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# THE FUNCTION OF CTLA-4 IN THE MECHANISM OF NEGATIVE REGULATORY COSTIMULATION OF TCR MEDIATED SIGNALS

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DISSERTAÇÃO DE CANDIDATURA AO GRAU DE DOUTOR EM CIÊNCIAS BIOMÉDICAS, ESPECIALIDADE DE IMUNOLOGIA APRESENTADA AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR DA UNIVERSIDADE DO PORTO.

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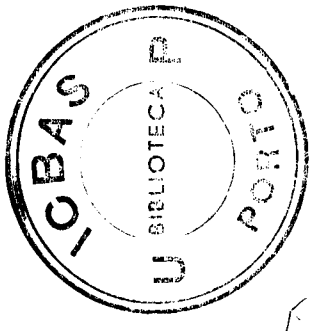
PORTO, 2002

*Aos meus pais*
*e avôs*



Nesta tese são incluídas as seguintes publicações nas quais, ao Artigo Oitavo do Decreto-Lei número 388/70 fui responsável pela sua execusão dos trabalhos experimentais e pela interpretação, discussão e redacção dos resultados apresentados:

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*"Tem o viajante, quem diz este, diz outro, a boa justificação de ser de belezas e grandezas a sua busca."*

*José Saramago*
*Viagem a Portugal*

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# Abstract

T cell costimulation provides the necessary tools to ensure a cooperate effort in TCR mediated signalling. Many studies have been dedicated in isolating and identifying hematopoetic specific proteins that link upstream phosphorylation events with downstream effectors. It's been well established that the interaction of the TCR with MHC bound to antigen and the subsequent coaggregation of the CD4/CD8 coreceptor brings p56lck or p59fyn into proximity to the non-covalently associated CD3 chains Phosphorylation of the TCR zeta chain creates a docking site for the Syk family tyrosine kinase ZAP-70 via its SH2 domain. Activated ZAP-70 can in turn activate downstream molecules such as MAPK and JNK via phosphorylation of the upstream targets adaptor proteins SLP-76 and the transmembrane protein LAT. As different T cell related functions require upregulation of different receptors at specific stages of the immune response, isolation and characterisation of surface molecules involved in regulating processes of activation and differentiation has been of prime interest. CD28 and ICOS costimulatory molecules are described as being positive regulators for cytokine production and T helper cell differentiation. The signalling mechanism by which CD28 operates has been extensively studied in many in vitro as well as in vivo models. It has provided much needed information and understanding on how upstream activation of intracellular pathways may lead to gene transcription in the nucleus. The costimulatory molecule CTLA-4, on the other hand, was shown to antagonise T cell activation while engaging support for T cell differentiation. The negative role of CTLA-4 in T cell homeostasis was clearly demonstrated by the dramatic lethal phenotype of CTLA-4 deficient mice. An aggressive lymphoproliferative disease and subsequent infiltration of T cells ensuing destruction of internal organs marked the significance that CTLA-4 may have in certain pathological situations. Thus, the study of the role of CTLA-4 in downmodulation of the immune response is of great importance, especially when providing immunotherapy in the context of graft rejection, tumour immunity and autoimmune diseases.

Despite its potential application in induction of immunotolerance and its central regulatory role in T cell activation and differentiation, little is known about the mechanism of CTLA-4 mediated signals. The complex nature of CTLA-4 surface expression and the limited binding of signalling molecules to the cytoplasmic tail have lead to contradictions between experimental data from different groups and unsatisfactory mechanistical models. The present study was designed to evaluate the signalling mechanism of CTLA-4 and analyse the role of upstream signalling pathways involved in the negative function of

CTLA-4. The models used in this study were based on two mouse hybridoma cell lines. A mouse hybridoma cell line DC27.10 was stably transfected with wild type human CTLA-4 and a CTLA-4 mutant of the YVKM cytoplasmic motif where the tyrosine is substituted for a phenylalanine. In addition, a mouse hybridoma T8.1 cell line expressing a human recombinant TCR recognising tetanus toxin peptide in the context of HLA-DR*1102 was stably transfected with human CTLA-4.

Results from this study have contributed to expanding the knowledge concerning CTLA-4 and redefining a new model for CTLA-4 negative signalling. In Chapter 2, I show that CTLA-4 may promote cytoskeletal changes, namely filopodia and lamellipodia formation. These changes are associted with decreased motility and increased adhesion. These cytoskeletal changes mediated by CTLA-4 may require activation of the Rho family of GTPase Rac. In addition, CTLA-4 may promote cytoskeletal changes in a context of antigen presentation. In Chapter 3, I describe that CTLA-4 can actively inhibit apoptosis in the hybridoma model and plays a role in regulating early stages of TCR mediated signalling, prior to the bifurcation of signals leading to IL-2 production and apoptosis. In addition, CTLA-4 can successfully block CD3 mediated signals without CD28 costimulation. In Chapter 4, I present evidence that PI3-kinase is important but not essential for CTLA-4 blockage of IL-2 production. The YVKM motif modulates the strength of CTLA-4 inhibition and moreover, the strength of CTLA-4 inhibition is directly dependent on the strength of the TCR stimulus.

Therefore, based on the results of this study, a new model for CTLA-4 signalling emerges. While CTLA-4 can still inhibit certain functions related to TCR activation such as IL-2 production, it also plays an active role, as opposed to having a static inhibitory function, in modulating TCR signals in the context of cell shape remodelling. These results may have important future consequences in the regulation of immune related pathological conditions.

# Resumo

A coestimulação de células T assegura a cooperação necessária para a activação da cascata de sinalização do receptor da célula T (TCR). O isolamento e a identificação de proteínas hematopoiéticas envolvidas nestas vias de transdução de sinal, tem sido objecto de estudo exaustivo. A interacção do TCR com o complexo de histocompatibilidade (MHC), quando ligado ao péptido antigénico, e a subsequente coagregação dos coreceptores CD4 e CD8 conduz ao recrutamento das quinases p56lck e p59fyn para a vizinhança das cadeias não-convalentemente associadas do CD3. A fosforilação da cadeia zeta do TCR constitui, através do seu domínio SH2, um local de ancoragem para a ZAP-70, uma tirosina quinase pertencente a família Syk. A forma activa da ZAP-70 activa por sua vez moléculas como o MAPK e JNK. Esta activação ocorre através da fosforilação de proteínas que se localizam a montante da cascata de transdução de sinal gerada pelo CD3, como é o caso da proteína adaptadora SLP-76 e proteína transmembranar LAT. As diferentes funções das células T obrigam a expressão de diferentes receptores associados a fases específicas da resposta imunológica. Consequentemente o isolamento e a caracterização das moléculas de superfície envolvidas na regulação dos processos de activação e diferenciação das células T tem sido uma prioridade na investigação nesta área. Os receptores de coestimulação CD28 e ICOS contribuem positivamente para a produção de citoquinas e para a diferenciação das células T helper. O mecanismo de sinalização pelo qual o CD28 opera tem sido estudado em detalhe recorrendo a modelos ***in vivo*** assim como ***in vitro***. Estes estudos têm contribuído para uma melhor compreensão dos mecanismos de activação a montante de cascatas intracelulares conducentes à transcrição de genes no núcleo. Por outro lado, a molécula coestimuladora CTLA-4 antagoniza a activação das células T, ao mesmo tempo que participa na diferenciação dos linfocitos T. O CTLA-4 tem também um papel essencial na manutenção da homeostase destas células conforme demonstrado pelo fenótipo letal observado em ratinhos deficientes em CTLA-4. Estes ratinhos apresentam intensas patologias linfoproliferativas e subsequente infiltração de células T originando a deterioração dos orgãos internos. Tal facto sugere um envolvimento do CTLA-4 em algumas patologias. Consequentemente, o estudo do papel do CTLA-4 na modulação negativa da resposta imunológica é de extrema importância, especialmente no âmbito das terapias imunológicas usadas na rejeição de transplantes, em imunidade tumoral e doenças autoimunes.

O mecanismo de actuação do CTLA-4 é desconhecido, apesar da sua aplicação na indução de tolerância imunológica e na regulação da activação e diferenciação das células T. A complexidade do padrão de expressão do CTLA-4 à superfície e a capacidade limitada de ligação das moléculas de sinalização à sua cauda citoplasmática conduziram a resultados contraditórios e à ausência de um modelo mecanístico satisfatório. Pretendeu-se com este trabalho avaliar o mecanismo de sinalização do CTLA-4 assim como identificar as cascatas de sinalização inibitória activadas por este receptor. Como modelo de estudo usaram-se duas linhas celulares de hibridomas de ratinho. A linha celular DC27.10 foi transfectada com o CTLA-4 humano e com um mutante no qual a tirosina do motivo citoplasmático YVKM foi substituída por uma fenilalanina. A segunda linha celular usada, T8.1, expressa um receptor de célula T (TCR) humano recombinante que reconhece um péptido derivado da toxina do tétano ligado ao complexo de histocompatibilidade HLA-DR*1102 e também foi transfectada com CTLA-4 humano.

Os resultados obtidos neste estudo contribuíram para a melhor compreensão das funções do CTLA-4 e para a definição de um novo modelo de sinalização negativa mediada pelo CTLA-4. No segundo capítulo, demonstra-se o envolvimento do CTLA-4 na formação de filopodias e lamelipodias após estimulação por anticorpos dirigidos para o TCR e o CTLA-4. A formação destas estruturas resulta numa perda de mobilidade e aumento de adesão celular. GTPases pertencendo a família Rho, nomeadamente Rac, parecem estar envolvidas neste processo. Estas alterações do citoesqueleto foram também observadas num modelo de apresentação de antigénio usando células T8.1 transfectadas. No capitulo 3, demonstra-se o papel activo do CTLA-4 tanto na inibição de apoptose bem como na produção do IL-2. Consequentemente, o CTLA-4 parece estar envolvido nas fases iniciais da cadeia de sinalização do TCR que antecedem a bifurcação de sinais conducentes à produção de IL-2 e à apoptose. Por outro lado, também ficou demonstrado que o CTLA-4 pode bloquear a sinalização mediada pelo CD3 sem necessitar da co-estimulação do CD28. No capitulo 4, demonstra-se que a quinase PI-3 é importante mas não é essencial para o bloqueio da produção de IL-2 pelo CTLA-4. O motivo YVKM modula o grau de inibição de CTLA-4, dependendo este directamente do estímulo do TCR.

Assim, com base nos resultados aqui apresentados foi possível estabelecer um novo modelo de sinalização pelo CTLA-4. Para além do papel inibitório na produção de IL-2 e apoptose o CTLA-4 também intervem na modulação dos sinais do TCR no âmbito de modificações do citoesqueleto. Estes resultados poderão ter importantes

consequências no desenvolvimento de terapias mais eficazes para patologias dos sistema imune.

# Résumé

La costimulation des lymphocytes T fournit les outils nécéssaires pour assurer une coopération dans la signalisation apporté par le récepteur T de l'antigène (TCR). De nombreuses études ont été consacrées à isoler et identifier des protéines hematopoiétiques spécifiques qui joignent des événements en amont de la cascade de phophorylation avec des effecteurs en aval de ce processus. Il est bien établi que l'interaction du TCR avec le complexe majeur d'histocompatibilité (CMH) lié à l'antigène suivie da la coaggrégation du corécepteur CD4/CD8 rapproche les protéines tyrosines kinases p56lck ou p59fyn à proximité des chaînes CD3, ce qui entraîne la cascade de signaux intracellulaires declenchés par la phosphorylation des tyrosines du module ITAM, plus spécifiquement les modules ITAM de la chaîne zeta du TCR. Ceci crée un site d'ancrage pour la tyrosine kinase ZAP-70 (appartenant à la famille des kinases Syk) par l'intermédiaire de son domaine SH2. ZAP-70 peut alternativement phosphoryler les molécules en aval da la cascade de phosphorylation telles que MAPK et JNK par l'intermédiaire de la phosphorylation des protéines en amont de la cascade des signaux contrôlés par le TCR comme l'adaptateur SLP-76 et la phosphorylation de la protéine transmembranaire LAT. Puisque différents lymphocytes T sont associés à diverses fonctions qui exigent l'augmentation de différents récepteurs à des étapes spécifiques de la réponse immunitaire, il a été d'intérêt principal d'isoler et de caractériser les récepteurs à la surface de la cellule impliquée dans ce processus de régulation d'activation et de différentiation. Les molécules costimulatrices CD28 et ICOS sont décrites comme étant les régulateurs positifs pour la production de cytokines et pour la différentiation de cellules T helper. Le mécanisme de signalisation par lequel CD28 fonctionne a été intensivement étudié aussi bien dans des modèles ***in vitro*** que des modèles ***in vivo***. Il a permis de mieux comprendre de quelle façon l'activation des voies intracellulaires en amont de la cascade peut conduire à la transcription de gènes dans le noyau. D'autre part, la molécule costimulatrice CTLA-4 a montré un effet antagoniste sur l'activation des lymphocytes T tout en engageant un soutien pour leur différentiation. Le rôle de CTLA-4 dans l'homéostasie des cellules T a été clairement démontré par le phénotype létal des souris déficientes en CTLA-4. Une maladie lymphoproliférative agressive et la destruction des organes internes causée par l'infiltration des lymphocytes T ont montré l'importance que CTLA-4 peut avoir dans certaines situations pathologiques. Ainsi, l'étude du rôle de CTLA-4 dans la modulation négative de la réponse immunitaire est très importante, particulièrement dans les situations concernant l'administration d'immunothérapies dans le contexte du rejet de greffe, de l'immunité des tumeurs et des maladies autoimmunes.

En dépit de son application potentielle dans l'induction de l'immunotolérance et de son rôle de régulateur central dans l'activation et la différentiation de cellules T, le mécanisme des signaux intracellulaires géré par CTLA-4 demeure peu connu. La nature complexe de l'expression de CTLA-4 à la surface du lymphocyte T ainsi que le nombre limité de molécules recrutées à son domaine cytoplasmique ont mené à la publication de données expérimentales contradictoires entre différents groupes et donc à des modèles de mécanisme insatisfaisants. Cette étude a été conçue dans le but d'évaluer le mécanisme de signalisation de CTLA-4 et pour analyser le rôle des voies de signalisation, en amont de la cascade déclanchée par le récepteur pour l'antigène, impliquées dans la fonction négative de CTLA-4. Les modèles utilisés dans cette étude ont été basés sur deux lignées cellulaires d'hybridomes de souris. La lignée cellulaire de souris DC27.10 a été transfectée de manière stable avec le gène sauvage du CTLA-4 humain et un gène mutant CTLA-4 où la tyrosine du motif cytoplasmique YVKM est substituée par une phénylalanine. De plus, un hybridome de souris T8.1 exprimant un TCR humain recombiné spécifique pour le peptide de la toxine tétanique présenté dans le contexte de HLA-DR*1102 a été transfecté de manière stable avec le gène du CTLA-4 humain.

Les résultats de cette étude ont contribué à mieux comprendre le rôle du CTLA-4 dans l'hinibition de la transduction des signaux et à redéfinir un nouveau modèle pour la signalisation négative apportée par le CTLA-4. Dans le chapitre 2, je mets en évidence que CTLA-4 peut favoriser des changements du cytosquelettes, à savoir la formation de filopodes et de lamellipodes. Ces changements sont associés à une motilité diminuée et une augmentation de l'adhérence des lymphocytes T. Ces changements cytosquelettiques médiés par le CTLA-4 exigent l'activation de GTPases de la famille Rho, entre autre l'activation de la protéine Rac. De plus, je décris que non seulement CTLA-4 peut favoriser les changements cytosquelettiques par la stimulation des récepteurs par des anticorps mais également dans un contexte de présentation de l'antigène. Dans le chapitre 3, je montre que CTLA-4 peut activement empêcher l'apoptose dans notre modèle et joue un rôle en régulant les premières étapes de la signalisation médiée par le TCR, précédent la séparation des signaux menant à la production d'IL-2 et à l'apoptose. CTLA-4 peut aussi bloquer les signaux transmis par le CD3 sans avoir recours à la costimulation par le CD28. Dans le chapitre 4, je mets en évidence que la kinase PI3 est importante mais non essentielle pour le bloquage de la production d'IL-2 par le CTLA-4. Le motif de YVKM module la force de l'inhibition par le CTLA-4 qui d'ailleurs dépend directement de la force du stimulus provenant du TCR.

Basé sur les résultats de cette l'étude, un nouveau modèle de signalisation par le CTLA-4 émerge. Tandis que CTLA-4 peut inhiber certaines fonctions liées à l'activation du TCR, tel que la production IL-2 et l'apoptose, il joue également un rôle actif. Au lieu d'avoir simplement une fonction inhibitrice statique, il module les signaux provenant du TCR en modifiant le cytosquelette et en remodellant la forme des cellules. Ces résultats peuvent avoir d'importantes implications dans le traitement des conditions pathologiques reliées au système immunitaire.

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# List of Abbreviations

| | |
|---|---|
| TCR | T cell receptor |
| Ig | Immunoglubulin |
| V5 | DC27.10 cells transfected with pSRα vector |
| CTLA-4WT | DC27.10 cells transfected with human CTLA-4 |
| hCTLA-4 | human CTLA-4 |
| Ttox | Tetanus toxin |
| AICD | Antigen Induced cell death |
| PTP'ase | protein tyrosine phosphatase |
| d | day |
| mAb | monoclonal antibody |
| CTL | Cytotoxic T lymphocyte |
| LCMV | lymphocytic choriomeningitis virus |
| SEB | Staphylococcal enterotoxin B |
| DC | dendritic cells |
| GVHD | Graft versus host disease |
| CTLA-4 | Cytotoxic T lymphocyte antigen-4 |
| Cdk | cyclin dependent kinase |
| MAPK | mitogen activating protein kinase |
| ERK | extracellular signal regulated protein kinase |
| GEMs | glycolipid enriched membrane |
| MHC | major histocompatibility complex |
| GM-CSF | granulocyte macrophage colony stimulating factor |
| OVA | Ovalbumin |
| APC | antigen presenting cells |
| Th | T helper cells |
| Ko | knock out |
| T reg | T regulatory suppressor cells |
| 2-ME | beta-mercaptoethanol |
| DTT | αDithiothreitol |
| MTOC | microtubule organising centre |
| TNP | trinitrophenol |

# Chapter 1. General Introduction

## 1.1. Background

The immune system is a unique network where orchestration of different cell types, lymphoid and non-lymphoid cells, is required for production of an efficient and specific response to foreign antigens. Whereas the innate arm of the immune system composed of basophils, eosinophils, mast cells and complement surveys and rapidly irradicates invaders by means of directed cytotoxic effects, the adaptive arm, composed of T and B cells, is required for differential recognition of self and non-self antigens and prolonged long-term memory (Janeway and Medzhitov, 2002). T cells recognise antigen via the T cell receptor (TCR) which is composed of the immunoglobulin-like supergene family $\alpha$ and $\beta$ chains. The TCR is selected in the thymus and has a potential repertoire of $2.5 \times 10^7$ different types of receptors. Transduction of intracellular signals is primarily generated via the associated CD3 chains $\gamma$, $\delta$ , $\varepsilon$, and $\zeta$ where phosphorylation and recruitment of adaptor proteins to the plasma membrane can signal to a downstream effector in the nucleus for transcription of genes (da Silva et al., 1997; Geng and Rudd, 2002; Iwashima et al., 1994; Rudd, 1999; Samelson, 2002; Weiss and Littman, 1994). Activation through the TCR promotes a cascade of events which includes phosphorylation of downstream effectors and regulation of transcriptional activity of the IL-2 gene.

