

## Invited Review

# Differential signaling through the T cell receptor: from biochemistry to transplantation tolerance

J. Madrenas and A.I. Lazarovits

The John P. Robarts Research Institute, and the Departments of Microbiology and Immunology and Medicine, The University of Western Ontario, London, Ontario, Canada

**Summary.** Recent advances in our understanding of the structural nature of T cell activation and signal transduction from the T cell receptor for antigen make possible the development of new tolerogenic strategies. Here, we summarize the evidence supporting a critical role for the co-receptor molecule (CD4 or CD8) and CD45 in determining the pattern of T cell receptor-mediated signaling. The consequences of this differential signaling can range from T cell proliferation and cytokine production to the establishment of a state of proliferative unresponsiveness known as T cell anergy. Inducing T cell anergy can be an alternative approach for the establishment of transplantation tolerance.

**Key words:** Signal transduction, Transplantation, T cell tolerance

### Introduction

T lymphocytes are critical regulatory and effector cells of the adaptive immune response. Their function becomes apparent upon clone-specific activation following engagement of their surface receptor for antigen (TCR) with its natural ligand, a transmembrane complex formed by an immunogenic peptide bound to the groove of a major histocompatibility complex (MHC) molecule on the surface of an antigen-presenting cell (APC). Full T cell activation results in activation of transcription of multiple genes, including the interleukin-2 (IL-2) gene. This will result in IL-2 production which will ultimately lead to proliferation and differentiation of the T cells into effector cells.

The signals transduced upon specific interaction between the TCR and its ligand, grouped as 'signal 1', are not sufficient for full T cell activation. Additional signals, known altogether as costimulation or 'signal 2', are required and delivered by different receptors on the surface of the T cell upon engagement with their ligands

(e.g. CD28-CD80, CD28-CD86, adhesion molecule-ligand interactions or cytokine-cytokine receptor interactions) (Schwartz, 1990). This two-signal paradigm for T cell activation has been very useful in providing a framework to understand T cell activation and to dissect the functional contribution of recognition and costimulatory signals.

A prediction of the two-signal model is that either one of the two signals alone will not induce T cell proliferation and differentiation, and this is indeed the case. However, a remarkable observation made during studies on the requirements for T cell activation was that TCR engagement is not ignored by the T cell in the absence of costimulation but results in a state of proliferative unresponsiveness to subsequent T cell in the absence of costimulation, rechallenge with antigen in the presence of costimulation (Jenkins and Schwartz, 1987). This state was termed T cell anergy, by analogy to the unresponsive state described on B cells (Nossal, 1993, 1996). The operational definition of T cell anergy initially emphasized the absence of costimulation as the crucial event leading to T cell unresponsiveness. However, recent evidence has expanded this definition by demonstrating that T cell anergy may be the result of TCR engagement that fails to induce IL-2 production, even when costimulation is available (Schwartz, 1996).

The significance of T cell anergy in immunobiology has been a controversial issue. Although the *in vitro* induction of T cell anergy is a well established phenomenon, some view it as a restricted *in vitro* phenomenon devoid of any biological relevance. This may in part be due to difficulties in demonstrating its involvement in the *in vivo* maintenance of peripheral tolerance. For example, it is not yet established *in vivo* that T cells become anergic upon recognizing antigen presented by non-professional APC, which resembles the original model of recognition in the absence of costimulation. However, two recent developments have renewed interest on T cell anergy. First, better understanding in signal transduction mechanisms has resulted in better biochemical characterization of the state of T cell anergy (reviewed in (Quill, 1996; Schwartz, 1996)). Thus, it is now feasible to analyze signal transduction in

primary T cells from different *in vivo* models of tolerance or disease and re-examine the involvement of anergy as a mechanism. Second, the demonstration that the TCR complex can signal in different patterns, some of these resulting in the induction of T cell anergy, has revived the interest for *in vivo* conditions in which this may occur and for strategies that could result in the induction of T cell anergy (Sloan-Lancaster and Allen, 1995; Madrenas and Germain, 1996).

In this paper, we will review the evidence implying that the TCR is a versatile signaling complex. As such, the TCR is capable of transducing in a variety of signaling patterns that will result in the development of different effector T cell responses, ranging from T cell pro-liferation and cytokine production to T cell anergy. We will then discuss the current information on the structural basis for this versatility that translates into differential signaling from the TCR. In particular, we will focus on the role that the co-receptor molecule (CD4 or CD8), and CD45 may have in differential signaling from the TCR. Finally, we will examine how this information may impact on the development of new therapeutic strategies for the induction of anergy-based T cell unresponsiveness.

### T cell activation through the TCR

The TCR is a molecular complex formed by a clonotypic  $\alpha\beta$  or  $\gamma\delta$  heterodimer non-covalently associated to four non-polymorphic CD3 chains (two CD3  $\epsilon$ , one CD3  $\gamma$ , and one CD3  $\delta$ ), and two TCR- $\zeta$  chains or its variant  $\eta$  chain. The  $\alpha\beta$  or  $\gamma\delta$  heterodimer is responsible for antigen recognition while the CD3-TCR- $\zeta$  chains are responsible for signal transduction. The fine stoichiometry of the TCR complex in basal conditions is not known. Recent crystallographic data on a TCR engaging a peptide:MHC molecule indicates that this interaction involves multiple regions of the membrane-distal domains of the TCR and requires an appropriate orientation of TCR engagement as well as congruency between the receptor and its ligand (Garboczi et al., 1996; Garcia et al., 1996a). Kinetic data using soluble TCR and its peptide:MHC molecule ligand indicate that the affinity of this interaction is low, with a fast dissociation rate (Matsui et al., 1991, 1994; Weber et al., 1992; Corr et al., 1994; Alam et al., 1996; Lyons et al., 1996).

The earliest detectable event upon T cell activation from recognition of specific peptide:MHC molecule complexes is tyrosine phosphorylation at Yxx(L/I)x(6-8)Yxx(L/I) motifs, known as immune receptor tyrosine-based activation motifs (ITAMs) (reviewed in (Wange and Samelson, 1996)). These motifs are located in the cytoplasmic tails of the TCR  $\zeta$  chains (3 in each chain) and CD3  $\gamma$ ,  $\delta$ , and  $\epsilon$  chains (1 in each chain). Phosphorylation of the tyrosines in these motifs is the primary function of two *src*-related protein tyrosine kinases (PTK): p56<sup>lck</sup> and p59<sup>fyn</sup>. These kinases have distinct ways to associate with the activating complex:

most lck is primarily associated to the C terminus of the co-receptor (CD4 or CD8) molecules (Veillette et al., 1988), while fyn can associate directly to the TCR (Samelson et al., 1990). The specific contribution of each one of these *src*-kinases in TCR-mediated signaling in developing and mature T cells is still controversial. During T cell development both *src*-kinases are essential (Groves et al., 1996; van Oers et al., 1996), although the defect caused by deletion of the lck gene is more intense than the corresponding one from fyn knock out mice (Appleby et al., 1992; Molina et al., 1992). In mature  $\alpha\beta$  T cells, lck seems to play an essential role although fyn may be required in a subset of mature T cells. Through the action of either one or both of them, the tyrosine residues on the ITAMs are phosphorylated and serve as a recruitment signal for cytosolic PTKs.

