express LFA-1 which does not bind sICAM-1-coated microspheres and is more evenly distributed. The mechanisms responsible for maintaining some LFA-1 molecules on L cells in a constitutively avid state are not yet known.

CHAPTER 4

The role of LFA-1 cytoplasmic domains in binding to ICAM-1 and in post-receptor cell spreading

The results presented in this chapter appear in the following publication:

AM Pyszniak, C Carpenito and F Takei (1997) The Role of LFA-1 (CD11a/CD18) Cytoplasmic Domains in Binding to Intercellular Adhesion Molecule -1 (CD54) and in Post-receptor Cell Spreading. *Exp Cell Res* 233:78.

4.1) INTRODUCTION

The data described in chapter 3 of this thesis suggested that the cell surface distribution of LFA-1 may constitute one means by which adhesion to ICAM-1 may be regulated. A non-random distribution of high-avidity LFA-1 at the cell surface could be achieved through the interaction of its cytoplasmic domains with intracellular elements. In fact, several studies have implicated the cytoplasmic domains of integrins as being important in the regulation of adhesiveness. Truncation of the β_3 subunit cytoplasmic domain abrogates cell spreading mediated by $\alpha_{IIb}\beta_3$ without affecting its binding affinity for fibrinogen (Ylanne et al., 1993), whereas truncation of the α_{IIb} cytoplasmic domain increases the binding affinity for fibrinogen over that of the wild type molecule (O'Toole et al., 1991). Experiments demonstrating that chimeric integrins consisting of heterologous integrin cytoplasmic domains can function differently from their wild type counterparts further underscore the importance of cytoplasmic domains in integrin regulation (O'Toole et al., 1991; O'Toole et al., 1994). In the case of the leukocyte integrins, truncation of the cytoplasmic domains of Mac-1 results in an increased capacity of transfected COS cells to bind iC3b ligand (Rabb et al., 1993), whereas partial truncation of the β_2 cytoplasmic domain of LFA-1 completely abolishes binding to ICAM-1 (Hibbs et al., 1991b). In addition, it has recently been reported that the β_2 cytoplasmic domain may affect certain post-receptor events such as the localization of the receptor to focal contacts, and cell spreading (Peter & O'Toole, 1995). In contrast to the data available regarding the β_2 cytoplasmic domain, relatively little is known about the contribution of the α_L cytoplasmic domain to LFA-1 regulation.

This chapter examines the role of LFA-1 cytoplasmic domains in adhesion, post-occupancy cell spreading, and cell surface distribution. LFA-1 lacking either or both the α_L and β_2 cytoplasmic domains were transfected into the murine fibroblasts TNR-2. Our choice of TNR-2 cells stemmed from the inherent problems associated with using either the parental NIH3T3 cells or L cells (see section 4.3). TNR-2 transfectants were tested for their ability to adhere and spread out following contact with ICAM-1 immobilized in microwells. In addition, the binding of GPI-anchored and soluble forms of LFA-1 to ICAM-1 was investigated. The results presented in this chapter demonstrate the importance of the LFA-1 α - and β - chain cytoplasmic domains in regulating adhesion to ICAM-1, as well as in the induction of cell spreading following ICAM-1 ligation.

4.2) RESULTS

4.2.1) Expression of truncated and GPI-anchored LFA-1 on transfected TNR-2 cells

As an initial step in examining the roles of the cytoplasmic domains of LFA-1 in adhesion to ICAM-1 and in post-binding events, we generated various mutant forms of α_L and β_2 (Fig. 4.1a). Recombinant cDNAs lacking the entire cytoplasmic domains of both LFA-1 chains were generated by PCR, as were chimeric cDNAs encoding the extracellular domains of α_L and β_2 fused with the GPI-anchoring signal derived from the heat stable antigen. The resulting molecules, along with the wild type chains, were transfected into murine TNR-2 fibroblasts in various combinations (Fig. 4.1b), and their expression was evaluated by flow cytometry.

a

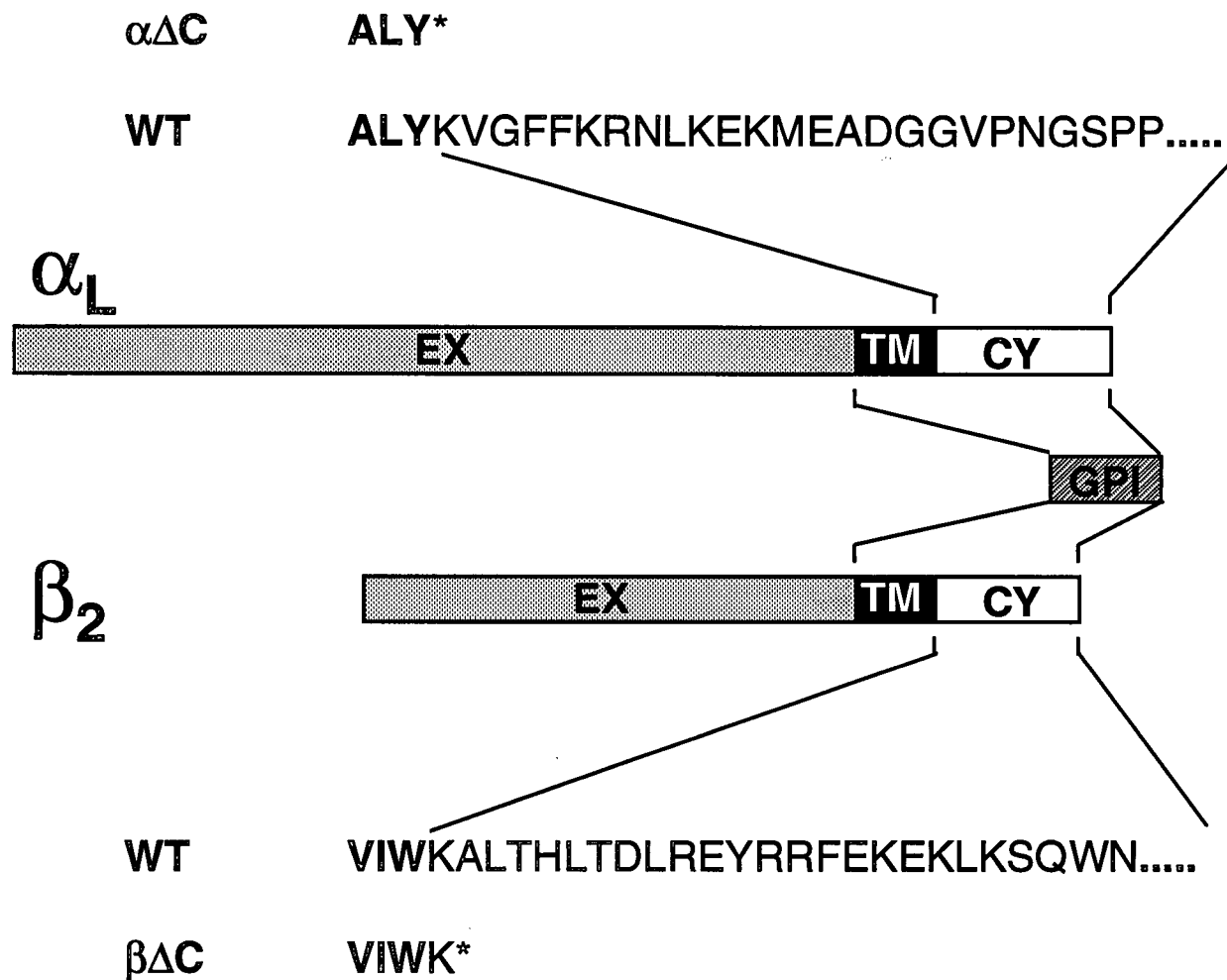
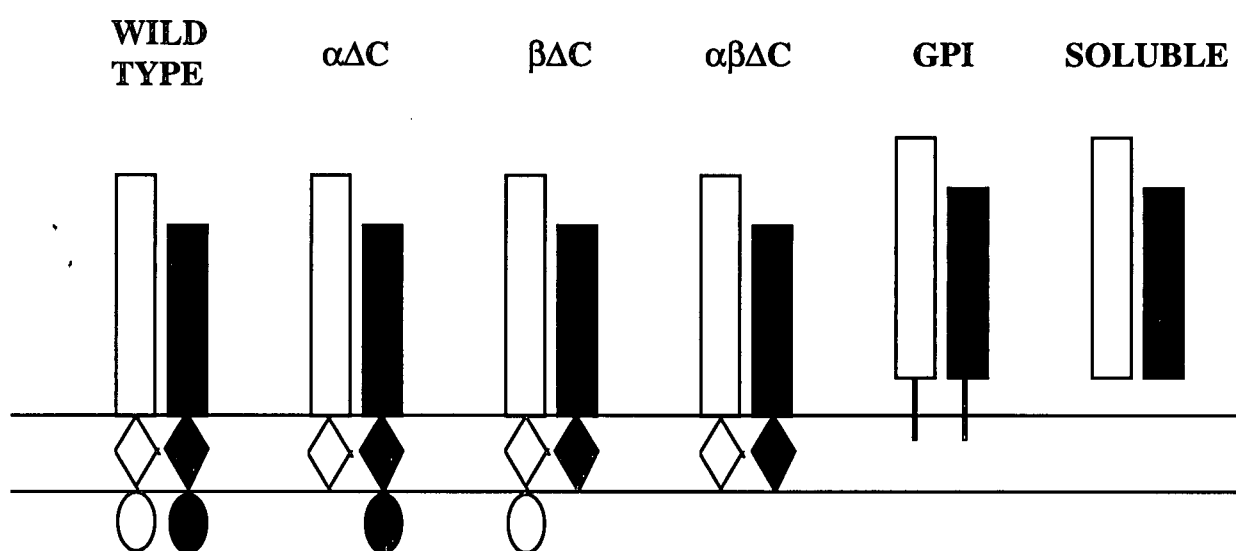


Figure 4.1. Schematic diagrams of LFA-1 cytoplasmic domain truncations and transfected cell lines used in this study. (a) The extracellular (EX), transmembrane (TM) and cytoplasmic (CY) domains of the wild type LFA-1 α_L and β_2 subunits are shown, along with their respective cytoplasmic truncation mutants. Truncated α_L and β_2 , as well as GPI-anchored forms of both subunits, were generated by PCR reactions and were verified by nucleotide sequencing. (b) Various combinations of wild type and mutant LFA-1 subunits were co-transfected into TNR-2 fibroblasts, and cell lines expressing LFA-1 were established by FACS sorting. * = stop codon.

b



The expression levels of the different forms of LFA-1 expressed on transfected TNR-2 cells were similar to each other (Fig. 4.2a), although the level of wild type LFA-1 was generally higher than that of the truncated forms. The expression of LFA-1 containing a truncated α cytoplasmic domain ($\alpha\Delta C$) was variable and decreased as the cells were cultured for a prolonged time. For the experiments described below, transfectants expressing comparable levels of LFA-1 were utilized. To confirm that LFA-1-GPI expressed on the transfected cells was indeed GPI-anchored, we treated the cells with PI-PLC. Approximately 90% of the LFA-1 molecules on these cells were sensitive to PI-PLC treatment, whereas wild type LFA-1 was resistant (Fig. 4.2b).

To further verify that the appropriate LFA-1 constructs were being expressed by the various transfected lines, we immunoprecipitated LFA-1 using anti- α_L or anti- β_2 antibodies, and separated the co-precipitated proteins by SDS-PAGE. Based on the number of amino acids comprising the truncated α and β chains, the expected molecular masses of the proteins are approximately 125 kD and 80 kD respectively. The results indicate that the various cell lines generated in this work express LFA-1 chains of the appropriate size (Fig. 4.3). No proteins were recovered from untransfected TNR-2 cells.

4.2.2) Adhesion of LFA-1 transfectants to ICAM-1

The effect of truncating LFA-1 cytoplasmic domains was first assessed in a cell adhesion assay measuring the ability of TNR-2 transfectants to adhere to ICAM-1 immobilized in microwells. Previous studies have demonstrated that this assay is suitable for detecting the upregulation of LFA-1 avidity, because resting leukocytes expressing LFA-1 adhere only marginally to ICAM-1 without prior activation (Diamond & Springer, 1994). This observation

