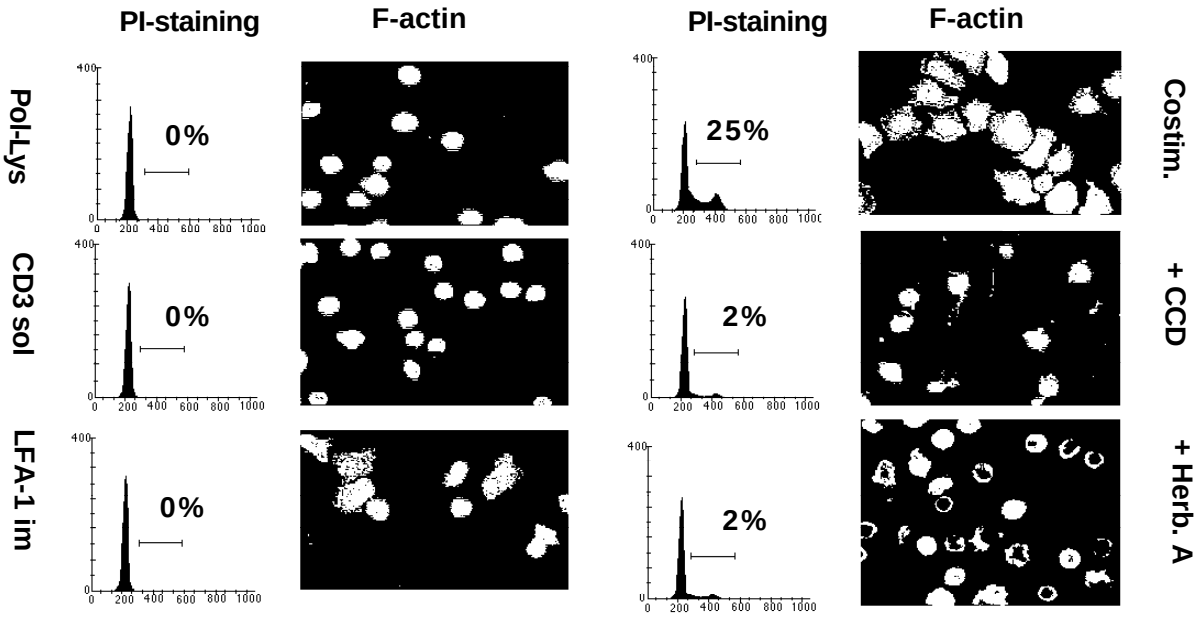
3. Results

3.1 Proliferation in response to TCR cross-linking requires LFA-1-dependent spreading in quiescent human T cells.

In order to address the role of integrin-dependent costimulation in cell cycle progression of antigen-stimulated primary T cells, we utilized anti-CD3 mAbs, in soluble or immobilized form, as a substitute for the cognate ligand of the T cell receptor complex. The immobilized ligand, ICAM-1 or the anti-LFA-1(αL) mAb TS1.22 was used to engage the αL/β2 integrin. In some experiments the stimulation was performed in the presence of soluble, function-blocking (inhibit ligand binding) anti-LFA-1 mAbs (anti-αL and anti-β2).
The dependence of T cell proliferation on receptor-stimulation and the actin-based cytoskeleton is shown in Figure 3.1a: While the stimulation of either LFA-1 with an immobilized mAb alone, which lead transiently to cytoskeletal rearrangement in a fraction of cells, or of TCR aggregation with soluble anti-CD3 mAbs in the absence of spreading did not induce S phase entry, the combination of the two stimuli, which lead to efficient prolonged spreading of all cells, induced proliferation. Proliferation of costimulated cells was not observed when cytoskeletal rearrangement was either inhibited with Cytochalasin D (CCD) or when cells were stimulated with both mAbs cross-linked in solution (not shown), demonstrating that S phase entry induced by the two receptors is dependent on the integrity of the actin-based cytoskeleton and a spread cell shape and not just on receptor aggregation. Furthermore, pre-treating cells with the tyrosine kinase inhibitor Herbimycin A prevented as well both spreading and proliferation, suggesting that tyrosine phosphorylation is critically involved in LFA-1-dependent proliferation of T cells.

Figure 3.1a: Requirements for TCR-stimulated proliferation of primary T cells. *Purified T cells were incubated in poly-L-lysine-coated dishes (Pol-Lys) in the absence or presence of soluble, cross-linked anti-CD3 mAbs (CD3 sol) or with immobilized anti-LFA-1 mAbs (LFA-1 im). Cells were further stimulated with both immobilized anti-LFA-1 mAbs and soluble anti-CD3 mAbs (Costim.) in the absence or presence of 10μM Cytochalasin D (+ CCD) or 1μM Herbimycin A (+ Herb A). Proliferation was assessed by propidium iodide staining after 72 hours of stimulation. Numbers indicate the fraction of cells in the S/G2/M-phases of the cell cycle. F-actin staining with labelled Phalloidin visualizing cytoskeletal rearrangement was performed after 20 minutes of stimulation as described in 2.5. Shown is one representative experiment out of five performed (see next page).*



As shown in Figure 3.1b, cells were further stimulated with immobilized anti-CD3 mAbs and β2-integrins were either functionally blocked with soluble anti-LFA-1 mAbs or co-engaged with co-immobilized ICAM-1: In contrast to soluble anti-CD3 mAbs, immobilized anti-CD3 mAbs induced some cellular proliferation and suboptimal spreading, the latter was shown to involve dynamic interactions between integrins and the actin-based cytoskeleton even in the absence of direct integrin engagement (Pardi et al., 1992). However, the proliferation induced by immobilized anti-CD3 mAbs was strongly enhanced by co-engaging LFA-1 and nearly completely prevented by blocking β2-integrin function, showing that proliferation induced with immobilized anti-CD3 mAbs is as well LFA-1-dependent under the used conditions. When the anti-CD3 mAbs and ICAM-1 were co-immobilized, cells remained spread and isolated from each other during the entire stimulation period (right panel). Conversely, when the immobilized anti-CD3 mAbs were used alone, cells only initially adhered to the dishes (middle panel), gradually rounded up after two to four hours, subsequently detached from the dish and formed tight homotypic clusters (right panel), probably due to upregulated integrin function. Surprisingly, the presence of the function-blocking anti-LFA-1 mAbs did not significantly interfere with the initial spreading induced by immobilized anti-CD3 mAbs (compare middle panels), but lead to an earlier rounding-up and detachment (not shown) and completely inhibited both the formation of the cell clusters and proliferation. It therefore appears that the soluble anti-LFA-1 mAbs limit the time cells are allowed to spread, and that LFA-1-dependent homotypic cell clusters may be required for proliferation induced by immobilized anti-CD3 mAbs since they allow cells to maintain a spread cell shape for a prolonged period.

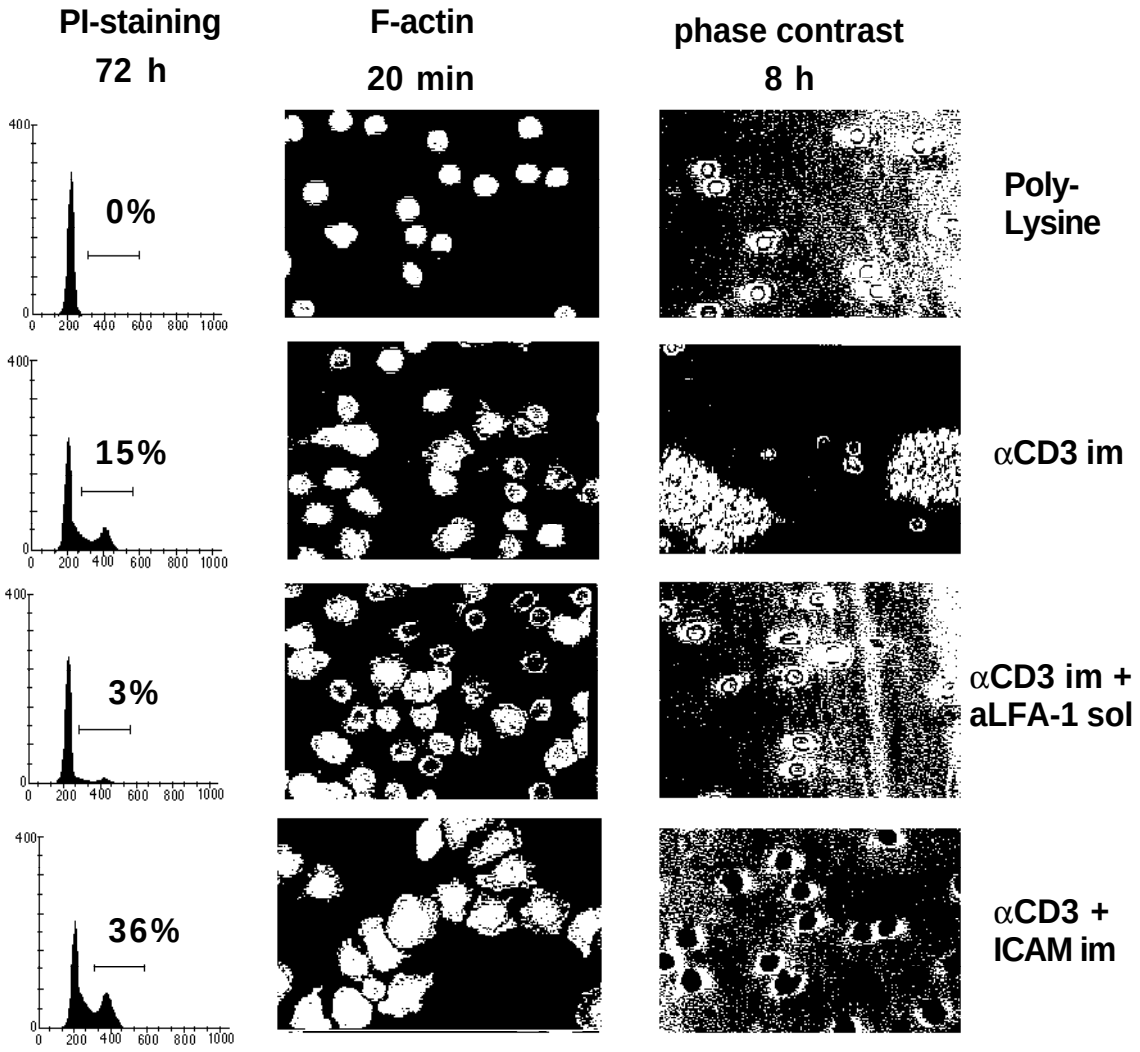


Figure 3.1b: Proliferation induced with immobilized anti-CD3 mAbs is LFA-1-dependent. *Cells were stimulated with immobilized anti-CD3 mAbs (αCD3 im) in the absence or presence of soluble, function-blocking anti-LFA-1 mAbs (αCD3 im + αLFA-1 sol) or with co-immobilized anti-CD3 mAbs and recombinant ICAM-1 (αCD3 + ICAM im). Left panels: Proliferation was assessed by propidium iodide staining after 72 hours of stimulation. Numbers indicate the fraction of cells in the S/G2/M-phases of the cell cycle. Statistic analysis of 10 experiments revealed that the fraction of cells in G2/S/M was 1 +/- 1% (Poly-Lysine), 15 +/- 7% (αCD3 im), 3 +/- 2% (αCD3 + αLFA-1 sol) and 36 +/- 10% (αCD3 +ICAM im), respectively. Middle panels: F-actin staining with labelled Phalloidin visualizing cytoskeletal rearrangement was performed after 20 minutes of stimulation as described in 2.5. Right panels: Phase contrast microscopic images were taken 8 hours after stimulation.*

The co-engagement of other costimulatory receptors, which is certainly possible under conditions in which large homotypic clusters are formed, is

unlikely to be primarily responsible for the observed phenomena, since co-immobilized anti-CD3 mAbs and ICAM-1 most efficiently stimulate proliferation in the absence of cell cluster formation (right panel). In summary, these findings demonstrate that in the absence of alternative receptor-ligand interactions, proliferation induced by TCR-triggering critically depends on an integrin-mediated spread cell shape, similar to what has been reported for the anchorage-dependent replication of adherent cell types in response to growth factors.