In recent times, much work has been dedicated in isolating and identifying hematopoetic specific proteins that link upstream phosphorylation events with downstream effectors. It's been well established that the interaction of the TCR with MHC bound to antigen and the subsequent coaggregation of the CD4/CD8 coreceptor brings p56lck or p59fyn into proximity to the non-covalently associated CD3 chains (Barber et al., 1989; Rudd et al., 1988; Veillette et al., 1989). This interactions results in phosphorylation of the tandem tyrosine motifs YXXL (ITAM motifs), typically separated by 6-8 amino acid, of the zeta chain of the CD3 component. Phosphorylation of the TCR zeta chain creates a docking site for the Syk family tyrosine kinase ZAP-70 via its SH2 domain. Activated ZAP-70 can in turn activate downstream molecules such as MAPK and JNK via phosphorylation of the upstream targets adaptor proteins SLP-76 (Bubeck Wardenburg et al., 1998; da Silva et al., 1997; Raab et al., 1997) and the transmembrane protein LAT (Ishiai et al., 2000; Shan et al., 2001). SLP76 has been involved in promoting IL-2 production as well as serving as a scaffolder protein for linking the Grb-2 like adaptor Gads, Vav and NCK to the cytoskeleton (Bubeck Wardenburg et al., 1998; Coppolino et al., 2001; Ishiai et al., 2000). SLP-76 has been shown to specifically be required to activate PLC$\gamma$1 since SLP-76-deficient cells show a selective loss of phospho-PLC$\gamma$1

(Yablonski et al., 1998). The phosphorylation of PLCγ1 catalyses the generation of second messenger IP3 which increases intracellular calcium levels. This increase stimulates calmodulin and calcineurin activity which will dephosphorylate NFAT and thus allow translocation into the nucleus. The Vav SH2 domain interacts with SLP-76 (Onodera et al., 1996; Wu et al., 1996), by binding to the two YESP sites on SLP-76 (Raab et al., 1997). Vav possesses GEF activity for Ras-related proteins Rho and Rac1. These GTP-binding proteins regulate the assembly of actin into stress fibres and structural changes. In addition, SLP-76 binds to another adaptor protein ADAP (FYB/SLAP-130) via its SH2 domain. ADAP has recently been shown to be crucial for TCR mediated integrin clustering and adhesion of mast cells (Geng and Rudd, 2001; Griffiths et al., 2001; Peterson et al., 2001). The LAT transmembrane protein exhibits a short extracellular domain with a tyrosine rich intracellular tail. It has been shown to associate with PLCγ1, Grb-2, PI 3-kinase and src kinases (Buday et al., 1994; Fukazawa et al., 1995; Sieh et al., 1994; Weber et al., 1992). LAT-deficient T cells show profound defects in pre-TcR and TcR signalling with defects in $Ca2^{+}$ mobilization, MAP kinase activation and CD69 expression, as well as in AP-1 and NFAT-driven cytokine gene transcription (Finco 1998, Zhang 1999).

In addition to TCR signals, coreceptors, such as CD28 and ICOS (Aicher et al., 2000; Aruffo and Seed, 1987; Beier et al., 2000; Hutloff et al., 1999), set the threshold for TCR activation (Gonzalo et al., 2001). CD28 has been shown to generate costimulatory signals, providing synergism and complementarity to CD3 mediated signals. However, to limit the response to non-self antigens, the immune system is designed to constrain itself by way of negative regulators expressed on the cell surface and terminate ongoing responses. PD-1 and CTLA-4 have been described as negative regulators of the immune response. The coreceptor CTLA-4 (cytotoxic T lymphocyte associated antigen-4) is structurally related to CD28 and is transiently expressed in activated T cells (Brunet et al., 1987; Harper et al., 1991; Lindsten et al., 1993; Linsley et al., 1991; Ostrov et al., 2000; Schwartz et al., 2001; Stamper et al., 2001). CTLA-4 is well documented in its ability to regulate immune responses against pathogens and modulate immunotherapeutic effects against tumours, graft-vs-host and autoimmune diseases (Anderson et al., 1999; Chambers et al., 2001b; Gomes and DosReis, 2001; Khoury et al., 1999; Salomon and Bluestone, 2001). Both CD28 and CTLA-4 coreceptors bind to the same ligands, CD80 (B7-1) and CD86 (B7-2) that are mainly expressed on activated antigen presenting cells (APC), monocytes and activated B cells (McAdam et al., 1998). Careful balance between the interplay of membrane bound receptors and soluble factors in the activating

intracellular scheme of the response is critical for determining the success of the outcome of the response (June et al., 1990; Valitutti et al., 1995).

## 1.2. The Role of CTLA-4 as a negative Regulator of Activation

### 1.2.1. Knockout mice

The generation of CTLA-4 deficient mice demonstrated the role of CTLA-4 in T cell homeostasis. CTLA-4 deficient mice showed early lethality at 3-4 weeks of age due to extensive lymphoproliferation and cellular infiltration of non-haematopoetic tissues (Tivol et al., 1995; Waterhouse et al., 1995). The mice suffered from destructive myocarditis and pancreatitis with infiltration of mononuclear cells. CTLA-$4^{-/-}$ mice exhibited massive splenomegaly and lymphadenopathy, having a 10-fold increase in the number of T cells. The TCR$\alpha\beta$ profile of CTLA-$4^{-/-}$ showed a massive expansion of T cells having a normal relative proportion of V$\beta$6-, V$\beta$8-, V$\beta$14 and V$\alpha$2-positive cells compared to normal littermates. In vitro studies showed that CTLA-$4^{-/-}$ cells proliferate spontaneously and are capable of producing increased amounts of IFN$\gamma$, IL-4 and GM-CSF compared to normal cells. In addition, serum levels of IgG2a, IgG2b, IgG3 and IgM are increased by 10 fold while IgG1, IgA and IgE are increased 100-1000 fold (Waterhouse et al., 1995). Interestingly, the lethal phenotype can be rescued by administering the soluble fusion protein CTLA-4Ig, which blocks binding of CD28 to B7 ligands (Tivol et al., 1997). However, the CTLA-4Ig treatment lacks long-term benefit as cellular infiltration and tissue damage started 14d after CTLA-4Ig withdrawal. In addition, mice lacking B7-1, B7-2 and CTLA-4 do not show a lymphoproliferative disorder (Mandelbrot et al., 1999). These results clearly shows that the CTLA-4 deficient phenotype is primarily caused by unregulated costimulation by CD28 together with TCR (Chambers et al., 1997a; Khattri et al., 1999; Masteller et al., 2000). Since the lymphoproliferative disease observed in the CTLA-4 deficient mice was very reminiscent of autoimmune diseases, it was hypothesized that CTLA-4 may play a role in balancing T helper differentiation and cytokine production. To better understand the significance and the underlying mechanism for the lethal phenotype in the CTLA-4 deficient mice, T cells were harvested from the mice before the onset of the disease and analysed in vitro (Oosterwegel et al., 1999b). It was found that CTLA-$4^{-/-}$ T cells demonstrated an altered cytokine profile. While the CTLA-$4^{-/-}$ T cells exhibited a spontaneous proliferation and altered peptide response, there was a dramatic

increase in IL-4 production, suggesting that the lack of CTLA-4 was skewing T cells to undergo an unhindered Th2 differentiation.

The outcome of a T cell response to antigen is partly dictated by the avidity and binding efficiency of TCR to the peptide encountered in the presence of the MHC molecule. It has long been determined that peptides that generate a positive signalling event could be classified as either agonists or weak agonists, depending upon their binding strength. It is well known, for example, that naïve and memory cells have different requirements for activation (Byrne et al., 1988; Croft et al., 1994; Farber et al., 1997). Costimulatory molecules such as CD28 have shown to be able to lower the threshold of activation by enhancing JNK activation (Su et al., 1994). Thus, the inhibitory role of CTLA-4 on the threshold of T cell activation was examined. CTLA-4 deficient mice readily showed an increased proliferative capacity before and after CD3 engagement, suggesting that CTLA-4 may act to keep T cells in a resting state. Furthermore, using peptide based assay against TCR transgenic deficient cell for CTLA-4, it was observed that a shift in the dose-response curve was responsible for increased proliferation and cytokine production at lower concentrations of the available peptide (Chambers et al., 1999; Oosterwegel et al., 1999a). In vivo, the lack of CD28 prolonged survival of allogeneic heart transplants (Rathmell et al., 1998) and treatment with CTLA-4Ig or blocking anti-CTLA-4 antibody accelerated graft rejection compared to control animals. These data clearly suggest that the loss of CTLA-4 signalling may decrease the threshold of TCR activation. It is conceivable that the cycling of intracellular stores of CTLA-4, subsequent polarisation of CTLA-4 to the cell surface at the site of TCR contact and accumulation at the immunological synapse may regulate peptide-MHC interactions and alter the requirements for activation (Egen and Allison, 2002; Linsley et al., 1996).

Optimal T cell activation is dependent on antigen presentation by APCs, which express ligands for a variety of surface receptors on T cells such as ICAM and B7 ligands. To further analyse the role of CTLA-4 in the context of antigen presentation, a variety of transgenic mice expressing specific $CD4^+$ or $CD8^+$ restricted TCR have been crossed with CTLA-$4^{-/-}$ mice. To examine the role of CTLA-4 on the function of $CD4^+$ T cells, CTLA-4 deficient mice were crossed onto an MHC class II-restricted TCR transgenic strain (AND TCR) that is selected on MHC class II $A^b$ background (Chambers et al., 1999)[1] whereas to examine the role of CTLA-4 in regulating peptide-specific responses by $CD8^+$ T cells,

[1] AND TCR (Vβ3Vα11) reacts with a peptide derived from the pigeon cytochrome C ($PCC_{88-104}$) presented in the context of $I\text{-}E^k$ (Kaye and Hedrick, 1988)

CTLA-4$^{-/-}$ mice were crossed to transgenic mice expressing MHC class-I restricted 2C TCR (Chambers et al., 1998)[2]. A RAG null mutation was introduced in these mice to generate transgenic RAG$^{-/-}$CTLA-4$^{-/-}$ mice in order to express only the specified transgenic TCR. Using the MHC class-II restricted T cell receptor AND in a RAG1$^{-/-}$ background introduced into CTLA-4 deficient mice, it was shown that CD4$^+$ T cells did not become activated in a manner observed in CTLA-4$^{-/-}$ mice (Chambers et al., 1999). These cells, however, did have an increased proliferation and shift in the dose response curve in response to the agonist peptide $PCC_{88-104}$ and $MCC_{93-103}$ during the primary as well as secondary stimulation. Thus, these results suggested that the repertoire of CD4$^+$ T cells influences the incidence of the lymphoproliferative disease observed in the CTLA-4 deficient mice and that the magnitude and breadth of the response may be controlled by CTLA-4 specifically by lowering the threshold of signaling and increasing responses to possible autoantigens. To investigate the role of CTLA-4 in CD8$^+$ T cell responses, MHC class-I restricted 2C TCR transgenic mouse strain was used to determine T cell mediated cytotoxic responses. It was found that CTLA-4 played a limited role in regulating primary cytotoxic responses where the amplitude of the primary response remained unchanged compared to normal controls. LCMV/CTLA-4$^{-/-}$ mice were used as well to study in vivo CTL responses in CD8$^+$ T cytotoxic responses (Bachmann et al., 1998). It was shown that CTLA-4 deficient mice expressing the LCMV transgene were still able to mount a successful cytolytic response (Bachmann et al., 1998). There seemed to be no abnormalities reported in terms of cytolytic activity, proliferation and clearance of T cells with either agonistic or partial agonistic peptides. In addition, the lack of CTLA-4 had no effect on peripheral deletion mechanisms of CD$^+$8 T cells in response to viral load. Thus, CTLA-4 negative signaling may affect CD4+ and CD8+ populations differentially.

The presence of CTLA-4 expression during thymocyte maturation and differentiation suggested that CTLA-4 may play a prominent role in thymic differentiation (Wagner et al., 1996). Several groups addressed this question by investigating thymocyte differentiation in CTLA-4 deficient mice at 2-3 weeks of age, before the onset of the lymphoproliferative disease. It was initially found that thymi from CTLA-4 deficient mice had a 10 fold decrease in the percentage of double positive CD4$^+$CD8$^+$ thymocytes and a 5 fold increase of double negative CD4-CD8- thymocytes (Tivol et al., 1995). However, the CD4/CD8 ratio remained normal (Waterhouse et al., 1995). It became clear that the

[2] 2C TCR (Vβ8.2 Dβ2 Jβ2.4 Cβ2;Vα3 Jα58 Cα) reacts with an endogenous 8-mer peptide derived from the Krebs cycle protein αketoglutarate dehydrogenase (p2Ca: LSPFPFDL) associated with MHC class-I H-2L$^d$ (Ukada et al., 1993, Sha et al., 1988).

increases in the number of T cells in the thymus might be a direct consequence of the massive expansion and infiltration of activated T cells from the periphery. To further examine the possibility of whether CTLA-4 played a direct role in thymocyte development, the thymi of fetal and newborn mice were removed and analysed for any phenotypic changes during ontogeny (Chambers et al., 1997b). It was found that there were no alterations in thymocyte subsets during early thymic differentiation in the fetal or newborn stages, and the thymocytes remained normal prior to the onset of the lymphoproliferative disorder. In relatively older mice, the phenotypic changes were predominantly observed in the parathymic lymph nodes that border the thymus. Infiltration of these cells into the thymus was perceived as the causal link.

The ability of the TCR to send maturational signals for thymocyte differentiation was also assessed. First, it was shown that there was no difference in intracellular calcium flux or the induction of apoptosis by immobilized anti-CD3 in CTLA-4 deficient thymocytes compared to the normal littermate. More extensive studies were pursued using TCR transgenic H-Y/CTLA4$^{-/-}$ mice[3] (Waterhouse et al., 1997). There appeared to be no differences in the proportion of double positive and single positive subset between the negatively selecting (male) or positively selecting (female) background of the transgenic males and their wild type counterpart. To confirm these results, an LCMV/CTLA-4$^{-/-}$ transgenic mouse was used to further study the influence of peptide density and agonist strength on negative selection. Using a weak and a strong agonist peptide, it was determined that deletion of thymocytes was equivalent in the LCMV/CTLA-4$^{-/-}$ mice compared to normal littermates. Thus, it is henceforth concluded that CTLA-4 does not have any apparent role in thymocyte selection and differentiation.

### 1.2.2. T cell responses and T helper cell differentiation

It is now clear that the major influence of CTLA-4 negative signalling arises in the peripheral compartment of T cells. It has been shown that CD28 costimulation can polarize T cells towards a Th2 subset (King et al., 1996; Rulifson et al., 1997; Webb and Feldmann, 1995). Thus, it was thought that CTLA-4 may regulate differentiation of cells towards a Th1 phenotype. To address this question, normal naïve CD4$^+$ T cells were isolated and primed under selective conditions to promote T helper differentiation (Kato and Nariuchi, 2000). When CTLA-4 was allowed to bind to its ligand B7-1, the cells were

[3] TCR (Vα(T3.70)Vβ8) recognises a male antigen presented on H2-D$^b$. Thymocytes are negatively selected in male H-2$^b$ but positively selected in female H-2$^b$.

polarized towards a Th1 phenotype, namely, an increase in IL-2 and IFNγ during the secondary response. In addition, TGFβ1 was induced after CTLA-4 co-crosslinking. Addition of neutralising antibodies to TGFβ1 in the cultures reverted polarization towards Th2 subset. Thus, it is speculated that CTLA-4 may promote Th1 polarization through production of TGFβ1. This result would lead one to believe that CTLA-4 may predominantly function in downregulation of Th1 mediated responses. Thus, to address this question, the functional properties of CTLA-4 in Th1 and Th2 derived clones was assessed (Alegre et al., 1998; Doyle et al., 2001). Contrary to what was expected, CTLA-4 was expressed at higher levels in Th2 rather than Th1 clones. This finding was confirmed at the level of mRNA and protein expression. In some way, this discrepancy may be resolved since it is known that CD28 costimulation is necessary to upregulate CTLA-4 expression as well as promote Th2 differentiation. The blocking ability of CTLA-4 on cytokine production was established in both Th1 and Th2 subsets. Surprisingly, proliferation was marginally increased in both Th1 and Th2 clones. Therefore, the lack of CTLA-4 expression may polarise T helper cells into a Th2 differentiation pathway. However, CTLA-4 signalling occurs both in Th1 and Th2 cells. It is still unknown whether CTLA-4 provides the same qualitative or/and quantitative signals in both type of helper T cells.

## 1.3. The role of CTLA-4 in tolerance induction

The appreciation of tolerance induction and T cell non-responsiveness to particular antigens, such as in the case of self-antigen recognition, has been an invariably complicated but consistent force in the need to understand the fundamental nature and structure of the immune response. The 2-step model for T cell activation recognised that, in the first instance, TCR distinguishes peptide in the context of the appropriate MHC and, secondly, that a costimulatory molecule, which in most cases was supplemented by CD28, provided a secondary additional signal. Absence of costimulation and recognition of antigen alone consequently lead to a state of unresponsiveness. This notion of the anergic state arose mainly from the study of cell lines in vitro. However, it became clear that the model for non-responsiveness was more complex in an in vivo situation. The concept of T cell peripheral tolerance had remained elusive and poorly defined until the emergence of negative regulatory molecules such as CTLA-4. Based on previous findings of CTLA-4 downregulating T cell activation, it was of interest to study CTLA-4 in the perspective for the requirement of tolerance induction.

The role of CTLA-4 in the induction of anergy was assessed using in vivo models. Clues relating to the involvement of costimulation in the induction of unresponsiveness came with the finding that blocking CD28 binding to its ligand induced a refractory state of the T cell (Boussiotis et al., 1996; Harding et al., 1992; Kang et al., 1992; Li et al., 1996; Tan et al., 1993). However, it was soon to be discovered that the lack of IL-2 production was responsible for this phenotype (Boussiotis et al., 1994; Boussiotis et al., 1997). The role of CTLA-4 in IL-2 production had been established, however it was still unknown whether CTLA-4 could be also be directly responsible for induction of anergy. To investigate the role of CTLA-4 in the induction of antigen-specific T cell tolerance, an in vivo model was used where DO11.10 transgenic cells were adoptively transferred into syngeneic recipient (Perez et al., 1997). Induction of tolerance was achieved by injecting a large dose of antigen intraperitoneally. It was shown that T cells from tolerised animals did not produce IL-2 or IFNγ and essentially showed no proliferation. In addition, administration of a tolerogenic form of the antigen did not induce IL-4, IL-5 and IL-10 secretion, suggesting that the cells had not differentiated into a Th2 phenotype. While B7 interaction was shown to induce functional inactivation, treatment with anti-CTLA-4 mAb was able to prevent the induction of T cell tolerance. These results were then later confirmed by using DO11.10 mice lacking CTLA-4 (Greenwald et al., 2001). Immunization with a tolerogenic dose of $OVA_{323-339}$ peptide of adoptively transferred DO11.10/$RAG2^{-/-}$ /$CTLA\text{-}4^{-/-}$ T cells showed an expansion of KJ1-26 T cells in vivo marked by increase in cdk2 enzymatic activity, decreases expression of p27kip and an increased production of IL-2 in vitro compared to the control DO11.10/$RAG2^{-/-}$ cells.

Interestingly, CTLA-4 has also been involved in the induction of tolerance in other models. During costimulation deficient antigen presentation, CTLA-4 was able to ligate to neighboring T cells expressing the B7-1 molecules (Chai et al., 2000). CTLA-4 was also shown to be involved in induction to tolerance against superantigens (Walunas and Bluestone, 1998) where SEB treatment of $CD4^{+}V\beta8$ T cells accompanied with blocking anti-CTLA-4 mAb were more responsive to a secondary rechallenge. The role of CTLA-4 was further demonstrated in a model for the induction of tolerance with low densities of agonistic peptide (Mirshahidi et al., 2001). Despite such a growing amount of evidence, it was surprising to find that induction of anergy could still be established in primary 2C TCR transgenic T cells which are CTLA-4 deficient, thus suggesting that other mechanisms may also be involved in induction of anergy (Frauwirth et al., 2000).