a

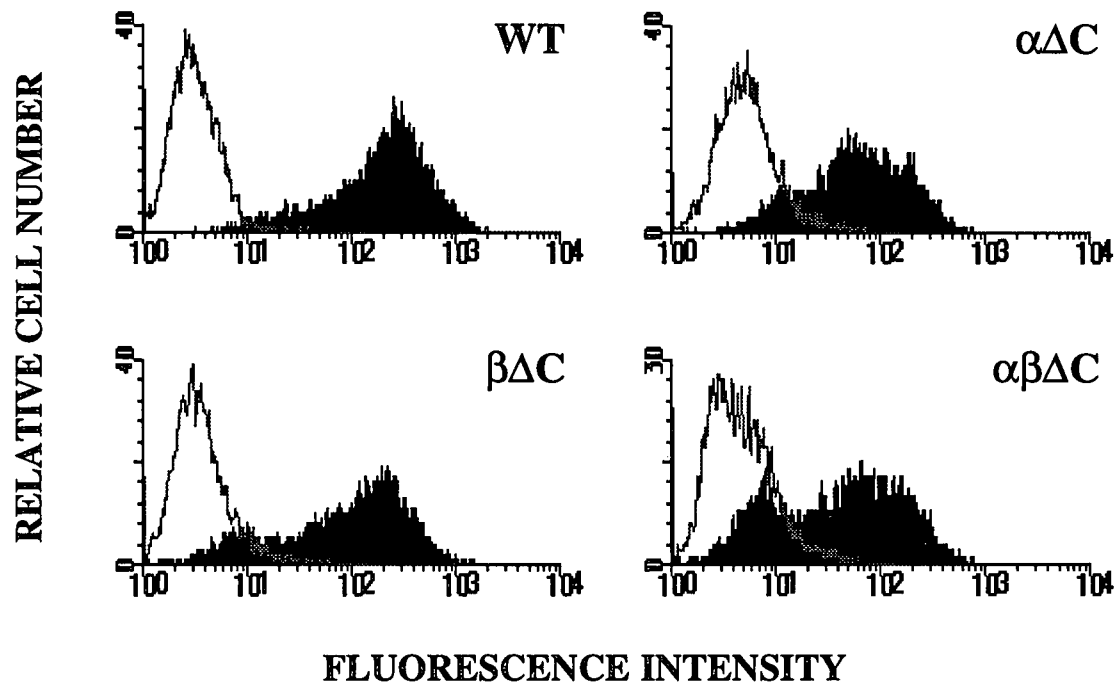
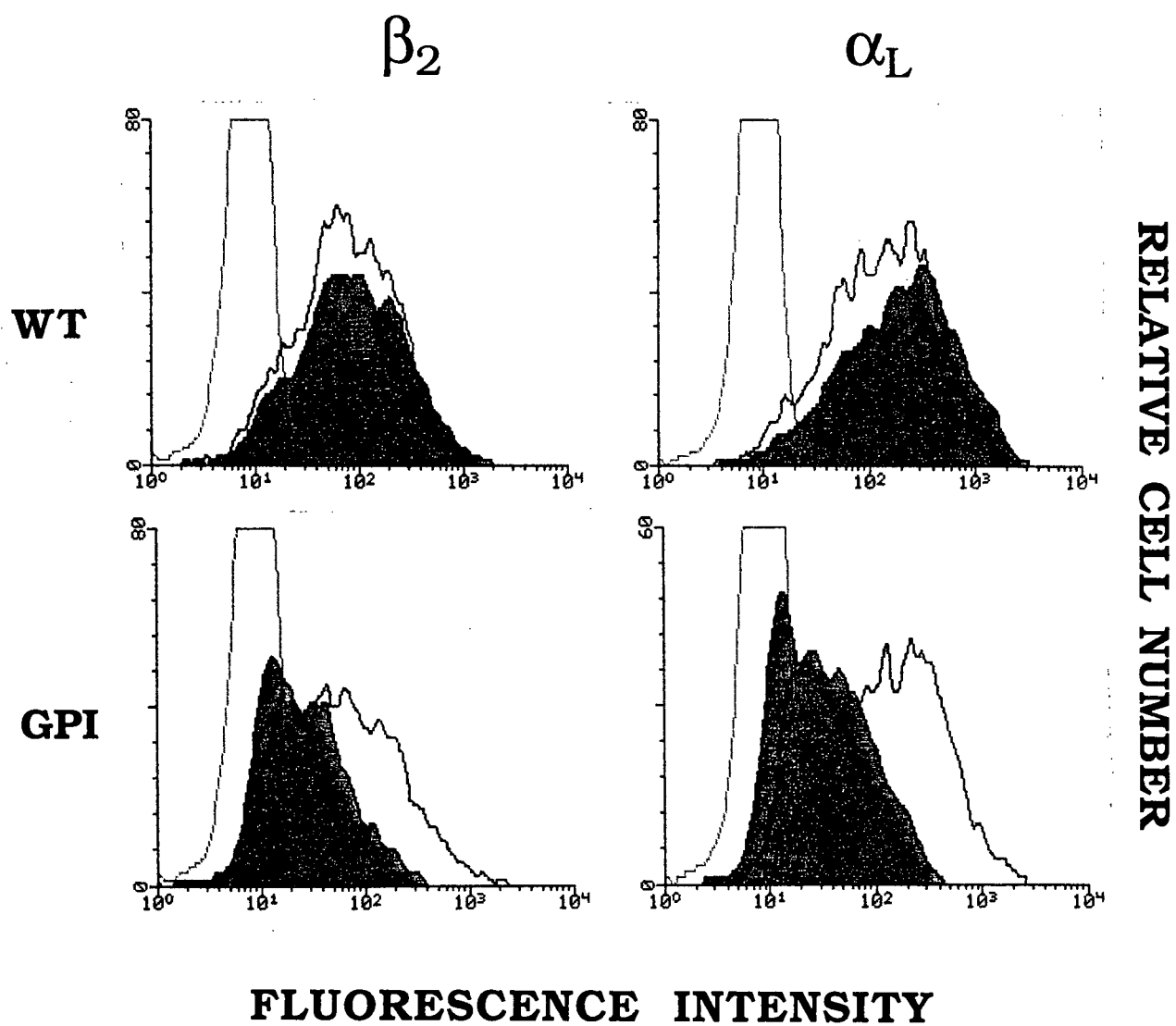


Figure 4.2. Flow cytometric analysis of LFA-1-transfected TNR-2 cells. (a) The transfected TNR-2 cell lines were stained with anti- α_L (shaded histograms) or, as a control, anti-ICAM-1 (open histograms) mAb, followed by FITC-conjugated secondary antibody. Parental untransfected TNR-2 cells were also analyzed as controls (data not shown). Representative FACS profiles are shown. (b) LFA-1-GPI-transfected TNR-2 cells were treated with PI-PLC, fluorescently stained, and analyzed by flow cytometry. TNR-2 cells transfected with the wild type LFA-1 (WT) were also analyzed. The shaded histograms represent fluorescent staining after treatment with PI-PLC whereas open histograms represent staining of untreated cells. The peaks closest to the vertical axis show the staining of control TNR-2 cells expressing no LFA-1.

b



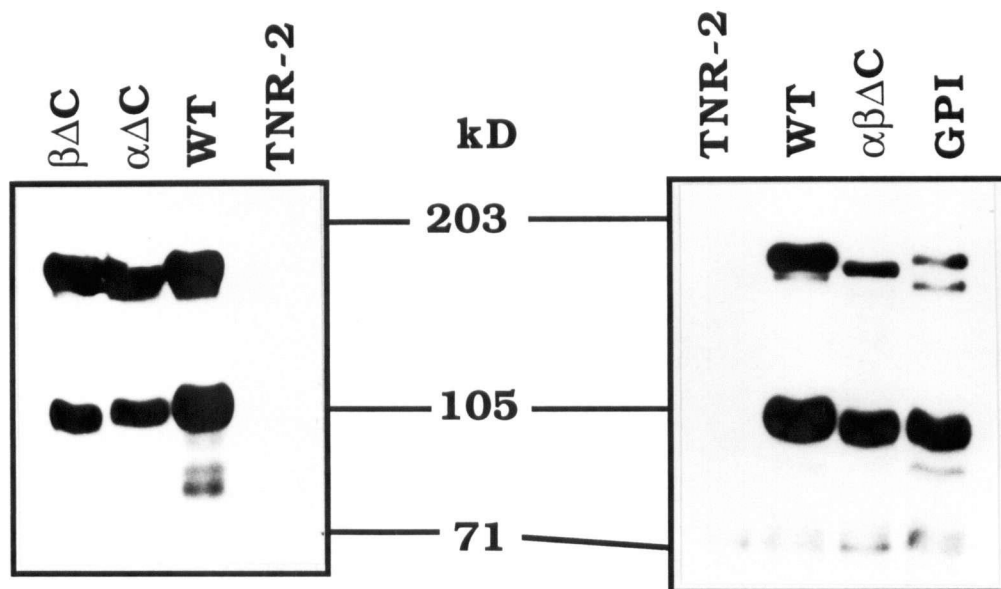


Figure 4.3. Immunoprecipitation of LFA-1 from TNR-2 transfectants. LFA-1 was immunoprecipitated from surface- biotinylated LFA-1-transfected TNR-2 cells using anti- α_L and anti- β_2 , separated by SDS-PAGE (7.5%) and detected by Western blotting using peroxidase-conjugated streptavidin. Immunoprecipitates from TNR-2 cells transfected with all forms of LFA-1, as well as from the parental untransfected TNR-2 cells, are shown. The molecular mass standards (in kD) are indicated.

is corroborated by TNR-2 cells expressing wild type LFA-1. These cells bound weakly to ICAM-1, and the adhesion was significantly enhanced by pre-treatment with phobol ester (Fig. 4.4a). Cells expressing doubly truncated LFA-1 ($\alpha\beta\Delta C$), LFA-1-GPI, and $\beta\Delta C$ bound to ICAM-1 to a higher basal level than did the wild type recombinant cells, and PMA treatment had no substantial effect on this adhesion. In contrast, $\alpha\Delta C$ showed very low basal adhesion that was weakly upregulated by PMA treatment. However, the adhesion of $\alpha\Delta C$ and $\alpha\beta\Delta C$ to ICAM-1 was greatly enhanced in the presence of Mn^{2+} (Fig. 4.4b). Interestingly, manganese only marginally enhanced the adhesion of wild type LFA-1 and $\beta\Delta C$. The basal levels of adhesion demonstrated by unstimulated cells expressing the different forms of LFA-1 (Fig. 4.4a) did not correlate with the degree of LFA-1 expression on these cells. None of the cell lines adhered to control wells coated with ovalbumin, and parental, untransfected TNR-2 cells did not adhere to ICAM-1 (data not shown) indicating that the cell adhesion in this assay is dependent on the expression of functional LFA-1 on the cell surface.

4.2.3) Adhesion of ICAM-1⁺ cells to soluble LFA-1

We extended these binding studies by asking whether the soluble form of LFA-1 also binds to ICAM-1. To generate soluble LFA-1, TNR-2 cells expressing LFA-1-GPI were treated with PI-PLC, and the resultant soluble LFA-1 was immunoprecipitated. Both α_L and β_2 were immunoprecipitated with either anti- α_L or anti- β_2 mAb from the supernatant of PI-PLC-treated TNR-2 cells expressing LFA-1-GPI (Fig. 4.5), indicating that soluble α_L and β_2 associate with each other to form heterodimers. No LFA-1 was immunoprecipitated from the supernatant of PI-PLC treated TNR-2 cells expressing the wild type LFA-1 (Fig. 4.5).

a

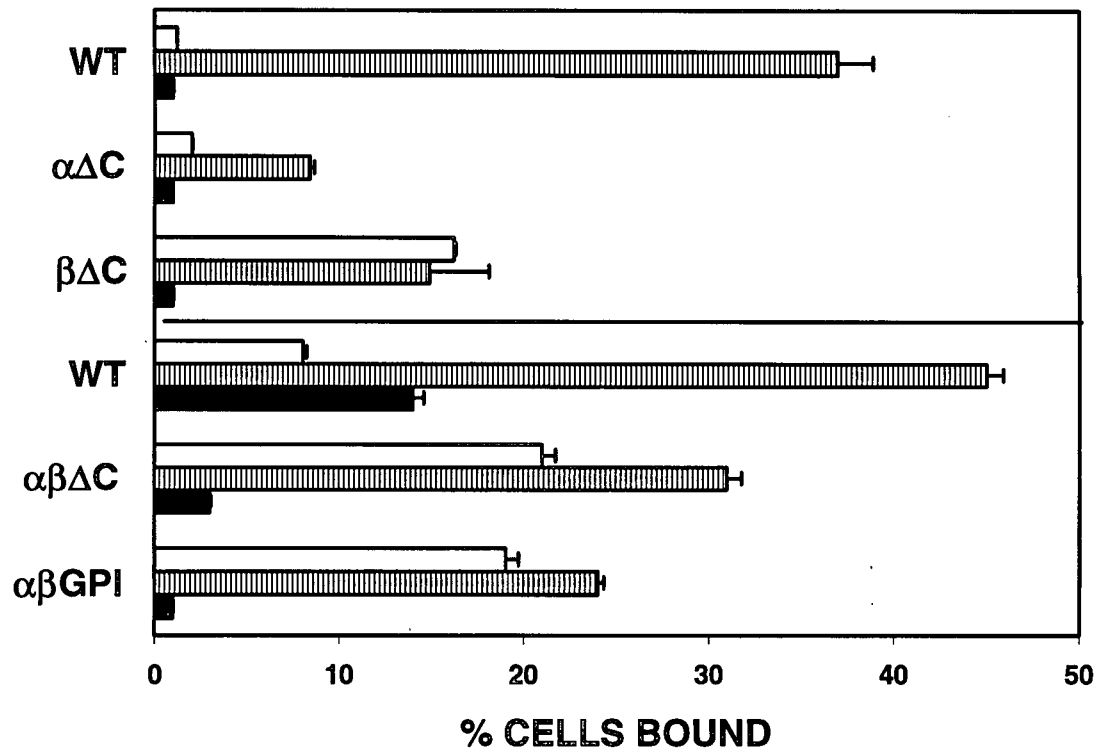
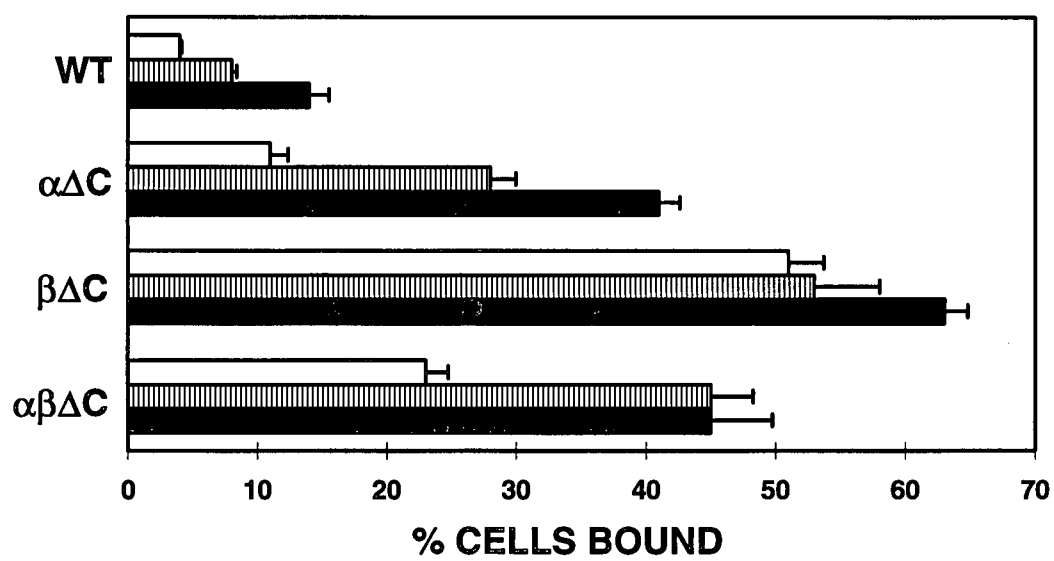


Figure 4.4. Adhesion of LFA-1-transfected TNR-2 cells to immobilized ICAM-1. (a) TNR-2 cells transfected with the various LFA-1 constructs were pre-incubated with (hatched bars) or without (clear bars) 50 ng/ml phorbol myristate acetate for 30 minutes, before addition to microwells coated with ICAM-1. Anti- α_L (black bars) was also added to PMA-stimulated cells to test the specificity of adhesion. (b) The effect of 0 (clear bars), 0.5 (hatched bars), and 5 mM (black bars) manganese on the binding of unstimulated TNR-2 transfectants to ICAM-1 is shown. All results are representative of at least three independent experiments and are shown as the percentages of input cells remaining in the wells after washing.