Tyrosine phosphorylation of ITAMs is representative of a general mechanism for the recruitment of signaling molecules. These molecules often contain conserved domains that function to colocalize other molecules (usually kinases) with their substrates or with adapters that will bind to the substrates (Pawson, 1995). The following types of domains have been so far identified: SH2, PTB, SH3, PH, WW, PDZ, and LIM. These domains are involved in the recognition of tyrosine phosphorylated motifs (SH2, PTB, and LIM), of proline-rich regions (SH3 and WW), interaction with phospholipids (PH), and valine-C terminus motifs (PDZ). This modularity in the signaling molecules increases their versatility, and contributes to the specificity of the signaling cascades in which they may be involved.

One of the early tyrosine kinases recruited to the TCR by tyrosine phosphorylation of the TCR subunits is ZAP-70. This PTK plays a critical role in TCR-mediated signaling as demonstrated by the severe defect in T cell activation observed in ZAP-70 knock out mice and in patients homozygous for mutations of the ZAP-70 gene (Chan et al., 1992, 1994; Arpaia et al., 1994; Elder et al., 1994; Negishi et al., 1995). Upon recruitment of ZAP-70 to the TCR, this PTK is tyrosine phosphorylated and activated. Only two substrates of ZAP-70 have been clearly identified so far: SLP-76, and perhaps HS1 (reviewed in Wange and Samelson, 1996). An alternative group of PTKs that may be involved in TCR-mediated signaling is the *Itk*/*Tec* group, as indicated by the severe deficit in T cell development and function in *Itk* knock out mice (Liao and Littman, 1995).

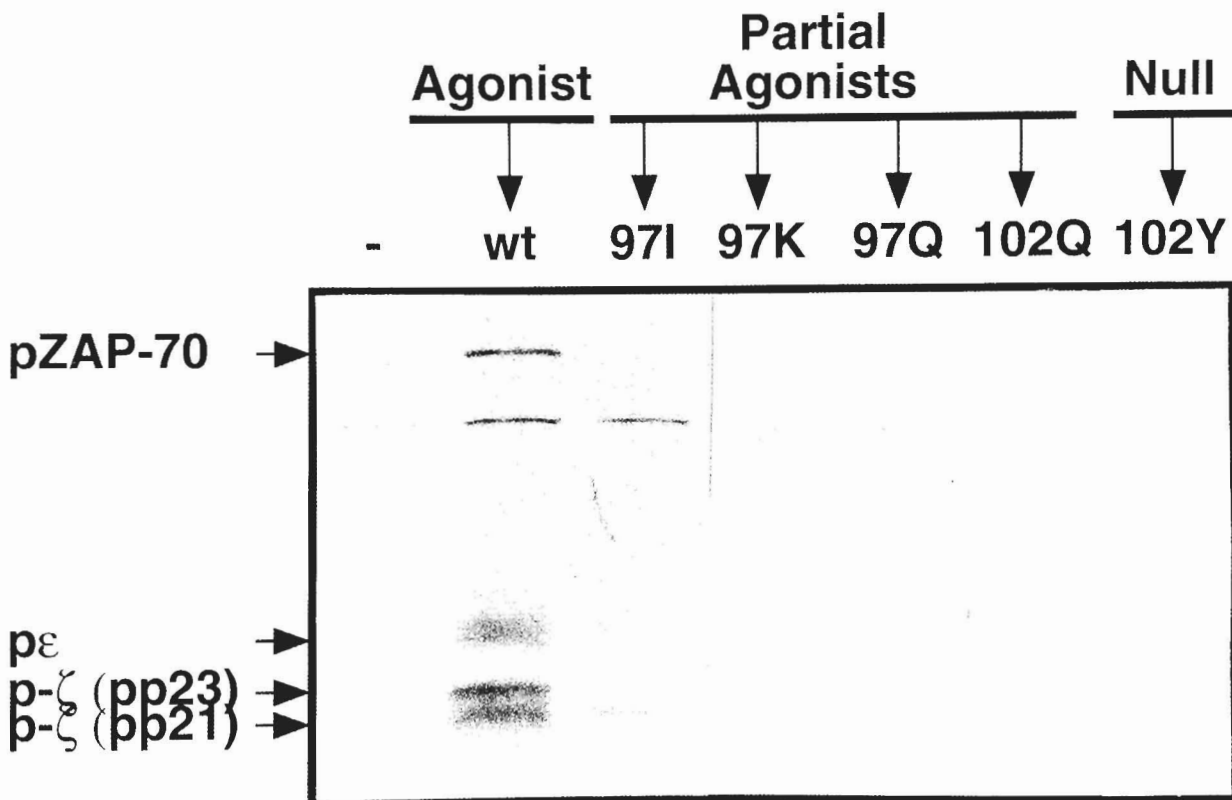
As a result of activation of these kinases, there is triggering of a cascade of downstream events involving at least three different signal transduction pathways. One pathway involves the activation of phospholipase C  $\gamma$  1 and subsequent hydrolysis of inositol phosphates leading to the generation of IP<sub>3</sub> and DAG. IP<sub>3</sub> will in turn induce Ca<sup>++</sup> influx and DAG will activate protein kinase C. Another is the p21 ras-MAPK-MAPKK pathway that will lead to the translocation of kinases to the nucleus where they will ultimately phosphorylate and activate transcription factors. The third pathway, partially characterized, includes the activation of PI3 kinase.

What activates the *src*-related PTKs? Structural characterization of the *src* family PTKs has shown the presence of a tyrosine residue (505) in their C terminus that plays a negative regulatory role on the kinase activity of the PTK when phosphorylated. From a functional point of view, activation of the kinase function requires dephosphorylation of such tyrosine residues, as indicated by constitutive activation of *lck* when this tyrosine residue is mutated into phenylalanine (reviewed in Peri and Veillette, 1994; Zenner et al., 1995). Thus, activation of the *src*-family of PTKs requires the activity of a phosphatase. An attractive candidate for this role is CD45.

