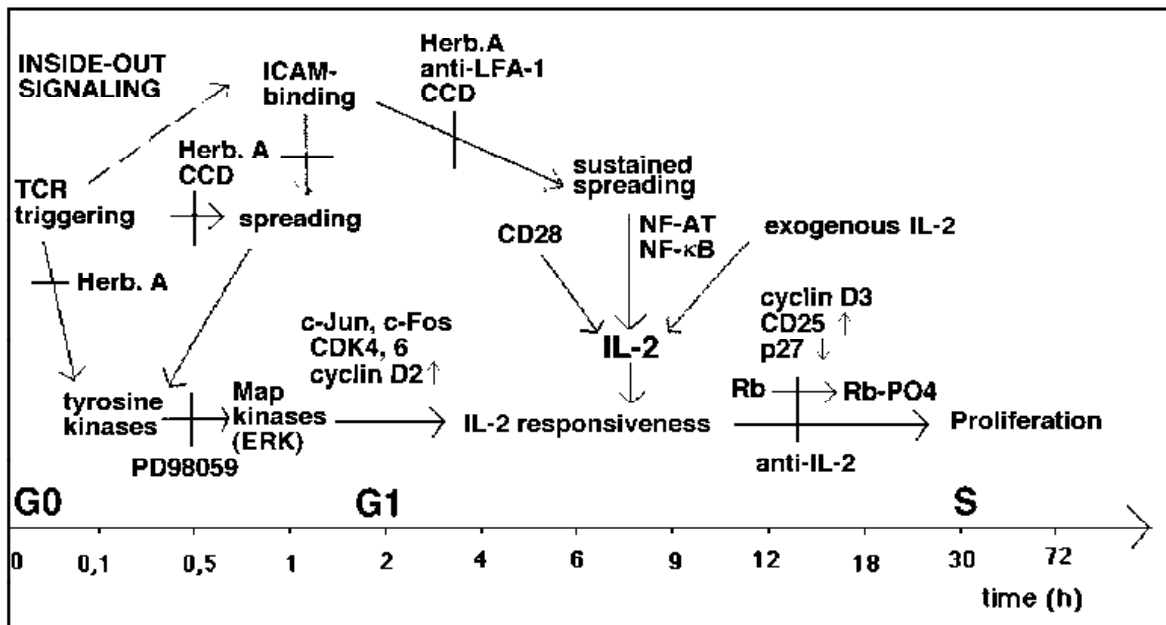# 4. Discussion

4.1 Summary

In this study, the role of the αL/β2-integrin leukocyte function antigen-1(LFA-1) in the proliferation of T cell antigen receptor(TCR)-stimulated primary human T lymphocytes was analyzed. Co-engagement of LFA-1 was found to be a prerequisite for proliferation in TCR-stimulated cells in the absence of other receptor-ligand interactions. The effect of LFA-1 on cell cycle progression can not simply be explained with enhanced adhesion-dependent TCR triggering, but is mediated by TCR-independent signal transduction. As reported for non-lymphoid, anchorage-dependent cell types, the integrin-mediated signal transduction and proliferation critically depend on an intact actin-based cytoskeleton and a spread cell shape rather than on receptor aggregation. The pro-mitotic effects of LFA-1 act at two distinct points on cell cycle progression: In the Gap 0 phase of the cell cycle, LFA-1-mediated spreading synergizes with the TCR on tyrosine phosphorylation, leading to enhanced mitogen-activated protein(Map) kinase activation, immediate early gene expression, cell cycle entry and the induction of responsiveness to the T cell growth factor Interleukin 2 (IL-2). However, LFA-1 was found to be as well a required late component of TCR-dependent proliferation: Prolonged LFA-dependent spreading, in the context of intercellular contact, is a prerequisite for the production of the mitogenic cytokine Interleukin-2. LFA-1-dependent IL-2 production was found to be cyclosporin A-resistant and was paralleled by the enhanced binding of transcription factors to the NF-AT and the CD28RE/AP-1 site of the IL-2 promoter *in vitro*. The alternative costimulatory receptor CD28 is able to bypass this step in an adhesion-independent way. IL-2 in turn triggers the expression of the α-chain of the IL-2 receptor (CD25), and leads to the activation of cyclin-dependent kinases (CDKs), probably due to enhanced cyclin D3 expression and to the downregulation of the CDK inhibitor p27^{kip1}. The activation of the CDKs leads to the inactivating phosphorylation of the retinoblastoma protein (pRb) and to the progression into the synthesis(S) phase of the cell cycle. Anchorage-dependent T cell growth is therefore characterized by a sequential action of signals conveyed by integrins which together with the activating antigenic stimulus effects both cell cycle entry and G1 to S transition.