b



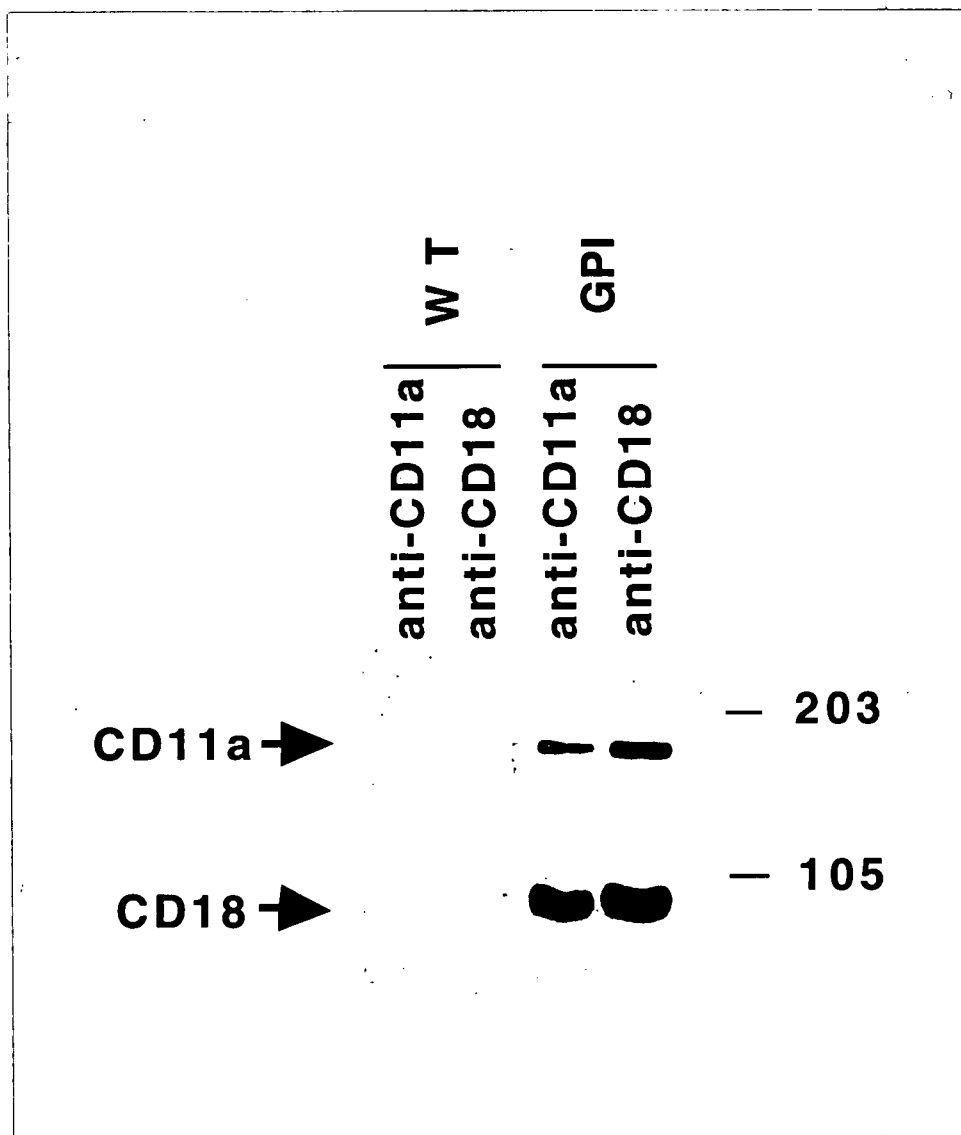


Figure 4.5. Detection of soluble LFA-1 by immunoprecipitation. LFA-1-GPI-transfected TNR-2 cells were surface-biotinylated and then treated with PI-PLC. The supernatants from the treated cells were subjected to immunoprecipitation with anti- α_L (CD11a) and anti- β_2 (CD18) as described in Figure 4.3. TNR-2 cells transfected with the wild type LFA-1 (WT) were used as a control.

Soluble LFA-1 was isolated on a larger scale by antibody affinity chromatography and elution from the affinity column in the presence of 2 mM MgCl₂. The soluble LFA-1 was then immobilized in microwells, and the ability of ICAM-1-transfected T28 cells to adhere to the immobilized soluble LFA-1 was tested. The transfected T28 cells expressing a high level of ICAM-1 (Fig. 4.6), but not the parental ICAM-1⁻ T28 cells, adhered to immobilized soluble LFA-1 in a fashion that was efficiently inhibited by anti-ICAM-1 antibody (Fig. 4.7), indicating that soluble LFA-1 is functional.

4.2.4) Role of LFA-1 cytoplasmic domains in post-receptor spreading

In addition to mediating the binding to its counterreceptors, LFA-1 is thought to play a role in outside-in signalling. To elucidate the role of LFA-1 cytoplasmic domains in post-receptor binding events, we examined the effects of truncating the LFA-1 cytoplasmic domains on cell spreading following binding to ICAM-1. LFA-1-transfected TNR-2 cell lines were taken through adhesion assays as described above, and after washing away unbound cells, the remaining bound cells were further incubated at 37° C to permit cell spreading. The cells expressing wild type LFA-1 readily exhibited spreading under the prevailing experimental conditions, and this spreading was unaffected by stimulation with PMA (Fig. 4.8). In contrast to wild type LFA-1, all the truncated forms, including those with individual cytoplasmic domain truncations, were unable to mediate spreading following adhesion to ICAM-1. Therefore, both the α and β cytoplasmic domains seem to be required for cell spreading. Moreover, although manganese significantly enhanced the adhesion of truncated LFA-1 to ICAM-1, it had no effect on cell spreading (data not shown).

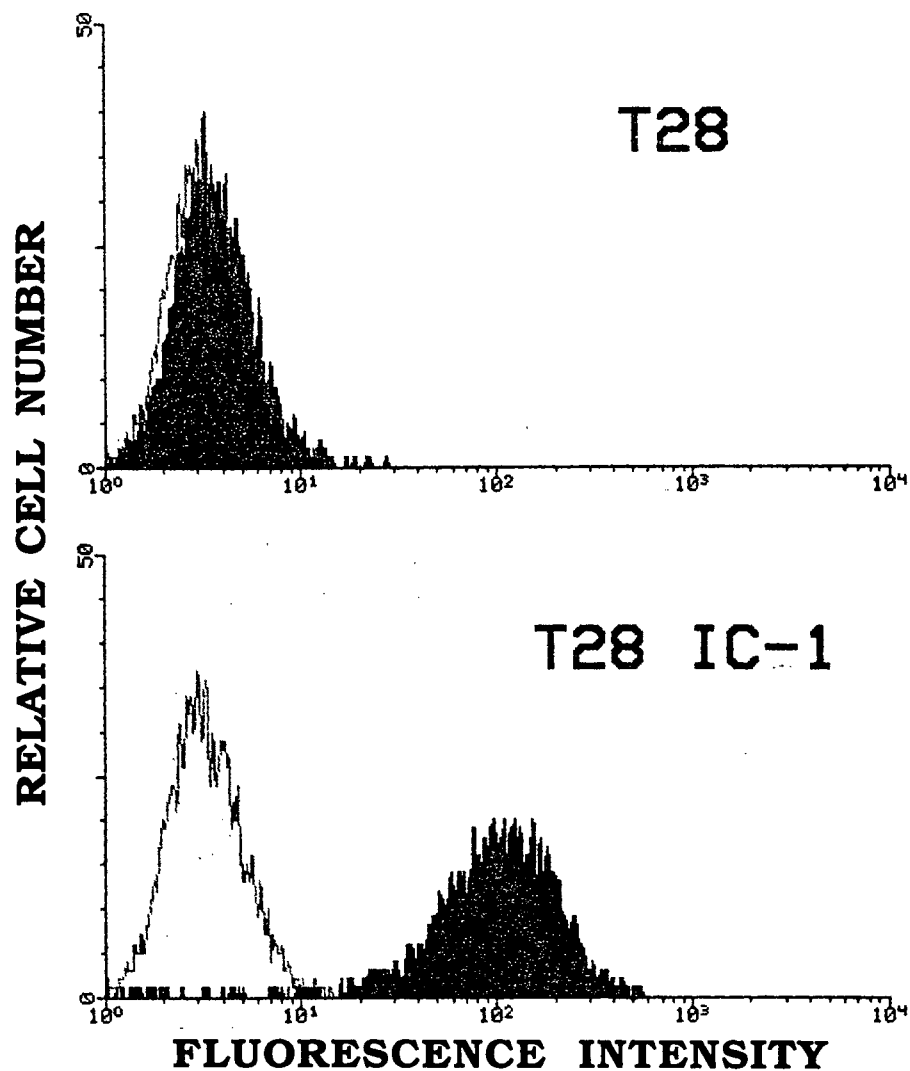


Figure 4.6. Flow cytometric analysis of T28 cells expressing murine ICAM-1. The murine T hybridoma line T28 expressing ICAM-1 was generated by electroporation of murine ICAM-1 cDNA. The ICAM-1⁺ T28 (T28 IC-1) and the parental T28 cells (T28) were stained with anti-ICAM-1 antibody and analyzed by flow cytometry. The shaded histograms show anti-ICAM-1-stained cells whereas the open histograms show staining with an irrelevant control antibody.

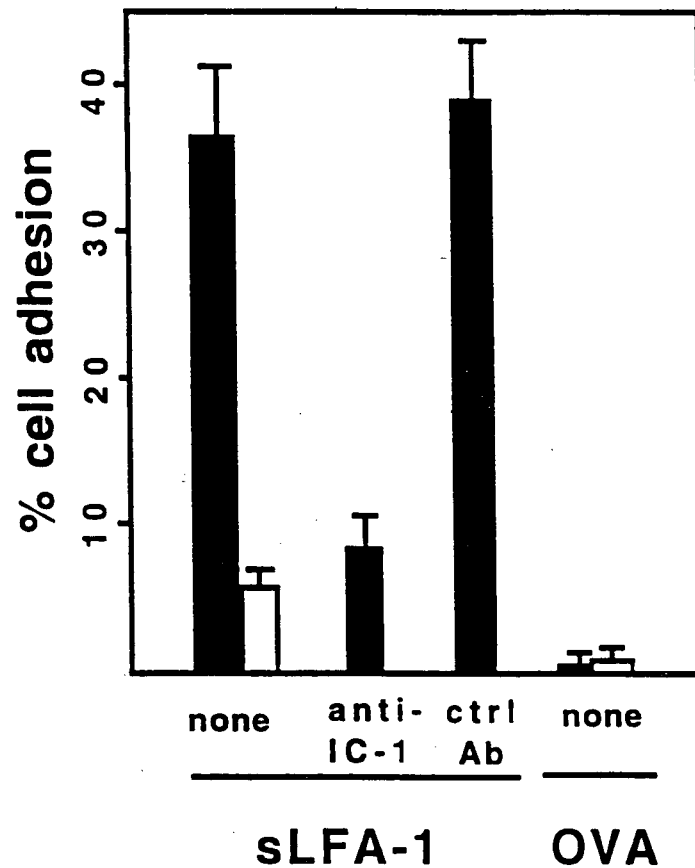


Figure 4.7. Adhesion of ICAM-1-transfected T28 cells to immobilized soluble LFA-1. Soluble LFA-1, isolated by treating LFA-1-GPI-transfected TNR-2 cells with PI-PLC, was purified by anti-LFA-1 antibody affinity chromatography, and immobilized in microwells. The adhesion of calcein-labeled ICAM-1⁺ T28 cells (filled bars), and untransfected T28 cells (ICAM-1⁻, open bars) to soluble LFA-1- and ovalbumin-coated microwells is shown, as are the effects of anti-ICAM-1 and control antibodies on this adhesion.

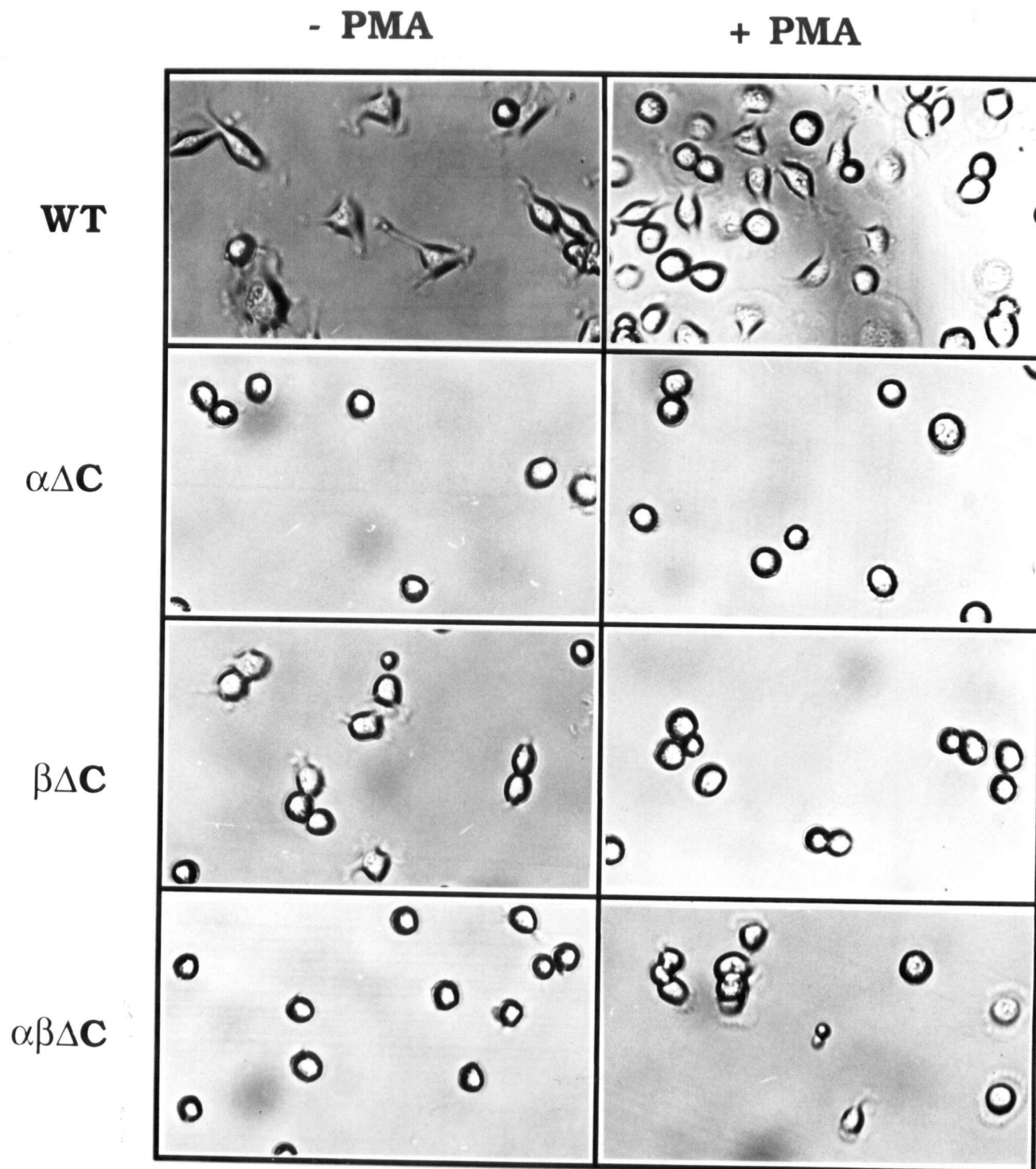


Figure 4.8. Spreading of LFA-1-transfected TNR-2 cells. Transfected TNR-2 cells were taken through adhesion assays as described in Figure 4.4, and bound cells were further incubated to permit spreading. Both PMA-stimulated and unstimulated cells were used in these experiments.