CD45 is a transmembrane tyrosine phosphatase expressed by leukocytes (Trowbridge and Thomas, 1994; Okumura and Thomas, 1995). The ligand(s) of CD45 on the APC is still an enigma. A group has reported that CD45 can interact with the B cell specific receptor CD22 (Stamenkovic et al., 1991). The heavy glycosylation of the N terminus of CD45 also suggests that this phosphatase can interact with many molecules through carbohydrate-mediated contact. At least four different isoforms have been described on T cells, as a result of alternative splicing of the RNA coding for the CD45 A, B, or C exons. These isoforms differ in their N terminus and are known as CD45RO, CD45RA,

CD45RB, and CD45RC. The distinct functions of these different isoforms are currently unknown. However, it is possible that these different isoforms may have different abilities to transduce signals. As mentioned above, CD45 is able to dephosphorylate the *src*-family *lck* and *fyn* kinases on the negative regulatory 505 tyrosine. The CD45 molecule contains two phosphatase domains in the intracellular region of the molecule. The phosphatase activity seems to be concentrated though on the first of these domains. In the absence of CD45 expression, T cells lose the ability to become activated through the TCR. The *in vivo* relevance of this finding is shown by the severe defect in thymocyte development seen in mice unable to express CD45.

CD45 phosphatase activity can be increased by phosphorylation on tyrosine and serine residues located on the second phosphatase domain of CD45. Interestingly, the  $p50^{csk}$  tyrosine kinase, that negatively regulates TCR-mediated signaling (Chow et al., 1993) probably by tyrosine phosphorylation of  $p56^{lck}$  (Bergman et al., 1992), also phosphorylates CD45 causing an increase in its phosphatase activity (Mustelin, 1994). In addition, CD7 also phosphorylates CD45 on tyrosine residues (Lazarovits et al., 1994). This is just a good example of regulation of TCR-mediated signaling by phosphatases, a whole new area starting to be



**Fig. 1.** Distinct patterns of TCR-mediated signaling upon TCR engagement with agonist or partial agonist ligands. T cells were stimulated with peptide and antigen presenting cells for 10 minutes. Then, T cells were harvested and lysed. Cell lysates underwent immunoprecipitation of CD3- $\epsilon$  with monoclonal antibodies and were immunoblotted with a monoclonal antibody against phosphotyrosine.

unraveled (Marengere et al., 1996; Pani et al., 1996; Plas et al., 1996).

### **Evidence for differential signaling from the TCR**

Until recently, the TCR was considered a transducing complex with an all-or-none function depending on occupancy of the receptor above an affinity threshold. According to this simple model, signals transduced by the TCR were qualitatively identical and could only be quantitatively regulated by manipulation of the level of occupancy of the receptor. Several lines of evidence have recently challenged this view and have provided strong support to the concept that the receptor is capable of sensing fine differences in the quality of the ligand that will result in differential signaling patterns.

The first piece of evidence came from observations of split function or inhibitory effect upon engagement of the TCR with variant ligands (reviewed in Evavold et al., 1993; Germain et al., 1995; Jameson and Bevan, 1995). These variant TCR ligands were obtained after slight modifications of the peptide or the MHC molecule at the secondary TCR contact residues. Depending on the system, CD4<sup>+</sup> or CD8<sup>+</sup> T cells lost the ability to proliferate but still up-regulated some cell surface receptors and were able to produce some cytokines but not others, or lost some effector function such as cytotoxicity. In addition, the interaction between a variant TCR ligand and the TCR can induce activation of the APC and subsequent activation of some functions of the T cell (Matsuoka et al., 1996). Using the pharmacological nomenclature for different types of receptor ligands, these altered peptide:MHC ligands have been classified as partial agonists or antagonists depending on whether they induce only some functions but not others or they are capable of inhibiting activation by agonist ligands.

A critical observation made during these studies was that some partial agonist ligands were capable of inducing T cell anergy in conditions in which costimulation was not limiting (Sloan-Lancaster et al., 1993). The cellular characteristics of T cell anergy induced by variant TCR ligands are indistinguishable from T cell anergy induced by TCR engagement in the absence of costimulation: it is cyclosporin-sensitive, implying a Ca<sup>++</sup>-dependent pathway, and can be blocked by the induction of T cell proliferation with exogenous IL-2 (Sloan-Lancaster et al., 1993; Madrenas et al., 1996).

The plethora of biological effects of variant TCR ligands was soon followed by biochemical evidence of differential signaling induced by these ligands. As expected, most changes occur during the early stages of TCR-mediated signaling at the level of tyrosine phosphorylation of TCR subunits. Different signaling patterns are observed when tyrosine phosphorylation of TCR subunits is examined after engagement of the TCR with agonist or partial agonist/antagonist ligands (Fig. 1) (Sloan-Lancaster et al., 1994; Madrenas et al., 1995).

Agonists of the TCR induce the appearance of two forms of tyrosine phosphorylated TCR  $\zeta$  chain, and tyrosine phosphorylation of CD3  $\epsilon$ . In contrast, TCR partial agonists and antagonists predominantly induce the appearance of the p21 form of phospho-TCR  $\zeta$ , with much less or no phospho-p23 TCR  $\zeta$  and very little or no phospho- $\epsilon$ . This differential pattern of tyrosine phosphorylation of TCR subunits translates into differential downstream activation. Specifically, TCR agonists induce recruitment, tyrosine phosphorylation, and activation of ZAP-70. In contrast, TCR partial agonists and antagonists induce the recruitment of ZAP-70 to the TCR complex but fail to induce tyrosine phosphorylation and activation of this kinase.

The differential downstream effects induced by partial agonists of the TCR is consistent with the activation of some signaling pathways but not others. Fine characterization of these pathways is elusive. Variant TCR ligands can induce acid release (a measure of cell activation through different pathways), cytosolic Ca<sup>++</sup> fluxes, and signaling through the calcineurin-dependent pathway as indicated by the sensitivity to cyclosporin of their ability to induce T cell anergy (Sloan-Lancaster et al., 1993, 1996; Rabinowitz et al., 1996a,b). However, each of these responses have some distinctive features. For example, the levels of cytosolic Ca<sup>++</sup> responses induced by TCR partial agonists are lower and shorter than those seen with agonist ligands (Sloan-Lancaster et al., 1996). In addition, it has not been possible to demonstrate increases in the generation of IP<sub>3</sub> by variant TCR ligands (Ruppert et al., 1993; Racioppi et al., 1996), raising the possibility that (if the sensitivity of IP<sub>3</sub> detection is similar to that of Ca<sup>++</sup> influxes) there are IP<sub>3</sub>-independent pathways of Ca<sup>++</sup> release.