3.2 The antibody-induced internalization of the TCR does not correlate with LFA-1-dependent proliferation

A proposed role for LFA-1 and the cytoskeleton is that they enhance the strength and duration of TCR-signalling by providing the necessary adhesion strength for a stable intercellular contact required for efficient TCR triggering (Bachmann et al., 1997; Valitutti et al., 1995). In order to investigate if the observed differences in proliferation could be caused by enhanced, adhesion-dependent TCR triggering, the internalization rate of the antibody-stimulated antigen receptor complex in the absence or presence of LFA-1 co-engagement was assessed as a well-established read-out of TCR triggering.

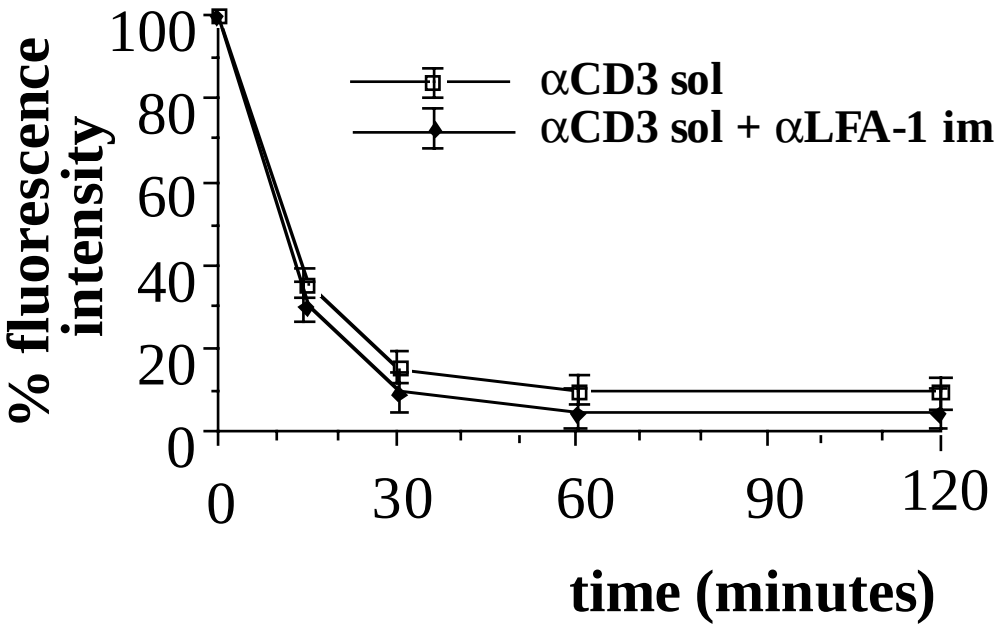


Figure 3.2a: The rate of TCR internalization is not altered by integrin co-engagement. Figure 3.2a: *Cells were stimulated with soluble cross-linked, anti-CD3 mAb (CD3 sol) in the absence or presence of immobilized anti-LFA-1 mAbs (LFA-1 im) and TCR expression assessed. Shown is the mean of three performed experiments.*

Figure 3.2a shows that co-engagement of LFA-1 did not alter the internalization rate of the soluble stimulating anti-CD3 mAbs, in spite of the fact that co-engaging LFA-1 is a prerequisite for proliferation induced by soluble anti-CD3 mAbs (see 3.1). Figure 3.2b shows that contact with saturating amounts of immobilized anti-CD3 mAbs induced a steady internalization of the receptor complex, which approximates 90% of the entire surface pool by 90 minutes post-stimulation. Co-engaging LFA-1 or blocking LFA-1 function did not have any significant effect on the rate of TCR-internalization. In contrast, suboptimal concentrations of anti-CD3 lead to a slower and incomplete downmodulation of the TCR. As reported for the internalization of the TCR following the recognition of its cognate ligand, this partial, antibody-induced receptor down-regulation was significantly enhanced by co-engaging LFA-1. Conversely, the presence of soluble function-blocking anti-LFA-1 mAbs in the culture did not significantly alter the extent or the kinetics of internalization of the TCR at any anti-CD3 concentration tested (1-10µg, shown for 10µg).

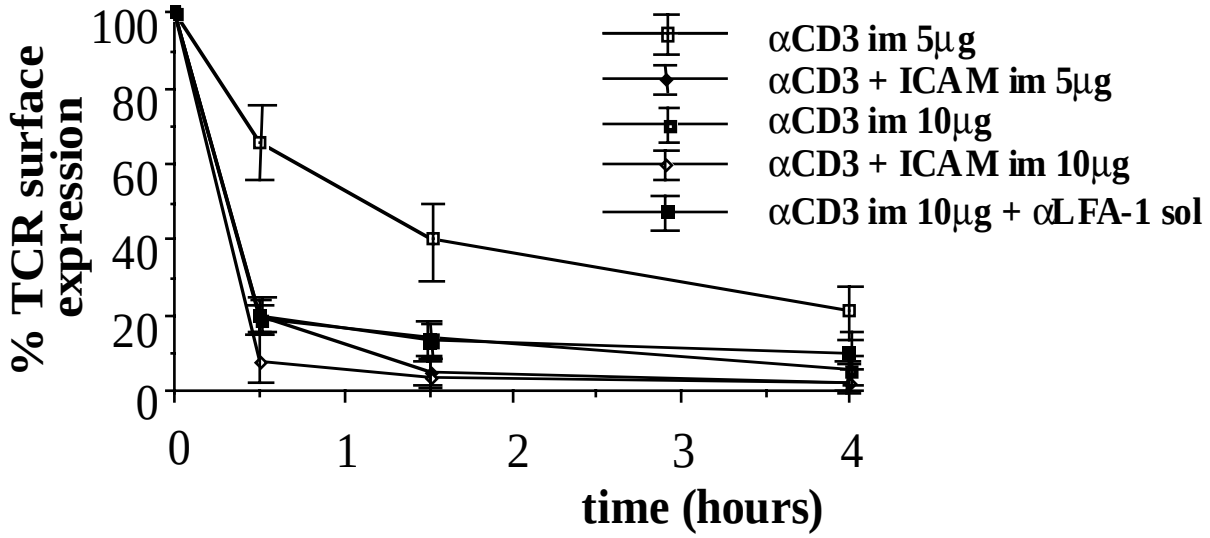


Fig. 3.2b: *Cells were stimulated either with immobilized, anti-CD3 mAbs (αCD3 im) at the indicated concentration with or without adding soluble anti-LFA-1 mAbs (+ αLFA-1 sol) or with co-immobilized anti-CD3 mAbs and ICAM (Costimulation) for the indicated time periods and TCR expression assessed as described in 2.4. Results are expressed as % reduction of mean fluorescence levels compared to untreated cells and show the mean of three separate experiments.*

Since our model of costimulation is based on the use of saturating amounts of anti-CD3, and since the amount of internalized TCRs is believed to reflect TCR-triggering and therefore TCR-dependent signal transduction (Viola and Lanzavecchia, 1996), these findings suggest that enhanced TCR-dependent signal transduction is not responsible for the observed integrin-dependent S phase entry. They further confirm that TCR downregulation can be

completely uncoupled from T cell proliferation (Cai et al., 1997; Salio et al., 1997).

3.3 Tyrosine phosphorylation is synergistically induced by TCR triggering and LFA-1-mediated spreading

One of the first detectable events in T cell signalling is the activation of tyrosine kinases, which leads to enhanced phosphorylation of proteins on tyrosine residues. T cell activation critically depends on the activation of tyrosine kinases and is completely prevented by tyrosine kinase inhibitors (June et al., 1990). Consistent with this, as shown in Figure 3.1a, the tyrosine kinase inhibitor Herbimycin A completely prevented TCR/LFA-1-dependent T cell proliferation. Tyrosine phosphorylation can be detected by phosphotyrosine-immunoblotting, which is a kind of fingerprint of early signal transduction since enhanced tyrosine phosphorylation potentially reflects multiple activation pathways. As shown in Figure 3.3a, stimulating cells with soluble anti-CD3 mAbs lead to enhanced phosphorylation on tyrosine residues of several proteins which peaks at 10 minutes and rapidly declined thereafter correlating thus closely with TCR internalization (see Figure 3.2a). Costimulating cells with both soluble anti-CD3 mAbs and immobilized anti-LFA-1 mAbs lead to a more sustained phosphorylation of apparently mostly the same proteins, in spite of the fact that TCR internalization was not altered by co-engaging LFA-1.