To ascertain whether the cell surface distribution of the expressed LFA-1 molecules could explain the observed differences in adhesion assays and cell spreading, we fixed the cells with paraformaldehyde and immunofluorescently labelled LFA-1 on the cells using anti- α_L antibodies followed by rhodamine-conjugated secondary antibodies. All forms of LFA-1 exhibited a similar punctate distribution on these cells (Fig. 4.9) suggesting that the cytoplasmic domains do not determine the distribution of LFA-1 on the cell surface.

4.3) DISCUSSION

In this study, we have examined the role of LFA-1 cytoplasmic domains in regulating adhesion to ICAM-1 and in post-receptor cell spreading using murine TNR-2 fibroblasts transfected with various recombinant forms of LFA-1. The results demonstrate the importance of both the α - and β -chain cytoplasmic domains in the regulation of LFA-1-mediated cell adhesion to ICAM-1 (inside-out signalling) as well as in post-adhesion cell spreading (outside-in signalling). The adhesion of TNR-2 cells transfected with wild type LFA-1 to ICAM-1 was inducible by PMA treatment. In contrast, truncated LFA-1 lacking the entire cytoplasmic domains of both the α and β chains, as well as GPI-anchored LFA-1 that lacks the cytoplasmic and transmembrane domains, supported constitutive basal adhesion to ICAM-1, with PMA treatment having little effect. Furthermore, soluble LFA-1, which is, by definition, free of cellular regulatory mechanisms, was capable of mediating the adhesion of ICAM-1⁺ cells. These results indicate that the cytoplasmic domains of LFA-1 are not required for the binding of LFA-1 to ICAM-1, but that they are important for the PMA-induced activation of LFA-1 (inside-out signalling). Truncation of individual cytoplasmic domains had differential effects on cell adhesion. TNR-2 transfectants expressing LFA-1 lacking only the α cytoplasmic domain

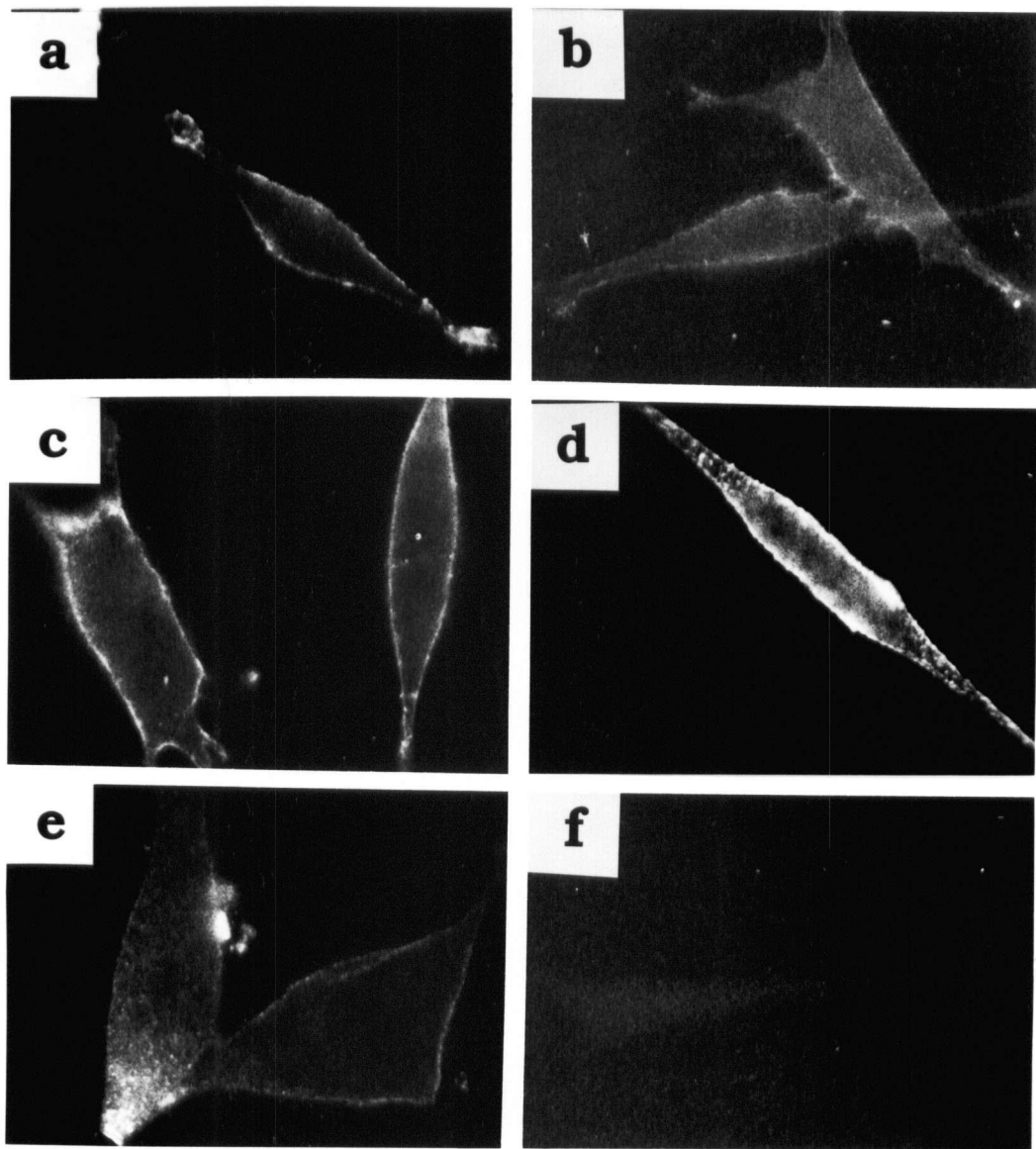


Figure 4.9. Fluorescent staining of LFA-1 on transfected TNR-2 cells. The distribution of LFA-1 on the various transfected TNR-2 cells fixed in paraformaldehyde was determined by staining with anti-LFA-1 antibody followed by TRITC-conjugated secondary antibody. (a) wild type, (b) $\alpha\Delta C$, (c) $\beta\Delta C$, (d) $\alpha\beta\Delta C$, (e) LFA-1-GPI, and (f) parental untransfected TNR-2 cells.

adhered to a considerably lower level than did the other transfected lines, including $\alpha\beta\Delta C$, and PMA exhibited weak upregulation of this adhesion. However, the adhesion of $\alpha\Delta C$ to ICAM-1 was greatly enhanced by manganese, indicating that $\alpha\Delta C$ can be activated, but seems to be non-responsive to inside-out signalling. In contrast, the effects of the β cytoplasmic domain truncation on adhesion to ICAM-1 were similar to those observed with $\alpha\beta\Delta C$. Therefore, both the α and β cytoplasmic domains are important in regulating cell adhesion to ICAM-1, but they seem to play different roles.

In addition to their possible roles in inside-out signalling, the cytoplasmic domains of both the α and β chains appear to be important in outside-in signalling, which is thought to be necessary in establishing firm secondary adhesion after initial cellular contact with, and arrest on, endothelium and subsequent transendothelial migration. We used cell spreading following adhesion to immobilized ICAM-1 as an indication of outside-in signalling. Cell spreading was rapid and dependent on the binding of LFA-1 to ICAM-1. LFA-1-transfected TNR-2 cells readily adhered to poly-L-lysine-coated microwells but no detectable cell spreading was observed within the incubation period used in this study (data not shown). We have found that truncation of either cytoplasmic domain of LFA-1 abrogates the ability of the transfected cells to spread out after binding to ICAM-1, supporting the contention that LFA-1 cytoplasmic domains are involved in transducing signals that lead to cell activation. The association of LFA-1 with the cytoskeleton has also been previously documented (Burn et al., 1988; Kupfer et al., 1990; Pardi et al., 1992b), and in one recent report, the adhesion of a chimeric LFA-1 consisting of the extracellular domains of $\alpha_{Ib}\beta_3$ and cytoplasmic domains of $\alpha_L\beta_2$ was shown to be modulated through cytoskeletal association (Peter & O'Toole, 1995). Furthermore, the punctate appearance

of fluorescently stained LFA-1 on TNR-2 transfectants implies that a microclustering of LFA-1 exists in the membrane, and this clustering may stem from an association with the cytoskeleton. It remains to be determined which cytoplasmic domain is responsible for signalling and/or association with the cytoskeleton.

In a previous report, truncated human LFA-1 lacking most of the cytoplasmic domain of the α chain was difficult to express on the surface of transfected COS cells (Hibbs et al., 1991b), suggesting that the α chain cytoplasmic domain is important for the assembly of the heterodimer or for its transport to the cell surface. It has been suggested that the KXGFFKR sequence conserved among all integrin α chains is required for the proper expression of some integrins (Kawaguchi & Hemler, 1993) but this is not the case for others (O'Toole et al., 1994). In our study, we have been able to get LFA-1 lacking the entire α and β chain cytoplasmic domains expressed on the cell surface at a level similar to that of wild type LFA-1. Therefore, the contribution of the α chain cytoplasmic domain to the proper expression of LFA-1 on the cell surface does not appear to be significant. It is of interest that truncation of the α cytoplasmic domain alone inhibits PMA-induced adhesion to ICAM-1 whereas truncation of both the α and β cytoplasmic domains together does not. It is possible that the β cytoplasmic domain associates with an inhibitory molecule that maintains LFA-1 in the low-avidity state whereas the α cytoplasmic domain is involved in the PMA-induced upregulation of LFA-1 avidity for ICAM-1. The inability of manganese to upregulate adhesion mediated by wild type LFA-1 is consistent with the notion of LFA-1 being "locked" into a low-avidity state. Truncation of the α cytoplasmic domain may release LFA-1 from this state, thereby permitting upregulation only through direct intervention by manganese, but not through physiological signalling pathways.

Our observation that LFA-1 lacking the cytoplasmic domains constitutively binds to ICAM-1 is consistent with a previous report on human LFA-1. COS cells transfected with mutant LFA-1 lacking most of the β chain cytoplasmic domain were able to adhere to ICAM-1 and were not activated with PMA (Hibbs et al., 1991b). Furthermore, our results are in agreement with the demonstration that truncation of the Mac-1 leukocyte integrin cytoplasmic domains resulted in enhanced binding of transfected COS cells to iC3b ligand relative to the wild type molecule (Rabb et al., 1993). Soluble Mac-1 has also been generated and was found to bind ligand (Dana et al., 1991). However, it is difficult to generalize the effects of integrin cytoplasmic domain truncation on the adhesive capacities of the integrins. Different integrins appear to be differentially regulated within a particular host cell type, and individual integrins may be regulated differently in different host cells. For example, in a previous study (Pyszniak et al., 1994), we reported that wild type LFA-1-transfected murine L fibroblasts constitutively adhere to ICAM-1, whereas the TNR-2 cells expressing wild type LFA-1 used in the present work required activation with PMA to overcome weak basal adhesion to ICAM-1. Our choice of TNR-2 (N-Ras-transformed NIH3T3) fibroblasts as an experimental system stemmed from the difficulty in using non-transformed 3T3 cells. The expression levels of the various LFA-1 constructs on these cells, and the adhesion of the transfected cells to ICAM-1 were highly variable, possibly due to contact inhibition during cell growth. L cells were also unsuitable because they cannot synthesize N-acetylglucosamine-phosphatidylinositol, the first intermediate in GPI anchor biosynthesis, and therefore do not express GPI-linked proteins (Kamitani et al., 1993). It is possible that, as a result of N-ras expression in TNR-2 cells, LFA-1 may be subject to different regulatory effects than in non-transformed cells. It was recently demonstrated that the expression of activated H-Ras suppressed the activation of $\alpha_{\text{IIb}}\beta_3$ chimeric integrins in CHO

cells (Hughes et al., 1997). However, another group reported that activated R-Ras enhanced cell adhesion to ECM proteins (Zhang et al., 1996). The effect of N-Ras on LFA-1 function is currently unknown.

Despite the limitations associated with TNR-2 cells, our current results support the notion that LFA-1 on some cells is maintained in a low-avidity form through an association of the cytoplasmic domains with putative negative regulatory elements. However, this model is not consistent with experiments demonstrating that overexpression of integrin β_3 or β_1 subunit cytoplasmic domains reduces the affinity of $\alpha_{\text{Lb}}\beta_3$ as measured by the binding of an affinity state-sensitive monoclonal antibody (Chen et al., 1994). An alternative explanation which cannot be discounted at the present time is that the truncation of LFA-1 cytoplasmic domains may influence the association of the two chains such that the extracellular domains adopt an "activated" conformation permitting binding to ligand.

Perhaps the most compelling evidence supporting the idea that LFA-1 is in a default high-avidity state in the absence of regulatory mechanisms is the finding that GPI-anchored and soluble LFA-1, which lack both the cytoplasmic and transmembrane domains, can constitutively bind to ICAM-1. Immunoprecipitation experiments failed to detect any co-precipitating proteins that may be associated with LFA-1. Therefore, soluble LFA-1 in this study is apparently free of cellular regulatory mechanisms, and yet is capable of mediating binding of ICAM-1⁺ cells. This observation is consistent with a previous report showing that LFA-1 purified from the cell surface is able to constitutively bind to ICAM-1 (Dustin & Springer, 1989; Dustin et al., 1992).

CHAPTER 5

**Dominant negative effect of the LFA-1 β (CD18)
cytoplasmic domain on leukocyte adhesion to ICAM-1 and
fibronectin**

5.1) INTRODUCTION

The intracellular events leading to increased LFA-1-mediated adhesion are currently poorly understood. Recent attention has focused on the integrin cytoplasmic domains as the moieties potentially responsible for functional regulation, and the previous chapter presented data demonstrating the importance of the LFA-1 cytoplasmic domains in regulating adhesion to ICAM-1. Various other experiments using integrins containing truncated cytoplasmic domains or heterologous cytoplasmic domains from other integrins have also lent credence to this theory (Diamond & Springer, 1994). For LFA-1, the CD18 subunit has been shown to be particularly important in regulating adhesiveness (Hibbs et al., 1991a,b; Peter & O'Toole, 1995). It is likely that modulation of adhesion through cytoplasmic domains occurs upon the interaction of these domains with other cytoplasmic factors (Chen et al., 1994; O'Toole et al., 1994; Kolanus et al., 1996; Hannigan et al., 1996).