Figure 4.1: LFA-1-dependent events leading to S phase entry in TCR-stimulated cells. Shown are the investigated events and their causal and/or temporal relationship as judged by the use of the tyrosine kinase inhibitor Herbimycin A (Herb. A.), the inhibitor of the ERK-cascade (PD98059), of cytoskeletal rearrangement (Cytochalasin D, CCD) and of function-blocking anti-IL-2 and anti-LFA-1 antibodies.

```
INSIDE-OUT
SIGNALING          ICAM-        Herb.A
                   binding      anti-LFA-1
                                CCD

       Herb. A
TCR    CCD                                    sustained
triggering    →  spreading                    spreading

    Herb. A                CD28    NF-AT      exogenous IL-2
                                   NF-κB
                                                    cyclin D3
          c-Jun, c-Fos                              CD25 ↑
          CDK4, 6                                   p27 ↓
          cyclin D2↑              IL-2
tyrosine    Map                                Rb → Rb-PO4
kinases  → kinases  ─────→ IL-2 responsiveness ───────────→ Proliferation
           (ERK)
      PD98059                                  anti-IL-2

G0              G1                       S
0  0,1  0,5  1   2    4    6    9   12   18   30   72
                                                  time (h)
```

4.2 The experimental system

The growth response of transformed cell lines to growth factors and anchorage is variably dysregulated. Proliferation of both T cell lines and T cell clones appear indeed to be mostly adhesion-independent, since they require only stimulation with serum or with soluble anti-CD3 antibodies and/or recombinant IL-2 for their replication, respectively. We therefore chose primary, resting human T lymphocytes in order to investigate the costimulatory role of LFA-1 in antigen-dependent proliferation. Although the results obtained with resting T cells should be more relevant for the understanding of physiological growth regulation, the choice of this cell type has several disadvantages: T cells isolated from peripheral blood are heterogeneous and show individual differences in their activation requirements (Croft and Dubey, 1997). The physiological ligand of a specific TCR is a unique combination of a polymorph MHC bound to an antigenic peptide and differs therefore from cell to cell, making it impracticable to stimulate the whole population with the physiological ligands. In the mouse model, this can be bypassed by generating TCR-transgenic mice; in humans anti-CD3 antibodies are used as a surrogate ligand for promiscuous TCR-dependent activation. However, engaging the TCR with anti-CD3 mAbs is a rather unphysiologic way of stimulation: Kinetic analysis have revealed that the TCR-MHC/peptide interaction is characterized by a high dissociation-rate, and the equilibrium dissociation constant is approximately 10^{-7} M (Corr et al., 1994), it was therefore proposed that many TCRs may be serially engaged by few MHC/peptide complexes (Valitutti et al., 1995). In contrast, the dissociation constant of the monovalent fragment (Fab) of the used mAb OKT3 was calculated 5×10^{-9} M, and the bivalent binding of OKT3 results in a hardly detectable dissociation (Marano et al., 1989). Anti-CD3 mAbs are

therefore not able to serially trigger TCRs and soluble, crosslinked anti-CD3 mAbs are very rapidly internalized and lead to only a transient burst of signal transduction (see Fig. 3.3a). Resting T cells are further not transfectable with expression vectors coding for dominant negative or constitutively activated mutants of proteins, which allows the direct evaluation of the relative importance of an investigated signal transduction pathway for gene expression in cell lines. This study was therefore mostly limited to the combined use of specific inhibitors and correlations with the important exception of IL-2, which can be either added to the culture medium and blocked with specific antibodies.

We used two different in-vitro systems to investigate the role of LFA-1 and the actin-based cytoskeleton in proliferation. They are based on the use of soluble or immobilized anti-CD3 mAbs to mimic antigenic stimulation, respectively, and they evidence different aspects of LFA-1-dependent signalling and growth. The in-vitro system based on soluble anti-CD3 mAbs was designed to investigate whether T cells show adhesion-requirements for proliferation similar to constitutively adherent cell types. Under the experimental conditions used to characterize anchorage-dependent growth, the stimulation of growth factor receptors is completely adhesion-independent, while adhesion and subsequent spreading is exclusively integrin-mediated. In T cells, TCR stimulation is required for- and contributes to integrin-mediated cell adhesion (Corr et al., 1994; Dustin and Springer, 1989), the dissection of TCR-dependent and integrin-dependent events proved therefore to be difficult (Udagwa et al., 1996; Van Seventer et al., 1992). However, performing TCR-triggering with soluble anti-CD3 mAbs and engaging LFA-1 with an immobilized ligand allows the complete dissection of TCR- and integrin-dependent events in T cells. Soluble, cross-linked anti-CD3 mAbs induce a number of events attributed to TCR stimulation such as complete TCR downregulation, tyrosine phosphorylation and Map kinase activation, showing that TCR-dependent signalling is indeed induced by this treatment. Stimulating cells in this way clearly shows that TCR-stimulated S phase entry critically depends on integrin-mediated spreading in normal T cells (3.1), similar to the anchorage-dependent replication of constitutively adherent cells in response to growth factors. It was reported that the integrin-mediated rearrangement of the actin-based cytoskeleton and a spread cell shape, rather then integrin aggregation *per se* is required for both signal transduction leading to ERK activation and for growth factor-dependent S phase entry (Bohmer et al., 1996; Chen et al., 1997; Hansen et al., 1994; Zhu and Assoian, 1995). This suggests that anchorage-dependent growth actually reflects a dominant role of the actin-based cytoskeleton in growth factor-induced signalling and proliferation. Concordant with this, LFA-1-mediated effects on signal transduction and proliferation were completely cell shape-dependent. The use of soluble anti-CD3 mAbs allowed further the identification of a TCR- and a spreading-dependent component of tyrosine phosphorylation, Map kinase activation and immediate early gene expression.