What is the determining factor for differential TCR signaling upon engagement with variant ligands? Recent evidence indicates that the TCR partial agonist and antagonist ligands examined so far have lower affinities for soluble TCR than the agonist ligands (Alam et al., 1996; Kersh and Allen, 1996; Lyons et al., 1996). This difference becomes more striking when one considers the much faster dissociation rates observed for partial agonists and antagonists (Alam et al., 1996; Lyons et al., 1996). In the context of these data, it is likely that the mechanism of action of these variant TCR ligands can be explained by an affinity model (McKeithan, 1995; Madrenas and Germain, 1996). According to this model, the affinity of the TCR for its ligands determines the length of engagement of the receptor. The time of engagement will determine the assembly of the appropriate signaling machinery for full signaling to occur. Lower affinities will correlate with shorter times of engagement and partial signaling with induction of some early signals (e.g. appearance of phospho-p21 TCR  $\zeta$  form and recruitment of ZAP-70) but not others (e.g. tyrosine phosphorylation and activation of ZAP-70) (Beeson et al., 1996). This model is also consistent with the findings of McConnell's group pointing to a

hierarchy in T cell responses that would start with TCR  $\zeta$  phosphorylation and proceed to partial  $\text{Ca}^{++}$  fluxes, acid release, full intracellular  $\text{Ca}^{++}$  release, and T cell proliferation depending on the duration or intensity of TCR engagement (Rabinowitz et al., 1996a,b). It is not known whether these responses would be causally related or would be occurring in parallel. If the latter is true, then one may claim differential amplification of the different pathways to proximal responses, and preservation of some responses but not others.

We cannot ignore that the measurement of TCR affinities was done in solution, using soluble components, and that only two specific TCR partial agonist-antagonist ligand systems have been examined. Therefore, it may be not entirely representative of TCR: peptide:MHC molecule interactions occurring *in vivo* between T cell and APC and with participation of multiple accessory molecules, or of other partial agonist-antagonist systems. In the latter cases, the differences in affinities and dissociation rates may be minor, and one may have to claim other models to explain their mechanism of action e.g. allosteric or architectural interference of TCR signaling induced by the variant TCR ligand (reviewed in Madrenas and Germain, 1996).

#### **Involvement of the CD4/CD8 coreceptor molecule in differential signaling from the TCR**

Most mature T cells express either CD4 or CD8 molecules on their surface. These molecules play a co-receptor function by binding respectively non-polymorphic regions of the class II or class I MHC molecules interacting with the TCR. The co-receptor function in T cell activation is twofold. On one hand, they are involved in the stabilization of the TCR: peptide:MHC molecule complex, and in this way contribute to the overall affinity of the TCR interaction with the peptide:MHC molecule complex (Luescher et al., 1995; Garcia et al., 1996b). On the other hand, they participate in the early signaling process by their non-covalent association with the *src*-kinase *lck* (Veillette et al., 1988). Given these two features of the co-receptor function, it is plausible to suggest that the co-receptor molecule plays a crucial role in differential signaling by variant TCR ligands by linking the affinity of the TCR: ligand interaction with the activation of intracellular signaling pathways. The evidence currently available supports such a role.

The level of co-receptor expression can determine the type of T cell response to specific variant TCR ligands. Increased CD4 expression can convert an antagonist ligand into an agonist ligand, and a null ligand into an antagonist ligand (Vidal et al., 1996). In the context of the kinetic model of differential signaling, one can explain these findings by claiming that the increase in CD4 expression is associated with more rapid colocalization of the CD4 molecules with the engaged TCR, allowing for TCR-CD4 co-engagement with peptide:MHC molecule, and the formation of more

stable activating complexes.

The role of co-receptor molecules in determining the pattern of T cell responses to peptide:MHC molecule complexes can also be evidenced using blocking monoclonal antibodies against CD4 or CD8. For example, blockage of CD8 function with monoclonal antibodies converted a poor antagonist into a very powerful antagonist (Jameson et al., 1994), and antibodies against CD4 converted a partial agonist ligand into an antagonist ligand (Mannie et al., 1995). We have recently demonstrated that blockage of CD4 function with monoclonal antibodies or by mutation of the CD4 binding site on the MHC class II molecule can lead to the conversion of an agonist response into a partial agonist response, even though the ligand is not altered (Madrenas et al., 1997). This response is characterized by a distinct pattern of signaling from the TCR with predominant appearance of the p21 tyrosine phosphorylated form of TCR  $\zeta$  but not of phospho23 or CD3- $\epsilon$  and by failure to activate ZAP-70. In addition, as described for responses to TCR partial agonists, TCR engagement with agonist ligands in the presence of CD4 blockage caused a split in cytokine production, with inhibition of IL-2 production but relative preservation of IL-3 production, and the induction of T cell anergy. Similar results were obtained using heterofunctional antibodies against CD3 alone compared with CD3 and CD4 antibodies. Thus, while TCR-CD4 co-engagement with peptide:MHC molecule complex determines an agonist-type of response, failure to induce such co-engagement determines a partial agonist-type of response. These conclusions are consistent with recent data demonstrating the quantitative contribution of the co-receptor molecule to the stabilization of TCR: peptide:MHC molecule (Garcia et al., 1996b).

The link between the efficiency of TCR-CD4 co-engagement and the TCR-dependent signaling pattern most likely involves changes in the activity of *lck*. It has been recently reported that the kinase activity of the CD4-associated fraction of *lck* is increased upon TCR engagement with antagonist ligands, similar to what is seen after TCR engagement with agonist ligands (Racioppi et al., 1996). The association of antagonist-induced increase in *lck* kinase activity with decreased antagonism in the presence of monoclonal antibodies against CD4 has led to the proposal that the CD4-associated fraction of *lck* may have a negative signaling effect on the TCR-mediated activation process. This would not be occurring when *lck* is sequestered with the anti-CD4 monoclonal antibodies. Alternatively, one could claim that the antibodies against CD4 may further decrease the affinity of interaction between the TCR and the antagonist ligand or may have some agonistic effects upon activation of *lck* and subsequent activation through the TCR in agonist mode. This alternative hypothesis is supported by data demonstrating that decreased expression and activity of *lck* leads to partial TCR-mediated signaling that translates into severe defect in T cell proliferation but preserves cytokine production (Al-

Ramadi et al., 1996).