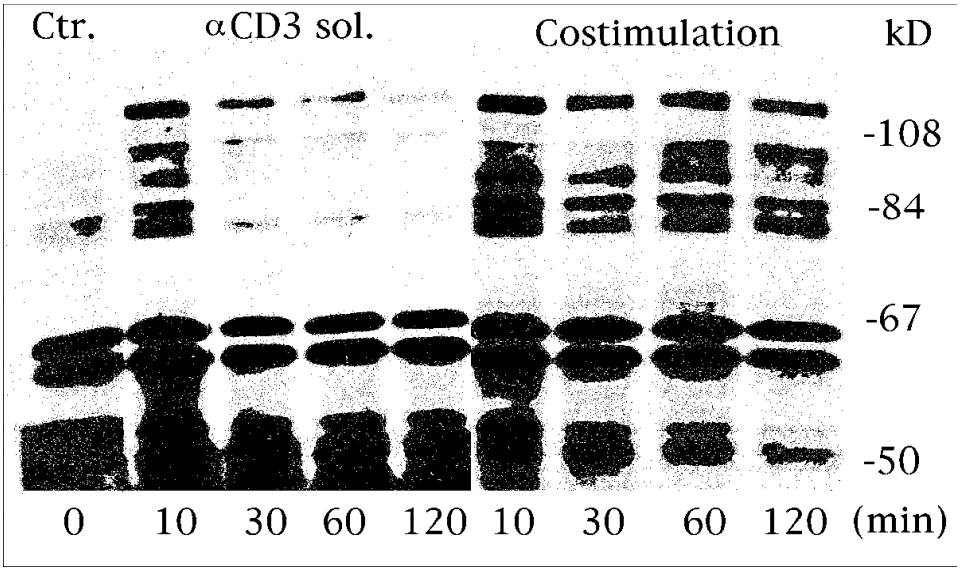
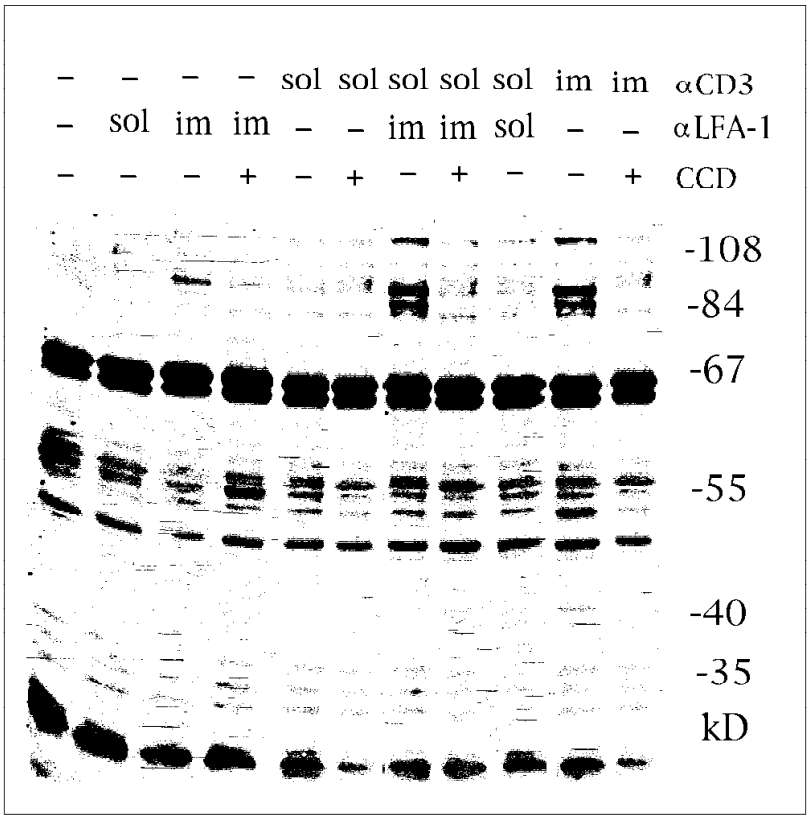


Figure 3.3a: Costimulation of T cells with LFA-1 leads to prolonged TCR-induced tyrosine phosphorylation.*Cells were left untreated (Ctr) or stimulated with soluble, cross-linked anti-CD3 mAbs either alone (αCD3 sol) or in the presence of immobilized anti-LFA-1 mAbs (Costimulation) for the indicated times. Phosphotyrosine-blotting was performed as described in 2.10.*

Densitometric analysis revealed that at least five phosphoproteins were affected in their phosphotyrosine content by costimulation, they migrated with an apparent molecular weight of approximately 80, 85, 90, 100 and 120 kD. Although no identification of the respective phosphoproteins was done, the phosphotyrosine blot clearly shows that co-engagement of LFA-1 synergizes with TCR-mediated signal transduction. It further appears that, at the onset of T cell activation, LFA-1-mediated spreading leads predominantly to a sustained activation of the pathways which are as well transiently activated by TCR aggregation rather than activating completely independent pathways. In order to address the role of spreading in LFA-1 stimulated tyrosine phosphorylation, the effect of Cytochalasin D (CCD) or of stimulating cells with soluble, cross-linked anti-LFA-1 mAbs on the observed phosphorylation was investigated. As shown in Figure 3.3b, tyrosine phosphorylation induced by immobilized mAbs to LFA-1 but not by TCR aggregation was inhibited by pretreating cells with CCD. CCD-pretreatment prevented further the synergistic tyrosine phosphorylation induced by costimulation. Moreover, LFA-1 aggregation with soluble antibodies failed to induce or costimulate tyrosine phosphorylation. This demonstrates that LFA-1- but not TCR-mediated signalling critically depends on a spread cell shape. Interestingly, stimulating cells with immobilized anti-CD3 mAbs, which induce spreading in the absence of LFA-1 engagement (see 3.1), bypassed LFA-1 requirement for synergistic tyrosine phosphorylation. Since the enhanced tyrosine phosphorylation observed under this condition was as well prevented by CCD pre-treatment and since CCD appears not to interfere with TCR triggering as assessed by TCR-downregulation (Cai et al., 1997), we concluded that spreading induced by immobilized anti-CD3 mAbs was responsible for the different signal transduction capacities of soluble and immobilized anti-CD3 mAbs, even if the very rapid downregulation of the soluble anti-CD3 mAbs following stimulation may contribute as well to the difference.

These results show that LFA-1 mediated signalling, as reported for other integrins, critically depends on spreading rather than on receptor aggregation. Furthermore, spreading and TCR-dependent signalling synergize on tyrosine phosphorylation. It further appears that cytoskeletal rearrangement and spreading-dependent signalling events are not exclusively inducible by LFA-1 engagement in our system.

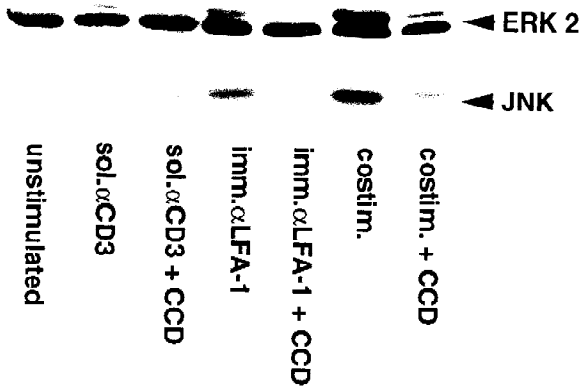
Figure 3.3b: Tyrosine phosphorylation induced by LFA-1 depends on cytoskeletal rearrangement and spreading. *Cells were left untreated or stimulated for 30 minutes with soluble (sol) or immobilized (im) anti-LFA-1 and/or anti-CD3 mAbs in the absence or presence of 10µM Cytochalasin D (CCD) as indicated. Phosphotyrosine-blotting was performed as described in 2.10 (see next page).*



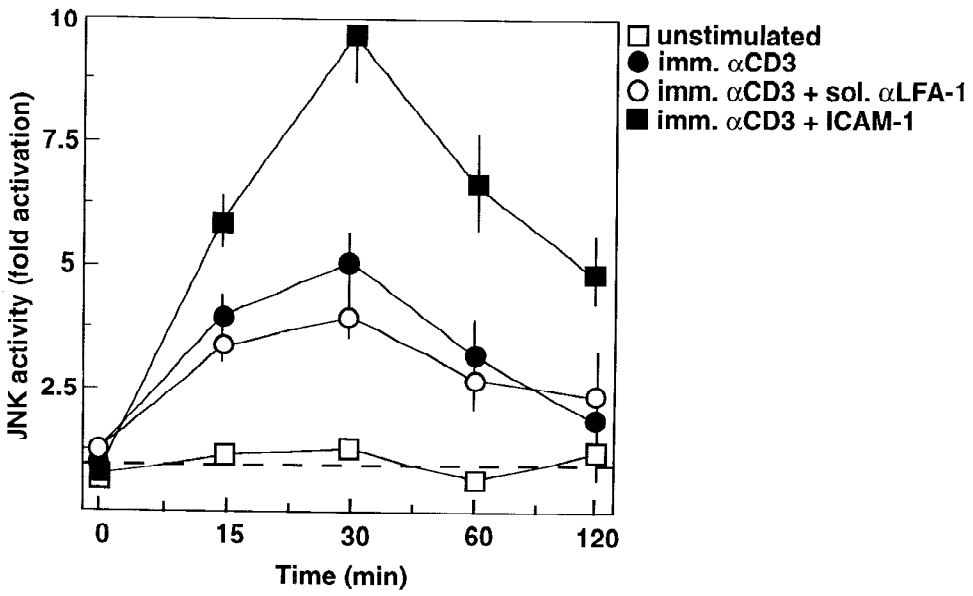
3.4 Regulation of MAP kinases by LFA-1 and the TCR in primary human T cells

In most cell types, mitogenic signals are transduced into the nucleus through the activation and nuclear translocation of MAP kinases (Karin, 1996). Previous studies in non-lymphoid cells have shown that integrins, alone or in combination with growth factors, contribute to Map kinase activation (Chen et al., 1994; Mainiero et al., 1997; Miyamoto et al., 1996; Morino et al., 1995; Zhu and Assoian, 1995). Synergistic activation of Map kinases, depending on an integrin-mediated spread cell shape of growth factor-stimulated cells has therefore been proposed to link integrin-mediated signalling to anchorage-dependent cell cycle progression. Moreover, JNK was proposed to be involved in the costimulation-dependent IL-2 production in the Jurkat cell line (Su et al., 1994). The regulation of the Map kinases ERK and JNK by TCR-triggering and LFA-1 mediated spreading was therefore analyzed. Figure 3.4a shows that, at the time of maximal stimulation, both ERK and JNK activities are potentiated when the TCR and LFA-1 were co-engaged in quiescent T cells. As observed for tyrosine phosphorylation, the integrin-dependent, but not the TCR-dependent component of MAP kinase activation appears to require an intact actin cytoskeleton and spreading, as judged by its complete inhibition by CCD (Fig. 3.4a, panel A) and by the failure of soluble anti-integrin antibody to induce enzyme activation (not shown).