The substitution of anti-LFA-1 mAbs with recombinant ICAM-1 revealed the following limitations of this approach: T cells do not adhere to ICAM-1 in the absence of TCR triggering (Dustin and Springer, 1989) unless the incubation is performed in the presence of EGTA and $MgCl_2$ (Stewart et al., 1996). Although this treatment allowed us to confirm the signalling capacities of LFA-1, inhibiting calcium-dependent signals prevents T cell proliferation (Kumagai et al., 1987) and chelating calcium with EGTA is therefore not suited for the investigation of proliferation requirements. Furthermore, although TCR triggering with soluble anti-CD3 mAbs lead to firm adhesion to ICAM-1, spreading and to a synergistic activation of early signal transduction, the induced adhesion was transient and was therefore often not sufficient to induce detectable proliferation. The transience of spreading under this condition is probably due to a lack of co-localization of ICAM-1 and the anti-CD3 antibodies (Berg and Ostergaard, 1995). and, as mentioned before, to the rapid antibody-induced internalization of the TCRs terminating the inside-out signal transduction required for integrin-mediated adhesion.

The in vitro system based on the use of immobilized anti-CD3 mAbs mimics better an intercellular contact and allows the substitution of anti-LFA-1 mAbs with recombinant ICAM-1, but TCR- and spreading-dependent events are partially overlapping: The TCR was stimulated with saturating concentrations of immobilized anti-CD3 mAbs, and LFA-1 was either co-engaged with co-immobilized ICAM-1 or blocked with soluble, function-blocking anti-LFA-1 mAbs. In order to address the role of the alternative costimulatory receptor CD28 we used either soluble, crosslinked anti-CD28 mAbs or the co-immobilized ligand, B7.2. The super-physiologic stimulation of the TCR with saturating amounts of anti-CD3 mAbs lead to spreading in the absence of LFA-1 engagement (Fig. 3.1, (Pardi et al., 1992)); the high concentration of anti-CD3 mAbs was chosen because lower amounts of anti-CD3 lead to adhesion-dependent TCR triggering (Fig. 3.2b). This in-vitro system confirmed the importance of LFA-1 and spreading for Map kinase activation and S phase entry in TCR-stimulated T cells, but it showed as well that synergistic Map kinase activation alone can not account for LFA-1-dependent S phase entry: While the co-engagement of ICAM lead to both synergistic Map kinase activation and to enhanced IL-2 production, the presence of soluble anti-LFA-1 antibodies did not alter early signalling or immediate early gene expression but selectively affected IL-2 expression. Concordant with our model, cytoskeletal rearrangement induced by the immobilized anti-CD3 mAbs was not affected by the soluble anti-LFA-1 antibodies, but they limited the spreading period of the TCR-stimulated cells to approximately 4 hours.

In summary, the two in-vitro systems illustrate the role of LFA-1 in proliferation at different checkpoints of the cell cycle: While TCR triggering in the absence of LFA-1-mediated spreading appears not to be sufficient to cause cell cycle entry and the acquisition of responsiveness to IL-2 (3.8), inhibition of LFA-1 function and spreading in G1 selectively prevents IL-2 production and the subsequent S phase entry of the competent cells.

4.3 The role of tyrosine phosphorylation in LFA-1-dependent signalling and proliferation

Figure 3.3a and b show that LFA-1 co-engagement synergizes with the TCR on tyrosine phosphorylation as a consequence of the LFA-1 induced spread cell shape. However, spreading induced by immobilized anti-CD3 mAbs was able to substitute LFA-1 engagement. It is questionable if binding of the TCR to a physiological ligand is as well able to induce spreading in the absence of adhesion molecules like LFA-1, since, as mentioned earlier, the interaction is weak and the dissociation rapid (Corr et al., 1994). Moreover, the TCR is recruited to an inner circle of receptors when a T cell interacts with an APC, while LFA-1 is excluded from this zone (Monks et al., 1998). Spreading results in a prolonged activation of tyrosine phosphorylation, suggesting that either different sets of tyrosine kinases are activated or that the prolonged activity of the kinases activated by the TCR depend on a reorganized cytoskeleton. Since most of the bands detected by tyrosine blotting appear to be commonly induced by the two receptors, the latter mechanism seems to be more likely. A prolonged kinase activation may be mediated by the cytoskeleton-dependent enrichment of tyrosine kinase activators or the exclusion of their inhibitors or both. The compartmentalization of signalling molecules and kinases seems to be a general mechanism of signal transduction regulation (Mochly-Rosen, 1995): The stimulation of integrins, growth factor receptors and the TCR results in phosphorylation-dependent recruitment of many proteins to the respective receptors (see Introduction). Further, mutations of Raf-1 (Stokoe et al., 1994) and of the PI 3-kinase (Klippel et al., 1996) leading to their constitutive membrane localization, result in constitutive kinase activity. Targeting kinases to specialized areas may lead to activating conformational changes and/or locally enriched enzyme and substrate concentration and therefore to more efficient substrate phosphorylation. One possible explanation how LFA-1 and the cytoskeleton mediate the prolonged TCR-dependent signal transduction is therefore, that signalling molecules are recruited to the integrin-linked cytoskeletal protein complexes, where they can be activated by the TCR or co-operate with TCR-dependent signalling pathways. Interestingly, two independent reports using human T cell clones stimulated with transfected fibroblasts, showed that CD28-dependent costimulation but not antigenic stimulation alone resulted in the association of p56^{lck} and ZAP-70 with the CD3 complex (Boussiotis et al., 1996; Sloan-Lancaster et al., 1994), providing thus a link between early tyrosine kinase localization and productive immunity in this model.

