Differential effects of low molecular weight inhibitors on the conformation and the immunologic function of the adhesion receptor LFA-1

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Abbreviations

ALEXA-647	Novel ALEXA based fluorescence dye for flow cytometry EX/EM 630/680nm
APC	Antigen presenting cell
BRET	Bioluminescence Resonance Energy Transfer
BD	Becton Dickinson
CPM	Counts per minute
CsA	Cyclosporin A
C-terminal	Carboxy-terminal
CTLs	Cytotoxic T-lymphocytes
DMSO	Dimethylsulfoxide
EA-REMA	Expression/Activation - Receptor Epitope Monitoring Assay
EDTA	Ethylenediaminetetraacid
FITC	Fluorescein isothiocyanate
fMLP	N-Formyl-L-Methyl-L-Leucyl-L-Phenylalanin
FRET	Fluorescence resonance energy transfer
h / hrs	Hour / Hours
i.v.	Intravenous
ICAM-1,-2,-3	Intra cellular adhesion molecules 1, 2, 3
ICAP-1	Integrin cytoplasmic domain associated protein-1
I domain	Inserted domain
Ig	Immunoglobulin
IgSF	Immunoglobulin superfamily
I-like domain	Inserted like domain
IL-1,-2,-6	Interleukin 1, 2, 6
INFα	Interferon alpha
JAM-1	Junctional adhesion molecule 1
kDa	Kilo Dalton
L-site	Lovastatin binding site
LFA-1	Lymphocyte function-associated antigen 1 (CD11a/CD18, αLβ2)
LMW	Low molecular weight
mAb	Monoclonal antibody
Mac-1	Macrophage differentiation antigen 1 (CD11b/CD18, αMβ2)

Abbreviations continued

MFI	Mean fluorescence intensity
MIDAS	Metal ion-dependent adhesion site
min	Minute
mL	Milliliter
MLR	Mixed lymphocyte reaction
mM	Millimolar
mTOR	Mammalian target of rapamycin
MW	Molecular weight
N-terminal	NH2-terminal
NK-cell	Natural killer cell
nM	Nanomolar
NMR	Nucleic magnetic resonance
p.o.	Per os
PBS	Phosphate buffered saline
PBLs	Peripheral blood lymphocytes
PD	Pharmacodynamic
PE	Phycoerythrin
PerCP	Peridium chlorophyll protein
PI3	Phosphatidylinositol 3
PKC	Protein kinase C
PMA	Phorbol-12-myristate-13-acetate
REMA	Receptor epitope monitoring assay
RT	Room temperature
SEB	Staphylococcus enterotoxin B
TCR	T cell receptor
TNFα	Tumor necrosis factor α
TRIS	Trishydroxylmethylaminoethane
µl	Microliter
µM	Micromolar

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Aims of this thesis

It was the aim of this thesis to investigate the effects of low molecular weight inhibitors on the conformation and the function of the adhesion receptor LFA-1. Moreover, the aim was to provide novel methods to enable the pharmacodynamic characterization of low molecular weight LFA-1 inhibitors.

Summaries

The β2 integrin lymphocyte function-associated antigen-1 (LFA-1, αLβ2, CD11a/CD18) is a conformationally flexible heterodimeric receptor which is expressed on the surface of all leukocytes. LFA-1 mediates cell adhesion, migration and costimulatory signaling events which are vital for immune- and inflammatory responses. Classic inside-out signaling events or extracellular cations are required to switch LFA-1 from a non-ligand binding to a ligand binding state. During this activation process the entire receptor and the ligand binding domain (αL I domain) undergo remarkable conformational changes. It was the aim of this thesis to contribute to the understanding how low molecular weight LFA-1 inhibitors block the function of LFA-1. The thesis is divided into three major parts: The **introduction**, **chapter 1** and **chapter 2**.

The **introduction** of this thesis is intended to review the current understanding of LFA-1 as an adhesion receptor. The recent molecular models of LFA-1 activation and signaling are described. Furthermore, the **introduction** provides an overview of preclinical and clinical data that support the rationale of LFA-1 as a therapeutic target. Chemical entities that are currently pursued as low molecular weight LFA-1 inhibitors are outlined.

The work described in **chapter 1** focused on the characterization of low molecular weight (LMW) LFA-1 inhibitors of different chemical classes. In particular their effect on the molecular receptor conformation was studied.

The aim of the work of **chapter 2** was to study effects of LFA-1 inhibitors in whole blood. In addition to characterizing the degree of target occupancy by LFA-1 inhibitors in whole blood, we investigated whether LFA-1 receptor occupancy corresponds to a modulation of the activation and proliferation of human T-lymphocytes in whole blood cultures.

Summary chapter 1

Previous studies of our group [1,2] and by others [3] on the isolated ligand binding domain of LFA-1 (αL I domain) have suggested that some LFA-1 inhibitors act allosterically while other inhibitors were proposed to competitively block the LFA-1/ligand interaction [4]. We postulated that LMW LFA-1 inhibitors allosterically alter the LFA-1 receptor conformation, resulting in shielding or neo-expression of epitopes recognized by monoclonal antibodies (mAbs) mapping to regulatory domains of the αL or β2 chains.

Results of chapter 1:

Our data revealed that LFA-1 inhibitors can be differentiated according to their mode of action on the receptor level.

The first group of lovastatin-derived LFA-1 inhibitors strongly induced conformational changes within the αL I domain. This was detected by the potent inhibition of the binding of the mAb R7.1 (anti CD11a, αL I domain specific) to either purified LFA-1 or LFA-1 expressed on Jurkat T-cells. The degree of epitope reduction by the LFA-1 antagonists tested, correlated well with the potency in inhibition of the LFA-1/ICAM-1 interaction. These LFA-1 inhibitors had no effect on the binding of mAbs directed to other domains within LFA-1.

In contrast, one lovastatin-derived inhibitor (LFA703) induced epitope changes in the αL I domain and also in the β2 I-like domain, a regulatory domain located on the β2 chain of LFA-1. This effect became evident by the reduced binding of mAb IB4 (anti CD18; β2 I-like domain specific) to cation-activated LFA-1 in the presence of LFA703. These results demonstrated that amongst lovastatin-derived inhibitors subclasses exit, which exert differential effects on the LFA-1 receptor conformation. Moreover, the antibody binding patterns observed on native LFA-1 receptors in the presence of various inhibitors demonstrated that upon receptor activation a conformational interaction between the αL I domain and the β2 I-like domain is

formed. These findings have meanwhile been confirmed by others in more comprehensive biochemical studies [5].

For the first time our results provided strong evidence that the β2 I-like domain embodies a target for allosteric LFA-1 inhibition similar to the well established regulatory L-site in the αL I domain. XVA143, a suggested ICAM-1 mimetic, which was proposed by the inventors to be a competitive αL I domain inhibitor [4], blocked the binding of the β2 I-like domain specific mAb IB4 with nM potency. XVA143 had no effect on the binding of mAb R7.1 or other anti CD11a mAbs under all experimental conditions, and did not bind to the αL L-site as determined by NMR studies. Furthermore, we showed that the target of XVA143 is most probably located on the β2 chain, as the compound also blocked the binding of mAb IB4 to purified Mac-1 (αMβ2) and inhibited the interaction of purified Mac-1 with ICAM-1. The compound typifies therefore a novel class of LFA-1 inhibitors with a distinct, probably allosteric mode of action. These findings provided evidence that the β2 I-like domain could represent a new target for potent inhibition of adhesion receptors of the β2 integrin subgroup. Potent LMW inhibitors like XVA143 may open new opportunities for specific intervention with the function of β2 integrins. These inhibitors could be therapeutically useful in transplantation, autoimmune diseases and inflammatory disorders.

Compellingly, the combined use of various LFA-1 inhibitors and selected monitoring mAbs contributed to the understanding of the mode of action of LFA-1 inhibitors and the function of β2 integrins on a molecular level. In addition, our findings show that currently available LFA-1 inhibitors can be differentiated into two major groups according to their mode of action on the receptor level: the αL L-site inhibitors and the putative β2 I-like domain inhibitors.

Chapter 1 was published in the Journal of Biological Chemistry in 2002 [6].

Summary chapter 2

LMW LFA-1 inhibitors may soon enter clinical trials. For their pharmacological and safety evaluation in clinical studies, it will be mandatory to provide, in addition to pharmacokinetic (PK) measurements, insights in the pharmacodynamic (PD) properties of these potentially immunosuppressive and anti-inflammatory compounds. The aim of the studies described here was to develop the methodology for studying the effect of LFA-1 inhibitors on receptor occupancy, receptor expression and T-cell function in whole blood. These studies are intended as a basis for the pharmacodynamic characterization of LFA-1 inhibitors in clinical trials. Furthermore, the effect of LFA-1 inhibitors on T-cell function was compared to the immunosuppressants cyclosporine A and everolimus.

Results of chapter 2

LFA-1 inhibitors of different chemical classes were tested in novel whole blood receptor epitope monitoring assays (REMAs). We designate here REMAs as cytometric methods which use target-specific mAbs to detect receptor occupancy by LMW compounds in whole blood. The lovastatin-derived LFA-1 inhibitor LFA878 and the experimental COMPOUND X, a non lovastatin-derived LFA-1 inhibitor, blocked the binding of mAb R7.1 to leukocytes in undiluted blood with nM potencies. As expected, the putative β2 I-like domain inhibitor XVA143 was unable to alter the binding of mAb R7.1 to leukocytes in whole blood. In contrast, we found that LFA-1 receptor occupancy by XVA143 led to a significantly increased binding of the β2 chain, stalk region specific mAb MEM48 to whole blood leukocytes. These results demonstrated for the first time that LFA-1 inhibitors with different modes of action can interact with LFA-1 in undiluted human blood and that target occupancy can be monitored by selected mAbs.

The REMA principle was validated *ex vivo* by measuring LFA-1 receptor occupancy in blood of rabbits after i.v. administration of LFA878. LFA878 blocked the binding of the mAb R7.1 with transient duration of action. Dependent on the dose administered

the pharmacodynamic half-life was 0.6 h (11.5 mg/kg i.v.) or 3.3 h (50mg/kg i.v.). These data showed for the first time that the REMA can be applied to study pharmacodynamic effects of αL L-site inhibitors in rabbits *ex vivo*. Our results furthermore suggested that the αL L-site and the mAb R7.1 epitope are conserved between man and rabbit. The pharmacodynamic effects of XVA143 could not be investigated because the mAb MEM48 did not cross-react with LFA-1 of other species.

To allow the assessment of the effect of LFA-1 inhibitors on several T-cell parameters, we developed an anti CD3 (OKT3) mAb stimulated T-cell activation assay (CD69 readout) and combined it with the REMAs described above. The so-called EA-REMAs allowed us to quantify simultaneously receptor occupancy by LFA-1 inhibitors (REMA), the cell surface LFA-1 expression (E) and the upregulation of the activation marker CD69 (A) on individual T-lymphocytes after *in vitro* stimulation of 1:1 diluted blood with immobilized mAb OKT3.

LFA878, COMPOUND X and XVA143 completely blocked mAb OKT3 stimulated CD69 upregulation with IC_{50}s of 2 µM, 1 µM and 0.05 µM respectively, while pravastatin, a statin that does not bind to LFA-1, was completely inactive at 50 µM. The LFA-1 inhibitors tested were completely inactive in blood cultures stimulated with a combination of mAbs OKT3 and anti CD28, demonstrating the specific inhibition of LFA-1 dependent T-cell responses by the compounds tested.

An additional pharmacodynamic property of XVA143 was revealed by the EA-REMA. 22 h incubation of whole blood with XVA143 led to a partial (35-55%) downregulation of LFA-1 cell surface receptors on T-cells, a phenomenon not observed for the αL L-site inhibitors tested.

The compounds were then assessed on their effect on mAb OKT3 stimulated T-cell proliferation in 1:10 diluted blood. All LFA-1 inhibitors blocked mAb OKT3 stimulated

T-lymphocyte proliferation with nearly equal potencies than observed in the mAb OKT3 stimulated T-cell activation assay.

Applying these protocols, experimental evidence was obtained for the first time that LFA-1 receptor occupancy by LFA-1 inhibitors can translate into efficient blockade of *in vitro* stimulated T-cell activation and proliferation in whole blood. The correlation between receptor occupancy and blockade of T-cell activation and proliferation (response) revealed that a >85% receptor occupancy in whole blood is required by the αL L-site inhibitors tested for the suppression of T-cell responses in whole blood cultures by 50%. In contrast, an almost 1:1 correlation between receptor occupancy and the resulting suppression of T-cell responses was observed for the β2 I-like domain inhibitor XVA143.

A comparison of LFA-1 inhibitors with cyclosporin A (CsA) and everolimus in the whole blood assays suggested that the structurally different LFA-1 inhibitors could be useful as immunosuppressants. XVA143 blocked T-cell activation (0.05 µM) and proliferation (0.02 µM) with higher potency than CsA (0.8 µM; 0.15 µM respectively) and was nearly equipotent to everolimus (0.01 µM) in the whole blood proliferation assay. In contrast, αL L-site inhibitors were nearly as potent as CsA in the CD69 T-cell activation assay, but significantly less active in whole blood proliferation assays (1-2 µM). As expected, CsA or everolimus did not interfere with LFA-1 expression or the binding of the monitoring mAbs R7.1 or MEM48.

During the development of the EA-REMA we found that supplemental $MgCl_2$ strongly synergized with anti CD3 triggered T-cell activation in whole blood. This finding may suggest a new role for magnesium cations in the regulation of integrin dependent T-cell responses *in vivo*. We hypothesize that locally elevated (mM) concentrations of Mg^{2+} may regulate integrin adhesiveness and thereby strengthen cell to cell contacts leading to enhanced integrin dependent T-lymphocyte responses. Further

investigations are ongoing, to elucidate the effect of magnesium on the activation and function of immune cells.

In conclusion, we demonstrated that various LFA-1 inhibitors could occupy their target on leukocytes in whole blood and that LFA-1 occupancy by these inhibitors translated into potent suppression of *in vitro* stimulated blood T-lymphocytes. Our data are strongly suggesting that LFA-1 inhibitors, in particular inhibitors with the potency of XVA143, could be applicable as therapeutic immunosuppressants. In addition, our array of novel methods allowed us to generate an "*in vitro pharmacodynamic* " profile of LMW LFA-1 inhibitors with different modes of action in whole blood. These protocols may be applicable as pharmacodynamic assays for LFA-1 inhibitors in clinical studies and may assist therapeutic dose finding.

Parts of this work (REMA) will be soon published as

G. Weitz-Schmidt, K. Welzenbach, J. Dawson, J. Kallen: "Improved LFA-1 inhibition by statin derivatives: Molecular basis determined by X-ray analysis and monitoring of LFA-1 conformational changes in vitro and ex vivo", J. Biol. Chem. 2004, in press

A second manuscript publishing other findings of chapter 2 is in preparation.

1 Introduction

Since the discovery of lymphocyte function-associated antigen-1 (LFA-1) in 1981 by the group of T.A. Springer [7,8] more than 4300 scientific papers - from basic research through clinical reports - were published concerning the adhesion receptor LFA-1. This introduction briefly describes the integrin family (1.1), outlines the general characteristics of LFA-1 and its ligands (1.2) and gives a description of the biological functions of LFA-1 (1.3) and the molecular mechanisms of LFA-1 activation (1.4). The rationale for LFA-1 as a therapeutic target is outlined in the chapter 1.5. Due to the wealth of information obtained on LFA-1 this introduction can only serve as an **overview** and as the background information needed for this thesis. In most areas of LFA-1 research, even in fundamental research topics such as LFA-1 signaling and LFA-1 receptor activation, many details still remain to be discovered to complement the puzzle of the highly complex LFA-1 dependent processes that are vital to all higher organisms.

1.1 Integrins

Adhesive cell contacts within a tissue or between cells and the establishment and maintenance of tissue scaffolds by the extracellular matrix are fundamental for the development and physiological function of all multicellular organisms. Cell adhesion molecules are cell surface proteins that mediate these cell to cell or cell to matrix interactions. Intensive research over the past decades has led to substantial knowledge about the function of cell adhesion molecules. Several groups of adhesion molecules can be distinguished: the integrins, the selectins, the immunoglobulin superfamily (IgSF), the cadherins, the CD44 family and the transmembrane proteoglucans.

The most versatile and widely distributed cell adhesion molecules are the integrins. In 1979 the first cell surface protein which was later classified as an integrin was

discovered on mouse myeloid leukocytes by the binding of a monoclonal antibody. This antigen was named macrophage differentiation antigen 1 (Mac-1; αMβ2) [9].

The term “integrin” for an adhesion molecule was firstly used by Tamkun *et al* in 1986 who described an integral cell surface protein, termed “integrin”, that established a transmembrane link between the extracellular matrix protein fibronectin with actin structures of the cytoskeleton [10].

Integrins soon received particular attention in various fields of research due to their diverse cellular functions ranging from adhesion of egg to sperm receptors (α6β1 [11]) over blood clotting (αIIbβ3 [12]) to metastasis and tumor growth (α4β1, α4β7, αvβ3, αvβ5 [13]), and leukocyte migration and activation (α4β1, αLβ2, αMβ2 [14]).

Many insights in the function and the importance of integrins have evolved by studying the patho-biochemistry of several inherited diseases. Glanzmanns thrombasthenia is a disease with hemorrhagic symptoms and bleeding disorders caused by a reduced expression of αIIbβ3 on platelets [15]. The expression of aberrant β2 subunits causes reduced inflammatory and immune responses summarized as “leukocyte adhesion deficiency syndrome” (LAD I). The various LAD phenotypes will be described in section 1.5.1.

Integrins are heterodimeric cell surface proteins consisting of two distinct, non-covalently associated subunits termed the α and the β chain. The N-terminal glycosylated ectodomains of both subunits are involved in ligand binding and specificity for a ligand. The C-terminal enterodomains point into the cytosol and are responsible for signaling and receptor activation. A total of 18 α and 8 β subunits are identified to date which can form 24 known α β heterodimers [16]. Since the β subunit can associate with different α subunits, the classification of integrins was based on the common β chain. As illustrated in Figure 1.1, 8 integrin subgroups (β 1-8) are classified to date. Some of the α chains are selective for a particular β chain, while others can form heterodimers with several β subunits. The most interesting α subunit in this respect is the αv chain which can associate with 5 different β chains.

The integrin subgroups also distinguish themselves by their ligand specificity. While β2 integrins mainly bind cell surface anchorage ligands of the IgSF, the β1 integrins (VLAs, very late antigens) mainly bind extracellular matrix proteins such as collagens, fibronectin, vitronectin, tenascin or laminin. Integrins of the β3 subgroup mainly bind to proteins found in the extracellular matrix (fibronectin, gelatin, vitronectin) as well as in serum proteins (fibrinogen, von Willebrand factor, thrombospondin).

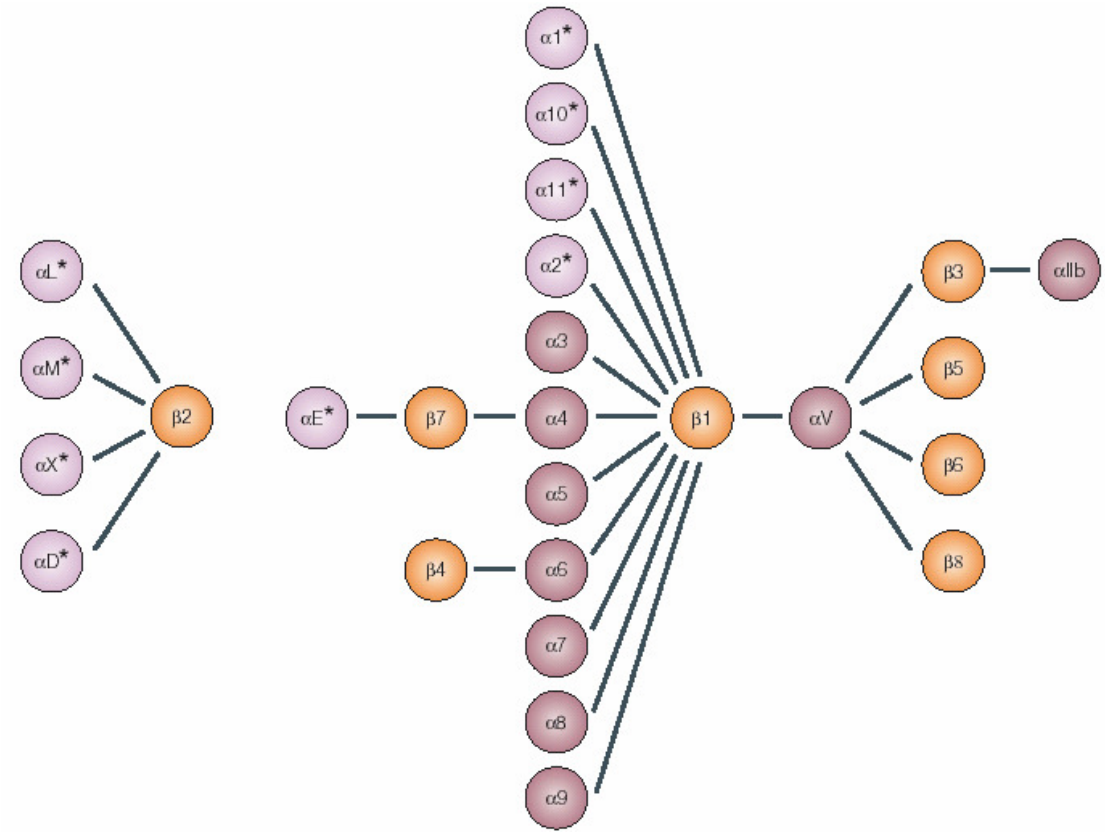


Figure 1.1 Integrins, a large family of adhesion receptors. Integrins are heterodimeric glucoproteins that consist of an α and a β chain. Fig. 1.1 illustrates the currently known possible associations between α and β chains. All chains with an asterix designate α chains that contain an inserted domain (I domain) in the N-terminal headpiece which is the major ligand binding domain of these integrins. Integrins are divided into subgroups according to their common β chains. Source: Shimaoka & Springer [16].

Integrins transduce signals after ligand binding in the classical “outside-in” direction triggering thereby cellular responses such as increased adhesiveness, changes in

cellular morphology, migration and anchorage dependent cell growth [17]. In the opposite way, cells are able to modulate the binding affinity for an integrin-ligand via intracellular signals (“inside-out“ signaling). It became clear, that many fundamental cellular processes base on the inside-out and outside-in signaling pathways and that these signals orchestrate the dynamics of integrin functions. The total integrin mediated cellular adhesiveness (**avidity**) is suggested to be dependent on the intrinsic strength of integrin-ligand bond (**affinity**) and on the number of these bonds (**valency**). It is today’s dogma that the dynamic regulation of integrin adhesiveness involves mutual modulation of these parameters [18].

1.2 LFA-1

1.2.1 LFA-1 history

LFA-1 (CD11a/CD18, αLβ2) was discovered in 1981 by the group of T.A. Springer who found that anti LFA-1 antibodies were able to block the killing activity of cytotoxic T-lymphocytes (CTLs) [8]suggesting a key function for LFA-1 in T-cell immunology [19]. Further studies showed that the molecule targeted by these antibodies participated also in NK-cell mediated killing as well as monocyte and granulocyte antibody dependent cellular cytotoxicity (ADCC) [20,21]. Later, it was demonstrated that LFA-1 mediated firm adhesion of leukocytes to the endothelium during transendothelial migration, a key process during lymphocyte homing and extravasation to sites of inflammation [22].

1.2.2 LFA-1 expression

LFA-1 is a heterodimer and consists of the αL (CD11a) and the β2 chain (CD18) and belongs to the β2 integrin subgroup which is selectively expressed by leukocytes. LFA-1 is expressed on all leukocytes subpopulations although on variable levels. The constitutive expression of LFA-1 appears to be dependent on the activation and differentiation state of a leukocyte. Naïve T-lymphocytes, for example, carry less LFA-1 on the cell surface than memory T-cells [23]. LFA-1 expression can be enhanced by cytokines. For example, IFNα was shown to increase αL expression on tissue

macrophages [24]. IL-4 upregulated LFA-1 expression on a B-lymphoma cell line [25]. In addition, viral products or oncogenes can affect the expression of LFA-1. CMV [26] and the oncogene *c-myc* [27] were shown to downregulate LFA-1 expression in certain cell types while EBV virus products upregulated mRNA and cell surface expression of αL [28].

The αL chain consists of 1145 amino acids (ca. 180 KDa) with 12 N-glycosylation sites [29]. The gene of the β2 chain (ca. 95 KDa) encodes 769 amino acids with 6 N-glycosylation sites. Next to differential expression of receptor numbers, cell type specific posttranslational modifications e.g. the number of glycosylations and the type of glycosylation introduce a degree of variation in the appearance of LFA-1 on the leukocyte cell surface. For instance, in thymocyte and spleen T-cells but not in monocytes or granulocytes or spleen B-cells the N-linked sugars are sulfated [30]. In addition, the LFA-1 receptors found on T-cells differ from that on B-cells or that on neutrophils by a the degree of sialic acid (B cell) [31] or CD15 (neutrophils) [32] expression. However, to which degree the posttranslational modifications of LFA-1 contribute to the functions of LFA-1 remains still to be elucidated.

1.2.3 LFA-1 ligands

The major ligands of LFA-1 are the three intercellular adhesion molecules -1, -2, and -3 (ICAM-1, -2, -3) [33]. ICAM-1,-2,-3 are structurally related glycosylated cell surface proteins which belong to the immunoglobulin superfamily (IgSF). ICAM-1 and ICAM-3 are constructed of five and ICAM-2 of two immunoglobulin (Ig) like domains from which the N-terminal domains 1 and 2 are responsible for binding to LFA-1 [34-37]. ICAM-1,-2,-3 can act as costimulatory signaling receptors by signaling via their cytoplasmic domains [38,39]. More specifically, LFA-1/ICAM interactions can trigger ICAM dependent signaling pathways leading to enhanced cellular activation and proliferation of T- and B-cells [40]. Besides, signaling via ICAM-1 was reported to trigger oxidative burst responses in monocytes [41].

LFA-1 binds with highest affinity to ICAM-1 followed by ICAM-2 and has the lowest affinity for ICAM-3. *In vitro* binding studies with soluble recombinant ICAM-1 and ICAM-3 showed binding affinities of 60 nM (ICAM-1) and 550 nM (ICAM-3) to purified

immobilized LFA-1 [42]. No affinity constant for the LFA-1/ICAM-2 interaction was found in literature.

ICAM-1

ICAM-1 (CD54, MW: 90kDa) was identified in 1986 [43]. The basal level of ICAM-1 expression is low on lymphocytes and endothelial-, epithelial cells and on fibroblasts and moderate on monocytes [23,43,44]. The cell surface expression of ICAM-1 is highly upregulated by numerous pro-inflammatory cytokines such as IFNα, TNFα, IL-6 or IL-1. There is experimental evidence that ICAM-1 is expressed on endothelial cells as a homodimer which may lead to enhanced valency and thus an increased binding strength to its counter receptors [45,46]. However, single monomeric ICAM-1 is a fully competent LFA-1 binding surface as shown by studies of Juen *et al* [47].

Serum levels of soluble ICAM-1 were found to be increased in different inflammatory and autoimmune diseases [45].

Mutational analyses defined that the amino acid residues Glu34, Lys39, Met64, Tyr66, Asn68, and Gln73 of ICAM-1 are crucial for the interaction with LFA-1. These 6 residues are suggested to form a rectangular binding surface for LFA-1 [48].

ICAM-2

ICAM-2 (CD102, MW: 55 kDa) is expressed on platelets, lymphocytes, monocytes and endothelial cells. ICAM-2 is the only leukocyte integrin ligand that is well expressed on resting endothelial cells. ICAM-2 is expressed only at low levels on not activated endothelium, the LFA-1/ICAM-2 interaction is suggested to be involved in the normal recirculation (homing) of lymphocytes through tissue endothelium. It has been shown that the LFA-1/ICAM-2 interaction augments T-cell receptor (TCR) mediated T-cell activation and triggers gene transcription processes [49]. In addition, soluble ICAM-2 was described to stimulate T-lymphocyte binding to ICAM-1 [50].

ICAM-3

ICAM-3 (CD50, MW: 110-140 kDa) is expressed only on leukocytes and absent on endothelial cells. It is highly and constitutively expressed on resting T-cells and antigen presenting cells (APCs) [51,52]. ICAM-3 is suggested to mediate the initial low affinity contact of a T-cell with an APC. This cell to cell contact is essential for coordinated T-cell activation [53]. Recently, experimental evidence has been established that solely cell-cell proximity is required to induce ICAM-3 clustering at the T-cell/APC contact region [53]. Furthermore, engagement of ICAM-3 can upregulate the affinity of LFA-1 for ICAM-1 [54]. Monoclonal antibodies to ICAM-1 and anti ICAM-3 have additive or synergistic effects in mixed lymphocyte reactions, proliferation and homotypic aggregation assays *in vitro* [51], again underlining the pivotal role of the ICAM-3 interaction with its ligands in T-cell immunology.

Additional but less characterized ligands for LFA-1 are ICAM-4 and the junctional adhesion molecule 1 (JAM-1).

ICAM-4 (MW: 42kDa) is identical to the LW (Landsteiner–Wiener) blood group antigen. The ICAM-4 glycoprotein contains two immunoglobulin domains of which the first domain is 30% identical to the first domains of ICAM-1, -2 and -3. The expression of ICAM-4 is restricted to erythrocytes and erythroid precursor cells and its function is restricted to erythropoiesis and apoptosis [55,56]. ICAM-4 is suggested to bind through novel motifs also to α4β1 and αIIbβ3 integrins [57,58].

JAM-1 (MW: 40 kDa) was established as an ligand for LFA-1 in 2002 [59]. JAM-1 belongs to the IgSF and consists of 2 Ig like domains. JAM-1 is expressed particularly at tight junctions of endothelial and epithelial cells, but can also be found at hematopoetic cell types. The LFA-1/JAM-1 interaction was suggested to guide and control chemokine induced transendothelial migration of T-cells and neutrophils [59].

1.3 Biological functions of LFA-1

Most functions of leukocytes are crucially dependent on coordinated sequences of adhesion events. LFA-1 is probably the most prominent adhesion molecule in the immune system, because it also establishes together with CD2/CD58 the adhesive contact between naïve T-cells and APCs. The establishment of this solid cross contact via LFA-1 and its ICAM-ligands is today recognized as the basis for effective T-cell activation [5]. LFA-1 was shown to be pivotal for T-cell effector function such as T-helper or killing function as well as processes such as transendothelial migration into sites of inflammation. LFA-1 mediated migration processes are fundamental for the lifecycle and function of all leukocytes.

As experiments shown in this thesis are largely concerned with the blockade of LFA-1 expressed on primary T-cells or T-cell lines, the following section focuses on the function of LFA-1 on T-lymphocytes.

1.3.1 LFA-1 as adhesion molecule in migratory processes

The adhesion molecule LFA-1 plays a pivotal role in the 3 major cellular migration processes that are crucial for T-lymphocytes: a) migration during cell development and differentiation, b) homing and c) transendothelial migration to sites of inflammation.

a) During T-cell development, the precursor T-cell has to migrate from the bone marrow to the thymus, where selection and maturation take place. T-cells migration from the thymus into the blood stream is mediated by adhesion molecules such as LFA-1.

b) Naïve T-lymphocytes continuously circulate from the peripheral blood to lymph nodes and secondary lymphoid organs and back into the blood stream. Naïve T-cells can migrate in an organ-specific manner. Specialized endothelial cells that line the high endothelial venules in lymph nodes and Peyer's patches constitutively express

so-called addressins, which support the homing of naïve lymphocytes, whereas other endothelial cells allow little or no leukocyte binding unless under inflammatory conditions [60]. Next to addressins, selectins and the integrins LFA-1, VLA-4 are α4β7 are involved in homing processes [61].

c) Inflammatory and immune disorders such as autoimmune diseases and graft rejection are largely mediated by activated T-lymphocytes. At the site of inflammation or in lymphoid organs, these T-cells drive and boost immunologic responses. A requirement for the inflammatory response is the migration of these T-cells from the blood stream into the target tissue thereby crossing the endothelial barrier. Lymphocytes which circulate in the blood generally can not establish an adhesive contact with endothelial cells because both endothelial and lymphocytes and their adhesion receptors are in a non activated state. Extravasation is initiated by an up-regulation of the endothelial ligands P- and E-selectin as well as ICAM-1, VCAM-1 and MAdCAM-1, which are triggered by inflammatory stimuli such as histamines, cytokines, endotoxins and complement fragments [62]. Primed effector T-cells express PSGL-1 and modified PSGL-1 which are the ligands for P- and E-selectin. The interaction between the selectins and their ligands allows to overcome the dynamic sheer forces of the blood stream and to tether lymphocytes which then start to roll on the inflamed endothelial cells. Recently, VLA-4 and α4β7 were shown to also mediate rolling of leukocytes [63] [64]. Carbohydrate trapped locally excreted chemokines such as MIP1α, MIP1β and Rantes can interact with chemokine receptors and activate the T-cell [65]. This leads to clustering and affinity upregulation of the adhesion molecules LFA-1, VLA-4, Mac-1 and α4β7 [60]. The latter bind to their ligands of the IgSF and mediate a firm arrest of the lymphocyte to the inflamed endothelium. Signaling events triggered by binding of chemokines to their receptors and signaling induced by LFA-1 and VLA-4 stimulate a change in cell morphology (flattening of the spherical shaped lymphocyte, building of the cellular uropod) and a rearrangement of surface receptors. The following migration process through the endothelium into the underlying tissue is a VLA-4, α4β7, Mac-1 independent, but LFA-1 dependent process. Junctional adhesion molecule 1 (JAM-1) is now recognized as the responsible ligand for LFA-1 for the trespassing through the borders of the

endothelial wall. The basic principle of transendothelial migration is exemplified in figure 1.2

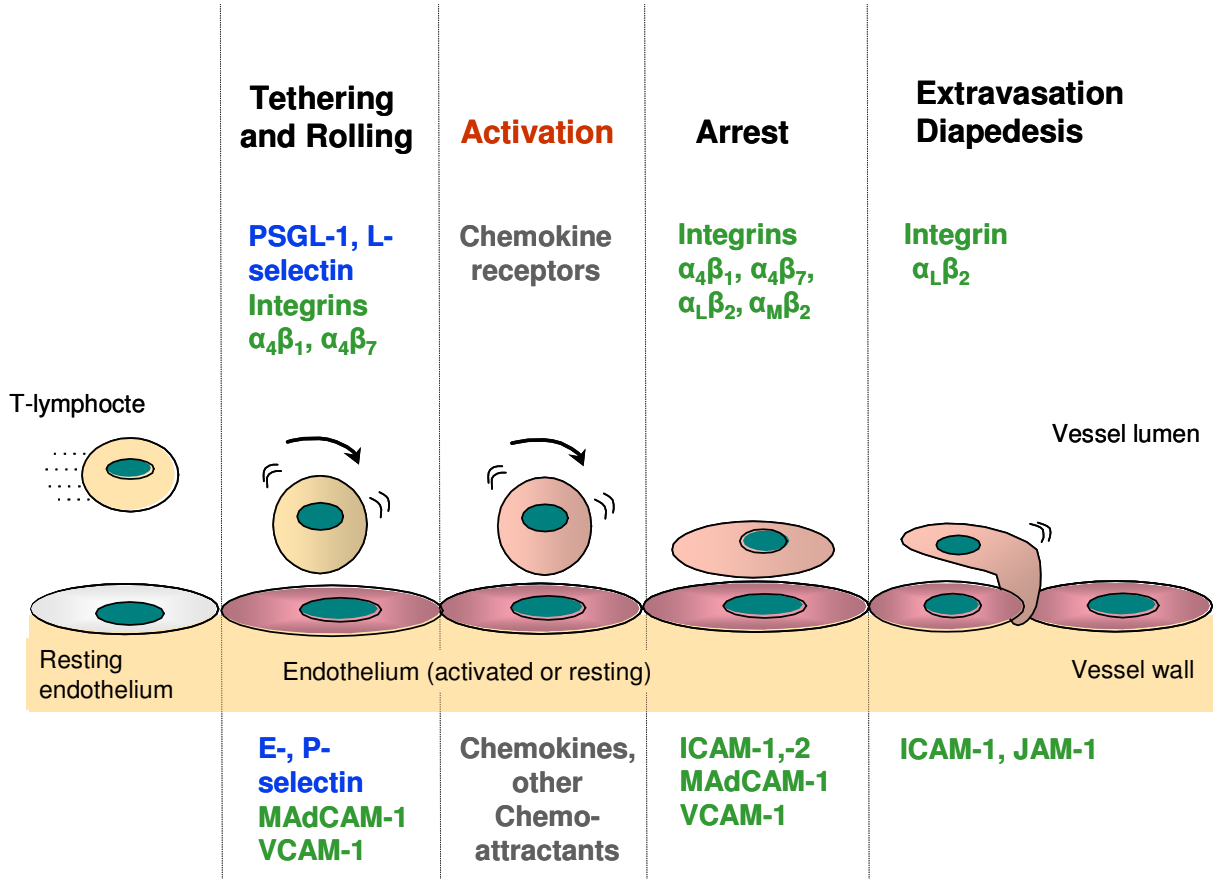


Figure 1.2 Basic principle of transendothelial migration of lymphocytes: Cell adhesion molecules of the selectin family and their ligands (blue), Integrins and members of the immunoglobulin superfamily (green), and chemokines and their receptors (grey), orchestrate the highly dynamic adhesive processes of leukocyte tethering and transendothelial migration. Endothelial transmigration can occur under inflammatory (activated endothelium) and not-inflammatory conditions (resting endothelium).