The results presented in the previous chapter demonstrated that truncation of LFA-1 cytoplasmic domains has significant effects on the ability of LFA-1 to bind ICAM-1, as well as on post-binding events. Based on these results, I hypothesized that overexpression of isolated LFA-1 cytoplasmic domains would disrupt the normal interaction of putative regulatory molecules with the cytoplasmic domains of endogenous LFA-1, and would therefore affect leukocyte adhesion to ICAM-1 and cell spreading following adhesion. To test this hypothesis, I constructed chimeric molecules consisting of the transmembrane and cytoplasmic domains of both LFA-1 subunits joined to the extracellular domain of CD4, transfected these molecules into leukocyte lines, and performed adhesion assays with the transfectants. The results show that overexpression of the CD18 subunit cytoplasmic domain abrogated both the adhesion to ICAM-

1 and cell spreading following adhesion, whereas overexpression of the CD11a cytoplasmic domain had no effect. Furthermore, the CD18 cytoplasmic domain inhibited the adhesion of leukocytes to fibronectin, suggesting a common regulatory apparatus for fibronectin receptors and β_2 integrins.

5.2) RESULTS

5.2.1) Overexpression of LFA-1 cytoplasmic domains in murine leukocytes

In order to study the effect of overexpressing LFA-1 cytoplasmic domains on adhesion to ICAM-1 and subsequent cell spreading, we generated chimeras consisting of the transmembrane and cytoplasmic regions of CD11a and CD18 joined to the extracellular portion of CD4 (Fig. 5.1). As a control, a truncated form of CD4 lacking all but the first four amino acids of the cytoplasmic domain was also constructed. The constructs were subcloned into the expression vector pBCMGSneo, and were introduced into murine B (A20A8) and T (EL-4) cell lines by electroporation. The transfected cell lines expressing the chimeric molecules were established by cell sorting using anti-CD4 antibodies. Flow cytometric analysis of the resulting lines indicated that all the lines expressed equivalent levels of control or chimeric molecules (Fig. 5.2). The expression of endogenous LFA-1 remained unaffected by the presence of the chimeras, and was similar among all the lines (Fig. 5.2). Based on the number of amino acids present in the chimeric molecules, the expected molecular masses were calculated to be approximately 51 kD for CD4/18, 55 kD for CD4/11, and 46 kD for CD4 Δ C. Immunoprecipitation of the chimeras from the various lines demonstrated that the sizes of the proteins expressed were somewhat larger than expected, but were consistent with post-translational modifications (e.g. glycosylation) of the chimeras (Fig. 5.3). The

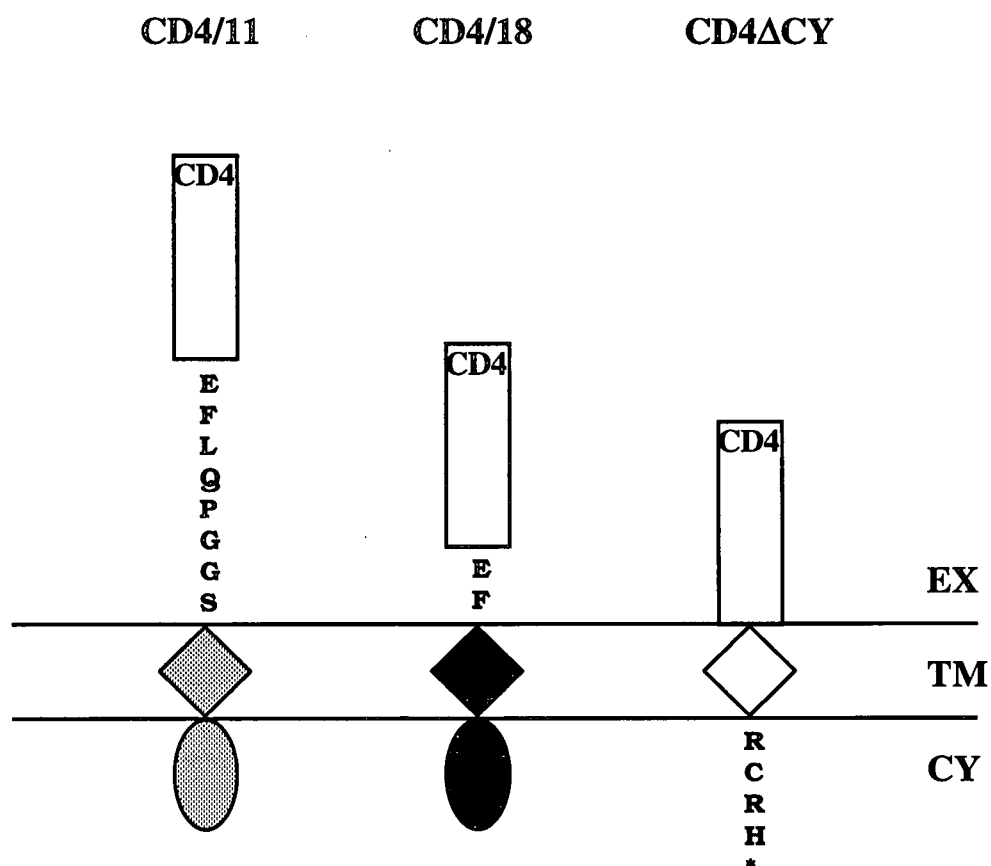


Figure 5.1. Generation of LFA-1 cytoplasmic domain chimeras used in this study. Constructs were generated by PCR as described in chapter 2. The amino acids present at the junction of CD4 and the respective LFA-1 transmembrane and cytoplasmic domains are shown. Amino acids in bold represent extraneous amino acids introduced during the subcloning procedure.

Figure 5.2. Flow cytometric analysis of LFA-1 chimera-transfected A20A8 and EL-4 cells. The expression levels of the CD4/LFA-1 chimeras as well as of endogenous LFA-1 was determined by direct immunofluorescence using, respectively, anti-CD4 and anti- β_2 antibodies conjugated to FITC.

A20A8

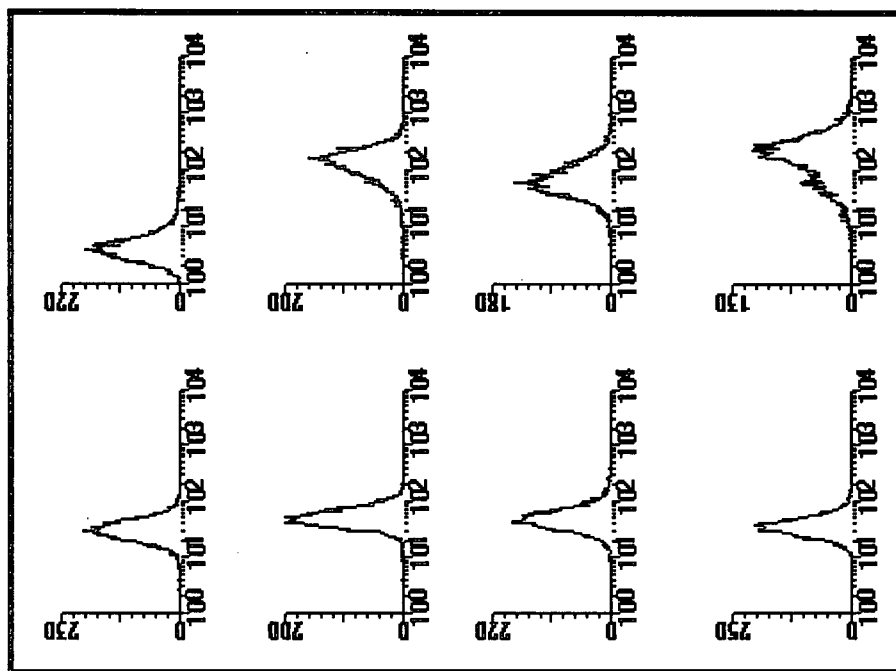
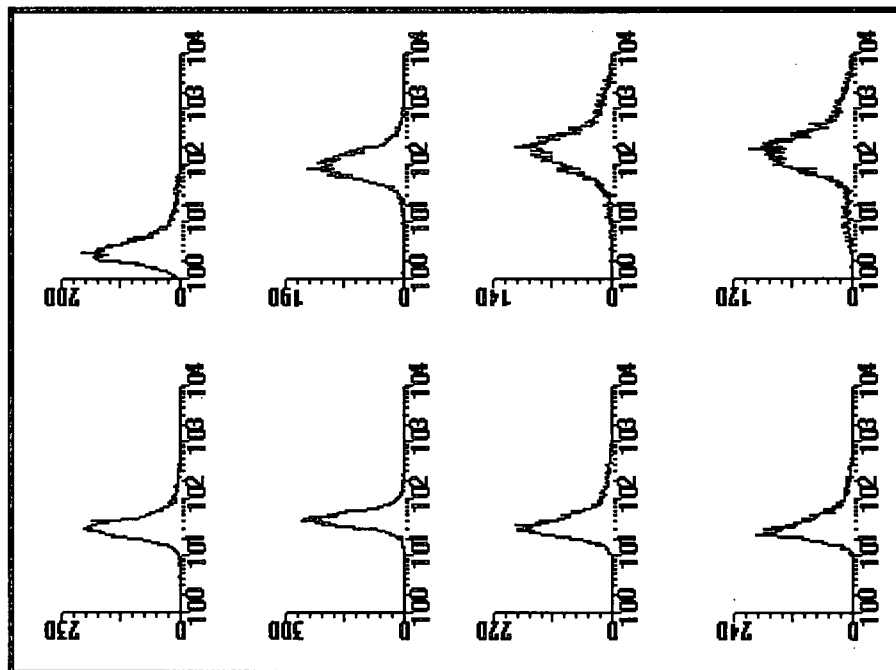
EL4

LFA-1

CD4

LFA-1

CD4



RELATIVE CELL NUMBER

FLUORESCENCE INTENSITY

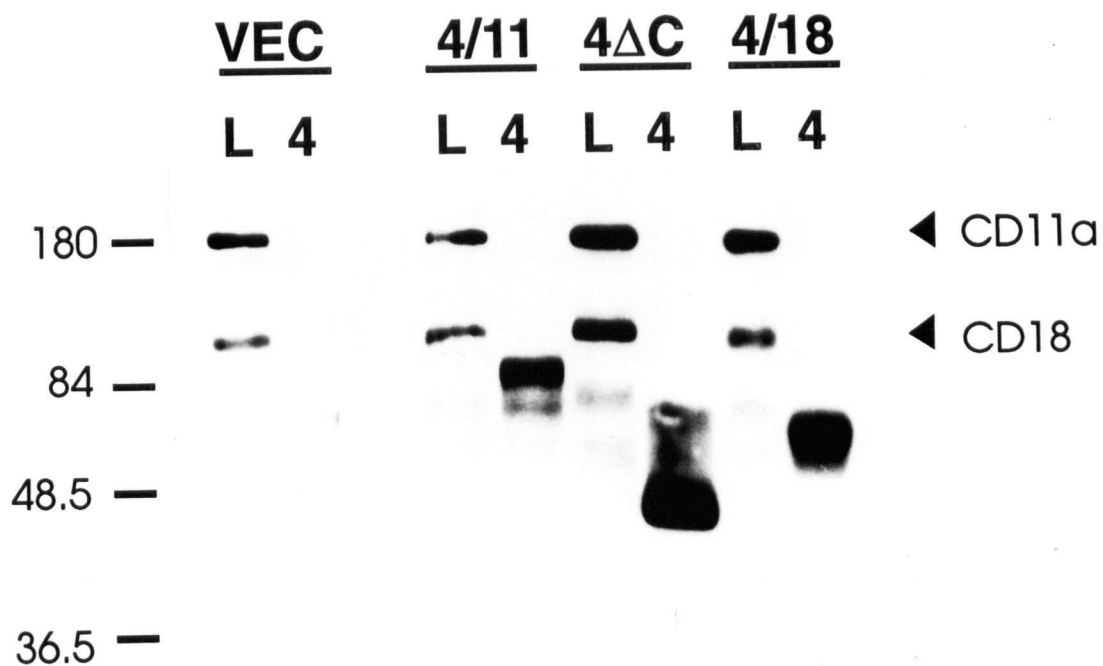


Figure 5.3. Immunoprecipitation of LFA-1 and of CD4/LFA-1 chimeras. Proteins were immunoprecipitated from surface-biotinylated A20A8 transfectants using anti- α_L (L) or anti-CD4 (4) primary antibodies, followed by rat anti-mouse Ig secondary antibodies conjugated to Affigel 10 beads. After separation by SDS-PAGE and electroblotting, proteins were detected by enzyme chemiluminescence using peroxidase-conjugated streptavidin.

apparent size of the CD4/11 chimera is considerably larger than expected, and is probably due to the constitutive phosphorylation of the CD11a cytoplasmic domain (Hara & Fu, 1986) in addition to glycosylation. Importantly, no association of the chimeras with endogenous LFA-1 subunits, or with other proteins was observed (Fig. 5.3).