The following observations indicate a central role of tyrosine phosphorylation in anchorage-dependent T cell growth and suggest that src-family tyrosine kinases are involved in mediating both LFA-1 and TCR-dependent signal transduction and proliferation: Activation of the src-family kinases is one of the earliest events in T cell activation (Perlmutter et al., 1993); the combined stimulation with phorbol esters and calcium ionophore,

which mimics the tyrosine kinase-dependent events PKC (and $p21^{ras}$)-activation and calcium mobilization, respectively, bypasses the anchorage-dependence of T cell growth, illustrated by the relative resistance of proliferation induced by this treatment to Cytochalasin D (not shown). The tyrosine-kinase inhibitor Herbimycin A, which at the used concentration has been reported to be specific for src-tyrosine kinases (June et al., 1990), prevents both Map kinase activation and proliferation induced by the two receptors. Herbimycin A inhibits further the induction of spreading, suggesting that cytoskeletal rearrangement is regulated by (src-) tyrosine kinase-dependent inside-out signalling. Herbimycin A still inhibits spreading and proliferation when added at 4 hours post-stimulation (not shown), indicating an important role for (src-) tyrosine kinases as well in sustained spreading and spreading-dependent IL-2 expression and/or IL-2-dependent signal transduction. Consistent with this, preliminary data indicates that the stimulation of both the TCR and LFA-1 can activate the src-kinases $p56^{lck}$ and $p59^{fyn}$ (not shown).

4.4 The role of spreading-dependent Map kinase activation in T cell proliferation

The TCR- and LFA-1 mediated signalling initiated by tyrosine kinases leads to the synergistic activation of Map kinases (3.4). Inhibition of spreading selectively inhibits the LFA-1 dependent component of Map kinase activation, confirming that signalling via LFA-1 depends on the reorganization of the actin-based cytoskeleton. The activation of Map kinase depends more than tyrosine phosphorylation on the extend of spreading and on the co-localization of the TCR and LFA-1: While ERK was most efficiently activated when cells were costimulated with both immobilized ligands, the phosphotyrosine pattern did not change significantly when spreading was induced either with immobilized anti-CD3 mAbs or anti-LFA-1 mAbs alone or with both co-immobilized mAbs. Notably, phosphotyrosine blots show only the major phosphorylation events and qualitatively important phosphorylations may therefore not be detected. Alternatively, pathways not requiring tyrosine phosphorylation may contribute to Map kinase activation in primary T cells. The use of mAbs as a substitute for the physiological ligand may lead to signal transduction artefacts (Nunes et al., 1994). We therefore controlled the specificity of spreading-dependent signal transduction for LFA-1: Immobilized mAbs specific for CD3 and LFA-1, but not for CD45 or CD5 induced band shifts in a dose-dependent manner (0.75-10μg/ml) of both ERK-2 and Raf-1 (not shown), which both reflect the activation of the ERK-cascade. Moreover, adhesion of cells to ICAM-1 but not to Poly-lysine in the presence of Mg^{2+} and EGTA induced activation of JNK, comparable to levels induced with mAb to LFA-1. Furthermore, adhesion to either ICAM or anti-LFA-1 mAbs but not to Poly-lysine enhanced the activation of ERK, p38 and JNK induced by both soluble and immobilized anti-CD3 mAbs (3.4). These observations together demonstrate, that the

observed spreading-dependent signal transduction can be indeed mediated by the interaction of LFA-1 with its physiological ligand and is not an artefact of an high-affinity antibody.

What may be the role of the LFA-1 dependent Map kinase activation? In resting T cells, LFA-1 is in its low-affinity state unless it becomes activated by inside-out signalling. Therefore an isolated LFA-1 stimulation leading to Map kinase activation and gene expression is probably never occurring *in vivo*, and the function of LFA-1 is probably to enhance TCR-mediated Map kinase activation. Concordant with this, spreading artificially induced with anti-LFA-1 mAbs was sub-optimal and transient in the absence of TCR triggering, confirming that quiescent T cells require TCR stimulation in order to turn to an adherent state.