1.3.2 LFA-1 as participant in the immunological synapse

The discovery of the phenomenon of the “immunological synapse” (IS) has gathered a lot of interest lately. The IS is a specialized circular arrangement of T-cell surface molecules formed during T-cell activation and is proposed to enable an optimal presentation of the T-cell receptor to the antigen-MHC complex of APCs. Alternative

functions of the IS may be the polarization of the secretion of cytokines or the regulation of the endocytosis of the TCR. These prerequisites for an efficient adaptive immune response are suggested to be fulfilled by compartmentalization of adhesion-, costimulatory, cytoskeletal and signaling molecules which are organized in so-called “supramolecular activation clusters” (SMACS). Two SMAC zones of an IS have been defined. The central zone of a SMAC cluster (central SMAC) bears next to other molecules the TCR/CD3 complex and CD28 on the T-cell side which are in contact with the APC presented ligands. In addition, signal transduction enzymes such as PKCθ, Lck, Jak2 or PI3 kinase are found in the cytoplasmic side of a central SMAC. The central SMAC is surrounded by a peripheral SMAC in which LFA-1 and the actin cytoskeleton are located [53,66]. LFA-1 builds a peripheral ring around the central zone of a SMAC [67]. LFA-1 may therefore stabilize the T-cell-APC contacts and provide additional costimulatory signals necessary for T-cell activation [68].

1.3.3 LFA-1 as signaling receptor

One property of the integrin family is their ability to mediate bidirectional signaling events. Many cell surface molecules including GPCRs such as chemokine receptors and tyrosine coupled T-cell receptors can send intracellular signals that impinge on the short cytoplasmic tails of integrins and trigger increased adhesiveness of the extracellular domain (inside-out signaling, reviewed in sections 1.4.1-1.4.3). *Vice versa,* ligand binding to the extracellular domains of integrins can induce signaling events (outside-in signaling) that can regulate cellular growth, proliferation, differentiation and apoptosis [69]. Although integrins are fundamental in development, immunity, wound healing and metastasis, the basic signaling mechanisms induced by these receptors remain to be elucidated. This section is intended to outline the insights gained in LFA-1 induced signaling.

Early studies have shown that stimulation of the TCR in combination with ICAM-1 led to T-cell proliferation. This suggested that LFA-1 is an adhesion molecule that can also act as a signaling receptor [70]. However, solid biochemical evidence was missing that LFA-1 can mediate unique signals that are exclusively dependent on the LFA-1

receptor and are not initiated via the TCR or co-receptors such as CD28. Recently, Perez *et al* demonstrated that binding of LFA-1 to ICAM-2 could trigger two key phosphorylating signaling events leading to the activation of gene transcription [49]. The interaction of ICAM-2 with LFA-1 activated selectively the δ isoform of PKC which led to a phosphorylation of the β2 cytoplasmatic tail and cytohesin-1. Cytohesin-1 was found earlier to specifically bind to the cytoplasmic tail of the β2 chain which suggested a regulatory function of cytohesin-1 for LFA-1 adhesiveness [71]. Yet, it was unclear, whether cytohesin-1 could trigger signaling pathways via the LFA-1/ICAM interaction [72,73]. The phosphorylation of cytohesin-1 by PKCδ resulted in the activation of the MAPKinases ERK1 and ERK2 which target c-Fos, a "nuclear translocator protein". Phosphorylation of the cytoplasmic tail of the β2 chain released the "Jun activating binding protein 1" (JAB-1), a transcriptional co-activator that translocates after LFA-1 activation to the nucleus and interacts with c-Fos for building the AP-1 transcriptional regulatory complex that is essential for gene transcription [49]. These data, generated in a Jurkat cell line, suggested that LFA-1 can trigger at least two independent signaling pathways that converge at the level of regulators of gene transcription.

The effects of LFA-1 signaling on naïve T-cells were also investigated by Perez *et al.* The addition of LFA-1 signals to TCR and CD28 mediated signaling resulted in accelerated IL-2 production and entry of naïve T-cells into the cell cycle [49]. In addition, LFA-1 mediated signals enhanced the number of Th1 type T-helper cells in a cytohesin-1 and JAB-1 depended manner. In summary, there is evidence today that LFA-1 mediated signaling can occur independently from the TCR and that LFA-1 mediated signals can act as qualitative modulators in T-cell immunity.

Most research regarding LFA-1 function is done using T-cells. Nevertheless, it should be emphasized that all leukocytes express LFA-1. Yet, very little is known about cellular activation or signaling processes that happen after engagement of LFA-1 on B-cells, dendritic cells, monocytes or granulocytes. It is very likely that signaling and activation events can also modulate the biological function of these cells.

1.4 Molecular mechanism of LFA-1 adhesion

Integrins such as LFA-1 have the unique property that their adhesiveness can be dynamically regulated by intracellular mechanisms. This regulation is pivotal for integrin dependent processes which require well coordinated sequential actions such as lateral motion or chemokine driven endothelial transmigration. The term “inside-out signaling” explains that extracellular receptors can trigger signals leading to rapid up- or downregulation of integrin adhesiveness. Many cell surface receptors are known to increase LFA-1 adhesiveness after engagement. However, only few are described that trigger a down-regulation of LFA-1 adhesiveness. *In vitro* LFA-1 affinity can be rapidly activated by extracellular activating antibodies (Table 1.1) or bivalent cations (section 1.4.3).

Two synergistic mechanisms permit to dynamically regulate the total binding strength (**avidity**) of LFA-1 under physiologic conditions. Firstly, the modulation of the **affinity** of the individual LFA-1 receptor to its ligands (section 1.4.1) and secondly, the number of these receptor ligand bonds (**valency**) (section 1.4.2). Despite of recent advances in the understanding of how these two mechanisms are regulated, an integrated model, taking all regulatory parameters into account which orchestrate dynamic cell adhesion or migration, has to date not been established.

The following section intends to review the current understanding of the LFA-1 receptor structure and how LFA-1 affinity can be regulated on a molecular basis.

1.4.1 The structure of LFA-1 and the regulation of receptor affinity

Affinity enhancement of cell surface integrins requires conformational changes in the extracellular domains. These changes in conformation have been evident by the identification of monoclonal antibodies (mAbs) that bind preferentially to the activated / or ligand occupied forms of integrins [74,75]. Several mAbs were described that recognize activated forms of LFA-1. The mAbs 24 [76,77], CBR LFA1/2 [78], 327A, 327C, and 330E [79] and MEM48 [80] detect neoepitopes on the β2 chain that become exposed

after activation of LFA-1. In particular mAb 24 has led to the first experimental evidences that cations can induce conformational changes in the extracellular LFA-1 structure and that these changes correspond to an enhanced ligand binding affinity of LFA-1 [76].

The current model of LFA-1 receptor activation

LFA-1 consists of the αL and the β2 chain. The extracellular N-terminal ends of both chains interact with each other and build together the ligand binding headpiece of LFA-1. The αL and the β2 chains contain furthermore extracellular stalk regions, transmembrane domains and short cytoplasmic tails (Fig. 1.3).

The head domain of the αL chain consists of seven 60-amino acid repeats which have been predicted to fold into a seven bladed β propeller domain [81]. The head domain of the αL chain contains an inserted domain the so-called “I domain”. The αL I domain is the major ligand binding domain of LFA-1 as shown by mutational - and antibody blocking studies [82].
The αL I domain consists of 200 amino acids and is located between the β sheets 2 and 3 of the putative β-propeller region on the αL subunit [83-85]. The αL I domain is highly homologous to other integrin I domains which can be found in 8 out of the known 18 α subunits [74] (Fig.1.1). The three-dimensional structures of I domains revealed that they can adopt conformations similar to small G proteins with one metal ion-dependent adhesion site (MIDAS) at the top of the domain where ligands are bound [86]. There is strong evidence that the metal-ion of the αL MIDAS is coordinated by an acidic glutamate residue (Glu34) from the domain 1 of ICAM-1 and thus directly participates in ligand binding [48,87].

It has recently been demonstrated that the αL I domain can be locked in an **open**, ligand binding and a **closed**, non-binding conformation. This has been shown by mutational introduction of disulfide bonds between the C-terminal helix and a central β sheet of the αL I domain. Stabilizing the activated form of the αL I domain by

disulphide bonds increased the affinity for ICAM-1 by 10000 fold (from 2 mM to 200 nM) [88,89]. The positions of the disulfide bonds were modeled according to the crystal structures of I domains of Mac-1 (αMβ2, CD11b/CD18) and VLA-2 (α2β1, CD49b/CD29). These I domains have been crystallized in two conformations which disclosed that major shifts in the positioning of the C-terminal helices can occur [87,90]. Moreover, transfectants expressing mutant LFA-1 with alanine or tryptophane substitutions in the C-terminal α7 helix region of the αL I domain showed impaired or constitutively active binding to ICAM-1 [2,91]. These studies suggested that the C-terminal α7 helix may be involved in regulating the conformation of the αL I domain.

Integrin β subunits contain regions structurally homologous to the I domains, the so-called I-like domains [92,93]. The existence of such I-like domains was recently confirmed by the crystal structure of the extracellular segment of the integrin αVβ3 [94]. The I-like domain of the LFA-1 β2 subunit (β2 I-like domain) is predicted to contact the β propeller of the αL subunit near β sheets 2 and 3 [95]. The β2 I-like domain contains 3 metal-ion binding sites. The central MIDAS is proposed to directly ligate to a putative intrinsic ligand on the αL chain. The outer ion-binding sites are termed as “ligand-induced metal-ion binding site” and the “adjacent MIDAS” based on findings with the integrin αvβ3 [16].

The current model of LFA-1 activation suggests that the conformation of the αL I domain is directly regulated by the β2 I-like domain [89]. The β2 I-like domain is proposed to undergo, analogous to the αL I domain, a conformational rearrangement that upregulates the affinity of the β2 I-like domain MIDAS for a putative intrinsic ligand by a downward movement of the β2 I-like domain C-terminal α-helix. The putative ligand for the β2 I-like domain MIDAS is probably the conserved glutamate residue 310 (Glu310) that is located close to the C-terminal α7 helix of the αL I domain [16]. The interaction between the β2 I-like domain and Glu310 may enable a downward pull of the C-terminal α7 helix of the αL I domain in which then leads to the open form (high affinity) of the αL domain [18]. The presence of regulatory elements in the β2 chain was further substantiated by the finding that all mAbs that triggered LFA-1 adhesiveness (Table 1.1) mapped to epitopes located on the β2 chain. In

agreement, almost all anti CD18 mAbs mapping to the β2 I-like domain, blocked LFA-1 adhesiveness and this presumably in an allosteric manner [89].

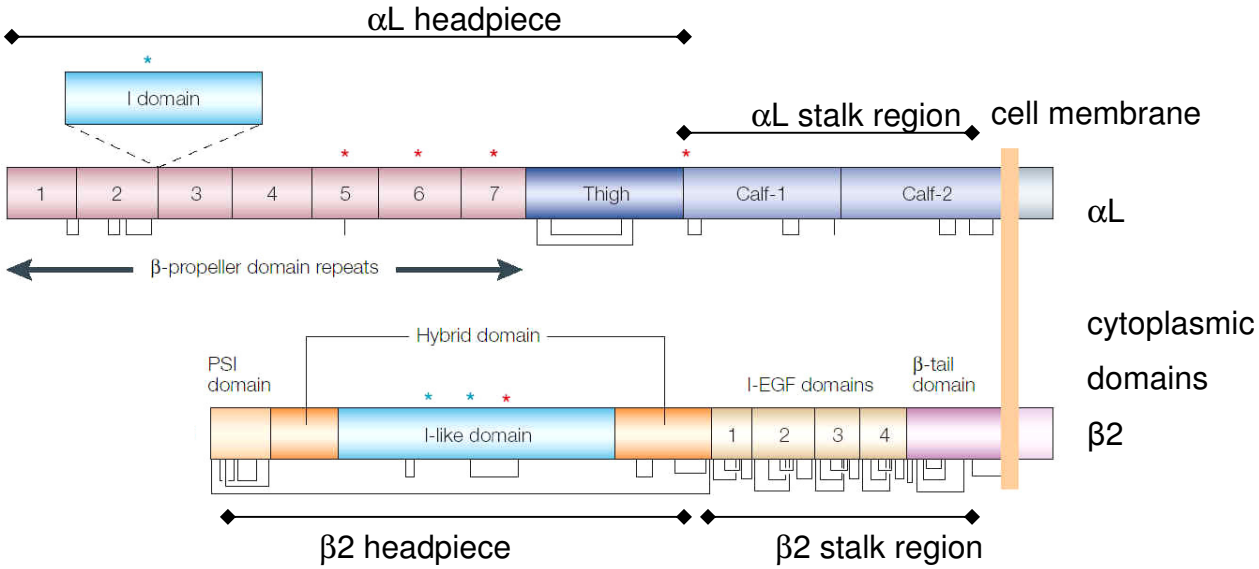


Figure 1.3 Schematic structure of LFA-1. The ligand binding domain (I domain) is inserted between the β propeller repeats as denoted by the broken lines. The β2 I-like domain is flanked by parts of the so-called hybrid domain. Cystein and disulphide bonds are shown as lines below the stick figures. Red and blue asterisks denote Ca^{2+}- and Mg^{2+}-binding sites, respectively. Each domain is color-coded: I-EGF: integrin-epidermal growth factor domain; PSI: plexin/semaphorin/integrin. Source: Shimaoka *et al* [16], modified drawing.

Figure 1.4 illustrates the present model of LFA-1 receptor activation showing only the ligand binding headpiece of LFA-1. It should be noted, that this model was not existing when the work for chapter 1 (in year 2001-2002) was conducted and that the work of chapter 1 has partially contributed to the proposed model.

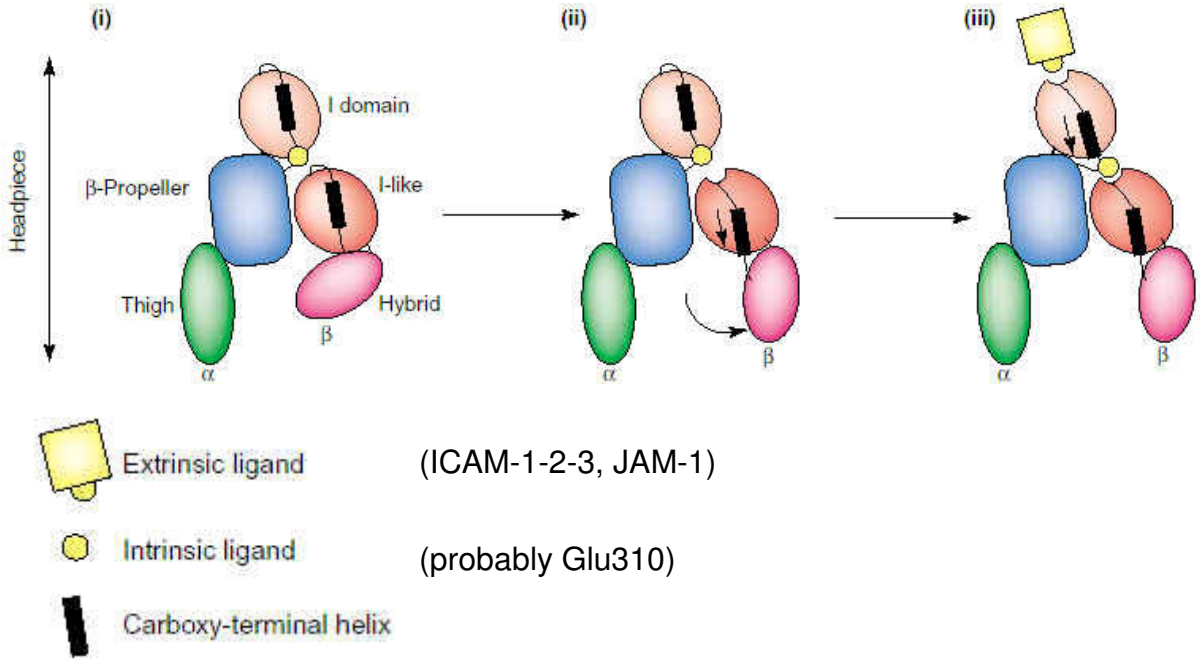


Figure 1.4 Activation of LFA-1 by conformational rearrangements in the headpiece of LFA-1: A black cylinder and curved line coming from the 'top' of the αL I domain represents its C-terminal α7 helix and the β6–α7 loop, respectively. In addition, the linker connecting the C-terminus of the α7 helix to the β-propeller domain is drawn as a curved line coming from the bottom of the αL I domain α7 helix and connecting to the β-propeller domain. The glutamate (Glu310) that is postulated to serve as an intrinsic ligand for the β2 I-like MIDAS is depicted as a yellow ball. (i) Closed head piece. (ii) Open headpiece transition. The hybrid domain of the β2 chain swings out and conformational changes in the β2 I-like MIDAS proceed. (iii) Open headpiece. The open β2 I-like MIDAS binds to the intrinsic ligand in the linker (Glu310), exerting a pull on the α7 helix. This causes the C-terminal α7 helix it to move down and the αL I domain MIDAS to shift into the high-affinity conformation. Source: Carman and Springer [18].

In addition to the conformational changes within the headpiece of LFA-1, large scale conformational changes in the entire heterodimer occur during activation and ligand binding. These rearrangements have been visualized by NMR, by electron microscopic studies and by atomic structures using purified extracellular LFA-1 domains and superimposing the results on the X ray structures of the αvβ3 integrin [96,97]. These studies revealed surprising details how integrins can alter their conformation. The latest model of conformational changes in whole dimeric integrins is reviewed by Carman and Springer [18].

It is the current understanding that LFA-1 has a full repertoire of different conformations ranging from a bent conformation (low affinity, closed form of the αL I domain) to intermediate conformations (extended conformation of the entire receptor but closed or intermediate form of the αL I domain) to a fully extended conformation (highest affinity for a ligand, open form of the αL I domain) (Fig.1.5). During LFA-1 activation, the entire receptor appears to switch from a bent to an extended conformation in a "switchblade" kind of motion. It can be assumed that the switchblade opening of the integrin may also serve to enhance the presentation of the αL I domain to the juxta-positioned ligands.

The conformational changes in the extracellular domains of LFA-1 are suggested to be regulated by the interaction between the cytoplasmic tails of the αL and the β2 chains. Experimental evidence for this interaction has been provided by two independent groups who showed that mutations in the membrane proximal sites of the cytoplasmatic domains of the LFA-1 resulted either in constitutive integrin activation or in an inactive LFA-1 [98,99] . In particular the GFFKR motif which is located on the αL cytoplasmic tail may play a major role in modulating LFA-1 function. The GFFKR motif is exposed after removal of the cytoskeleton linkage during activation. It is likely that this motive plays a role in activation of LFA-1 as the deletion of this region resulted in a constitutively activated LFA-1 [102,103].
In contrast, the introduction of an artificial clasp in the cytoplasmic domains resulted in an inactive LFA-1 [69]. The close association of the αL and β2 cytoplasmic and transmembrane domains may thus constrain a low affinity state of LFA-1. FRET analyses in living cells provided first evidences that spatial separation of the cytoplasmic domains of the αL and the β2 chains are responsible to control the LFA-1 affinity to ICAM-1 (inside-out) [69]. Figure 1.5 summarizes the knowledge about the switchblade model of LFA-1 receptor activation.

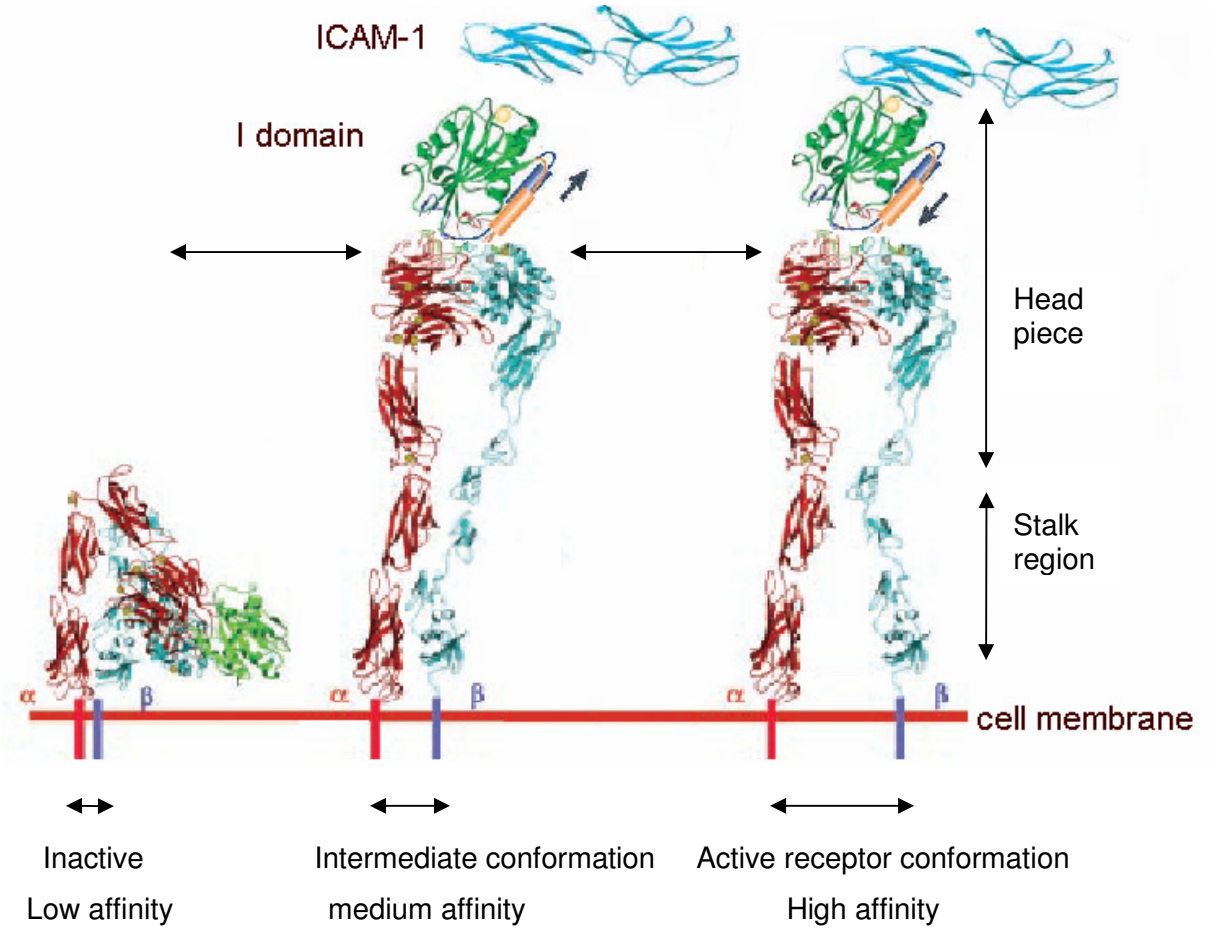


Figure 1.5 Current "switchblade" model of the LFA-1 receptor activation: Different conformations allow the regulation of LFA-1 affinity. Blue ribbons show the ICAM-1 (domains 1 and 2). Green ribbons indicate the αL I domain. Spatial separations of the α and β cytoplasmic chains (red and blue bars) initiate the switchblade–style opening of the LFA-1 receptor from a bent to an extended conformation. These arrangements may induce conformational changes in the β2 I-like MIDAS which regulate the conformation and ligand binding affinity of the αL I domain. The picture was assembled for this thesis from Shimaoka and Springer [16] and Kim *et al* [69].

There are two known distinct ways to induce the spatial separation of the LFA-1 cytoplasmic chains.

Firstly, the cytoplasmic domains of LFA-1 are known to interact with members of the cytoskeleton during inside-out signaling events. The cytoskeletal protein talin is known to associate with cytoplasmic domains of LFA-1 and other integrins. This association was suggested to modulate integrin adhesiveness. Isolated talin head

pieces were shown to associate with cytoplasmic domains of several chains [69]. Over-expression of talin modulated LFA-1 affinity and suggested that the talin head piece can promote a spatial separation of the αL and the β2 cytoplasmic chains which stabilizes a high affinity conformation of LFA-1 [69]. Other proteins may be involved in regulating the high affinity conformation. Rap1, a small GTPase, has emerged as important effector protein for chemokines and cytokines. Rap1 was shown to act as a regulator for several integrin subgroups [104]. Over-expression of dominant negative forms of Rap1 blocked TCR induced upregulation of LFA-1 adhesiveness [105] while a defective regulation of RAP1 led to leukocyte adhesion deficiency syndrome in man [106]. RAPL was recently identified as effector molecule of Rap1 [105]. It is still speculation, however, whether the Rap1/RAPL interaction directly promotes the active conformation of LFA-1 or whether these regulators enhance the valency (*vide* 1.4.2) of the interaction. Integrin cytoplasmic domain associated protein-1 (ICAP-1) may be another candidate for intracellular LFA-1 affinity regulation. ICAP-1 is suggested to act synergistically with talin head domains of β1 integrins [107-110].

Secondly, ICAM-1 binding to extracellularily activated LFA-1 was found to induce significant spatial separations of the cytoplasmic domains which may lead to the triggering of “outside-in” signals [69]. The transmission of signals by integrins across the plasma membrane may therefore occur bi-directionally and may be mediated by coupling spatial separation of cytoplasmic domains to conformational changes of the extracellular chains of LFA-1. The study which was published 2003 in Science by Kim *et al* was the first biochemical evidence for a distinct molecular mechanism how information can be transmitted bi-directionally across the plasma membrane [69].

1.4.2 LFA-1 clustering

Early studies revealed that LFA-1 and the ICAM ligands can form clusters or patches upon cellular activation [111,112]. LFA-1 clusters were recognized to increase the overall binding strength (avidity) of LFA-1 dependent adhesion processes. Receptor- or ligand clustering can be induced by processes such as random diffusion,

oligomerisation, ligand dependent redistribution, intracellular trafficking or polarization.

Recently, the terminology was redefined to categorize the different modes of integrin reorganization leading to increased valency [18]. These can be differentiated into a) large scale LFA-1/ligand reorganizations detectable by fluorescence microscopic techniques and b) microcluster formation on submicroscopic scale which can only be visualized by sophisticated methods such as FRET or BRET technology.

Both large reorganizations and microcluster formation can be triggered via ligand binding or occur in a ligand independent way. Clustering in a ligand dependent way requires the presence of locally clustered or mulitmeric ligands. For the ICAM ligands of LFA-1 clustering was shown to be induced via chemotactic and proinflammatory chemokines such as RANTES, MCP-1, MIP-1 α, MIP-1 β, and IL-8 [65] as well as other stimulators (Table 1.1)

1.4.3 Activation of LFA-1 mediated adhesion

The activation of LFA-1 via inside-out signaling pathways has been the focus of numerous studies. Crosslinking of various cell surface molecules by antibodies or treatment of lymphocytes with protein kinase C activators rapidly induced LFA-1 mediated adhesion to ICAM-1 (Table 1.1). This “inside-out” activation of LFA-1 requires intact and metabolically active cells. *In vitro* studies with inside-out activators suggested that only a minority of LFA-1 receptors undergo conformational changes as measured by the expression of activation epitopes on the β2 chain. Beals *et al* suggested that 20% of LFA-1 receptors are activated after PMA activation while only 10% of the LFA-1 receptors show the expression of activation related neoepitopes after stimulation of T-lymphocytes with mAb CD3 [79]. It can be assumed that *in vivo* only a minor number of LFA-1 receptors per cell are in a high affinity conformation. Table 1.1 summarizes the methods to induce LFA-1 dependent adhesion *in vitro*. Activation of LFA-1 with cations is generally used in *in vitro* binding assays thereby circumventing the need for metabolically active and intact cells.

Table 1.1 Inside-out and extracellular modulators of LFA-1 adhesiveness

Stimulus	Comment	Ref
Monoclonal antibodies		
Anti CD2	Persistent activation of LFA-1 dependent adhesion	113
Anti CD3	Transient activation of LFA-1 dependent adhesion	79,113
Anti TCR	Transient activation of LFA-1 dependent adhesion	114,115 116
Anti CD4	Antigen independent T-cell adhesion	117,118
	Dephoshorylation of CD4 by CD45 may negatively regulate LFA-1 mediated signaling	119
Anti CD7	Crosslinking of the T-cell-specific accessory molecules CD7 modulates T-cell adhesion.	120
Anti CD14	LPS binds to CD14 activates monocytes and their LFA-1 mediated adhesion	121
Anti CD28	Crosslinking of the T-cell-specific accessory molecules CD28 modulates T-cell adhesion.	120
Anti CD44	Crosslinkage with anti CD44 mAb leads to Mg^{2+} and Ca^{2+} independent activation pathway	
Anti CD45	Enhanced adhesiveness only in activated but not in resting T-cells	122,123
Receptor ligand interactions		
fMLP	As for IL-8 long lasting adhesiveness	124
IL-2&IL-12,	Synergistic effect of IL-2&IL-12;	125,126
	IL-12 upregulates CCR5 (inside-out receptor)	
IL-8	Sub nanomolar IL-8 stimulated rapid redistribution of active LFA-1, but not Mac-1 on neutrophils.	127
	Sub nanomolar IL-8 stimulated LFA-1 dependent neutrophil adhesion to ICAM-1	128
Chemokines/ Chemokine receptors	In principle all chemokines and the engagement of their receptors should utlimatively lead to integrin dependent adhesion and migration. Which integrin is activated is for many chemokines not depicted yet. Rantes/CCR5, SLC (CCL21), SDF-1 (activates Rap-1), MIP1α and MIP1β have been shown to mediate LFA-1 dependent effects.	65 126,129,130 65,131
ICAM-2 and ICAM-3	ICAM-2/-3 interact with LFA-1 and may regulate the LFA-1/ICAM-1 cell adhesion	50,132

Table 1.1 continued

Chemical agents		
Ionomycin	Ca^{2+} ionophor	133
PMA	PKC activator	79
Extracellular LFA-1 activators (directly acting on the LFA-1 receptor)		
Cations		
Calcium ($CaCl_2$)	Extracellular Ca^{2+} modulates LFA-1 cell surface distribution on T-lymphocytes and consequently affects cell adhesion. Role of Ca^{2+} is controversial.	134
	Ca^{2+} competes with magnesium activation	135
Magnesium ($MgCl_2$)	Usually used at 2 mM in adhesion assays	136 135 116
Manganese ($MnCl_2$)	Usually used at 1-2 mM with or without additional 2 mM $MgCl_2$ for adhesion assays	135
LFA-1 antibodies that increase ligand binding		
Anti CD18	Clone KIM185 and KIM127	137 138 69
Anti CD18	Clone MEM83	139
Anti CD18	Clone CBR LFA-1/2	140
Anti CD11a	Clone NKI L16 Induces LFA-1 dependent cell aggregation.	139 141 134

1.5 LFA-1 as a therapeutic target

The characterization of the phenotype and the underlying cause of disease in LAD I (1.5.1) patients suggested LFA-1 as a potential target to interfere with inflammatory immunological pathways. Besides, the development of LFA-1 knockout mice (1.5.2) allowed studying the effect of absent LFA-1 on the immune system. Support for LFA-1 as a therapeutic target for several indications was established in rodent models of disease using blocking anti LFA-1 antibodies (1.5.3). Today, LFA-1 is well established as therapeutic target for treatment of immunologic disorders. Anti LFA-1 antibodies have been clinically tested and one (efalizumab) has been approved recently for clinical use (1.5.4). Several chemical classes of low molecular weight LFA-1 inhibitors are described in this section (1.5.5).

1.5.1 Leukocyte adhesion deficiency (LAD) syndrome

One hallmark of this rare disease is the recurrence of life-threatening bacterial infections and impaired would healing. According to the molecular reason for impaired leukocyte adhesion three types of LAD diseases are distinguished to date:

LAD I: Individuals afflicted with LAD I lack the expression of the β2 integrins LFA-1 , Mac-1 and p150/95 on the cell surface of leukocytes due to autosomal recessive inherited mutations within the β2 chain encoding gene INTG2 [142,143]. The severity of this disease has been linked to the degree of β2 integrins expressed on the leukocyte surface. Interestingly, in LAD I patients, T helper - or B-cell responses, and the susceptibility to viral infections appeared to be nearly normal [142]. This was surprising as LFA-1 contributes to T-cell driven humoral and cellular defense mechanisms. Maybe redundant mechanisms can substitute missing β2 integrins and foster T-cell and B-cell dependent immune responses in these patients.

LAD II patients have a defect in the gene encoding a putative GDP-fucose transporter [144]. This leads to hypofucosylated lactosaminoglycans such as sialyl-Lewis^x which are ligands for the selectin adhesion receptor family [145]. Impaired interaction of selectins with their ligands causes leukocyte adhesion defects which result in immunodeficiency syndromes similar as seen in LAD I patients. LAD II was reviewed by Wild *et al* [145].

Patients with **LAD III** disease show normal surface expression of integrins and selectin ligands, but have a defect in chemokine receptor induced activation of β2 integrins as described by Alon *et al* [146,147]. These patients appeared to have a defect in the regulation of the small GTPase, Rap1 which is a key regulator of inside-out integrin activation [106].

1.5.2 Phenotype of LFA-1 knockout mice

Targeted deletion of the αL chain of LFA-1 in mice resulted in a phenotype of decreased lymphocyte recirculation, decreased leukocyte adhesion to the ligand intercellular adhesion molecule ICAM-1, and decreased delayed-type hypersensitivity

reactions. αL deficiency caused a selective defect in the induction of peripheral immune responses whereas responses to systemic viral infections were described as normal. In LFA-1 (-/-) mice it was found that α4β1 (VLA-4) and α4β7 have a hitherto unrecognized ability to compensate for the lack of LFA-1 in migration to peripheral lymph nodes [148].