5.2.2) Cell adhesion and spreading of chimera-transfected lines

We tested the effect of LFA-1 cytoplasmic domain overexpression by measuring the ability of leukocytes transfected with CD4/LFA-1 chimeras to adhere to soluble ICAM-1 adsorbed onto plastic. Both A20A8 (Fig. 5.4a) and EL-4 (Fig. 5.4b) cells transfected with vector alone bound to immobilized ICAM-1, and PMA upregulated this adhesion significantly. Cells transfected with the CD4/11 chimera, as well as those transfected with truncated CD4, bound to a similar extent. In contrast, cells expressing the CD4/18 chimera bound to ICAM-1 at significantly reduced levels regardless of PMA stimulation. The absolute degree of binding displayed by the various lines was variable between experiments, but the observed trend in binding was consistent. Over three experiments, the binding of unstimulated A20A8 cells expressing the CD4/18 chimera ranged from 3-13% whereas cells expressing the other constructs displayed 23-58% binding. For PMA-stimulated cells, binding ranged from 7-33% for CD4/18⁺ cells, and 48-71% for the other lines. Thus, the CD4/18 chimera exhibited a dominant negative effect on LFA-1-mediated adhesion to ICAM-1 in both B cells and T cells. As an extension to these studies, we asked whether the dominant negative effect of the CD18 cytoplasmic domain was specific for adhesion to ICAM-1. Therefore, we adsorbed whole fibronectin in microwells and tested the binding of A20A8 control and transfected cells to it.

a

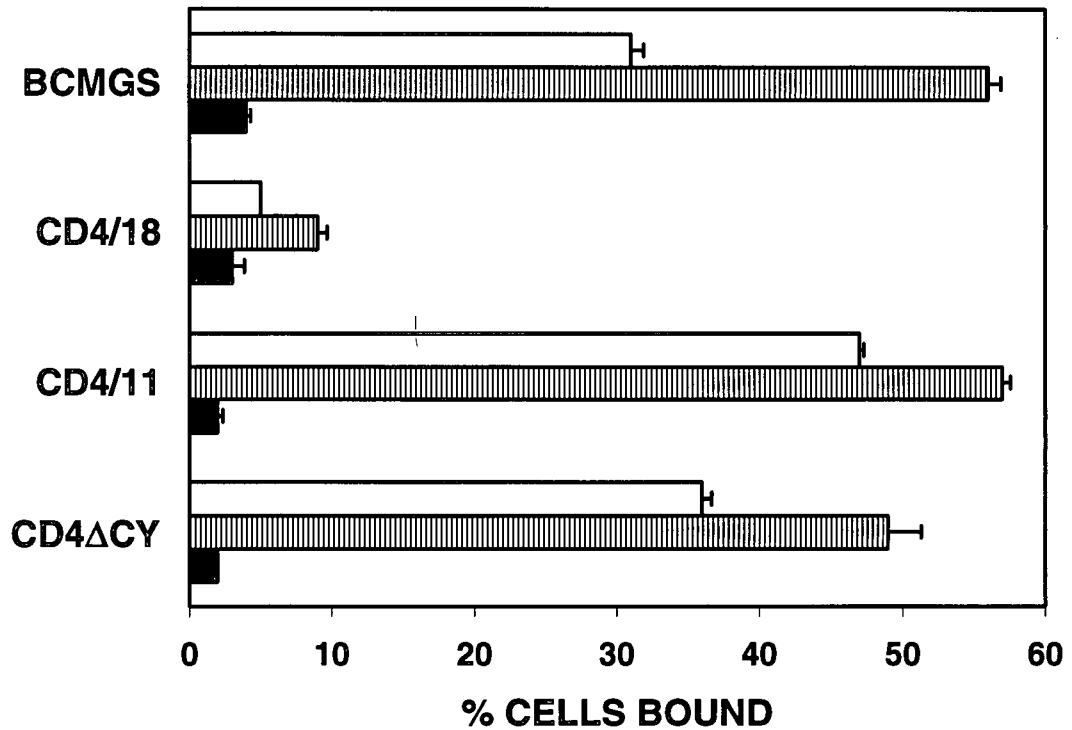
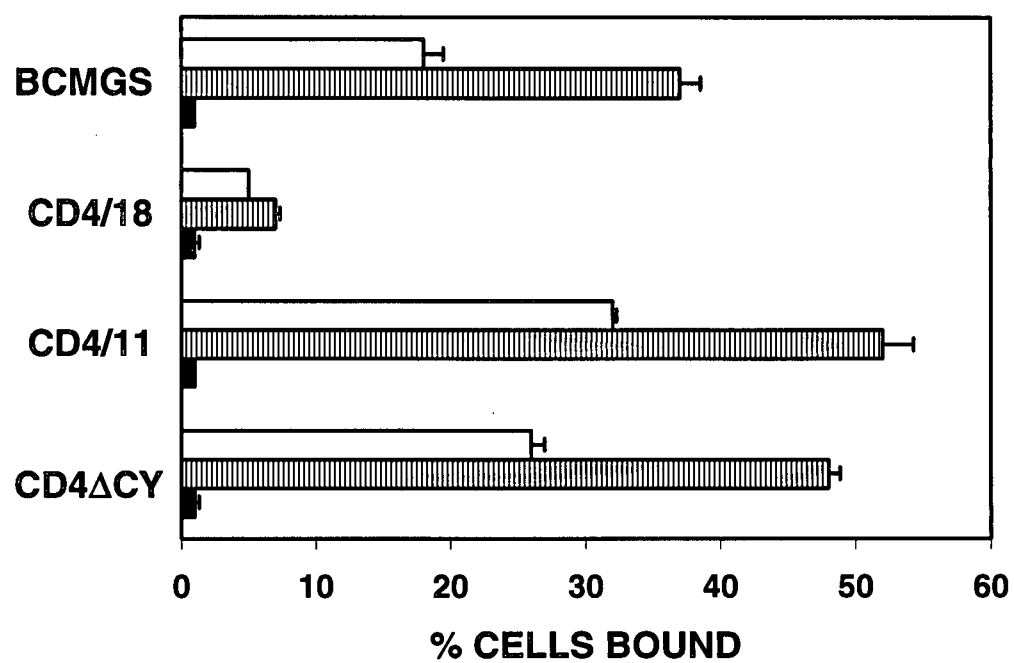


Figure 5.4. Adhesion of chimera-transfected A20A8 (a) and EL-4 (b) cells to immobilized ICAM-1. For each line, 10^5 PMA-activated (vertically hatched bars) or unstimulated (clear bars) cells labelled with BCECF-AM were added to microwells coated with soluble ICAM-1. As a test of specificity, anti- α_L was also added to PMA-activated cells (dark bars). Results are expressed as the mean of triplicate measurements \pm SEM, and are representative of at least two independent experiments. BCMGS = vector alone.

b



The adhesion of A20A8 to fibronectin was generally of lower degree than that to ICAM-1, but interestingly, the CD4/18 chimera drastically reduced this adhesion whereas CD4/11 had no effect (Fig. 5.5). The PMA-induced adhesion to fibronectin was completely inhibited by RGD peptide, but not by control RGE peptide, suggesting that this adhesion is mediated by integrins. These results suggest that the mechanisms regulating β_2 integrin and fibronectin receptor function share a common feature. Finally, we investigated whether overexpression of either LFA-1 cytoplasmic domain would affect post-binding events in the form of cell spreading following adhesion to ICAM-1. We performed adhesion assays using A20A8 cells, and after washing away unbound cells, we incubated the remaining cells for a further 20 minutes at 37° C. Cells transfected with vector alone, CD4/11, and CD4 Δ CY readily spread out on ICAM-1 (Fig. 5.6). In contrast, cells expressing CD4/18 did not spread.

5.3) DISCUSSION

The work presented herein was carried out to test the hypothesis that cytoplasmic regulatory factors interact with LFA-1 cytoplasmic domains to modulate adhesion to ICAM-1. Overexpression of the CD18 cytoplasmic domain in two leukocyte lines dramatically reduced the LFA-1-mediated adhesion of these lines to sICAM-1 immobilized in microtiter wells. In contrast, overexpression of truncated CD4 or of the CD11a cytoplasmic domain had no effect on adhesion as compared to control cells transfected with vector alone. The observed reduction in adhesion was not due to an alteration in the level of LFA-1 expression on the transfected cells, and immunoprecipitations of the chimeras failed to co-precipitate specifically bound proteins. Thus, it appears that the excess CD18 cytoplasmic domain competitively alters the binding of endogenous LFA-1 to ICAM-1 in a dominant-negative fashion. These results are consistent

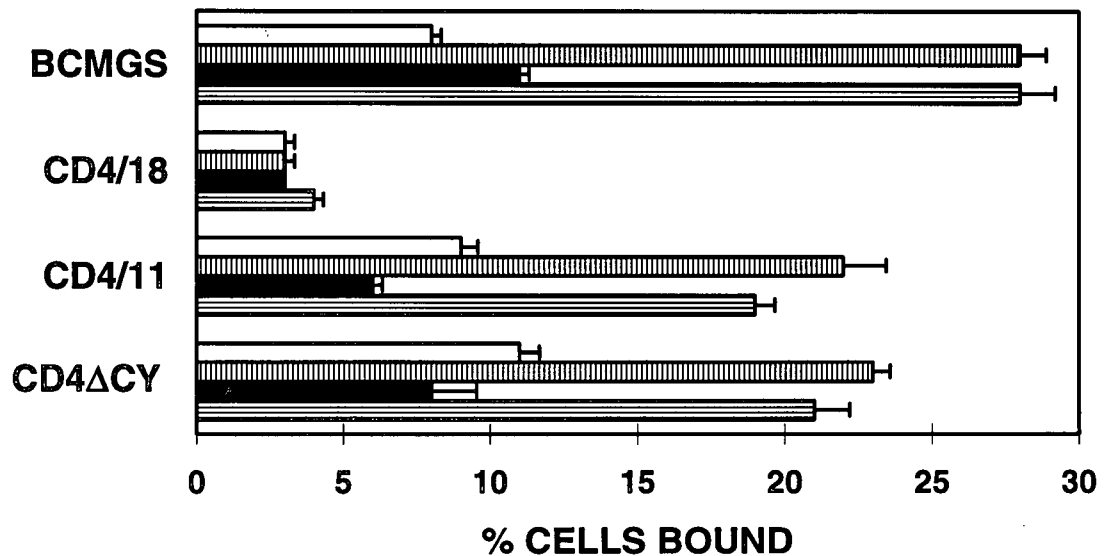


Figure 5.5. Adhesion of chimera-transfected A20A8 cells to fibronectin. Assays were performed as described in Figure 5.4. RGD and RGE peptides were added as tests for integrin activity. *Clear bars*, unstimulated cells; *vertically hatched bars*, PMA-activated cells; *black bars*, RGD peptide added to PMA-activated cells; *horizontally hatched bars*, RGE peptide added to PMA-activated cells. BCMGS = vector alone.

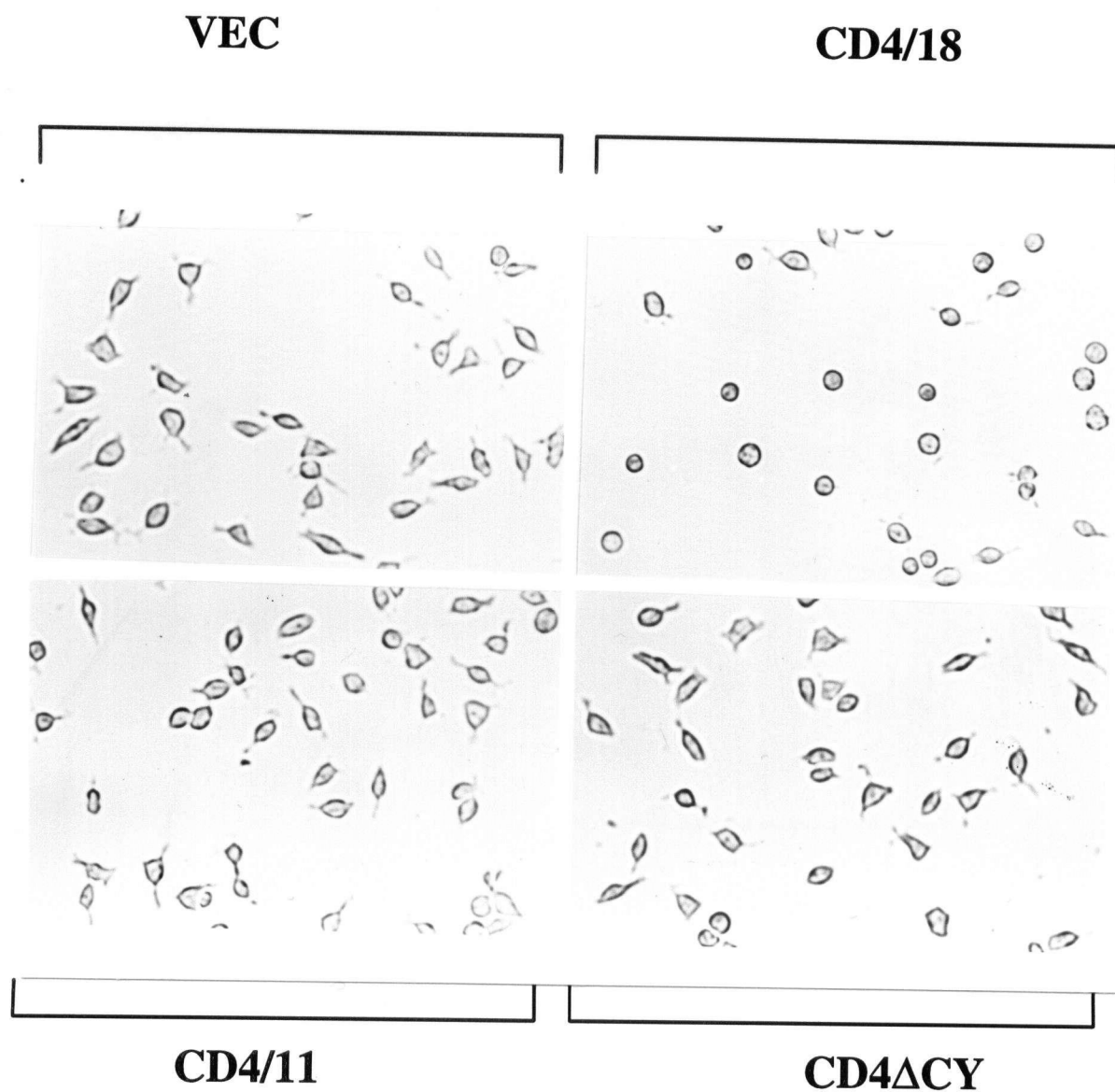


Figure 5.6. Spreading of A20A8 transfectants on ICAM-1. Cells were taken through conventional plate adhesion assays as described in chapter 2 with the exception that they were not labelled with BCECF-AM. After washing away unbound cells, the remaining cells were incubated for a further 20 min. Cells were photographed on an inverted microscope using a 10X objective. VEC, vector alone.

with a previous report demonstrating that overexpression of integrin β_1 or β_3 cytoplasmic domains reduced the binding affinity of platelet glycoprotein $\alpha_{IIb}\beta_3$ in transfected CHO cells (Chen et al., 1994). The ability of the CD18, but not the CD11a, cytoplasmic domain to inhibit the adhesion of LFA-1 to ICAM-1 supports previous observations that suggested the importance of the CD18 cytoplasmic domain in regulating LFA-1-mediated adhesion (Hibbs et al., 1991b; Peter & O'Toole, 1995). In particular, alteration of a sequence of three threonines in the CD18 cytoplasmic domain has been correlated with impaired post-receptor functions, including cell spreading, cytoskeletal stress fiber formation, and localization of receptors to focal contacts (Peter & O'Toole, 1995). Since our results demonstrated a reduction not only in adhesion, but in cell spreading following binding to ICAM-1, it is tempting to speculate that the expression of excess CD18 cytoplasmic domain in leukocytes interferes with the normal "signals" linking the cytoskeleton to the cytoplasmic domains of endogenous LFA-1. In this scenario, expression of excess CD18 cytoplasmic domain sequences may mimic the effects of mutation of CD18 described by Peter and O'Toole (1995) by disrupting the cytoskeletal association with LFA-1.