A


ERK 2
JNK
unstimulated
sol.αCD3
sol.αCD3 + CCD
imm.αLFA-1
imm.αLFA-1 + CCD
costim.
costim. + CCD

B


JNK activity (fold activation)
10
7.5
5
2.5
0
15
30
60
120
Time (min)
unstimulated
imm. αCD3
imm. αCD3 + sol. αLFA-1
imm. αCD3 + ICAM-1

C

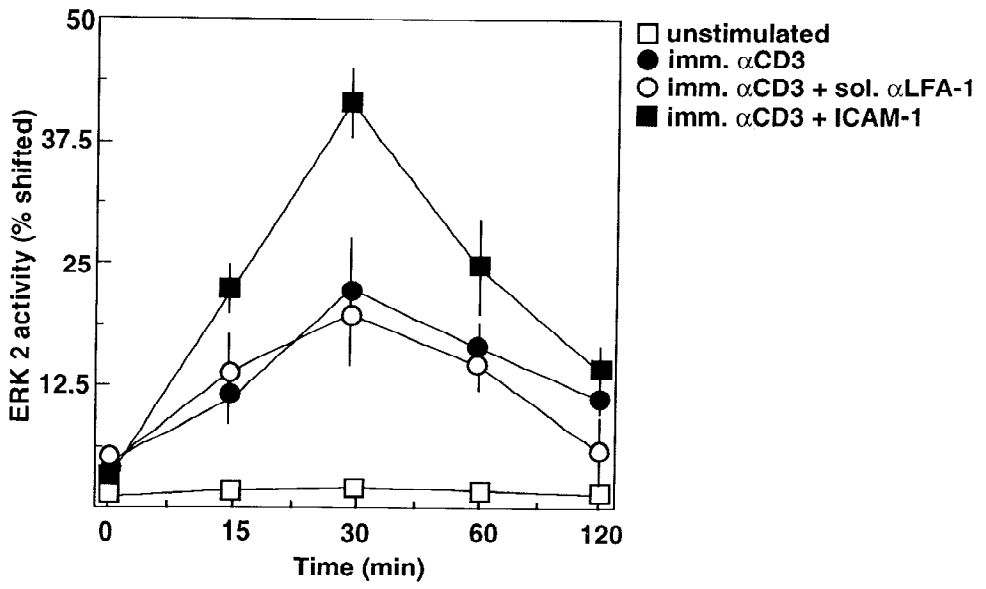

ERK 2 activity (% shifted)
50
37.5
25
12.5
0
15
30
60
120
Time (min)
unstimulated
imm. αCD3
imm. αCD3 + sol. αLFA-1
imm. αCD3 + ICAM-1

Figure 3.4a: ERK-2 and JNK activation by TCR- and LFA-1-dependent signals. *Panel A: Purified, resting T cells were stimulated for 20 minutes as indicated, with (lanes 3, 5 and 7) or without (lanes 1, 2, 4 and 6) pre-treatment (1 hour at 37°C) with 10µM Cytochalasin D (CCD). ERK activation was measured by a band shift assay (2.10) and JNK activity was assessed with an in vitro kinase assay (2.14). Panels B and C: Time-course of ERK-2 (C) and JNK (B) activation. Cells were left untreated (empty squares) or stimulated for the indicated times with immobilized anti-CD3 mAbs (filled circles), co-immobilized anti-CD3 mAb and ICAM-1 (filled squares) or immobilized anti-CD3 mAbs in the presence of blocking soluble anti-LFA-1 mAbs (empty circles). Results represent the mean of three separate experiments.*

In contrast to this, pre-treating cells with the tyrosine kinase inhibitor Herbimycin A prevented both the TCR- and the LFA-1-dependent component of ERK activation (not shown), confirming the importance of tyrosine phosphorylation for TCR- and LFA-1-dependent signalling. Interestingly, co-immobilization of anti-CD3 mAbs and ICAM-1 proved to be the more efficient way to cause synergistic ERK activation, confirming that co-localization of the TCR and LFA-1 is critical for LFA-1-dependent T cell activation (Berg and Ostergaard, 1995).

We compared further the activation levels and kinetics of ERK and JNK in cells stimulated with either immobilized anti-CD3 mAbs in the absence or presence of soluble anti-LFA-1 mAbs or with co-immobilized anti-CD3 mAbs and recombinant ICAM-1. Panels B and C show that, although the simultaneous engagement of the two receptors indeed resulted in a stronger activation of both kinases, the extent and kinetics of ERK and JNK activity were virtually superimposible in the other two conditions, in spite of the fact that S phase entry was completely prevented by the presence of the function-blocking anti-LFA-1 mAbs.

The activation of the p38 MAP kinase, which has recently been reported to be required for T cell proliferation (Crawley et al., 1997; Ward et al., 1997), seemed to be regulated similarly to ERK and JNK: Costimulation of the TCR and LFA-1 resulted in a stronger activation as compared to the stimulation with either soluble or immobilized anti-CD3 mAbs alone and the presence of soluble anti-LFA-1 mAbs did not significantly alter p38 kinase activity induced by immobilized anti-CD3 mAbs (not shown). These results suggest that in spite of the fact that all investigated Map kinases can integrate TCR- and LFA-1-dependent signals, none of the major eukaryotic MAP kinase pathways seems to primarily mediate the costimulatory effect of LFA-1 on T cell proliferation.

Nuclear translocation of MAP kinases is believed to be a key step in their ability to control transcriptional activation of target genes (Karin, 1996; Treisman, 1996). A selective extraction procedure was developed (described in 2.12), which allows to detect the translocation of proteins from the cytosol to the nucleus or to the membrane. For example, fractions of T cells

prepared as described in 2.12 evidenced a complete translocation of PKCα from the cytosolic to the nuclear and membrane-associated fractions upon stimulation with PMA (not shown). This procedure was used to investigate whether nuclear translocation of ERK-2 and JNK was differentially affected in TCR- and LFA-1-stimulated T cells. Figure 3.4b shows that both kinases were exclusively found in the cytosolic fraction in unstimulated cells. Enzyme activation by engagement of the TCR and LFA-1, alone or in combination, induced the nuclear translocation of a fraction of JNK, ranging from 2 to 5% of the total detectable enzymatic pool normalized for the amount of loaded proteins. Interestingly, stimulating cells with PMA, in contrast to costimulating them via the TCR and LFA-1, induced a band-shift of JNK. This reflects kinase activation since a kinase assay confirmed that PMA was about 10 times more potent in activating JNK as compared to costimulation (not shown), in spite of the fact that PMA does not cause proliferation. A significant nuclear translocation of ERK-2 (and ERK-1, not shown) could never be observed under any of the conditions tested in this model. This may reflect an insufficient sensitivity of this assay or the existence of differing kinetics of ERK activation versus its nuclear translocation. However, these results indicate that major differences in the extent of MAP kinase translocation into the nucleus are unlikely to be responsible for LFA-1-dependent costimulation of cell cycle progression in primary T cells.

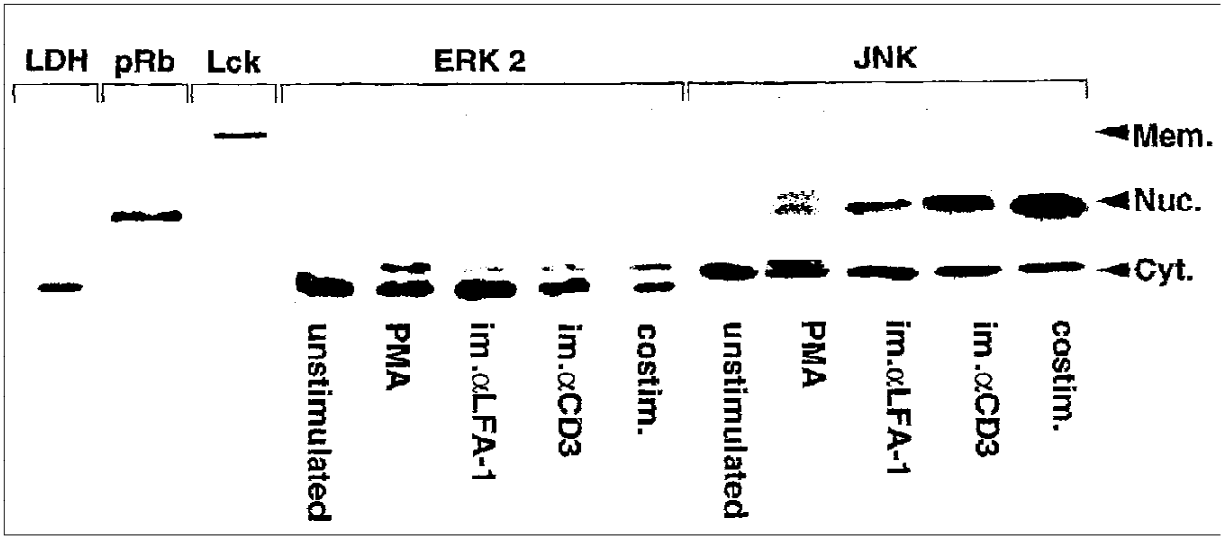


Figure 3.4b: Nuclear translocation of Map kinases in TCR- and LFA-1-stimulated T cells. *Membrane- (upper panel), nuclear- (middle panel) and cytosolic-enriched (lower panel) fractions were prepared as described in 2.12. Purity of the various fractions was assessed using LDH (lane 1), pRb (lane 2) and $p56^{lck}$ (Lck, lane 3) as markers for cytosolic, nuclear and membrane-associated proteins, respectively. Since >80% of the total cellular proteins were extracted in the cytosolic fraction, 8-10 fold higher protein amount was loaded for the nuclear and membrane-associated fractions. Cells were left untreated or stimulated with either 50nM PMA or with immobilized*

mAbs to LFA-1, CD3 or both. LDH, p56^{lck}, pRb, ERK-2 and JNK were detected by immunoblotting using specific antibodies. Shown is one out of three experiments yielding comparable results.

3.5 Effects of LFA-1 on the expression of the immediate early genes *c-fos* and *c-jun*.

As a later readout for MAP kinase activation in TCR- and LFA-1-stimulated T cells, we analyzed the expression of the *c-jun* and *c-fos* immediate early genes, since transcription of both genes is thought to be a downstream event following the activation of JNK and ERK, respectively (Karin, 1996; Treisman, 1996). Analysis of gene expression by Northern blotting (Figure 3.5a) revealed that stimulation of the TCR and LFA-1, alone or in combination, induced transient transcriptional activation of both the *c-jun* and *c-fos* genes. As observed for Map kinase activation, *c-fos* was induced independently of the presence of soluble anti-LFA-1 mAbs by immobilized anti-CD3 mAbs. Surprisingly, cells costimulated with co-immobilized anti-CD3 and ICAM or anti-LFA-1 mAbs expressed only slightly enhanced levels of *c-jun* and *c-fos* as compared to anti-CD3 stimulated cells. However, as shown for tyrosine phosphorylation and Map kinase activation, the expression of *c-fos* induced by costimulation was partially inhibited by pre-treating cells with Cytochalasin D, indicating thus the existence of a spreading-dependent pathway from early tyrosine kinase activation to immediate early gene expression.

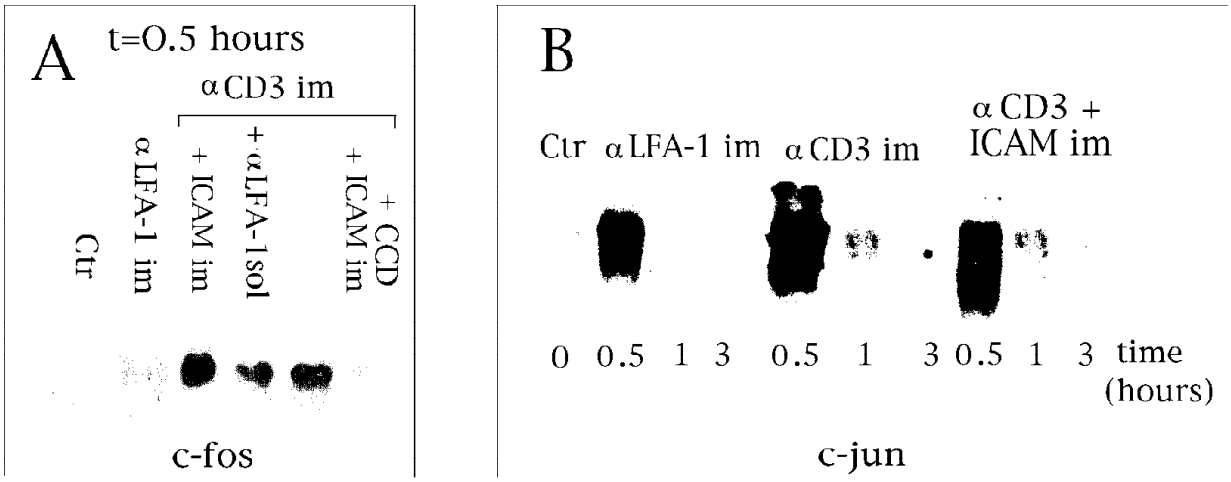


Figure 3.5a: Immediate early gene expression induced by the TCR and LFA-1. *Panel A: Cells were left untreated (Ctr) or stimulated with immobilized mAbs specific for LFA-1 (αLFA-1 im) or CD3 (αCD3 im). In anti-CD3 stimulated cells, LFA-1 was either functionally blocked (+ aLFA-1 sol) or co-engaged (+ ICAM im) in the absence or presence of Cytochalasin D (CCD)-pretreatment. Panel: B: Cells were stimulated for the indicated times with immobilized anti-LFA-1 mAbs (αLFA-1 im), with immobilized anti-CD3 mAbs*

(αCD3 im) or with co-immobilized anti-CD3 mAbs and ICAM-1 (αCD3 + ICAM im). C-jun and c-fos expression was analyzed by Northern Blotting as described in 2.15.