1.5.3 Synopsis of the effect of LFA-1 antibodies in rodent models of disease

Monoclonal anti LFA-1 antibodies (aCD11a mAbs) have been successfully employed in animal studies and have provided an early proof of concept that LFA-1 plays pivotal roles in these experimental models. These animal models built also the basis for potential indications for therapeutic LFA-1 inhibition. The most striking effects of aCD11a mAbs were provided in rodent models of transplantation. Treatment with aCD11a mAb alone led to prolongation of cardiac allograft survival [149]. In combination with an anti ICAM-1 mAb indefinite survival of the cardiac allograft was achieved after administration of the biologicals for 6 days [150,151]. This tolerance induction was, however, dependent on the combinations of mouse strains used [150]. In some animal models, particularly in transplantation models, aCD11a mAb therapy proved to be only efficacious if used as an induction therapy, while aCD11a mAbs were not able to block graft rejection in sensitized animals [152]. This indicated that aCD11a mAb treatment can block initial lymphocyte activation processes which may add next to the anti-migratory effects, an immunomodulatory property to a drug targeting LFA-1.

Further animal studies of integrin blockade with antibodies were reviewed in [153]. It should be noted that anti CD18 and anti CD11b (Mac-1) were found most efficacious in models of ischemia reperfusion injury indicating that Mac-1 and LFA-1 may play synergistic roles in these diseases. However, long term blockade of Mac-1 did result in nearly all experiments in increased susceptibility of the animals to fungal and bacterial infections, a phenotype mirrored in LAD I patients. Table 1.2 gives an overview about the rodent models in which anti LFA-1 antibodies have been successfully employed. Many of these models support LFA-1 as therapeutic target in inflammatory and autoimmune disorders as well as in several infectious diseases.

Table 1.2 Synopsis of the effect of anti LFA-1 antibodies in rodent models

Disease of animal model	Antibody	Effect	Refs.
Murine AIDS	aCD11a, aCD54	Inhibited disease development and lowered serum Ig levels	154
Cerebral malaria	aCD11a	Prevented specifically cerebral malaria aCD11b, aCD54 or aCD49d had no effect	155
Cerebral malaria	aCD11a (not CD54)	Abrogated cerebral malaria	156
Collagen induced arthritis	aCD11a	Suppressed arthritis	157
Experimental murine lupus	aCD11a	Suppressed auto-reactive antibody formation	158
LPS induced lethal shock	aCD11a	Protected from liver injury	159
EAE	aCD11a, aCD54	Contradictory effects: No effect Suppressed disease progression	160,161
Skin inflammation	aCD11a	Reduced dermal inflammatory reactions to LPS, zymosan IL-1 activated serum)	162
fMLP induced skin edema	aCD11a	Reduced edema, blockade of infiltrating leukocytes to sites of inflammation	163
DTH	aCD11a, aCD54	Decreased ear swelling	164 157
Antigen specific unresponsiveness	aCD11a, aCD54	Induced tolerance	150
Experimental autoimmune uveitis	aCD11a, aCD54	Reduced ocular inflammation	165
Thioglycolate induced peritonitis	aCD11a	Reduced neutrophil immigration	2
Graft versus host disease	aCD11a	Reduced severity and enhanced survival in allogenic mice	166
Acute graft rejection cardiac allograft	aCD11a	Prolonged allograft survival	150
Acute graft rejection cardiac allograft	aCD11a	Indefinite graft survival	152
Acute graft rejection cardiac allograft	aCD11a aCD54	Indefinite graft survival	151 150
Acute graft rejection: thyroid gland	aCD11a	Complete inhibition of rejection	167
Murine islet transplantation	aCD11a	Increased survival	168,169

1.5.4 Anti LFA-1 antibodies in clinical studies

While the development of humanized mAbs against LFA-1 ligands ICAM-1 (Enlimomab, Boehringer Ingelheim Inc.) [170,171] and ICAM-3 (ICOS Inc.) has been discontinued [172], two blocking anti LFA-1 mAbs (Odulimomab, Efalizumab) have shown benefits in clinical trials.

In 1989 the first anti human CD11a antibody named odulimomab (IMTIX-Sangstat, murine IgG1, clone 25.3) was in clinical trials for HLA mismatched bone marrow transplantation [173] in children and kidney transplantation as reviewed by M. Behrend [174]. Odulimomab was beneficial for the treatment of steroid resistant graft versus host disease [175,176] and on the rate of engraftment in children receiving a HLA non identical bone marrow graft [177] while it was therapeutically not successful in adults [178]. When used in combination with an anti CD2 antibody odulimomab prevented in a phase II clinical trial the rejection of partially incompatible bone marrow transplants in leukemic children [179]. Odulimomab showed a trend towards efficacy in renal transplantation when given as induction therapy [180] but was not effective in the treatment of acute kidney graft rejection [181]. The development of odulimomab was discontinued [182] after a clinical trial in phase III failed to show efficacy in kidney transplantation. Anti LFA-1 therapy with odulimomab was tolerated well in these studies.

Efalizumab (Raptiva®) was developed by Genentech Inc. and Xoma Ltd. and is the first humanized anti CD11a mAb (IgG1) that was tested in clinical studies. Efalizumab was tested is psoriasis, an inflammatory autoimmune diseases of the skin which is characterized by strong infiltration of leukocytes and a strong proliferation of keratinocytes. Psoriasis is recognized as a T-cell dependent disease which can be demonstrated by the therapeutic use of calcineurin inhibitors CsA and FK506, classic immunosupressants which strongly interfere with T-cell activation processes. Efalizumab treatment led to an internalization of LFA-1 on circulating leukocytes within a day of treatment [101]. A second pharmacodynamic effect of efalizumab was the increase in lymphocyte counts in the peripheral blood by a factor of 2. Recently, Gordon *et al* reported the outcome of a phase III trial on psoriatic patients

demonstrating efficacy of efalizumab in patients with moderate to severe disease. 27% (47%) of patients treated with efalizumab showed an improved PASI (DLQI) score vs. 4% (14%) placebo. Efalizumab was generally well tolerated [183]. Clinical data suggested that concentrations of efalizumab which correspond to saturated receptor occupancy by the antibody were essential for efficacy [184,185]. In October 2003 efalizumab has been granted FDA approval for the treatment of chronic, moderate-to-severe plaque psoriasis in systemic therapy.

Efalizumab was tested in renal transplantation for 24 weeks as adjunctive to the standard therapy (CsA plus MMF and steroids) in a phase I/II trial. There was only a trend towards a lower incidence of acute rejection; however, more extensive studies are necessary to show the significance of a benefit in renal transplantation [186].

1.5.5 Low molecular weight LFA-1 inhibitors

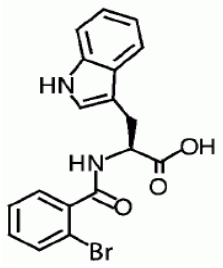
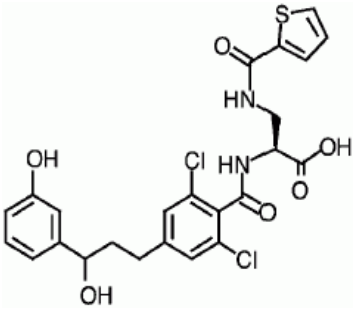
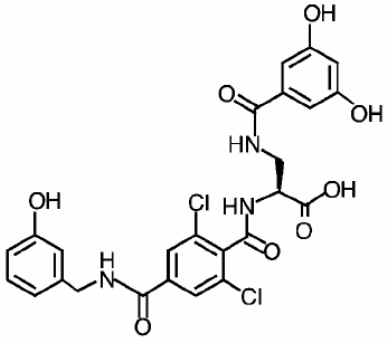
Administration of therapeutic anti LFA-1 mAbs requires delivery via injection or infusion. To circumvent this, strong efforts are ongoing to discover and develop orally available low molecular weight (LMW) inhibitors that can interfere with LFA-1 function. The blockade of the LFA-1/ICAM interaction was initially regarded as challenging as a LMW competitive inhibitors (Mol. Weight <1000) would have to interfere with the large protein-protein interactions (300-400 kDa). A good argument to foster the efforts to discover LFA-1 antagonists came from the discovery that ligand mimetics (RGD-mimetics) could effectively block the function of β1 and β3 integrins. αIIbβ3 (GPIIb-IIIa) blocking ligand mimetics (lamifiban, eptifibatide or tirofiban) have been shown efficacious in clinical studies [187] and have become important drugs in cardiology since a few years as i.v. anti-thrombotic agents [188]. Extensive clinical trials are ongoing with lefradafiban, sibrafiban and orbofiban as orally available αIIbβ3 blockers [188,189]. To date no LFA-1 specific LMW inhibitor has entered clinical studies. This section is intended to introduce currently followed structural classes of LMW LFA-1 inhibitors.

Compounds designed based on the ICAM-1 binding motif (ICAM-1 mimetics)

Many efforts that were undertaken to design competitive LFA-1 inhibitors based on the 6 amino acid residues of the ICAM-1 domain 1 that are mandatory for binding to LFA-1 [4,48]. Many groups have reported ICAM-1 derived peptides; however, no ICAM-1 domain 1 mimetic was further developed possibly due to low potency and limited bioavailability.

In 2002, a group of Genentech reported the successful translation of the LFA-1 binding site of ICAM-1 into a low molecular mimetic structure. Compounds were designed based on the 2 amino acid residues Glu34 and Tyr66 which are essential for the interaction with LFA-1 [4,48]. Based on an *ortho*-bromobenzoyl tryptophan backbone several compounds were synthesized that blocked ICAM-1 binding to purified LFA-1 with low nM potencies [4]. The most potent derivative described (e.g. compound #4, Table 1.3) inhibited the human mixed lymphocyte reaction (MLR) ca. 20 times more potently than cyclosporin A and 7 times more potently than anti CD11a Fab fragments [4]. In murine contact hypersensitivity models compound 4 showed activity after continuous infusion. The authors proposed that these compounds act as ICAM-1 proteomimetics, provided, however, no experimental evidence that these compounds have a competitive mode of action [4]. In addition, no information on the integrin specificity of this compound class was given in the patent or in the publication. Derivatives of this compound class (e.g. compound #5) were patented by Roche [190].

Table 1.3 Examples for LFA-1/ICAM-1 inhibitors based on the ICAM-1 domain 1

Structure			
Names	Compound #1 *ortho*-bromobenzoyl tryptophan	Compound #4	Compound #5 (XVA143)
Potency	LFA-1/ICAM-1 ELISA: 1.4 µM MLR: n.d.	LFA-1/ICAM-1 ELISA: 1.4 nM MLR: 3nM	LFA-1/ICAM-1 ELISA: n.d. MLR: n.d.
Company	Genentech	Genentech	Roche
References	[4]	[4]	[190]

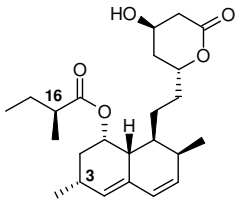
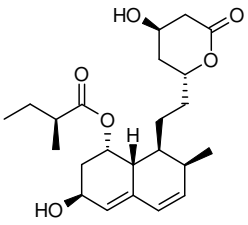
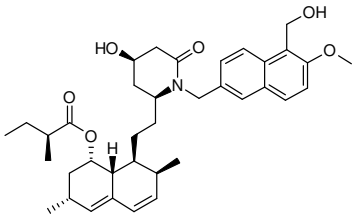
Lovastatin derivatives

The first LMW antagonist for LFA-1 was described by our research group in 1999. The group discovered by high throughput screening that the HMG CoA reductase inhibitor lovastatin interfered with LFA-1/ICAM-1 dependent cellular adhesion. X-ray analysis and crystallography of the lovastatin αL I domain revealed that lovastatin binds to a hitherto unknown binding site within the αL I domain. This region has not been implicated as part of the binding site for any of the ICAMs (ICAM-1 [191], ICAM-2 [192], ICAM-3 [193]) as well as mAbs that inhibit LFA-1 mediated adhesion map not to this site [191,194,195]. The site was thereafter termed “lovastatin binding site” (L-site) [2,196] . The αL L-site is identical with the “I domain allosteric site” (IDAS) described later by others [91]. Occupation of the αL L-site by lovastatin modulates the αL I domain conformation thereby allosterically modulating the adhesive function of LFA-1 [1]. Mutational analyses further substantiated that the αL L-site is a regulatory site for the

αL I domain as some point mutations completely abrogated binding to ICAM-1 while one mutation constitutively activated LFA-1 [2]. LMW inhibitors (lovastatin derivatives, hydantoins and cinnamides) were described to block the downward axial displacement of the C-terminal helix of the αL I domain and inhibited ICAM binding by stabilizing the αL I domain in a low-affinity (closed) conformation [1,89,196]. These inhibitors have thus the equal mode of action and can be regarded as αL L-site inhibitors.

Lovastatin and the ten times more potent derivative LFA703 were able to block adhesion to ICAM-1 and interfered with ICAM-1 dependent T-cell costimulation *in vitro*. The structurally similar HMG CoA reductase inhibitor Pravastatin did not interfere with LFA-1 function [2]. Lovastatin derivatives were shown to be highly active after oral administration in thioglycolate induced murine peritonitis models (ED_{50} 0.4 µg/kg) [2,89]. In a different study, LFA703 has recently been described as being protective in ischemia/reperfusion-induced leukocyte adhesion in the murine colon [197]. Lovastatin-derived LFA-1 inhibitors were found to act independently from HMG CoA reductase inhibition and were selective over the related integrins Mac-1 and VLA-4 [2]. Several other lovastatin derived inhibitors were synthesized within Novartis. LFA451 and LFA878 will be described in chapter 1 and 2 of this thesis.

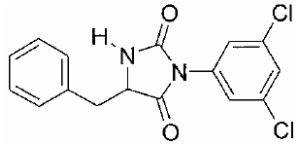
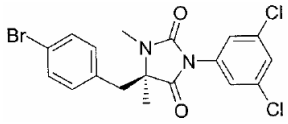
Table 1.4 Examples for lovastatin derived allosteric LFA-1 inhibitors/ Control

Structure			
Names	Lovastatin Mevacor™	Pravastatin Pravachol™	LFA703
Potency	ELISA: 2 µM	> 100 µM	0.2 µM
	Cell adhesion assay 25 µM	> 100 µM	0.7 µM
Company	Merck	Bristol-Myers Squibb	Novartis
References	2,6	2,6	2,6,198

Hydantoin derivatives

Hydantoin derivatives were first described as LFA-1 antagonists in 1999, briefly after the publication of Lovastatin as LFA-1 inhibitor. A group of Boehringer Ingelheim Inc. published BIRT 377, a compound which evolved from a lead structure discovered in an ELISA based high throughput screening assay using purified LFA-1. These compounds have a >35 fold increased *in vitro* potency compared to the lead structure. The binding site of BIRT 377 and its analogs maps to the αL L-site [199]. Selectivity of hydantoin derivatives for LFA-1 over Mac-1 was also reported by Kelly *et al* [3]. BIRT 377 showed *in vivo* efficacy after oral dosing. 10 or 3 mg/kg p.o. once daily for two weeks potently inhibited the production of human IgG in SCID mice engrafted with human peripheral blood mononuclear cells. The efficacy of BIRT 377 was comparable to that achieved with an anti LFA-1 antibody. BIRT 377 also inhibited SEB induced IL-2 induction in mouse after 25 and 50mg/kg-p.o. [3].

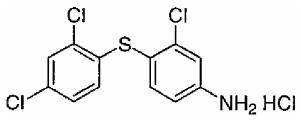
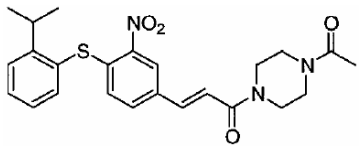
Table 1.5 Examples for hydantoin derived allosteric LFA-1 inhibitors

Structure		
Names	Lead structure	BIRT 377 (R)-5-(4-bromobenzyl)-3-(3,5-dichlorophenyl)-1,5-dimethylimidazolidine-2,4-dione
Potency	ELISA 3.5 µM	26 nM ELISA 2.6 µM cell adhesion assay
Company	Boehringer Ingelheim	Boehringer Ingelheim
References	3,200	3,200

Cinnamide derivatives

In 2000, Abbott Laboratories have reported a novel class of LFA-1 inhibitors. A cinnamide lead structure was identified by a time resolved fluorescence LFA-1/ICAM-1 binding assay. Intensive chemical modification led to molecule A-286982 which has a 38 fold increased potency compared to the lead compound. Cinnamide derivatives bind to the αL L-site (IDAS) as demonstrated by Liu *et al* [201]. No *in vivo* efficacy studies are described so far. However, pharmacokinetic data demonstrated that A-286982 was orally available [202].

Table 1.6 Examples for cinnamide derived allosteric LFA-1 inhibitors

Structure		
Names	Lead structure *p*-Arylthio Cinnamides	A-286982
Potency	ELISA LFA-1/ICAM-1 1.7 µM	ELISA LFA-1/ICAM-1 44 nM
Company	Abbott Laboratories	Abbott Laboratories
References	201,202	201,202

1.6 References

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2 Chapter 1

Low molecular weight inhibitors induce conformational changes in the αL I domain and the β2 I-like domain of lymphocyte function-associated antigen-1: Molecular insights into integrin inhibition

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by Welzenbach K., Hommel U. and Weitz-Schmidt G.

2.1 Introduction

Central to ligand binding by LFA-1 is an inserted domain of 200 amino acids at the N-terminus of the αL subunit, the so called αL I domain [1]. This LFA-1 I domain is highly homologous to I domains displayed on other integrin α subunits. Three-dimensional structures of I domains reveal that they adopt a dinucleotide-binding fold and contain a cation binding site designated metal ion-dependent adhesion site (MIDAS). There is strong evidence that the metal ion of the MIDAS is coordinated by an acidic residue from ICAM and thus directly participates in ligand binding [2]. The LFA-1 I domain is inserted between the β sheets 2 and 3 of a seven bladed putative β-propeller region on the αL subunit [3].

There is strong evidence that integrin β subunits contain regions structurally homologous to the I domains, the so-called β I-like domains [2,4]. The existence of such I-like domains was recently confirmed by the crystal structure of the extracellular segment of the integrin αVβ3 [5]. The β2 I-like domain of the LFA-1 β2 subunit is predicted to contact the putative β propeller of the αL subunit near β sheets 2 and 3 [6]. A recent study suggests that the β2 I-like domain has a regulatory role rather than a direct role in ligand binding [7].

As described for other integrins, the ligand binding activity of LFA-1 is tightly regulated [1,8]. LFA-1 is expressed on leukocytes in an inactive state and can be rapidly activated by cations or by intracellular signals [1,9]. Receptor clustering and conformational changes are proposed to be the major mechanisms by which LFA-1 is converted from a low to a high affinity form [1]. Several studies provide strong evidence that in particular the C-terminal helix of the αL I domain plays an important role in the activation process. It has been recently demonstrated that LFA-1 can be locked in an open, ligand binding and a closed non-binding conformation by mutational introduction of disulfide bonds between the C-terminal helix and a central

β sheet of the αL I domain [1,10]. The position of the disulfide bonds were modeled according to the crystal structures of Mac-1 (αMβ2, CD11b/CD18) and VLA-2 (α2β1, CD49b/CD29) I domains. These I domains have been crystallized in two conformations which differed by a major shift in the positioning of the C-terminal helix [1,11,12]. Moreover, transfectants expressing mutant LFA-1 with alanine or tryptophan substitutions in the C-terminal helix region of the αL I domain show impaired or constitutively active binding to ICAM-1 [12-14]. The importance of this region in controlling LFA-1 activity is further underlined by the fact that low molecular weight (LMW) LFA-1 inhibitors bind to a hydrophobic pocket between the C-terminal helix and the central β-sheet of the LFA-1 I domain, termed lovastatin binding site (L-site) [13-15]. This pocket has been also termed I domain allosteric site (IDAS) by others [13]. The first compound described to interact with the L-site was the HMG-CoA reductase inhibitor lovastatin [15]. Only recently it became evident that compounds of diverse chemical classes including hydantoin and cinnamide derivatives utilize the same pocket for inhibition of LFA-1 [16,17]. Since the L-site is distant from the MIDAS, it is thought that these compounds inhibit LFA-1 via an allosteric mechanism [15-18]. However, molecular details of this inhibition in context of the whole receptor are unknown. Similarly, the molecular details of the natural activation process are not well understood. Addressing both molecular mechanisms, LFA-1 activation and inhibition, is the objective of this study.

Here we investigate the effect of LMW LFA-1 inhibitors on the LFA-1 αL I domain, the putative β-propeller region and the β2 I-like domain using monoclonal antibodies (mAbs) mapped to these different regions of the receptor. We show that the inhibitors induce epitope changes in the I-domain, the β2 I-like domain or both domains depending on their chemical structure and binding site. For the first time we establish the β2 I-like domain on the β2 subunit as a molecular target for LFA-1 inhibition. Moreover, utilizing the native LFA-1 receptor, as compared to mutated LFA-1 studied previously [7], we provide strong evidence for a functional relationship between the αL I domain and the β2 I-like domain which is induced upon activation.

2.2 Methods

2.2.1 Reagents and antibodies

LFA-1 was purified from JY cells as described earlier [14]. Mac-1 was purified from peripheral blood leukocytes as described by Diamond *et al.* [19]. ICAM-1 mouse Cκ fusion protein (ICAM-1-mCκ) was produced and biotinylated as described [15]. ICAM-1-human Fc, ICAM-2 human-Fc and ICAM-3 human-Fc fragment fusion proteins were purchased from R&D Systems, Oxon, UK. 3'-O-Acetyl-2',7'-bis (carboxyethyl)-4 or 5-carboxyfluorescein diacetoxy-methyl ester (BCECF-AM) was purchased from Molecular Probes, Leiden, Netherlands. Streptavidin-peroxidase conjugate (SPOD) was purchased from Boehringer Mannheim GmbH, Germany. 2,2-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] was obtained from Bio-Rad, Hercules, California. All cell culture reagents were purchased from Life Technologies AG, Switzerland. Hybridoma cells producing mAbs TS2/4.1.1 (anti-CD11a) and TS1/22.1.3 (anti-CD11a) and mAbs 44aacb (anti-CD11b) and LM2/1 (anti-CD11b) were obtained from the American Type Culture Collection (ATCC). Antibodies were purified from culture supernatants using protein A sepharose (Amersham Pharmacia Biotech, Switzerland) separation. Amino-directed biotinylation of these antibodies was performed using NHS-biotin following manufacturers instructions. The biotinylated and the non-biotinylated anti-human LFA-1 mAb R7.1 (anti-CD11a) and R3.3 (anti-CD18) were purchased from BioSource International, Camarillo, CA. The biotinylated and the non-biotinylated mAb TS1/18 (anti-CD18) was from Endogen, Woburn, MA; the mAb IB4 (anti-CD18) was from Ancell Corp. Bayport, MN; YFC118.3 (anti-CD18) was from Serotech, UK. CLB-LFA-1/1 (anti-CD18) was from Caltag Laboratories, Burlingame, CA. Antibody 25.3.1 (anti-CD11a) was from Immunotech, Marseille, France. The biotinylated and the non-biotinylated mAb clone 38 (anti-CD11a), the mAb MEM48 (anti-CD18), isotype controls for mouse IgG1, IgG2a, IgG2b and rat IgG1 as well as goat anti-mouse IgG Cκ were from Southern Biotechnology Associates, Birmingham, AL. Alexa Fluor® 488 goat anti-mouse IgG (H+L) conjugate and Alexa Fluor®488 goat anti-rat IgG (H+L) conjugate were purchased from Molecular Probes, Leiden, Netherlands. Goat anti-human IgG-Fc fragment was

purchased from KPL, Gaithersburg, Maryland. All other assay reagents were bought from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

2.2.2 Cell culture

Jurkat E6-1 and HUT78 cell were obtained from ATCC and grown in RPMI 1640 medium supplemented with 10 % fetal calf serum, 1% Glutamax I and 1% non essential amino acids.

2.2.3 LFA-1/ICAM-1 ELISA-type binding assay

LFA-1 inhibitors were tested in a LFA-1/ICAM-1 ELISA-type binding assay which was performed as previously described [14]. Briefly, 96 well microtiter plates (Nunc Maxisorb) were coated with 2-10 µg/ml of purified human LFA-1 and blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 hour (h). After washing the plates compounds dissolved in dimethyl sulfoxid (DMSO) and diluted in 2 mM $MgCl_2$ and 0.5% FCS, pH 7.4 (assay buffer) were added to the plates. After 15 minutes of incubation, ICAM-1-mCκ in assay buffer (4 µg/ml) was added and incubated at 37°C for 1 h. After several washing steps SPOD was added 1:5000 diluted in assay buffer and incubated at 37°C for 35 min. After removal of unbound SPOD by washing, ICAM-1-mCκ was quantified using 2,2-azino-bis[3-ethylbenzthiazoline-6-sulfonic acid] as substrate.

2.2.4 Biotinylated antibody binding to purified LFA-1

The binding of biotinylated anti-CD11a and anti-CD18 mAbs to purified LFA-1 was investigated in the presence of the LFA-1 inhibitors. The compounds were dissolved in DMSO and serially diluted with PBS containing 2 mM $MgCl_2$ and 0.5% FCS, pH 7.4 (assay buffer). The inhibitors were added to LFA-1 coated and blocked microtiter plates (see above). After 15 min incubation at room temperature (RT), biotinylated anti-LFA-1 mAbs in assay buffer (0.1 µg/ml) were added and incubated at RT on a shaker for 30 min. After washing the plates, SPOD was added, 1:5000 diluted in assay buffer and incubated at RT for 35min. After a washing step, bound anti-LFA-1 mAbs were quantified using 3,3,5,5-tetramethyl-benzidine (TMB) as substrate. For experiments in the absence of cations the immobilized LFA-1 was washed three

times with 1 mM EDTA in PBS and the assay buffer was switched to PBS, 0.5% BSA, pH7.4 containing either 0.1 mM or 1 mM EDTA.

2.2.5 Mac-1/ICAM-1 ELISA-type binding assay

Purified Mac-1 was immobilized at 2-10 μg/well onto 96-well microtiter plates (Nunc Maxisorb) and blocked with 5% non-fat dry milk in PBS. The inhibitors were dissolved in DMSO and serially diluted in Tris-buffered saline (TBS) containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $MnCl_2$ and 0.2% non-fat dry milk, pH 7.4 (assay buffer). The compounds were added to the Mac-1 coated plates. After 15 min incubation biotinylated ICAM-1-mCκ in assay buffer (2 μg/ml) was added and incubated at 37°C for 1 h. After four washing steps with assay buffer, SPOD diluted 1:5000 in assay buffer was added and incubated at 37 ^{0}C for 35 min. After washing, bound ICAM-1-mCκ was quantified using TMB as substrate.

2.2.6 Biotinylated antibody binding to purified Mac-1

The binding of the anti-CD18 mAb IB4 to purified Mac-1 was tested in the presence of LFA-1 inhibitors dissolved in DMSO and diluted in Tris-buffered saline containing 150 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM $MnCl_2$ and 0.2% non-fat dry milk, pH 7.4 (assay buffer). The compounds were added to the microtiter plates coated with Mac-1 and incubated at RT for 15 min. Then biotinylated mAb IB4 was added (0.1 μg/ml) in assay buffer. After 30 min incubation, bound antibodies were detected by the streptavidin-peroxidase reaction as described above.

2.2.7 Cell-based adhesion assays

LFA-1 dependent adhesion of HUT78 cells to immobilized ICAM-1-Fc, ICAM-2-Fc and ICAM-3-Fc fusion proteins was carried out as described earlier for the HUT78/ICAM-1 mCκ assay [15]. Briefly, 96 well microtiter plates were coated with goat anti-human IgG-Fc fragment in PBS, pH 7.8 (5 μg/ml) followed by a blocking step with 1.5% BSA in TBS. After washing with TBS, ICAM-1-Fc, ICAM-2-Fc or ICAM-3-Fc were added at 0.1-0.3 μg/ml, 0.3 μg/ml or 1 μg/ml respectively in TBS containing 150 mM NaCl, 1.5% BSA, 5 mM glucose, 2 mM $MgCl_2$ and 2 mM $MnCl_2$ (assay buffer). BCECF-AM labeled HUT78 cells (1.25 $\times 10^5$ cells/well) in assay buffer were

transferred to the plates and incubated together with the inhibitors at 37°C for 30 min. The plates were then gently washed with assay buffer and bound cells were quantified by measuring fluorescence using a VICTOR2 microplate reader (Wallac, Finland).

2.2.8 Flow cytometry

The effect of the LFA-1 inhibitors on the conformation of cell expressed LFA-1 was tested by monitoring the binding of anti LFA-1 mAbs to Jurkat E6-1 cells using flow cytometry. Jurkat E6-1 cells were harvested and washed two times with TBS containing 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $MnCl_2$, 0.5% BSA, pH 7.4 (assay buffer). For experiments in the absence of cations, cells were washed three times with TBS containing 150 mM NaCl, 1 mM EDTA and the assay buffer was replaced by TBS containing 150 mM NaCl, 0.5% BSA, 0.1 mM EDTA, pH 7.4.

3 x 10^5 cells were pre-incubated with the LFA-1 inhibitors at a final concentration of 50 µM in assay buffer at RT for 20 min followed by the addition anti-CD11a and anti-CD18 mAbs (1-2 µg/ml). After 25 min incubation, cells were washed two times with assay buffer and counter stained with Alexa Fluor® 488 goat anti-mouse IgG (H+L) conjugate or Alexa Fluor® 488 goat anti-rat IgG (H+L) conjugate diluted 1:175 in assay buffer for 30 min. After a washing step, antibody binding was immediately analyzed by flow cytometry on a FACScan (Becton-Dickinson, San Jose, CA).

Control experiments at low temperatures were performed as described above except that the antibody binding steps were carried out on ice.

Mean fluorescence intensities (MFI, geometric mean) were calculated using the CellQuest software. MFIs were corrected for background staining by subtracting the MFI of the appropriate isotype-matched negative control. MFIs of the solvent controls were set as 100 %. Inhibition of anti-LFA-1 mAb binding induced by inhibitor treatment was expressed as percentage of these controls.

2.2.9 NMR binding assay (conducted by Dr. U. Hommel)

The NMR binding assay was performed as previously described [15]. Briefly, {^{1}H, ^{15}N}-HSQC spectra were recorded on a 600 MHz Bruker Avance spectrometer (Bruker AG, Karlsruhe) at 23 ºC. Uniformly ^{15}N-labelled αL I domain was titrated with increasing amounts of ligand dissolved in DMSO. The protein concentration was 100 µM and the maximal ligand concentration varied between 200 µM and 600 µM depending on the ligand. The change in the ^{15}N-resonance frequency for the cross-peak corresponding to the amide group of Thr291 was followed for a qualitative assessment of the binding affinity. For the assessment of compound binding to the αM I domain, 1D-^{1}H spectra of the isolated Mac-1 I-domain were recorded. The αM I domain (residues 131-321) was first cloned into a pET9a vector using standard molecular biology procedures [20]. The protein was expressed as His-tagged fusion protein and purified using NTA and size exclusion chromatography. The N-terminal His-tag was thereafter cleaved from the purified protein using thrombin.

2.3 Results

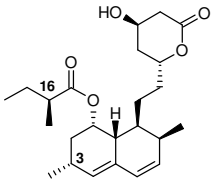
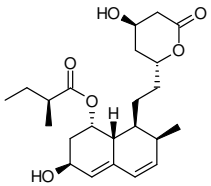
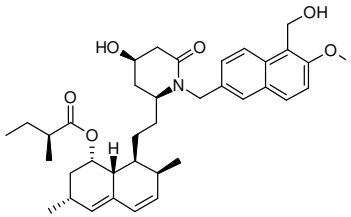
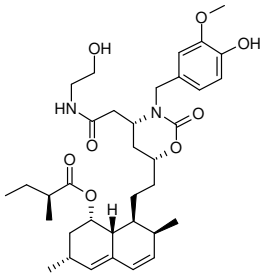
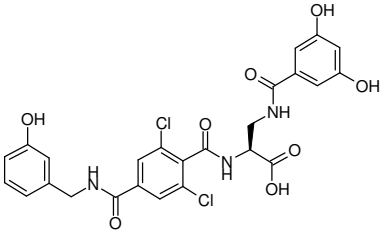
2.3.1 Compounds of two different chemical classes inhibit LFA-1 function

Table 2.1 summarizes the properties of LMW LFA-1 inhibitors of two different chemical classes. Lovastatin and the optimized lovastatin-derived LFA-1 inhibitors LFA703 and LFA451 blocked ICAM-1 binding to purified immobilized LFA-1 with IC_{50}s of 2.1, 0.2 and 0.04 μM, respectively (Table 2.1). The inhibitors not only interfered with the LFA-1/ICAM-1 but also with the LFA-1/ICAM-2 and LFA-1/ICAM-3 interaction as shown in T-cell adhesion assays (Table 2.1). ICAM-3 mediated adhesion was more potently inhibited by the compounds than ICAM-1 and ICAM-2 mediated adhesion (Table 2.1). This result is in agreement with previous studies showing that LFA-1 binds to ICAM-1 and ICAM-2 with stronger affinity than to ICAM-3 [21]. The IC_{50} values determined for the LFA-1 inhibitors in the HUT78/ICAM-1 adhesion assay were found to be similar to IC_{50} values determined in an ICAM-1 adhesion assay utilizing the human lymphoma cell line Jurkat (data not shown). Jurkat/ICAM-1 or Jurkat/ICAM-3 adhesion assays were not performed. As shown by nuclear magnetic resonance (NMR) spectroscopy the inhibitors were able to interact with the L-site of the αL I domain (Table 2.1). A close analogue of the lovastatin-derived inhibitors, pravastatin, does not inhibit LFA-1 function and has only marginal affinity for the αL I domain (Table 2.1). This compound was included in the study as a negative control (Table 2.1).

A unique property of the lovastatin-derived LFA-1 inhibitors is their specificity for LFA-1. The function of other integrins including the β2 integrin Mac-1 (αMβ2, CD11b/CD18) was not affected by the compounds (Table 2.1). In contrast, a LFA-1 inhibitor recently described by others was less specific [22]. This inhibitor, which we termed XVA143, is a suggested peptidomimetic with an unknown LFA-1 binding site [22]. XVA143 inhibited LFA-1 and Mac-1 with IC_{50}s < 20 nM (Table 2.1). Based on the presence of a carboxylic acid function in the structure of XVA143 (Table 2.1) it could

be speculated that the inhibitor binds to the MIDAS pocket of the I domains of LFA-1 and Mac-1. Thus, the binding of XVA143 to both, the LFA-1 and Mac-1 I domain was qualitatively assessed using NMR. In general, I domain binding of lovastatin and some close derivatives are associated with large chemical shift changes in protein spectra of the αL I domain [15]. These changes are observed for many residues of the central β-sheet as well as helices α7 and α1 indicating ligand induced structural rearrangements in solution [15]. The same was observed for the lovastatin-derived inhibitors LFA703 (Fig. 2.1A) and LFA451 (not shown) at equimolar concentrations of protein and ligand. In contrast, XVA143 influenced the protein spectra to a lesser extent even at high concentrations (Fig. 2.1B). We note, that none of the residues associated with the LFA-1 MIDAS motif was affected by XVA143 ruling out the possibility that inhibition of LFA-1 is mediated by competitive binding of the compound to the MIDAS region implicated in ICAM-1 binding (Fig 2.1B). Likewise, protein spectra of the αM I domain acquired in the presence and absence of XVA143 did not show changes in the chemical shift of resonances affected by magnesium, i.e. residues located in the vicinity of the MIDAS pocket (data not shown). These results indicate that LFA-1 and Mac-1 inhibition by XVA143 is not mediated by the engagement of the α chain MIDAS pocket. The data further suggest that the molecular target for XVA143-driven inhibition of LFA-1 and Mac-1 is different from the L-site utilized by the lovastatin-derived LFA-1 inhibitors.