## 1.4. Generation of Suppressor T cells

To explore the possibility that the production of suppressive cytokines or a suppressive T cell population may mediate tolerance, CTLA-4 was investigated in the context of "naturally" occurring subpopulations of peripheral blood T cells (Maloy and Powrie, 2001; Mason and Powrie, 1998; Read and Powrie, 2001). By analysing the phenotype of different populations of cells in naïve mice, it was found that CTLA-4 could be expressed at very low levels in 7-10% of peripheral non-activated T cells and in thymus derived $CD4^+CD25^+$ T cells. These cells have been isolated from human peripheral blood (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Jonuleit et al., 2001; Stephens et al., 2001; Taams et al., 2001) as well as in mouse spleen (Takahashi et al., 2000; Thornton and Shevach, 1998). $CD4^+CD25^+$ T cells had been shown to inhibit colitis in SCID transfer models of colitis (Groux and Powrie, 1999; Powrie et al., 1996; Read et al., 2000). It became clear that these cells had special properties that rendered them capable of inducing anergy in vivo and in vitro, thus they became known as T regulatory suppressive $CD4^+CD25^+$ cells (Ts or Treg) (Levings et al., 2001; Shevach et al., 2001; Suri-Payer et al., 1998; Thornton and Shevach, 2000). Human Treg cells (hTreg) have also shown to have the same properties as their mouse counterpart, suggesting that a common underlying evolutionary mechanism may be involved in suppression (Read et al., 1998; Taams et al., 2001). The hTreg cells are also found to express intracellular levels of CTLA-4 (Dieckmann et al., 2001; Jonuleit et al., 2001). These cells are characterized by having a low proliferative response to antigen or anti-CD3 stimulation and fail to produce IL-2. In addition, co-culturing of Treg with $CD4^+CD25^-$ cells showed that Treg cells were able to suppress proliferation of $CD4^+CD25^-$ in a cell-contact dependent manner (Nakamura et al., 2001; Thornton and Shevach, 1998) It was thought that CD28 costimulation, IL-10, IL-4 and TGFβ contributed to the development of Treg cells (Asseman et al., 1999; Jonuleit et al., 2000; Yamagiwa et al., 2001). However, data emerged that $CD4^+CD25^+$ T cells from $CD28^{-/-}$, $IL\text{-}10^{-/-}$ and $IL\text{-}4^{-/-}$ mice were also able to induce suppression. Moreover, blocking antibodies to IL-10, IL-4, TGFβ, IFNγ had no effect on suppression. By contrast, blockage of CTLA-4 signalling had a profound effect on the suppressive activity of Treg cells (Takahashi et al., 2000). It was shown that Treg cells from $CTLA\text{-}4^{-/-}$ mice could exhibit only a weak suppressive activity compared to wild type mice. However, blocking CTLA-7/B7 pathway did not completely abrogate the suppressive property of hTreg cells (Baecher-Allan et al., 2001).

Thus, the function of CTLA-4 in the suppressive mechanism of $CD4^+CD25^+$ T cells is still unclear. Because of the consistency in finding that suppression is mediated and dependent on cell-contact, it is speculated that CTLA-4 may compete for B7 ligand and attenuate the response. It is interesting to note that $RAG2^{-/-}$ chimeric mice reconstituted with half normal and half CTLA-$4^{-/-}$ derived bone marrow prevent the lymphoproliferative disease observed in CTLA-4 deficient mice (Bachmann et al., 2001; Bachmann et al., 1999). One may speculate that CTLA-4 expressing Treg cells derived from normal bone marrow may dampen intrinsic activation. There is also evidence that B7-1 and B7-2 expression on the surface of DC can be downregulated by $CD4^+CD25^+$ T cells, although the mechanism has not yet been elucidated (Cederbom et al., 2000). In addition, TGFβ was also found to be present on the cell surface of $CD4^+CD25^+$ T cells in high amounts. In contrast to previous studies, one study showed that addition of anti-CTLA-4 to anti-CD3/CD28/IL-2 stimulated Treg cells lead to an increase in T cell proliferation and an enhanced production of TGFβ1 (Yamagiwa et al., 2001). Blocking anti-TGFβ inhibited the suppressive activity. Detection of TGFβ was observed in freshly isolated and upregulated in stimulated Treg cells. Thus, it was concluded that membrane bound TGFβ might potentially act to inhibit T cell proliferation. The authors argue that the disparity between their results and previous studies is based on the experimental protocol and the level of secreted as well as membrane bound active form of TGFβ in the cultures.

Other mechanisms for induction of anergy, besides CTLA-4 and TGFβ, may operate. Using an in vitro assay, it was described that $CD4^+CD25^-$ T cells can upregulate GRAIL, a novel gene involved in induction and maintenance of anergic state, after co-incubation with $CD4^+CD25^+$ T cells (Ermann et al., 2001).

### 1.2.5. CTLA-4 regulation of cytokine production

The hallmark of CTLA-4 mediated TCR inhibition is the loss of IL-2 production. Initial studies utilising soluble Fab fragment antibodies against CTLA-4 or B7 showed an augmentation of IL-2 production by normal T cells, suggesting that blocking interaction of CTLA-4 augmented the responses by removing an inhibitory signal (Krummel and Allison, 1995; Krummel and Allison, 1996; Walunas et al., 1994). CTLA-4 was found to counteract the costimulatory activity of CD28 (Azuma et al., 1993; Chambers and Allison, 1997c; Chen et al., 1994; Coyle et al., 2000; Damle et al., 1994; Hathcock et al., 1993; Lenschow et al., 1993; Linsley et al., 1992). In vitro studies using crosslinking

anti-CTLA-4 antibodies on antibody coated microspheres have revealed that CTLA-4 can block TCR/CD3/CD28 mediated activation, proliferation and IL-2 production. Addition of exogenous IL-2 reversed IL-2Rα downregulation and restored T cell proliferation. The effects of CTLA-4 on cytokine production were also investigated at a molecular level. It was found that CTLA-4 crosslinking inhibited the accumulation of IL-2 mRNA (Finn et al., 1997) but did not influence the rate of decay of IL-2 mRNA, which is normally stabilised by CD28 (Brunner et al., 1999). Interestingly, CTLA-4 could inhibit IL-2 mRNA transcription in normal T cells but did not inhibit $bclX_L$ mRNA after 4h of culture with anti-CD3, anti-CD28 and anti-CTLA-4 (Blair et al., 1998). Thus, these results provided ample evidence that CTLA-4 specifically blocks the signalling pathway leading to IL-2 production. Using 2C transgenic mice deficient for CD28, it was shown that CTLA-4 was able to inhibit IL-2 and IFNγ production from in vivo primed T cells restimulated in vitro with APCs (Fallarino et al., 1998). Thus this data suggested that CTLA-4 inhibits T cell activation by early attenuation of signals delivered by the TCR as well as CD28. CTLA-4 was also shown to antagonise upregulation of ICOS as well as ICOS costimulation of TCR signalling on preactivated cells (Riley et al., 2001). In addition to blocking IL-2, CTLA-4 costimulation was found to induce TGFβ production (Chen et al., 1998) in murine $CD4^+$ T cells. Using anti-TGFβ neutralising antibodies or $TGF\beta^{-/-}$ $CD4^+$ T cells, it was shown that TGFβ was necessary for the downregulation of the response initiated by CTLA-4 crosslinking. Interestingly, $TGF\beta^{-/-}$ mice also suffer from a lymphoproliferative disease which is reminiscent of the $CTLA\text{-}4^{-/-}$ phenotype (Kulkarni et al., 1993; Shull et al., 1992). This data would support a link between CTLA-4 mediated inhibition via costimulation of TGFβ. However, using $TGF\beta1^{-/-}$ and $Smad3^{-/-}$ mice, it was shown that CTLA-4 could inhibit proliferation compared to wild type strain (Sullivan et al., 2001). In addition, they could not measure induction of TGFβ production after co-culturing normal wild type cells with anti-CTLA-4 mAb. It is possible that the discrepancy could be attributed to different activating protocols.

To further study the pathway that may be involved in dowregulation of T cell activation and IL-2 production, several groups have investigated downstream effectors of the TCR signaling pathway that may be affected by CTLA-4. MAPK kinases are known to be involved in transducing signals from the cytoplasm to the nucleus via a cascade of phosphorylation involving the Jun NH2-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) (Blenis, 1993; Howe et al., 1992). It has been shown that JNK and ERK activation can be blocked by CTLA-4 ligation (Calvo et al., 1997).

The TCR and CD28 have been shown to induce activation of several nuclear factors involved in cytokine production. Since the IL-2 gene is tightly connected to CD28 costimulation, it was no surprise that the IL-2 promoter contains a CD28 responsive element (CD28RE) (Fraser et al., 1991). The Rel family of nuclear factors, RelA, c-Rel, NFκBp50 and p65 subunits associate with the CD28RE (Bryan et al., 1994; Lai et al., 1995). In resting cells, Rel family proteins associate with IκB or the ankyrin-containing cytoplasmic proteins such as NFκB1 (p105) and NFκB2 (p100) and remain sequestered in the cytoplasm until the cell is activated. After activation, proteolytic degradation of IκB proteins allows active NFκB to translocate into the nucleus. While anti-CD3 and anti-CD28 costimulation induces IκBα degradation, CTLA-4 coligation blocked the degradation of IκBα and restored its levels in the cytoplasm. In addition, RelA activity and translocation into the nucleus was increased after culturing the cells with anti-CD3 and anti-CD28, where this effect was counteracted by CTLA-4 stimulation (Pioli et al., 1999). A different group using NFκB luciferase reporter transgenic mice (Fraser et al., 1999) confirmed these results. IL-2 and other cytokine genes are also dependent on functional transcription factor AP-1 and NFAT complexes in order to be fully transcribed (Shapiro et al., 1998). The AP-1 complex is composed of the subunits of c-jun and c-fos where c-jun is a substrate of JNK (Chatta et al., 1994; Kyriakis et al., 1994). NFAT is translocated into the nucleus after it is dephosphorylated by the calcium dependent phosphatase Calcineurin (Rooney et al., 1995). Using luciferase reporter transgenic mice for AP-1 and NFAT, it was shown that $CD4^+$ T cells cultured in the presence of anti-CD3 and anti-CD28 induced significant AP-1 and NFAT transcriptional activity. Addition of anti-CTLA-4 blocked activity of both of these factors. The accumulation of AP-1 and NFAT complexes in the nucleus was also abolished by CTLA-4 ligation (Fraser et al., 1999; Olsson et al., 1999).

## 1.6. Inhibitory role of CTLA-4 in T cell proliferation and cell cycle progression

The role of CTLA-4 in T cell proliferation is well established in several mouse models. In vivo studies have shown that addition of soluble anti-CTLA-4 to T cell cultures enhanced CD28 costimulation of proliferation (Krummel and Allison, 1995); Walunas, 1996 #217; Krummel, 1996 #210]. This effect is thought to result from the blocking effect of the antibody. In fact, addition of anti-B7 or CTLA-4Ig in the presence of anti-CD28 increased proliferation, suggesting that blocking CTLA-4 interaction with its ligand removed its inhibitory function. Cell cycle analysis revealed that cultures stimulated in the presence of anti-CD28 resulted in an increase in the number of cells in the S/G2 phase, while cultures costimulated in the presence of anti-CTLA-4 were arrested a the G0/G1

phase. However, another study showed that no differences were observed in cell cycle progression at 24h in anti-CD3 and anti-CD28 stimulated cells compared to those in the presence of anti-CTLA-4. However, there was a difference observed at 72h in the S/G2 phase in the anti-CTLA-4 treated cultures (Walunas et al. 1996). Cyclins, cyclin-dependent kinases (cdk) and cell cycle inhibitors control cell cycle progression. As each phase of the cell cycle induces different pattern of protein expression, several groups investigated the role of CTLA-4 in the regulation of each of the cell cycle phase. There are two major checkpoints, the transition from G1/S and the later stage G2/M. After T cell activation, the cells enter the cell cycle from G0 to G1 phase. During this period, D-type cyclins and their cdks (cdk4 and cdk6) are increased. Although cdks are present throughout the cell cycle, expression of cyclins determine the enzymatic activation of the holoenzyme and the phase of the cell cycle. When the cycle reaches the G1 restriction point, cyclin E, cyclin A and cdk2 are highly expressed. Progression into S phase is marked by hyperphosphorylation of the retinoblastoma gene (Rb). Members of the INK family of inhibitors (p15INK4b, p16INK4a, p18INK4c and p19INK4d) and cip/kip inhibitors (p21kip1 and p57kip2) inhibit cell cycle progression by associating with their respective cyclins and cdks. It has been shown that transcription of the cell cycle dependent proteins cyclin D3, cdk4 and cdk6 as well as degradation of the cell cycle inhibitor p27kip were inhibited by CTLA-4 (Brunner et al., 1999). These results were also confirmed using an induced tolerance model for a transgenic TCR specific for OVA peptide (Greenwald et al., 2001) and a triple knockout mouse which lacks B7-1/B7-2 and CTLA-4$^{-/-}$ (Greenwald et al., 2002).

More recently, a study from our laboratory demonstrated that CTLA-4 might affect surface expression of membrane rafts during T cell activation which would lead to inhibition of proliferation (Martin et al., 2001). Ganglioside and cholesterol enriched microdomain (rafts, GEMs) serve as focal points that recruit signaling effectors such as p56$^{lck}$, LAT, Vav, SLP-76 and PKCθ (Sedwick and Altman, 2002). CD28 was shown to promote TCR/raft colocalisation, which correlated with increased proliferation of T cells (Viola et al., 1999). The adaptor LAT (linker of activation) was found to localise in the raft membrane fraction after activation. In contrast, CTLA-4 co-crosslinking abrogated proliferation and raft recruitment to the plasma membrane. These findings suggest that proliferation and T cell activation might be tightly controlled by surface expression of raft microdomains. Blockage of raft surface expression by CTLA-4 provides an alternative mechanism by which cell cycle progression may be inhibited.

Studies were also performed to analyse the role of CTLA-4 in cell cycle progression and induction of cell death. There was no increase in apoptosis in cultures stimulated with anti-CTLA-4 in the presence of anti-CD3/CD28 compared with anti-CD3 or anti-CD3/CD28 alone (Krummel and Allison, 1996). In fact, during the culture period, recovery of viable cells stimulated in the presence of anti-CTLA-4 was higher than in non-stimulated or cultures stimulated with anti-CD3 alone. In addition, using Fas deficient lpr mice, CTLA-4 was able to block proliferation in response to anti-CD3 and anti-CD28 and (Scheipers and Reiser, 1998). However, some studies suggested that CTLA-4 may play a role in AICD. In one study, using a T cell clone, it was shown that CTLA-4 ligation lead to induction of apoptosis (Gribben et al., 1995). Cell death was averted by supplying exogenous IL-2 to the cell culture, suggesting that the CTLA-4 induced AICD was an indirect effect. In a thymocytic model for CD3 induced negative selection, it was shown that intrathymical blockage of CTLA-4 dramatically inhibited CD3 depletion of $CD4^+CD8^+$ double positive immature thymocytes (Cilio et al., 1998); (Samoilova et al., 1997). This data suggested that CTLA-4 may regulate TCR mediated negative selection of thymocytes. However, it is unclear whether the in vivo administration of the antibody served as a "blocking" agent or as a stimulating antibody. One could interpret these results as CTLA-4 mediated blockage of CD3 induced apoptotic signals. In addition, it was shown that CTLA-4 may induce cell death in activated T cells, independently of a Fas mediated mechanism. The authors did not speculate further on other possible mechanisms to explain their data. Thus, the role of CTLA-4 in induction of apoptosis is still controversial. Taken together, this data suggests that CTLA-4 does not restrict proliferation by inducing apoptosis but by inhibiting cell cycle progression.

## 1.7. Mechanism of CTLA-4 signalling

Despite its pivotal role in attenuating activation and the knowledge acquired from the extensive number of functional in vitro and in vivo studies here cited, little is known regarding the mechanism responsible for intracellular CTLA-4 inhibitory signaling. Progress in the field of signal transduction has been largely hampered by the fact that CTLA-4 and CD28 bind to the same ligands, namely B7-1 and B7-2, as well as by the fact that CTLA-4 peak expression is detected at 40 h after stimulation (Perkins et al., 1996). In

addition, the complex and peculiar pattern of CTLA-4 expression (low levels of surface expression but high levels of intracellular expression) has been the basis for much discussion on the interpretation of the results obtained (Alegre et al., 1996; Iida et al., 2000). Nevertheless, the analyses of transfected cell lines, with various mutant forms of CTLA-4, have provided an insight into possible models for signalling mechanisms.

### 1.7.1. Role of the CTLA-4 cytoplasmic tail

The cytoplasmic tail of CTLA-4 consists of a short intracellular peptide sequence of 36 amino acids and possesses two tyrosine residues at position Y201 and Y218, located in the motifs YVKM and YFIP, respectively (Leung et al., 1995) (Figure 1). These tyrosines can be phosphorylated by the src kinases p56lck, p59fyn (Bradshaw et al., 1997; Chuang et al., 2000; Miyatake et al., 1998) and the Tec kinase Rlk (Schneider et al., 1998). While phosphorylation of the tyrosine in the YVKM motif was reported to recruit PI 3-kinase and the Src homology 2 domain-containing tyrosine phosphatase (SHP-2) (Chuang et al., 1999; Cilio et al., 1998; Lee et al., 1998; Marengere et al., 1996; Schneider et al., 1995), the clathrin adaptor complexes AP-1 and AP-2 bind to the non-phosphorylated version of the motif (Bradshaw et al., 1997; Chuang et al., 1997; Schneider et al., 1999; Shiratori et al., 1997; Zhang and Allison, 1997). Phosphorylation of this residue prevents binding of AP-2 to CTLA-4 and internalization of the receptor. Recently, the phosphatase PP2A has been reported to bind to CD28 and CTLA-4. Although its function in the generation of signals is still unclear (Chuang et al., 2000), it is hypothesized that PP2A may inhibit signaling via CD28. Phosphorylation of the cytoplasmic tail of CD28 may act to relieve binding of the negative regulatory molecule. The role of PP2A binding to CTLA-4 may have analogous effects, although it has not yet been proven experimentally. As of yet, there have been no proteins reported to bind to the Y218 motif. Several groups have proposed models where negative signaling can be generated in a tyrosine-independent manner by sequestration of the B7 ligands (Baroja et al., 2000; Cinek et al., 2000; Nakaseko et al., 1999), while others maintain that negative

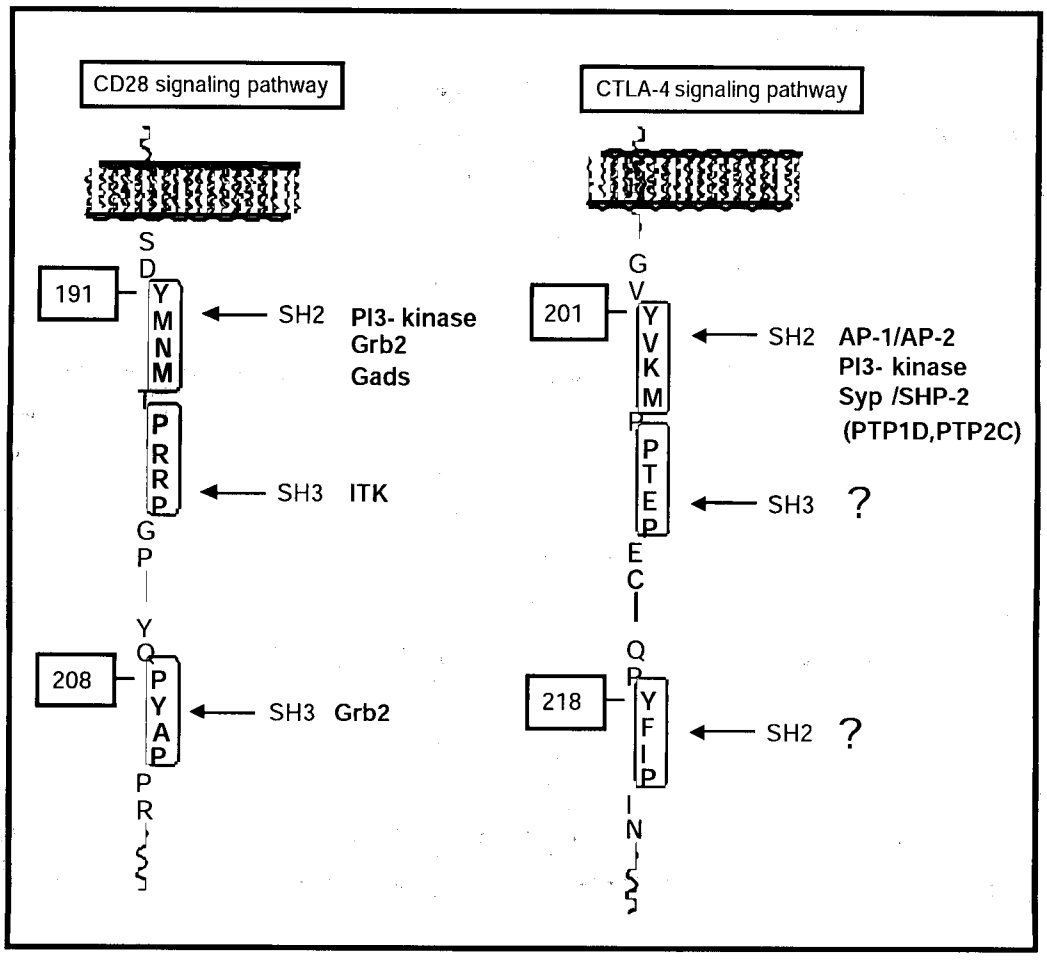


Figure 2: Comparison between CD28 and CTLA-4 cytoplasmic tails. CD28 cytoplasmic tail is composed of 41 amino acids. The tyrosine motifs at 191 and 208 can be phosphorylated by p56lck and p59fyn and binds to PI3-kinase, Grb2 and Gads. The proline rich mitof has been shown to bind to the SH3 domain of ITK. CTLA-4 cytoplasmic tail is composed of 36 amino acids. The YVKM motif can be phosphorylated by p56lck and p59fyn and has been shown to bind to AP-1/AP-2, PI3-kinase and SHP-2. The 218 motif is still undefined.

signalling still requires phosphorylation of the YVKM motif (Marengere et al., 1996; Miyatake et al., 1998).