In order to understand if LFA-1 may enhance c-Jun and c-Fos protein expression at the post-transcriptional level, the expression of both transcription factors was monitored as well by western blotting. Figure 3.5b demonstrates that the stimulation with immobilized anti-CD3 antibodies lead to a rapid up-regulation of both proto-oncogene products, reaching a plateau at 3 to 6 hours post-stimulation and gradually decreasing at later time points. The expression levels of both proteins were again only slightly enhanced by coengaging LFA-1, particularly at late time points after stimulation. Moreover, the abrogation of integrin-dependent homotypic clustering did not significantly affect the kinetics and the absolute expression levels of either proto-oncogene product in TCR-stimulated cells. In summary, the expression of the *c-fos* and *c-jun* genes sustains the conclusion that LFA-1 mediated Map kinase activation leading to immediate early gene expression *per se* is not sufficient to explain the observed LFA-1-dependence of S phase entry in TCR-stimulated primary T cells.

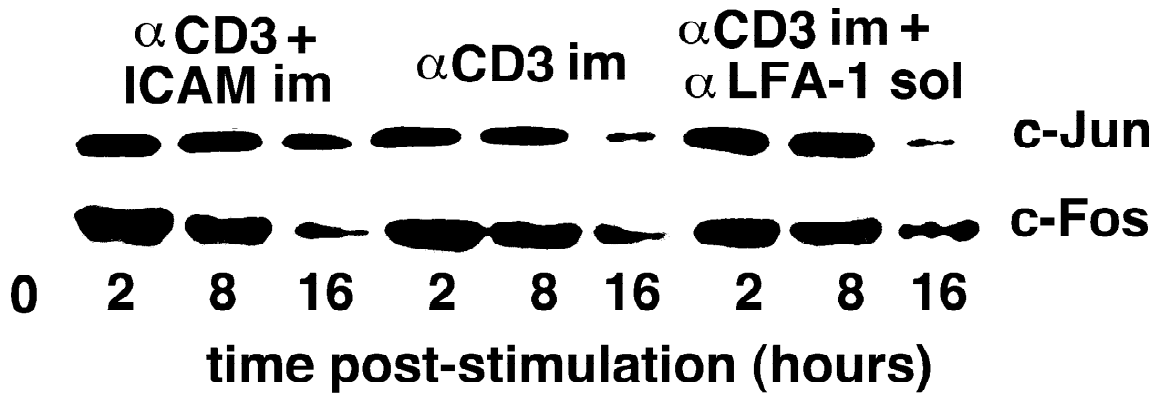


Figure 3.5b: LFA-1-dependent G1 to S transition does not involve the modulation of c-Fos and c-Jun protein levels. *Cells were stimulated with immobilized anti-CD3 mAbs in the absence or presence of soluble, function-blocking anti-LFA-1 mAbs for the indicated times. C-Jun and c-Fos protein levels were assessed by Immunoblotting. Similar results were obtained in four separate experiments.*

3.6 LFA-1-mediated spreading is a required late component for S phase entry in TCR-stimulated cells

The experiments reported above suggest that although LFA-1-dependent cell spreading is necessary for optimal MAP kinase activation, a critical component of integrin-mediated signalling is acting at a later step in the

sequence of events leading to the onset of DNA replication in TCR-stimulated cells. To more precisely map the LFA-1-dependent requirement in G1, we abrogated LFA-1 mediated spreading at various time points by adding CCD to costimulated cells. The Map kinase kinase (MKK) inhibitor PD98059, which specifically inhibits ERK, but not significantly JNK and p38 (Figure 3.6, panel B) was used as a probe for ERK function within the same time period. The results in Figure 3.6, panel A show that pre-treatment of cells with either CCD or PD98059 completely prevents proliferation, confirming the importance of spreading and Map kinase activation for T cell proliferation. Nevertheless, CCD but not PD98059 prevented proliferation even when added at 4-8 hours post-stimulation, showing that an intact actin-based cytoskeleton and a spread cell shape through G1 is required independently of ERK activity for proliferation. This prolonged spreading-requirement is very similar to that reported for non-lymphoid, anchorage-dependent cells (Bohmer et al., 1996).

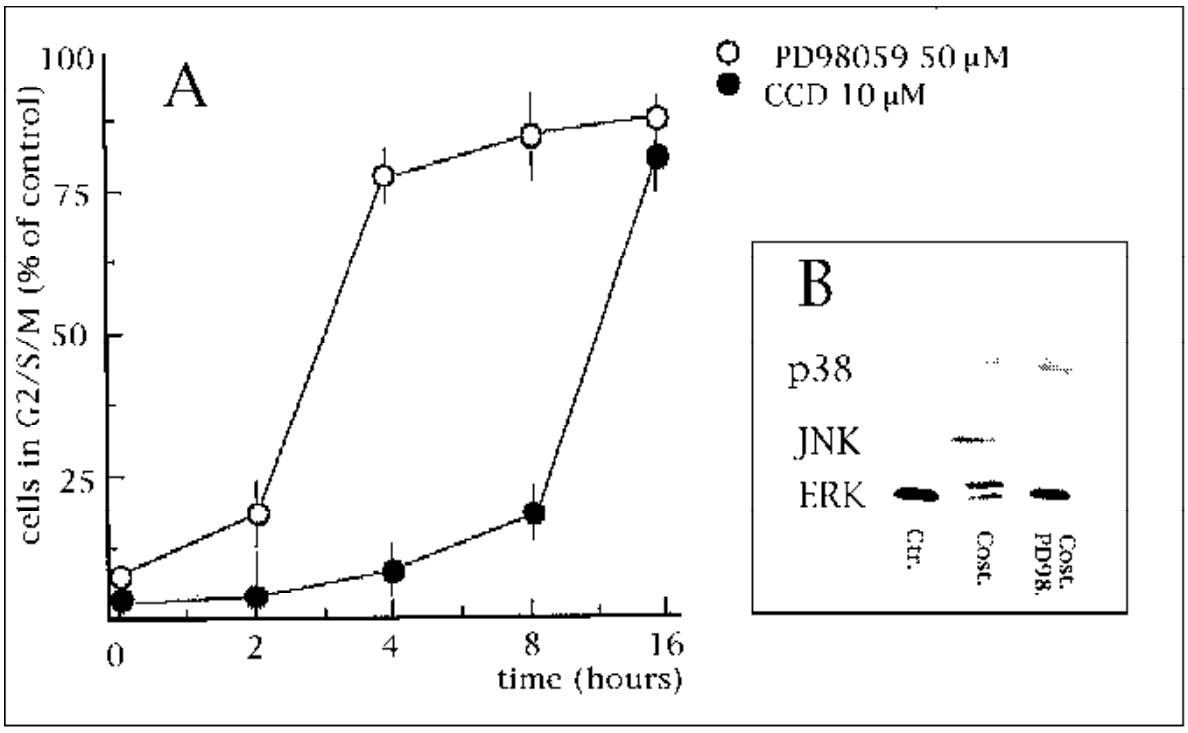


Figure 3.6: A sustained integrin-dependent spread cell shape is required throughout G1 to initiate DNA replication in TCR-stimulated T cells. *Panel A: The MKK inhibitor PD98059 (50µM) or Cytochalasin D (CCD, 10µM) were added at the indicated time points to T cells stimulated with co-immobilized anti-CD3 mAbs and ICAM-1. Progression into the cell cycle was quantified by propidium iodide staining, and the results expressed as % of S/G2/M cycling cells compared to untreated controls. Values represent the mean of four separate experiments. Panel B: Specificity of the Inhibitors PD98059. Cells were left untreated (Ctr.) or stimulated with co-immobilized anti-CD3 mAbs and ICAM-1 (Cost.) in the*

absence or presence of the MKK-inhibitor PD98059. Map kinase activation was measured as described before.

The specificity of CCD for inhibiting LFA-1-function was controlled as follows: the proliferation of TCR- or co-stimulated cells was as well abrogated when cells were gently detached at 4 to 8 hours post-stimulation with a pipette, when nearly all TCRs had been triggered and internalized (see Figure 3.2b). Allowing the detached cells to re-adhere in dishes pre-coated only with ICAM-1 however nearly completely restored proliferation (not shown). The respective importance of JNK and p38 for T cell proliferation was tried to elucidate as follows: The p38 Map kinase was inhibited with the Inhibitor SB203580 (see 2.2 b)). Pre-treatment with SB203580 resulted as well in a complete block of proliferation, but in contrast to ERK, p38 activity seemed to be required for G1 to S transition, since the inhibitor continued to block proliferation when added at 4 to 8 hours post-stimulation (not shown). It is therefore difficult to evaluate the respective importance of the initial, spreading-enhanced activation in G0 and the probably IL-2 dependent activation in G1 (Crawley et al., 1997; Ward et al., 1997) for proliferation (see Discussion). It was as well tried to inhibit JNK: Cells were permeabilized by means of electroporation in the presence of excess GST-c-Jun, which acts as a competitive inhibitor for JNK (see 2.17). Although the control of the electroporated cells by immunoblotting with a mAb specific for the amino-terminal of c-Jun revealed that considerable amounts of the inhibitor indeed entered the cells, no inhibition of proliferation was observed (not shown). Nevertheless, since we could not evaluate the achieved inhibition this does not rule out an important role of JNK in LFA-1-dependent T cell proliferation. In summary, these results demonstrates that abrogating LFA-1-mediated spreading in G1, when TCR-delivered signals are exhausted, inhibits S phase entry independently of early signal transduction, and that LFA-1-ICAM interactions are necessary and sufficient for G1 to S transition in the absence of alternative ligand-receptor interactions.