The nature of the putative linkage between LFA-1 cytoplasmic domains and the cytoskeleton is currently unknown. Several components of the cytoskeleton, including α -actinin (Pavalko & LaRoche, 1993), talin (Burn et al., 1988), and filamin (Sharma et al., 1995) have been shown to interact with CD18. It is possible, therefore, that cytoskeletal association is mediated directly through these proteins. Alternatively, other cytoplasmic proteins may bridge the integrin with the cytoskeleton. In any case, if the observed effect of overexpressing CD18 cytoplasmic domains in leukocytes is the result of competition between endogenous native LFA-1 and excess CD18 cytoplasmic domains, then the putative regulatory molecules are likely to exist in limiting quantities. Furthermore, the inhibition of LFA-1-mediated adhesion to ICAM-1

was almost complete in our assay system, rather than being partially inhibited. This implies that regulatory factors preferentially associate with the chimeric CD18 cytoplasmic domain. It is conceivable that the CD18 cytoplasmic domain displays a higher affinity for such factors when it is removed from close proximity with the CD11a subunit in the native receptor. Another explanation for the complete inhibition is that the chimera does not preferentially bind the regulatory factor, but sequesters enough of it to prevent a certain threshold level from becoming associated with native LFA-1, and the adhesive phenotype may depend on attaining this threshold level.

It has become apparent in recent years that the regulation of integrin function is a complex process that is dependent on the specific integrin and cell types studied. However, some similarities likely exist in the regulation of different integrins since most of them can be activated in a similar fashion. Whether or not LFA-1 shares common regulatory mechanisms with other integrins is currently unknown. We have shown that expression of the CD4/18 chimera in leukocytes abrogates their binding to whole fibronectin immobilized on plastic. This binding is most likely mediated by integrins since it was inhibited by RGD peptide, but not by RGE control peptide. These results suggest that the pathways regulating the fibronectin receptor and β_2 integrins share a common component. Although we were unable to determine which integrin is responsible for mediating the binding of A20 cells to fibronectin, it seems likely that this receptor is a member of the β_1 or β_7 families. Candidate receptors include VLA-4 (Wayner et al., 1989; Guan & Hynes, 1990) and $\alpha_4\beta_7$ (Chan et al., 1992a; Ruegg et al., 1992).

The inhibition by chimeric CD18 cytoplasmic domains of not only β_2 integrin function, but also that of other integrins has important practical implications. Since lymphocyte adhesion

to endothelial cells appears to involve LFA-1 as well as VLA-4 and $\alpha_4\beta_7$ (Ruegg et al., 1992; Springer, 1994; Alon et al., 1995; Altevogt et al., 1995; Berlin et al., 1995), it is conceivable that the CD18 cytoplasmic domain chimeras could be utilized as a generalized and efficient therapy in modulating this stage of the inflammatory response.

CHAPTER 6

Summary and Discussion

The last few years have witnessed a tremendous advance in our understanding of integrin regulation. At the time that this thesis work was initiated, the importance of the LFA-1: ICAM-1 interaction in normal immune function had been established by antibody inhibition experiments, as well as by studying the effects of leukocyte integrin deficiency in LAD patients. It was also known that LFA-1-mediated adhesion to ICAM-1 was associated with cell activation, and that increased adhesion was the result of qualitative, rather than quantitative changes in LFA-1. However, little was known about the actual mechanisms involved in the conversion of LFA-1 from a non-adhesive to an adhesive state. It is now appreciated that the regulation of LFA-1-mediated adhesion is a complex process involving a network of intracellular events and pathways that mediate the bi-directional traffic of information through the integrin, and, in turn, allow the regulated interaction of leukocytes with other cells.

The main objective of this thesis work was to elucidate the mechanisms involved in the regulation of LFA-1 function. In Chapter 3, polystyrene microspheres coated with sICAM-1 were shown to bind specifically to high-avidity LFA-1. The beads bound in a non-random fashion to T28 and LFA-1-transfected L cells, suggesting that the cell surface distribution of high-avidity LFA-1 may be localized on some types of cells. However, staining with fluorescently-labelled anti-LFA-1 mAb demonstrated that LFA-1 was still present on the entire cell surface. These results suggest that LFA-1 in multiple activation states can co-exist on the cell surface, consistent with what has been observed for LFA-1 on activated T cells (Lollo et al., 1993) and for Mac-1 on neutrophils (Diamond & Springer, 1993). The distribution of high-avidity LFA-1 could not be determined on primary T cells because of their small size, and because PMA activation of these cells in the presence of sICAM-1-coated beads induced the formation of large complexes of cells and beads. The fact that treatment with cytochalasin B

inhibited the adhesion of activated primary T cells to ICAM-1 immobilized on plastic, but not to sICAM-1-coated beads implies that the cytoskeleton is not involved in the initial activation and binding of LFA-1 to ICAM-1, but that it is important for the firm secondary adhesion of cells to ICAM-1 immobilized on plastic. Furthermore, the data raise the possibility that different patterns of LFA-1 distribution influence adhesiveness through this receptor. Theoretically, a high local concentration of LFA-1 at the site of cell contact could augment the strength of adhesion regardless of the affinity between individual receptor:ligand pairs. An interesting related question is whether the observed non-random distribution of high-avidity LFA-1 precedes ligand binding, or whether it occurs as a result of ligand binding. In this regard, the aggregation of Mac-1 has been shown to occur in the absence of ligand upon phorbol ester stimulation, and this aggregation correlates with an enhanced capacity to bind ligand (Detmers et al., 1987). On the other hand, it has recently been suggested that clustering of $\alpha_{\text{Lb}}\beta_3$ can be induced by ligand binding (Fox et al., 1996).

Several reports have suggested that multimerization of LFA-1 is a prerequisite for strong adhesion to ICAM-1 (van Kooyk et al., 1994; Peter & O'Toole, 1995; Lub et al., 1997). The mechanisms influencing the surface distribution of LFA-1 are currently unclear, but one possibility is that the cytoplasmic domains, particularly that of CD18, play an important role. Several studies have demonstrated an association of cytoskeletal proteins with the CD18 cytoplasmic domain (Burn et al., 1988; Pavalko & LaRoche, 1993; Sharma et al., 1995). Furthermore, mutation of three adjacent threonine residues in the human CD18 cytoplasmic domain affected cytoskeletal organization in transfected COS cells, which correlated with the inability of LFA-1 to multimerize on these cells (Peter & O'Toole, 1995) and others have shown that the β_1 cytoplasmic domain was responsible for the localization of β_1 integrins to focal

contacts (Solowska et al., 1989; Hayashi et al., 1990; Marcantonio et al., 1990; Reszka et al., 1992). These data suggest that receptor distribution may be determined via a linkage with the cytoskeleton. The inhibitory effect of cytochalasin B treatment on T cell binding to immobilized sICAM-1 (Chapter 3) is consistent with this idea, though it was not demonstrated that such treatment influenced the distribution of LFA-1. Recently, Lub et al. (1997) suggested that activation of LFA-1 may be governed by regulated interactions with the cytoskeleton. These authors reported that treatment of resting primary T cells with cytochalasin D results in enhanced cell binding to immobilized ICAM-1, and that this binding is further enhanced by stimulating the cytochalasin D-treated cells with PMA. The authors postulated that cytochalasin D uncouples LFA-1 from the cytoskeleton, thereby permitting the clustering of LFA-1, and leading to the initial increase in binding to ICAM-1. PMA treatment synergizes with this effect by converting individual LFA-1 receptors into high affinity states. These results, however, are not consistent with the data presented in chapter 3 which clearly demonstrate that the combined treatment of cytochalasin B and PMA stimulation abrogated primary T cell binding to immobilized ICAM-1. The reason for this discrepancy is unknown.

In addition to interacting with cytoskeletal elements, the cytoplasmic domains of integrins have been suggested to bind to other putative regulatory factors. Of significance to the study of LFA-1 regulation has been the identification of cytohesin-1, a cytoplasmic protein consisting of both a positive regulatory domain, which is structurally similar to the yeast SEC7 gene product, and a negative regulatory domain, which is homologous to the pleckstrin domain (Kolanus et al., 1996). This indicates that association with the cytoskeleton may be only one means of regulating LFA-1. Indeed, it is generally accepted that conformational changes are induced in integrin extracellular domains upon cell activation, and that these changes influence the affinity

of the integrin for ligand. The biochemical pathway leading to changes in integrin conformation remains elusive, but it is possible that associations with the cytoskeleton or with other cytoplasmic factors, such as cytohesin-1, may contribute to this phenomenon. At any rate, it is reasonable to postulate that LFA-1 cytoplasmic domains play important roles in transmitting conformational changes to the extracellular domain.

The results in chapter 4 indicate that the presence of the CD18 cytoplasmic domain correlates with a non-adhesive form of LFA-1 regardless of whether it is associated with full-length or truncated CD11a. Truncation of this domain results in an increased binding capacity to ICAM-1 that is unaffected by treatment with PMA (i.e. the binding is constitutive). This result supports what has been observed for Mac-1 (Rabb et al., 1993) and for human LFA-1, where partial truncation of the CD18 cytoplasmic domain abrogated binding to ICAM-1, but near-entire truncation supported binding (Hibbs et al., 1991b).

In contrast to the situation with CD18, virtually nothing is known about the function of the CD11a cytoplasmic domain. A previous attempt to probe this question using a truncation of the entire cytoplasmic domain was unsuccessful because the construct was not expressed at the cell surface (Hibbs et al., 1991b). Thus, my successful attempt to express LFA-1 lacking the entire CD11a cytoplasmic domain provided an excellent opportunity to shed light on the role of this domain in regulating LFA-1-mediated adhesion. Truncation of the CD11a cytoplasmic domain and its expression with a wild type CD18 subunit results in a receptor that is expressed at somewhat lower levels compared to the other receptor subunit combinations, and that binds only weakly to ICAM-1 even after PMA stimulation. It is possible that the lower expression level of this construct may, in part, explain the reduced adhesion to ICAM-1. However, by extension to the results presented in chapter 3, the measurement of LFA-1 expression levels may

be of limited value since this does not account for the functional status of individual molecules. In any event, the fact that Mn^{2+} treatment upregulated the adhesion to ICAM-1 to a level similar to that of PMA-stimulated cells expressing wild type LFA-1 suggests that the receptor is functional, and that the CD11a cytoplasmic domain is involved in the upregulation of adhesion by physiological signalling pathways (see below). Interestingly, when the CD18 cytoplasmic domain was truncated along with that of CD11a, the mutant receptor bound to ICAM-1 in a constitutive fashion. In light of these results, one can speculate on the events involved in LFA-1 activation in TNR-2 cells. Both LFA-1 cytoplasmic domains appear to play important, but different, roles (Fig. 6.1). Truncation of both cytoplasmic domains simultaneously removes the integrin from intracellular signalling pathways, and results in a receptor that is in an active state by default (Fig. 6.1a). This default high-avidity state may stem from an alteration in the association of the two LFA-1 chains upon truncation. However, it should be noted that intact (i.e. non-truncated) LFA-1 purified from the cell membrane also appears to be active (Dustin & Springer, 1989; Dustin et al., 1992). If LFA-1 is active by default when removed from the intracellular milieu, this suggests that intracellular factors may function to maintain the receptor in a non-binding state, and that PMA may release it from this negative regulation (Fig. 6.1b). This is observed in TNR-2 cells transfected with wild type LFA-1. Since truncation of the CD18 cytoplasmic domain results in constitutively active LFA-1, the putative negative regulation may occur through this chain. The nature of this negative regulatory signal is unknown, but an intriguing possibility is that association with the cytoskeleton is responsible. Support for this idea comes from experiments demonstrating that PMA induced an alteration in neutrophil morphology when the cells were in suspension, presumably via an effect on the cytoskeletal network (Lawrence & Springer, 1991). Furthermore, the lateral mobility of LFA-1

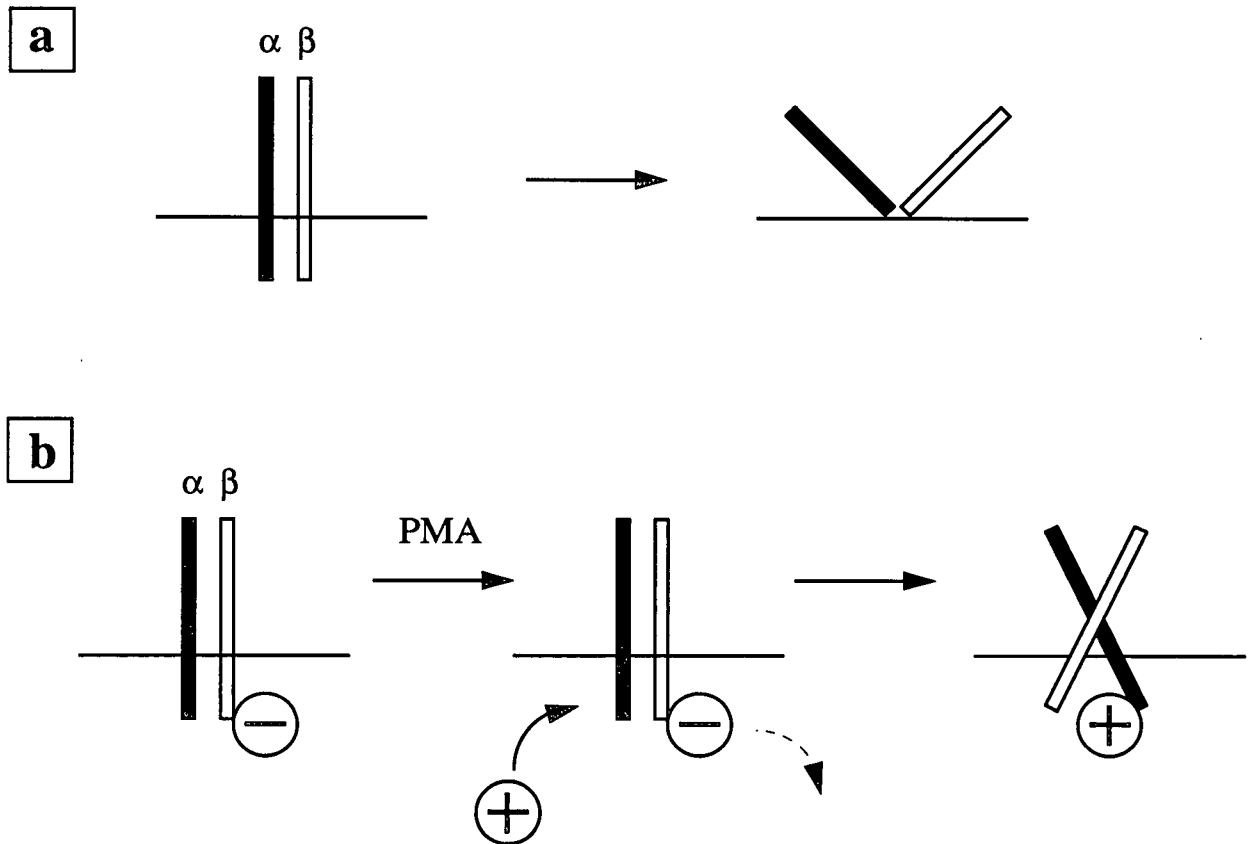


Figure 6.1. Hypothetical model of LFA-1 activation in TNR-2 cells. (a) Truncation of both LFA-1 cytoplasmic domains yields the default high-avidity state. (b) A negative regulator, acting via the β_2 subunit, maintains LFA-1 in an inactive state. Upon stimulation with PMA, a putative positive regulator mediates the activation of LFA-1, possibly by associating with the α_L chain. See text for details.

in the plane of the membrane is enhanced upon PMA stimulation, suggesting that release from cytoskeletal constraints may be a determinant in the upregulation of binding (Kucik et al., 1996), possibly by assisting in the conversion of LFA-1 into a high-affinity state, and by permitting a redistribution of LFA-1 at the cell surface (Lub et al., 1997). This would be consistent with the results presented in chapter 3. However, my attempt to determine the distribution of LFA-1 on TNR-2 cells using fluorescence microscopy did not resolve this question. The resolution provided by fluorescence microscopy may not be adequate to show differences in distribution that may be functionally significant. It would be interesting, therefore, to revisit this issue using confocal or electron microscopy.