### 1.7.2. The role of SHP-2 in CTLA-4 signalling

Several models have been proposed to explain CTLA-4 mediated negative signaling. One model proposes that negative signaling through CTLA-4 occur by binding to a phosphatase. SHP-2, but not SHP-1 (Chambers and Allison, 1996; Cilio et al., 1998; Frearson and Alexander, 1998; Lee et al., 1998; Marengere et al., 1996), was found to associate to CTLA-4. It was found that CTLA-4 deficient mice displayed constitutively activated forms of the tyrosine kinases FYN, LCK, ZAP-70 and activation of the ras pathway. Immunoprecipitation studies showed that CD3ζ and the adaptor Shc are associated and were hyperphosphorylated in CTLA-4$^{-/-}$ T cells and that SHP-2 could associate in CTLA-4 control cells. Using glutathione S-transferase (GST) fusion protein, it was determined the SHP-2 bound to the phosphorylated YVKM motif. Subsequently, it was demonstrated that CTLA-4 crosslinking during restimulation with anti-CD3 decreased tyrosine phosphorylation of CD3ζ and the adaptor LAT (Lee et al., 1998). They also found that CTLA-4 could associate with the CD3ζ chain without the need for phosphorylation of the CTLA-4 molecule. However, the molecular nature of the association between CD3ζ chain and CTLA-4 molecule was not elucidated. Despite studies showing less recruitment of SHP-2 binding to phosphorylated CTLA-4 after antigen stimulation (Gajewski et al., 2001), there is some evidence supporting this model (Lee et al., 1998; Waterhouse et al., 1995). Nonetheless, this model presents several caveats. In lieu of a negative effect of SHP-2 on signaling, SHP-2 is an essential component in positive signaling by many other growth factors such as IL-2 receptor and the TCR/CD3 complex (Frearson and Alexander, 1998; Gadina et al., 1998; Hadari et al., 1998). In addition, SHP-2 binds to an ITIM motif, which is missing in the tail of CTLA-4. It is noteworthy to mention that several attempts made by other groups to immunoprecipitate SHP-2 associated with CTLA-4 in different cell lines were unsuccessful in finding an association (Cinek et al., 2000; Schneider and Rudd, 2000). There is also data suggesting that Jak2 can phosphorylate the YVKM motif and bind to the box 1-like proline rich sequence 205-209 PPTEP of CTLA-4 (Chikuma et al., 2000). The functional relevance of this association has yet to be determined.

### 1.7.3. Competitive ligand binding

Several groups have proposed a revised model where CTLA-4 negative signalling can be generated in a tyrosine-independent manner. This alternative model was initially based on experiments that showed that inhibition of IL-2 production did not require the cytoplasmic tyrosine residues in CTLA-4. Using LCK or ZAP-70 deficient cells transfected with CTLA-4 (Baroja et al., 2000) and Jurkat cells expressing various single substitution and truncated mutants of CTLA-4 (Cinek et al., 2000), it was demonstrated that surface trafficking of CTLA-4, but not inhibition of IL-2 production or Erk activation, was affected. Moreover, a truncated mutant with only a membrane proximal region of 7 amino acids left on the cytoplasmic tail was able to fully inhibit T cell activation (Nakaseko et al., 1999). These results lead to the hypothesis that a negative signal could be generated without the need for the tyrosine motifs and, for that matter, most of the cytoplasmic tail. Further examination of a non-signalling truncated mutant using B7-Ig coated beads revealed that CTLA-4 when expressed at high levels could sequestrate B7 binding from CD28 and thus, dampening T cell activation (Carreno et al., 2000).

## 1.8. Introduction to experimental model used

Professor Rudd's group has been investigating the molecular mechanism of CTLA-4 negative signalling. The low level of CTLA-4 expression after activation, as well as the intracellular pattern of expression, are the main constraints which render CTLA-4 a difficult molecule to study. To bypass this problem, I initially used the hybridoma DC27.10 cells which were stabily transfected with human CTLA-4. Dr. Hui Hu produced these cells in the laboratory as a model to study the inhibitory role of CTLA-4 in T cell activation. Different clones were generated of the DC27.10 transfected hybridomas expressing CTLA-4 as well as a mutant with a substituted tyrosine residue at position 201 for a phenylalanine. Using this model, the lab identified several proteins that bind to CTLA-4. PI-3 kinase was shown to bind to the phosphorylated YVKM motif and AP-1 and AP-2 to the non-phosphorylated form. However, SHP-2 binding to CTLA-4 was never observed in the CTLA-4 transfectants (Schneider and Rudd, 2000). The reason for this is unknown. It is possible that the hybridoma used as a model does not permit this association. It is also possible that the interaction between SHP-2 and CTLA-4 is not direct since the

cytoplasmic tail of CTLA-4 is missing the consensus ITIM motif for binding (V/IXYXXL/V). This finding was also reported recently by another group (Cinek et al., 2000). In addition, I generated stable T8.1 transfectants (TCR restricted for tetanus toxoid peptide (Ttox) in the context of HLA-DR*1102) also expressing high levels of CTLA-4 to use as a model to study CTLA-4 signalling pathways in the physiological context of antigen presentation.

### 1.8.1 Aims of the project

My thesis project involves several key questions in finding the role of CTLA-4. The aim of my project revolved on the following fundamental questions:

1) Which level does CTLA-4 inhibit TCR mediated signals?
2) What is the role of the tyrosine motifs in the cytoplasmic tail of CTLA-4?
3) What is the molecular mechanism involved in the inhibitory function of CTLA-4?
4) Does the cytoskeleton play a role in the negative function of CTLA-4?

By elucidating these questions, we expect a better understanding of the molecular nature of CTLA-4 negative signalling which will serve to grasp the complexity of signalling pathways regulating T cell activation. This knowledge would have profound implications in manipulating the immune response applied to areas involved in modulating immunotherapy and immune dysfunctions.

# Chapter 2.
# CTLA-4 regulates morphological changes in T cells

# Cell shape remodelling induced by CTLA-4

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key words: CTLA-4 , filopodia, cytoskeleton

## 2.1. Abstract

CTLA-4, which is a member of the CD28 costimulatory family, has been extensively studied as a negative regulatory molecule of the immune system. Its effect on downmodulation of cytokine production and possible involvement in the suppressive activity of regulatory T cells suggests that it may be responsible for the induction of peripheral tolerance. Several hypotheses have been placed forth to try to explain the mechanism of CTLA-4 signalling since it is still largely unknown. It has been proposed that CTLA-4 may act indirectly by blocking availability of ligands by competitive binding, by induction of immunosuppressive cytokines or by blocking surface expression of rafts. It may also act directly by dampening TCR mediated signalling. In this study, we show that CTLA-4 is able to induce actin polymerization and promote the formation of structures such as filopodia along with lamellipodia. An increase in adhesion and decreased motility in T cells accompany these cytoskeletal changes. Moreover, the cytoskeletal changes are paralleled with a decrease in IL-2 mRNA transcription and an increase in Rac activity indicating that not all signals generated by the TCR are inhibited simultaneously. These results suggest that the role of CTLA-4 as a negative regulator of activation should be reconsidered and regarded instead as a modulator of TCR signals that reprograms cellular events which results in repressed IL-2 production rather than an inhibitor of signalling.

## 2.2 Introduction

The two-step signalling model for T cell activation initially proposed by Bretscher & Cohn (Bretscher and Cohn, 1970) suggested that a molecular complex comprising of the MHC bound with peptide interacting with a highly specific TCR was insufficient for the successful development of an effective response. Instead, a second signal was proposed to be necessary for differentiation of T cells into effector cells. Absence of this costimulatory signal could render T cells refractory to further signalling or lead to apoptosis (Boussiotis et al., 1996; Harding et al., 1992; Kang et al., 1992; Li et al., 1996; Tan et al., 1993). In the last few years, the discovery of a number of costimulatory molecules and their binding partners led to the recognition that specific stages of activation are compartmentally as well as temporally regulated. Costimulatory molecules on T cells include CD28, ICOS and CTLA-4 (Chambers, 2001; Hutloff et al., 1999). CD28 and CTLA-4 bind to the same ligand B7-1 (CD80) and B7-2 (CD86), while ICOS binds to the B7h (also known as B7-H2, B7-RP1 and B7h) ligand. CD28 and ICOS have both been described as positive regulators of the immune response by enhancing TCR signals, or indirectly providing help in the form of cytokine secretion for the differentiation of T cells (Greenwald et al., 2002; Hutloff et al., 1999; Tamura et al., 2001; Wang et al., 2000). While CD28 has been shown to be required for IL-2 production, proliferation and increased cytotoxicity by T cells, ICOS has been demonstrated to be required for IL-4, IL-5, IL-10 secretion and differentiation of Th2 cells (Gonzalo et al., 2001; McAdam et al., 2000).

CTLA-4 has been described as a negative regulator of the immune response (Azuma et al., 1993; Chambers and Allison, 1997c; Chen et al., 1994; Coyle et al., 2000; Damle et al., 1994; Hathcock et al., 1993; Lenschow et al., 1993; Linsley et al., 1992). Both in vivo and in vitro studies have suggested that CTLA-4 ligation leads to decreased TCR mediated activation and IL-2 secretion. Confirmation of its negative role was ascertained by deficient mice that showed pronounced lymphoproliferation and lethality by 3-4 weeks of age (Tivol et al., 1995; Waterhouse et al., 1995). However, blocking CD28 costimulation could reverse the phenotype indicating that the primary phenotype of CTLA-$4^{-/-}$ mice is caused by unregulated costimulation by CD28 together with TCR (Chambers et al., 1997a; Khattri et al., 1999; Masteller et al., 2000).

Interestingly, mice reconstituted with chimeric bone marrow derived from CTLA-4 -/- and CTLA-4 +/- did not progress through disease. This finding puts into question whether CTLA-4 only regulates tolerance in a cell autonomous manner. Recently, new

focus on a subpopulation of T cells termed regulatory CD4+CD25+T cells which expresses high levels of intracellular CTLA-4 has gained particular attention as it has been discovered to be an important tool in the development of suppressive function of T cells. Although still controversial, it has been shown by some that CTLA-4 can mediate the negative function of this population. Nevertheless, the molecular mechanism of negative signalling by CTLA-4 has still to be elucidated. There are several models, which have been proposed to account for CTLA-4 negative function such as competition for ligand binding, induction of suppressive cytokines or sequestration of intracellular signalling molecules. The most widely accepted model involves the reduction of phosphorylation levels the adaptor LAT by the phosphatase SHP-2.

## Actin cytoskeleton

The cytoskeleton plays an important role in T cell signalling. There are two main events that underscore the importance of cytoskeletal rearrangement in T cell activation. As shown by numerous studies, a specialised microdomain termed the immunological synapse (IS) is formed at the cell to cell contact site during a T:B interaction and conjugate formation is the opening step required for assembly of surface receptors and recruitment of intracellular proteins involved in the initiation of the response. It has been proposed that TCR induced actin polymerisation is necessary to generate direct contact to antigen presenting cells (APCs) (Delon et al., 1998). In addition, segregation and clustering of the TCR to supramolecular activation clusters (SMACs) was shown to be dependent on movement of actin filaments by a myosin dependent mechanism (Grakoui et al., 1999; Monks et al., 1998). Polarisation of the cytoskeleton and the microtubule network are also important for targeted delivery of cytokines to APCs (Kupfer and Dennert, 1984; Lowin-Kropf et al., 1998; Wulfing and Davis, 1998). Secondly, TCR engagement has also been shown to induce actin rearrangement. Inhibitors for actin polymerisation have been shown to block TCR mediated signals. In addition, the loss of WASP, a regulator of actin polymerisation, and Vav, an exchange factor for the Rho family of GTPases, weakens T cell activation and proliferation due to defective actin polymerization and clustering of TCRs into patches and caps. In addition, specific T cell adaptors regulate rearrangement of the cytoskeleton. SLP-76 was found to play a role as a scaffolding molecule that can recruit Nck and Vav to the plasma membrane to facilitate actin polymerisation (Bubeck Wardenburg et al., 1998). Nonetheless, little is known on the influence of costimulatory molecules on the process and function of cytoskeletal rearrangement in T cell activation. Therefore, TCR signalling and actin remodelling are

inseparable forces regulating a process which has great potential outcome for T cell activation and effector function. Little is known, however, about the complex mechanism of TCR induced actin reorganisation and its function in TCR signalling.

In this paper, we analysed the effect of CTLA-4 regulation on the process of TCR induced actin polymerisation and cytoskeletal rearrangement. We show that CTLA-4 can have a modulatory function in TCR mediated control of cell shape, adhesion and cytoskeletal reorganization. We also show that while CTLA-4 is capable of inhibiting IL-2 production, it also induces changes in the cytoskeleton with a high percentage of the cells showing filopodia and lamellipodia formation. These changes are accompanied by increased adhesion and decreased motility in cells over a prolonged period of time. These results were confirmed using an antigen presenting system where tetanus toxin is presented to a hybridoma expressing the restricted TCR. These results support a model where CTLA-4 modulates TCR mediated responses. It also suggests that an active stop signal delivered by CTLA-4 may target the cell to increase APC contact and redirect signalling processes that are not needed at the moment of conjugate formation to changes in morphology. The negative function of CTLA-4 may have to be reconsidered in light of these results.

## 2.3. Materials and Methods

### 2.3.1. Cells, reagents and antibodies

The murine T cell hybridoma DC27.10 was cultured in complete RPMI 1640 medium, supplemented with 5% FCS and 50 μM 2-mercaptoethanol. DC27.10 cells were stably transfected with human CTLA-4 as described (Schneider et al. 1999). Anti-hCTLA-4 (BNI3), anti-hCTLA-4PE was purchased from Coulter, anti-mCD28 (37.51), anti-mCD3 (145-2C11), anti-mCD3-FITC (145-2C11), anti-TNP was purchased from Pharmingen (san Diego, CA). Phalloidin-FITC and Phalloidin-TRITC was purchased from Sigma (P5282, P1951). Anti-alpha tubulin (clone B-5-1-2), anti-mouse TRITC (T7657) and the PKH67 green fluorescent cell linker kit (MINI-67) were purchased from Sigma. Anti-hCD28 (9.3) and anti-hCD3 (OKT3) was purchased from the American Type Culture Collection (Manassas, VA). Triazol was purchased from GibcoBRL (15596-026). T8.1 cells were cultured in DMEM supplemented with 10% FCS, 5mM glutamine, 5mM pen/step, 10 mM Hepes, 400nM methotrexate, 50 μM 2-mercaptoethanol and 1mg/ml geneticin (Sigma) (G418).

### 2.3.2. Immunofluorescence

To analyse cytoskeletal changes induced by CTLA-4, coverslips were coated with the designated antibodies at the following concentrations: anti-mCD3 (1μg/ml), anti-hCTLA-4 (10μg/ml), anti-mCD28 (10μg/ml) and anti-TNP was used to equalise the amount of antibody. The cells were activated at 37C for 3.5hrs or otherwise at the indicated time. The cells were then fixed with 2% paraformaldehyde for 20min and either stained for surface antigens or permeabilized with 10 mM Hepes, 3 mM $MgCl_2$, 50 mM NaCl, 300 mM Sucrose, 0.5% TritonX-100. The cells were washed twice with PBS and blocked for 30 min with blocking buffer (PBS with 10%FCS). The cells were then stained with the appropriate primary antibodies for 1.5hr at 37C. The cells were then washed with blocking buffer and stained with the secondary antibody along with phalloidineFITC in the dark at 37°C for 1hr. Hoechst was added in the final 5 min of incubation. The cells were extensively washed and mounted on a slide with glycerol gelatine (Sigma #GG-1).

### 2.3.3. Flow Cytometry

To analyse surface expression of CTLA-4 and CD3, DC27.10 cells stably transfected with hCTLA-4 were stained with anti-hCTLA-4PE and anti-mCD3FITC with staining buffer (PBS, 1% BSA and 0.01% NaN3). The cells were washed three times and analysed on a Becton Dickinson FACSCalibur machine and Cell Quest software. Isolated peripheral blood cells by Ficoll Paque were stimulated with platebound anti-hCD3 (5μg/ml) and anti-hCD28 (5μg/ml) for 40hr. The cells were harvested and stained with anti-hCD3 FITC and anti-hCTLA-4PE with staining buffer. Cells were washed three times and analysed.

### 2.3.3. Activation assay

To analyse cytokine expression, the cells were activated at a density of 2.5 X $10^5$ in 96 flatbottom wells previously coated with antibodies, anti-mCD3 (1μg/ml), anti-CTLA-4 (10 μg/ml), anti-CD28 (10 μg/ml). Anti-TNP was used to equalise the amount of antibodies. The cells were activated for 3.5 hrs at 37°C in complete medium and harvested for RNA extraction using the phenol-chloroform extraction method.

### 2.3.4. Immunoprecipitation and PAK assay

For immunoprecipitation, cells were lysed in ice-cold lysis buffer containing 1% TritonX-100 in 20 mM Tris-HCL, pH 8.2, 150 mM NaCl. The lysis buffer contained phosphatase inhibitor 1mM sodium vanadate and the protease inhibitors 1mM PMSF, 1 mM sodium fluoride. Postnuclear lysates were incubated for 1 hour with the indicated antibody. Protein A-Sepharose beads (40 μl Pharmacia) were added and incubated for 1 hour at 4°C. The eluted proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. For immunoblotting, the membranes were blocked with 5% milk or 2% gelatine in TBS (10 mM Tris-HCL, pH 7.6, 150 mM NaCl) and incubated with the indicated antibody. Bound antibody was revealed with the appropriate secondary antibody, and protein was visualised by enhanced chemilluminescence (ECL, Amersham). The Rho GTPases activity was assayed as described by Sander et al., (1998), based on the interaction of the GTP-bound GTPase to the PAK CRIB domain. To prepare the GST-PAK CRIB domain fusion protein, ***Escherichia coli*** BL21 cells transformed with the GST-

PAK-CD construct were harvested and resuspended in lysis buffer (50 mM Tris-Hcl, pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100, 1 mM DTT, 10 µg/ml leupeptin, 0.4 µg/ml pepstatin, 0.1 TIU/ml aprotinin, 1 mM PMSF) and sonicated. After centrifugation, the cell lysate supernatant was incubated with glutathione-coupled Sepharose 4B beads (Amersham Pharmacia, Biotech) for 30 min or overnight at 4°C. The beads were washed three times in lysis buffer, and the amount of bound fusion protein was estimated after separation by SDS-PAGE and Coomassie blue staining. The supernatant was incubated with GST-PAK-CD bound to glutathione–Sepharose beads (Amersham Pharmacia, Biotech) at 4°C for 30 min. The beads were washed three times with an excess of lysis buffer, and bound proteins were eluted in Laemmli sample buffer and analyzed for the presence of Cdc42 or Rac1 molecules by Western blotting using antibodies specific HA.

### 2.3.5. Acquisition of images and measurement of cell interactions

The Vector and CTLA-4WT cells were activated using 96 flat bottom plates which had previously been coated overnight with the indicated antibodies. The wells were washed twice with medium and were blocked by incubation with RPMI supplemented in 10% FCS. Experiments were visualised using an inverted Nikon Diaphot 300 fluorescence microscope connected to a JVC TK-C1360B colour video camera and recorded on a Panasonic AG-6730 S-VHS video recorder. For each experiment, recording commenced after 5 minutes after cells had reached the bottom of the 96 well plate while still in 5% $CO_2$ incubator. The recording consisted of a 15-sec recording at each 3 min during the period of 1h using either 40X or 60X objective.