Table 2.1 Characterization of LFA-1 inhibitors and a dual LFA-1/Mac-1 inhibitor in different assays

Assay	Lovastatin	Pravastatin	LFA703	LFA451	XVA143 (Genentech/Roche)
					
LFA-1/ICAM-1: IC_{50} [µM]	2.1 ± 0.8 (*n=8*)	> 100 (*n=5*)	0.2 ± 0.1 (*n=5*)	0.04 ± 0.010 *(n=6)*	0.020 ± 0.008 *(n=5)*
HUT78/ICAM-1: IC_{50} [µM]	25.4 ±13.3 (*n=5*)	> 100 (*n=3*)	0.7 ± 0.5 (*n=7*)	0.40 ± 0.07 *(n=7)*	0.005 ± 0.004 *(n=4)*
HUT78/ICAM-2 IC_{50} [µM]	36.2 ± 22.7 *(n=4)*	> 100 *(n=2)*	1.2; 1.7	0.6 ± 0.251 *(n=3)*	0.007, 0.007
HUT78/ICAM-3 IC_{50} [µM]	16, 3.5	> 100 *(n=2)*	0.4, 0.1	< 0.1, 0.06	0.002, 0.001
I-domain binding	+	-	+	+	-
Mac-1/ICAM-1: IC_{50} [µM]	> 100 *(n=3)*	> 100 *(n=3)*	> 100 *(n=3)*	> 100 *(n=3)*	0.002 ± 0.001 (n=3)

Values represent independent experiments or the mean ± SD; in parentheses, number of independent experiments performed; #uniformly ^{15}N-labelled I-domain was titrated with increasing amounts of compound dissolved in DMSO. The change in the ^{15}N-resonance frequency for the peak corresponding to Thr291 was followed and analyzed. + and - correspond to a change > 50 Hz and < 5 Hz at a concentration for the I-domain and compound of 100 µM, respectively.

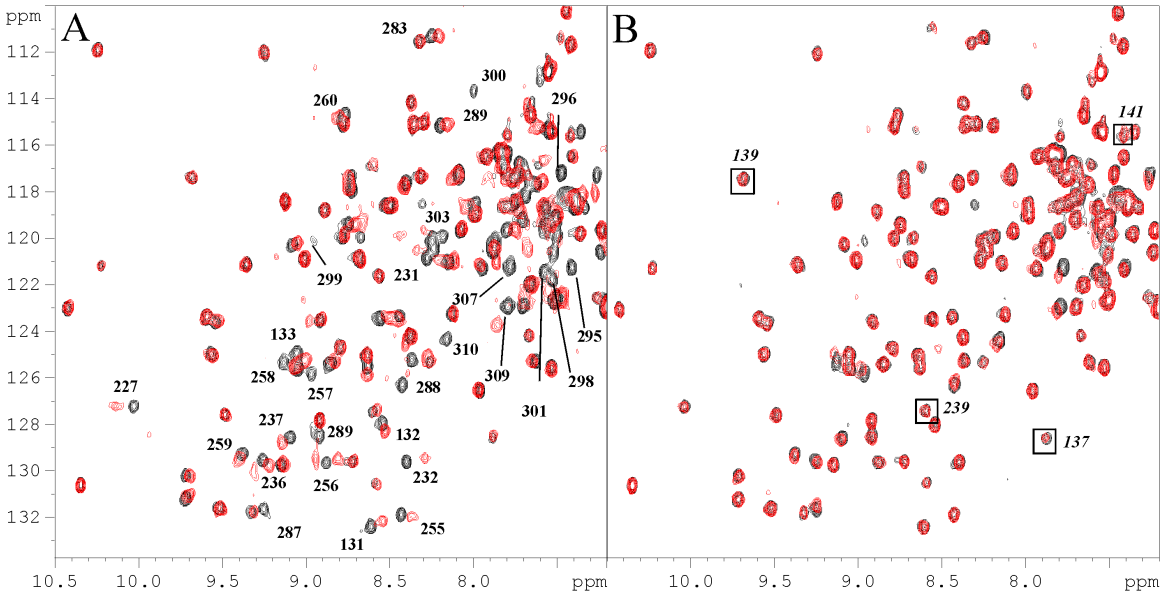


Figure 2.1 Effect of the LFA-1 inhibitors LFA703 and XVA143 on the LFA-1 αL I domain. [^{1}H,^{15}N] HSQC spectra are shown for the αL I domain in the absence (black) and presence (red) of A) LFA703 and B) XVA143. The protein concentration was 0.1 mM and ligand concentrations were 0.2 and 0.6 mM for LFA703 and XVA143, respectively. In A) cross-peaks experiencing strong chemical shift changes upon ligand binding are labeled by their sequence numbering. In B) cross peaks corresponding to residues of the conserved MIDAS motif are indicated by boxes and labeled. (Data of Dr. U. Hommel)

2.3.2 Selection of mAbs specific for different LFA-1 domains

To investigate the effect of the inhibitors described above on the different domains of LFA-1 the binding of mAbs to LFA-1 expressed on Jurkat cells and to purified LFA-1 was determined in the presence and absence of compounds. The selected mAbs are reported to map to different regions of the αL chain and the β2 chain of LFA-1. MAbs TS1/22, 25.3.1, R7.1 and clone 38 bind to the αL I domain [3,23,24]. The epitopes of mAb R7.1 and mAb clone 38 on the αL I domain are unknown whereas mAbs TS1/22 and 25.3.1 map to a region in the close vicinity of the L-site [3] (Fig. 2.2). MAb TS2/4 maps outside the αL I domain recognizing the putative β-propeller of the αL chain [3]. MAbs TS1/18, YFC118.3, IB4 and R3.3 are reactive with the putative β2 I-like domain [4,25] According to the recognized epitopes consisting of at least two non-contiguous sequences the anti- β2 I-like domain mAbs can be divided into two groups: the first group includes mAbs TS1/18 (Leu154-Glu159 and Glu344-Asp348) and YFC118.3 (Leu154-Glu159, Glu344-Asp348, and His354-Asn358) and the

second group includes mAbs IB4 and R3.3 (Arg144-Lys 148 and Pro192-Glu197) [4]. All mAbs which map to the αL I domain or the putative β2 I-like domain of LFA-1 are reported to block LFA-1 binding to ICAM-1 [24,4,23,25,26]. In contrast, mAb TS2/4.1.1 (TS2/4) only marginally inhibits LFA-1 function [26].

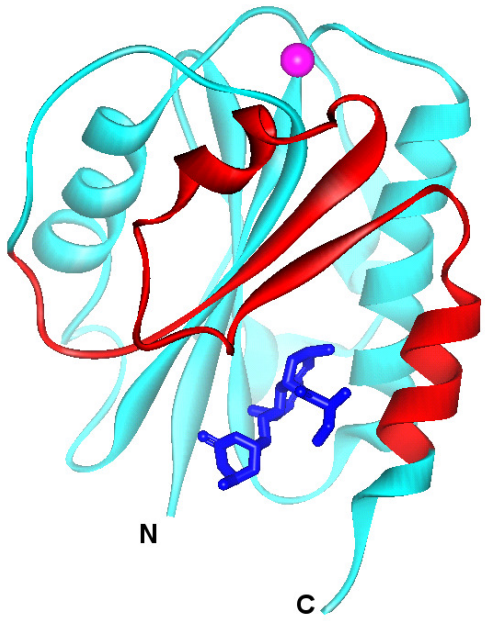


Figure 2.2 The function-blocking anti-LFA-1 mAbs TS1/22 and 25.3.1 bind adjacent to the allosteric L-site of the LFA-1 I-domain. The X-ray structure of the lovastatin-I domain complex is shown. The region recognized by the function-blocking anti-LFA-1 mAbs TS1/22 and 25.3.1 (residues 250 – 303) is shown in red.

2.3.3 The lovastatin-derived LFA-1 inhibitors induce epitope changes in the αL I domain and β2 I-like domain of LFA-1 on Jurkat cells

We first utilized Mn^{2+}/Mg^{2+} activated LFA-1 expressed on the surface of Jurkat cells to investigate the effect of the lovastatin-derived inhibitors on the binding of the mAbs to the β2 integrin. Lovastatin and the inhibitors LFA703 and LFA451 prevented the binding of the anti-αL I domain mAbs to different extents (Table 2.2). It can be assumed that this compound effect is due to the alteration of epitopes induced by compound binding to the αL L-site rather than the direct interaction of the compounds with the various antibody epitopes. We found that the epitope recognized by mAb R7.1 was most prominently altered by the compounds as demonstrated by 80% to

100% inhibition of antibody binding (Table 2.2, Fig. 2.3A). The epitopes recognized by mAbs TS1/22 and 25.3.1 were only partially or not affected by the compounds (Table 2.2). Interestingly, αL L-site engagement by lovastatin also influenced the presentation of epitopes displayed on the β2 I-like domain. This phenomenon became even more pronounced in the presence of the lovastatin-derived LFA-1 inhibitor LFA703 (Table 2.2). The compound potently blocked the binding of mAbs IB4, TS1/18 and YFC118.3 shown to recognize two distinct regions of the β2 I-like domain [4]. This finding indicates that LFA703 is able to alter epitopes in the β2 I-like domain of the CD18 chain by binding to the L-site of the αL chain (Table 2.2). In contrast, the effect of the inhibitor LFA451 on the β2 I-like domain was much less evident. Only the epitope recognized by mAb YFC118.3 was marginally affected by the inhibitor (Table 2.2). The fact that LFA451 and LFA703 show different effects on mAb binding to the β2 I-like domain was not entirely unexpected. The compounds differ in their pattern of chemical modifications on the parent lactone ring of lovastatin. While LFA703 is a N-substituted lactame with a modification in position 2' of the lactam ring, LFA451 is modified in position 3' of the corresponding carbamate ring (Table 2.1). These chemical modifications, in conjunction with the accompanying changes in the stereochemistry of the lactam/carbamate ring were expected to result in a different orientation of the newly introduced groups with respect to the native LFA-1 receptor. This assumption was confirmed by the crystal structure determination of the LFA451 and LFA703 αL I domain complexes (not shown). Our data suggest that the naphthyl part of LFA703 interacts with pockets of the αL L-sites which are not reached by the vanillyl group of LFA451. Likewise, the recently described LMW LFA-1 inhibitor BIRT 377 does not extent into pockets of the αL L-site used by LFA703 as indicated in the modeled structure published recently [16]. Consistent with this observation, BIRT 377 was found to inhibit the binding of mAbs to the αL I domain, but not the β2 I-like domain [23]. Taken together these results provide evidence that conformational changes of the β2 I-like domain are induced by the unique interaction of the naphthyl part of LFA703 with the αL L-site.

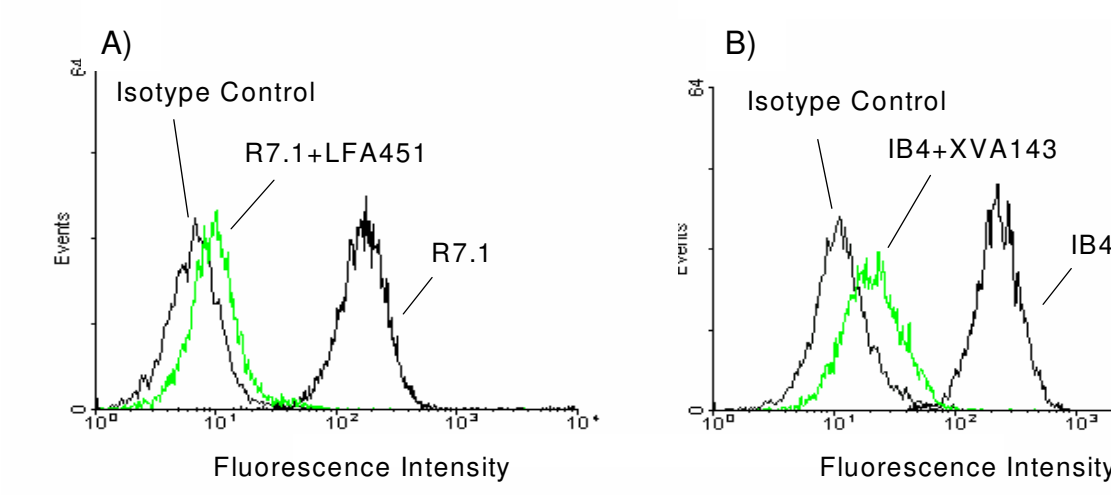


Figure 2.3 Inhibition of mAb R7.1 or mAb IB4 binding to LFA-1 expressed on Jurkat cells by LFA-1 inhibitors: data of a representative flow cytometry experiment.Jurkat cells were incubated with the mAbs R7.1 (anti- αL I domain) or mAb IB4 (anti-β2 I-like domain) in the presence and absence of the LFA-1 inhibitors LFA451 (A) and XVA143 (B). The experiment was performed in the presence of cations. Bound antibody was detected by the addition of Alexa Fluor® 488 conjugated anti-IgG followed by flow cytometry analysis as described under Methods.

In contrast to the αL I domain and β2 I-like domain, the putative β-propeller region of LFA-1 was not affected by the binding of the lovastatin-derived LFA-1 inhibitors (Table 2.2). As expected pravastatin did not inhibit antibody binding to LFA-1 (Table 2.2). To exclude the possibility that internalization of antibodies in the presence of compounds led to the effects described above all experiments were also performed at 4 ^{0}C. The results at low temperatures were comparable to those generated at room temperature (data not shown).

2.3.4 Lovastatin-derived LFA-1 inhibitors induce epitope changes in the αL I domain and β2 I-like domain of purified LFA-1

To further substantiate our findings we investigated the interaction of mAb R7.1 and mAb IB4 to immobilized purified LFA-1 in the presence of the LFA-1 inhibitors. The analysis was performed with the Mg^{2+}-activated form of LFA-1. Consistent with the flow cytometry experiments described above lovastatin, LFA703 and LFA451 inhibited the binding of the mAb R7.1 in a dose-dependent manner whereas

pravastatin had no effect (Fig. 2.4A). Interestingly, the IC_{50} values determined for the compounds in the LFA-1/mAb R7.1 binding assay (Fig. 2.4A: Lovastatin: IC_{50}: 2.9 µM, LFA703: IC_{50}: 0.14 µM, LFA451: IC_{50}: 0.03 µM) correlated well with those generated in the cell-free LFA-1/ICAM-1 binding assay (Table 2.1). Moreover, LFA703 blocked the binding of the mAb IB4 to purified LFA-1 in a dose-dependent manner confirming the effect of LFA703 on the β2 I-like domain of the CD18 chain (Fig. 2.4B). In contrast, lovastatin and LFA451 did not inhibit the interaction of mAb IB4 with purified LFA-1 corroborating that their binding to the αL L-site does not affect the β2 I-like domain to the same extent as observed for LFA703 (Fig. 2.4B). As expected pravastatin showed no effect on antibody binding (Fig. 2.4 A and B).

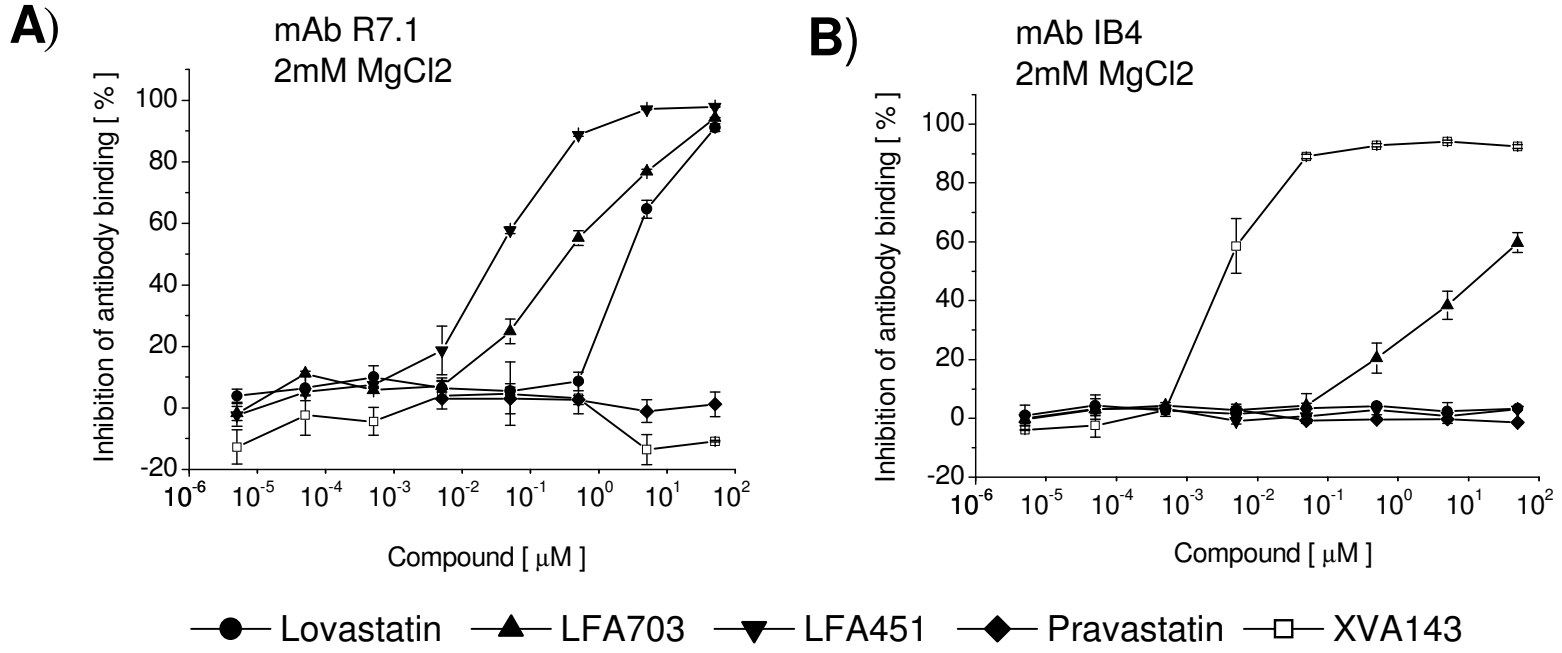


Figure 2.4 Effect of LFA-1 inhibitors on the binding of mAbs to cation-activated purified LFA-1:Purified LFA-1 was immobilized onto 96 well plates and incubated with biotinylated mAb R7.1 (anti- αL I domain) (A) or biotinylated mAb IB4 (anti-β2 I-like domain) (B) in the presence and absence of increasing concentrations of indicated LFA-1 inhibitors. The experiment was performed in the presence of 2 mM $MgCl_2$. Bound antibody was detected via streptavidin-peroxidase as described under Methods. Compound induced inhibition of mAb binding to LFA-1 is expressed as the percentage of solvent control. Each value represents the mean ± SD of triplicates. A representative experiment out of two is shown.

2.3.5 The LFA-1 inhibitor LFA703 does not interact with the β2 I-like domain

To exclude that LFA703 directly binds to the β2 I-like domain of the CD18 chain and by this inhibits the interaction of the mAbs with the β2 I-like domain, we tested the effect of LFA703 on Mac-1 which shares the β2 chain with LFA-1 [9]. Purified Mac-1 was immobilized on microtiter plates and the binding of the mAb IB4 was measured in the presence of LFA703 (Fig. 2.5). The compound did not inhibit the binding of mAb IB4 to purified Mac-1 at a concentrations of 50 µM (Fig. 2.5) or 200 µM (not shown) suggesting that LFA703 does not bind to the β2 I-like domain and indeed has to bind to the L-site on the αL chain to influence epitopes on the β2 chain. Likewise, lovastatin and LFA451 were inactive in the Mac-1/mAb IB4 binding assay confirming the high selectivity of the lovastatin-derived LFA-1 inhibitors for LFA-1 as compared to Mac-1.

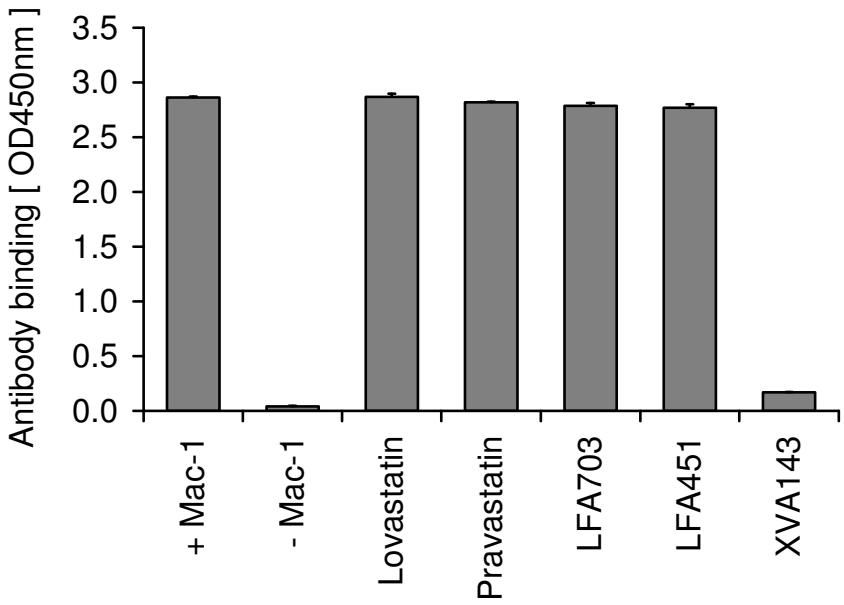


Figure 2.5 Effect of LFA-1 inhibitors on the binding of mAb IB4 to cation-activated purified Mac-1: Purified Mac-1 was immobilized onto 96 well plates and incubated with biotinylated mAb IB4 (anti-β 2 I-like domain) in the presence of different LFA-1 inhibitors (50 µM). Binding of the antibody in the absence of compounds is indicated as '+ Mac-1' and background binding in the absence of immobilized Mac-1 is indicated as '- Mac-1'. The experiment was performed in the presence of cations. Bound antibody was quantified via streptavidin-peroxidase. Each bar represents the mean ± SD of triplicates. A representative experiment out of two independent experiments is shown.

2.3.6 The LFA-1/Mac-1 inhibitor XVA143 induces epitope changes in the β2 I-like domain of LFA-1 on Jurkat cells and purified LFA-1

In contrast to the lovastatin-derived molecules, XVA143 had no effect on the binding of mAbs to the αL I domain of cation-activated LFA-1 (Table 2.2 and Fig. 2.4A). Instead, the compound potently blocked the interaction of the mAbs TS1/18, IB4 and YFC118.3 with the β2 I-like domain of LFA-1 (Table 2.2, Fig. 2.3B, Fig. 2.4B). Moreover, XVA143 was active in the Mac-1/mAb IB4 binding assay indicating that the effect of XVA143 on the β2 I-like domain is independent from the type of α chain associated with the β2 chain (Fig. 2.5). In contrast, the binding of the mAb TS2/4 to LFA-1 was not impaired in the presence of XVA143 (Table 2.2). These findings provide strong evidence that the binding site of XVA143 is located on the β2 I-like domain of the CD18 chain shared by LFA-1 and Mac-1. Moreover, the IC_{50} values determined in the LFA-1/ICAM-1 and the Mac-1/ICAM-1 assays suggest that the engagement of this binding site allows very efficient β2 integrin inhibition (Table 2.1).

2.3.7 Effect of the LFA-1 inhibitors on inactive LFA-1 on Jurkat cells

We also investigated the effect of the inhibitors on the inactive form of LFA-1. To keep LFA-1 in its inactive state Jurkat cells were washed several times with 1 mM EDTA before compounds and antibodies were added. The experiments were performed in the presence of 0.1 mM EDTA. Under these conditions, LFA-1 did not interact with ICAM-1 (data not shown). The antibodies utilized for the study bound the active and inactive form of LFA-1 equally well (data not shown). As observed for active LFA-1, lovastatin, LFA703 and LFA451 most prominently inhibited the binding of the anti-αL I domain mAb R7.1 to inactive LFA-1 on Jurkat cells (Table 2.2). This suggests that the L-site ligands interact with the L-site irrespective whether the receptor resides in its ligand binding or non-binding state. However, the effect of LFA703 on the reactivity of anti-β2 I-like domain mAbs with the inactive receptor was less pronounced than observed for the active receptor (Table 2.2). This result indicates, that the conformational linkage between the αL I domain and the β2 I-like domain observed in the active receptor is modified upon inactivation of LFA-1 by

cation removal. In contrast to the lovastatin-derived LFA-1 inhibitors, XVA143 had almost no effect on the mAb epitopes of inactive LFA-1 expressed on Jurkat cells (Table 2.2). As observed for activated LFA-1, the inhibitors did not affect or only marginally affected the β-propeller region of inactive LFA-1 recognized by mAb TS2/4 (Table 2.2).

Table 2.2 Effect of LFA-1 inhibitors on the binding of different mAbs to LFA-1 expressed on Jurkat cells

Inhibition of antibody binding [%]

Target of mAbs	Lovastatin		LFA703		LFA451		Pravastatin		XVA143	
aL I domain	+ Mg/Mn	- Mg/Mn + EDTA	+ Mg/Mn	- Mg/Mn + EDTA	+ Mg/Mn	- Mg/Mn + EDTA	+ Mg/Mn	- Mg/Mn + EDTA	+ Mg/Mn	- Mg/Mn + EDTA
TS1/22	22 ± 22 n.s.	44 ± 33 *	58 ± 2 *	52 ± 31 *	11 ± 10 n.s.	39 ± 22 n.s.	5 ± 11 n.s.	22 ± 25 n.s.	-4 ± 15 n.s.	8 ± 4 n.s.
25.3.1	37 ± 7 *	43 ± 14 n.s.	55 ± 4 *	48 ± 9 n.s.	22 ± 25 n.s.	53 ± 16 n.s.	-1 ± 5 n.s.	1 ± 24 n.s.	-12 ± 27 n.s.	18 ± 11 n.s.
R7.1	80 ± 12 ***	81 ± 10 **	99 ± 3 ***	78 ± 12 **	103 ± 2 ***	97 ± 3 **	6 ± 4 *	16 ± 19 n.s.	11 ± 11 n.s.	22 ± 12 *
clone 38	23 ± 8 *	20 ± 2 *	34 ± 7 *	28 ± 3 *	6 ± 2 n.s.	17 ± 9 n.s.	1 ± 8 n.s.	4 ± 2 n.s.	8 ± 21 n.s.	5 ± 5 n.s.
β-propeller: TS2/4	27 ± 8 n.s.	22 ± 2 *	32 ± 10 n.s.	27 ± 5 *	6 ± 10 n.s.	10 ± 13 n.s.	1 ± 2 n.s.	0 ± 5 n.s.	4 ± 14 n.s.	5 ± 4 n.s.
β2 I-like domain										
IB4	32 ± 22 *	21 ± 3 **	88 ± 8 ***	27 ± 6 **	16 ± 22 n.s.	13 ± 5 *	5 ± 6 n.s.	4 ± 1 ***	96 ± 6 ***	21 ± 14 **
TS1/18	21 ± 22 n.s.	23 ± 16 n.s.	75 ± 6 ***	25 ± 18 n.s.	5 ± 19 n.s.	13 ± 21 n.s.	-1 ± 17 n.s.	4 ± 6 n.s.	72 ± 6 ***	12 ± 7 *
YFC118.3	39 ± 13 **	29 ± 4 *	84 ± 8 ***	35 ± 3 n.s.	31 ± 15 **	17 ± 4 n.s.	15 ± 20 n.s.	15 ± 14 n.s.	86 ± 11 ***	14 ± 8 n.s.
R3.3	30 ± 5 **	19 ± 3 *	38 ± 7 **	26 ± 1 **	14 ± 10 n.s.	12 ± 14 n.s.	4 ± 7 n.s.	-3 ± 2 n.s.	15 ± 12 n.s.	2 ± 4 n.s.

Table 2.2. Jurkat cells were incubated with indicated mAbs in the presence or absence of LFA-1 inhibitors (50 µM). The experiment was either performed in the presence of 2 mM Mg/Mn or in the presence of 0.1 mM EDTA combined with the absence of Mg/Mn. Bound antibody was quantified by immunofluorescence flow cytometry as described under Methods. Compound induced inhibition of mAb binding to LFA-1 is expressed as the percentage of solvent control. Each value presents the mean ± SD of more than 3 independent experiments. The statistical significance of the compound effects on antibody binding versus controls was tested using the paired t-test, where *$p<0.05$, **$p<0.01$ and ***$p<0.001$ are considered significant, very significant and highly significant, respectively.

2.3.8 Effect of the LFA-1 inhibitors on inactive purified LFA-1

LFA-1 immobilized on microtiter plates was washed several times with 1 mM EDTA and antibody binding experiments were performed in the presence of 0.1 mM EDTA or 1 mM EDTA to further examine the effect of the compounds on the inactive integrin. At both EDTA concentrations, lovastatin, LFA703 and LFA451 inhibited the interaction of mAb R7.1 with the αL I domain in a dose-dependent manner whereas XVA143 and pravastatin had no effect on mAb R7.1 binding (Fig. 2.6A and B). This result is in agreement with the data described above for inactive LFA-1 expressed on Jurkat cells. In the mAb IB4/LFA-1 assay at 0.1 mM EDTA, lovastatin and LFA451 were inactive whereas XVA143 and LFA703 significantly influenced the interaction of the antibody with the inactive receptor (Fig. 2.6C). At 1 mM EDTA, however, the lovastatin-derived inhibitors and XVA143 had no effect or only a marginal effect on the IB4 epitope (Fig. 2.6D). The latter data, at 1 mM EDTA, are consistent with the flow cytometry experiment involving LFA-1 on Jurkat cells in 0.1 mM EDTA. This may indicate that EDTA more effectively removes cations from the membrane-bound receptor than from the purified receptor. The results show that the binding of XVA143 to LFA-1 is highly cation-dependent. This property is not shared by the lovastatin-derived LFA-1 inhibitors. We note that the binding of the mAbs directed against the β2 I-like domain of LFA-1 was not altered by the removal of cations (data not shown). This clearly indicates that the effects of XVA143 on the antibody/LFA-1 interaction are not due to competitive inhibition but a consequence of epitope alterations .

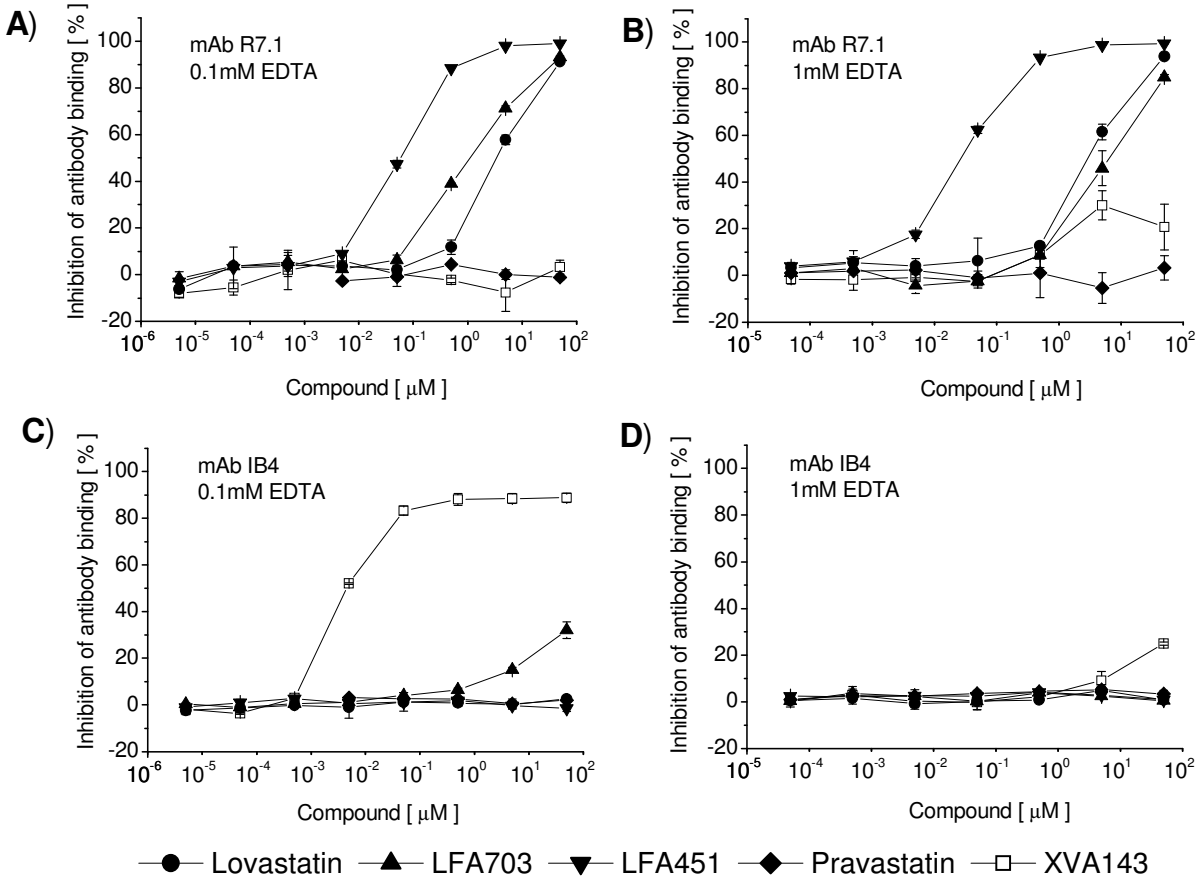


Figure 2.6. Effect of LFA-1 inhibitors on the binding of mAbs to purified LFA-1 in the presence of EDTA. Purified LFA-1 was immobilized onto 96 well plates and incubated with the biotinylated anti-αL I domain mAb R7.1 (A and B) or the biotinylated the anti-β2 I-like domain mAb IB4 (C and D) in the presence and absence of increasing concentrations of indicated LFA-1 inhibitors. The experiment was performed in the presence of 0.1 or 1 mM EDTA. Bound antibody was detected via streptavidin-peroxidase as described under Methods. Compound induced inhibition of mAb binding to LFA-1 is expressed as the percentage of solvent control. Each value represents the mean ± SD of triplicates. A representative experiment out of two is shown.

2.4 Discussion

The data reported here demonstrate that LMW LFA-1 inhibitors can induce conformational changes in both, the αL chain and β2 chain of the LFA-1 receptor. The nature of these changes is dependent on the binding site of the compound and its chemical structure. This is schematically illustrated in Figure 2.7 for two of the inhibitors analyzed. All lovastatin-derived LFA-1 inhibitors shown to bind to the αL L-site and to be highly LFA-1 specific, lead to the partial or total loss of epitopes on the αL I domain as monitored by impaired binding of several αL I domain specific antibodies. This finding suggests that αL L-site ligands induce conformational changes in the αL I domain resulting in LFA-1 inhibition and is consistent with earlier data showing that the αL I domain is directly involved in ICAM-1 binding [24].

Dependent on the binding mode, some inhibitors targeting the αL L-site were found to induce conformational changes in both the α chain I domain as well as the β2 chain I-like domain. This result is in agreement with a recent study showing that mutational activation or inactivation of LFA-1 at the IDAS/L-site results in enhanced or reduced binding of a novel mAb recognizing an activation epitope on the β2 chain [27]. Further, Lu *et al.* demonstrated that LFA-1 locked in an active form by introducing a disulfide bond within the αL L-site region is resistant to inhibition by blocking mAbs to the β2 I-like domain, and conclude that a functional relationship between the αL I domain and β2 I-like domain exists [7]. The data reported here using αL L-site ligands demonstrate that indeed an interaction between the I domains exists in the native LFA-1 receptor.

Interestingly, the additional involvement of the β2 I-like domain by certain lovastatin-derived LMW inhibitors does not correlate with increased inhibitory activity as exemplified by the profile of the lovastatin-derived inhibitor LFA451. Although LFA451 is a more potent inhibitor of LFA-1 than the other lovastatin-derived inhibitors, epitope changes induced by LFA451 are restricted to the αL I domain. This result indicates that the β2 I-like domain indirectly rather than directly contributes to ligand binding to the I-domain of the αL chain as has been proposed before [7].