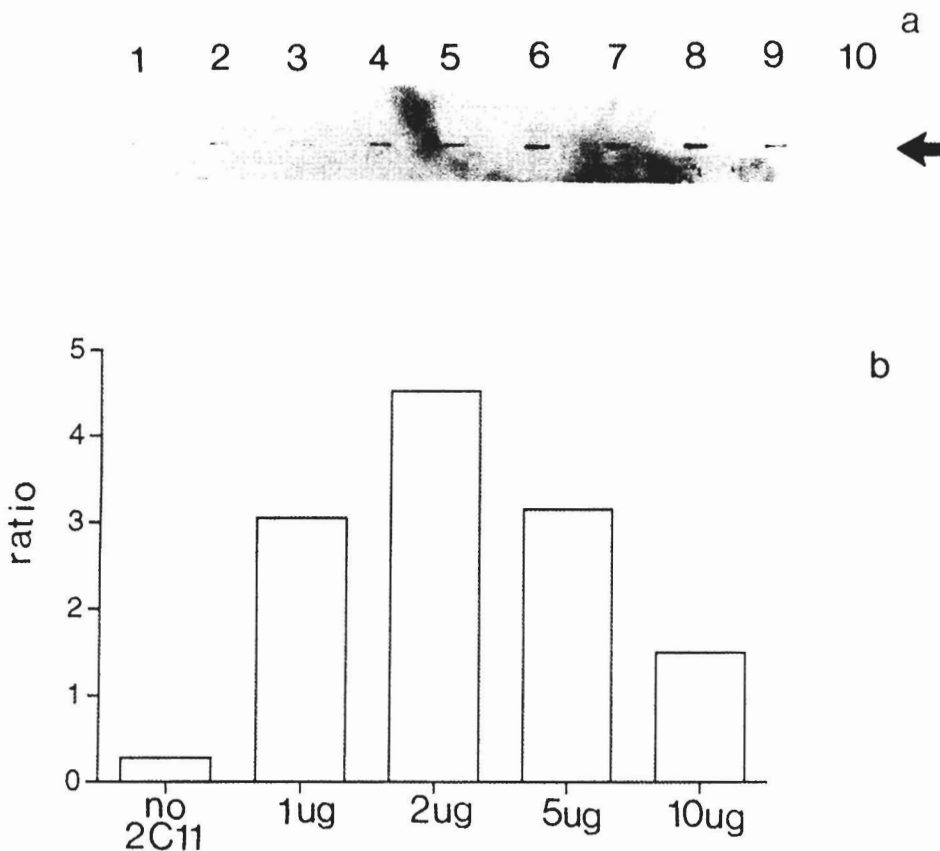
The information presented above provides us with a new base to evaluate the evidence supporting a therapeutic window for the use of monoclonal antibodies against CD4 and/or CD8. It is well documented that, in rodent models of autoimmunity and transplantation tolerance, monoclonal antibodies against CD4 and CD8 can induce a long lasting state of antigen-specific T cell unresponsiveness, and we would claim that this is due to the induction of T cell anergy by differential TCR-mediated signaling (Shizuru et al., 1992; Alters et al., 1993; Waldmann and Cobbold, 1993). Experiments trying to address the underlying mechanism of this phenomenon are currently underway.

#### Contribution of CD45 in differential signaling from the TCR

As previously discussed, CD45 plays a crucial role in lymphocyte signal transduction. Thus, it is important to examine the effects blockage of CD45 on T cell function. Our interest has initially focused on the CD45RB isoform of this molecule. We have recently reported that mice receiving a kidney allograft and treated with a monoclonal antibody against the CD45RB molecule (MB23G2) have a significant prolongation of graft survival to levels comparable to the isografted mice (Lazarovits et al., 1996). Remarkably, a different

monoclonal antibody against CD45RB (MB4B4) was no better than the vehicle alone at preventing rejection. Using syngeneic and allogeneic skin transplants, we were able to demonstrate that antigen specific tolerance had been induced by the effective antibody against CD45RB. In addition, the effective antibody against CD45RB was useful to reverse kidney allograft rejection.

The mechanism of action of the effective anti-CD45RB antibody is still unclear. Several possibilities may be considered. First, the CD45RB monoclonal antibody may interfere with TCR-mediated signal transduction perhaps by steric hindrance, or by preventing interaction of the extracellular domain of the CD45RB protein with its natural ligand. Second, the CD45RB monoclonal antibody could physically interfere with the interaction of CD3-TCR with the allogeneic histocompatibility molecule since CD45 is known to be physically linked with CD3-TCR and CD7 (Lazarovits et al., 1994). These possibilities are not mutually exclusive. With these possibilities in mind and with the knowledge that CD45 is a protein tyrosine phosphatase (Trowbridge and Thomas, 1994), we designed experiments to test the hypothesis that the mechanism of induction of tolerance by CD45RB antibody is related to an alteration in tyrosine phosphorylation of T cell substrates necessary for signal transduction to occur.



**Fig. 2.** MB23G2 monoclonal antibody augments the tyrosine phosphorylation of phospholipase C  $\gamma$ -1. The A1.1 T cell hybridoma was stimulated with anti-CD3  $\epsilon$  mAb 2C11 for 5 minutes at 37 °C at a concentration of 1  $\mu$ g/ml (lanes 2 and 6), 2  $\mu$ g/ml (lanes 3 and 7), 5  $\mu$ g/ml (lanes 4 and 8), 10  $\mu$ g/ml (lanes 5 and 9), or they were left in the absence of 2C11 stimulation (lanes 1 and 10). For lanes 6 to 10, the cells were incubated in the presence of 50  $\mu$ g/ml of MB23G2. The cells were lysed and lysates underwent immunoprecipitation with phosphotyrosine mAb PY72 and immunoblotted with antibodies to PLC  $\gamma$ -1 (panel A). Location of PLC  $\gamma$ -1 is indicated by the arrow. Panel B represents measurements obtained by densitometry from the blot in panel A. The histograms are ratios of adjusted volumes of MB23G2 and 2C11 coincubation: 2C11 alone or no 2C11 as the case may be.

As shown in figure 2, in the presence of the effective MB23G2 monoclonal antibody, there is an increase in tyrosine phosphorylation of phospholipase C (PLC)  $\gamma$ -1. The ineffective antibody MB4B4 had no effect on tyrosine phosphorylation of PLC  $\gamma$ -1. It is interesting to point out that anergic T cells have an increase in the levels of tyrosine phosphorylation of PLC  $\gamma$ -1 (Gajewski et al., 1994) as well as of p59<sup>fyn</sup>, with increased functional activity of p59<sup>fyn</sup> (Quill et al., 1992; Bhandoola et al., 1993; Cho et al., 1993). In addition, anergic T cells show a decrease in tyrosine phosphorylation of p56<sup>lck</sup> and ZAP-70 with decreased activity of these PTKs, probably as a result of decreased and/or altered TCR-mediated signaling leading to decreased tyrosine phosphorylation of certain CD3 polypeptides (Quill et al., 1992; Bhandoola et al., 1993; Cho et al., 1993; Migita et al., 1995). Our data are provocative, and suggest that certain stimuli delivered through CD45RB induce tolerance by rendering the alloreactive T cells anergic.