### 2.3.6. Adhesion studies

Flat-bottomed tissue culture-treated 96-well plates were coated with antibodies as described in PBS at 4°C overnight. Wells were washed once with PBS, then unoccupied sites were blocked by incubating with media containing 10% (wt/vol) FCS at 37°C for 1 hr. The wells were washed twice with PBS and once with RPMI. Transfectants grown overnight were harvested from flasks and resuspended at a concentration of 1.5 x $10^6$ cells/ml. 200 µl was added to the wells and incubated for various time intervals at 37°C. Unbound cells were removed by aspiration, and wells were washed twice with complete RPMI. At the indicated time points, the cells were submitted an initial vigorous shaking on

a platform rocker for 1 min. The cells were washed twice and submitted to low shaking to allow further detachment. This step was repeated twice. A colorimetric assay previously described (Denizot, 1996), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to quantify the number of bound cells. Briefly, 25 µl of MTT (2 mg/ml; Sigma) solution was added to each well. The cells were incubated at 37°C for 3 hr, and the plates were centrifuged at 1500 rpm for 10 min (Sorvall RT6000, DuPont). After the supernatant was removed, 100 ml of DMSO (Sigma) was added to each well. Formazan was dissolved by shaking and OD at 595 nm was measured by an ELISA reader (Bio-Rad).

### 2.3.7. Antigen Presentation Assay

T8.1 cells were labelled with PKH67-FITC as followed by manufacture's instruction. Briefly, cells were washed in complete medium without FCS and resuspended in diluent C in a concentration of 2 x $10^7$ cells/ml. 2x PKH stock solution was added to the cells and incubated for 3 min at room temperature. 2vol of FCS was added to stop the reaction and incubated for 1 min. The cells were washed 3 times with complete medium and resuspended in a concentration of 3 x $10^5$ cells/ml. The cells were preincubated with 25 µg/ml of anti-CTLA-4 antibody on ice for 1h and crosslinking antibody was added 5 min prior to addition to APCs. Tetanus toxin (Ttox) (2.5mg/ml) (QYIKANSKFIGITE) was added to adherent APCs cultured overnight on glass coverslips. The peptide was incubated for 2h at 37°C and washed with complete supplemented medium. T8.1 cells were added to the APCs and incubated for 6h period at 37°C. The cells were extensively washed with PBS to remove unbound cells and fixed in 2% paraformaldehyde.

### 2.3.8. Generation of Transfectants

To generate T8.1CTLA-4 stable transfectants, full-length human CTLA-4 cDNA was cloned into the pSRα mammalian expression vector containing a sequence encoding the neomycin resistant gene. T8.1 cells ($10^7$) in 0.4 ml of Dulbeco's modified of Eagle's medium (DMEM) were electroporated with 20 µg of CTLA-4 together with 3µg of pBABE containing a puromycin resistance gene (generously provided by Prof. Junia, Melo) with an electroporation apparatus from BTX (San Diego) at 800 µF, 250 V, 200_. Stable transfectants were selected in complete medium containing 0.5 mg/ml for 2 weeks and 1

mg/ml for 1 week of puromycin. Stable lines overexpressing CTLA-4 were determined by flow cytometry. Single clones were subcloned by limited dilution. For transient transfection, 1.5 x $10^7$ Vector and CTLA-4WT cells were electroporated with 30 μg of Rac-HA tagged or cdc-42HA tagged DNA at 960 μF, 260 V, 480Ω. The cells were cultured overnight in compete RPMI medium supplemented with 10% FCS. The cells were ficolled the next day to remove debris and incubated on ice for 1h before activation.

## 2.4. Results

### 2.4.1. CTLA-4 Co-ligation Changes T-cell Morphology

Previous studies have documented the ability of CTLA-4 to negatively regulate aspects of T-cell function such as cytokine production and proliferation (Azuma et al., 1993; Krummel and Allison, 1995; Krummel and Allison, 1996; Lenschow et al., 1993; Linsley et al., 1992; Walunas et al., 1994). However, despite this, CTLA-4 has also be shown to bind to positive regulatory signalling proteins such as PI 3K (Chikuma et al., 2000; Frearson and Alexander, 1998; Gadina et al., 1998; Schneider et al., 1998). It was therefore of interest to assess whether CTLA-4 might possess the capacity to re-direct aspects of T-cell functions in addition to its role in blocking certain activation properties. In this regard, we investigated the possibility that the co-receptor might have a specialised role in altering other aspects of T-cell function such as morphology. To this end, we used as a model a hybridoma DC27.10 that had been stably transfected either with the human form of CTLA-4 (WT) or with a vector control (vector) as well as activated peripheral T-cells (Cefai et al., 1996; Hu et al., 2001; Schneider et al., 2001). Cells were ligated with combinations of anti-CD3, anti-CD28 and/or anti-CTLA-4 immobilized on plates and examined for changes in cells shape by differential interference contrast (DIC) microscopy as described in the ***Materials and Methods***. As shown in Figure 1, vector and CTLA-4 WT cells expressed CD3, CTLA-4 and CD28 at comparatively high levels (panel A). The level of CTLA-4 expression was higher than observed on activated T-cells (panel B), but some 10-fold lower than CD3 (panel A, upper and lower panels). CD28 expression was somewhat lower than both CD3 and CTLA-4 and appeared to show reduced expression with the expression of CTLA-4. By initially using over-expressing DC27.10 cells, we attempted to establish a phenotype that could be replicated in normal peripheral T-cells.

DIC microscopy showed that non-stimulated cells were well rounded with no apparent distinctive structures (Figure 2A, panel a'). Likewise, stimulation by anti-CD3 did not alter cell shape at a macroscopic level (panel b'). Stimulation by anti-CD3/anti-CD28 showed a moderate flattening of the cells which occurred within the first few minutes after activation (panel c'). By contrast, cells stimulated with anti-CD3/anti-CTLA-4 showed extensive and pronounced conformational shape changes, with the cells spreading and flattening on the glass slide within 30 mins of activation (panel d'). A higher 100X magnification of cells at 3 hours showed extensive spreading of cells with extended pseudopodia (adjacent panel e'). CTLA-4WT cells were followed during the course of 6h activation and found that 50% the cells were showing shape changes on the glass slide

within 30 min of activation after stimulation with anti-CD3/anti-CTLA-4 (Figure 2B). Documentation of the percentage of cells that undergone spreading during a 6 hour time course showed that some 30 percent of the population showed spreading by 1 hour, 50 percent by 2 hours and 60-70 percent by 3 hours. Some 70 percent of T-cells had underwent the same conformational change by 4hrs and remained constant and stable following at least the next 2hrs. By contrast, neither anti-CD3 induced shape changes. Anti-CD3/anti- CD28 induced some changes in morphology over the time-course (10-20%), but far less extensive than observed for anti-CD3/anti-CTLA-4. As controls, anti-CTLA-4 was found to inhibit interleukin 2 production in the assays (Figure 2C), in a dose dependent manner. These data suggested that CTLA-4 co-ligation provided potent co-signals that direct changes in cell shape.

### 2.4.2 CTLA-4 Induces the Appearance of Filopodia/Lamellipodia

To further determine the nature of the cytoskeletal and morphological changes induced by CTLA-4, DC27.10 and peripheral T-cells were stained for F-actin with phalloidin-FITC and visualised by fluorescence microscopy (Figure 3, panels A, B, respectively). While non-stimulated vector and CTLA-4WT cells showed no distinctive features (panels a and a'), CTLA-4WT cells stimulated with anti-CD3/anti-CTLA-4 induced dramatic changes in morphology with the appearance of numerous microspikes (panels d,d'). Stimulation by anti-CD3 or anti-CD3/anti-CD28 showed the formation of distinct clusters of actin polymerization throughout the cells (panels b,b' and c,c', respectively). Stimulation with anti-CD3 and anti-CD3/anti-CD28 showed, however, distinctive formation of clusters of actin polymerisation in both transfectants suggesting that CD3 engagement can dynamically activate the formation of actin rich focal clusters. In contrast, CTLA-4WT demonstrated a substantial change in overall morphology when stimulated with anti-CD3/anti-CTLA-4. The cells spread on the coverslip and formation of actin rich filaments and cytoplasmic protrusions was observed. As a control, vector transfected cells did not show any of these changes (panels a-d). Different cell structures are required for different functions where lamellipodia formation at the leading edge of a cells is required for cell movement and migration (Boudreau and Jones, 1999; de Curtis, 2001; Jones et al., 1998; Kjoller and Hall, 1999; Ridley, 2001; Sanchez-Madrid and del Pozo, 1999; Schmitz et al., 2000). In addition, contact between T cell and APC requires the involvement of actin polymerisation to recruit and cluster a multitude of receptors.

In order to determine the type of changes induced in the actin cytoskeleton by CTLA-4, the different morphological structures were classified and analysed to establish

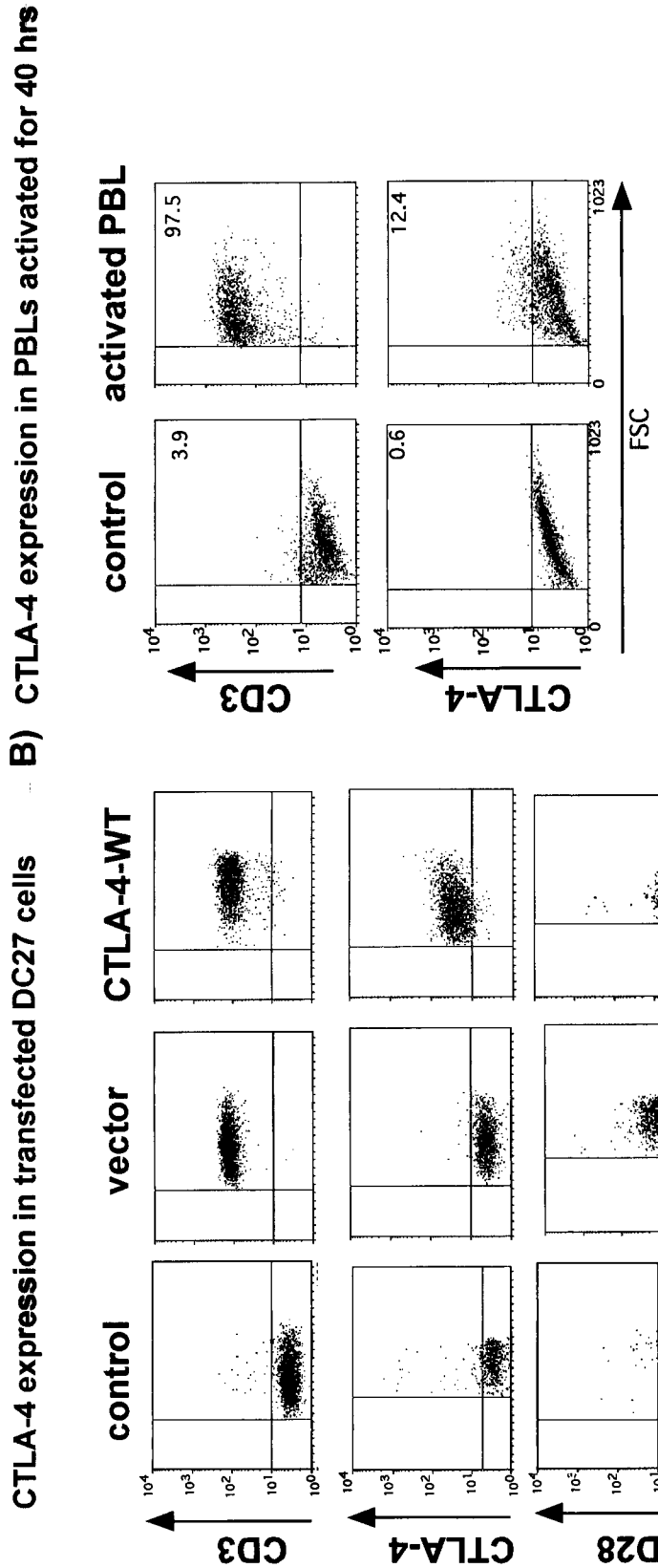



Figure 1: FACS profile of Vector control, CTLA-4WT and activated peripheral blood leukocytes.
(A) Vector and CTLA-4WT cells were stained with antibodies against mCD3, mCD28 and hCTLA-4 and analysed by flow cytometry.
(B) PBLs were activated with platebound anti-CD3 and anti-CD28 for 40 h. The cells were recovered and fixed immediately. 106 cells were stained for surface hCD3 and permeabilized for intracellular staining with hCTLA-4.

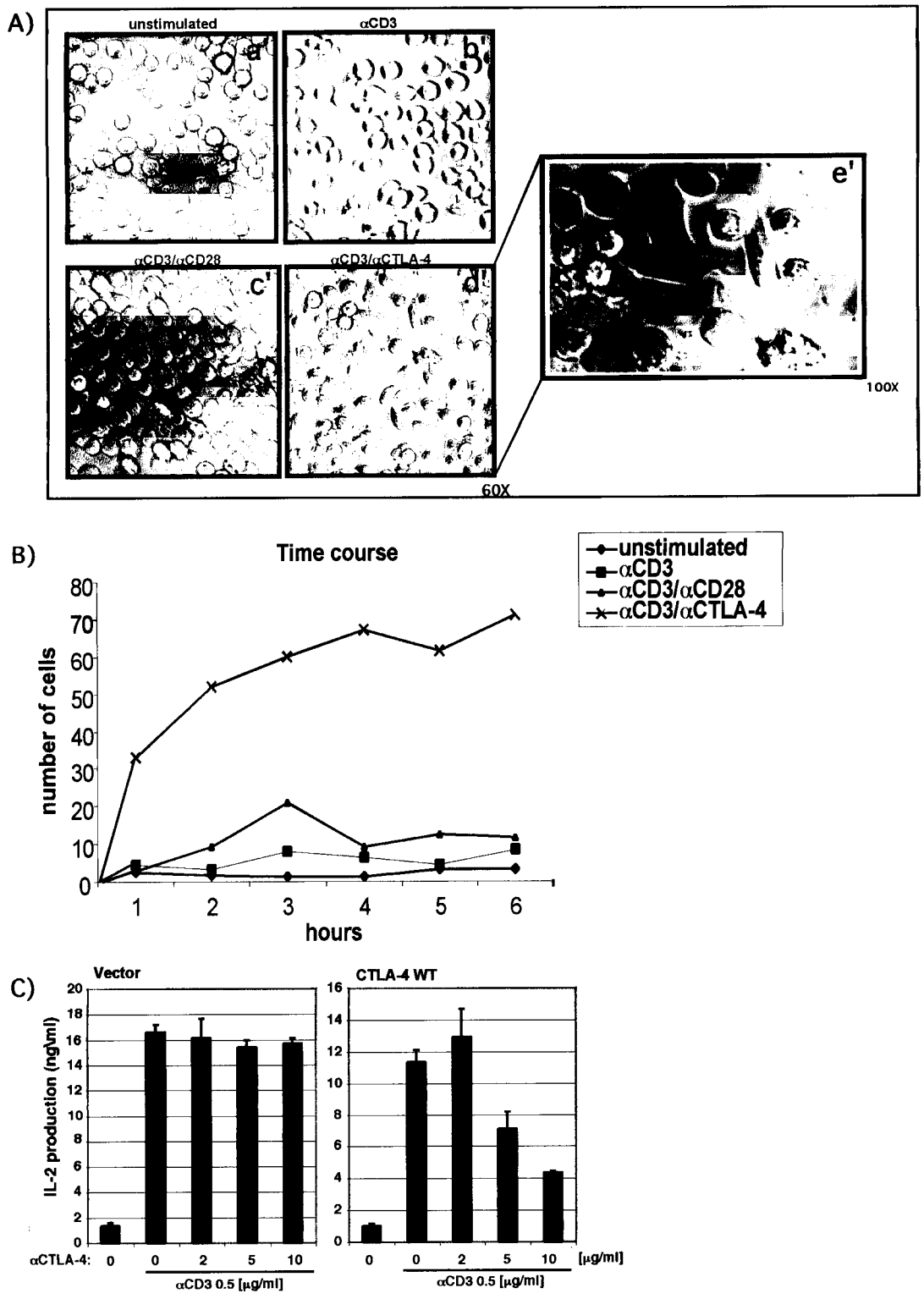



**Figure 2: Costimulation with CTLA-4 induced morphological changes while still inhibiting IL-2 production.**
**(A) CTLA-4WT cells were stimulated via platebound antibodies for 3h as described in materials and methods. The cells were stimulated with i) media ii) anti-CD3 iii) anti-CD3/anti-CD28 and iv) anti-CD3/anti-CTLA-4 and imaged in a 24 well plate using an inverted light microscope. A 100X magnification of the 4th panel (v) shows detailed structural changes.**
**(B) Time course of shape changes occurring during a 6h time period. Cells in (A) were followed for each stimulatory condition and counted every hour for 6h for any morphological changes.**
**(C) Vector control (left panel) and CTLA-4WT cells (right panel) were activated with platebound anti-CD3 concentration and varying anti-CTLA-4 concentrations (0, 2, 5, 10 μg/ml). Anti-TNP control antibody was used to equalise the total amount of antibody bound to the plate (10, 8, 5, 0 μg/ml). The supernatant was harvested at 24h after stimulation and frozen at –20°C for later analysis of IL-2 production by ELISA.**

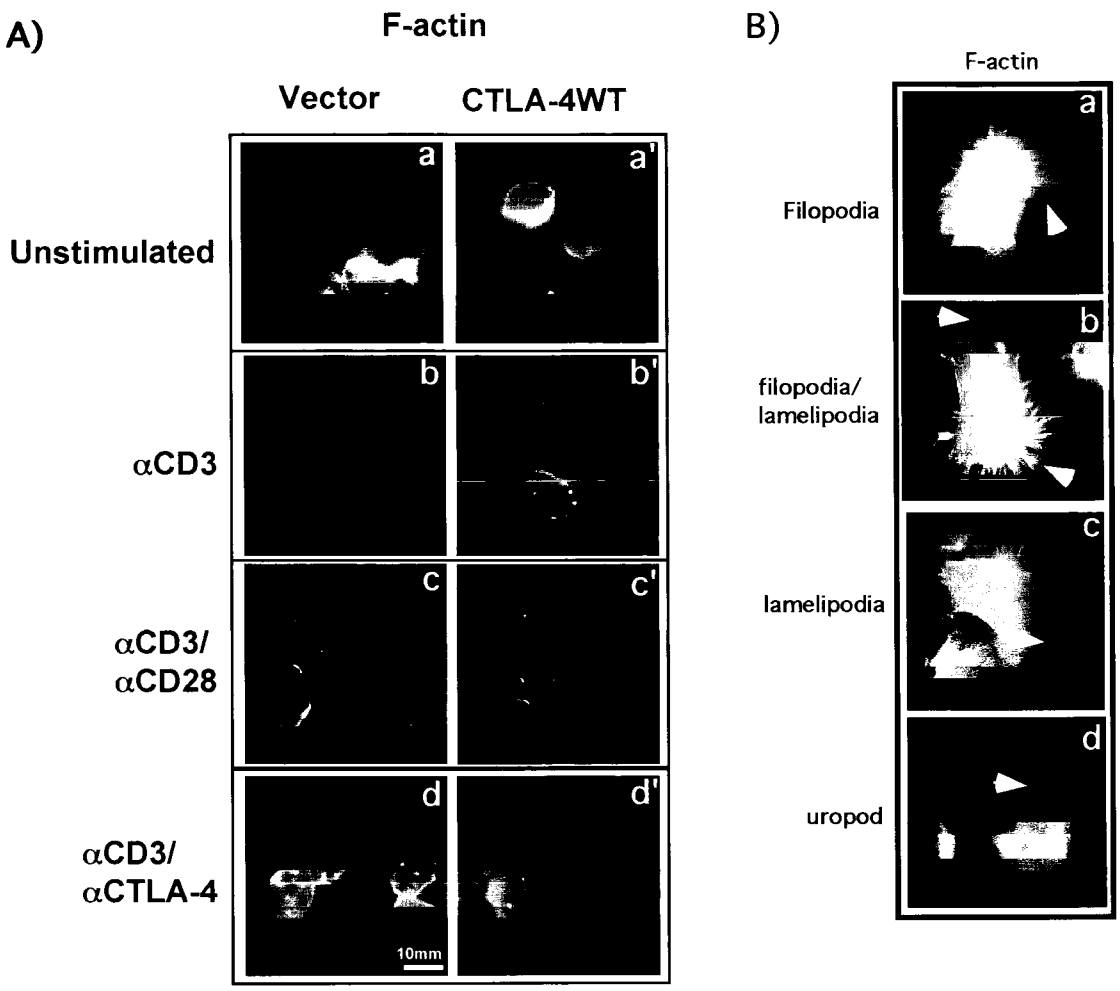



**Figure 3: Actin staining following stimulation of Vector and CTLA-4WT cells.**
**(A) Vector and CTLA-4WT cells were left unstimulated or stimulated (a, a') with coverslips coated with anti-CD3 (b, b'), anti-CD3/CD28 (c, c') and anti-CD3/CTLA-4 (d, d'). The cells were subsequently fixed and stained with phalloidin-FITC.**
**(B) Representative cells are shown based on consistent assessment of the average behaviour. The cells were characterised based upon staining with phalloidin-TRITC and the differentiation and appearance of a)filopodia b)filopodia/lamellipodia c)lamellipodia and d)uropod. Categorisation of structural changes is based upon previous published reports and expert analysis of the morphological changes.**
**(C) Numbers of cells falling within a category were calculated based on three independent experiments. A total of approximately 850 cells or more were characterised for each of the stimulatory conditions.**

whether CTLA-4 induced a preponderance of a specific cell shape change (Figure 3B). Cells were classified under the following categories: round, filopodia (a), filopodia/lamellipodia (b), lamellipodia (c) and uropod (d). The white arrows highlight each of the structures. CTLA-4WT cells were activated as previously described and stained for phalloidin-TRITC to visualise the actin microfilaments and counted based on the previous described classification. As shown in Figure 3C, almost all unstimulated cells showed primarily a round morphology, while CTLA-4WT cells activated with either anti-CD3 or anti-CD3/anti-CD28 showed a small induction of specialised structures. By contrast, activation with anti-CD3/anti-CTLA-4 induced a large number of cells to form filopodia (35%) and the combination of filopodia/lamellipodia (37%). In addition, formation of lamellipodia (10%) and uropod (8%) is also observed. These results suggest that CTLA-4 may influence mainly the formation of filopodia although it does not limit the induction of other specific morphological changes.