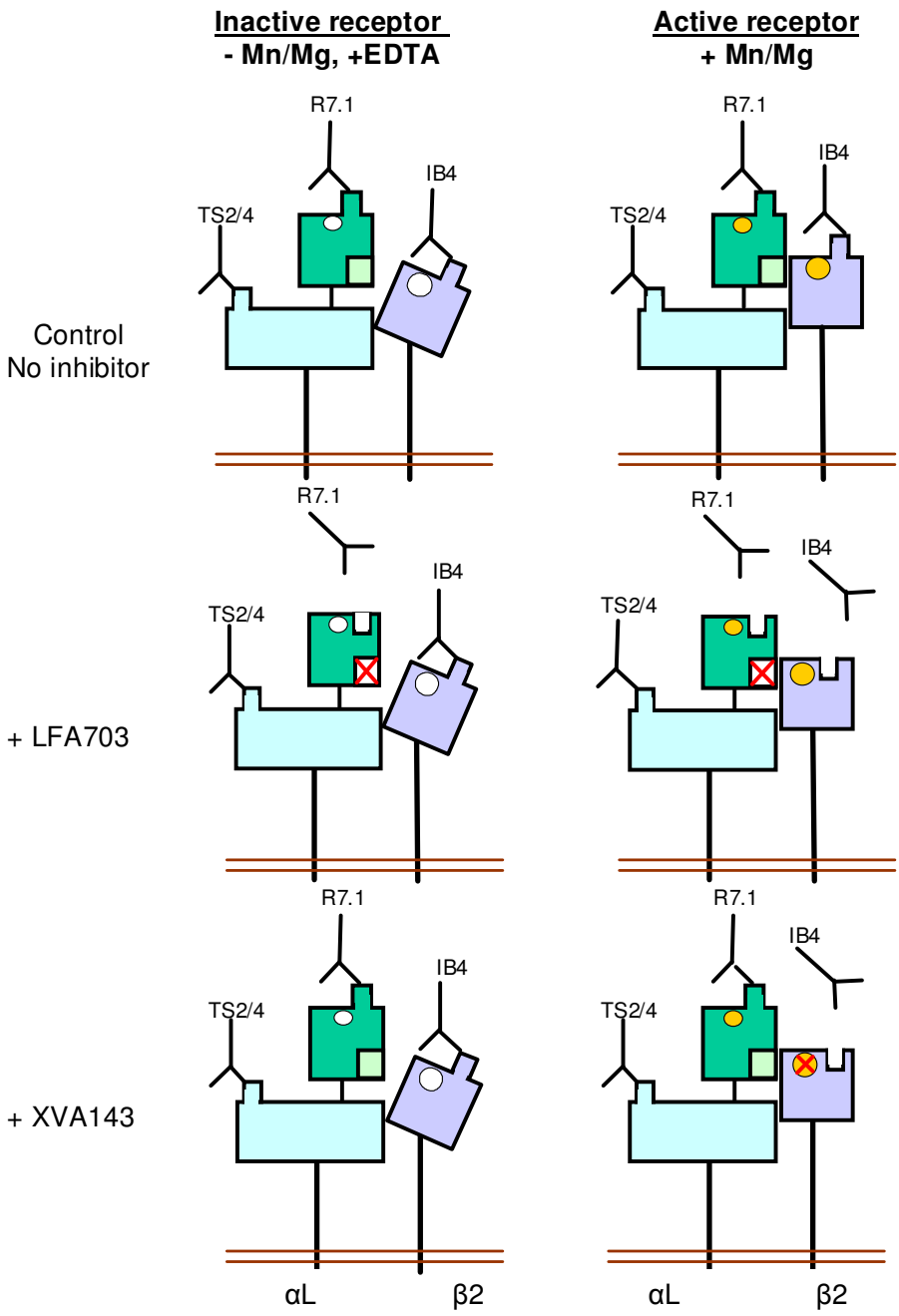
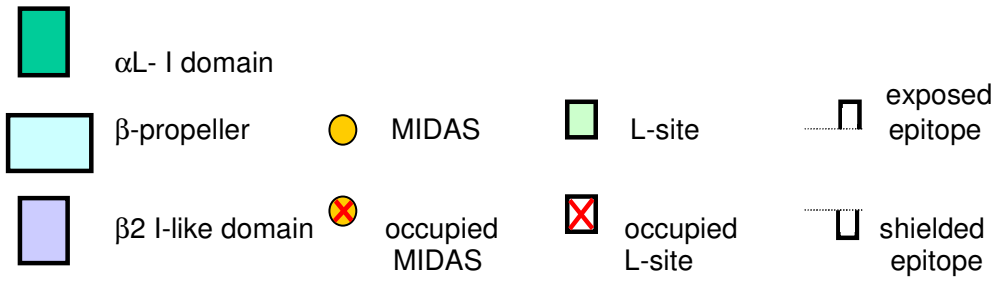


Figure 2.7 Schematic diagram of the LFA-1 αL chain and the β2 chain domains in the absence or presence of the LFA-1 inhibitors LFA703 or XVA143. Inhibitor induced epitope changes within the domains are detected by reduced antibody binding. A conformational linkage between the αL I domain and β2 I-like domain induced by receptor activation via cations is illustrated by a movement of the β2 I-like domain towards the αL L-site of the αL I domain.

Legend to Figure 2.7



For the first time we show that the dual LFA-1/Mac-1 inhibitor XVA143 induces epitope changes in the β2 I-like domain of the LFA-1 CD18 chain. Most interestingly, the I domain on the αL chain of LFA-1 is not affected by the compound. The cation dependency of XVA143 binding suggests that the inhibitor directly interacts with the putative metal-ion in the MIDAS-like motif of the β2 I-like domain or a putative calcium binding site adjacent to the MIDAS-like motif. Such a calcium binding site has recently been identified within the β3 I-like domain of the integrin αVβ3 [5]. It remains unknown, however, whether this putative interaction of XVA143 with LFA-1 inhibits ICAM-1 binding via a direct or indirect mechanism. Our data (see above) and a recent study investigating the role of the β2 I-like domain of LFA-1 [7] provide evidence in favor of an indirect mechanism. Thus, we hypothesize that the binding of XVA143 to the β2 I-like domain induces conformational changes which disturb the interaction between αL I domain and β2 I-like domain necessary for ICAM-1 binding. This hypothesis is supported by a study analyzing the inhibition of the αIIbβ3/fibrinogen interaction by RGD containing peptides. This study indicates that inhibitor binding to the β3 I-like domain induces the dissociation of fibrinogen from its binding site located on the α chain of αIIbβ3 via an allosteric mechanism [28]. Taken together, these data provide strong evidence that β2 I-like domains on integrin β subunits represent targets for allosteric integrin inhibition, similar to the well established allosteric αL L-site on the αL chain [14-17].

We also examined the effect of the different LFA-1 inhibitors on inactive LFA-1 in the absence of cations. The interaction of XVA143 with the inactive receptor was found

to be very weak as indicated by marginal changes of a few mAb epitopes. This observation is in agreement with the assumption that XVA143 interacts with a cation binding site on the β2 I-like domain. In contrast, the lovastatin-derived LFA-1 inhibitors mediated epitope changes in the αL I domain of LFA-1 in absence of cations indicating that the αL L-site is accessible in the inactive and active receptor and that inhibitor binding to the αL L-site is not dependent on cations. However, αL L-site engagement in the inactive receptor did no longer result in conformational alterations in the β2 I-like domain of the β chain. This finding indicates that the conformational interaction between the αL I domain and β2 I-like domain observed in the active receptor is cation-dependent, and does not take place in the inactive receptor. Similarly, a conformational interaction between the αL I domain of the LFA-1 α chain and the stalk region of the LFA-1 β chain was abolished in the inactive receptor in the absence of cations (K.W. and G.W., unpublished data). These results suggest that during the LFA-1 activation process cation-dependent domain linkages are built up which allow inter-subunit communication. Our data imply that the putative β propeller region of LFA-1 is less affected by the activation process although this region is thought to be in close contact with both I domains.

In conclusion using mAbs directed against different domains of LFA-1 we were able to analyze how inhibitor binding to the αL L-site or the β2 I-like domain triggers epitope changes within and across LFA-1 domains. For the first time we can demonstrate these processes in the native receptor without introducing mutations. Our study demonstrates that both, the αL L-site and the β2 I-like domain constitute suitable targets for the design of integrin inhibitors which could be used to prevent or treat a wide range of diseases.

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3 Chapter 2

Low molecular weight inhibitors differentially affect the conformation and the immunologic function of the adhesion receptor LFA-1: Studies in whole blood

One manuscript publishing parts of this chapter (REMA) was accepted for publication:

"Improved LFA-1 inhibition by statin derivatives: Molecular basis determined by X-ray analysis and monitoring of LFA-1 conformational changes in vitro and ex vivo", J. Biol. Chem. 2004, in press by G. Weitz-Schmidt, K. Welzenbach, J. Dawson, J. Kallen

A second manuscript publishing further data of this chapter is in preparation

3.1 Introduction

T-cell migration and T-cell activation are fundamental for immune responses but can also account for graft rejection and for the development and worsening of autoimmune diseases [1]. Currently, in clinical transplantation and severe autoimmune diseases, therapeutic inhibition of T-cell activation is achieved with calcineurin phosphatase inhibitors such as cyclosporin A (Neoral™) or ascomycin derivatives (Prograf™, Elidel™). Alternatively rapamycin derivatives (Certican™, Rapamune™) are therapeutically used to block T-cell functions such as activation or clonal proliferation [2]. However the current immunosuppressive regimens are often associated with severe side effects such as lymphomas, nephrotoxicity and hypertension (CsA), diabetes mellitus (steroids) and hyperlipidemia as well as thrombocytopenia (rapamycin derivatives) [1,3-5]. Strong efforts are ongoing to circumvent these side effects by either using combination of additively and synergistically acting drugs, or to develop compounds or biologicals with new mode of actions [6,7]. Novel, first in class, and potentially safer drugs for the prevention of graft rejection and to treat autoimmune disorders are therefore medical needs [8]. One approach for a novel therapeutic intervention is the inhibition of the β2 integrin LFA-1. This receptor is pivotal in adhesion and migration processes of T-cells and acts as a costimulatory molecule essential for T-cell activation and T-cell function [9,10]. LFA-1 is a clinically validated target. Efalizumab, a humanized monoclonal antibody (mAb) which blocks LFA-1 function has been shown to alleviate the symptoms of psoriasis in clinical trials and has recently been approved as a biopharmaceutical [11-13]. To overcome the hurdle of administration by injection or infusion, several classes of low molecular weight (LMW) inhibitors are under preclinical development. Today these compounds can be differentiated into two groups according to their mode of action on the receptor level. One group of antagonists which we termed αL L-site inhibitors, binds to the αL L-site located underneath the C-terminal α7 helix of the αL I domain and blocks the downward axial displacement of the α7 helix, thereby stabilizing the αL I domain in an inactive state [14]. Our work of chapter 1 has led to the definition of the

second group of LFA-1 inhibitors (e.g. XVA143 and derivatives), the β2 I-like domain inhibitors. These compounds appear to interfere with regulatory domains on the β2 chain and act independently of the αL I domain [15]. Our hypothesis that the compound class of XVA143 may bind to the MIDAS of the regulatory β2 I-like domain was further substantiated by recent, more detailed studies by Shimaoka *et al*[16]. These studies suggested that XVA143 may act as ligand mimetic for the β2 I-like domain MIDAS, stabilizes the β2 I-like domain in the liganded state and induces a conversion from the bent to the extended integrin conformation. In addition, XVA143 may disrupt the conformational communication between the regulatory β2 I-like domain and the αL chain which is required for affinity regulation of the αL I domain [16]. In summary, both classes of LFA-1 inhibitors can induce conformational changes in regulatory domains of LFA-1 which allosterically prevent the structural rearrangements within the headpiece of LFA-1 required for ligand binding. Our results of chapter 1 have demonstrated that these compound-induced conformational changes can be monitored by the altered binding behavior of specific mAbs which allowed us to differentiate between the different modes of action.

LFA-1 inhibitors of several chemical entities are presently in the late phase of preclinical development and may approach clinical studies. As for many “first in class” drug candidates, an early evaluation of a promising LFA-1 inhibitor in man is desirable before laborious and costly drug development efforts are undertaken. An early clinical assessment of a novel drug candidate aims to gain first insights on drug absorption, distribution, metabolism and excretion (ADME) and preliminary tolerability. However, these pharmacokinetic (PK) measurements do not reflect the pharmacological effects of the drug *in vivo*. Particularly in life supporting immunosuppressive therapies such as those used in transplant recipients, both sub-therapeutic concentrations and overdosing of a immunosuppressive drug can have devastating results. Sub-therapeutic dosage increases the risk of transplant rejection, and overdosing can lead to lymphomas [3], infections [4] and/or drug-specific side effects [17]. The assessment of pharmacodynamic activity (PD) of immunosuppressive drug candidates in early clinical phases gains thus more and more interest. There are

two major advantages to PD measurements. Firstly, PD activity may help to predict adequate immunosuppression in the clinical setting. Furthermore, blood levels of drug candidates and their metabolites often do not correlate with drug efficacy due to inter-subject variability in sensitivity towards an immunosuppressant drug [18]. PD assay systems which demonstrate that a compound can indeed impair immune cell function in a subject treated may facilitate drug development and may guide clinical decision on the therapeutic dose [19].

In addition, the technical feasibility of PD assays has significantly improved over the last decade. Methods such as flow cytometric techniques have evolved, and allow us today to study immune cell function on individual cells requiring only microliter quantities of blood. These methods are essentially used to assess T-cell function of immune deficient individuals but some reports describe the assessment of PD effects of immunosuppressants *ex vivo* [18,19].

Currently strong efforts are ongoing to develop suitable methods to quantify PD activities for immunosuppressive compounds *ex vivo*.

A PD assay for the *ex vivo* characterization of a T-cell immunosuppressant ideally measures compound mediated effects on T-cell effector functions such as cytokine release, cytotoxic function or proliferation. The most commonly used *in vitro* method to test immunosuppressant compound effects on T-cell function is the mixed lymphocyte reaction (MLR). This method quantifies proliferation of peripheral blood lymphocytes (PBLs) after incubation with allogenic PBLs. For most drug candidates, these assays are not suitable as predictive *ex-vivo* PD assays: for example, during the isolation of PBLs the equilibrium of the immunosuppressant with serum proteins is altered; or the immunosuppressant is removed by the required washing steps. To overcome this, PD assays which measure T-cell function in whole blood are ideal.

The purpose of the work described in chapter 2 of this dissertation was to characterize pharmacodynamic effects of LFA-1 inhibitors on LFA-1 expressed on native leukocytes in whole blood. We intended to determine whether and to what extent LFA-1 inhibitors can occupy their target in whole blood as well as how these

compounds affect LFA-1 receptor expression. Furthermore, we thought to study whether LFA-1 inhibitors can impair T-lymphocyte activation and proliferation triggered in whole blood. The methodology developed should allow for the first time a broad *in vitro* pharmacodynamic characterization of LFA-1 inhibitors (αL L-site inhibitors and β2 I-like domain inhibitors) in whole blood. The methods developed were furthermore intended as a basis for the pharmacodynamic characterization of LFA-1 inhibitors in clinical trials.

The principle of measuring changes in LFA-1 receptor conformation by monitoring antibodies upon inhibitor binding (Chapter 1) was adapted to the whole blood situation. Two novel receptor epitope monitoring assays (REMAs) were developed to allow the monitoring of receptor occupancy by two distinct chemical classes of αL L-site inhibitors and the β2 I-like domain inhibitor XVA143 in whole blood. The methods were further advanced to simultaneously assess LFA-1 occupancy, the LFA-1 receptor density and T-cell activation on individual T-cells after *in vitro* activation of whole blood.

In vitro T-cell activation was triggered by the crosslinkage of the CD3/TCR complex with mAb OKT3. This *in vitro* stimulation represents a specific and relatively physiological way to activate T-cells [20] and is different from published protocols using phorbol ester and calcium ionophors for whole blood T-cell functional studies. mAb OKT3 triggers T-cell receptor (TCR) specific signaling pathways (Fig. 3.1) which lead to LFA-1 activation [20-23]. LFA-1 receptors interact with ICAM-1 and -3 on the neighboring leukocytes and transduce costimulatory signals (signal 2). Signal 1 (of the TCR) and costimulatory signaling (signal 2) are required for T-cell activation (e.g. CD69 upregulation), the formation of the “immunological synapse” [24] and functional T-cell responses such as cytokine (IL-2) mediated proliferation [21,24,25]. CD69 up-regulation as an early marker for T-cell activation was described earlier [26,27]. A model for this LFA-1 dependent T-cell activation is shown in figure 3.1.

For demonstrating LFA-1 dependency of the assay a combination of mAb OKT3 with an anti CD28 mAb was used. Crosslinking the CD28 receptor on the cell surface of

T-lymphocytes by antibodies induces strong T-cell specific costimulatory signals (signal 2) which lead to pronounced T-cell activation and proliferation [28] (not shown in Fig. 3.1). The combination of mAb OKT3 and anti CD28 mAbs (OKT3/aCD28) should lead to LFA-1 independent T-cell activation.

For the measurement of T-cell proliferation, a separate mAb OKT3 stimulated protocol was set up using the classic ^{3}H thymidine incorporation approach similar to that described by Wendelbo *et al.* [29].

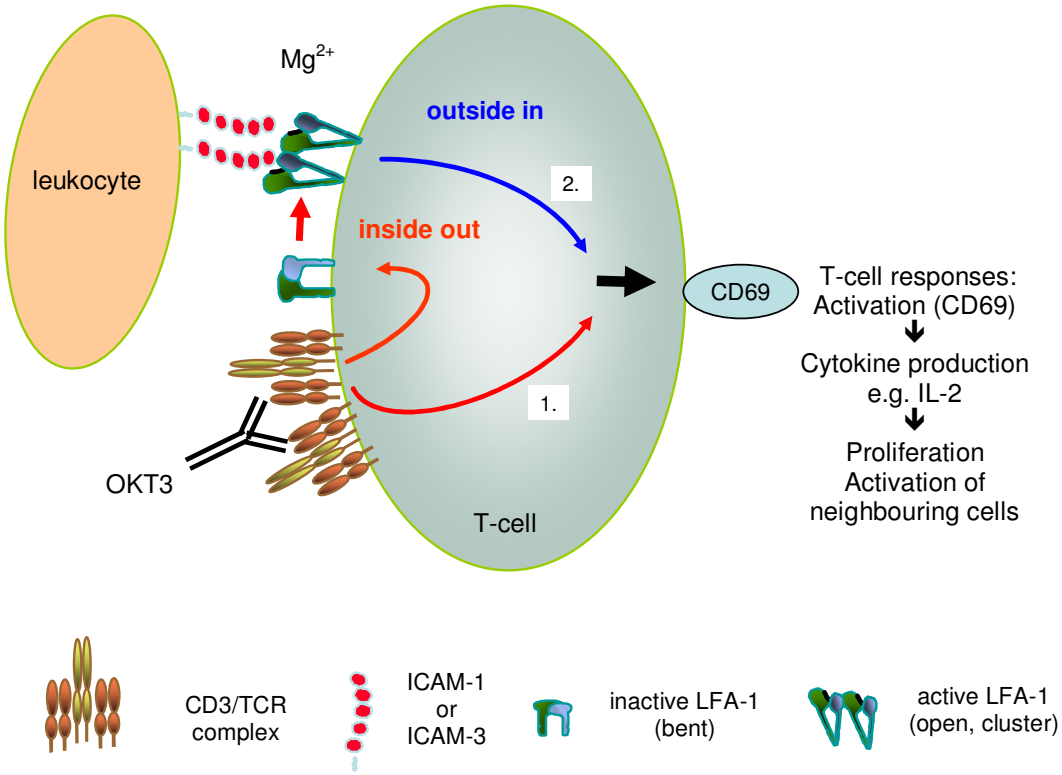


Figure 3.1 Principle of T-lymphocyte activation in the LFA-1 dependent whole blood T-cell activation /proliferation assays. T-lymphocytes are specifically activated with anti CD3 mAb (OKT3) which activates (amongst other pathways) LFA-1/ICAM binding and LFA-1/ICAM dependent costimulatory events. Both T-cell receptor (TCR) and LFA-1 dependent signals (signal 1+2) together result in T-cell activation (CD69 upregulation) and production of proliferation cytokines (IL-2 synthesis). An overview of LFA-1 signaling pathways is given in the first part of the dissertation.

Besides compounds described in chapter 1 (XVA143 and pravastatin), we included two new LFA-1 inhibitors, LFA878 and COMPOUND X in our studies. LFA878, a lovastatin-derived LFA-1 inhibitor, is similar to LFA451 (described in chapter 1) and

has almost equivalent *in vitro* characteristics. COMPOUND X was included to employ a different chemical class of LFA-1 antagonists. COMPOUND X is an exploratory compound which is not based on the lovastatin structure. COMPOUND X was found to bind to the L-site of the αL I domain and to allosterically modify the αL I domain conformation as reported for the lovastatin derivatives in chapter 1 (Novartis unpublished data).

Furthermore, two Novartis immunosuppressive drugs were assessed in the developed assays: Cyclosporin A (CsA, Sandimmune™, Neoral™) and everolimus (Certican™). CsA is widely used in transplantation and severe autoimmune diseases and impairs T-cell activation and T-cell proliferation by inhibiting the phosphatase calcineurin [30]. Everolimus is a rapamycin derivative similar to Rapamune™, which binds to the mammalian target of rapamycin (mTOR) and blocks cytokine mediated T-cell proliferation [2,31,32]. While CsA has been used in humans since 1983, everolimus was granted approval by the European authorities in 2003.

3.2 Methods

3.2.1 Materials

Antibodies (mAb)

FITC-conjugated anti human CD11a (clone R7.1) was obtained from BioSource, US; FITC-conjugated anti human CD18 (clone IB4) was purchased from SBA, US; FITC-conjugated anti human CD18 (clone MEM48) and FITC-conjugated anti human CD2 (clone MEM65) and all isotype controls (IgG1 and IgG2a) were obtained from Immunotools, Germany; phycoerythrin (PE) labeled anti human CD69 (clone L78) and PerCp-conjugated anti human CD3 (clone UCHT1) were purchased from Becton Dickinson, Switzerland. The hybridoma cell lines producing anti human CD11a (clone TS2/4.1.1) or anti human CD3 (clone OKT3) mAbs were obtained from the American Type Culture Collection (ATCC). The hybridoma cell line producing anti human CD28 (clone 15E8) was a kind gift of Prof. L. Aarden, Sanguin INC., Netherlands. Production and purification of mAbs were conducted within Novartis Pharma AG, Switzerland by standard protocols. mAb TS2/4.1.1 was labeled with ALEXA647 using an ALEXA647 antibody labeling kit (Molecular Probes, Netherlands) according to manufacturer's instructions.

Other materials

Erythrocyte FACS Lysing Solution was purchased from Becton Dickinson, Switzerland; Sodium heparin was obtained from B.Braun Medical AG, Switzerland; X-VIVO 10™ medium was obtained from BioWhittaker, US; Sterile Dulbecco`s phosphate buffered saline (PBS) (w/o Ca^{2+} and Mg^{2+} w/o sodium bicarbonate) and RPMI1640 were obtained from Invitrogen LifeTechnologies, US; Bovine serum albumin (BSA, protease and IgG free), Dimethylsulfoxide (DMSO), Ethylenediaminetetraacid (EDTA) and phytohemaglutinin (PHA) were purchased from Sigma-Aldrich, Switzerland; Scintillation liquid (Betaplate Scint) was purchased from Perkin-Elmer, Switzerland; ^{3}H methyl thymidine (^{3}H thymidine) was purchased

from Amersham Pharmacia Biotech, Switzerland; Prequillan was obtained from Fatro Inc, Italy.

Test compounds

LFA878, XVA143, COMPOUND X, CsA, everolimus were supplied by the Novartis department of chemistry. Pravastatin was obtained from Calbiochem, US.

3.2.2 Biohazard

All experimental steps involving blood samples were conducted in a laboratory classified as “BL2” according to Novartis research guidelines.

3.2.3 Blood collection

Human blood was collected from healthy volunteers by venipuncture (butterfly needle) at the Novartis Basle health care unit. Time of collection was in the morning (7:30 a.m.-8:35 a.m.) Sodium heparin (100 U/ml) was immediately added to the blood samples.

3.2.4 Dilution of test compounds

All test compounds were dissolved in DMSO at 10 mM. Stock solutions were stored at 4°C except everolimus which was stored in aliquots at -20°C. In indicated experiments the compounds were serially diluted in DMSO before direct addition to blood.

3.2.5 REMA (human/rabbit) for αL L-site inhibitors

Heparinized undiluted human or rabbit blood samples (198 µL) were mixed with the compounds or the DMSO solvent control (2 µL) and incubated for 30 min at room temperature (RT). The blood samples (90 µl/well) were then transferred to 96 well PP microtiter plates. The FITC-conjugated mAb R7.1 was diluted in PBS containing 0.5% BSA, pH 7.4 (PBS/0.5%BSA) and added (10 µL) to the blood samples (final concentration: 1 - 2 µg mAb/ml blood). After 25 min staining at RT the blood samples (100 µl) were transferred to polypropylene tubes containing FACS lysing solution (1

ml), mixed and incubated in the dark at RT for 10 min. The samples were centrifuged (200 x g) for 5 min, washed once with PBS/0.5% BSA and analyzed within 24 hours on a FACScan or a FACS-Calibur flow cytometer. Light scatter parameters and fluorescence 1 (FITC) were acquired. Data acquisition was stopped after the acquisition of 12000 lymphocyte events.

Background fluorescence of unstained lymphocytes, monocytes or granulocytes was deducted from all values. Mean fluorescence intensities (MFIs) of bound mR7.1 to whole blood leukocytes in solvent controls were set as 100 %. mAb R7.1 binding, in the presence of the test compounds was expressed as percentage of these controls. The values derived for the 3 subpopulations of leukocytes were averaged and standard deviation (SD) was calculated. Resulting curves were plotted and IC_{50} were analyzed with the dose response curve fitting tool of ORIGIN V. 7.0 (OriginLab Corporation).

3.2.6 REMA (human) for β2 I-like domain inhibitors

The REMA for the β2 I-like domain inhibitor XVA143 was conducted as described above for αL L-site inhibitors, except that the FITC-conjugated anti human CD18 mAb MEM48 (1.5 µg/ml) was used. In one experiment the anti human CD18 mAb IB4 was used instead of mAb MEM48. MFIs of bound mAb MEM48 to whole blood leukocytes in solvent controls were set as 100 %. Enhancement of mAb MEM48 binding to LFA-1 was expressed as percentage of these controls. The values derived for the 3 subpopulations of leukocytes were averaged and standard deviation (SD) was calculated. Resulting curves were plotted and EC_{50}s were analyzed with the dose response curve fitting tool of ORIGIN V. 7.0.

3.2.7 *Ex vivo* rabbit REMA for αL L-site inhibitors

Female russian dwarf rabbits received 0.1 ml/kg Prequillan (10 mg/ml) subcutaneously. LFA878 was dissolved in a mixture of Cremophor EL and ethanol (2:1/w:w) and then further diluted with 5% glucose (1:3/v:v). The compound or vehicle control was administered intravenously (i.v.) by bolus injection (right ear, 1.5 ml per

rabbit). Blood samples (200 µl) were taken from the left ear at indicated time points and collected into micro tubes containing sodium heparin (100 U/ml blood). The blood samples were stored on ice until analysis. The REMA was conducted as described above. The animal experiments were conducted according to the animal experimentation guidelines and laws laid down by the Swiss Federal and Cantonal authorities.

3.2.8 T-lymphocyte activation in whole blood

The activating anti human CD3 mAb OKT3 was immobilized onto a 96 well microtiter plate (Maxisorb, Nunc Inc, US) in PBS (0.01-30 µg/ml, 100 µL/well), pH 8.0 at 4°C over night. On the next day the plate was washed twice with sterile PBS/0.5%BSA. The plate was then blocked with sterile PBS/0.5%BSA for 1 h at 37°C. PBS (50 µL/well) with or without 4 mM magnesium chloride ($MgCl_2$) was added to the wells followed by the addition of undiluted human blood (50 µL/well). After briefly shaking, the plate was incubated at 37°C in a 5% CO_2 humidified atmosphere for 22 h. EDTA solution (20 mM, 15 µL/well) was added to each well, and incubated for further 15 min to loosen adherent leukocytes. 3 color flow cytometry was performed to assess the upregulation of extracellular CD69 on human CD2+ lymphocytes. PerCp-conjugated anti human CD4 (1.1 µL), PE-conjugated anti human CD69 (2.5 µL) and FITC-conjugated anti human CD2 (1.2 µL) were diluted with PBS/0.5% BSA and added to a 2 ml PP plate (20 µL/well). Activated blood samples (200 µL) were added to the antibody staining cocktail and incubated at RT in the dark for 15 min. To lyse red blood cells and fix the samples, FACS lysing solution containing formaldehyde was added (1.4 ml). After 10 min lysis, the plate was centrifuged (250 x g) at RT for 6 min. The samples were washed once with PBS/0.5% BSA and then either analyzed immediately or stored at 4°C in the dark overnight and analyzed on the next day.

Data acquisition was performed on a FACSCalibur (Becton Dickinson) after adjustment of compensation with single fluorochrome labeled whole blood lymphocytes. For data acquisition a live gate was employed to acquire 10000 CD2+ positive events. Data evaluation was carried out with Cellquest Pro (BD). The

leukocyte subpopulation corresponding to lymphocytes was gated (gate 1) according to the FSC/SSC light scatter properties. Gate 1 was applied to a dot plot to visualize and gate all CD2+CD4+ events (gate 2). In an additional histogram the CD69 expression of these T-cells was plotted applying both gate 1 and gate 2. CD69 positive cells were selected using the marker tool. Generally less than 2% of CD2+CD4+ lymphocytes of non-stimulated human blood samples were CD69 positive. Histogram statistics were performed and exported using the "Batch" processing module of Cellquest Pro.

3.2.9 Expression-Activation (EA) REMA for αL L-site inhibitors

Heparinized human blood (1 ml) was added to wells of 2 ml polypropylene (PP) deep well plates followed by the addition of pre-diluted compounds (2 µL). DMSO (2 µL) was added as solvent control. The test compounds were incubated for 1 h at RT.

mAb OKT3 (1 µg/ml) - or alternatively mAb OKT3 (0.1µg/ml) and anti CD28 (clone 15E8, 1 µg/ml) - were immobilized onto a 96 well microtiter plate (Maxisorb, Nunc Inc, US) in PBS, pH 8.0 (100 µL/well) at 4°C overnight. On the next day the plates were washed twice with PBS/0.5%BSA. The plates were then blocked with sterile PBS/0.5%BSA for 1 h at 37°C.

In experiments involving activation by mAb OKT3, PBS (50 µL/well) containing 4 mM $MgCl_2$ was added to the wells followed by the addition of the blood samples (50 µL/well). The plate was incubated at 37°C in a 5% CO_2 humidified atmosphere for 22 h. EDTA solution (20 mM, 15 µL/well) was added to each well, and incubated for further 15 min to loosen adherent leukocytes. 2-3 individually activated blood samples were pooled.

In experiments involving the combination of mAb OKT3 and the anti CD28 (OKT3/aCD28) for stimulation, the assay was conducted as described for the mAb OKT3 stimulation but without additional $MgCl_2$.

200 µL of activated pooled blood was transferred to a 2 ml polypropylene deep well plate (Becton Dickinson, CH) and stained with PBS/0.5% BSA (20 µL/well) containing

FITC-conjugated mAb R7.1 (1.5 µL), PE-conjugated anti human CD69 (2.5 µL), PerCp-conjugated anti human CD3 (1.3 µL) and ALEXA-647-conjugated anti human CD11a mAb TS2/4.1.1 (1 µL). The concentrations used were saturating for TS2/4.1.1 and non-saturating for mAbs R7.1 as determined by titration (not shown). Blood samples were stained at RT in the dark for 15 min. Erythrocytes were lysed with Facs lysing solution (1.4 ml). After 10 min lysis, the plate was centrifuged (250 x g) at RT for 6-7 min. The samples were washed once with PBS/0.5% BSA and then either analyzed immediately or stored at 4°C in the dark overnight and analyzed on the next day.

For data acquisition a live gate was employed to acquire 10000 CD3 positive events. Data evaluation was carried out with Cellquest Pro (BD). The leukocyte subpopulation corresponding to lymphocytes was gated (gate 1) according to the light scatter properties. Gate 1 was applied to a histogram plotting all CD3 positive events. A second gate (gate 2) was drawn to select all CD3 positive lymphocytes. In three histograms the fluorescence signals of mAb R.71, anti CD69 and mAb TS2/4.1.1 were plotted applying both gate 1 and gate 2. Geometric means of the fluorescence intensities of mAbs R7.1 and TS2/4.1.1 binding to CD3 positive T-lymphocytes were calculated. The percentage of CD69 positive cells was determined using the marker tool. Histogram statistics were performed and exported using the "Batch" processing module of Cellquest Pro.

IC_{50} values were obtained by fitting the percentage of CD69 positive T-cells using the dose response curve fitting tool of ORIGIN V. 7.0 (OriginLab Corporation). The percentage of CD69 positive CD3+ events of non-stimulated blood and the averaged percentage of CD69 positive T-cells after stimulation without an inhibitor were used as the fitting range. 6-7 different concentrations per compound were tested to generate concentration response curves.

IC_{50}s for the effect of LFA-1 inhibitors on mR7.1 binding were obtained using unprocessed MFI data. Solvent controls and the MFI of an isotype control were used as fitting range.

3.2.10 EA-REMA (human) for the β2 I-like domain inhibitors XVA143

The EA-REMA for the β2 I-like domain inhibitor XVA143 was performed as described for the EA-REMA measuring αL L-site inhibitors, except that the FITC-conjugated anti human CD18 mAb MEM48 (1 µg/ml) was used instead of mAb R7.1. EC_{50} values were calculated with the dose response curve fitting tool of ORIGIN V. 7.0 . To calculate the effect of XVA143 on the LFA-1 surface expression, the mean fluorescence intensities of mAb TS2/4.1.1 binding to T-lymphocytes in the absence of an inhibitor was set as 100%.

3.2.11 Whole blood lymphocyte proliferation

Microtiter plates coated with mAb OKT3 or the combination of mAb OKT3 and anti CD28 mAb (OKT3/aCD28) were prepared as described for the EA-REMA.

Compounds were added to undiluted human blood as described for the EA-REMA. After 1 h incubation of the blood with the test compounds, blood samples (20 µl/well) were transferred to X-VIVO 10™ medium (180 µl/well) and added to antibody coated microtiter plates. Replicas of 3-4 wells were generated per compound concentration. The plates were put on a shaker for mixing and incubated for 72 h in a 5% CO_2-humidified air incubator. Wells were then pulsed with ^{3}H-methyl thymidine (1 µCi/ well) and incubated for additional 22 h. Blood samples were then transferred to a cellulose filter using a BETAPLATE® 96 well harvester (Perkin-Elmer). The filter was washed with distilled water, dried and counted after the addition of liquid scintillation fluid using a BETAPLATE® Liquid scintillation counter (Perkin-Elmer).

Alternatively, blood samples were stimulated with the lectin phytohemaglutinin (PHA) (3µg/ml). Proliferation experiments in 1:10 diluted blood lymphocytes were conducted as described above except that un-coated sterile microtiter plates (Costar, US) were used.

IC_{50} values were calculated by fitting the raw data of the ^{3}H thymidine incorporation assays with the dose response curve fitting tool of ORIGIN V. 7.0. 6 to 7 different

concentrations were used to generate concentration response curves. Triplicates or quadruplicates were used per concentration.

