4. DISCUSSION

T cell receptor (TCR) recognition of antigenic peptide bound to major histocompatibility complex (MHC) is critical for both T cell activation and development. However, efficient T cell activation requires a signal amplification, which is provided by ligand-binding of antigen-nonspecific costimulatory molecules on the T cell, i.e. induction of T cell proliferation as well as T cell apoptosis by engagement of the TCR/CD3 complex will be greatly enhanced by a second signal provided by a costimulatory molecule (Schwartz, 1992; Green and Thompson, 1994; Croft and Dubey, 1997). Recently it has been debated whether these costimulatory molecules exert their function by initiating signal transduction independently of the TCR/CD3 complex or whether they strengthen the TCR/CD3 complex initiated signaling cascade by stabilizing the TCR-MHC interaction and/or recruiting signal transducing molecules towards the side of TCR ligand interaction (Shaw and Dustin, 1997; W lfing and Davies, 1998, Viola et al., 1999).

It has been suggested and experimentally supported that CD44 is involved in lymphocyte activation by functioning as a costimulatory molecule (Shimizu et al., 1989; Denning et al., 1990; Conrad et al., 1992; Galluzzo et al., 1995). Yet, the pathway of signal transduction via CD44, like that of other costimulatory molecules (Dustin and Springer, 1989; Chambers and Allison; 1997; Zuckerman et al., 1998), remained elusive. There have been reports on the association of CD44 with the cytoskeleton, particularly via ankyrin and the ERM family (Bourguignon et al., 1992; Tsukita et al., 1994). It also has been described that a strong and prolonged Ca^{++} influx will be observed after cross-linking of CD44 (Bourguignon et al., 1993; Galandrini et al., 1994; Galluzzo et al., 1995) and that cross-linking of CD44 may be accompanied by phosphorylation of lck, fyn and ZAP-70 (Ilangumaran et al., 1998; Taher et al., 1996; Dianzani et al., 1999).

Here, I report on two phenomena which provided further support for costimulatory activity of CD44: i. cross-linking of CD44 supports T cell activation as well as T cell apoptosis and ii. IL-2 secretion after antigenic stimulation is significantly reduced in the presence of a competitive CD44-RG. Based on these observations, I started to explore the mechanisms underlying the costimulatory function of CD44. All experimental evidences strongly favor the interpretation that CD44 exerts its costimulatory function by enhancing/facilitating TCR/CD3 signaling without initiating a independent TCR/CD3-signaling cascade. The mechanism by which CD44 potentiates T cell activation most likely involves recruitment of src-family kinases to the TCR-complex, redistribution of signaling molecules into lipid rafts and/or actin cytoskeletal rearrangements.

4.1 Costimulatory function of CD44 in T cell proliferation and apoptosis

First to mention, this study provides evidence that CD44 can function as a costimulatory molecule under the physiological situation of an antigenic stimulation. This conclusion was drawn from the observation that T cell activation, as monitored by IL-2 secretion of the hemagglutinin specific Th line IP12-7, by peptide pulsed antigen presenting cells (APCs) can be inhibited by a CD44-receptor globulin (CD44-RG).

Further *in vitro* studies revealed that under identical experimental conditions cross-linking of CD44 synergistically increases and accelerates TCR-mediated proliferation of peripheral T cells as well as TCR-induced apoptosis of thymocytes and a Th line. CD44-induced proliferation was accompanied by an increased IL-2 production and a concomitant upregulation of CD25, the high avidity α chain of the IL-2 receptor. Furthermore, coligation of CD44 with CD3 on peripheral T lymphocytes resulted in an upregulation of the early T cell activation marker CD69, an TCR-triggered activation event, which is thought to involve the p21ras/ERK signaling pathway (D'Ambrosio et al., 1994). Depending on the differentiation state of the responding T cell, engagement of the TCR may not only induce cellular proliferation, but also apoptosis. Overlapping signal transduction pathways are involved in both types of responses (Winoto, 1997). Several studies have shown that one of the key events in activation induced cell death (AICD) is the expression and interaction of CD95/CD95L (Ju et al., 1995; Alderson et al., 1995). TCR-induced apoptosis, as characterized by PI-uptake, DNA-degradation and loss of membrane asymmetry, correlated with a functional upregulation of CD95 and CD95L, which could be greatly enhanced by costimulation via CD44.

The next hint on CD44 functioning as a costimulatory molecule in a spatially dependent manner was derived from the observation that cross-linking via the CD44s-specific mAb IM7 supported T cell proliferation and apoptosis, while KM81 did not. Both antibodies bind strongly to $CD44^+$ cells and recognize a comparable number of surface molecules. KM81 is known to block the hyaluronic acid (HA) binding site, while IM7 does not interfere with hyaluronan binding and does not compete with KM81 (Zheng et al., 1995). The observation, that the agonistic function of cross-linked CD44 depends on the engaged CD44 epitope is in line with a recent report investigating the requirements for signal delivery through CD44 by the use of CD44-Fas chimeric proteins (Ishiwatari-Hyasaka et al., 1999). This sudy describes that CD44-specific antibodies recognizing the HA binding site failed to generate signaling, while antibodies binding to other CD44 regions were capable to transduce signals.

Importantly, engagement of CD44 is only effective when supporting co-localization of CD44 with the TCR/CD3 complex, since mixtures of beads coated with either anti-CD3 or anti-CD44s did not induce, while beads coated with both antibodies supported T cell proliferation. T cell proliferation was hardly induced when plates were coated with anti-CD3 and anti-CD44 was added to the culture medium (data not shown). The observation that for CD44-mediated costimulation to work, CD44 must come into close proximity of the TCR/CD3 complex, suggests that CD44 does not transmit global signals into the cell, but rather acts to enhance/facilitate TCR/CD3-mediated signaling. Similar findings have been described for other important costimulatory molecules, such as CD28 and LFA-1, optimal effects being only achieved when the costimulatory molecule was colocalized with the TCR/CD3 complex (Berg and Ostergaard, 1995; Viola et al., 1999).