Although the relationship between the changes in tyrosine phosphorylation induced by the MB23G2 monoclonal antibody against CD45RB and the induction of long-term graft acceptance is not known, it is reasonable to speculate that the latter is the result of a pattern of partial T cell activation induced by this antibody and similar to that caused by some TCR partial agonists. Given the upstream role that CD45 plays on activation of lck, one could argue that this may occur by causing co-sequestration of CD45 and CD4 or any other molecule critical for full TCR-mediated signaling (Lazarovits et al., 1992, 1994), and leading to a partial activation of the CD4-associated fraction of this kinase. Alternatively, one could claim that the antibody blocks the phosphatase activity of CD45 or any other function that may regulate TCR-mediated signaling. As a result of partial T cell activation, T cells become anergic. A major issue that needs to be addressed is why this effect is observed with this particular antibody against the CD45RB isoform and it is not shared by another antibody against the same isoform.

### From biochemistry to transplantation tolerance

An alternative vision of TCR-mediated signaling is emerging. This involves a versatile signal transduction machine, able to sense small changes in the quality of the ligand, and translating those changes in differential activation of T cells. This results in different responses ranging from full T cell activation with cell proliferation, and cytokine production, to inhibition of on-going responses, to T cell anergy. This versatility of the TCR may be operational in physiological events such as during development of the T cell repertoire in the thymus through positive and negative selection, or in the maintenance of peripheral T cell tolerance.

The current challenge is to apply this new knowledge in therapeutically feasible strategies. Structural information from dissection of TCR epitopes implies

that there are some general principles for peptide binding to MHC molecules (Ljunggren and Thorpe, 1996; Nelson et al., 1996) and these may be valid for the generation of variant TCR ligands with partial agonist and antagonist properties in high frequency (as high as 50% of all substitutions in a particular residue (Chen et al., 1996; Hsu et al., 1996)). However, it is still unclear how restrictive TCR recognition will be as to limit the validity in polyclonal or even in oligoclonal responses. In this sense, the knowledge coming from studies manipulating the formation of activating complexes with monoclonal antibodies against CD4, CD8, or CD45 may be more applicable in the short term to reproduce the effects seen with engagement of the TCR with variant TCR ligands in *in vivo* situations. The establishment of reliable and reproducible protocols to attain this outcome will certainly be a major achievement.

---

*Acknowledgments.* We wish to thank our colleagues in our laboratories, at the Robarts Research Institute, in the Multi-Organ Transplant Service, and in our departments for helpful comments and criticisms that have been very useful in the development of the ideas expressed in this review. We also acknowledge the many contributions of scientists whose work has not been referenced due to space limitations. Research presented in this review paper has been funded by the Kidney Foundation of Canada, the Medical Research Council of Canada, and the Multi-Organ Transplant Service of the London Health Sciences Centre at the University of Western Ontario.

---

### References

- Al-Ramadi B.K., Nakamura T., Leitenberg D. and Bothwell A.L.M. (1996). Deficient expression of p56<sup>lck</sup> in Th2 cells leads to partial TCR signaling and a dysregulation in lymphokine mRNA levels. *J. Immunol.* 157, 4751-4761.
- Alam S.M., Travers P.J., Wung J.L., Nasholds W., Redpath S., Jameson S.C. and Gascoigne N.R.J. (1996). T-cell-receptor affinity and thymocyte positive selection. *Nature* 381, 616-620.
- Alters S.E., Song H.K. and Fathman C.G. (1993). Evidence that clonal anergy is induced in thymic migrant cells after anti-CD4-mediated transplantation tolerance. *Transplantation* 56, 633-638.
- Appleby M.W., Gross J.A., Cooke M.P., Levin S.D., Qian X. and Perlmutter R.M. (1992). Defective T cell receptor signaling in mice lacking the thymic isoform of p59fyn. *Cell* 70, 751-763.
- Arpaia E., Shahar M., Dadi H. Cohen A. and Roifman C.M. (1994). Defective T cell receptor signaling and CD8+ thymic selection in humans lacking zap-70 kinase. *Cell* 76, 947-958.
- Beeson C., Rabinowitz J., Tate K., Gütgemann I., Chien Y., Jones P.P., Davis M.M. and McConnell H.M. (1996). Early biochemical signals arise from low affinity TCR-ligand reactions at the cell-cell interface. *J. Exp. Med.* 184, 777-782.
- Bergman M., Mustelin T., Oetken C., Partanen J., Flint N.A., Amrein K. E., Autero M., Burn P. and Alitalo K. (1992). The human p50csk tyrosine kinase phosphorylates p56<sup>lck</sup> at Tyr-505 and down regulates its catalytic activity. *Embo J.* 11, 2919-2924.
- Bhandoola A., Cho E.A., Yui K., Saragovi H.U., Greene M.I. and Quill H. (1993). Reduced CD3-mediated protein tyrosine phosphorylation in anergic CD4+ and CD8+ T cells. *J. Immunol.* 151, 2355-2367.