3.2.12 Calculations

For all calculations the compound concentration added to undiluted blood samples was used. This facilitates the back calculation of sensitivities of these assay systems.

3.2.13 Correlation receptor occupancy with T-cell responses

The inhibition of CD69 upregulation and T-cell proliferation by the test compounds was calculated [%] and correlated with the percentage of inhibition (mAb R7.1) or induction (mAb MEM48) of monitoring antibody binding. Alternatively the natural logarithm (ln) of the mean fluorescence intensities (MFIs) of the mAb R7.1 signals were used. Regression analysis was performed using ORIGIN V.7.0 statistic software.

3.2.14 ANOVA

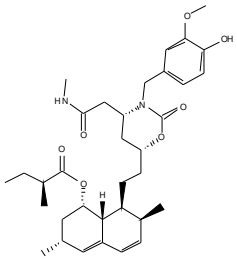
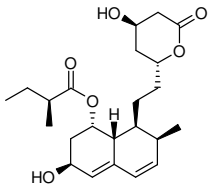
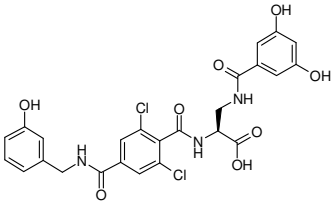
ANOVA was performed using the statistical software program “Graphpad INStat” version 3.05 and the Tukey-Kramer multiple comparison test.

3.3 Results

3.3.1 *In vitro* profile of LFA-1 inhibitors

Table 3.1 shows the *in vitro* profile of the LFA-1 inhibitors tested in chapter 2. The results were generated by applying the methods described in chapter 1 [15]. LFA878 (a lovastatin-derived LFA-1 inhibitor) and COMPOUND X (non lovastatin-derived LFA-1 inhibitor) blocked ICAM-1 binding to purified immobilized LFA-1 with IC_{50}s of 0.05 and 0.03 µM respectively (Table 3.1). No effect on ICAM-1 binding to purified Mac-1 was observed, demonstrating the selectivity of these compounds for LFA-1. The adhesion of the T-cell line HUT78 to immobilized ICAM-1 was inhibited with average IC_{50}s of 0.3 and 0.8 µM respectively (Table 3.1). The results for Pravastatin and XVA143 were already described in chapter 1.

Table 3.1 *In vitro* profile of the LFA-1 inhibitors and pravastatin

Assay	LFA878	Pravastatin (Control)	COMPOUND X	XVA143 (Genentech/Roche)
			experimental compound, unrelated to Lovastatin structure	
LFA-1/ICAM-1: IC_{50} [µM]	0.048 ± 0.013	> 100	0.034 ± 0.01	0.020 ± 0.008
HUT78/ICAM-1: IC_{50} [µM]	0.3 ± 0.2	> 100	0.8 ± 0.7	0.005 ± 0.004
I domain binding	+ *	- [15]	+ *	- [15]
Mac-1/ICAM-1: IC_{50} [µM]	> 100	> 100	> 100	0.002 ± 0.001

Values represent the mean ± SD of at least 3 independent experiments.
* Novartis unpublished observation. Methods see chapter 1

3.3.2 Receptor occupancy by LFA-1 inhibitors in human whole blood

The flow cytometric method used in chapter 1 to monitor LFA-1-inhibitor-induced conformational changes within the LFA-1 receptor was adapted to establish a method to measure LFA-1 receptor occupancy in whole undiluted blood. Different LFA-1 inhibitors were added to undiluted heparinized human blood at various concentrations. After 30 min of incubation the effect on the binding of the FITC-conjugated monoclonal antibodies (mAbs) R7.1 (anti CD11a), IB4 (anti CD18) or MEM48 (anti CD18) to whole blood leukocytes was measured. Leukocyte subpopulations were distinguished by their light scatter properties (Fig. 3.2a). We termed this simple and straight forward method to measure compound induced conformational changes within LFA-1 Receptor Epitope Monitoring Assay (REMA). The REMA utilizes the readout of only one fluorescence parameter and can be conducted in less than 2 h on any basic flow cytometer. A typical example for a REMA experiment is shown in figure 3.2a. In this experiment the αL L-site inhibitor LFA878 was tested. The compound almost completely abrogated the binding of mAb R7.1 to the major leukocyte subpopulations in human whole blood at 10 µM. The inhibition of mAb R7.1 binding was concentration dependent as shown in figure 3.2b (IC_{50} 0.4 µM). The potency of LFA878 was equal on the different leukocyte subpopulations as shown by the small standard deviation (Fig. 3.2b). Figure 3.2a also shows that lymphocytes contain subpopulations with distinct LFA-1 receptor densities, while monocytes and granulocytes demonstrated a homogenous LFA-1 expression pattern.

As expected, XVA143, which was shown in chapter 1 to affect the β2 I-like domain but not the aL I domain, was inactive in the mAb R7.1 REMA (Table 3.2).

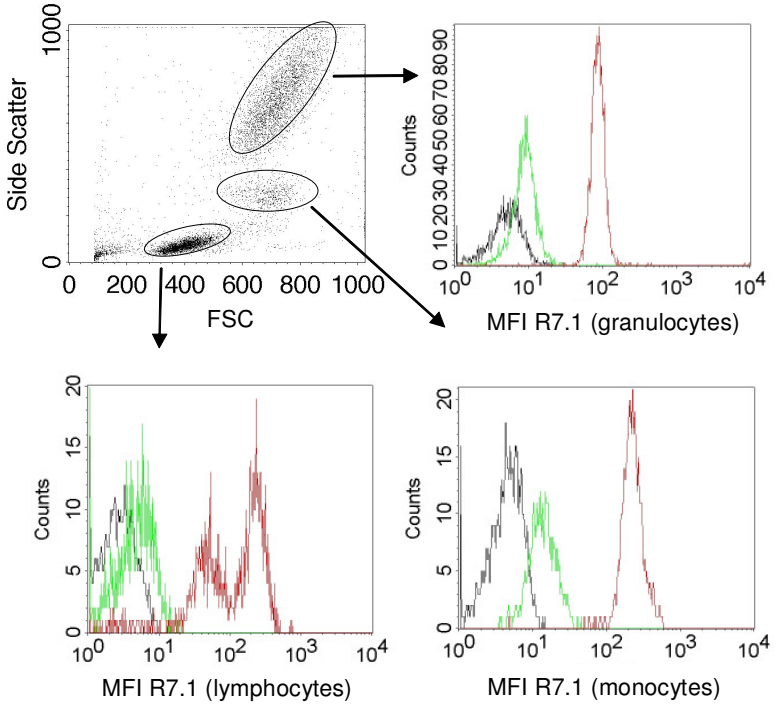


Figure 3.2a REMA: LFA878 induced conformational changes within LFA-1 receptors expressed on leukocytes in human whole blood. LFA878 efficiently blocked binding of anti CD11a mAb R7.1 to leukocytes in human whole blood. Dot-Plot: Leukocyte subpopulations were discriminated according to light scatter properties as granularity (side scatter) and cell size (forward scatter, FSC). Histograms: Red line: Binding of R7.1-FITC in the absence of LFA878. Green line binding of R7.1-FITC in the presence of 10 µM LFA878. Black line: Fluorescence of isotype control (IgG1).

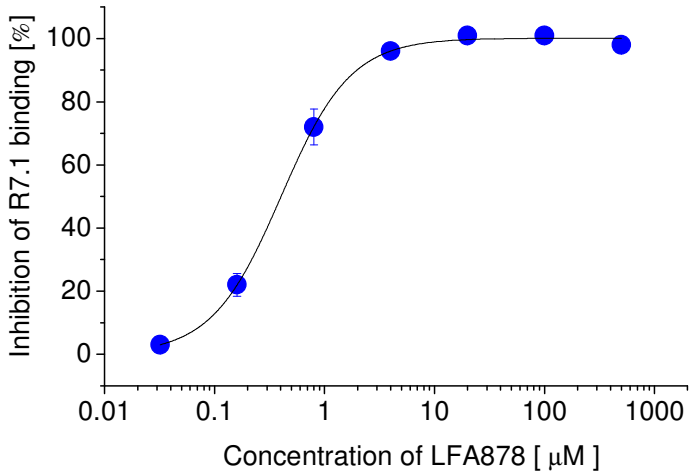


Figure 3.2b Binding of the CD11a mAb R7.1-FITC was completely abrogated by LFA878 at concentrations ≥10 µM in whole undiluted human blood. Experiment was carried out as described under Methods. Inhibition of mAb R7.1 binding to lymphocytes, monocytes and granulocytes was individually calculated. Data shown are means ± SD of these inhibitions.

As shown in chapter 1, the β2 I-like domain inhibitor XVA143 potently blocked the binding of mAb IB4 (anti CD18) to cation activated LFA-1 on Jurkat T-cells or purified LFA-1 [15]. The effect of XVA143 on the binding of mAb IB4 to native whole blood leukocytes was tested. To our surprise XVA143 did not or only marginally block the binding of mAb IB4 to human whole blood leukocytes even at high concentrations (50 µM) (Fig. 3.3a). As we demonstrated in chapter 1 XVA143 is a β2 integrin inhibitor and alters the conformation of the β2 I-like domain in a cation dependent manner. To investigate whether the absence of the activating cation manganese prevented XVA143 from interacting with LFA-1 we supplemented human whole blood with 1-2 mM $MnCl_2$. XVA143 remained inactive on mAb IB4 binding in the presence of $MnCl_2$ (data not shown). mAb IB4 was therefore not suitable for measuring receptor occupancy by XVA143 in whole undiluted human blood for reasons that need further investigation.

In contrast, XVA143 potently induced the binding of the anti CD18 mAb MEM48 to whole blood leukocytes (Fig. 3.3b). This agonistic effect of XVA143 on mAb MEM48 binding and its effect on the LFA-1 receptor conformation was dependent on the blood concentration of XVA143. (Fig. 3.3b: EC_{50} 0.014 µM). XVA143 enhanced the binding of FITC-conjugated mAb MEM48 by ≥ 2.5 fold (250%) at concentrations above 0.2 µM compared to solvent controls (100%). These findings demonstrate for the first time that XVA143 can interact with LFA-1 receptors in human blood and suggest that the degree of mAb MEM48 epitope exposure is dependent on the concentration of XVA143.

mAb MEM48 has been mapped to the cysteine rich repeat 3 flanked on the stalk region of the β2 chain of LFA-1 [33]. In addition, mAb MEM48 was shown to bind preferably to epitopes in the stalk region of the β2 chain that become exposed after LFA-1 activation [33]. Our data from the experiments with XVA143 in whole blood confirm our earlier, but not reported findings that XVA143 strongly induced conformational changes in domains on the β2 chain that are not directly involved in ligand binding (chapter 1 discussion). Furthermore, our data confirm meanwhile

published insights that XVA143 can induce an extended conformation of the β2 stalk region which is similar to the conformation found in the stalk region of an fully activated LFA-1 receptor (Fig. 1.5 [16]). Since anti CD18 mAbs such as IB4 and MEM48 are not specific for a particular β2 integrin, the data shown in Figs 3.3 a&b may also reflect the effect of XVA143 on the β2 integrins Mac-1 and possibly CD11c/CD18 which are significantly expressed on monocytes, granulocytes, NK cells and weakly on minor subgroups of T and B lymphocytes [34].

None of the αL L-site inhibitors altered the binding of mAb MEM48 in human blood (Table 3.2).

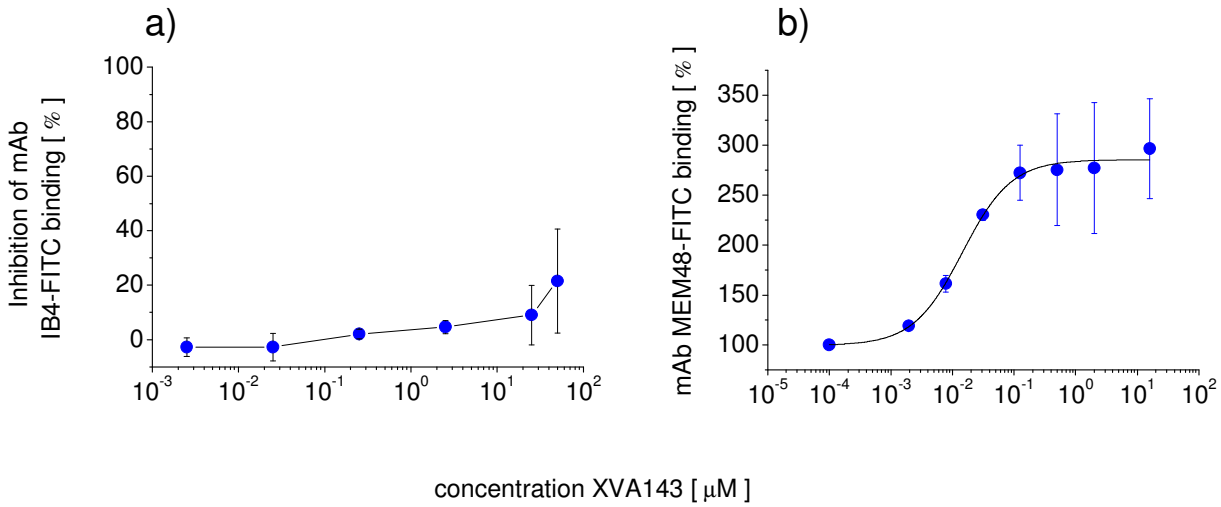


Figure 3.3 Effect of XVA143 on the binding of mAb IB4 and mAb MEM48 to human whole blood leukocytes: a) Binding of FITC-conjugated mAb IB4 to whole blood leukocytes is not significantly impaired by the indicated concentrations of XVA143. b) XVA143 strongly induced the epitope recognized by the anti CD18 mAb MEM48 (EC_{50}: 0.014 µM) on leukocytes inn whole blood. Experiments were carried out as described in Methods using undiluted whole blood. Inhibition of mAb IB4 or induction of mAb MEM48 binding to lymphocytes, monocytes and granulocytes were individually calculated. Data shown are means ± SD. One representative experiment out of 3 is shown.

3.3.3 Binding of mAbs R7.1 and MEM48 to LFA-1 of different species

The mAbs R7.1 and MEM48 were tested on cross species reactivity by flow cytometry. The experiments were performed as described for the REMA except that heparinized blood of other species was used. mAb R7.1 did not cross react with LFA-1 on leukocytes of mice and rats. In contrast, mAb R7.1 bound to leukocytes of dogs (beagles), monkeys (rhesus and cynomolgus) (data not shown) and rabbits (russian dwarf). All LFA-1 inhibitors that were active in the human REMA (R7.1 binding) also potently inhibited the binding of mAb R7.1 to leukocytes of the cross-reactive species. LFA878 for example blocked the binding of the mAb R7.1 to leukocytes in undiluted rabbit (russian dwarf) blood with an average IC_{50} of 0.34±0.18 µM (n=7, data not shown) equally potent as in the human REMA (Fig.3.2b). These findings indicate that the epitopes recognized by mAb R7.1 may be conserved amongst higher mammalian species and that the REMA can be applied in these species to study pharmacodynamic receptor occupancy by αL L-site inhibitors. In contrast to mAb R7.1, mAb MEM48 did not bind to LFA-1 of mice, rats, dogs, monkeys or rabbits (± XVA143, data not shown). The antibody was therefore not suitable for the *ex vivo* assessment of receptor occupancy by XVA143 or its derivatives in these species.

3.3.4 *Ex vivo* receptor occupancy assessment in rabbits

The REMA was validated by measuring the receptor occupancy by LFA878 in rabbits after i.v. administration of 11.5mg/kg or 50mg/kg LFA878. As shown in figure 3.4 LFA878 almost completely blocked the binding of the mAb R7.1 to whole blood leukocytes immediately after application. The pharmacodynamic effect of LFA878 was dependent on the dose administered and time point of blood sampling. Half-maximal blockade of mAb R7.1 binding was reached after 0.6 h (11.7mg/kg) or 3.3 h (50mg/kg). mAb R7.1 binding returned to levels of vehicle controls after 4 h (11.7mg/kg) and after 6 h (50mg/kg) respectively. These data indicate that the REMA is well suited to monitor pharmacodynamic effects of αL L-site inhibitors *ex vivo*. The PK/PD relationship for LFA878 after i.v. administration was measured in separate

experiments in rabbits and demonstrated that the pharmacokinetic measurements of LFA878 correlated well with the PD effect measured by the REMA (data not shown).

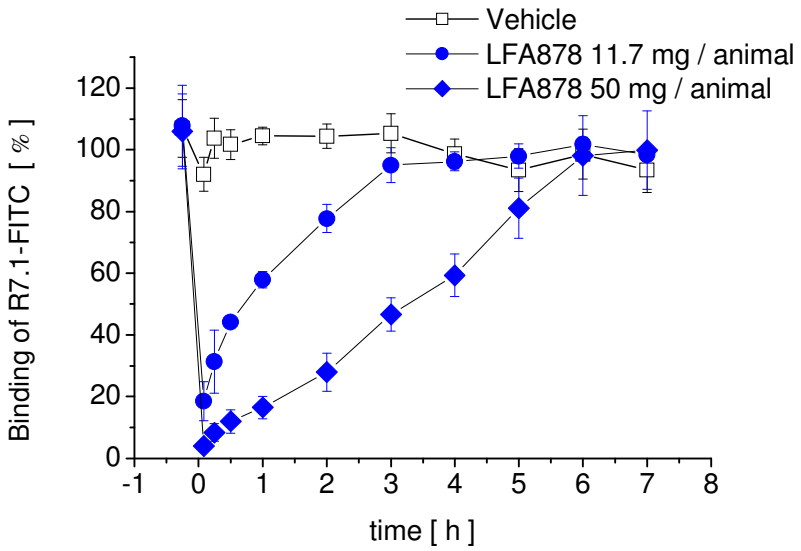


Figure 3.4 *Ex vivo* pharmacodynamic assessment of LFA-1 receptor occupancy by LFA878 in blood samples of rabbits. Compound or vehicle were applied i.v. at the indicated dose. Blood samples were taken at indicated time points. The REMA assay was performed as described in the Methods section. In this experiment the data represent the mean ± SD of individually calculated binding of mAb R7.1 to lymphocytes, monocytes and granulocytes in one blood sample. Binding of mAb R7.1 to leukocytes of a blood sample drawn before the application were set as 100%. One representative experiment out of 2 is shown.

3.3.5 Experimental set-up of the human whole blood T-cell activation and proliferation assays

After the establishment of the REMAs for LFA-1 inhibitors we intended to study whether LFA-1 receptor occupancy on blood lymphocytes translates into an impairment of T-cell activation or proliferation. The *in vitro* characterization of the effect of LFA-1 inhibitors on T-cell activation and proliferation in whole blood necessitated the *de novo* establishment of several test systems. Undiluted blood samples were spiked with the test compounds. T-lymphocyte activation and

proliferation was then triggered by immobilized anti human CD3 mAb OKT3 in 1:1 or 1:10 diluted blood samples respectively. The blood samples were then incubated in for either 22 h in the presence of 2 mM additional $MgCl_2$ (T-cell activation) or for 96 h (proliferation) after which different readouts were measured. T-cell activation was quantified by measuring upregulation of the activation marker CD69. Assessment of proliferation was done by quantifying incorporated radioactive ^3H thymidine. As a control stimulus mAb OKT3 was used in combination with anti CD28 mAb (OKT3/aCD28). Figure 3.5 shows the experimental principle of the whole blood T-cell activation and proliferation test systems.

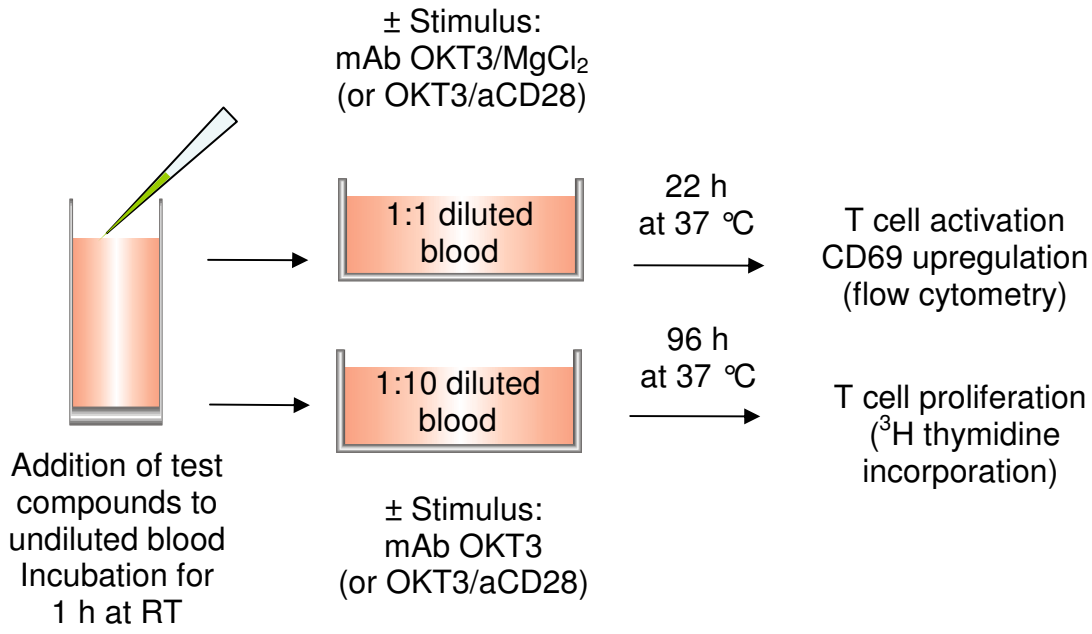


Figure 3.5 Experimental principle of whole blood T-cell activation and proliferation assays: Compounds were added to undiluted heparinized human blood and incubated for 1 h at RT. Aliquots of the blood samples were then transferred to wells of microtiter plates coated with the mAb OKT3 (anti CD3) or a combination of mAb OKT3 and anti CD28 (OKT3/aCD28). The effect of the inhibitors on CD69 upregulation as marker for T-cell activation was measured by flow cytometry after 22 h of incubation. Effects on T-cell proliferation were measured by 3H thymidine incorporation after a total incubation time of 96 h.

3.3.6 mAb OKT3 stimulated T-cell activation in whole blood is augmented by supplementary $MgCl_2$

Initial experiments revealed that extended incubation (3-4 days) of undiluted human blood at 37°C resulted in clotting of blood samples of several donors (data not shown). A 50% dilution of the heparinized blood with PBS and a 22 h activation allowed further assay development without observable coagulation. Furthermore, our initial data revealed that mAb OKT3 stimulated T-cell activation in undiluted blood, as assessed by CD69 upregulation, was marginal and barely reproducible. In 50% diluted blood only a minor percentage of T-cells responded with the expression of the activation marker CD69 (<4% CD69+CD3+ lymphocytes; example shown in figure 3.6). We investigated thus the addition of 2 mM magnesium chloride ($MgCl_2$) to the blood cultures. We reasoned that additional magnesium ions could activate LFA-1 (*vide* Table 1.1) on T-lymphocytes which could result in LFA-1 binding to the ICAM ligands presented by neighboring leukocytes. This would enhance LFA-1 dependent costimulatory signaling and boost T-cell activation. We tested the effect of supplemental 2 mM $MgCl_2$ with blood samples of two different blood donors. In the experiments shown in figures 3.6a&b CD2 and CD4 were used in these particular experiments to define T-cells (CD2+CD4+, T helper cells) as increasing concentration of immobilized mAb OKT3 strongly internalized the CD3 antigen (not shown). In the experiments shown below, the CD3 antigen was suitable for the discrimination of T-cells by flow cytometry due to the reduced amount of immobilized mAb OKT3 used.

Supplementing the whole blood cultures with 2 mM $MgCl_2$ resulted in 3-4 times enhanced CD69 upregulation in T-lymphocytes of both blood specimens after mAb OKT3 stimulation compared to the absence of the additional $MgCl_2$. 2 mM $MgCl_2$ alone (no mAb OKT3) did not lead to upregulation of CD69 on T-cells (Fig. 3.6a&b lowest concentration indicated on X-axis). The augmenting effect of 2 mM $MgCl_2$ on T-cell activation was LFA-1 dependent as specific LFA-1 inhibitors blocked the activation (Fig. 3.10a, table 3.2) and was evident on both CD4+ and CD8+ (CTLs) T-lymphocytes (CD8 not shown).

Our data suggest that 2 mM $MgCl_2$ acts synergistically with mAb OKT3 during T-lymphocyte activation in blood cultures, possibly by enhancing LFA-1 dependent cell to cell adhesive contacts. The effect of this enhanced cell-cell contact by $MgCl_2$ may be the reason for pronounced costimulatory events and subsequent T-cell activation.

It should be noted that the addition of 2 mM $MgCl_2$ and the activation of whole blood with mAb OKT3 for 22 h did not lead to macroscopic blood clotting or hemolysis.

Figures 3.6a&b also demonstrate that the CD69 response of T-lymphocytes was depended on the concentration of immobilized mAb OKT3. Furthermore, when mAb OKT3 was replaced by an isotype control antibody no significant activation of T-lymphocytes was observed demonstrating the specificity of the activation by the anti CD3 antibody. For the final protocol 2 mM additional $MgCl_2$ and a concentration of 1 µg/ml mAb OKT3 (mAb OKT3/MgCl2) was chosen for stimulation.

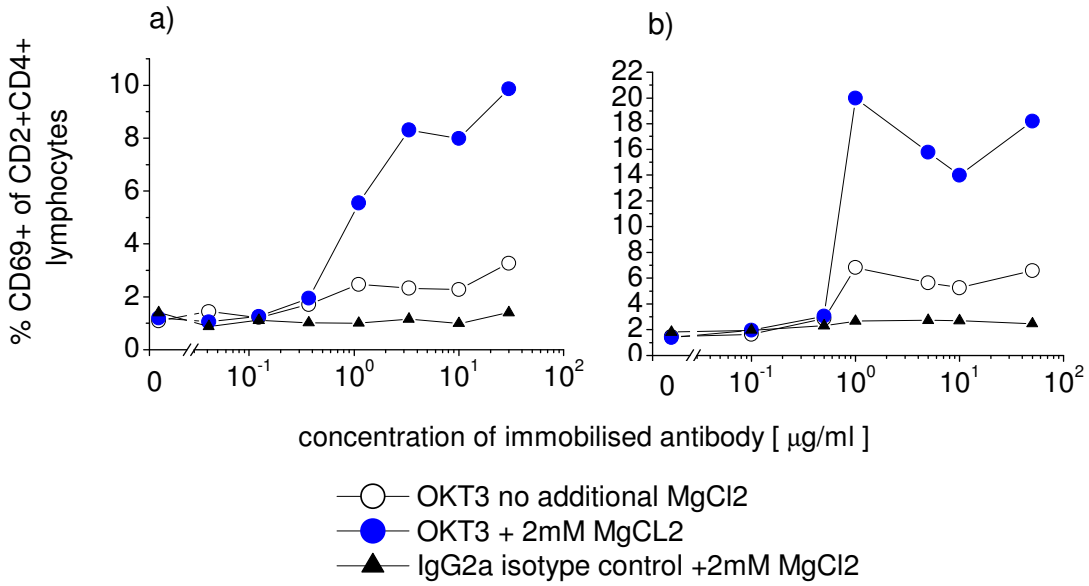


Figure 3.6 Addition of magnesium chloride enhanced mAb OKT3 stimulated T-lymphocyte activation in whole blood cultures: mAb OKT3 or an isotype control (IgG2a) antibody were immobilized to wells of 96-well microtiter plates at concentrations indicated in the graphs. At the lowest concentration shown on the axis no antibody was immobilized. Blood samples were diluted 1:1 with PBS w/w.o. 2 mM $MgCl_2$ and activated for 22 h. In this experiment 3 individually activated samples were pooled and stained for T-cell markers (CD2 and CD4) and CD69 expression. Data shown are the results of the pooled samples. Experiments were conducted with blood samples of 2 different donors (a) donor 1 and (b) donor 2. All samples were processed and analyzed by flow cytometry as described in Methods.

3.3.7 Principle of the EA-REMA

After optimization of T-cell activation via mAb OKT3 in whole blood cultures we combined the methods of T-cell activation and receptor epitope monitoring (REMA) to a single assay. In addition, the expression of LFA-1 receptors on the T-cell surface was measured using the non blocking anti CD11a mAb TS2/4.1.1 . mAb TS2/4.1.1 did not interfere with the binding of mAbs R7.1 or MEM48 (not shown). We termed our method EA-REMA (Expression-Activation Receptor Epitope Monitoring Assay). By utilizing either the anti CD11a mAb R7.1 (for αL L-site inhibitors) or the anti CD18 mAb MEM48 (for XVA143), the resulting method allowed to simultaneously assess the effect of different LFA-1 antagonists on receptor conformation (occupancy), LFA-1 receptor expression and T-lymphocyte activation (CD69 upregulation) on individual T-cells in 50% diluted blood. In figure 3.7 the principle of this four color flow cytometric method is visualized, depicting also the different fluorochrome combinations used.

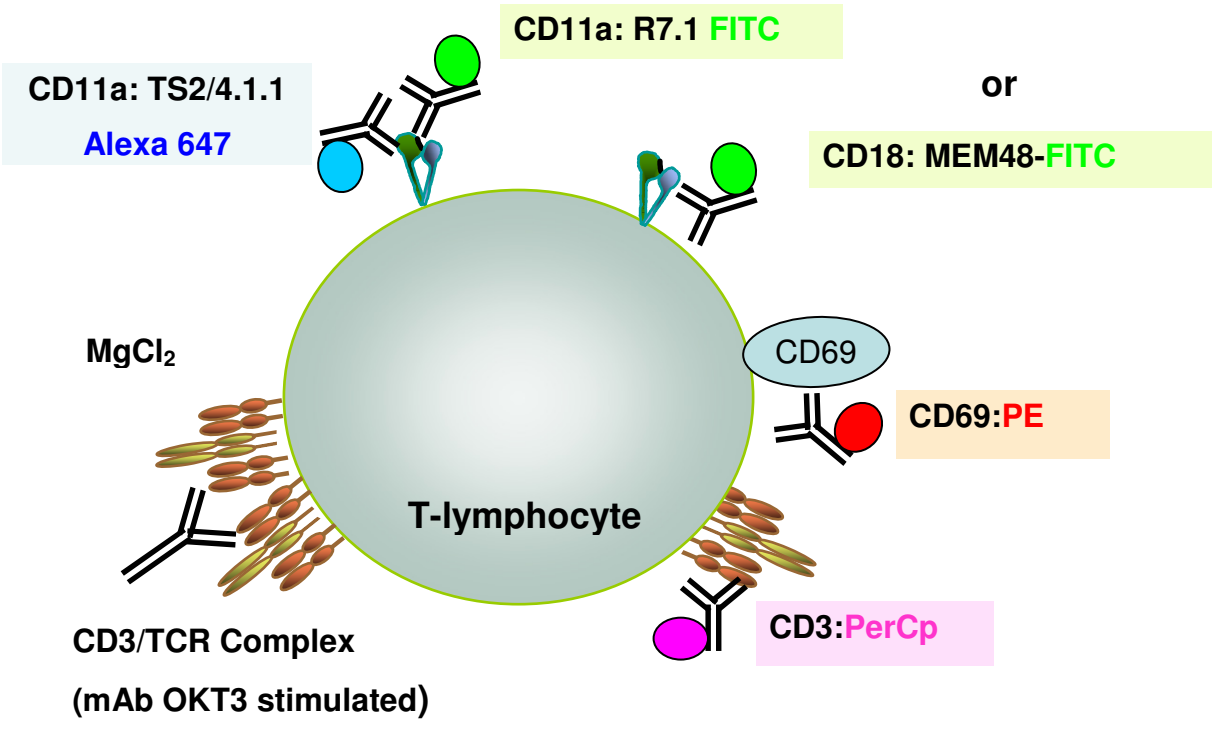


Figure 3.7 Principle of the EA-REMA: The EA-REMA is a four color flow cytometric method that allows to measures simultaneously the effect of LFA-1 inhibitors on T-cell activation (CD69 upregulation, mAb OKT3/MgCl2 stimulation shown), LFA-1 receptor expression (mAb TS2/4.1.1) and receptor occupancy (mAbs R7.1 and MEM48). The T-lymphocytes are detected by immunostaining the T-cell specific marker CD3.

3.3.8 Effects of LFA-1 inhibitors and control compounds in the EA-REMA

Figures 3.8 and 3.9 are examples for raw data derived from the two different EA-REMA assays. LFA878 (Fig. 3.8) and XVA143 (Fig. 3.9) were chosen as examples for LFA-1 inhibitors with the two different modes of action. In the experiments shown ca 6-7% of the T-lymphocytes were activated after 22 h stimulation with mAb OKT3/$MgCl_2$. In contrast, in the absence of mAb OKT3 1% of the peripheral blood lymphocytes were found positive for the CD69 antigen (Fig. 3.8 & 3.9) in the presence or absence of 2 mM $MgCl_2$.

Both LFA-1 inhibitors completely blocked mAb OKT3/$MgCl_2$ stimulated CD69 upregulation on T-cells at the tested concentrations (Figs. 3.8 & 3.9) indicating that both compounds were able to interfere with LFA-1 dependent T-cell activation in human whole blood cultures.

At a concentration of 10 µM LFA878 no mAb R7.1 binding was observed to T-cells indicating that at this concentration all LFA-1 receptors on T-lymphocytes were occupied (Fig. 3.8). At the same time LFA878 had no effect on the binding of mAb TS2/4.1.1 e.g. the expression of the LFA-1 receptors after 22 h incubation at 37°C (Fig. 3.8). Similar results were obtained with other αL L-site inhibitors such as COMPOUND X (Table 3.2).

Substituting the mAb R7.1 with mAb MEM48 enabled to apply the EA-REMA principle to assess the effects of the β2 I-like domain inhibitor XVA143 in human whole blood. In the experiment shown 2 µM XVA143 enhanced the binding of mAb MEM48 to whole blood T-cells by >3 fold (Fig 3.9). At the same time, XVA143 led to a significant downregulation (-52%) of LFA-1 surface receptors on T-cells as measured by the reduced binding of mAb TS2/4.1.1 (Fig 3.9).