T cell proliferation in response to PMA plus ionophore, as well as apoptosis of thymocytes in response to dexamethasone were both independent of cross-linking of CD44. Stimulation with PMA and ionophore is considered to mimic physiologic T cell activation, but bypasses early TCR-mediated events (Crabtree, 1989). The glucocorticoid dexamethasone induces cell death by a mechanism distinct from that activated by the TCR/CD3 complex (Zacharchuk et al., 1990). Since both of these types of stimulation are independent of the engagement of the TCR/CD3 complex, these results provide additional evidence for a cooperative activity between CD44 and the TCR/CD3 complex.

Support for cross-linking of CD44 strengthening signal transduction via the TCR/CD3 complex also derived from the observation that cross-linking of CD44 was ineffective in the presence of a sufficiently high amount of anti-CD3 to induce T cell activation/apoptosis. Important to note is that engagement of CD44 in the context of cross-linking subthreshold amounts of CD3 increases TCR-mediated functions without modifying the outcome of the response which would be elicited by stronger TCR-triggering in the absence of CD44 costimulation. As could have been expected and also has been described for other costimulatory molecules (Nunes et al., 1996; Croft and Dubey, 1997; Punt et al., 1997), neither T cell proliferation nor T cell apoptosis could be induced by cross-linking only CD44 via immobilized antibody.

Thus, coligation of CD44 with the TCR/CD3 complex generally increases the susceptibility of cells for the induction of TCR-mediated cellular functions. The CD44-mediated costimulatory mechanism essentially requires that both, CD44 and the TCR/CD3 complex, are engaged simultaneously in a close proximity. Notably, the final outcome of the response is only dependent on the state of T cell maturation/activation, i.e. is not modified by cross-linking of CD44. All these properties are well compatible with the idea of CD44 functioning by facilitating TCR/CD3-stimulated signaling events.

4.2 Costimulatory function of CD44 on CD3-induced signal transduction

In order to determine the mechansims underlying the costimulatory function of CD44 its signaling ability was elucidated. Efficient T cell activation requires engagement of the TCR and of costimulatory molecules. However, the contribution of costimulatory molecules in the integration of TCR-mediated signals still remains elusive. Some data suggest that CD28, which has become the archetype for costimulatory molecules, transduces signals which integrate with those delivered through the TCR/CD3 complex to specifically activate a mitogen activated protein (MAP) kinase, the Jun-N-terminal kinase (JNK), whose activation is required for the induction of cytokine gene transcription (Su et al., 1994). However, this view has been questioned by recent findings demonstrating the involvement of CD28 in more proximal events following TCR engagement, such as activation of ZAP-70 (Tuosto and Acuto, 1998) and reorganization of membrane microdomains (Viola et al., 1999). Therefore in this work it was carefully analyzed what the specific signaling role of CD44 is and at which point the TCR and CD44 signals converge.

4.2.1 Costimulatory function of CD44 on CD3-induced ERK and JNK activation

The investigation of how engagement of CD44 affects MAP kinases revealed, that cross-linking of CD44 synergistically increases TCR/CD3-mediated activation of ERK1/2 as well as JNK activation. The observation that cross-linking of CD44 enhances TCR/CD3-induced ERK activation is in line with the costimulatory effect of CD44 on the suface expression of CD69, which is believed to be mediated via the p21ras/ERK signaling cascade (D'Ambrosio et al., 1994). In addition, the p21ras/ERK (Izquierdo Pastor et al., 1995) and the JNK (Su et al., 1994) signaling pathways are known to be essentially involved in the induction of activator protein 1 (AP-1) transcriptional activity. AP-1 in turn is one of the most critical transcription factors in the regulation of IL-2 gene expression (Jain et al., 1995). Consistent with the effect on IL-2 production, which is a hallmark of T cell activation, cross-linking of CD44 was only effective in the context of CD3 stimulation, i.e. engagement of CD44 alone was not sufficient to induce activation of these MAP kinases. Furthermore, as also observed for the secretion of IL-2, the costimulatory function of CD44 on ERK and c-jun phosphorylation was even obvious at high levels of CD3 stimulation. The observed differences in the CD44-induced response at low levels of CD3 stimulation indicate that the ERK signaling pathway is activated more quickly, while activation of the JNK pathway requires more sustained signaling. Since theoretical models predict that MAP kinase cascades are designed to respond in a all-or-none fashion to stimuli once a particular threshold is reached (Ferrell, 1996), the

threshold for JNK activation might be higher than the threshold for ERK activation. Previous studies on the costimulatory function of CD28 have described similar observations for CD28-mediated coinduction of JNK activity in human T cells (Franklin et al., 1994; Rincon and Flavell, 1994; Su et al., 1994). In these experiments full activation of JNK required costimulation of T cells with antibodies to the TCR and CD28. In contrast, the MAP kinases ERK1 and ERK2 were not affected by CD28 crosslinkage, but rather seemed to be controlled only by the TCR/CD3 module. These observations have been interpreted in terms of a "two-signal-model", in which the TCR/CD3 complex and costimulatory molecules induce independent signaling cascades which, at least in case of CD28, converge at the level of JNK to induce T cell activation. However despite intensive efforts, to date no convincing costimulatory signaling pathway has been identified. Rather signaling via costimulatory molecules generally requires coengagement of the TCR. This has led to the counterproposal that costimulators function mainly to enhance or modify TCR signaling and may not transduce a unique signal by themselves (Shaw and Dustin, 1997). In support of this model is the recent study by Tuosto et al. (1998) demonstrating that CD28 engagement by its natural ligand regulates the TCR/CD3 signaling ability from a very early, TCR proximal level and in consequence affects both the ERK1/2 and the JNK signaling pathway. As cross-linking of CD44 also costimulates ERK1/2, as well as JNK activation, this argues for CD44 affecting TCR/CD3 induced signaling from a more proximal point.