Map kinases are thought to independently link signal transduction to immediate early gene expression. The fact that co-stimulation strongly enhances Map kinase activation but only slightly enhances *c-fos* and *c-jun* transcription suggests that either a threshold level of Map kinase activation is sufficient to induce immediate early gene expression, or that, alternatively, Map kinase-independent pathways are responsible for or contribute to the expression of *c-fos*. and *c-jun*. It has been proposed that, since ERK is mostly activated by mitogenic stimuli, its activation is probably required for proliferation; conversely, JNK and p38 are predominantly activated by stress as UV or high osmolarity ("stress-activated protein kinases"), and may induce apoptosis (Cano and Mahadevan, 1995; Chen et al., 1996; Ichijo et al., 1997; Xia et al., 1995). Consistent with this, T cells from JNK1 knock-out mice showed reduced activation-induced cell death (Dong et al., 1998). Moreover, cyclin D1 expression was reported to be stimulated by ERK and inhibited by the p38 Map kinase (Lavoie et al., 1996). However, it was reported that the diverse Map kinase cascades synergize in activating proto-oncogene transcription (Karin, 1996; Price et al., 1996; Whitmarsh et al., 1995), and studies in transformed T cell lines have proposed that JNK may link CD28-dependent costimulation to IL-2 gene expression (Su et al., 1994). Since important cell-type specific differences in Map kinase function and regulation exist, it seems likely that it depends on the cell type, the differentiation state and the kind of stimulation, if apoptosis or proliferation is favoured by one of the diverse Map kinase pathways (Cano and Mahadevan, 1995). In primary T cells it appears that all three Map kinases are synergistically activated by the TCR and spreading in G0 (see 3.4) and by IL-2 in G1 (Crawley et al., 1997; Crawley et al., 1996). Both ERK and the p38 Map kinase are required for proliferation as judged by the effects of their respective inhibitors (3.4). Although conclusions based on the use of inhibitors should be drawn with care, it seems that the used Map kinase inhibitors are quite specific for their targets ((Gould et al., 1995), Fig. 3.6, panel B). While the importance of the p38 kinase for cell cycle entry is not clear, it seems that p38 activity is required for S phase entry since its inhibitor blocked proliferation even when added 4 hours post-stimulation, when cells have already acquired IL-2 responsiveness. Consistent with this, it

was reported that IL-2 mediated S phase entry depends on the p38 Map kinase (Crawley et al., 1997), but not on ERK (Crawley et al., 1996). In fact, preventing ERK activity resulted only in the first 2 hours in the inhibition of proliferation (3.6). ERK activity seems to be required for the acquisition of competence in resting T cells, since co-stimulated cells failed to respond to exogenous IL-2 in the presence of the MKK-inhibitor. The role of JNK in T cell proliferation is not clear from the presented experiments. The introduction of a competitive JNK-inhibitor into the cells did not reduce proliferation. However, since we could not evaluate the extend of the achieved inhibition this does not exclude an important function of JNK in T cell activation. Interestingly, the analysis of JNK1 knock-out mice revealed hyperproliferation but normal IL-2 production of JNK1-deficient T cells (Dong et al., 1998), arguing therefore against a permissive role for JNK in T cell activation as is shown for ERK in Figure 3.6.

We were not able to demonstrate a nuclear translocation of ERK upon stimulation. Although the selective extraction procedure seemed to minimize protein loss from the nuclei, it may not be sensitive enough to detect nuclear translocation of ERK. Alternatively, ERK could mediate its mitogenic effect by activating the Kinase p90^{Rsk}, which in turn may translocate in the nucleus and activate transcription (Xing et al., 1996). We were further not able to address the question if ERK activation *per se* is sufficient to induce IL-2 responsiveness in this study, since the transfection of constitutively activated ERK is technically impossible in resting T cells. However, considering the complexity of signalling networks leading to gene expression in T cells, it seems more likely that activation of multiple pathways comprising diverse Map kinase cascades are required for cell cycle entry and the induction of competence. Although the TCR is able to activate all investigated Map kinase pathways in the absence of LFA-1 engagement or even spreading it appears that this weak activation is insufficient to induce IL-2 responsiveness: CD25 expression is upregulated on competent T cells in response to IL-2, CD25 is therefore a useful marker of IL-2 responsiveness when cells are stimulated in the presence of recombinant IL-2. Addition of exogenous IL-2 did not induce proliferation or CD25 expression in cells stimulated with soluble anti-CD3 mAbs and failed to stimulate CD25 expression in a fraction of cells stimulated with immobilized anti-CD3 mAbs. Moreover, the use of saturating amounts of immobilized anti-CD3 mAbs is probably a super-physiologic stimulation. This suggests that LFA-1 co-engagement is required for the induction of competence to respond to the mitogen at least in a fraction of cells and/or at low antigen concentration. Preliminary evidence suggests that this fraction is composed mostly of naive, CD4-positive T cells.

In summary, the activation of Map kinases dependent on LFA-1 co-engagement is probably importantly contributing to gene expression required for cell cycle entry and the acquisition of competence to respond to IL-2 in primary human T cells.