Truncation of the CD11a cytoplasmic domain alone renders LFA-1 refractory to stimulation by PMA, but not through non-physiological mechanisms (i.e. by Mn^{2+}), suggesting that this domain is involved in PMA-stimulated physiological signalling leading to receptor activation. A possible explanation for these observations is that a cytoplasmic positive regulator becomes associated with the CD11a cytoplasmic domain, or otherwise acts through this domain, upon stimulation with PMA. In the case of wild type LFA-1, this factor may overcome the negative effect of the CD18-associated factor, perhaps by displacing it, thereby permitting the conversion of the receptor to an active form (Fig. 6.1b).

If the cytoplasmic domains of LFA-1 associate with intracellular factors to effect regulation of adhesion, one might expect that expression of extra amounts of either cytoplasmic domain would affect the normal regulation of adhesion through the cytoplasmic domains of endogenous LFA-1. To test this hypothesis, we transfected the CD11a and CD18 transmembrane and cytoplasmic domains into B and T lymphocyte lines as chimeras with the extracellular domain of CD4, which acted as a reporter antigen for sorting and FACS analysis.

The results indicated that expression of extra CD18 cytoplasmic domains greatly reduced the degree of binding to sICAM-1 of both transfected cell lines, whereas equivalent expression of CD11a cytoplasmic domains had no effect on adhesion (chapter 5). These findings are consistent with what has been observed previously, with isolated β_1 and β_3 integrin cytoplasmic domains, but not those from α_5 or α_{IIb} subunits, reducing the affinity of $\alpha_{IIb}\beta_3$. Moreover, the results raise the possibility that the isolated CD18 cytoplasmic domain competes with native endogenous LFA-1 for a putative positive regulator of adhesion that, upon PMA stimulation, would associate with the CD18 subunit, but not with the CD11a cytoplasmic domain (Fig. 6.2). This "positive regulator" may be an association with the cytoskeleton, which was reported to be necessary for stabilizing cell adhesion (Lub et al., 1997), and would serve to convert LFA-1 into an active state. However, the degree of inhibition observed by expression of the CD18 cytoplasmic domain raises the question of whether simple competition for limited amounts of regulatory factors is adequate to explain the findings. A possible explanation for this observation is that CD18 cytoplasmic domains isolated from those of CD11a may exhibit a higher affinity for regulatory factors than does CD18 as part of the native LFA-1 molecule. It is unlikely that the chimera associates with other cell surface molecules as no such proteins specifically co-immunoprecipitated with it. Alternatively, since it is likely that only a subpopulation of LFA-1 is actually involved in binding to ligand at any given time, the CD18 chimeras would probably be expressed in excess relative to this subpopulation of LFA-1, and, therefore, would not be competing for regulatory factors with the entire complement of LFA-1 at the cell surface.

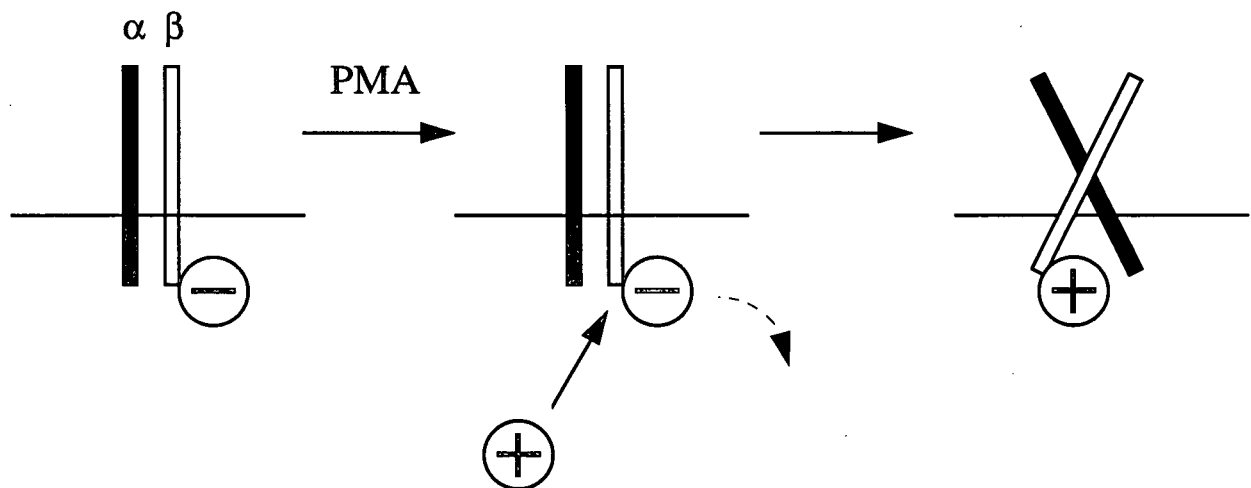


Figure 6.2. Hypothetical model to explain observations with LFA-1 chimeras. As in TNR-2 cells, a putative negative regulator may associate with the β_2 cytoplasmic domain to maintain LFA-1 in an inactive state. Upon PMA stimulation, a putative positive regulator may associate with the β_2 subunit, simultaneously displacing the negative regulator and resulting in LFA-1 activation. See text for details.

The hypothetical scenarios described above present some obvious discrepancies concerning the mechanism of inside-out signalling through LFA-1. For example, the CD18 cytoplasmic domain has been proposed to associate with both negative and positive regulators. Furthermore, the scenario described in Fig. 6.1 suggests that the expression of extra CD11a cytoplasmic domains might have an inhibitory effect on the ability of PMA to upregulate adhesion to ICAM-1, but no such effect was observed in the system studied. Perhaps isolated CD11a cytoplasmic domains require close proximity with CD18 in order to bind putative positive regulators. In this regard, it is interesting that cytohesin-1 has been suggested to contain both positive and negative regulatory domains (Kolanus et al., 1996). The presence of both these domains on the same protein may allow the interaction of cytohesin-1 with both LFA-1 cytoplasmic domains under the appropriate conditions. Alternatively, it is possible that the observed results reflect differences in regulatory pathways among the different cell lines studied. It is currently established that integrins appear to be regulated differently in different cell types. Furthermore, although the effect of N-Ras expression in TNR-2 cells is unknown, it may nevertheless confound the interpretation of the observed data. The obvious solution to these issues would be to standardize the experimental system used to study LFA-1 regulation. Thus, re-examining the effects of cytoplasmic domain truncations in a LFA-1⁺ leukocyte line would be appropriate. Conversely, examining the effects of LFA-1 cytoplasmic domain chimeras in TNR-2 cells may also help to clarify the issues.

Nevertheless, it is possible to propose a speculative model of LFA-1 activation that can reconcile the observed data. This model (Fig. 6.3), takes advantage of the interesting observation that the expression of isolated β_2 cytoplasmic domains consistently results in a severe reduction in LFA-1-mediated adhesion to ICAM-1. This is observed both in leukocytes

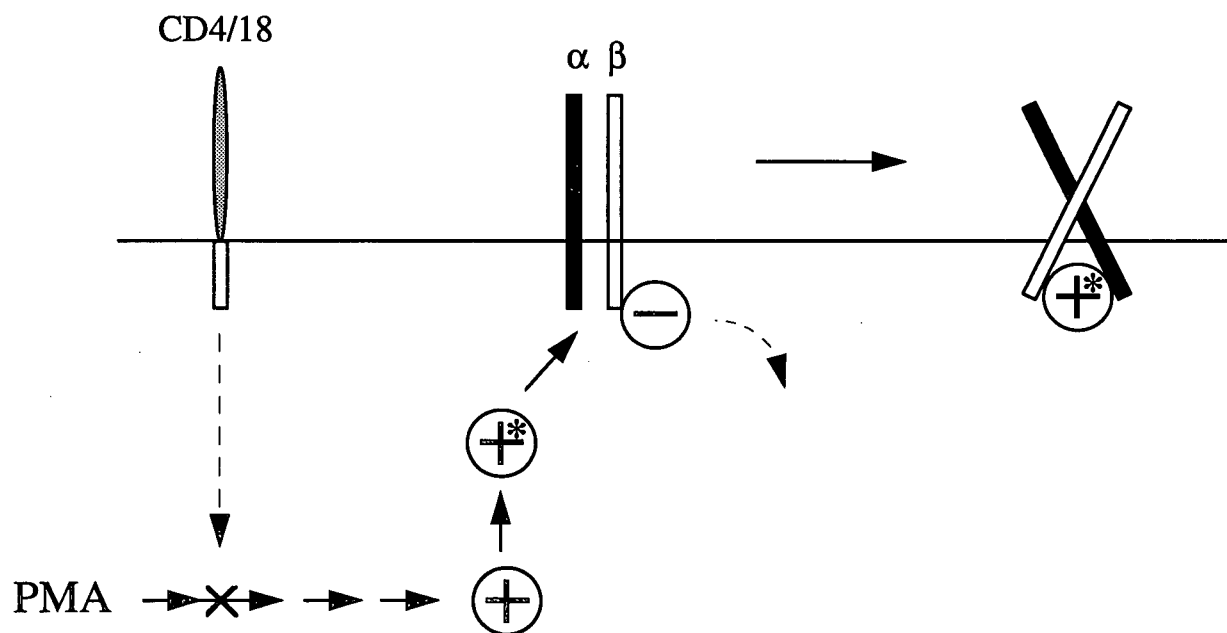


Figure 6.3. Schematic representation of possible events leading to LFA-1 activation. As described previously, a putative negative regulator maintains LFA-1 in an inactive state. Treatment with PMA initiates a signalling cascade that results in the conversion of a putative positive regulator from an inactive to an active form (represented by an asterisk). This activated positive regulator may then associate with LFA-1, thereby activating the receptor. In this model, the expression of an isolated β_2 cytoplasmic domain blocks the activation of the positive regulator. See text for details.

transfected with CD4/18 (chapter 5) and in TNR-2 cells expressing $\alpha\Delta C$ (chapter 4). In this scenario, putative inhibitor and activator molecules co-operate to modulate LFA-1-mediated adhesion. Thus, a negative regulator may associate with the CD18 cytoplasmic domain thereby maintaining LFA-1 in an inactive state. Treatment with PMA may initiate a signalling pathway that results in the conversion of a positive regulator from an inactive to an active form. The activated positive regulator may then associate with the LFA-1 cytoplasmic domains to overcome the negative regulator and enhance adhesion. Thus, the isolated CD18 cytoplasmic domain does not compete for a direct modulator of LFA-1 activity in this case. Rather, it interferes with the PMA-induced conversion of the positive regulator from the inactive to the active form, perhaps by transducing signals of its own, or by transiently interacting with an intermediary in the PMA-induced pathway. In the absence of an activated positive regulator, the negative regulator dominates, and LFA-1-mediated adhesion is not increased. Therefore, TNR-2 cells expressing wild type LFA-1 do not bind to ICAM-1 unless treatment with PMA yields an activated positive regulator. Cells expressing $\alpha\Delta C$ express an isolated CD18 cytoplasmic domain, and therefore resemble CD4/18⁺ leukocytes in the characteristics of their binding to ICAM-1. Both $\alpha\beta\Delta C$ and $\beta\Delta C$ in TNR-2 cells do not bind negative regulator, and therefore assume the default active state, similar to the situation in figure 6.1a. The appeal of this model resides in the fact that the effect of the isolated CD18 cytoplasmic domain on intracellular signalling pathways may also explain the observation that the adhesion of A20A8 cells to fibronectin is dramatically reduced. The implication is that the CD18 cytoplasmic domain exerts its effect at a stage of the signalling pathway that is common to both LFA-1 and fibronectin receptors.

This thesis has attempted to shed some light on the mechanisms involved in regulating LFA-1-mediated adhesion. While the details remain elusive, data presented herein suggests that LFA-1 can exist in multiple activity states at the cell surface, and that the distribution of high-avidity LFA-1 is non-random on several cell types. The corollary is that this distribution may be functionally significant. Furthermore, both LFA-1 cytoplasmic domains appear to participate in the regulation of inside-out, as well as outside-in, signalling. The data concerning CD11a is particularly interesting in that little was known about its role in LFA-1 function. The dominant negative effect of the CD18 cytoplasmic domain on LFA-1-mediated adhesion further suggests the importance of the LFA-1 cytoplasmic domains in regulating adhesion through this receptor, perhaps by interacting with cytoplasmic regulatory molecules that can modulate its function. The elucidation of the signalling pathways regulating integrin function would be useful in the design of therapeutic remedies for diseases involving integrins. Treatment of chronic and acute inflammation constitutes one major clinical application that would benefit from knowledge of integrin regulation. In this regard, it is particularly interesting that the CD18 cytoplasmic domain inhibits not only LFA-1, but also the fibronectin receptor(s) (see Chapter 5). Future studies aimed at elucidating the crystal structures of active and inactive forms of integrins would shed light on the nature of the conformational changes that are believed to occur during integrin activation. Further study of intracellular molecules interacting with integrin cytoplasmic domains, and of their precise roles in co-ordinating the bi-directional signalling through integrins is certainly warranted. The field of integrin regulation promises to be fruitful for the foreseeable future.

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