4.2.2 Costimulatory activity of CD44 acts on TCR-proximal signaling events

The initiation of a relatively well characterized protein tyrosine phosphorylation cascade is the earliest detectable biochemical event relaying the TCR/CD3 complex to downstream signaling pathways such as Ca^{++}/calcineurin and ras/raf-1/MAPK (Weiss and Littman, 1994; Wange and Samelson, 1996; Qian and Weiss, 1997). The analysis of early tyrosine phosphorylation events confirmed that the pathway of activation of signal transduction molecules by cross-linking of CD44 in the presence of suboptimal amounts of anti-CD3 largely resembled the one initiated by appropriate engagement of the TCR/CD3 complex. This accounted for CD44 facilitated T cell activation as well as apoptosis. Cross-linking of CD44 with agonistic antibodies synergized with and stabilized TCR/CD3 induction of tyrosine phosphorylation of numerous cellular proteins, including the ζ chain, ZAP-70, LAT, PLCγ and $p95^{vav}$. As the initial event that occurs after engagement of the TCR is phosphorylation of CD3 and ζ ITAMs and their association with ZAP-70, which then becomes tyrosine-phosphorylated and activated, the data provide evidence for a role of CD44 in enhancing the earliest stages of T cell activation.

Together with lck/fyn, activated ZAP-70 is thought to phosphorylate a number of downstream substrates, such as LAT, PLCγ and $p95^{vav}$ (Leeuwen and Samelson, 1999). This is entirely consistent with the experimental data presented here, demonstrating a dramatic increase in tyrosine phosphorylation of these cellular substrates by the coengagement of CD3 with CD44. Tyrosine phosphorylation of PLCγ and $p95^{vav}$ contributes to the activation of their intrinsic enzymatic activity (Secrist et al., 1991; Weiss et al., 1991; Crespo et al., 1997; Han et al., 1997). For PLCγ, this results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP2) to inositol-1,4,5-triphosphate (IP3) and diacylglycerol. IP3 generation induces a sustained increase in intracellular Ca^{++}, while diacylglycerol promotes the activation of protein kinase C (PKC). PKC may subsequently activate ras by suppressing the activity of a rasGTPase-activating protein (Downward et al., 1990). Vav tyrosine phosphorylation stimulates its guanine nucleotide exchange factor (GEF) activity towards the rho family of small GTPases (Crespo et al., 1997; Han et al., 1997) and thereby may be implicated in the activation of JNK (Olson et al., 1996).

Cross-linkage of CD44 in the context of CD3 stimulation not only enhanced early tyrosine phosphorylation events, but under the same experimental conditions also induced a mobility shift of lck. This activation induced shift in the mobility of lck has been used as an indicator of biologically TCR/CD3 receptors (Sancho et al., 1993) and likely reflects post-translational modification of lck by both PKC and MAP kinases (Soula et al., 1993; Watts et al., 1993; Winkler et al., 1993). Together with the results on ERK and JNK activation, this data clearly indicate that the requirements for CD44 affecting TCR proximal tyrosine phosphorylation events parallel the requirements for CD44-mediated costimulatory activity on the subsequent downstream signaling pathways.

The coengagement of CD44 with the TCR/CD3 complex resulted in a long lasting induction of tyrosine phosphorylation in T cells. Such a sustained signaling is required for MAP kinase activation and functional T cell activation (Valetutti et al., 1995; Berg et al., 1998). Previous evidence has shown that the cytoskeleton is necessary for T cell effector functions (O'Rourke et al., 1991) and is essentially required for sustained signaling (Valetutti et al., 1995). As previously described for the TCR signaling pathway, the CD44-mediated potentiating effects on tyrosine phosphorylation were totally abolished in the presence of cytochalasin B (data not shown). The cytoskeleton, either directly or indirectly, is likely needed for receptor clustering and the assembly of signaling molecules into multimeric complexes necessary for complex T cell functions. Recently it has been reported that upon TCR-triggering the ζ subunit of the TCR gets phosphorylated and associates with a Triton X-100 detergent-insoluble fraction (Rozdzial et al., 1995). The association between ζ and the insoluble fraction is broken by treatment of T cells with actin-disrupting drugs, cytochalasins D and B, suggesting that the ζ subunit might interact directly with the actin cytoskeleton. The present

study demonstrates that CD44 coengagement with the TCR/CD3 complex leads to a specific enhancement of cytoskeleton-associated tyrosine phosphorylated ζ. Since, actin polymerization and TCR ζ tyrosine phosphorylation and cytoskeletal association have been correlated with TCR activation of downstream functions (Rozdzial et al., 1995; Valetutti et al., 1995; Caplan et al., 1995), this observations further strengthens the idea of CD44 affecting the whole TCR-induced signaling cascade by interfering at the earliest levels of TCR-mediated signal transduction.

Another essential component of the early part of the TCR/CD3 signaling pathway is the recently cloned T cell protein LAT (Linker for Activation of T cells), a 36-38 kD integral membrane protein that after T cell activation is heavily phosphorylated on multiple tyrosine residues by ZAP-70/syc-family PTKs (Zhang et al., 1998). LAT functions as an adaptor protein which is essentially required for coupling the TCR to the PLCγ/calcineurin and the ras/MAPK pathway (Finco et al., 1998). As demonstrated here, cross-linking of CD44 strongly upregulates CD3-induced tyrosine phosphorylation of a 36-38 kD protein, most likely to represent LAT, in glycolipid-enriched microdomains (GEMs). Localization and phosphorylation of LAT in GEMs has been demonstrated to be required for T cell activation (Zhang et al., 1998; Lin et al., 1999). Therefore the data indicate, that the CD44-mediated upregulation of early TCR-induced protein tyrosine kinase activity is, at least in part, coupled to further downstream signaling pathways via the adaptor protein LAT involving lipid raft microdomains.