4.5 Costimulation mediates cyclosporin A-resistant G1 to S transition: The role of Interleukin-2

Several lines of evidence lead to the conclusion, that synergistic Map kinase activation and subsequent immediate early gene expression *per se* can not account for LFA-1-dependent proliferation: Several different stimuli lead to a comparable activation and nuclear translocation of Map kinases and *c-jun* and *c-fos* expression in spite of the dramatic differences in the proliferative outcomes. Concordant with this, while ERK activation was shown to be required only in the first 2-4 hours of stimulation, spreading was required for up to 12 hours. Further, when cells were stimulated with immobilized anti-CD3 mAbs in the presence of function-blocking anti-LFA-1 mAbs, G1 to S transition was blocked but Map kinase activation, immediate early gene expression and even the expression of the G1 cell cycle regulators cyclin D2, CDK4 and 6 were not significantly affected. The expression of IL-2 is known to be a crucial event in T cell activation (Croft and Dubey, 1997). and the LFA-1-dependent IL-2 expression appears to be both necessary and sufficient to trigger G1 to S transition: The observed differences in CD25 and cyclin D3 expression, p27^{kip1} protein stability and CDK-mediated pRb-hyperphosphorylation have been reported to be IL-2-dependent events (Evans et al., 1992; Firpo et al., 1994; Nourse et al., 1994; Turner, 1993; Ullman et al., 1990). IL-2 secretion induced by stimulating cells with immobilized anti-CD3 mAbs was strongly enhanced by costimulating LFA-1 and nearly completely inhibited by preventing the LFA-1-dependent cell clusters. This was paralleled by the enhanced induction of specific binding activity to the CD28RE/AP-1 and NF-AT site of the IL-2 promoter (3.11). Furthermore, exogenous IL-2 bypassed the inhibition of proliferation caused by soluble anti-LFA-1 mAbs in TCR-stimulated cells and lead to CD25 expression and pRb-hyperphosphorylation comparable to the levels observed in cells stimulated in the absence of soluble anti-LFA-1 mAbs. Excess anti-IL-2 antibodies added to the culture completely blocked the above events induced by recombinant IL-2 even at high doses and strongly inhibited proliferation induced by endogenously produced IL-2. The incomplete block of endogenous IL-2 may be due to an efficient autocrine mechanism, which allows a fraction of IL-2 to bind to its receptor even in the presence of anti-IL-2 antibodies in the culture medium (for example they could associate before they reach the cell surface). Alternatively, the production of other cytokines may be as well LFA-1-dependent and induce proliferation in the presence of threshold levels of IL-2. Consistent with our findings, studies from IL-2 knock-out mice suggest that IL-2 is necessary for T cell activation *in vitro*. (Kundig et al., 1993). However, the *in vivo* T cell activation was found to be normal, suggesting that alternative cytokines, probably not produced by T cells, can substitute IL-2 *in vivo*. CD28 aggregation can lead to probably IL-2-dependent G1 to S transition independently of LFA-1. The mechanisms used by the two receptors seem to be different, since CD28-induced S phase entry was adhesion-independent. We can therefore not rule

out that the cell-cluster-dependent IL-2 expression is at least partially CD28-mediated. Notably, the observed IL-2 expression can be mediated exclusively by LFA-1-ICAM interactions, since costimulating cells with co-immobilized anti-CD3 mAbs and ICAM most efficiently stimulates IL-2 secretion in the absence of cell cluster formation. Furthermore, detaching TCR- or co-stimulated cells after 4-8 hours from the dishes completely blocks proliferation unless cells are replated on ICAM or grown in the presence of recombinant IL-2. This control clearly shows that LFA-1-ICAM binding is sufficient to cause S phase entry and rules out the possibility that uninvestigated early LFA-1-dependent signal transduction events are required for LFA-1-dependent IL-2 production. In summary the data clearly shows that, once cells achieve competence, IL-2 is both necessary and sufficient to trigger the onset of DNA replication in our system.

A considerable body of evidence points to CDK-dependent pRb-inactivation as the main molecular switch leading to S phase entry in eukaryotic cells. In human T cells pRb-inactivation, and therefore the passage through the restriction point of the cell cycle appears to be mediated by IL-2-induced CDK activation (3.8). It is believed that, while CDK4 and CDK6 mediate the first wave of pRb-hyperphosphorylation in mid-G1, CDK2 is most active at the G1/S boundary, probably mediating a second wave of pRb-phosphorylation and/or events downstream of pRb-inactivation. Concordant with the literature (Modiano et al., 1994; Nourse et al., 1994; Turner, 1993), our data points to an important role for p27^{kip1} degradation and cyclin D3 expression for the IL-2-dependent CDK4/6 activation, but the expression levels of p21 and the INK4 family members may as well contribute to this step. The expression of the high-affinity IL-2 receptor, CD25, is as well IL-2-dependent and seems to represent a feed-forward mechanism, which allows pRb-inactivation and subsequent proliferation in response to threshold levels of IL-2.

IL-2 secretion induced by LFA-1 appears to be mediated at least in part at the transcriptional level: The binding activity specific for the NF-AT and CD28RE/AP-1 site of the IL-2 promoter induced by stimulating cells with immobilized anti-CD3 mAbs is enhanced by LFA-1-co-engagement.

Since co-engagement of LFA-1 was shown to induce prolonged increase of intracellular calcium (Van Seventer et al., 1992), the enhanced DNA-binding of NF-AT, which translocates into the nucleus in a calcium-dependent manner, is not really surprising. The inducible LFA-1-modulated complexes binding to the CD28RE/AP-1 contained c-Rel, RelA/p50, c-Jun and c-Fos. If the composition of the complexes binding to the CD28RE/AP-1 site is affected by costimulation remains to be determined. Both the NF-AT and the CD28RE/AP-1 site appear to be occupied in stimulated T cells *in vivo* (Garrity et al., 1994) and mutating the CD28RE/AP-1 or the NF-AT sites strongly reduces IL-2 transcription in TCR-stimulated primary T cells (Hughes and Pober, 1996). These findings together suggest that the LFA-1-mediated induction of NF-AT and CD28RE/AP-1 components are important for the transcriptional activation of the IL-2 gene. The importance of NF-AT for IL-2

transcription was questioned by reports showing that mice lacking members of the NF-AT family (NF-ATc/p) showed normal IL-2 production (Hodge et al., 1996; Ranger et al., 1998). However, redundancy of function between NF-AT family members may compensate for the lack of one single member.