3.7 Sustained LFA-1-dependent spreading promotes pRb-inactivation

The observed anchorage-dependence for T cell growth coincides with the time required for TCR-stimulated cells to transit through G1 and express cell cycle-related proteins that trigger the initiation of DNA replication (Assoian and Zhu, 1997). It is believed that the main molecular switch in eukaryotes which allows mitogen-stimulated cells to enter S phase is the inactivating phosphorylation of the retinoblastoma tumor suppresser protein (pRb), which occurs in mid-to-late G1 (Bartek et al., 1996). The hyperphosphorylation of pRb is effected by G1 phase cyclin-dependent kinases (CDKs), whose catalytic activity is tightly regulated by complexed G1 cyclins and CDK-inhibitors.

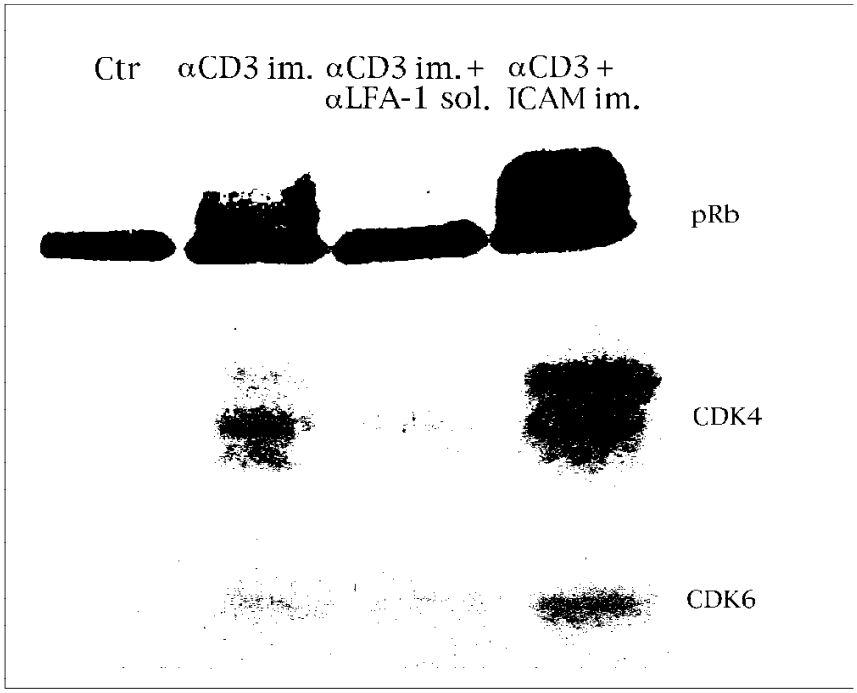


Figure 3.7a: LFA-1-dependent CDK-activation and pRb-inactivation in TCR-stimulated primary T cells. *Cells were left untreated (Ctr) or stimulated with either immobilized anti-CD3 mAbs alone (αCD3 im) in the absence or presence of soluble anti-LFA-1 antibodies (αCD3 im + αLFA-1 sol) or with co-immobilized anti-CD3 mAbs and ICAM-1 (αCD3 + ICAM im) for 20 hours. PRb-inactivation is visualized with a band shift assays, activation of CDK4 and CDK6 is assessed by an in-vitro kinase assay.*

As shown in Figure 3.7a, pRb-hyperphosphorylation, detectable by the appearance of slower migrating bands after 12-6 hours of stimulation, closely correlated with S phase entry (compare Figure 3.1b). This was paralleled by the activation of the two major G1 pRb-kinases, CDK4 and CDK6 as judged by their ability to phosphorylate recombinant pRb *in vitro*. In contrast, only weak CDK2-dependent kinase activity could be detected in mid G1 (not shown), indicating that the first wave of Rb-phosphorylation is mediated predominantly by CDK4 and 6.

In order to understand which event may mediate LFA-1-dependent CDK activation and subsequent pRb-inactivation, the expression levels of the G1 CDKs (2, 4 and 6) and their regulators (D-type cyclins and the CDK inhibitors $p27^{kip1}$ and $p16^{INK4}$) were monitored by immunoblotting. Figure 3.7b shows that T cells displaying significant pRb-hyperphosphorylation in response to immobilized anti-CD3 mAbs showed a marked, progressive increase in the levels of both D-type cyclins and a moderate increase in the levels of the pRb-kinases CDK4 and CDK6, independently of the presence of soluble anti-LFA-1 mAbs. Only cyclin D3 expression was reduced in cells were LFA-1-ICAM interactions were prevented as compared to the reference protein ERK-2. The pleiotropic CDK inhibitor $p27^{kip1}$ was reduced 2 to 3 fold at 16 hours post-stimulation as compared to the level of unstimulated cells or cells stimulated

in the presence of function-blocking anti-LFA-1 mAbs. Cells which were stimulated with co-immobilized anti-CD3 mAbs and ICAM expressed similar amounts of CDK4, CDK6 and cyclin D2, but expressed higher levels of cyclin D3 and downregulated $p27^{kip1}$ even more efficiently as compared to anti-CD3 stimulated cells. The CDK-inhibitor $p16^{INK4}$ was not detectable in primary T cells ((Tam et al., 1994), not shown). In summary, the most important events correlating with LFA-1-dependent pRb-inactivation by CDKs and the subsequent entry into S phase, is a marked decrease in the levels of the CDK-inhibitor $p27^{kip1}$ and the enhanced expression of cyclin D3.

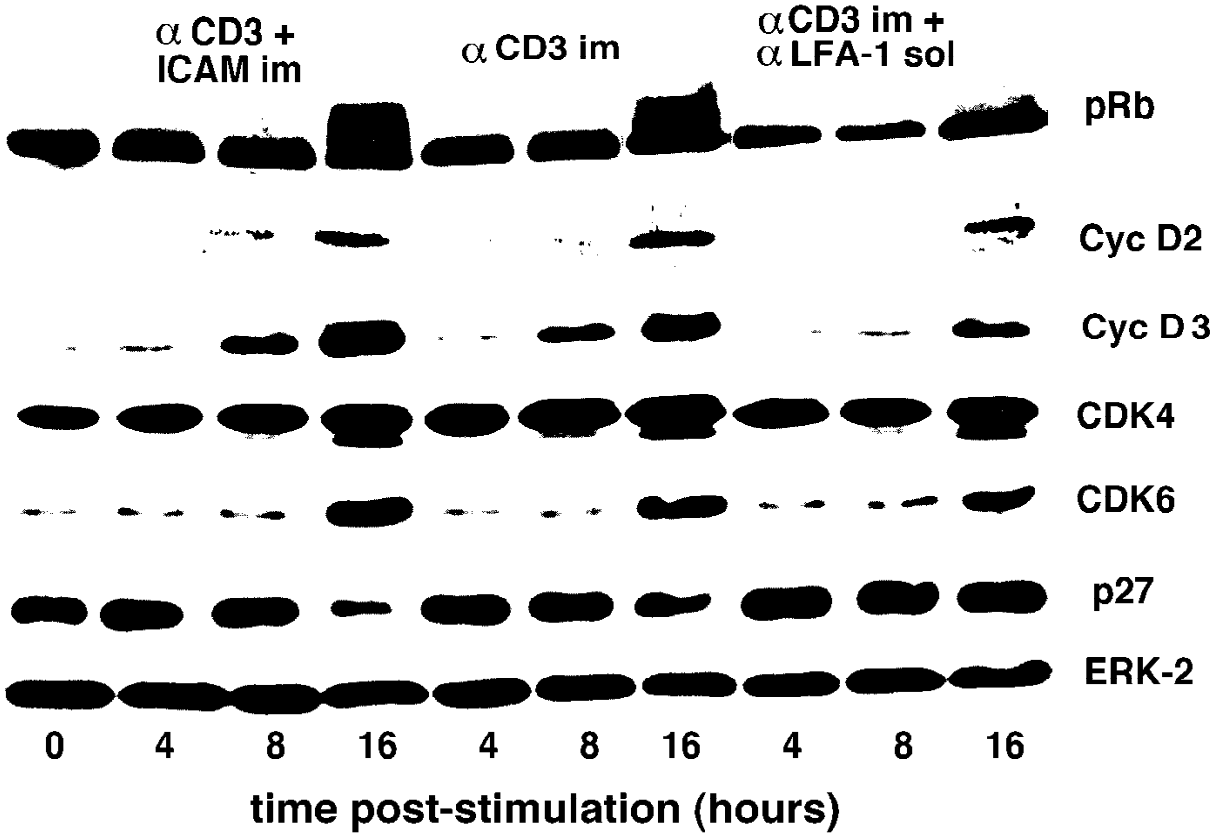


Figure 3.7b: Expression of G1 CDK regulators as a function of LFA-1 engagement in TCR-stimulated T cells. *T cells were stimulated with immobilized anti-CD3 mAbs in the absence or presence of function-blocking, soluble anti-LFA-1 mAbs or with co-immobilized anti-CD3 mAbs and ICAM for the indicated time periods, followed by immunoblot analysis with antibodies specific for the indicated proteins. Results are representative of four separate experiments.*

3.8 LFA-1-dependent Interleukin-2 production mediates G1 to S transition.

Independent reports have proposed that both D-type cyclin expression and $p27^{kip1}$ degradation in T lymphocytes is mediated by Interleukin-2 (IL-2)

(Ajchenbaum et al., 1993; Fero et al., 1996; Firpo et al., 1994; Nourse et al., 1994; Turner, 1993), and may represent the main biochemical event underlying S phase entry in IL-2 responsive T cells. It was therefore investigated if the observed integrin-dependent pRb-inactivation was an indirect consequence of LFA-1-dependent IL-2 secretion. Figure 3.8 shows that co-engagement of the TCR and LFA-1 indeed results in a marked increase of IL-2 production. Moreover, the inhibition of integrin-dependent homotypic clustering with soluble anti-LFA-1 mAbs nearly completely abrogated the secretion of IL-2 observed in anti-CD3 stimulated cells.