- Chan A.C., Iwashima M., Turck C.W. and Weiss A. (1992). ZAP-70: a 70 kd protein-tyrosine kinase that associates with the TCR zeta chain. *Cell* 71, 649-662.
- Chan A.C., Kadlecik T.A., Elder M.E., Filipovich A.H., Kuo W.L., Iwashima M., Parslow T.G. and Weiss A. (1994). ZAP-70 deficiency in an autosomal recessive form of severe combined immunodeficiency. *Science* 264, 1599-1601.
- Chen Y.Z., Matsushita S. and Nishimura Y. (1996). Response of a human T cell clone to a large panel of altered peptide ligands carrying single residue substitutions in an antigenic peptide. Characterization and frequencies of TCR agonism and TCR antagonism with or without partial activation. *J. Immunol.* 157, 3783-3790.
- Cho E. A., Riley M. P., Sillman A.L. and Quill H. (1993). Altered protein tyrosine phosphorylation in anergic Th1 cells. *J. Immunol.* 151, 20-28.
- Chow L.M., Fournel M., Davidson D. and Veillette A. (1993). Negative regulation of T-cell receptor signalling by tyrosine protein kinase p50csk. *Nature* 365, 156-160.
- Corr M., Slanetz A.E., Boyd L.F., Jelonek M.T., Khilko S., Al-Ramadi B.K., Kim Y.S., Maher S.E., Bothwell A.L. and Margulies D.H. (1994). T cell receptor-MHC class I peptide interactions: affinity, kinetics and specificity. *Science* 265, 946-949.
- Elder M.E., Lin D., Clever J., Chan A.C., Hope T.J., Weiss A. and Parslow T.G. (1994). Human severe combined immunodeficiency due to a defect in ZAP-70, a T cell tyrosine kinase. *Science* 264, 1596-1599.
- Evavold B.D., Sloan L.J. and Allen P.M. (1993). Tickling the TCR: selective T-cell functions stimulated by altered peptide ligands. *Immunol. Today* 14, 602-609.
- Gajewski T.F., Qian D., Fields P. and Fitch F.W. (1994). Anergic T-lymphocyte clones have altered inositol phosphate, calcium and tyrosine kinase signaling pathways. *Proc. Natl. Acad. Sci. USA* 91, 38-42.
- Garboczi D.N., Ghosh P., Utz U., Fan Q.R., Biddison W.E. and Wiley D.C. (1996). Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature* 384, 134-141.
- Garcia K.C., Degano M., Stanfield R.L., Brunmark A., Jackson M.R., Peterson P.A., Teyton L. and Wilson I.A. (1996a). An  $\alpha\beta$  T cell receptor structure at 2.5 Å and its orientation in the TCR-MHC complex. *Science* 274, 209-219.
- Garcia K.C., Scott C.A., Brunmark A., Carbone F.R., Peterson P.A., Wilson I.A. and Teyton L. (1996b). CD8 enhances formation of stable T-cell receptor/MHC class I molecule complexes. *Nature* 384, 577-581.
- Germain R.N., Levine E.H. and Madrenas J. (1995). The T-Cell Receptor as a diverse signal transduction machine. *Immunologist* 3, 113-121.
- Groves T., Smiley P., Cooke M.P., Forbush K., Perlmutter R.M. and Guidos C.J. (1996). Fyn can partially substitute for lck in T lymphocyte development. *Immunity* 5, 417-428.
- Hsu B.L., Donermeyer D.L. and Allen P.M. (1996). TCR recognition of the Hb(64-76)/I-E<sup>k</sup> determinant. Single conservative amino acid changes in the complementarity-determining region 3 dramatically alter antigen fine specificity. *J. Immunol.* 157, 2291-2298.
- Jameson S.C., Hogquist K.A. and Bevan M.J. (1994). Specificity and flexibility in thymic selection. *Nature* 369, 750-752.
- Jameson S.C. and Bevan M.J. (1995). T cell receptor antagonists and partial agonists. *Immunity* 2, 1-11.
- Jenkins M.K. and Schwartz R.H. (1987). Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness *in vitro* and *in vivo*. *J. Exp. Med.* 165, 302-319.
- Kersh G.J. and Allen P.M. (1996). Structural basis for T cell recognition of altered peptide ligands: A single T cell receptor can productively recognize a large continuum of related ligands. *J. Exp. Med.* 184, 1259-1268.
- Lazarovits A.I., Poppema S., White M.J. and Karsh J. (1992). Inhibition of alloreactivity *in vitro* by monoclonal antibodies directed against restricted isoforms of the leukocyte-common antigen (CD45). *Transplantation* 54, 724-729.
- Lazarovits A.I., Osman N., Le Feuvre C.E., Ley S.C. and Crumpton M.J. (1994). CD7 is associated with CD3 and CD45 on human T cells. *J. Immunol.* 153, 3956-3966.
- Lazarovits A.I., Poppema S., Zhang Z., Khandaker M., Le Feuvre C.E., Singhal S.K., Garcia B.M., Ogasa N., Jevnikar A.M., White M.H., Singh G., Stiller C.R. and Zhong R.Z. (1996). Prevention and reversal of renal allograft rejection by antibody against CD45RB. *Nature* 380, 717-720.
- Liao X.C. and Littman D. (1995). Altered T cell receptor signaling and disrupted T cell development in mice lacking Itk. *Immunity* 3, 757-769.
- Ljunggren H.G. and Thorpe C.J. (1996). Principles of MHC class I-mediated antigen presentation and T cell selection. *Histol. Histopathol.* 11, 267-274.
- Luescher I.F., Vivier E., Layer A., Mahiou J., Godeau F., Malissen B. and Romero P. (1995). CD8 modulation of T-cell antigen receptor-ligand interactions on living cytotoxic T lymphocytes. *Nature* 373, 353-356.
- Lyons D.S., Lieberman S.A., Hampl J., Boniface J.J., Chien Y., Berg L.J. and Davis M.M. (1996). A TCR binds to antagonist ligands with lower affinities and faster dissociation rates than to agonists. *Immunity* 5, 51-59.
- Madrenas J. and Germain R.N. (1996). Variant TCR ligands: new insights into the molecular basis of antigen-dependent signal transduction and T cell activation. *Semin. Immunol.* 8, 83-101.
- Madrenas J., Wange R.L., Wang J.L., Isakov N.A., Samelson L.E. and Germain R.N. (1995).  $\zeta$  phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science* 267, 515-518.
- Madrenas J., Schwartz R.H. and Germain R.N. (1996). Interleukin 2 production, not the pattern of early T-cell antigen receptor-dependent tyrosine phosphorylation, controls anergy induction by both agonists and partial agonists. *Proc. Natl. Acad. Sci. USA* 93, 9736-9741.
- Madrenas J., Chau L.A., Smith J., Bluestone J.A. and Germain R.N. (1997). The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J. Exp. Med.* 185, 219-230.
- Mannie M.D., Rosser J.M. and White G.A. (1995). Autologous rat myelin basic protein is a partial agonist that is converted into a full antagonist upon blockade of CD4. Evidence for the integration of efficacious and nonefficacious signals during T cell antigen recognition. *J. Immunol.* 154, 2642-2654.
- Marengere L.E., Waterhouse P., Duncan G.S., Mittrucker H.W., Feng G.S. and Mak T.W. (1996). Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. *Science* 272, 1170-1173.
- Matsui K., Boniface J.J., Reay P.A., Schild H., Fazekas de St-Groth B.