Taken together, the results on the induction of early tyrosine phosphorylation events strongly support a model in which CD44 does not signal independently, but rather cooperates with the TCR/CD3 complex during the earliest phases of the signaling cascade resulting in enhanced and sustained TCR-signaling. As a consequence, CD44 influences all subsequent TCR-initiated signaling pathways, such as Ca^{++}/calcineurin, p21ras/ERK1/2 and JNK1/2 cascades, which finally culminate in T cell activation. This model of CD44-mediated costimulation is consistent with the necessity of a spatial vicinity between CD44 and CD3 for costimulatory activity to work and also explains how cross-linking of CD44 can enhance such different cellular functions as TCR-mediated proliferation and TCR-induced apoptosis (Figure 39). Similar findings on costimulatory molecules affecting proximal TCR signal transduction events have been reported for the involvement of LFA-1 (Berg and Ostergaard, 1995), CD48 (Moran and Miceli, 1998) and CD28 (Tuosto and Acuto, 1998; Viola et al., 1999) in T cell activation, suggesting that the enhancement of early TCR/CD3 signaling might represent a general mechanism for the action of costimulatory molecules.

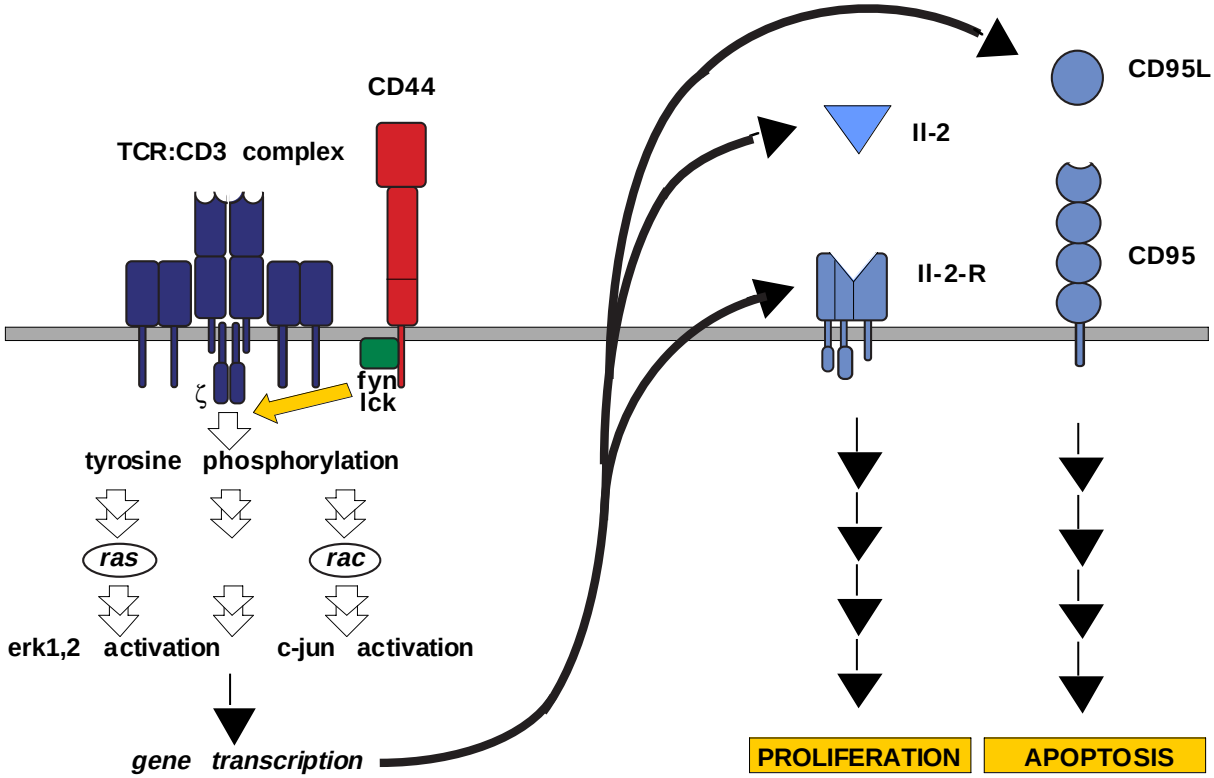

Figure 39

Model for the costimulatory function of CD44
CD44 supports TCR-mediatd proliferation, as well as TCR-induced apoptosis by enhancing signal transduction via the TCR/CD3 complex. The coligation of CD44 with the TCR/CD3 complex results in an increase of early tyrosine phosphorylation events and subsequent activation of downstream signaling pathways, such as the ras/ERK and the JNK/jun signaling cascades, probably resulting in gene activation. Depending on the state of T cell maturation/differentiation, this leads to the induction of different ligands (e.g. IL-2 or CD95L) and different receptors (e.g. IL-2R or CD95), which finally mediate cell proliferation or apoptosis.

4.3 Mechanism of CD44-mediated costimulatory activity

A central issue raised by the results of this study is through which mechanism CD44 strengthens signaling via the of TCR/CD3 complex. There are at least 4 possible explanations: i. CD44 functions as an adhesion molecule which allows for prolonged contact between the TCR/CD3 complex and its ligand; ii. cross-linking of CD44 is accompanied by reorganization of the cytoskeleton contributing to the organization of focal actin-scaffold stuctures required for efficient T cell activation; iii. cross-linking of CD44 supports its own reorganization towards the contact area of the TCR/CD3 complex, where it provides PTKs allowing for tyrosine phosphorylation of the ITAM motifs of CD3 and ζ-subunits; iv. cross-linking is accompanied by a reorganization of lipid microdomains and/or association with additional membrane receptors and their accumulation in the contact area between the TCR and its ligand.