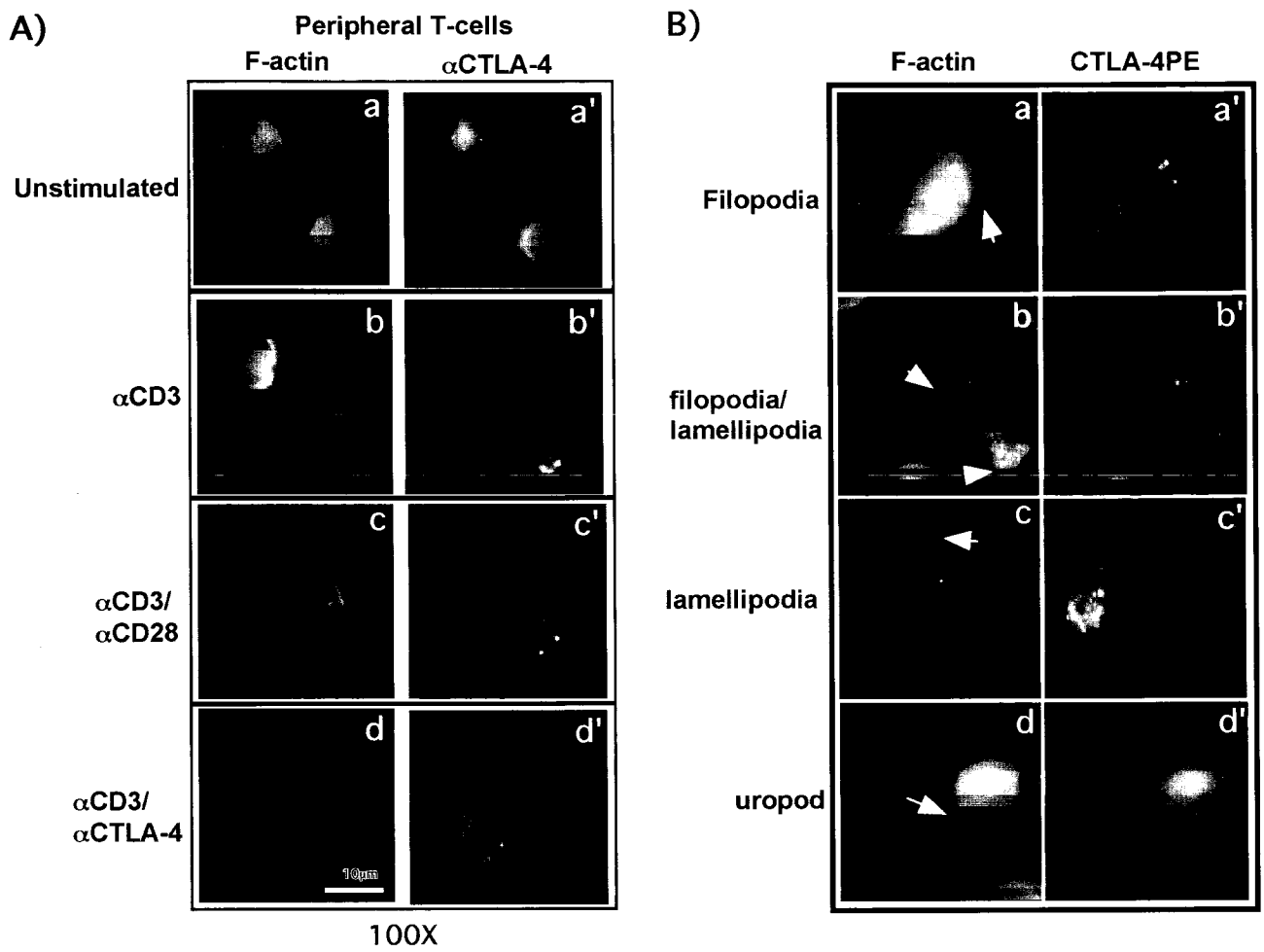
### 2.4.3. CTLA-4 Induces Filopodia/Lamellipodia in Peripheral T-cells

So far, our analysis focussed on the use of transfected cell lines (Figures 1-3). To determine whether these changes also occur in normal peripheral T-cells, PBLs were activated for 40h with anti-CD3 and anti-CD28 to up-regulate of CTLA-4 expression, and then restimulated with anti-CD3, anti-CD3/CD28 or anti-CD3/CTLA-4. Cells were then stained with phalloidin and anti-CTLA-4PE (i.e. for detection of the presence of endogenous CTLA-4). As shown in Figure 4A, pre-activated T cells under unstimulated conditions appeared round with no particular marked feature (panels a,a'). Stimulation with anti-CD3 and anti-CD3/anti-CD28 resulted in a flattening of the cells, while still maintaining a round morphology (panels b, b' and c, c' respectively). In contrast, anti-CD3/anti-CTLA-4 stimulated cells produced the appearance of extensive microspikes, filopodia and lamellipodia (panels d, d'). The changes were similar to that observed with the CTLA-4WT cells. In addition, cells that expressed highest levels of CTLA-4 had greater capacity to induce these morphological changes. The cells were classified and counted as previously described (Figure 4B). The majority of the cells were found to form filopodia (47%) and lamellipodia (20%), which is in accordance to that observed with the CTLA-4WT (Figure 4C). These results confirmed the previous findings with the CTLA-4WT transfectants and acknowledge a common pathway utilised by CTLA-4 in relation to TCR signalling events leading to actin polymerisation.

## 2.4.4. CTLA-4 induced morphology changes are accompanied by increased adhesion

TCR ligation is known to increase adhesion of T cells to substratum (Borroto et al., 2000). These events tended to be short-lived where the peak response was observed at 5 min and started to decrease by 20 min (Bunnell et al., 2001). Extensions such as filopodia and podosomes are often accompanied by an increase in T-cell adhesion. We therefore next investigated whether the changes in morphology induced by anti-CD3/anti-CTLA-4 was accompanied by decreased motility and an increase in adhesion (Figure 5A). Vector and CTLA-4 WT cells were activated under the same conditions as previously described. After 6h stimulation, the unbound cells were vigorously washed away and level of adhesion was measured by assaying for viability using an MTT assay. Cells stimulated with varying concentrations of anti-CD3/anti-CD28 did not show increased adherence compared to unstimulated or anti-CD3 stimulated cells in vector and CTLA-4WT cells alike. In contrast, anti-CD3/anti-CTLA-4 or anti-CD3/anti-CD28/anti-CTLA-4 stimulated CTLA-4WT cells showed a marked increase in adhesion to the substratum. A time-course showed that adhesion of CTLA-4WT cells to the activating substratum occur within the first hour of stimulation and remain constant throughout the measuring period (Figure 5B). This data demonstrates that CTLA-4 costimulation increased adhesion and induced formation of tight contacts to the coverslip. This data suggests that CTLA-4 may initially increase adhesion in a first phase of the response, which could support and facilitate the formation of filopodia and lamellipodia. It also suggests that this event may not involve de novo transcription of genes since adhesion starts occurring within the first hour of stimulation.

It is not yet known by which mechanism a T cell increases its chances for sustained signalling by encountering the appropriate antigen on the surface of the APC. One possible mechanism would be to increase the surface contact area with which the T cell can explore the surface of the APC. We therefore measured the surface area of the CTLA-4WT cells after stimulation. As shown in Figure 5C, the area occupied by CTLA-4WT cells remains constant after stimulation with anti-CD3 or anti-CD3/anti-CD28 compared to unstimulated cells. After stimulation with anti-CD3/anti-CTLA-4 however, the area of contact between the T cells and the substratum increases by approximately 3 fold. A time course experiment showed that the contact area increases during a 3h period and remains stable during the course of at least 6h (Figure 5D). This data shows that while CTLA-4 ligation can cooperate in increasing cell spreading induced by the TCR, this initial contact is stabilised for a prolonged period of time. Thus, cell spreading may allow for



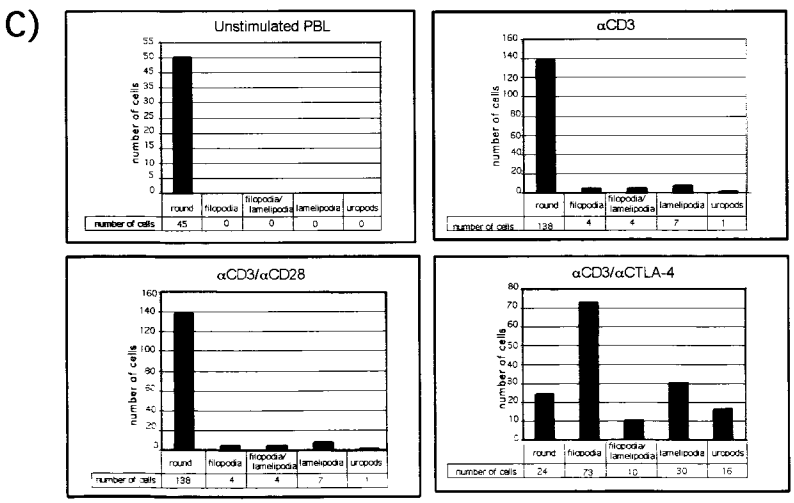
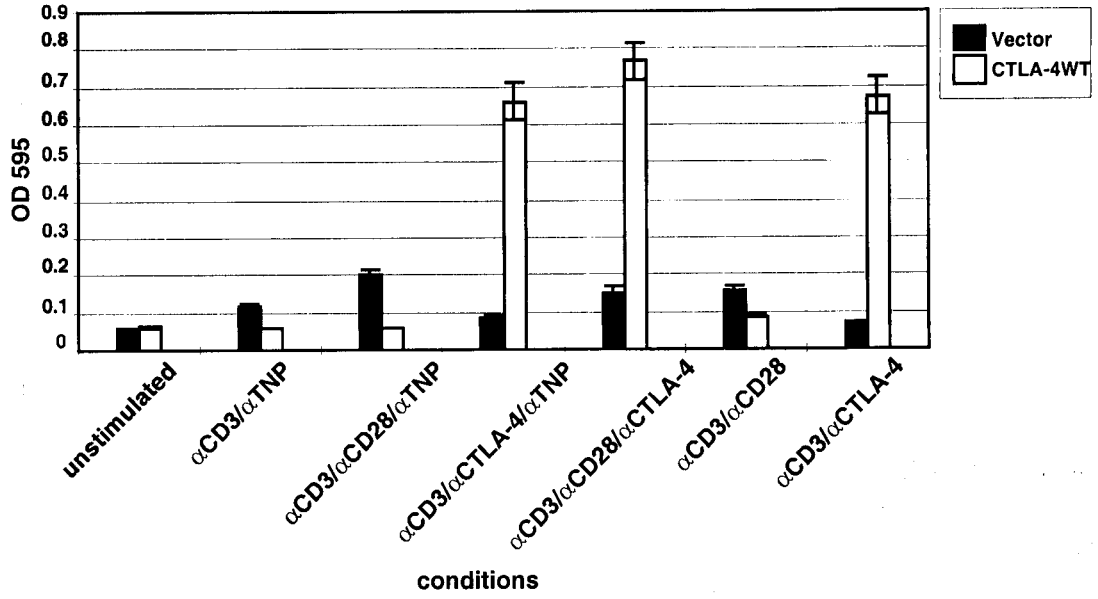




**Figure 4: Actin staining following stimulation of preactivated PBL.**
**(A) Peripheral T cells were stimulated for 40 h with anti-CD3/CD28 prior to resting (a, a') or restimulation with coverslips coated anti-CD3 (b, b'), anti-CD3/CD28 (c, c') and anti-CD3/CTLA-4 (d, d'). The cells were subsequently fixed and stained with phalloidin-FITC (left panel) and hCTLA-4PE (right panel).**
**(B) Representative cells are shown based on consistent assessment of the average behaviour of hCTLA-4 expressing cells only. The cells were characterised based upon staining with phalloidin-FITC and the differentiation and appearance of a, a') filopodia, b, b')filopodia/lamellipodia, c, c') lamellipodia and d, d')uropod. Categorisation of structural changes is based upon previous published reports and expert analysis of the morphological changes.**
**(C) Numbers of cells expressing hCTLA-4 and falling within a category were calculated based on two independent experiments. A total of approximately 150 cells or more were characterised for each of the stimulatory conditions.**

A)

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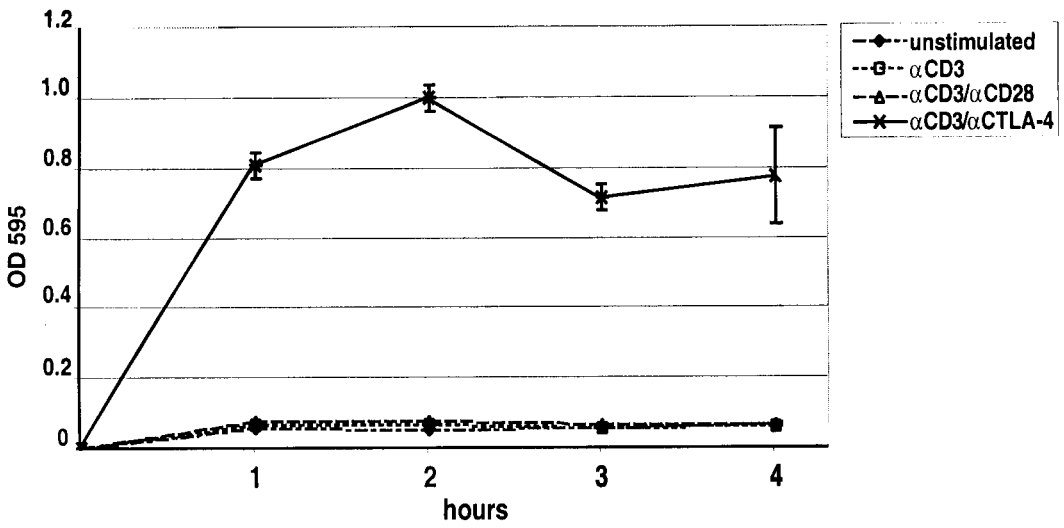
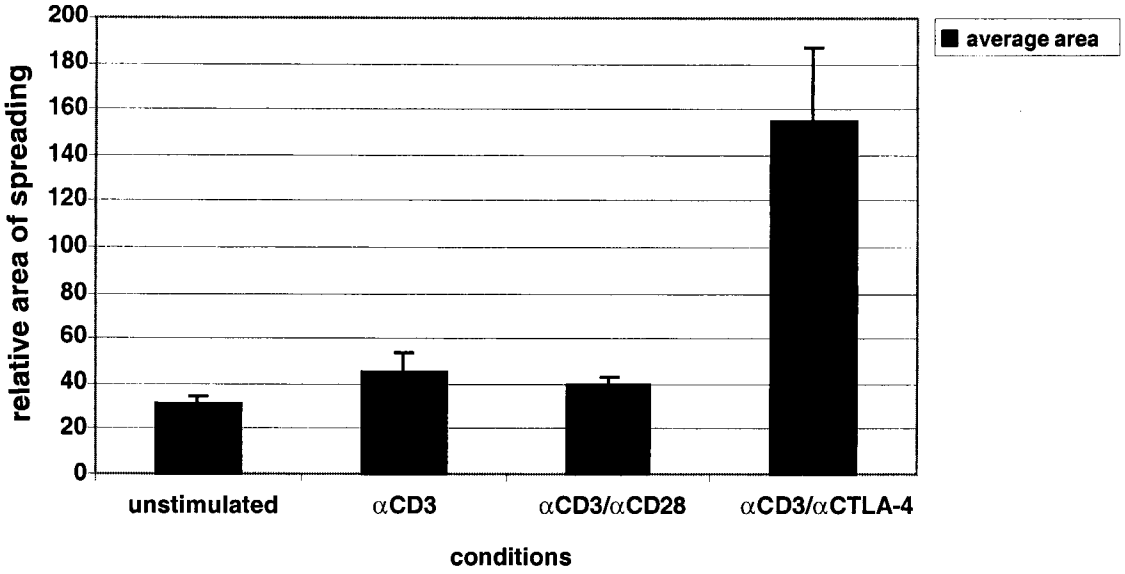
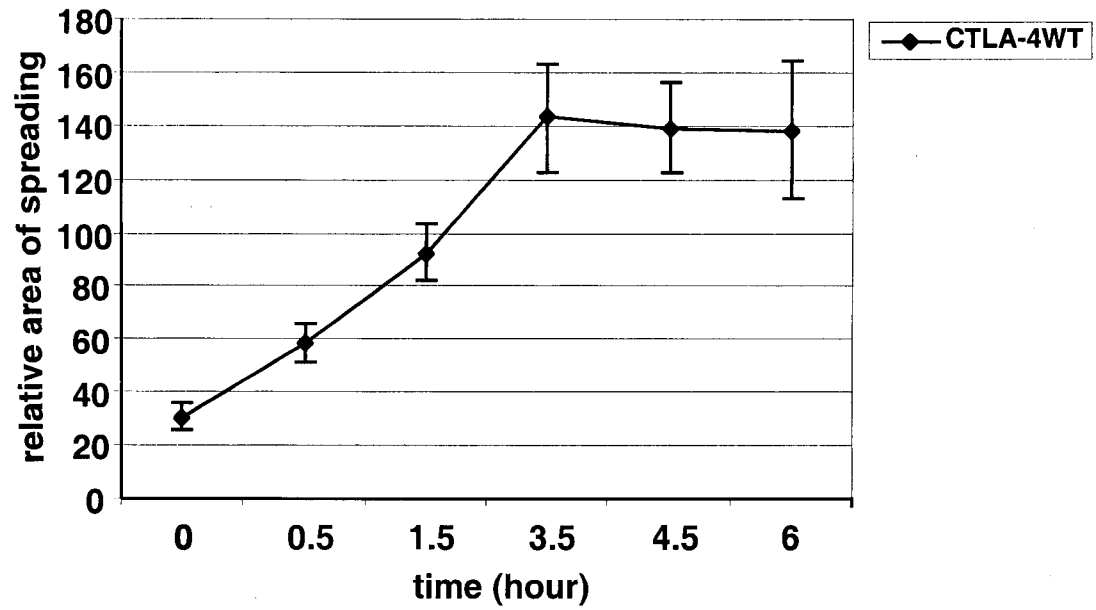



**Figure 5: CTLA-4 can promote adhesion to the substratum and increase cell spreading following stimulation. Cells were stimulated as described in materials and methods. The extent of cell adhesion was examined by quantifying the adherent cells with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay read and measured by ELISA reader at 595 nm. The mean OD of adhered cells at 6 h along with the SDs derived from triplicates are presented. A representative experiment from three is shown.**
**(A) Vector control and CTLA-4WT were stimulated and compared as to their ability to adhere after stimulation. The cells were stimulated with the following concentrations: 0.5 μg/ml αCD3: 10 μg/ml αTNP; 0.5 μg/ml αCD3: 5 μg/ml αCD28: 5 μg/ml αTNP; 0.5 μg/ml αCD3: 5 μg/ml αCTLA-4: 5μg/ml αTNP; 0.5 μg/ml αCD3: 5 μg/ml αCD28: 5 μg/ml αCTLA-4; 0.5 μg/ml αCD3: 5 μg/ml αCD28; 0.5 μg/ml αCD3: αCTLA-4. B)Time course using CTLA-4WT cells.**
**(B) CTLA-4 transfectant or vector control transfectant were allowed to adhere to antibody-coated plates for various time periods. The mean OD of adhered cells along with the SDs derived from triplicates is presented. A representative experiment from five is shown.**

C)




D)




**(C) CTLA-4WT cells were stimulated with 0.5 μg/ml αCD3: 10 μg/ml αTNP; 0.5 μg/ml αCD3: 10 μg/ml αCD28; 0.5 μg/ml αCD3: 10 μg/ml αCTLA-4 for 4 hours, fixed and and stained with phalloidin-FITC. The spreaded area was measured by quantitative microscopic analysis using Adobe Photoshop and calculating the total fluorescent area .**

**(D)Time course measuring the area in contact with the substratum after CTLA-4WT stimulation with anti-CD3/CTLA-4. The cells were stimulated as described with 0.5 μg/ml αCD3: 10 μg/ml αCTLA-4, fixed at the indicated times and stained with phalloidin-FITC. The area of spreading was measured by quantitative microscopic analysis.**

greater opportunity in extending the contact between the TCR and its ligand and possibly support a role in delivery of more efficient transmission of signals by facilitating sustained signalling.