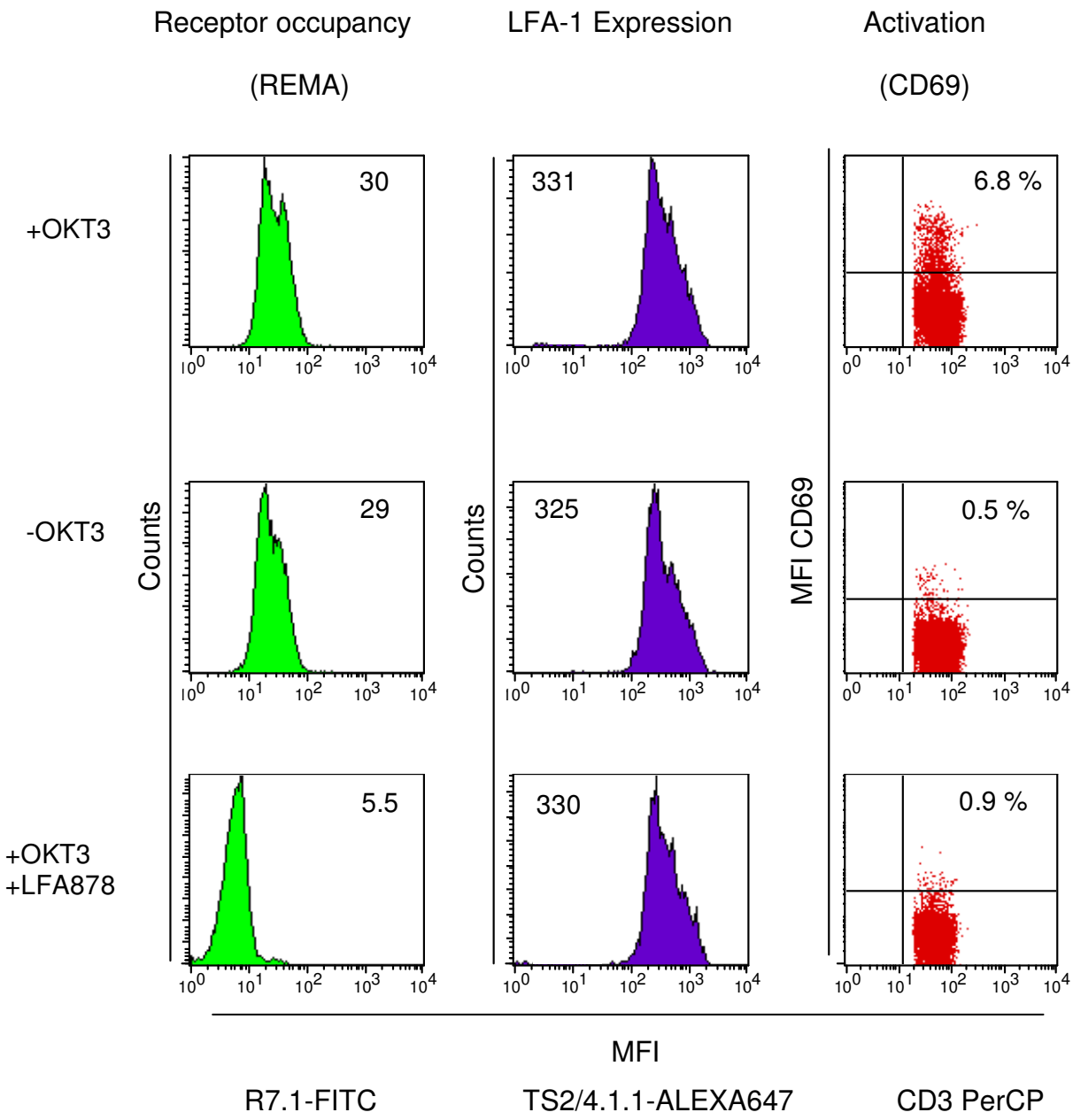


Figure 3.8 Effects of the αL L-site inhibitor LFA878 on T-cell activation, LFA-1 receptor occupancy and LFA-1 expression in whole blood (EA-REMA). 50% diluted blood samples were incubated in the presence of 2 mM MgCl2 with (+ OKT3) or without (- OKT3) immobilized mAb OKT3. One blood sample (+LFA878) contained 10 µM LFA878. mAb R7.1 was used to assess LFA-1 receptor occupancy by LFA878. mAb TS2/4.1.1-Alexa647 was employed to measure the effect on receptor expression. CD69 upregulation was measured to quantify the effect on T-cell activation. Experiment was carried out as described in Methods. One representative experiment out of more than 3 is shown. MFI: Mean fluorescence intensity. Numbers in the left and middle column indicate the MFIs; numbers in the right column the percentage of CD69+CD3+ T-cells.

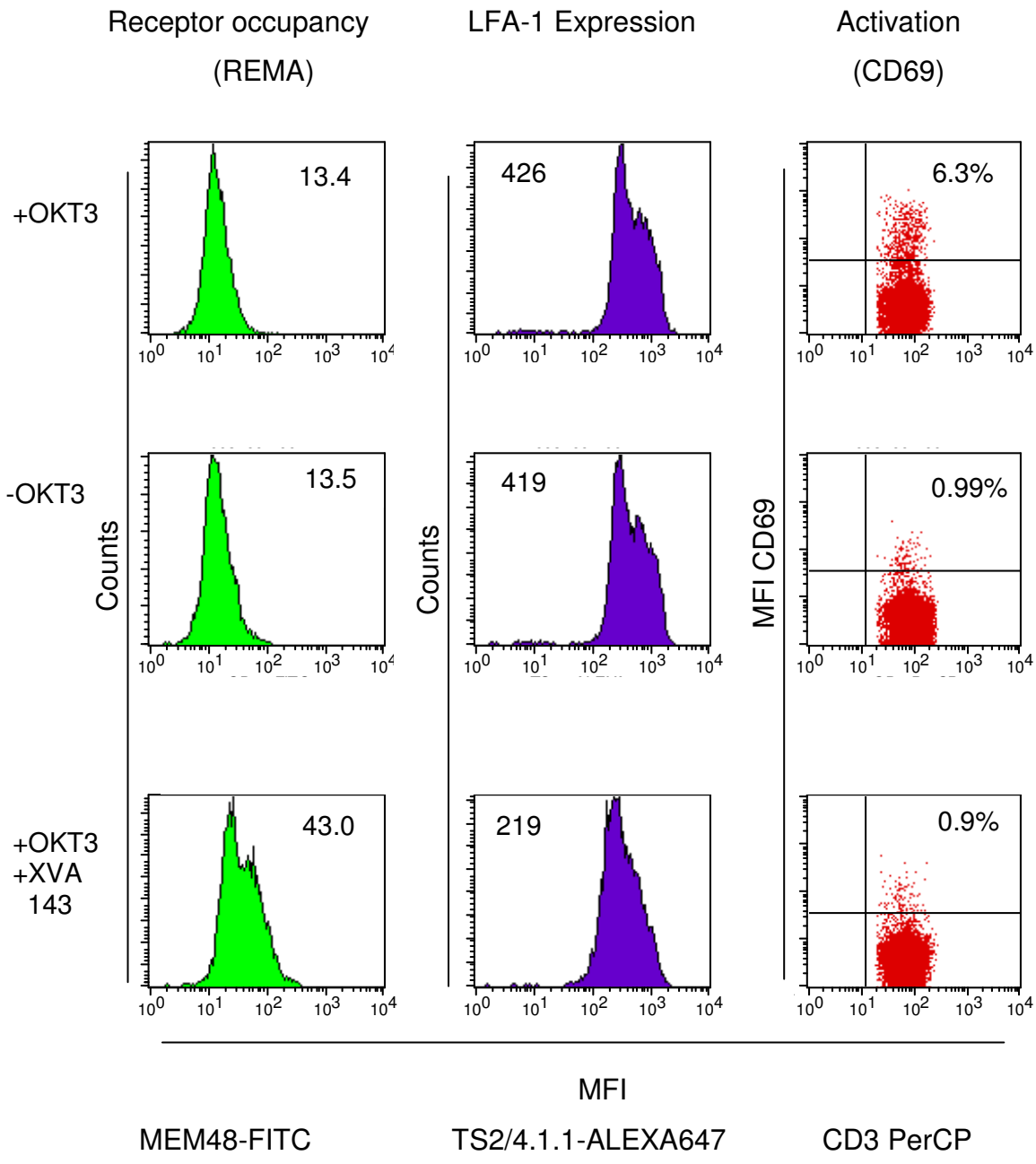


Figure 3.9 Effects of the β2 I-like domain inhibitor XVA143 on T-cell activation, LFA-1 receptor occupancy and expression in whole blood (EA-REMA). 50% diluted blood samples were incubated in the presence of 2 mM MgCl2 with (+ OKT3) or without (- OKT3) immobilized mAb OKT3. One blood sample (+ XVA143) contained 2µM XVA143. mAb MEM48 was used to assess LFA-1 receptor occupancy by XVA143. mAb TS2/4.1.1-Alexa647 was employed to measure the effect on receptor expression. CD69 upregulation was measured to quantify the effect on T-cell activation. Experiment was carried out as described in Methods. One representative experiment out of more than 3 is shown. Numbers in the left and middle column indicate the MFIs; numbers in the right column the percentage of CD69+CD3+ T-cells.

Figures 3.10-3.13 illustrate the effects of the LFA-1 inhibitors LFA878, COMPOUND X and XVA143 in the different EA-REMA readouts. These compounds were compared in mAb OKT3/$MgCl_2$ (LFA-1 dependent) and OKT3/aCD28 (CD28 dependent) stimulation. Pravastatin, which does not block LFA-1 (chapter 1) was included in the shown experiments as a control. All tested LFA-1 inhibitors, but not pravastatin, blocked mAb OKT3/$MgCl_2$ triggered T-lymphocyte activation (Fig. 3.10a) with different potencies in a concentration dependent manner. The inhibitory effects were clearly dependent on LFA-1 signaling pathways as the inhibitors failed to block CD69 upregulation when the T-lymphocytes were stimulated with OKT3/aCD28 (Fig. 3.10b), even though LFA-1 receptors were fully occupied by the inhibitors as measured by the REMA readout (Fig. 3.12b). These data provide strong evidence that low molecular weight (LMW) LFA-1 inhibitors can potently block LFA-1 dependent activation of T-lymphocytes in the whole blood environment. The data also demonstrate the high potency of the β2 I-like domain inhibitor XVA143. XVA143 blocked T-cell activation with the highest potency (IC_{50}: 48 nM), followed by COMPOUND X (IC_{50} 1.0 µM). LFA878 blocked T-cell activation with the lowest potency (IC_{50} 2.6 µM). All results of the whole blood assays are summarized in Table 3.2.

Figures 3.11a&b illustrate the effect of the test compounds on the expression of LFA-1 receptors on whole blood T-lymphocytes after 22 h at 37°C. The results provide strong evidence that prolonged incubation of XVA143 leads to a significant reduction of LFA-1 cell surface molecules (35% in the experiment shown). The downregulation of LFA-1 surface receptors was confirmed by using other anti LFA-1 mAbs such as mAb R7.1 (Fig. 3.12a) or mAb IB4 (not shown). This effect of XVA143 was highly reproducible and also observed in non-stimulated blood samples after 22 h at 37°C. In addition, the downregulation of LFA-1 by XVA143 correlated well with the potency of XVA143 in the mAb OKT3 stimulated T-cell activation assay (Table 3.2). However, since XVA143 did only induce a partial downregulation (maximal ≤55% of total LFA-1 staining) it is unlikely that the high potency of XVA143 in the mAb OKT3 mediated T-cell activation assay is solely a result of the diminished numbers of LFA-1 receptors present at the cell surface. Since LFA-1 is not reported to be proteolytically cleaved, it

is plausible that XVA143 caused internalization of LFA-1. In independent experiments we have observed complete internalization of LFA-1 receptors from the cell surface after 22 h incubation with crosslinking anti LFA-1 antibodies (data not shown) which indicates that upon receptor occupancy LFA-1 receptors can be internalized. The partial nature of the LFA-1 downregulation mediated by XVA143 could indicate that two subgroups of LFA-1 may exist which differ by their ability to internalize upon receptor occupancy by XVA143. The almost constant ratio of 35-55% downregulation of LFA-1 by XVA143 after 22 h further substantiates this speculation. In separate experiments the effect of XVA143 on the binding of mAbs R7.1, IB4 and TS2/4.1.1 was studied after 1 h incubation at 37°C without stimulation. The short term incubation of whole blood lymphocytes with XVA143 did not to affect the binding of these mAbs to T-lymphocytes suggesting that downregulation of LFA-1 receptors by XVA143 required prolonged incubation (data not shown). In contrast to XVA143, all other test compounds did not induce a downregulation of LFA-1 surface receptors on T-lymphocytes under these experimental conditions again showing the completely different mode of action of these compounds.

A comparison of the effect of the test compounds on the receptor conformation e.g. LFA-1 receptor occupancy under EA-REMA conditions is shown in Figures 3.12 and 3.13. It should be noted, that neither mAb OKT3/$MgCl_2$ nor OKT3/aCD28 stimulation (Fig. 3.12&3.13) for 22 h or the dilution of the blood by 50 % (EA-REMA versus REMA) had a significant effect on the potency of the compounds to reduce or induce binding of mAbs R7.1 and MEM48 respectively. The αL L-site inhibitors LFA878 and COMPOUND X reduced the binding of mAb R7.1 to whole blood T-lymphocytes with nM potencies while pravastatin was inactive (Fig. 3.12a&b;Table 3.2). The downregulation of cell surface LFA-1 by XVA143 was confirmed by the reduced binding of mAb R7.1 (Fig 3.12a). As expected XVA143 potently induced mAb MEM48 binding to whole blood T-cells while the αL L-site inhibitors did not have an effect on the expression of the mAb MEM48 epitope (Figs. 3.13a&b) demonstrating again the ability of the monitoring antibodies to detect the two modes of action of the tested LFA-1 inhibitors.

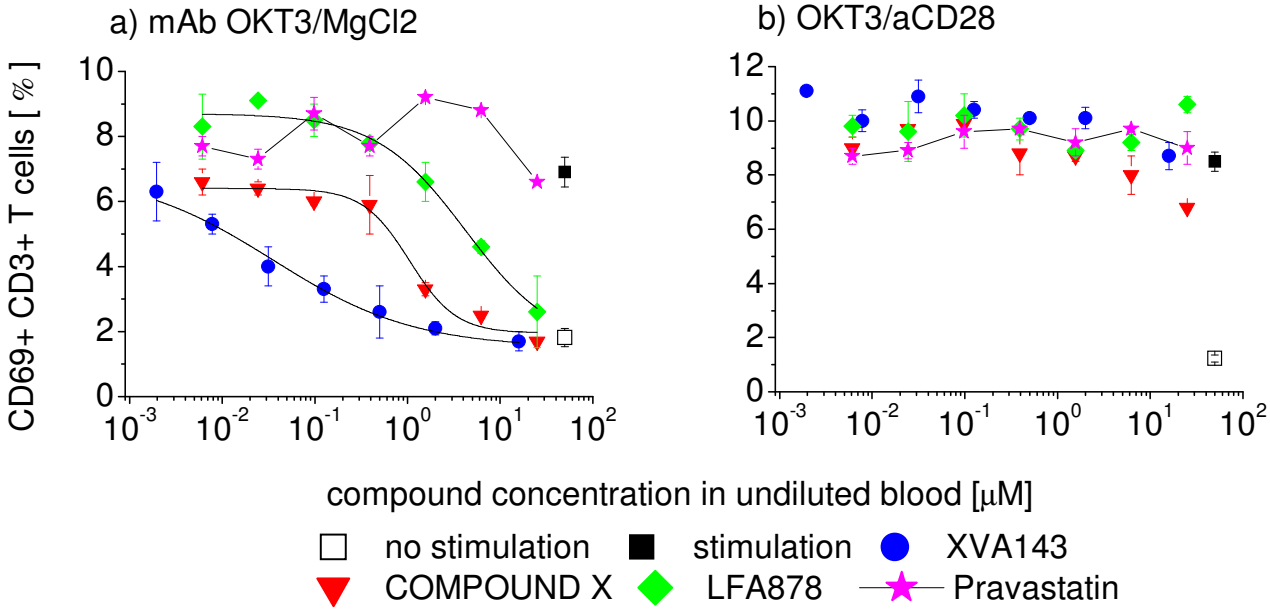


Figure 3.10 Effect of LFA-1 inhibitors and pravastatin on T-lymphocyte activation in whole blood cultures (EA-REMA): Whole undiluted blood was spiked with the compounds at concentrations indicated in the graphs. CD69 upregulation was quantified on CD3+ T-lymphocytes after 22 h stimulation of 1:1 diluted blood with a) mAb OKT3/$MgCl_2$ or b) OKT3/aCD28. 4 individually activated samples were pooled. Two of these pooled samples were stained and measured as independent samples. Data shown are means of these duplicates. Standard deviation is shown to indicate range of data. The EA-REMA was conducted as described in Methods. One representative experiment out of more than 3 is shown.

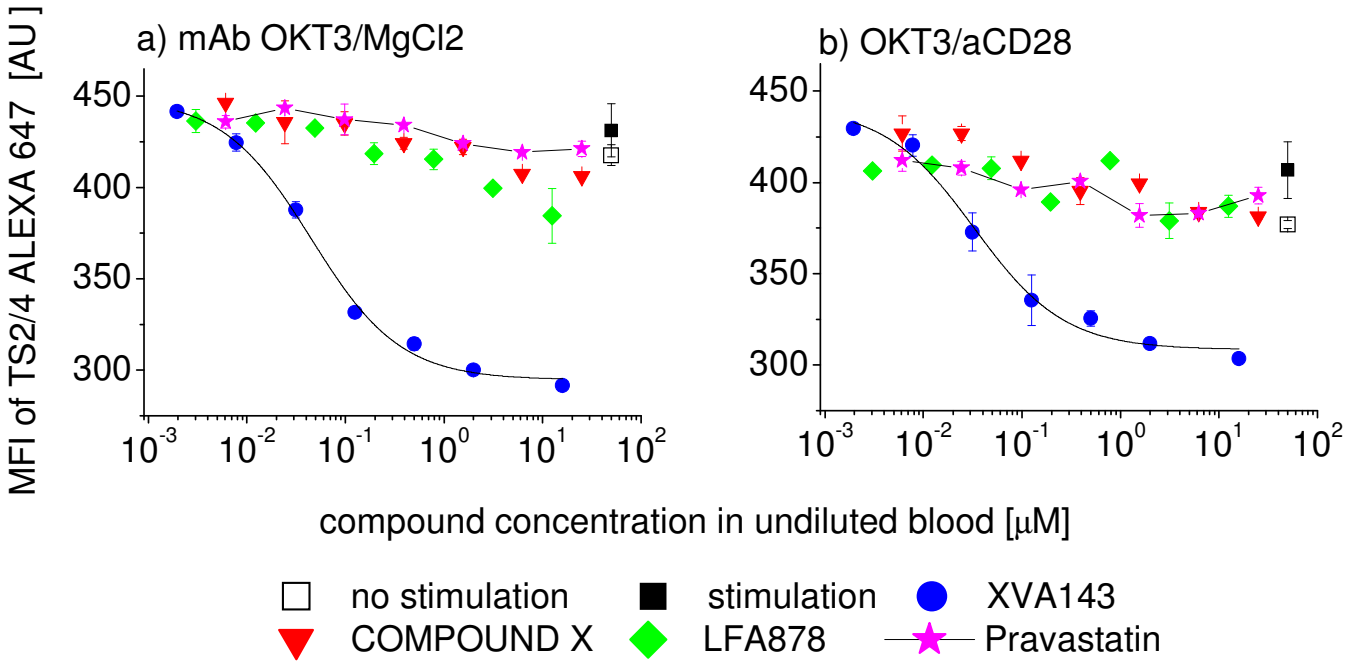


Figure 3.11 Effect of LFA-1 inhibitors and pravastatin on the cell surface expression of LFA-1 on T-lymphocytes in whole blood cultures (EA-REMA): LFA-1 cell surface expression was quantified by binding of mAb TS2/4.1.1 to CD3+ T-lymphocytes after 22 h stimulation of 1:1 diluted blood with a) mAb OKT3/$MgCl_2$ or b) OKT3/aCD28. 4 individually activated samples were pooled, stained and measured as two independent samples. Data shown are means of these duplicates. Standard deviation is shown to indicate range of data. The EA-REMA was conducted as described in Methods. One representative experiment out of 3 is shown.

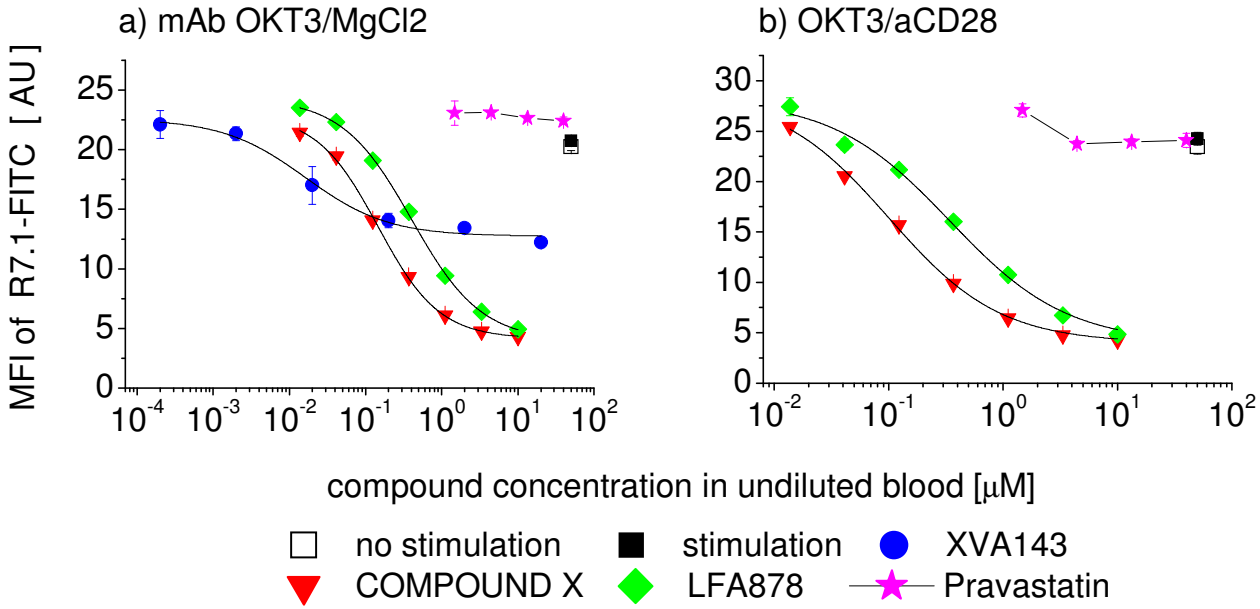


Figure 3.12 Effect of LFA-1 inhibitors and pravastatin on the binding of the monitoring antibody R7.1 to LFA-1 on T-lymphocytes in whole blood cultures (EA-REMA): Whole undiluted blood was spiked with the compounds at concentrations indicated in the graphs. mAb R7.1 binding was quantified on CD3+ T-lymphocytes after 22 h stimulation of 1:1 diluted blood with a) mAb OKT3/$MgCl_2$ or b) OKT3/aCD28. 4 individually activated samples were pooled. Two of these pooled samples were stained and measured as independent samples. Data shown are means of these duplicates. Standard deviation is shown to indicate range of data. The EA-REMA was conducted as described in Methods. One representative experiment out of 3 is shown. XVA143 was not measured in the OKT3/aCD28 (b) experiment.

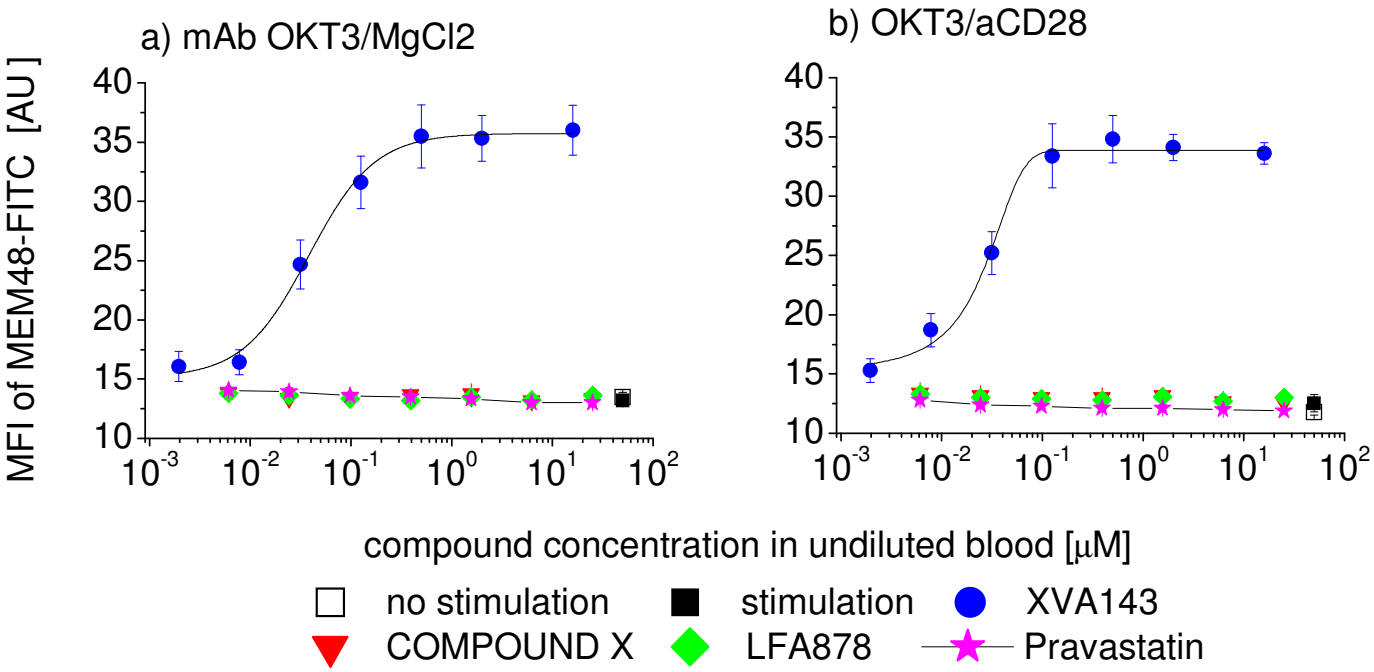


Figure 3.13 Effect of LFA-1 inhibitors and pravastatin on the binding of the monitoring antibody MEM48 to LFA-1 on T-lymphocytes in whole blood cultures (EA-REMA): Whole undiluted blood was spiked with the compounds at concentrations indicated in the graphs. mAb MEM48 binding was quantified on CD3+ T-lymphocytes after 22 h stimulation of 1:1 diluted blood with either a) mAb OKT3/$MgCl_2$ or b) OKT3/aCD28. 4 individually activated samples were pooled. Two of these pooled samples were stained and measured as independent samples. Data shown are means of these duplicates. Standard deviation is shown to indicate range of data. The EA-REMA was conducted as described in Methods. One representative experiment out of 3 is shown.

3.3.9 Development of T-cell proliferation assays in human whole blood

Initially we attempted to test the effect of LFA-1 inhibitors on human blood T-lymphocytes proliferation in 50% blood. Stimulation of lymphocytes with mAb OKT3 for 96 h in the presence or absence of $MgCl_2$ was, however, not successful to trigger significant proliferation in 50% diluted whole blood cultures (data not shown). Dilution of blood samples by 1:10 and the use of the novel serum free culture medium X-VIVO 10™ resulted in significant and reproducible proliferation of mAb OKT3 stimulated T-cells as measured by ^3H thymidine incorporation after 96 h of incubation. In addition, the use of X-VIVO 10™ medium resulted reproducibly in ≥3 times stronger proliferation compared to the standard cell culture medium RPMI 1640 (data not shown). The beneficial effect of X-VIVO 10™ medium on lymphocyte proliferation in diluted blood cultures was recently described [29]. As shown in figure 3.14 the degree of proliferation of whole blood lymphocytes was dependent on the concentration of immobilized mAb OKT3 and to a lesser extent on the blood donor. Whole blood lymphocytes of donor 1 started to proliferate at low concentrations of mAb OKT3 (≤0.3µg/ml, Fig. 3.14a) while no ^3H thymidine incorporation was observed for lymphocytes of donor 2 at this concentration (Fig. 3.14b). At 1 µg/ml immobilized mAb OKT3, T-lymphocytes of both donors proliferated maximally. This concentration was routinely used in our proliferation assays. 1 µg/ml of mAb OKT3 stimulation resulted in reproducible proliferation also using blood lymphocytes of other donors (not shown). Interestingly, at higher concentrations of immobilized mAb OKT3 a strong decline in proliferation was observed (Fig. 3.14a&b). It is likely that lymphocytes underwent activation-induced cell death (AICD) [37,38] as a result of overstimulation by the high mAb OKT3 concentration [39]. The isotype control IgG used (IgG2a) did not induce ^3H thymidine incorporation under the same experimental conditions demonstrating that the mitogenic stimulation measured was specific for mAb OKT3 (Fig. 3.14a&b).

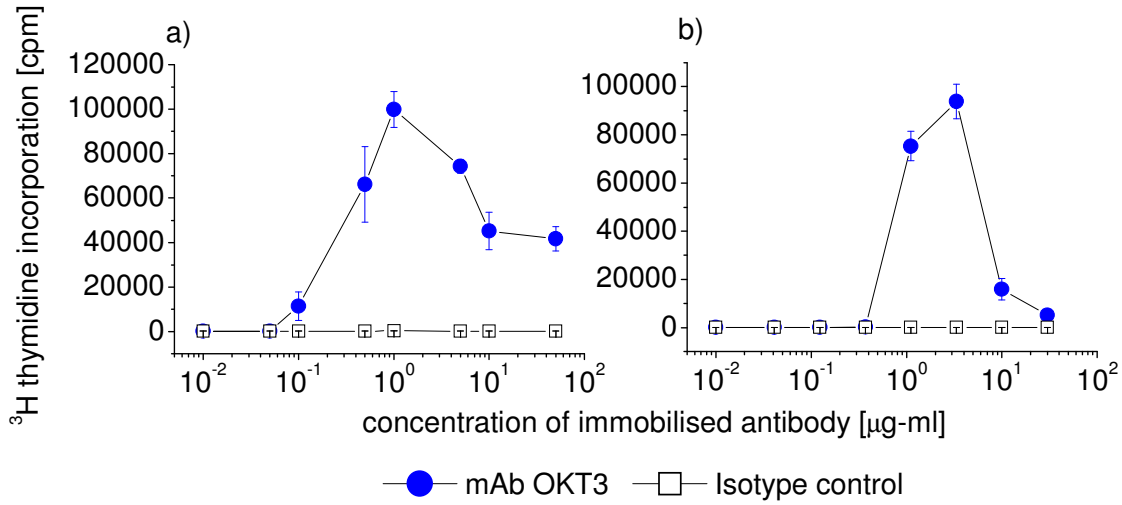


Figure 3.14 Proliferation induced by mAb OKT3 was dependent on the blood origin and the amount of mAb OKT3 coated. Experiments were carried out with the blood samples of 2 donors (a) and (b). Blood samples were diluted 1:10 with X-VIVO 10™ medium and incubated for 96 h at 37°C. ^{3}H thymidine incorporation was measured after 96 h of incubation with mAb OKT3 or the isotype control mAb (IgG2a) as described in Methods. Data are means of quadruplicates ±SD.

3.3.10 Effect of LFA-1 inhibitors and control compounds on T-cell proliferation in human blood

Figure 3.15a exemplifies the *in vitro* effects of LFA-1 inhibitors LFA878, COMPOUND X, XVA143 and the control compound pravastatin on mAb OKT3 stimulated proliferation of lymphocytes in diluted blood. All LFA-1 inhibitors potently blocked mAb OKT3 stimulated lymphocyte proliferation (Fig 3.15a). In contrast, the compounds showed no effect on OKT3/aCD28 stimulated proliferation (Fig 3.15b) which again demonstrated the LFA-1 pathway specific effect of the tested inhibitors. As paralleled in the T-cell activation assay, XVA143 was significantly more potent (≥40 fold) than LFA878 or COMPOUND X (Table 3.2). These findings show for the first time that LFA-1 inhibitors can impair CD3 stimulated T lymphocyte proliferation in whole blood cultures *in vitro.* As observed before in the T-cell activation assays pravastatin had no effect in the whole blood proliferation assays.

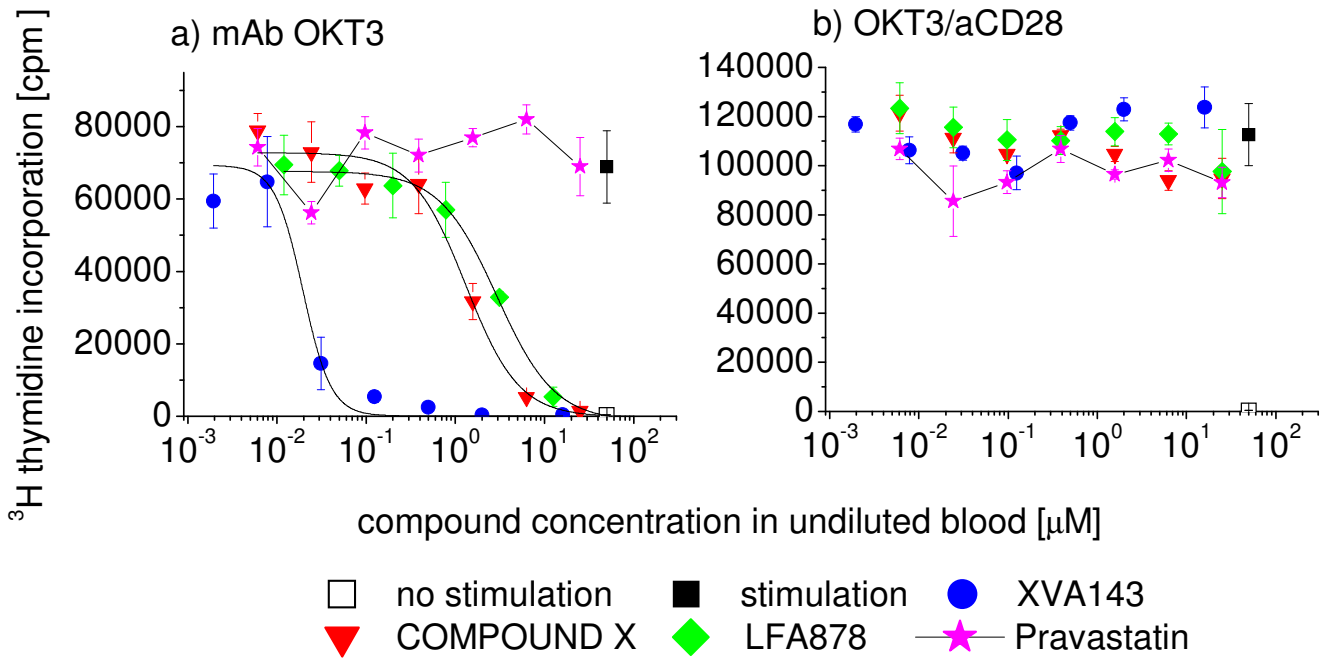


Figure 3.15 Effect of LFA-1 inhibitors and pravastatin on the proliferation of lymphocytes in whole blood. Undiluted whole blood was spiked with the compounds at concentrations indicated in the graphs. The blood was then diluted 1:10 with XVIVO-10 medium and incubated with a) mAb OKT3 or b) OKT3/anti CD28 for 96 h at 37°C. 3H thymidine was added for the last 22 h of incubation. 3H thymidine incorporation was quantified as described in Methods. Data are means ± SD of triplicates. One representative experiment out of 3 is shown.

3.3.11 Correlation between receptor occupancy and inhibition of T-cell activation and proliferation in whole blood cultures

The relationship of receptor occupancy by the LFA-1 inhibitors and the effect on mAb OKT3 stimulated T-cell activation (CD69) and proliferation was studied.

Despite of the 1:10 dilution of the blood samples in the mAb OKT3 proliferation assay the receptor occupancy by the tested inhibitors (REMA) was not significantly altered in comparison to undiluted blood e.g. equal IC_{50}s were obtained in the REMAs in 1:10 diluted and undiluted blood (not shown). This allowed us to compare the data generated in the different assays.

As an example for αL L-site inhibitors COMPOUND X showed a non linear relationship between the inhibition of mAb R7.1 binding and the inhibition of T-cell activation (Fig. 3.16a) and proliferation (Fig. 3.16b). Our results show that >85% of receptor occupancy is needed (e.g. >85 % inhibition of mAb R7.1 binding) to reach half-maximal inhibition of T-cell responses in whole blood. For COMPOUND X this corresponded to a blood concentration of 0.9 µM. The logarithmic values of the antibody binding fluorescence negatively correlated (R= ≥-0.94) with the T-cell responses (Fig. 3.16c&d). LFA878 demonstrated the same correlation between receptor occupancy and the inhibition of T-cell responses (data not shown). Our data suggest that the logarithmic values of the mAb R7.1 binding signals can be used as a marker to estimate the effect of αL L-site inhibitors on *in vitro* stimulated functional T-cell responses.