CsA completely abrogated the nuclear translocation of NF-AT and significantly reduced the binding to both the NF-κB- and the CD28RE/AP-1-site in both TCR- and costimulated cells in spite of the respective susceptibility and resistance of the IL-2-dependent proliferation observed. The inhibitory effect of CsA on the proliferation of TCR-stimulated cells could be reverted with recombinant IL-2, confirming that CsA specifically interferes with IL-2 production. The inhibitory effect of CsA on the synergistically induced transcription factors binding to the IL-2 promoter indicate that the most important contribution of LFA-1 (and CD28) on IL-2 production may not be mediated at the transcriptional level. Enhance IL-2 secretion caused by CD28 co-engagement is known to be due to both enhanced transcription and to the stabilization of the IL-2 m-RNA (Jain et al., 1995). Costimulation-mediated IL-2 m-RNA stabilization could therefore allow IL-2 production and subsequent cell cycle progression even when IL-2 transcription is strongly reduced. However, the transcription of IL-2 is not only regulated by the binding of transcription factors to the IL-2 promoter, but as well by post-translational modifications of them. Notably, the CD28-dependent post-translational modification of AP-1 required for AP-1-dependent transcriptional activation in primary T cells was shown to be susceptible to CsA (Rincon and Flavell, 1994). In order to understand the relative contribution of transcription and m-RNA stabilization to CsA-resistant IL-2 production, the IL-2 m-RNA stability in TCR- and co-stimulated cells in the presence of CsA should therefore be determined.

In summary, the late LFA-1 requirement for proliferation has been identified as the LFA-1-dependent secretion of IL-2. The obtained results confirm the modified two-signal model, which predicts that accessory molecules first augment the TCR-dependent signal transduction (LFA-1) and that costimulatory signals in G1 (CD28, LFA-1) are acting primarily on IL-2 expression and cell growth (Croft and Dubey, 1997). They further suggest that not only CD28, but as well LFA-1 are both "real" costimulatory receptors, since costimulation provided by their ligands qualitatively alters T cell activation as shown by the induction of CsA-resistant growth.

4.6 The presumed role of LFA-1 in T cell proliferation in a physiological intercellular contact

The obtained results show that spreading is a prerequisite for antigen-driven T cell proliferation in our system and that LFA-1 exerts its pro-mitotic effect through a cytoskeleton-dependent signalling mechanism. It is less clear, however, if exclusively LFA-1 mediates spreading in a physiological cell-cell contact. Immobilized mAbs to CD3 (see 3.1) and to CD2 for example induce spreading in the absence of LFA-1 engagement (Pardi et al., 1992). Although

integrins are recruited to the cytoskeleton under this conditions, and this response was partially inhibited in T cells from LAD patients, it seems that LFA-1 is not the only receptor involved in cytoskeletal rearrangement in T cells. It was further reported that cytoskeletal reorganization upon TCR triggering with anti-CD3 mAbs leads to phosphorylation-dependent linkage of the CD3-zeta chain to the actin-based cytoskeleton (Rozdzial et al., 1995). However, results obtained with antibodies as surrogate ligands should be interpreted with caution. In our system, spreading is strongly enhanced when the ligand of LFA-1 is co-immobilized with saturating concentrations of anti-CD3 mAbs (see Fig. 3.1b), and spreading is completely LFA-1-dependent when ICAM is co-immobilized with low amounts (1μg/ml) of anti-CD3 mAb (not shown). The use of low amounts of immobilized anti-CD3 mAbs is probably the more physiological way of T cell stimulation since an APC is believed to present only about hundred MHC-peptide complexes for one specific TCR; the interaction is further of low affinity. A recent report analysed the three-dimensional distribution of receptors and intracellular proteins that cluster at the contacts between T cells and APCs during antigen-specific interactions (Monks et al., 1998). Surprisingly, instead of showing uniform oligomerization, these proteins clustered into segregated three-dimensional domains within the cell contacts. The TCR, the co-receptors CD4 and CD8 and CD2, CD28 were redistributed to an inner circle of the cell-cell contact, while LFA-1 (and CD45) were excluded from this region, forming an outer circle of receptors involved in receptor -ligand interactions. Clearly all receptors limited to the inner circle can not directly induce a spread cell shape, since cells interacting with a limited surface remain rounded (Chen et al., 1997). These findings together strongly suggests that the interaction of LFA-1 with its physiological ligand is indeed important for the induction of a spread cell shape in a physiological T cell-APC interaction and that spreading induced by immobilized anti-CD3 mAbs may be an artefact. The low avidities of CD28 and of the TCR for their respective ligands suggest that the major contribution of both receptors to adhesion and the induction of spreading is not due to ligand binding, but is the consequence of inside-out signalling (Dustin and Springer, 1989; Turcovski-Corrales et al., 1995): The induction of an avidity shift of LFA-1, VLA-4 (and CD2) for their respective ligands leading to ligand binding, firm adhesion and cytoskeletal reorganization. However, CD28-engagement was shown to be able to functionally substitute LFA-1 in the absence of the induction enhanced spreading (see 3.9 and 3.10), consistent with this it was shown that proliferation induced by CD28 but not by LFA-1 costimulation was resistant to CCD (Cai et al., 1997). In another report it was shown that both CCD and anti-LFA-1 mAbs blocked the intracellular calcium increase in an intercellular contact between an APC and a T cell without affecting intercellular adhesion. Since CCD did not block TCR-dependent signal transduction (see Figures 3.3b and 3.4) and was reported not to interfere with TCR-downregulation (Cai et al., 1997), this strongly suggests that the interruption of LFA-1-mediated spreading was responsible for the observed inhibition. The expression pattern and the