4.3.1 CD44 functioning as an adhesion molecule

T cell activation occurs when the TCR is engaged by its ligand, antigenic peptide bound to a major histocompatibility complex (MHC) molecule on the surface of an APC (Babbitt et al., 1985). However, complex T cell functions, such as T cell proliferation, require sustained TCR engagement which faces many barriers. The TCR has a relatively low affinity towards antigenic MHC-peptide (Davis et al., 1998), the number of antigenic complexes on the APC can be very low (Wang et al., 1997) and the movement of T cells work against sustained recognition of antigen (Springer, 1994). In addition, for the initiation of the interaction between T cells and APCs, the cells must overcome their tendency to repeal each other owing to their net negative surface charge (Springer et al., 1987). The attractive force necessary to overcome this barrier is mediated largely by the action of adhesion molecules. Thus, it is not surprising that important costimulatory receptors on T cells, such as CD28, LFA-1 and CD2, have originally been regarded as adhesion molecules. Since CD44 is a well characterized cell adhesion molecule (Haynes et al., 1989; Lesely et al., 1993; Herrlich et al., 1993; Aruffo et al., 1990) it is possible that the initial costimulatory role of CD44 is to favor the adhesion between the T cell and the APC, thereby stabilizing the contact area allowing sufficient time for TCR recognition of appropriate antigen. CD44 could enhance cell-cell adhesion by directly binding to its ligand on the APC or, as previously reported, by potentiating integrin activity as observed after trigggering of CD44 on T cells (Koopman et al., 1990; Vermot-Desroches et al., 1995). A similar effect has been described for CD28, which upon cross-linking with antibodies enhances the activity of β1-integrins (Shimizu et al., 1992). The data presented

here do not support a passive adhesion model, because differences in the epitope binding region of anti-CD44 antibodies do not explain distinct efficiencies in costimulatory activity, if based simply on passive adhesiveness. The costimulatory function of CD44 in T cell activation rather relies on active processes, possibly involving a reorganization of the T cell cytoskeleton.

4.3.2 CD44 contributes to cytoskeletal rearrangements

Cross-linking of CD44 via immobilized IM7, but not KM81, induced profound morphological changes in T cells, as characterized by increased adhesion to the substrate, cell flattening and cell spreading. This behavior was demonstrated to be energy dependent and was specifically orchestrated by the T cell actin cytoskeleton, since it was accompanied by the formation of F-actin bundles and could be inhibited by actin cytoskeletal poisons, such as cytochalasin B. Furthermore, clustering of CD44 into receptor caps in IP12-7 cells induced the formation of cortical F-actin accumulations at the site of the CD44 caps. On a variety of different stages, the activity of T lymphocytes is associated with changes in cell morphology that depend on a functional cytoskeleton, e.g. lymphocytes migrate through blood and lymph vessels, transgress vessel walls and extracellular matrix spaces. It is known for a long time that CD44 facilitates homing of lymphocytes into lymph nodes by binding to high endothelial venules (Jalkanen et al., 1988; Berg et al., 1989; Miyake et al., 1990; Scheeren et al., 1991). CD44 also is required for the extravastion of activated T cells into infammatory sites (DeGrendele et al., 1997). Taking these features and the observations I reported, it becomes very likely that triggering of CD44 may contribute to cytoskeletal rearrangements and morphologic changes essentially required for these functions.

In addition, accumulating data support a role for the cytoskeleton as an integral component of T lymphocyte activation. A crosstalk between the CD44-induced cytostructural changes and costimulatory activity is suggested by the coordinated modulation of these two phenomena. Comparing the effects of different CD44-specific mAbs, a strict correlation between their ability to induce morphological changes and their costimulatory activity in T cell activation/apoptosis was observed. A functional relationship between the actin regulated cytoskeletal reorganization and receptor clustering for the induction of physiological T cell responses has first been suggested by studies with drugs that disrupt actin filament assembly, i.e. cytochalasins. Cytochalasins were shown to inhibit TCR capping and T cell shape changes required for sustaining and facilitating antigen-specific T cell/antigen presenting cell interactions and cytokine production (Geppert and Lipsky, 1990; Matsuyama et al., 1991; Rozdzial et al., 1995; Valetutti et al., 1995). Treatment of T cells with cytochalasin D

prevents Ca^{++} mobilization, IL-2 production, T cell proliferation, as well as peptide/MHC-mediated thymocyte apoptosis (Holsinger et al., 1998, Kong et al., 1998). In this context, it has been proposed that the specific reorganization of the actin cytoskeleton upon cell activation could either enable the formation of coordinated structures composed of receptors and signaling molecules in order to reduce the threshold required for full activation, or prolong essential activation signals, or both (Valetutti et al., 1995). Thus, CD44-induced cell spreading could strengthen cell-cell adhesion by broadening the cell contact, enhancing the number of TCR/CD3 complexes engagement and reducing bond strain on the more fragile TCR-MHC interactions. The CD44-mediated focal reorganization of the actin cytoskeleton could also favor the clustering of cell surface receptors, which is an essential step in T cell activation. Furthermore, since many signaling molecules are associated with cytoskeletal elements, CD44-mediated actin reorganization may provide cytoskeletal structure and scaffold geometries required to sustain TCR signaling and coordinate downstream signaling pathways. This concept is further supported by the observation that coengagement of CD44 with CD3 specifically targets tyrosine phosphorylated ζ to the actin cytoskeleton. Phosphorylation of the ITAM motifs of TCR ζ is followed by docking and activation of ZAP-70, building a complex which then can couple the signal to several downstream signaling cascades (Weiss and Littman, 1994; Wange and Samelson, 1996). In this respect, the actin cytoskeleton provides the three-dimensional scaffold structures which direct the formation of the multimolecular signaling complex required for effective activation of T lymphocytes. Interestingly, T cell activation with ionomycin and PMA, which bypasses receptor proximal signaling events, is not affected by cytochalasins (Holsinger et al., 1998). The idea of CD44 facilitating T cell activation by favoring cytoskeletal reorganization events is in line with this observation, since T cell proliferation induced by stimulation with PMA plus ionophore was independent of CD44 cross-linkage.