In contrast to the experiments described above, we found a linear relationship between the induction of mAb MEM48 binding and the inhibition of T-cell activation and proliferation by the β2 I-like domain inhibitor XVA143. The agonistic activity of XVA143 on mAb MEM48 binding strongly correlated with the blockade of CD69 up-regulation (Fig. 3.17a) and proliferation (Fig. 3.17b) (R≥0.96). Receptor occupancy by XVA143 translated therefore directly into suppression of *in vitro* T-cell functional responses. These results suggest that receptor occupancy monitoring (REMA) can

also be a valuable tool to predict the potency of β2 I-like domain inhibitors in *in vitro* stimulated functional T-cell responses in whole blood.

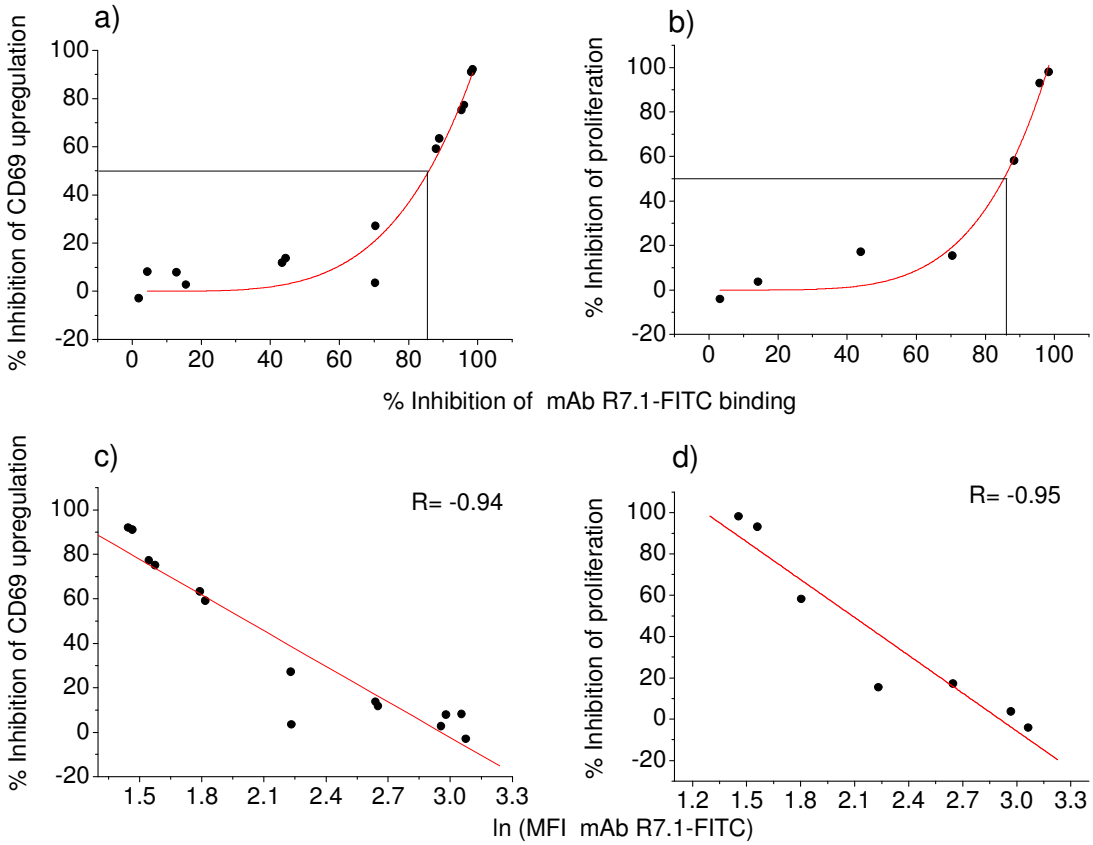


Figure 3.16 Receptor occupancy at different concentrations of COMPOUND X was measured by the inhibition of mAb R7.1-FITC binding to whole blood T-lymphocytes using the EA-REMA method. Data were correlated to the inhibition of a) mAb OKT3/$MgCl_2$ induced CD69 upregulation (EA-REMA) and b) the inhibition of mAb OKT3 triggered T-cell proliferation as described in Methods. Regression analysis was performed to fit the curve and to calculate the R value. Data are means of duplicates. One of 3 independent experiments is shown.

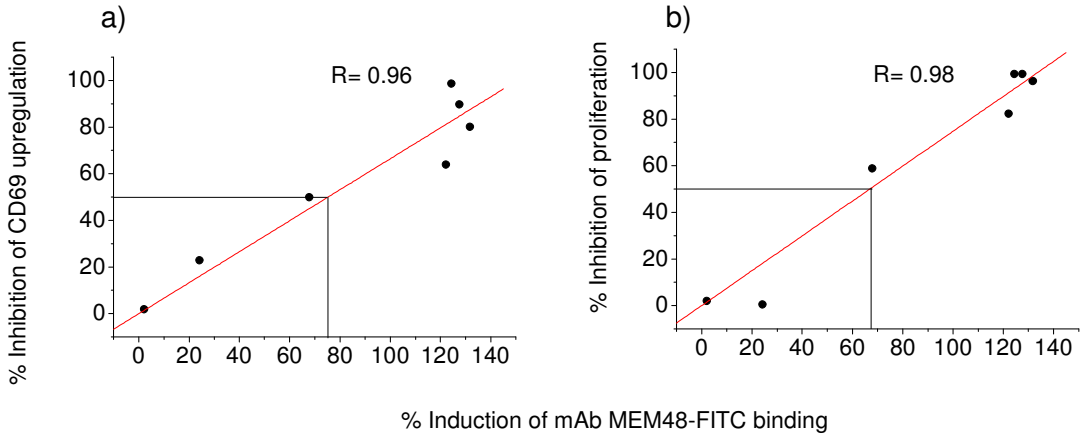


Figure 3.17 Correlation between receptor occupancy and the inhibition of mAb OKT3 mediated T-cell activation and proliferation by XVA143. Receptor occupancy by XVA143 was measured by induction of mAb MEM48 binding to whole blood T-cells using the EA-REMA method. Data were correlated to a) the inhibition of mAb OKT3/$MgCl_2$ induced CD69 upregulation (EA-REMA) and b) the inhibition of mAb OKT3 triggered T-cell proliferation as described in Methods. Regression analysis was performed to fit the curves and to calculate the R value. Data are means of duplicates.

3.3.12 LFA-1 inhibitors are inactive in phytohemaglutinin stimulated proliferation of whole blood T-lymphocytes

As already observed for the OKT3/aCD28 stimulated expression of the activation marker CD69 and proliferation, the activity of LFA-1 antagonists in whole blood was dependent on the type of stimulus used. Proliferation of whole blood lymphocytes induced by the T-cell stimulating lectin phytohemaglutinin (PHA [60]) was not (COMPOUND X, LFA878) or only marginally (XVA143, 35%) blocked by LFA-1 inhibitors demonstrating that PHA stimulated proliferation is insensitive towards extracellular inhibition of LFA-1 function (Table 3.2).

3.3.13 Effect of cyclosporine A (CsA) and everolimus in the whole blood assays and comparison to LFA-1 inhibitors

To better understand the results of the whole blood assays in the context of immunosuppression and to validate the test systems, the calcineurin inhibitor CsA and the mTOR inhibitor everolimus were tested. Both compounds are therapeutically used immunosuppressants which block T-cell activation (CsA [40]) and proliferation (CsA and everolimus [2,32]) via interfering with intracellular pathways.

As expected CsA or everolimus had no effect on the binding of the mAbs R7.1, MEM48 or TS2/4.1.1 to T-lymphocytes in whole blood (Table 3.2).

CsA inhibited mAb OKT3/$MgCl_2$ stimulated T-cell activation (CD69 upregulation) in whole blood equipotent (IC_{50}: 0.8 µM) to COMPOUND X (Table 3.2). To our surprise the LFA-1 inhibitor XVA143 showed a 16 fold higher potency than CsA demonstrating the essential requirement of β2 integrin function in this assay system. Everolimus did not interfere with mAb OKT3 stimulated T-cell activation (Table 3.2). These data indicate that LFA-1 inhibitors can be as potent or can even more potently interfere with T-lymphocyte activation in whole blood cultures as classical immuno-suppressants. It should be noted however, that CD69 is only one of many possible T-cell activation markers and other markers (e.g. CD25, CD71 or intracellular cytokines) may have a higher sensitivity towards calcineurin inhibition. We have tested these markers (data not shown) but were unable to establish robust and LFA-1 inhibitor sensitive T-cell activation protocols in whole blood cultures.

CsA and everolimus inhibited both mAb OKT3 or PHA stimulated whole blood lymphocyte proliferation with nM potencies (Table 3.2). Again XVA143 was significantly more potent in the mAb OKT3 stimulated proliferation assay than CsA and nearly equipotent than everolimus. In contrast, CsA or everolimus inhibited mAb OKT3 stimulated proliferation with significantly higher potency than the αL L-site inhibitors LFA878 and COMPOUND X.

Interestingly OKT3/aCD28 stimulated T-lymphocyte activation and proliferation were largely resistant to inhibition by CsA. Only minor inhibition of lymphocyte activation

and proliferation was observed in the presence of CsA (Table 3.2). Everolimus was unable to block OKT3/aCD28 stimulated activation. Proliferation induced by OKT3/aCD28 was potently but only partially inhibited. The maximum inhibition by everolimus measured under our assay conditions was <75%. Similar observations were made with CsA [41,42] and everolimus in OKT3/aCD28 mediated T-cell response assays using purified lymphocytes or T-cell lines (Novartis in-house unpublished observation). These properties of CsA and everolimus in the OKT3/aCD28 stimulated assays suggest, that the nature of signaling events induced by the crosslinking antibodies may base, at least in part, on calcineurin and mTOR independent mechanisms.

3.3.14 Summary of the effects of the tested compounds in the whole blood assays

Table 3.2 Summary: Profile of LFA-1 inhibitors and control compounds in the human whole blood assays

Compound	Stimulus	mAb OKT3/$MgCl_2$ or OKT3/aCD28			mAb OKT3/$MgCl_2$	mAb OKT3	OKT3/aCD28	OKT3/aCD28	PHA
	Readout	CD11a REMA‡	CD18 REMA‡	LFA-1 expression‡	CD69 upregulation‡	^{3}H thymidine incorporation‡‡	CD69 upregulation‡	^{3}H thymidine incorporation‡‡	^{3}H thymidine incorporation‡‡
	Target	(R7.1)	(MEM48)	(TS2/4.1.1)					
LFA878	αL (L-site)	0.5±0.2	>10	>10	2.6±1.7 **	2.6±0.5 ***	>10	>10	>10
COMPOUND X	αL (L-site)	0.15±0.06	>10	>10	1.0±0.2 ***	1.0±0.38 **	>10	>10	>10
XVA143	β2-I-like domain	>50	EC_{50}: 0.031±0.007	35-55% ‡‡‡ at 10 µM 0.045±0.01	0.049±0.016	0.023±0.001	>10	>10	≤35% inhibition at 10 µM
Pravastatin	HMG CoA reductase	>40	>40	>10	>40	>40	>40	>40	>40
CSA	Calcineurin phosphatase	>10	>10	>10	0.8±0.26 **	0.15±0.09 *	≤35% inhibition at 10 µM	≤55% inhibition at 10 µM	0.05±0.03
Everolimus	mTOR	>10	>10	>10	≤35% inhibition at 2 µM	0.01±0.005 n.s.	≤35% inhibition at 2 µM	0.03±0.02 ≤75% inhibition at 2 µM	0.015±0.014

All results were obtained in whole human blood cultures by the EA-REMA and proliferation assays described in Methods. Values represent the mean ± SD of more than 3 independent experiments. Concentrations, IC_{50}s and EC_{50} are in µM. Concentrations shown are the highest concentrations tested. No significant effects at these concentrations were observed except if indicated. ‡: readout after 22 h incubation at 37°C; ‡‡: readout after 96 h incubation at 37°C;‡‡‡: receptor internalization %. The statistical significance of the compound effects versus XVA143 on activation and proliferation was tested using the unpaired t-test, where *p<0.05, **p<0.01 and ***p<0.001 are considered significant, very significant and highly significant, respectively.

3.4 Discussion

Affinity regulation of LFA-1 and the transmission of post ligand binding events requires conformational flexibility and three dimensional conformational changes to occur within the quaternary receptor structure. Differential conformational states within LFA-1 induced by receptor activation or by allosteric LFA-1 inhibitors were demonstrated to be detectable by the binding pattern of specific monoclonal antibodies (mAbs). The binding of the anti CD11a mAb R7.1 or mAb R3.1 to the LFA-1 αL I domain was shown by us and others to be significantly impaired by different αL L-site inhibitors (chapter 1 [15,43]). In the work described here, we show for the first time that two structurally different αL L-site inhibitors LFA878 and COMPOUND X blocked the binding of mAb R7.1 to leukocytes in undiluted blood (REMA) with nanomolar potencies. These potencies were similar to the IC_{50}s measured in the *in vitro* cellular adhesion assay (HUT78/ICAM-1, Table 3.1 and 3.2).

The REMA employing mAb R7.1 was found to be simple, robust and was applicable to different mammalian species which facilitated to study receptor occupancy in animal blood. The REMA was tested and validated in rabbits and it was demonstrated that the assay could be applied to study pharmacodynamic receptor occupancy of αL L-site inhibitors *ex vivo*. During the documentation of this work a method was published using a similar approach to measure receptor occupancy by αL L-site inhibitors in blood of humans and monkeys. This publication suggested that receptor occupancy measurements can be achieved using a different anti CD11a mAb and hydantoin derived αL L-site inhibitors [44]. The authors further speculated that the binding of their utilized mAb (clone R3.1) may be competitively inhibited by their LFA-1 inhibitor. It remains open, however, whether the published method can be applied to LFA-1 inhibitors others than hydantoins. Furthermore, the mode of action by which the binding of mAb R7.1 (REMA) is inhibited by various classes of αL L-site inhibitors is not investigated yet.

We have furthermore developed a protocol that allowed for the first time to measure target occupancy by the putative β2 I-like domain inhibitor XVA143 in human whole blood. XVA143 induced conformational changes in the β2 chains (CD18) expressed

on leukocytes in whole blood. The effects of XVA143 on the three dimensional structure of LFA-1 was detected by the enhanced binding of the anti CD18 mAb MEM48. The agonistic potency (EC_{50}) of XVA143 on mAb MEM48 binding in whole blood was ≥5 times less compared to the potency (IC_{50}) measured in the cellular adhesion assay (Table 3.1 and 3.2). This difference was not evident on purified PBLs (not shown). These findings may indicate that XVA143 was bound to serum proteins in whole blood. Due to lack of cross-species reactivity of mAb MEM48 this CD18 REMA variant could not be validated *ex vivo*. Other mAbs such as clones KIM127 and m24 were recently described to detect XVA143 induced conformational changes in LFA-1 [16]. These mAbs may be tested for their suitability to assess receptor occupancy by XVA143 or derivatives in blood of other species than man.

The modulation of mAb binding to integrins by small molecules is rather exceptional. Binding of anti-CLIBS (cation/ligand-induced binding sites) antibodies (reviewed by Bazzoni & Hemmler [45]) has been shown to be enhanced in the presence of LMW antagonists against other, non β2, integrins such as αIIbβ3 [46,47] or VLA-4 (α4β1) [48]. In this view mAb MEM48 could be accounted to the category of anti CLIBS antibodies while the mAb R7.1 differs from this category in that its binding is impaired, not enhanced in the presence of LFA-1 inhibitors. Antibodies with reduced target binding in the presence of a LMW antagonist have been successfully employed to study target receptor occupancy by xemilofiban, an orally available αIIbβ3 antagonist in human blood *ex vivo* [49]. This study provided evidence that receptor occupancy by integrin inhibitors can be studied in clinical trials using selected mAbs.

To date no studies were reported demonstrating that LFA-1 occupancy by an inhibitor translates into suppression of functional responses of native human T-cells. Furthermore, the important question remained to which extent cell surface receptors must be occupied in order to block LFA-1 mediated functional T-cell responses. To answer these questions, we studied the effect of LFA-1 inhibitors on T-cell activation (CD69) and proliferation in human blood and correlated it to receptor occupancy.

Both, the principle to activate T-lymphocytes in 1:1 diluted blood by mAb OKT3 and supplemental $MgCl_2$, as well as the method to assess T-cell activation together with receptor expression and occupancy in one sample (EA-REMA), are described here for the first time. The EA-REMA made it possible to conveniently assess the effects of LFA-1 inhibitors on receptor occupancy, receptor expression and T-cell activation on individual T-cells stimulated in whole blood cultures. The EA-REMA and the mAb OKT3 stimulated whole blood T-cell proliferation assay allowed to establish a broad "*in vitro* pharmacodynamic" profile of LFA-1 inhibitors on human blood T-cells.

We could show that receptor occupancy by LFA-1 inhibitors correlated with the inhibition of *in vitro* T-cell activation and proliferation in whole blood. Interestingly, the degree of receptor occupancy required for suppressing T-cell responses was different for the αL L-site inhibitors and the putative β2 I-like domain inhibitor XVA143. For the two αL L-site inhibitors LFA878 and COMPOUND X > five fold differences between the IC_{50}s in the mAb R7.1 binding- and the mAb OKT3 stimulated T-cell activation or proliferation assays were observed. Nearly complete receptor occupancy by these inhibitors was thus required to achieve half-maximal inhibition of the LFA-1 dependent T-cell functions. In contrast, XVA143 induced the binding of mAb MEM48 (REMA) and blocked equipotently T-cell activation and proliferation in whole blood. Our data strongly suggest that the conformational changes induced by XVA143 directly translate into blockade of immunologically relevant LFA-1 (or Mac-1) dependent pathways.

An additional distinguishing property of XVA143 was revealed by the assessment of LFA-1 cell surface expression in the EA-REMA. Prolonged incubation of whole blood with XVA143 led to a partial downregulation of LFA-1 cell surface receptors on T-cells in whole blood and also on purified lymphocytes (not shown), a phenomenon which was not observed for the tested αL L-site inhibitors. This property of XVA143 was not reported for any LFA-1 inhibitor before and underlines again the differential effects of αL L-site inhibitors and the putative β2 I-like domain inhibitor on native LFA-1. Our finding suggests that XVA143 may have amongst other integrin inhibitors a

unique mode of action. The downregulation of the target receptor by an LMW integrin inhibitor has not been described so far. LMW integrin inhibitors against αIIbβ3, such as xemilofiban [49,50], or the LMW α4β1 (VLA-4) inhibitor BIO5192 [51] were described to not affect the cell surface expression of their target receptors.

During the assay development for this thesis we found that supplementary magnesium cations can have substantial synergistic effects on the CD69 upregulation on whole blood T-cells triggered by mAb OKT3. The effect of magnesium on CD69 upregulation on T-cells was confirmed using mAb OKT3 or allogenic cells as stimulators for purified PBLs (K.W. unpublished observation). Furthermore, the magnesium effect not evident when T-cells were activated by SEB (data not shown) indicating that supplemental magnesium cations may strengthen required cell to cell contacts and/or LFA-1 dependent costimulatory signaling. However, it was proposed that during early stages of wound healing elevated magnesium concentrations found in the wound fluid could serve to stimulate migration of macrophages, keratinocytes and endothelial cells [52]. Our findings provide for the first time experimental evidence that elevated extracellular magnesium cations can enhance LFA-1 dependent T-cell activation in human whole blood. Our data give raise to the speculation that locally elevated magnesium concentrations could be involved in the regulation of β2 integrin dependent functions of immune cells *in vivo*. Further studies, however, are indispensable to support the physiological relevance of the regulatory function of magnesium.

The technical simplicity and robustness of the here described receptor epitope monitoring assays (REMAs) suggest that they could be applicable without major optimizations as PD assays in clinical studies for LFA-1 inhibitors. LFA-1 expression on T-cells may be altered during courses of stress [53], malignancies or autoimmune disorders [54,55] by medication (e.g. statins [56], thalidomide [57]) or by the investigated drug (e.g. XVA143 class). Potential alterations of LFA-1 expression would have an

impact on the readout of the REMAs and should be considered, in particular, in later phases of clinical development of LFA-1 inhibitors. The mAb TS2/4.1.1 component of the EA-REMA is intended to detect these alterations in LFA-1 expression on peripheral leukocytes. This antibody can be employed in experiments were only receptor occupancy and expression measurements and not T-cell activation are desired.

The REMAs as methods for pharmacodynamic (PD) receptor occupancy studies may have several benefits for clinical application. Next to the simplicity and speed of analysis, the insights in target receptor occupancy by the drug administered is of high value. This is particularly important if active metabolites are generated, if the inhibitor investigated is substantially bound to serum proteins or if a compound has a rapid clearance, but due to a strong target affinity (high k_{on}) remains biologically active. In these cases, PK would not correlate with PD effects. The REMAs may complement PK measurements during the pharmacological characterization of LFA-1 inhibitors and may guide dose finding in early phases of clinical development. For preclinical research efforts, the REMA (mAb R7.1) enables new possibilities to conduct pharmacodynamically controlled animal models. In addition, REMAs may give insights into systemic effects of locally administered compounds or are useful for the assessment of PK/PD relationships in animal models. All these studies could help to accelerate the selection and characterization of LFA-1 inhibitors.

The clinically used immunosuppressants CsA and everolimus inhibited the mAb OKT3 stimulated whole blood proliferation assay with IC_{50}s of 0.15 and 0.01 µM respectively. Patients that are on immunosuppressive therapy with these drugs have average blood levels of 0.1-0.2 µM (CsA) [58] or 3-16 nM (everolimus, combination therapy with CsA) [59]. A comparison of the blood levels of CsA and everolimus necessary for immunosuppression with the IC_{50}s determined in the mAb OKT3 stimulated proliferation assay suggests that the sensitivity of this assay would allow its application as PD assay in clinical trials. All LFA-1 inhibitors tested demonstrated considerable inhibitory properties in this assay. Moreover, LFA-1 inhibitors can be

expected to impair leukocyte extravasation from the blood stream and block migration processes which may add an additional immunomodulatory effect. Based on these considerations LFA-1 inhibitors may have the potential to be promising immunomodulatory agents.

In contrast to the robust receptor occupancy assays, the T-cell activation or the proliferation assays described here may need extensive optimization and validation before being applicable as PD assays in clinical trials. We have observed that inter-donor variation concerning the T-cell response to mAb OKT3 exist (Fig. 3.6). In addition, we encountered significant intra-day and inter-assay variability (not shown) in the CD69 upregulation and proliferation assays suggesting that mAb OKT3 stimulated T-cell response assays in whole blood may have a considerable degree of variation. However, once optimized and properly validated, these assays could provide valuable insights into the inter-individual T-cell sensitivity towards a LFA-1 inhibitor which may again guide dose finding.

There are, however, certain considerations for the clinical application of the here described methods as PD assays.

There is no *in vitro* stimulation of T-cell immune responses that realistically reflects all the mechanisms and cell types involved in an *in vivo* immune response. The most prominent location where immune responses take place *in vivo* are the lymph nodes and lymphoid tissue. These compartments contain distinct micro environments than peripheral blood. Peripheral blood may therefore not represent all cell types involved in an immune response.

Besides, it is generally accepted that *in vitro* whole blood PD assays can not reflect the tissue distribution of a drug. This may have a significant impact on the efficacy and dose finding, in particular, if a compound shows tissue specific accumulation.

Most of the pharmacodynamic T-cell function assays reported in literature lack a direct correlation with efficacy. For therapeutics in immunologic diseases (transplant

rejection, autoimmune diseases) it is difficult to demonstrate a direct correlation of a T-cell function PD readout to efficacy as these tests assess the responsiveness of T-cells to *in vitro* stimulants. These results are largely dependent on the degree and the nature of the stimulation, the PD readout and the sensitivity of the stimulated pathways towards the tested drug. The results of such PD assays can therefore over-estimate the compound efficacy or be under-predictive. The outcome of *ex vivo* stimulated T-cell responses must thus be considered with caution.

For all these reasons, it needs to be determined in clinical trials, to which degree the LFA-1 PD assays described here can really reflect the immunosuppressive potential of LFA-1 inhibitors.

3.5 References

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4 Final conclusion

The work of this PhD thesis elucidated that low molecular weight (LMW) LFA-1 (αLβ2) inhibitors block the function of LFA-1 with different modes of actions. Accordingly, these inhibitors can be designated into two major groups: The αL L-site inhibitors and the putative β2 I-like domain inhibitors.

αL L-site inhibitors encompass meanwhile several chemical classes. These inhibitors induce conformational changes within αL I domain of the native LFA-1 receptor which can be monitored using monoclonal antibodies. We provided further evidence that lovastatin-derived αL L-site inhibitors can be further distinguished by their effect on the conformation of the β2 I-like domain on the neighboring β2 chain.

The definition of the second major class of LFA-1 inhibitors based on our finding that the compound class of XVA143 inhibits β2 integrin adhesive function by inducing conformational changes in the regulatory I-like domain as well as the stalk region of the β2 chain of LFA-1. We demonstrated for the first time that the β2 chain contains motives that allow potent, probably allosteric, β2 integrin inhibition by LMW compounds. The requirement of the β2 I-like domain for the activity of XVA143 was confirmed meanwhile by others [16]. However, a crystal structures of XVA143 with the β2 I-like domain or NMR studies are mandatory to finally prove our hypothesis that XVA143 binds to the β2 I-like domain. We therefore designated this compound class as putative β2 I-like domain inhibitors.

Furthermore, our studies provided next to the methodology, insights into the potential pharmacodynamic (PD) effects of LFA-1 inhibitors of both compound classes. These studies demonstrated that LFA-1 inhibitors occupy their target in whole blood and that receptor occupancy can translate into potent suppression of T-cell function. The distinct modes of action of the tested LFA-1 inhibitors were not only evident by their effects on the LFA-1 receptor conformation but were also reflected by differential effects on T-cell function and LFA-1 cell surface expression. Furthermore, our findings strongly suggest that regulatory domains in the β2 chain are novel and promising targets for T-cell suppression approachable with LMW inhibitors. However, further investigations, backed by *in vivo* testing and the assessment of other β2 I-like

domain inhibitors are required to allow a comparison of the immunosuppressive concepts between αL L-site and β2 I-like domain inhibitors.

The here described methods may serve as a basis to assess PD effects of LFA-1 inhibitors in clinical studies. The results of these studies will broaden our understanding whether *ex vivo* PD effects - measured by the here described techniques – allow a correlation with therapeutic efficacy of LFA-1 inhibitors.

5 Curriculum vitae

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Education

03/2003 – 10/2004	**Ph.D.** in Pharmaceutical Sciences, University Basle, Switzerland, with Prof. Dr. S. Krähenbühl; Prof. Dr. P. Herrling *summa cum laude*
10/1994 – 10/1995	European **Master of Science** program in Biotechnology, University of Teesside, Middlesborough, UK. Title: "MSc."
10/1989 – 06/1994	Studies of **Biotechnology**, Fachhochschule Weihenstephan, Freising, Germany, Title: "Dipl. Ing. (FH) Biotechnology"
07/1988 – 09/1989	**National service**, Artillery battalion, Regensburg, Germany
1988	German "**Abitur**": qualification for university entrance (includes *Graecum* and *Latinum*)

Experience

06/1996 – present
Investigator, Novartis Pharma AG, Basle, Switzerland. Discovery and preclinical development of LFA-1 inhibitors for the prevention of graft rejection and/or the treatment of auto-immune diseases. Characterization of bioactive lysophospholipids (FTY720 derivatives). Development and validation of methods for the pharmacodynamic assessment of novel immunomodulators in clinical trials.

12/1995 – 05/1996
Research Associate, **University of Birmingham**, UK**.** Characterization of cell growth, productivity and apoptotic behavior in cell cultures by innovative flow cytometric methods.

10/1994 – 10/1995
Master of Science Thesis, **University of Teesside**, Middlesborough, UK; scholarship granted by the German Academic Exchange Service (DAAD). Evaluation of novel chromatographic media for the affinity-separation of immunoglobulins from cheese whey.

09/1993 – 06/1994
Diploma Thesis, **CIBA GEIGY AG**, Basel, Switzerland. Development of an innovative device to on-line monitor productivity and cellular physiology in medium/large scale tissue cultures.

02/1992 – 07/1992

2nd practical semester, **Trent University of Nottingham**, UK. Investigations on the influence of tissue-transglutaminase on tumour proliferation, metastatic potential and programmed cell death (*apoptosis*).

09/1991 -01/1992

1st practical semester, Central Research Laboratories of **MBB**/**DASA** (German NASA), Munich, Germany. Investigation on microbiologically induced corrosion in aircraft materials:

Patents

- Case 4-31613P1/USN - Priority patent filing (2000): R. Albert, J. Dawson, C. Ehrhardt, S. Wattanasin, G. Weitz-Schmidt, K. Welzenbach: Organic compounds (Diazepane-dione class as drugs for anti inflammatory and autoimmune diseases)

- Case 4-31155P1/USN Priority patent filing (1999): R. Albert, J. Meingassner, C. Ehrhardt, U. Hommel, J. Kallen, S. Wattanasin, G. Weitz-Schmidt, K. Welzenbach: Organic compounds (Diazepane class as drugs for anti inflammatory and autoimmune diseases)

Awards

- Structure activity relationship of lead structure derivatives in inhibiting LFA-1/ICAM-1 interaction Bänteli R.; Bauer W.; Cottens S.; Ehrhardt C.; Hommel U.; Kallen J.; Weitz-Schmidt G.; Welzenbach K.

- Identification of small molecule antagonists of the beta 2 integrin LFA-1 with strong anti-inflammatory effects in vivo; Weitz-Schmidt G.; Welzenbach K.; Bruns C.; Meingassner J.; Bauer W.; Cottens S.

Both posters were within the top 10 of 600 submitted posters presented at the Novartis International Research Conference in Florence 1999.

Positions of Responsibility

- Organisation, documentation and executive tasks in a preclinical research lab at Novartis Pharma AG (CH)
- Supervision of diploma students and trainees at Novartis Pharma AG (CH) and at the University of Birmingham (UK)
- Co-founder and Vice Chair of INTERLINK, the Society for International Students in Middlesborough (UK)

Further Qualifications

- 4 weeks research visit at TSRI (Scripps), San Diego, USA (1999)
- Attendance of several international research conferences and workshops
- Course for biosafety officer acknowledged by the government of Oberbayern, Germany (1994)

Languages & Computing

German (native speaker), English (fluent writing and speaking), Spanish (beginner)
Computing: Expert level on MS-Office applications, design and programming of MS-Access databases

Reference

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Publications

Improved LFA-1 inhibition by statin derivatives: Molecular basis determined by X-ray analysis and monitoring of LFA-1 conformational changes in vitro and ex vivo
Weitz-Schmidt G., Welzenbach K., Dawson J., Kallen J. *J Biol Chem in press* (2004)

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Ullrich T., Baumann K., Welzenbach K., Schmutz S., Camenisch G., Meingassner JG., Weitz-Schmidt G. *Bioorg Med Chem Lett.* May 17;14(10): 2483-7 (2004)

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Wattanasin S, Albert R, Ehrhardt C, Roche D, Sabio M, Hommel U, Welzenbach K, Weitz-Schmidt G. *Bioorg Med Chem Lett*. 13(3):499-502 (2003)

Small molecule inhibitors induce conformational changes in the I domain and the I-like domain of lymphocyte function-associated antigen-1: Molecular insights into integrin inhibition
Welzenbach K., Hommel U., Weitz-Schmidt G.; *J Biol Chem*.277(12):10590-8 (2002)

Lovastatins selectively inhibit leukocyte function antigen-1 by binding to a novel regulatory integrin site
Weitz-Schmidt G., Welzenbach K., Brinkmann V., *et al*: *Nature Medicine*, 7:687-692 (2001)

Structural basis for LFA-1 Inhibition upon lovastatin binding to the CD11a I-Domain
Kallen J., Welzenbach K., Cottens S., Weitz-Schmidt G., Hommel U. *J. Mol. Biol.,* (292) 1-9 (1999)

Assembly of binding loops on aromatic templates as VCAM-1 mimetics
Peri F., Grell D., Welzenbach K., Weitz-Schmidt G., Mutter M., *J. Peptide Sci*., 5: 313-322 (1999)

A rapid method for evaluation of cell number and viability by flow cytometry
Al-Rubeai M., Welzenbach K., Lloyd D.R, Emery A.N., *Cytotechnology,* , 24, 161-168, (1997)

Recent Abstracts (selection)

A novel and rapid pharmacodynamic (PD) assay measures complex immunosuppressive drug effects in rats, monkeys and humans Burkhart C.; Morris, RE.; Weckbecker, G.; Weitz-Schmidt G.; Welzenbach K. Novartis Institutes for BioMedical Research research conference, Vienna (2004)

FTY720 is phosphorylated stereospecifically in vivo: Chemical proof and biological characterization of two enantiomers of FTY720-phosphate Albert, R.; Brinkmann, V.; Hinterding, K.; Welzenbach, K.;
Novartis Institutes for BioMedical Research research conference, Vienna (2004)

Efficacy of lovastatin-derived LFA-1 inhibitors in animal models of inflammation and allograft rejection
Weitz-Schmidt G, Bauer W, Joergensen J, Weckbecker G, Welzenbach K, Dawson J, Meingassner J, American Transplant Congress, Washington DC, (2003)

LFA878, a lovastatin-derived LFA-1 inhibitor with high potency against rodent allergic contact dermatitis
Meingassner J, Bauer W, Welzenbach K, Baumann K, Weitz-Schmidt; Society of Investigative Dermatology, Miami, (2003)

The I-domain and the I-like domain as targets for allosteric inhibition of lymphocyte function-associated antigen-1
Welzenbach K., Hommel U., Weitz-Schmidt, G. Keystone Symposia on molecular mechanisms of leukocyte trafficking, Colorado, (2002)

Development of a 384 well ELISA-type Mac-1/ICAM-1 binding assay: Excellent performance in screening and rapid identification of inhibitors. Welzenbach K., Link M.,Trifilieff E., Scheel G., Weitz-Schmidt G., Novartis Research Conference, Florence (2001)

Selective Inhibition of the LFA-1/ICAM-1 Binding by HMG-CoA-Reductase Inhibitors: Identification of a novel binding site. Weitz-Schmidt G., Brinkmann V., Cottens S., Hommel U., Kallen J., Welzenbach K. AST Meeting Transplant 2000, Chicago, USA, (2000)

Attendent courses, symposia and seminars as PhD student

26.5.03 Novartis, Basel	Prof. Dr. Scapozza ETH Zürich	Solving immunogenicity of suicide gene in suicide gene therapy	Seminar
11.7. 2003 Novartis, Basel	Dr. Marti	Potential collaboration on gene expression profiling of chronically rejecting renal grafts	Seminar
5-6.9. 2003 Basel		Pre-Congress Symposium, Transplantation Immunotherapy, International Association of Therapeutic Drug Monitoring and Clinical Toxicology (IATDM/CT)	Symposium
17-20.9.2003 Munich		ISFC Symposium/Workshop on Antigen specific T-cell activation	Symposium/Workshop
15-18.10. 2003 Kraków, Poland		Polish-Austrian-German-Hungarian-Italian Joint Meeting on Medicinal Chemistry	Poster presentation/ Conference
Novartis, Basel	Dr. J. Klupp	Optimization of pharmacodynamic assays in humans	Seminar
Geneva 15-17.04. 2004		Annual meeting Swiss Society for Allergology and Immunology	Conference
18-19.04.2004 Geneva		Cellular interactions in the immune system	Conference
Novartis, Basel		Several Lunch time seminars of Novartis Institutes for BioMedical Research, Basel,	Seminars
Novartis, Basel		Several journal club seminars, Transplantation / Immunology, Novartis Institutes for BioMedical Research, Basel	Seminars
University Basel		Several seminars on Drug Discovery	Seminars