cellular redistribution of surface receptors favour CD45, whose ligands remain to be identified, or the recently identified αD/β2 integrin (Corbi, 1996) as candidates for substituting LFA-1 in the induction of spreading in an cell-cell contact with an APC, because the expression of the ligand of the β1-integrin VLA-4 is apparently limited to endothelial cells (Stewart et al., 1995).

This point of view is supported by the results obtained from LAD patients (Corbi, 1996) and from LFA-1 knock-out mice (Bachmann et al., 1997; Schmits et al., 1996; Shier et al., 1996): Although LFA-1 deficient T cells from LAD patients and from the knock-out mice show indeed selected defects in some *in vitro* proliferative responses, LFA-1 is clearly not required in all T cell dependent immunological processes: In LAD patients the expression of the β2-chain is affected, therefore not only LFA-1, but as well the expression of the other β2 integrins expressed mostly by phagocytes is lost or reduced. The most severe defects in LAD patients such as insufficient response to bacteria have been attributed to impaired phagocyte function. Interestingly, it has been proposed that in LAD patients phagocyte functions are more severely inhibited than lymphocyte functions, because lymphocytes have additional adhesion receptors like VLA-4 and CD2, which may be able to functionally substitute LFA-1 (Corbi, 1996).

In the knock-out mice, not the β2-chain but the LFA-1-specific αL-chain is not expressed, resulting in LFA-1-specific defects. Some *in vivo* responses such as defence against virus or thymocyte maturation appear to be unaffected in these mice, while others such as tumor rejection and delayed-type hypersensitivity are impaired. However, since LFA-1 is expressed on nearly all leukocytes and participates as well in other important lymphocyte functions such as transendothelial migration to inflammatory sites and cytotoxicity, it is not possible to attribute these defects exclusively to impaired T cell activation. Notably, the use of knock-out mice in order to investigate the activation requirements of mature T lymphocytes has as well its limitations: T cell development in the thymus is critically dependent on TCR surface expression, its affinity for MHC molecules and on costimulatory molecules such as LFA-1 and CD28 (Kishimoto et al., 1996). Since LFA-1 can lower the threshold of antigen required for T cell activation (Bachmann et al., 1997; Cai et al., 1997), selection of LFA-1-deficient thymocytes may result in a population of mature T cells, which have a higher affinity for MHC molecules thus compensating partially for the loss of LFA-1. A recent paper compared the respective roles of CD28 and LFA-1 in T cell proliferation, making use of T cells from TCR-transgenic mice deficient for one of the two costimulatory receptors stimulated with MHC-transfected fibroblasts and exogenously added antigenic peptide. They reported that, in the presence of antigen-presenting cells, high but not low doses of antigenic peptide could completely bypass the LFA-1 deficiency, leading to proliferation comparable to that observed with the LFA-1 expressing control T cells (Bachmann et al., 1997). Since the authors observed a reduced TCR internalization in T cells derived from LFA-1- but not from CD28-knock-out animals at low antigenic

peptide concentrations, they proposed that LFA-1 is only required for adhesion-dependent TCR triggering at low antigen concentrations. However, this conclusion is only partially justified by their own data, since proliferation induced by intermediate antigen concentrations is still not observed in LFA-1-deficient T cells, in spite of the fact that the TCR is completely down-regulated. Furthermore, the presence of antigen-presenting cells allows the stimulation of additional receptor-ligand interactions which may substitute LFA-1 at high antigen concentrations.

Taken together these findings strongly suggest that LFA-1 co-engagement is indeed important for the induction of spreading in a physiological APC-T cell contact and therefore for spreading-dependent gene expression required for proliferation. It further appears that, since IL-2 is not absolutely required for a T cell response *in vivo* (Kundig et al., 1993), other cytokines possibly not secreted by the T cell itself and/or not depending on LFA-1 may substitute LFA-1-mediated, IL-2-dependent G1 to S transition *in vivo*. In summary, TCR-induced proliferation of non-transformed T cells depends on an integrin-mediated spread cell shape in the absence of other receptor-ligand interactions, similar to the growth factor-stimulated replication of constitutively adherent cells. However, other features, such as a surface receptor-bound growth-factor 1 (antigen), which regulates and contributes to integrin-dependent adhesion, the anchorage-dependent expression of growth factor 2 (IL-2) and an alternative mechanism (CD28), which allows S phase entry independently of adhesion seem to be tissue-specific and characterize T cells as a peculiar case of anchorage-dependence. It appears further that, dependent on the antigenic concentration and the expressed alternative costimulatory molecules on the antigen-presenting cell, T cell activation may depend on LFA-1 in some physiological situations (low antigen and little alternative costimulation) while LFA-1-ICAM interactions may be partially or even completely substituted by alternative activation pathways in others (high antigen and high alternative costimulation or alternative cytokines).