Recently it has been demonstrated that the forces driving receptor clustering require actin polymerization in the T cell but not in the APC (W lfing et al., 1998). Thus, it has been suggested that a signaling mechanism must first activate actin polymerization resulting in the accumulation of cortical actin at the contact area and assembly of associated signaling molecules (Penninger and Crabtree, 1999). However, many questions regarding the molecular mechanisms that connect the signal at the T cell plasma membrane to the assembly of actin filaments into an integrated well orchestrated response are still unanswered. The results of the present study demonstrate that CD44 is a potent candidate for mediating cytoskeletal reorganization processes in T cells. In further experiments, important signaling elements involved in CD44-induced morphological changes / reorganization of the actin cytoskeleton could be identified.

The use of specific pharmacological inhibitors revealed the requirement of src-family kinase activity for CD44-mediated cell spreading. Src-family tyrosine kinases have also been implicated in adhesive processes in other systems, such as integrin-mediated spreading and migration (Beggs et al., 1994; Thomas et al., 1995; Meng et al., 1998). Interestingly, CD44 is constitutively associated with the src-family kinases $p56^{lck}$ and $p59^{fyn}$ in T cells, suggesting that these kinases are directly involved in the very proximal CD44-mediated signaling events leading to cytoskeletal restructuring. PI3-kinase has been reported to influence actin dynamics following engagement of accessory receptors such as CD2 or CD28 (Shimizu et al., 1995; Woscholski et al., 1994). However, PI-3 kinase activity does not seem to be required for the morphological changes mediated via CD44.

Key regulators of the actin cytoskeleton also involve small GTPases of the rho subfamily (e.g. rho, rac1, and cdc42), which have been shown to control adhesion, morphology and motility of cells, and in addition are able to regulate signal transduction pathways in some systems (Hall, 1998; Mackay et al., 1998). Rho proteins act as molecular switches that when in the active GTP-bound state regulate diverse biological functions. Immunofluorescence studies revealed that rac1 colocalized with CD44-receptor caps. Moreover, triggering of CD44 via immobilized IM7, which induces cell spreading, specifically targeted rac1 to the leading edge of the cell. Expression of a dominant negative mutant form of rac1, but not of rho, completely inhibited CD44-mediated morphological changes. These findings provide clear evidence for a functional role of rac1 in CD44-induced actin polymerization and cytoskeletal rearrangements. In fibroblasts, rac can be activated by a distinct set of agonists (e.g. growth factors, phorbol ester), leading to the assembly of actin filaments at the cell periphery to produce lamellipodia and membrane ruffles (Ridley et al., 1992). Besides of its relatively well characterized role in mediating actin cytoskeletal changes in fibroblasts (Ridley et al., 1992; Nobes and Hall, 1995; Mackay et al., 1997), rac has recently also been implicated in integrin-mediated cell adhesion and cell spreading in T lymphocytes (D'Souza-Schorey et al., 1998; Price et al., 1998). Among the downstream targets of rac, phophatidylinositol 4-phosphate 5 (PI4P5)-kinase is likely to play a central role in mediating actin polymerization, since the product of PI4P5-kinase activity, phosphatidylinositol-4,5-bisphosphate (PIP2), is known to affect actin filament assembly (Hartwig et al., 1995). Another important target of rac is the serine-threonine kinase $p65^{PAK}$, which is supposed to interact with a molecular complex that controls actin polymerization (Hall, 1998). Several lines of evidence also implicate $p65^{PAK}$ in the activation of the JNK pathway (Manser et al., 1994; Bagrodia et al., 1995; Brown et al., 1996). By affecting JNK activation, rac could be directly involved in the signal transduction events that regulate T cell activation. However, other groups have failed to find a role for $p65^{PAK}$ in JNK activation (Teramoto et al., 1996; Tapon et al., 1998). In T cells, triggering of CD44 induces profound cytoskeletal rearrangements, which require activation of rac1, but triggering of CD44

alone does not result in c-jun phosphorylation. These data indicate that CD44-mediated rac1 activation is insufficient to induce JNK activity in T cells and argue against CD44 affecting T cell activation by directly activating a rac1/p65^{PAK}/JNK signaling pathway. A recent report has shown that ERM proteins are essential for rho- and rac-induced cytoskeletal effects (Mackay et al., 1997). Furthermore, ERM proteins are associated with CD44 and rhoGDI in BHK cells (Tsukita et al., 1994; Hirao et al., 1996). It has been proposed that ERM proteins behave as regulatable scaffold proteins that anchor actin filaments to cell surface receptors (e.g. CD44) and that this is an essential prerequisite for rho and rac to induce cytoskeletal changes (Hall, 1998). The absence of ezrin and rhoGDI in CD44-immunoprecipitates of T cell lysates (data not shown), however, did not confirm this hypothesis. In T cells, an important upstream activator of rac is p95^{vav}. This protein displays a phosphotyrosine-dependent guanine nucleotide exchange activity (GEF) for rho-family GTPases (Crespo et al., 1997). Vav phosphorylation appears to be mediated by src-family kinases, particularly by p59^{fyn} (Michel et al., 1998). Interestingly, vav deficient T cells exhibit defects in cap-formation, TCR-induced actin polymerization and association of TCR ζ with the actin cytoskeleton. Furthermore, in the absence of vav, TCR-mediated proliferation and IL-2 production, as well as antigen receptor-mediated thymocyte apoptosis are impaired (Fischer et al., 1998; Holsinger et al., 1998; Kong et al., 1998). It is tempting to speculate, that upon CD44-cross-linkage, CD44-associated fyn induces the activation of p95^{vav}, which in turn could activate rac1, leading to actin cytoskeletal changes necessary for T cell activation/apoptosis. As vav phosphorylation can be controlled by antigen-receptors and costimulatory molecules (Collins et al., 1997), this dual control might reflect an important regulatory mechanism in lymphocyte activation.