*T-cell signaling and transplant tolerance*

- and Davis M.M. (1991). Low affinity interaction of peptide-MHC complexes with T cell receptors. *Science* 254, 1788-1791.
- Matsui K., Boniface J.J., Steffner P., Reay P.A. and Davis M.M. (1994). Kinetics of T-cell receptor binding to peptide/I-Ek complexes: correlation of the dissociation rate with T-cell responsiveness. *Proc. Natl. Acad. Sci. USA* 91, 12862-12866.
- Matsuoka T., Kohrogi H., Ando M., Nishimuta Y. and Matsushita S. (1996). Altered TCR ligands affect antigen-presenting cell responses. *J. Immunol.* 157, 4837-4843.
- McKeithan T.W. (1995). Kinetic proofreading in T-cell receptor signal transduction. *Proc. Nat. Acad. Sci. USA* 92, 5042-5046.
- Migita K., Eguchi K., Kawabe Y., Tsukada T., Ichinose Y. and Nagataki S. (1995). Defective TCR-mediated signaling in anergic T cells. *J. Immunol.* 155, 5083-5087.
- Molina T.J., Kishihara K., Siderovski D.P., Ewijk van W., Narendran A., Timms E., Wakeham A., Paige C.J., Hartmann K.U., Veillette A., Davidson D. and Mak T.W. (1992). Profound block in thymocyte development in mice lacking p56lck. *Nature* 357, 161-164.
- Mustelin T. (1994). T cell antigen receptor signaling: Three families of tyrosine kinases and a phosphatase. *Immunity* 1, 351-356.
- Negishi I., Motoyama N., Nakayama K., Nakayama K., Senju S., Hatakeyama S., Zhang Q., Chan A.C. and Loh D.Y. (1995). Essential role for ZAP-70 in both positive and negative selection of thymocytes. *Nature* 376, 435-438.
- Nelson C.A., Viner N.J. and Unanue E.R. (1996). Appreciating the complexity of MHC class II peptide binding: lysozyme peptide and I-Ak. *Immunol. Rev.* 151, 81-105.
- Nossal G.J. (1993). Tolerance and ways to break it. *Ann. NY Acad. Sci.* 690, 34-41.
- Nossal G.J. (1996). Clonal anergy of B cells: a flexible, reversible and quantitative concept. *J. Exp. Med.* 183, 1953-1956.
- Okumura M. and Thomas M.L. (1995). Regulation of immune function by protein tyrosine phosphatases. *Curr. Opin. Immunol.* 7, 312-319.
- Pani G., Fischer K.D., Mlinaric-Rascan I. and Siminovitch K.A. (1996). Signaling capacity of the T cell antigen receptor is negatively regulated by the PTP1C tyrosine phosphatase. *J. Exp. Med.* 184, 839-852.
- Pawson T. (1995). Protein modules and signalling networks. *Nature* 373, 573-580.
- Peri K. G. and Veillette A. (1994). Tyrosine protein kinases in T lymphocytes. *Chem. Immunol.* 59, 19-39.
- Plas D.R., Johnson R., Pingel J.T., Matthews R.J., Dalton M., Roy G., Chan A.C. and Thomas M.L. (1996). Direct regulation of ZAP-70 by SHP-1 in T cell antigen receptor signaling. *Science* 272, 1173-1176.
- Quill H. (1996). Anergy as a mechanism of peripheral T cell tolerance. *J. Immunol.* 156, 1325-1327.
- Quill H., Riley M.P., Cho E.A., Casnellie J.E., Reed J.C. and Torigoe T. (1992). Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. *J. Immunol.* 149, 2887-2893.
- Rabinowitz J.D., Beeson C., Lyons D.S., Davis M.M. and McConnell H.M. (1996a). Kinetic discrimination in T-cell activation. *Proc. Natl. Acad. Sci. USA* 93, 1401-1405.
- Rabinowitz J.D., Beeson C., Wülfing C., Tate K., Allen P.M., Davis M.M. and McConnell H.M. (1996b). Altered T cell receptor ligands trigger a subset of early T cell signals. *Immunity* 5, 125-135.
- Racioppi L., Matarese G., D'Oro U., De Pascale M., Masci A.M., Fontana S. and Zappacosta S. (1996). The role of CD4-Ick in T-cell receptor antagonism: Evidence for negative signaling. *Proc. Natl. Acad. Sci. USA* 93, 10360-10365.
- Ruppert J., Alexander J., Snoke K., Coggeshall M., Herbert E., McKenzie D., Grey H.M. and Sette A. (1993). Effect of T-cell receptor antagonism on interaction between T cells and antigen-presenting cells and on T-cell signaling events. *Proc. Natl. Acad. Sci. USA* 90, 2671-2675.
- Samelson L.E., Phillips A.F., Luong E.T. and Klausner R.D. (1990). Association for the Fyn protein tyrosine kinase with the T-cell antigen receptor. *J. Immunol.* 87, 4358-4362.
- Schwartz R.H. (1990). A cell culture model for T lymphocyte clonal anergy. *Science* 248, 1349-1356.
- Schwartz R.H. (1996). Models of T cell anergy: Is there a common molecular mechanism? *J. Exp. Med.* 184, 1-8.
- Shizuru J.A., Alters S.E. and Fathman C.G. (1992). Anti-CD4 monoclonal antibodies in therapy: Creation of nonclassical tolerance in the adult. *Immunol. Rev.* 129, 105-130.
- Sloan-Lancaster J. and Allen P.M. (1995). Significance of T-cell stimulation by altered peptide ligands in T cell biology. *Curr. Opin. Immunol.* 7, 103-109.
- Sloan-Lancaster J., Evavold B.D. and Allen P.M. (1993). Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature* 363, 156-159.
- Sloan-Lancaster J., Shaw A.S., Rothbard J.B. and Allen P.M. (1994). Partial T cell signalling: Altered phospho- $\zeta$  and lack of Zap70 recruitment in APL-induced T cell anergy. *Cell* 79, 913-922.
- Sloan-Lancaster J., Steinberg T.H. and Allen P.M. (1996). Selective activation of the calcium signaling pathway by altered peptide ligands. *J. Exp. Med.* 184, 1525-1530.
- Stamenkovic I., Sgroi D., Aruffo A., Sy M.S. and Anderson T. (1991). The B lymphocyte adhesion molecule CD22 interacts with leukocyte common antigen CD45RO on T cells and alpha 2-6 sialyltransferase, CD75, on B cells. *Cell* 66, 1133-1144.
- Trowbridge I.S. and Thomas M.L. (1994). CD45: an emerging role as a protein tyrosine phosphatase required for lymphocyte activation and development. *Annu. Rev. Immunol.* 12, 85-116.
- van Oers N.S.C., Lowin-Kropf B., Finlay D., Connolly K. and Weiss A. (1996).  $\alpha\beta$  T cell development is abolished in mice lacking both Ick and fyn protein tyrosine kinases. *Immunity* 5, 429-436.
- Veillette A., Bookman M.A., Horak E.M. and Bolen J.B. (1988). The CD4 and CD8 T cell surface antigens are associated with the internal membrane tyrosine-protein kinase p56lck. *Cell* 55, 301-308.
- Vidal K., Hsu B.L., Williams C.B. and Allen P.M. (1996). Endogenous altered peptide ligands can affect peripheral T cell responses. *J. Exp. Med.* 183, 1311-1321.
- Waldmann H. and Cobbold S. (1993). The use of monoclonal antibodies to achieve immunological tolerance. *Immunol. Today* 14, 247-251.
- Wange R.L. and Samelson L.E. (1996). Complex complexes: signaling at the TCR. *Immunity* 5, 197-205.
- Weber S., Traunecker A., Oliveri F., Gerhard W. and Karjalainen K. (1992). Specific low-affinity recognition of Major Histocompatibility Complex plus peptide by soluble T-cell receptor. *Nature* 56, 793-796.
- Zenner G., zur Hausen J.D., Burn P. and Mustelin T. (1995). Towards unraveling the complexity of T cell signal transduction. *BioEssays* 17, 967-975.