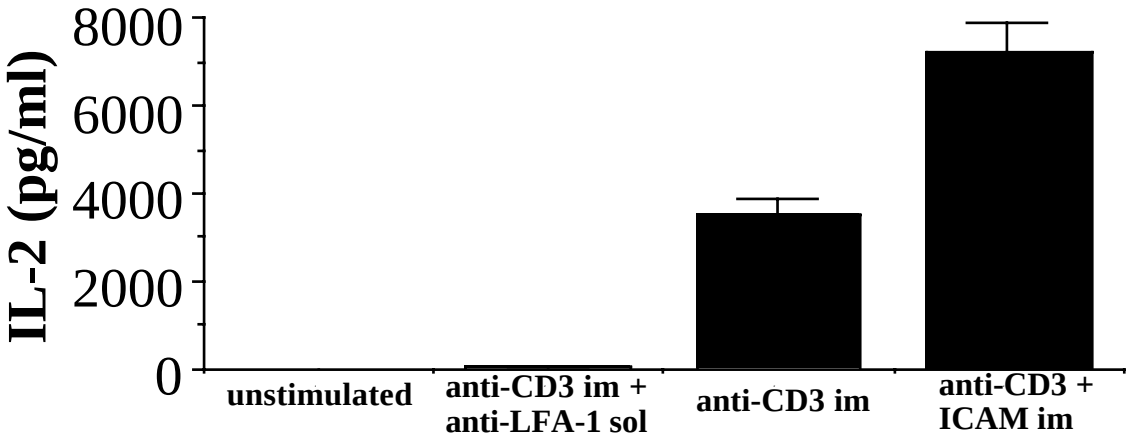


Figure 3.8: Interleukin-2 secretion is critically affected by LFA-1 engagement in primary T cells. *Resting T cells were left untreated or stimulated for 8 hours with immobilized anti-CD3 mAbs. LFA-1 was either co-engaged or functionally blocked with soluble anti-LFA-1 mAbs as indicated. IL-2 was quantified in the culture supernatants by ELISA. Shown are the mean values of three experiments.*

To further confirm that IL-2 production was a limiting step in the LFA-1-dependent G1 to S transition of TCR-stimulated T cells, complementa- tion experiments were performed in which low doses of exogenous IL-2 (20U/ml, approximately the amount IL-2 secreted by costimulated cells) were added to the cultures under the various conditions. Table 3.8 clearly demonstrates that the addition of exogenous IL-2 is able to completely rescue S-phase entry of T cells in which integrin-dependent homotypic clustering was blocked with soluble anti-integrin antibodies. The ability of exogenous IL-2 to revert the inhibition of cell cycle progression induced by blocking LFA-1-dependent cell clusters correlated with pRb-hyperphosphorylation (not shown). Notably, exogenous IL-2 did not induce cell cycle progression in T cells stimulated with soluble anti-CD3 antibodies or via LFA-1 alone, indicating that TCR triggering or aggregation of integrins *per se* is not sufficient to induce responsiveness to the mitogen. Further, stimulating cells in the presence of

excess blocking anti-IL-2 antibodies completely prevented proliferation induced by recombinant IL-2 and inhibited the same events to about 60-70% when IL-2 was produced endogenously by the cells. Another important event in T cell activation is the expression of the α-chain of the Interleukin-2 receptor (CD25), resulting in a higher affinity of the constitutively expressed IL-2 receptor for its ligand. As shown in Table 3.8, CD25 expression , similar to pRb-inactivation, closely correlates with S phase entry under the investigated conditions and is regulated by IL-2 (Ullman et al., 1990):

Table 3.8: IL-2 mediates integrin-dependent G1 to S transition and CD25 expression.

Condition:	**w/o IL-2**	**+ IL-2**	**+ α-IL-2**
untreated	**1+/-1%** CD25$^+$:<5%	**2+/-1%** CD25$^+$:<5%	**n. d.**
α-CD3 sol	**2+/-1%** CD25$^+$:<5%	**2+/-1%** CD25$^+$:<5%	**n. d.**
α-LFA-1 im	**1+/-1%** CD25$^+$:<5%	**2 +/- 2** CD25$^+$:<5%	**n. d**.
α-CD3 im + α-LFA-1 sol	**3+/-2%** CD25$^+$:10 +/-10% (13)	**21+/-4** CD25$^+$:55 +/-9% (50)	**3+/-2%*** CD25$^+$:9+/-5% (10)*
α-CD3 im	**15+/-7%** CD25$^+$:45 +/-15% (50)	**24+/-5** CD25$^+$:60 +/-13% (58)	**6+/-3%** CD25$^+$:20 +/-10% (20)
α-CD3 + ICAM im	**36+/-10%** CD25$^+$:95+/-5% (90)	**41+/-8** CD25$^+$:95 +/- 5% (105)	**12+/-7%** CD25$^+$:80 +/-20% (30)

Purified, resting T cells were stimulated as indicated in the presence of 20 U/ml recombinant IL-2 and(*)/or 100µg/ml polyclonal anti-IL-2 serum. Cell cycle progression was assessed 72 hours post-stimulation by propidium iodide staining and flow cytometry. Bold numbers indicate the fraction of cells in the G2/S/M phases of the cell cycle. Indicated are as well the fraction of cells staining positive for the CD25 antigen (CD25+). Numbers in parenthesis indicate the mean fluorescence intensity of the CD25-positive cells. Results show the mean of at least three independent experiments. (n. d. : not determined)

Exogenous IL-2 did further not bypass the inhibition of proliferation induced by the inhibitor of the ERK-cascade, PD98059 (not shown), suggesting that ERK is not only contributing to cytokine production as reported for the Jurkat T cell line (Park and Levitt, 1993), but that ERK activity in primary T cells is further necessary for the induction of competence to respond to IL-2.

In summary, these results demonstrate that IL-2 production critically depends on LFA-1 in TCR-stimulated primary T cells and that IL-2 is both necessary and sufficient to mediate G1 to S transition under the used conditions.

3.9 CD28 aggregation can bypass the late LFA-1-dependent step of anchorage-dependent T cell growth

CD28 is considered to be the most important, "professional" costimulatory receptor; the interaction with its ligands B7-1 or -2 is thought to deliver an TCR-independent signal required for full T cell activation (Lenschow et al., 1996), while the lack of CD28 costimulation may result in functional unresponsiveness. The relative importance of CD28 aggregation and LFA-1-mediated spreading in TCR-stimulated primary T cells was therefore analyzed by costimulating CD28 with soluble, cross-linked mAbs, since the purified recombinant ligand was not available and immobilized mAbs were found to contribute to the induction of spreading. Table 3.9 shows the effect of CD28 aggregation on proliferation is very similar to that of recombinant IL-2: CD28 costimulation fails to induce proliferation or IL-2 responsiveness in cells stimulated with soluble anti-CD3 mAbs or immobilized anti-LFA-1 mAbs alone. In contrast, the late LFA-1-requirement for IL-2-mediated S phase entry of competent cells is bypassed by CD28 co-aggregation since TCR-stimulated cells proliferated then even in the absence of LFA-1-dependent cell cluster formation. These results show that CD28 aggregation can induce G1 to S transition independently of LFA-1 in TCR-stimulated T cells.

Table 3.9: CD28 aggregation induces LFA-1-independent G1 to S transition. Cells *were stimulated with soluble or immobilized mAbs specific for CD3, LFA-1 and CD28 in the absence or presence of 20 U/ml IL-2 as indicated and proliferation was assessed after 72 hours by propidium iodide staining. Numbers indicate the fraction of cells in G2/S/M and represent the mean of three independent experiments (n.d.: not determined).*

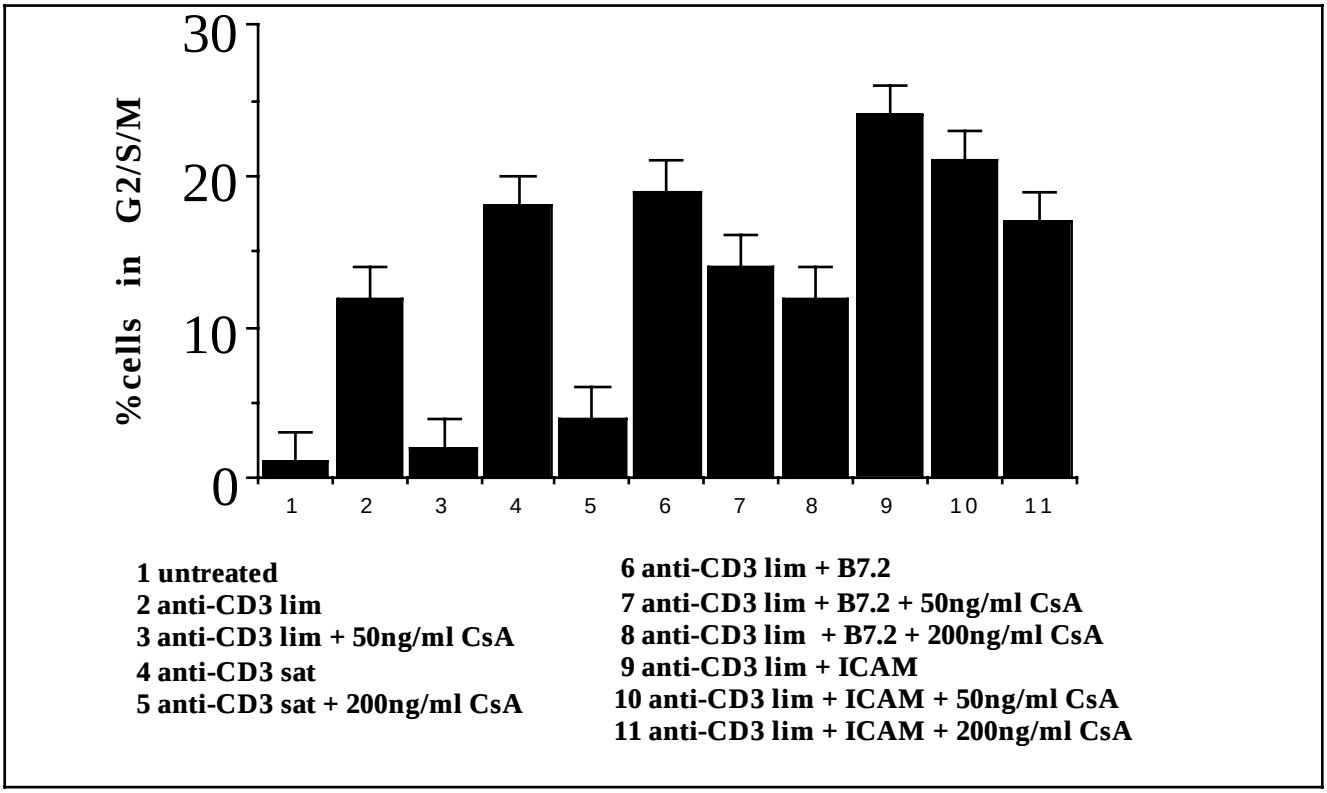
Condition:	w/o α-CD28	+ α-CD28	+ α-CD28 + IL-2
α-CD3 sol	1 +/- 1%	1 +/- 1%	2 +/- 1%
α-LFA-1 im	1 +/- 1%	1 +/- 1%	2 +/- 1%
α-CD3 im + α-LFA-1 sol	3 +/- 2%	19 +/- 5%	n.d.
α-CD3 im	15 +/- 7%	24 +/- 4%	n.d.
α-CD3 + ICAM im	36 +/- 10%	41 +/- 11%	n.d.