### 2.4.5. CTLA-4 decreases motility in adherent cells

The formation of few lamellipodia and uropod in anti-CD3/anti-CTLA-4 stimulated cells would suggest that CTLA-4 might regulate T cell motility. However to confirm these findings, live movement of cells is needed to be visualised and monitored after stimulation. To this end, we investigated the morphological changes during the initial contact with the stimulating antibody for a time period of 1h using time-lapse microscopy. As Figure 6A shows, unstimulated CTLA-4WT cells have a high capacity for locomotion. CTLA-4WT cells that have been stimulated by anti-CD3 or anti-CD3/anti-CD28 have the capability of displacing themselves within a certain area (indicated by the arrows), while still remaining round in shape. However, CTLA-4WT cells that were stimulated with anti-CD3/anti-CTLA-4 exhibited morphological changes while remaining stationary. The decrease in motility of the cells stimulated with anti-CD3/anti-CTLA-4 was confirmed by analysing the trails from 10 individual cells from each stimulation condition. As shown in Figure 6B, while unstimulated cells have a continued direction of travel, anti-CD3 or anti-CD3/CD28 stimulation causes changes in the cell direction and restricts the area of motion. However, anti-CD3/anti-CTLA-4 stimulation stopped cells from crawling and immobilised them to the substratum. The CTLA-4WT cells appeared flattened and did not have increased migration. In fact, their position did not change with time. The relative speed of the cells was measured and the results shown in Figure 6C confirmed that cells stimulated with anti-CD3/anti-CTLA-4 were moving at a much lower speed compared to unstimulated, anti-CD3 or anti-CD3/anti-CD28 stimulated cells. Therefore, these results suggest that CTLA-4 may in fact curb the effect of cell migration by permitting the cells to induce morphological changes and remain immobile.

### 2.4.6. CTLA-4 induced shape changes operates via the Rac/Cdc42 pathway

Cell morphology and adhesion can be regulated by the GTPases Rac1 and Cdc42 (Dharmawardhane and Bokoch, 1997; Evers et al., 2000; Nobes and Marsh, 2000; Ridley et al., 1999; Takenawa and Miki, 2001). To assess whether either of these GTPases were involved following activation of CTLA-4WT cells, the activity of Rho family of GTPases was measured (Etienne-Manneville and Hall, 2001). As shown in Figure 7A, binding of

Rac to a PAK-GST fusion protein was increased after stimulation with anti-CD3/anti-CTLA-4. Activity of cdc42 was found to be marginally increased compared to Rac activity following anti-CD3/anti-CTLA-4 (Figure 7C). Equal amounts of Rac and cdc42 lysate were loaded as shown in Figure 7B and Figure 7D. To assess the importance of GTPases in the generation of shape changes induced by CTLA-4, dominant negative (dn) dnRac and dncdc42 were used to block induction of shape changes. The cells were activated as previously described and stained for the presence of transfected Rac, cdc42 and the dominant negative of Rac and cdc42. As shown in Figure 7E, while the dominant negative Rac and cdc42 failed to localise at the filopodia and lamellipodia, respectively, after anti-CD3/CTLA-4, wild type Rac and cdc42 were able to do so. Wild type Rac (Figure 7 panel d) was shown to localize in the filopodia as well as in the microspikes in the leading edge. Localization of the wild type cdc42 was primarily seen in the membrane ruffles (Figure 7 panel d'). The inactive form of Rac and cdc42 failed to show specific localization following anti-CD3/anti-CTLA-4 stimulation (Figure 7 panel h, h' respectively). Expression of dnRac and dncdc42 however failed to inhibit adhesion of cells to substratum indicating that events leading to adhesion and shape changes may be independently regulated. These results confirm the role of CTLA-4 in regulating pathways involved in actin polymerisation.

### 2.4.7. CTLA-4 induced changes is independent of upstream signalling events

Although a mechanism for CTLA-4 mediated signalling operating from the cytoplasmic tail of CTLA-4 has yet to be found, it is clear that TCR signals are influenced by coligation of CTLA-4. In order to examine upstream signalling pathways originating from the TCR that might overlap with CTLA-4, we have developed an approach that permitted separate analysis and dissection of TCR mediated pathways. Two main pathways emanate following TCR mediated activation: activation of PKC isoforms and the release of intracellular Ca2+ stores from the ER. Independent activation of these two pathways can mimic TCR mediated activation. PKC will activate the mitogen-activated protein kinase pathway (MAPK) whereas Ca2+ will bind to calcineurin and activate the nuclear transcription factor NFAT. Inhibitors that block PKC and calcineurin, such as bisindolylmaleimide and cyclosporin respectively are capable of abrogating T cell activation. Thus we used the specific PKC activating phorbol ester PMA and the calcium ionophore Ionomycin to activate the two pathways separately. CTLA-4WT cells were activated with PMA or Ionomycin in conjunction with anti-CD3, anti-CD28 or anti-CTLA-4. Cells treated with PMA or PMA + anti-CD28 exhibited mild morphological changes (Figure 8 A panels a, c) with increased adhesion to the substratum, whereas PMA + anti-CD3 treated cells had in addition, a slightly more pronounced change in cell shape and some

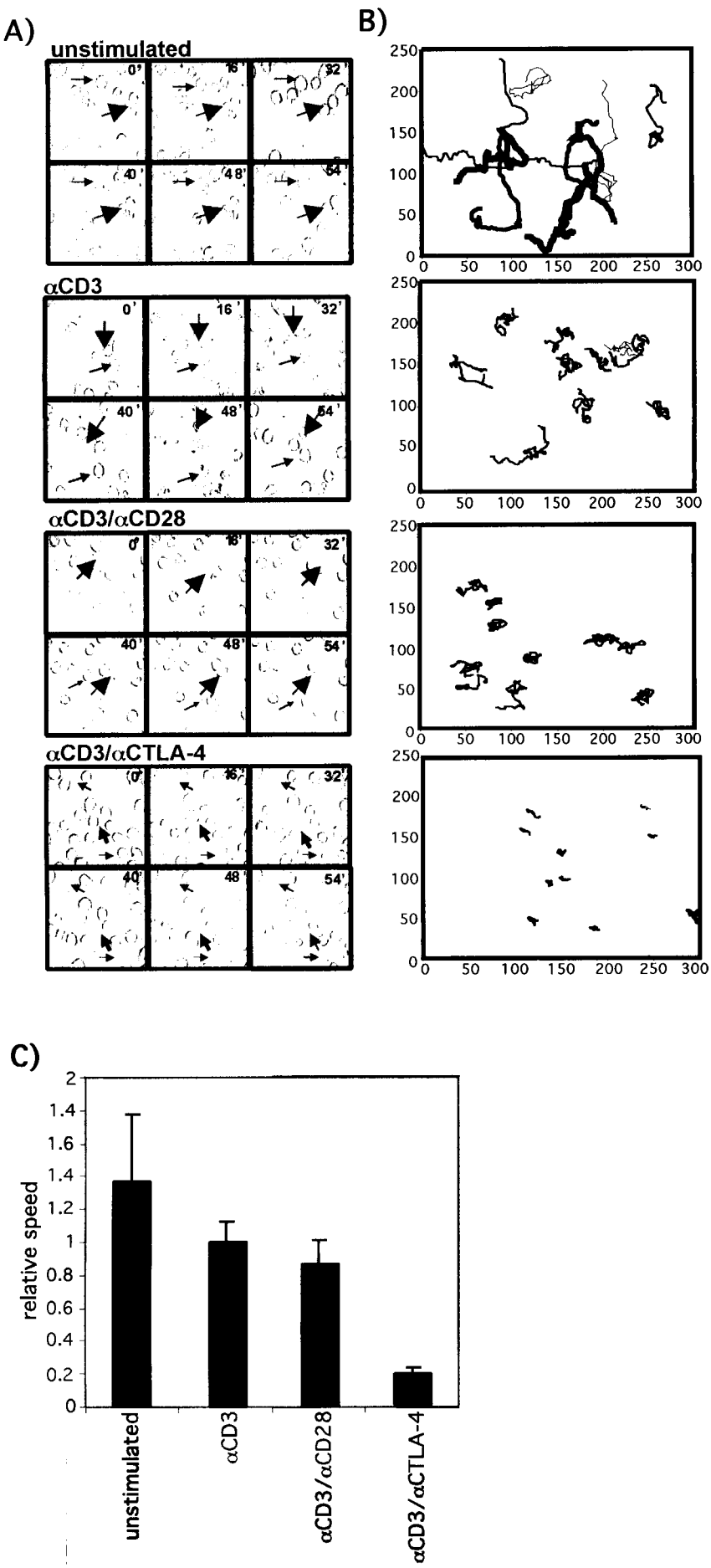



**Figure 6: Time lapse microscopy of cell movement. CTLA-4WT cells were stimulated as previously indicated in 96 well-plates and followed for a period of 1 hour. Recording was started 10 min after addition of cells to the wells.**
**(A) DIC images of cells at 37°C for a period of 1 hour under different stimulation conditions. Arrows indicate two cells that were followed and their movement recorded during a time period of 1h. Unstimulated cells show movement across the field of view. Anti-CD3 stimulated cells show increased crawling compared to non-stimulated cells. Anti-CD3/CD28 stimulated cell display decreased movement compared to non-stimulated. One of the cells appeared at a 40' after the start of the recording (fine arrow) while the other stayed in the same field of view during the recording (thick arrow). Anti-CD3/CTLA-4 stimulated cells show a decrease in motility. Cells indicated by an arrow remained mainly stationary and showed spreading accompanied by morphological changes. These cell shape changes are difficult to visualise since the cells become less refractile to light as they start spreading. Arrows show the movement of three different cells.**
**(B) Tracking profile of experiment measured in (A). A total of 10 cells were followed to track their relative distance of migration for each stimulation condition. Unstimulated cells mainly continued their original direction of travel while anti-CD3 and anti-CD3/CD28 induced crawling. Anti-CD3/CTLA-4 stimulation immobilized the cells.**
**(C) Average relative speed of cells measured in (B). While unstimulated, anti-CD3 and anti-CD3/CD28 stimulation show similar velocities, anti-CD3/CTLA-4 stimulation inhibited mobility.**

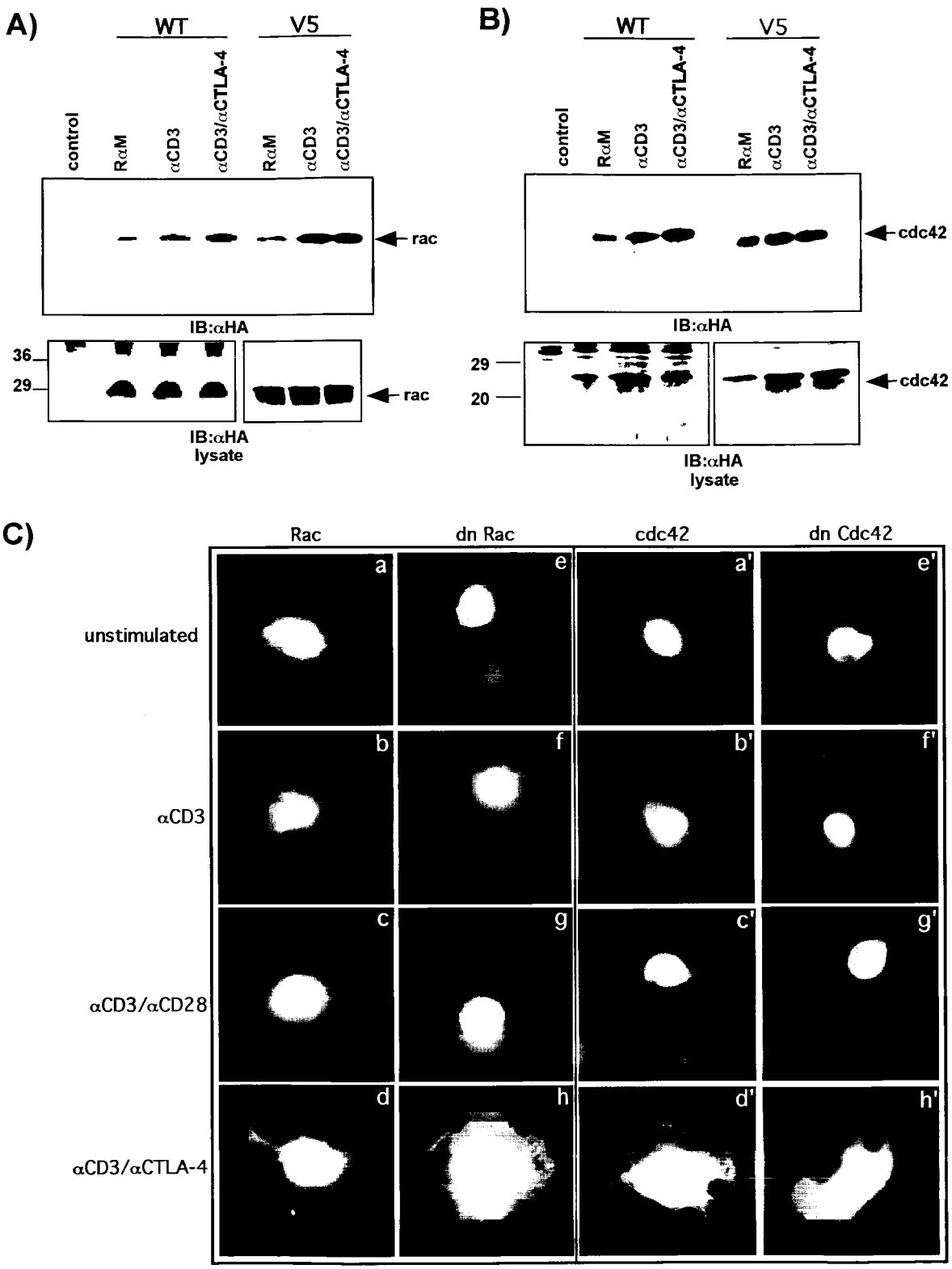



Figure 7: Rac and Cdc42 activation following CTLA-4 cross-linking. GST-Pak was used to precipitate the active form of transfected rac HA-tagged (A) and cdc42 HA-tagged (B) in CTLA-4WT cells. Cells were incubated with antibodies 2.5 μg/ml αCD3: 10 μg/ml αCTLA-4 for 1h on ice and crosslinked with 2.5 μg/ml of rabbit anti-mouse and mouse anti-hamster secondary antibodies. The cells were incubated at 37°C for 20 min and lysed with 0.1% TritonX-100 lysis buffer. Supernatant was used to precipitate active form of Rac and Cdc42. Cell lysate of Rac-HA and Cdc42-HA transfected CTLA-4WT cells showing equal amounts of protein in all samples. (C) Immunofluorescence of Rac-HA (a-d), dn Rac-HA (e-h), Cdc42-HA (a'-d') and dn Cdc42-HA (e'-h') transfected CTLA-4WT cells activated using the indicated platebound antibody.

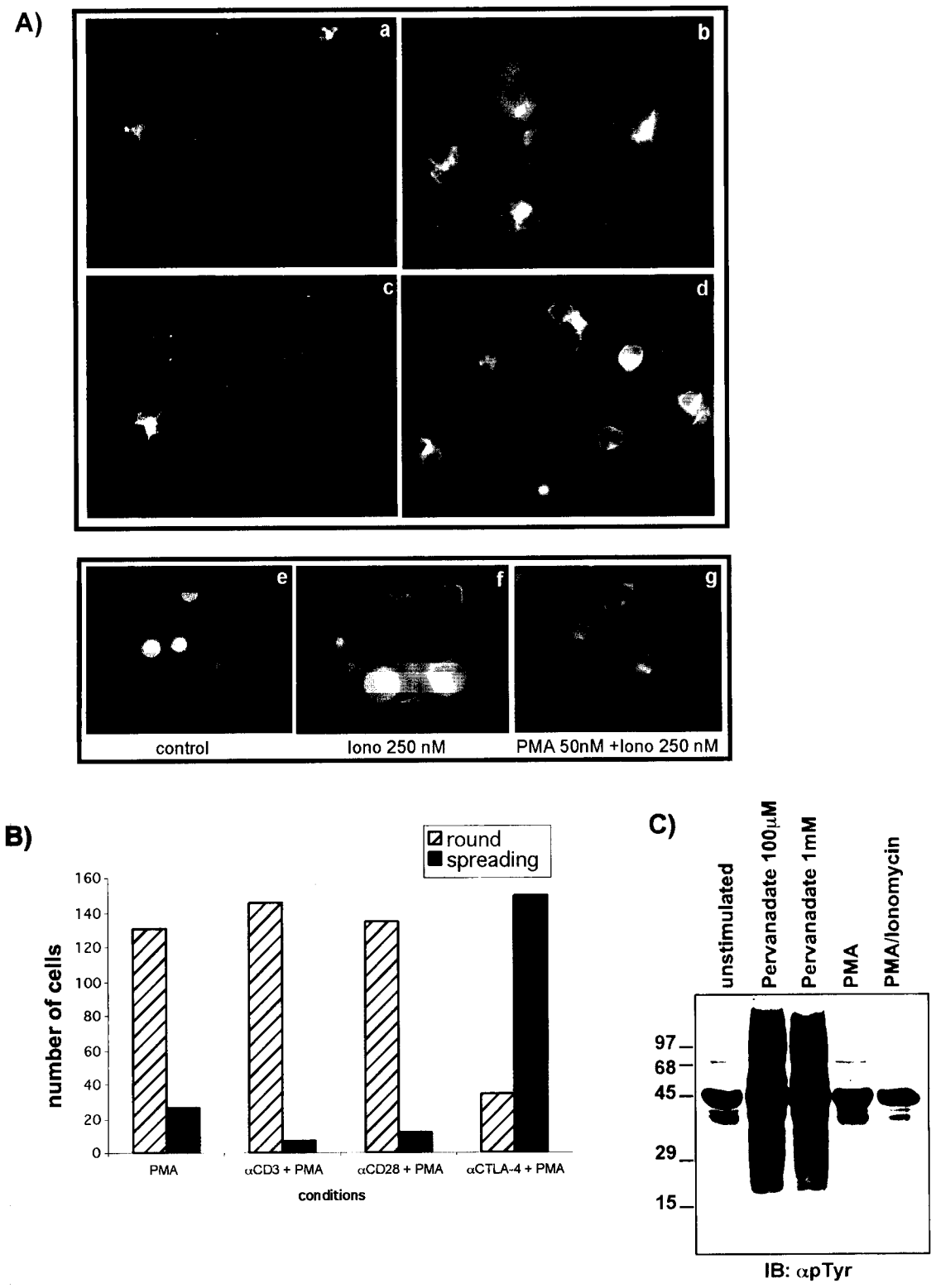



**Figure 8: Effects of PMA and Ionomycin on CTLA-4 induced cell shape changes. (A)CTLA-4WT cells were activated with 50 nM PMA in addition to medium (a), platebound 0.5 μg/ml αCD3 (b), 10 μg/ml αCD28 (c), 10 μg/ml αCTLA-4 (d). Cells were also activated with 250 nM Ionomycin + 10 μg/ml αCTLA-4 with (g) or without (f) PMA. Unstimulated cells were cultured with medium alone (e). Cells were activated for 4h, fixed and stained with phalloidin-FITC. (B) PMA + 10 μg/ml αCTLA-4 induced mostly lamellipodia formation. Approximately 170 cells were counted for lamellipodia formation for each stimulation as indicated. (C)Anti-phosphotyrosine immunoblot of cell lysates stimulated with PMA or PMA/Ionomycin shows no increased phosphorylation. Control pervanadate stimulation was shown to induce high levels of phosphorylation at two different concentrations.**

morphological changes associated with lamellipodia (Figure 8 A panel b). In PMA + anti-CTLA-4 treated cells however, a profound induction of lamellipodia was observed (Figure 8 A panel c). In most cases, CTLA-4WT cells displayed membrane ruffling suggesting that CTLA-4 ligation had a synergistic effect with PMA in lamellipodia formation (Figure 8 B). In contrast, anti-CTLA-4 + Ionomycin induced formation of microspikes and filopodia (Figure 8 A panel f), suggesting that CTLA-4 may regulate the formation of filopodia and lamellipodia at different levels and regulate separate pathways involved in cell shape morphology. This hypothesis was confirmed by treating CTLA-4WT cells with PMA, Ionomycin and anti-CTLA-4. The resulting phenotype was similar to that observed with TCR ligation, however, the formations of lamellipodia using this protocol overrode the effects of Ionomycin (Figure 8 A panel g). It is possible that by adjusting concentrations of PMA and Ionomycin to lower levels may achieve a similar threshold for filopodia formation such as that seen during TCR activation.