The CD44-induced morphological changes were not only inhibited by cytochalasin B and dominant negative mutant forms of rac, but also by treatment of cells with the microtubule-disrupting drug nocodazole. This observation implies, in addition to actin polymerization, a role for the microtubular system in the CD44-mediated cytoskeletal restructuring process. This is not surprising, since in many systems the cortical actin cytoskeleton and the microtubule cytoskeleton act in concert to induce morphological changes. The data are particularly consistent with a recent report describing a functional cooperation between microtubule growth, the activation of rac1 and actin polymerization (Waterman-Storer et al., 1999).

Taken together, the presented data demonstrate that cross-linking of CD44 on T cells can induce a rearrangement of the cytoskeleton which is correlated with dramatic alterations of cellular morphology and sustained signaling through TCR/CD3 engagement. The CD44-induced cytoskeletal changes are regulated by the actin cytoskeleton and also involve the microtubular system. The signal transduction molecules which are required to couple CD44 receptor triggering to the coordinated reorganization of the cytoskeleton include src-family kinases and rac1, a small GTPase of the rho-subfamily. The results suggest, that by affecting cytokeletal restructuring, CD44 could participate in T cell stimulation at least in two ways. First, cytoskeleton-dependent changes in cell shape could enhance contact formation between the T cell and the APC and increase the contact area with the APC, thereby enhancing the number of TCR complexes engaged. Second, through cytoskeleton mobilization, CD44 could recruit surface receptors and/or signaling molecules to the contact zone, resulting in a stabilization of the activation process.

4.3.3 Recruitment of src-family protein tyrosine kinases by CD44

Another possibility, which may be considered in connection to cytoskeletal rearrangements, is that CD44 potentiates T cell activation by supplying the TCR signaling complex with the addition of protein tyrosine kinases, which could significantly lower the threshold for initiation of signal transduction via the TCR/CD3 complex. The present study provides evidence that in murine T cells CD44 is constitutively associated with the src-kinase family members fyn and lck. This observation is in accordance with recent reports that CD44 forms a physical complex with lck and fyn in human cells (Taher et al., 1996; Ilangumaran et al., 1998). In addition to src-kinases, CD44 was also found to constitutively associate with an unidentified protein of about 32 kD. At the present time the function of this protein and its possible invovement in the activity of CD44 are completely unknown.

The initial TCR signals are mediated by lck and fyn, since these kinases phosphorylate the multiple ITAM motifs of the TCR complex. Upon phosphorylation of the ITAMs, ZAP-70 is recruited to the TCR and gets phosphorylated. Furthermore, lck and fyn have an additional, kinase independent role as adaptor proteins (Xu and Littman, 1993), which might be essential for the assembly of a transduction-competent signaling complex at the activated TCR. This signaling complex can then propagate the signal by phosphorylating adaptors, such as LAT and SLP-76, that couple it to several downstream signaling pathways (Weiss and Littman, 1994; Wange and Samelson, 1996; Qian and Weiss, 1997). The proximal TCR signaling events are significantly enhanced by MHC-induced coengagement of TCR with surface CD4/CD8 coreceptor molecules, since such coengagement serves to juxtapose TCR complexes with

coreceptor-associated lck molecules and by this means efficiently promotes phosphorylation of TCR ITAMs (Barber et al., 1989, Veillette et al., 1989) and activation of TCR-associated ZAP-70 molecules (Wiest et al., 1996). It has been suggested that the costimulatory molecules CD2 and CD28, which are associated with src-family kinase activity, may also favor the accumulation and activation of protein tyrosine kinases at the cell-cell contacts (Davis and van der Merwe, 1996; Holdorf et al., 1999). Since CD44 was found to be associated with the src-family protein kinases lck and fyn, it may be possible that following TCR-engagement, CD44 and the associated protein tyrosine kinases are recruited to the TCR/CD3-complex enhancing ITAM phosphorylation and ZAP-70 activation, thereby facilitating T cell activation. Recently, Zoltan Rozsnyay of our laboratory could identify an amino acid binding motif of CD44 responsible for the association with src-kinases (Rozsnyay, 1999). This sequence motif of the receptor is located at the internal face of the plasma membrane. The binding motif contains a critical cysteine residue in the putative transmembrane domain and three positively charged (arginine) residues in the putative cytoplasmic domain, which direct the selective interaction between CD44 and src-family kinases. Remarkably, similar sequences were found within a number of other molecules, including the coreceptors CD4 and CD8, as well as the accessory molecules CD2 and CD28. As already mentioned, the coactivating function of these receptors has been proposed to be, at least partly, due to the contribution of these molecules to the signal generation by recruiting src-family kinases to the TCR/CD3 complex. This kind of action of course would require coclustering of these surface receptors with the TCR in order to physically juxtapose lck and/or fyn with TCR/CD3 signaling complexes. For CD44, an association with CD4 and CD3 on the T cell surface has been reported, and this interplay seems to have functional consequences for T cell activation, probably involving signaling through $p56^{lck}$ (Dianzani et al., 1999). The findings further support the idea of CD44 potentiating T cell activation by increasing the actual amount of src-family kinases at the TCR/CD3 complex.