3.10 Coengagement of either CD28 or LFA-1 induces cyclosporin A-resistant proliferation

The immunosuppressant cyclosporin A (CsA) specifically inhibits T cell proliferation by blocking IL-2 transcription (Liu, 1993). Crosslinking of CD28 with antibodies in combination with either PMA or anti-CD3 mAbs can induce cyclosporin A(CsA)-resistant IL-2 production (Ghosh et al., 1996; Van Gool et al., 1993). However, how CsA-resistant T cell activation in a physiological situation is achieved is less clear. We therefore investigated the relative CsA-sensitivity of TCR-dependent proliferation in the absence or presence of costimulation provided by CD28 or LFA-1-coengagement with their recombinant ligands. As shown in Figure 3.10, limiting amounts of anti-CD3 mAbs induced some proliferation, which was nearly completely blocked by low doses (50ng/ml) of CsA. In contrast, costimulating cells with co-immobilized B7.2 or, even more efficiently, ICAM-1 rendered proliferation largely resistant to even intermediate doses (200ng/ml) of the drug. Since LFA-1 (but not CD28) can enhance TCR-triggering at low ligand densities ((Bachmann et al., 1997), see Figure 3.2b), we wondered if LFA-1-induced CsA-resistant growth could be simply due to enhanced TCR stimulation. But, as shown in Figure 3.11, the TCR-dependent proliferation remained even at saturating concentrations of anti-CD3 mAbs largely sensitive to CsA, showing that LFA-1-induced CsA-resistant growth can not simply be explained by enhanced TCR stimulation. The CsA-induced inhibition of proliferation could be nearly completely bypassed by adding recombinant IL-2 to the TCR-stimulated cells (not shown), confirming that CsA specifically inhibits IL-2 production and indicating that both CD28- and LFA-1-mediated CsA-resistant proliferation is due to CsA-resistant IL-2 production. Consistent with this, both pRb-inactivation and CD25 expression, which are both regulated by IL-2

(see 3.7/8,(Evans et al., 1992; Ullman et al., 1990)), closely correlated with the ability of CsA to block the TCR- and costimulation-induced proliferation, respectively (not shown).

Figure 3.10: Costimulation leads to CsA-resistant proliferation of TCR-stimulated cells. *Cells were pretreated with CsA and subsequently with limiting (5ng/10^6cells, "lim") or saturating (1µg/10^6cells, "sat") amounts of anti-CD3 mAbs as indicated. Cells were then plated on dishes which had been pretreated first with polyclonal goat anti-mouse Ig and rabbit anti-human Ig antibodies and subsequently with 500ng/ml protein A-ICAM-1 or B7.2-human IgG3 fusion proteins in RPMI, 1%FCS as indicated. Proliferation was assessed by propidium iodide staining and flow cytometry as described before. Values represent the mean of at least three independent experiments.*



These results show that both CD28- and LFA-1-mediated costimulation induce CsA-resistant T cell growth in TCR-stimulated cells. They suggest further that both CD28 and LFA-1 are able to transduce signals promoting IL-2 secretion which are qualitatively different from those triggered by the TCR.

3.11 The *in vitro* binding of transcription factors to the IL-2 promoter is not differentially affected by cyclosporin A in TCR- and co-stimulated cells.

IL-2 was shown to be necessary and sufficient for LFA-1-dependent cell cycle progression of TCR-stimulated cells (3.8) and CsA is believed to specifically block IL-2 transcription. CsA-resistant IL-2 production is therefore probably causing the CsA-resistant proliferation in costimulated cells. The expression of IL-2 is regulated both at the transcriptional and the post-transcriptional level. While CD28 costimulation was shown to enhance IL-2 production both at the transcriptional level and by m-RNA stabilization, the effects of LFA-1 on IL-2 remain to be determined. The transcriptional activation of the IL-2 gene is believed to depend on the DNA-binding of multiple transcription factors of the NF-AT, AP-1, NF-κB/Rel and OCT-families. CsA specifically inhibits IL-2 transcription by inhibiting the nuclear translocation of NF-AT (Lieu, 1993) and of NF-κB (c-Rel, p50 and RelA/p65, (Marienfeld et al., 1997)); CD28 costimulation of PMA-stimulated cells was shown to result in CsA-resistant nuclear translocation of NF-AT (Ghosh et al., 1996). CsA was reported to inhibit as well CD28-induced AP-1-dependent transcriptional activity. This inhibition was however not mediated at the level the DNA binding of AP-1, but appeared to depend on post-translational modifications (Rincon and Flavell, 1994). In order to address if costimulation-induced, CsA-resistant IL-2 production could be mediated at the transcriptional level, electrophoretic mobility shift assays visualizing the DNA binding of transcription factors to the IL-2 promoter were performed as described in 2.18. The four regulatory elements of the human IL-2 promoter chosen were shown to be the most important ones for the inducible IL-2 expression in TCR-stimulated primary human T cells (Hughes and Pober, 1996). They contain the consensus sites for AP-1/OCT, NF-AT, CD28-Response Element(CD28RE)/AP-1 and NF-κB, respectively. As shown in Figure 3.11, nuclear extracts of unstimulated cells contained specific binding activity only for the OCT/AP-1 fragment, detectable by a retardation of the labelled oligonucleotides in the gel electrophoresis. TCR-stimulated cells displayed additional binding activities specific for the NF-κB-, the CD28RE/AP-1- and for the NF-AT-site, while binding to the OCT/AP-1-site was not altered. The binding activities were sequence-specific since they were competed by an 100-fold excess of the unlabelled oligonucleotide, but not by an oligonucleotide with an unrelated sequence (not shown). Costimulating cells with immobilized anti-CD3 mAbs and ICAM-1 significantly enhanced the inducible binding activity to the NF-AT and to the CD28RE/AP-1 site, but not significantly those specific for the NF-κB- or to the OCT/AP-1-site. Preliminary results indicate that costimulating cells with B7.2 is less efficient in enhancing the binding activity to the NF-AT and the CD28RE/AP-1 site (not shown). Supershift studies with specific antibodies were performed in order to identify the inducible proteins binding to the three promoter fragments

(not shown): The slowest migrating complex binding to the NF-κB site was thus identified as a mixture (approximately 3:1) of p50/RelA and p50/c-Rel-heterodimers and the fastest one as the p50-homodimer. The constitutive binding activity migrating between the two was not supershifted by any of the three antibodies used. The slower migrating complex binding to the CD28RE/AP-1 site contained c-Jun, c-Fos, c-Rel and possibly other AP-1 family members. The faster migrating complex was identified as the RelA/p50(NF-κB)-heterodimer. The NF-AT-specific complexes were supershifted with anti-NF-ATc and to a lesser extend with anti-NF-ATp antibodies. CsA as expected inhibited completely the NF-AT-specific binding activity and reduced the binding of both CD28RE/AP-1- and NF-κB-specific complexes in TCR-stimulated cells. Surprisingly, no significant difference between the binding pattern of the IL-2 promoter fragments of TCR- and LFA-1- or CD28-costimulated cells in the presence of CsA was observed (Figure 3.11 and not shown, respectively), in spite of the fact that IL-2-dependent T cell proliferation under the aforementioned conditions showed dramatic differences (see 3.10). This is in contrast with a report suggesting that CsA-resistant IL-2 production may be due to CsA-resistant nuclear translocation of NF-AT (Ghosh et al., 1996) and indicates that costimulation-induced, CsA-resistant IL-2 production may not be mediated at the transcriptional level but may be due to enhanced IL-2 m-RNA stability.

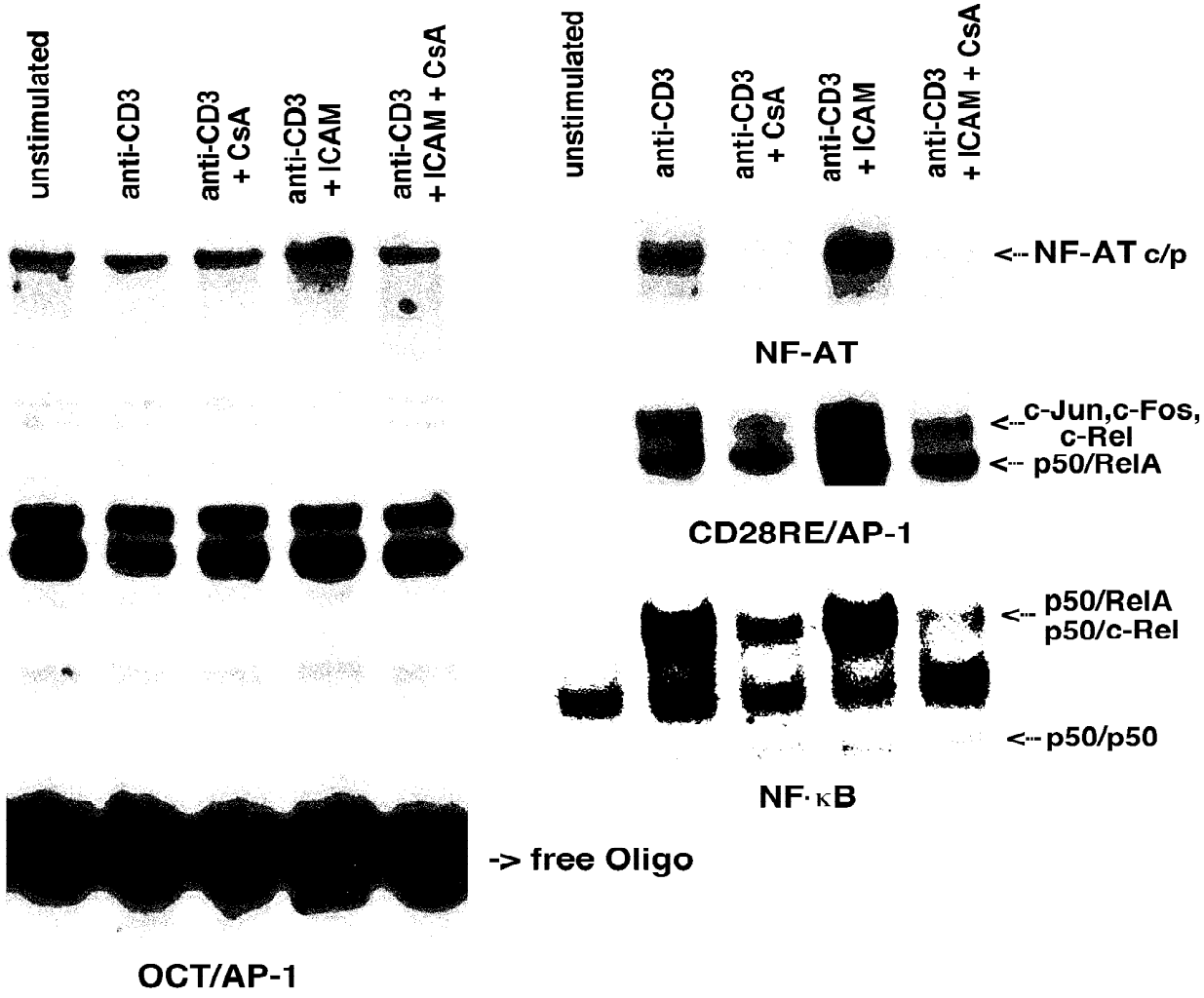


Figure 3.11: LFA-1 costimulation enhances the induction of factors specific for the CD28RE/AP-1- and for the NF-AT-site in a CsA-sensitive manner. *Cells were left untreated (Ctr) or stimulated for 8 hours with 50ng/10^6 cells anti-CD3 mAbs and plated on goat anti-mouse Ig-pretreated dishes in the absence or presence of co-immobilized ICAM-1 and/or 50ng/ml CsA as indicated. Nuclear extracts and electrophoretic mobility shift assays were performed as described in 2.18. Arrows indicate the major sequence-specific binding activities induced upon stimulation.*