4.3.4 Costimulatory function of CD44 by reorganization of membrane microdomains

It has been suggested that cellular stimulation via CD44 may proceed through the signaling machinery of GEMs, since CD44 has been found to selectively associate with active src-family protein tyrosine kinases in glycolipid-enriched membrane microdomains (GEMs) (Ilangumaran et al., 1998). Consistent with this observation, the detection of CD44-fyn complexes was significantly increased by solubilization of cells with the combination of 0.2 % saponin and 1 % Triton X-100, which solubulizes GEMs far better than Triton X-100 alone. Moreover, the present study extends previous findings by demonstrating that cross-linking of

CD44 specifically redistributes CD44 and src-family kinases into these lipid rafts. Recent data suggest, that costimulation modulates the signaling environment around the engaged TCR by affecting the distribution and organization of membrane microdomains (W lfing and Davis, 1998; Moran and Miceli, 1998; Viola et al., 1999). Therefore it is reasonable to speculate that the costimulatory function of CD44 might involve CD44-driven membrane compartmentation processes. GEMs are more and more emerging as platforms that function in signal transduction and actin cytoskeleton-driven membrane trafficking (Simons and Ikonen, 1997). They are enriched in signal transduction molecules (e.g. src-family kinases, monomeric and heterotrimeric G proteins, LAT, and phosphoinositides), actin, and actin-binding proteins (Lisanti et al., 1994; Harder et al., 1997). A role for lipid rafts in signal transduction involves receptor-induced recruitment and activation of PTKs, MAP kinase cascades, or generation of second messengers (Harder and Simons, 1997). The formation of GEM-dependent, higher order signaling complexes has also been demonstrated to play an essential role in T cell activation (Xavier et al., 1998; Montixi et al., 1998; Zhang et al., 1998). By specifically targeting the src-family kinases fyn and lck into lipid enriched rafts, CD44 could contribute to the assembly of such signaling complexes, thereby facilitating T cell stimulation. In such a model, CD44 would modify the composition of lipid rafts, which serve as signaling platforms for engaged TCRs. This localized accumulation of src-kinases may then facilitate TCR-induced tyrosine phosphorylation and subsequent activation of further downstream signaling cascades. It is important to note, that cross-linkage of CD44 did result in the specific localization of CD44 and src-kinases into lipid rafts, but apparently did not alter the activation/phosphorylation state of these kinases, i.e. triggering of CD44 only regulates the subcellular distribution of fyn and lck. This is in line with the observation that triggering of CD44 alone did not significantly induce tyrosine phosphorylation of cellular substrates, whereas coengagement of CD44 with the TCR/CD3 complex resulted in a strong increase in tyrosine phosphorylation of numerous proteins, including phosphorylation of the GEM-associated adaptor protein LAT. The precise mechanism which drives CD44 and src-kinases into the GEMs upon receptor engagement remains unknown at present, but it might involve cytoskeletal rearrangements. The hypothesis is supported by a recent report, which describes that in epithelial cells CD44 interacts with annexin II in lipid rafts and that these CD44-containing lipid microdomains are functionally coupled to the underlying actin cytoskeleton (Oliferenko et al., 1999). A link between raft-mediated signaling and the cytoskeleton has also been suggested by Harder and Simons (1999), who describe a tyrosine phosphorylation dependent accumulation of F-actin in lipid raft patches on T cells. This is strikingly similar to the CD44-mediated cytoskeletal rearrangements. Moreover, recent publications suggest that the coupling of rafts to the cytoskeleton may be part of a complex orchestration of membrane components in T cell activation. W lfing and Davis (1998) demonstrated that in T cells

costimulation by either CD28 or LFA-1 initiates an actin-myosin driven directional transport of protein and lipid domains to the T cell:APC contact zone, which correlates with enhanced signaling. Coengagement of CD28 with the TCR/CD3 complex essentially recruits all the GEMs to the contact area, leading to higher and more stable tyrosine phosphorylation of cellular substrates (Viola et al., 1999). Furthermore, similar to the effects seen after cross-linkage of CD44, engagement of CD48, a glycophosphatidylinositol-linked molecule, enhances TCR-mediated functions and increases cytoskeletal attachment of the TCR ζ-chain. This process has been demonstrated to involve lipid microdomains (Moran and Miceli, 1998). Thus, it can well be suggested that CD44-induced raft reorganizations and CD44-induced cytoskeletal rearrangements are interconnected and synergize to enhance TCR-signaling.
With respect to the hypothesis of CD44 affecting T cell activation by reorganizing lipid microdomains, it should also be pointed out that CD44 has been described to associate with integrins (Verfaillie et al., 1994) and tetraspanin molecules (Jones et al., 1996). Particularly the latter are known to function as molecular facilitators by co-clustering in specialized membrane domains with a multitude of membrane molecules (Maecker et al., 1997). Thus, signaling via the TCR could well be supported by additional membrane molecules co-trapped and concentrated via CD44 in the contact area.

Taken together, the rearrangement of membrane microdomains might represent a general mechanism by which costimulation can enhance T cell signal transduction, probably resulting from an increased recruitment of kinases and other signaling components. The costimulatory activity of CD44 may as well be attributed to a CD44-induced reorganization of lipid microdomains in T cells, probably involving a redistribution of src-family kinases and/or other signaling molecules into lipid rafts. This process of membrane compartmentation, which may be closely associated with CD44-mediated cytoskeletal rearrangements, may subsequently lead to an increase in TCR-induced tyrosine phosphorylation and activation of downstream signaling pathways, thereby affecting cellular activation.