### 2.4.8. CTLA-4 alters the nature of T-cell interaction with APCs

To further confirm these findings in a more relevant physiological context, we used an established antigen presenting system consisting of T8.1 cells that had been transfected with an antigen-receptor specific for tetanus toxoid peptide (Ttox) in the context of HLA-DR*1102 (Boitel et al., 1995; Michel and Acuto, 1996). Peptide is presented by murine fibroblast L625.7 that expresses the appropriate HLA-DR antigen. T8.1 cells were subsequently transfected with human CTLA-4 and stable transfectants were generated (see appendix 4) for characterisation of the T8.1CTLA-4 and T8.1 vector clones). As shown in Figure 9A, T8.1Vector and T8.1CTLA-4 cells express relatively the same amount of CD3 on the cell surface, while CD28 expression on both cell lines is not detectable. hCTLA-4 is expressed at high levels on the surface of the transfected T8.1 CTLA-4 cells, whereas almost 100 fold higher expression of hCTLA-4 is to be found in intracellular compartments. Both transfectants remained round when left unstimulated. Stimulation of T8.1Vector and T8.1CTLA-4 cells with anti-CD3 did not alter the morphology of the cells (Figure 9 B panel b, e). In contrast, stimulation with anti-CD3/anti-CTLA-4 promoted identical morphological changes as previously observed with CTLA-4WT cell line (Figure 9 panel f). Having confirmed that CTLA-4 can function as a modulator of cell morphology in this model, we next considered the hypothesis of whether the morphological changes demonstrated with activating antibodies to CD3 and CTLA-4 could be reproduced in a more physiological context as during antigen presentation. Thus, we used this model to test whether CTLA-4 ligation induced cell shape changes when

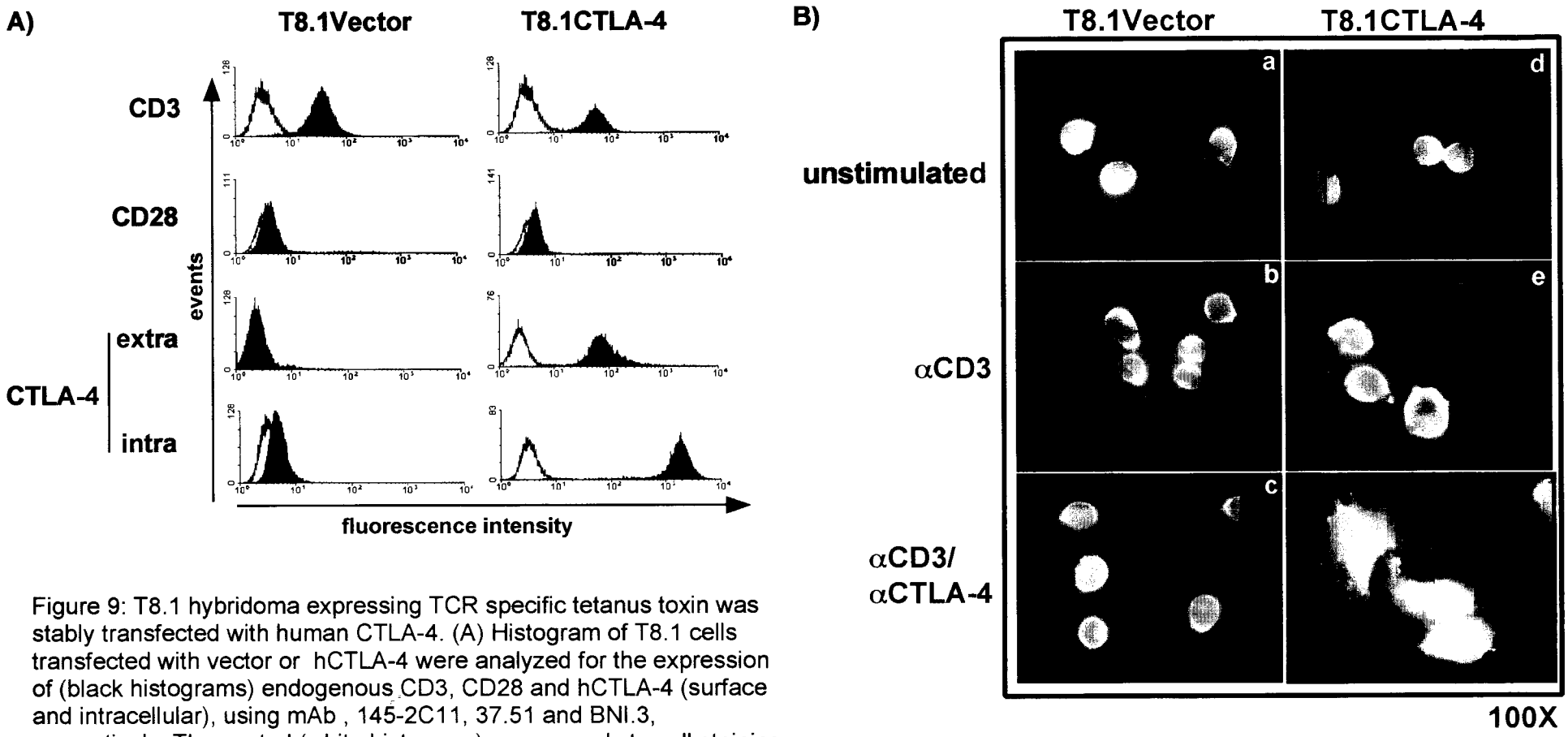



Figure 9: T8.1 hybridoma expressing TCR specific tetanus toxin was stably transfected with human CTLA-4. (A) Histogram of T8.1 cells transfected with vector or hCTLA-4 were analyzed for the expression of (black histograms) endogenous CD3, CD28 and hCTLA-4 (surface and intracellular), using mAb , 145-2C11, 37.51 and BNI.3, respectively. The control (white histogram) corresponds to cell staining with secondary antibody alone FITC-conjugated goat-anti-hamster mAb or PE-conjugated mouse IgG. (B) T8.1Vector and T8.1CTLA-4 cells were activated as previously described with platebound 0.5 μg/ml αCD3: 10 μg/ml αCTLA-4. Cells were fixed and stained with phalloidin-TRITC. Filopodia and lamellipodia were observed in T8.1CTLA-4 cells stimulated (f) compared to T8.1Vector stimulated (c) cells.

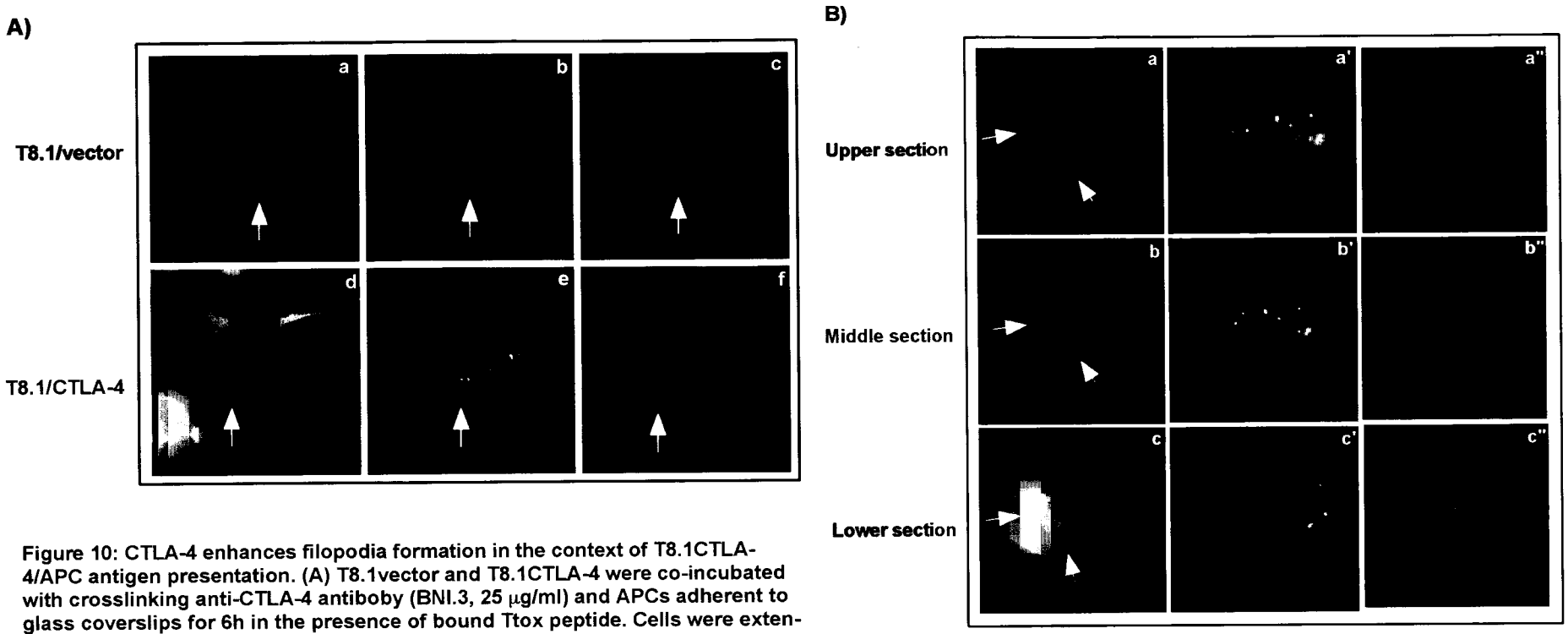



Figure 10: CTLA-4 enhances filopodia formation in the context of T8.1CTLA-4/APC antigen presentation. (A) T8.1vector and T8.1CTLA-4 were co-incubated with crosslinking anti-CTLA-4 antiboby (BNI.3, 25 μg/ml) and APCs adherent to glass coverslips for 6h in the presence of bound Ttox peptide. Cells were extensively washed with PBS to remove unbound cells, fixed with 2% paraformaldehyde and stained with phalloidin-TRITC. Cells on the coverslip were mounted on a slide and analysed by fluorescence microscopy. T8.1 cells, vector and CTLA-4 transfected, appear green (b, e) whereas actin filaments for both APC and T8.1 cell appear red (a, d). The nucleus was stained with hoechst (c, f). (B) Cross-sections of T8.1CTLA-4 cell stimulated with bound peptide to APC and anti-CTLA-4 antibody cell for 6h. The cells were treated as mentioned above. Filopodia extensions are denoted across the section, forming a tight junction and spreading in the contact region with the APC. T8.1CTLA-4 cell appears green (a', b', c') whereas actin filaments for both APC and T8.1 cell appear red (a, b, c). The nucleus was stained with hoechst (a'', b'', c'')

coligated to a tetanus toxin specific TCR in the presence of the agonistic peptide presented on an antigen presenting cell. T8.1Vector and T8.1CTLA-4 cells were labelled with PKH67 prior to coculture with the APCs + peptide and are shown in green whereas phalloidin-TRITC was used to stain for actin. The nucleus was stained with hoechst. As shown in Figure 10 A panel (a), T8.1Vector cells stimulated with peptide + anti-CTLA-4 and stained with phalloidin did not show any accentuated morphological changes. In contrast, T8.1CTLA-4 cells stimulated under the same conditions demonstrated a marked extension of filopodia (Figure 10 A, panel d). A view of 3 different sectional planes showed that the formation of filopodia enhanced T8.1 CTLA-4 adhesion to APC by promoting a tight interface between the T cell and the APC (Figure 10 B). This result shows that CTLA-4 ligation induces the same phenotypic changes whether in the context of antibody activation or peptide/TCR interaction.

## 2.5. Discussion

Here we present evidence that CTLA-4 can affect changes in shape morphology, adhesion and migration at a very early time point. Stimulation by anti-CD3 or antiCD3/anti-CD28 induces focal points of actin polymerisation. In contrast, stimulation of CTLA-4WT cells by anti-CD3/anti-CTLA-4 induced shape changes while still inhibiting IL-2 production (Figure 2C, 3A). Similarly, anti-CD3/anti-CTLA-4 costimulation of preactivated PBLs expressing a fair amount of CTLA-4 promoted spreading and extensions of the plasma membrane (Figure 4A). These changes are accompanied by the formation of specific structural changes, mainly filopodia formation (Figure 3B, C, 4B, C). It has been shown in other studies that TCR can induce these structural changes as well (Borroto et al., 2000; Bunnell et al., 2001; Lowin-Kropf et al., 1998; Parsey and Lewis, 1993; Sedwick et al., 1999; Wulfing et al., 2000). The reason that in our system we do not see these changes with the TCR may simply be a difference in stimulation regime and protocol used. Just as is the case with costimulation by anti-CD28, some cells can bypass the need for costimulation if the cells are stimulated under optimal conditions. Higher concentration of anti-CD3 failed to produce the same structural changes induced with TCR alone. However the tracking studies suggest some similarities (Negulescu et al., 1996). In addition to the shape changes, there is an increase of adherence, which subsequently increases the area of contact between the T cell and the stimulating antibody (Figure 5). The effect of CTLA-4 on migration was further analysed by examining the migratory behaviour of CTLA-4WT cells on the stimulating substratum. Anti-CD3/anti-CTLA-4 stimulation showed a decreased movement of the cells (Figure 6). This result suggested that CTLA-4 might send a restraining signal to cells that have encountered a stimulus. This model fits with several reported findings that CTLA-4 is highly expressed in the lymph nodes and tonsils where active and constant antigen presentation is taking place.

To reach a site of inflammation, T cells must become motile and migrate across the endothelial barrier to reach the site of inflammation or antigen presentation. The signals that are required for cells to gain motility or stop once they reached the suitable target are poorly defined. Chemokines and other soluble factors have been shown to attract cells to the sites of ongoing immunological activity (Bromley et al., 2001) but the mechanism by which cells, once they've reached their destination, become stable enough to accomplish their function, is unknown. These results would suggest that CTLA-4 might block withdrawal of T cells from antigen presenting sites and suspend migration for a prolonged period of time. Other systems have also been shown to induce the same type

of findings. Dustin et al., have shown that ICAM-1 can deliver a "stop signal" to block migration of T cells (Dustin et al., 1992; Dustin et al., 1996). However, they do not show if the stopping was achieved for a prolonged period of time. It is possible that CTLA-4 may be more efficient than ICAM-1 at suspending migration, especially in specific tissues during an immune response. ICAM-1 and B7-1/2 have a different tissue distribution. ICAM-1 is primarily expressed in endothelial and epithelial cells, highly expressed on activated endothelial cell and moderately expressed in activated T and B lymphocytes, while B7-1 and B7-2 molecules are restricted to haematopoetic tissues (dendritic cells and B cells). Thus, it may be possible that CTLA-4 plays a crucial role in T cell migratory events in lymphoid specific tissues.

To study the mechanism of CTLA-4-induced cell shape changes, Rho family of small GTPases were analysed for their participation in the induction of filopodia and lamellipodia formation. Here we show that Rac and Cdc42 activation is upregulated by CTLA-4 coligation (Figure 7). In addition, dnRac and dnCdc42 fail to localize when cells are stimulated with anti-CD3/anti-CTLA-4 suggesting that CTLA-4 is directly linked to activation of Rac and Cdc42 in promoting cell shape changes. In addition, the fact that CTLA-4 may cooperate with PMA or Ionomycin to induce shape changes suggests that the signalling pathway may intersect at a juncture which is independent of TCR phosphorylation and LCK activity (Figure 8A, C). Thus, the orchestration of these two pathways function to induce polymerisation of actin filaments and microtubules. As to my knowledge, this is the first evidence that CTLA-4 may function to convert signalling from the TCR in response to antigen. This result was confirmed by transfection of hCTLA-4 into a cell line expressing T cell receptor specific for tetanus toxin. Using this model to study signalling during antigen presentation, it was shown that crosslinking CTLA-4 on T cells in conjunction with stimulation via peptide bound to MHC presented on an APC provided the same support for signalling and induction of cell shape changes as in response to anti-CD3/anti-CTLA-4 stimulation (Figure 10A, B). The interaction between the T cell and the APC was tightly juxtaposed and wound around the APC providing much firmer and sustained signalling. Filopodia formation was easily detectable during this interaction. Thus, based on these two models for activation, CTLA-4 coligation contributes assistance in relaying TCR activating signals towards control of cell binding and cellular migration.

CTLA-4 has been extensively studied as a downmodulator of the immune system. The function of CTLA-4 has always been regarded as a negative regulator of signalling where setting of a higher threshold for TCR activation and dampening of early phosphorylation events were proposed as being the major mechanisms by which CTLA-4

operated. The first evidence of its inhibitory role in cytokine production and proliferation were confirmed by the generation of knockout mice that showed a fatal autoreactive disease characterised by extensive lymphoproliferation and infiltration of T cells into tissues (Tivol et al., 1997; Waterhouse et al., 1995). In addition, it provided clues as to its role in determining a balance between differentiation of cells into T helper 1 and T helper 2 cytokine profile which indicated that, although important as a negative regulator of the immune response, CTLA-4 could also provide signals which were able to promote the generation of specific effector cells perhaps by altering the threshold of signalling. CTLA-4 may differentially affect Th1 vs Th2 responses based on either high or low affinity for the ligands. More recently, CTLA-4 signalling has also been shown to regulate the suppressive activity of CD4+CD25+ regulatory T cells (Treg).

Nevertheless, the mechanism by which CTLA-4 may mediate these tolerogenic functions is still unknown. One of the main problems in working with in vivo models is the broader and intricate interactions that occur between different cell types. In addition, interaction of costimulatory molecules within lymph nodes and at the site of antigen presentation cannot be controlled. These variables can affect the results, which are seen in terms of days or weeks. Although informative in terms of bridging the knowledge acquired in the context of disease and pathology with functionality, it is difficult to reconcile these data with at the molecular level. Thus, this study encompasses both short term and long term analysis of CTLA-4 negative function. In this study, we show that CTLA-4 may have a negative effect on IL-2 production and migration, however, it occurs through a newly described mechanism of increased adhesion and filopodia/lamellipodia formation involving remodelling of actin filaments and microtubule polymerisation. It is likely that these events gain predominance during a secondary response to an antigen rechallenge since CTLA-4 is upregulated and expressed in memory cells. According to this hypothesis, one would predict that CTLA-4 expressing cells may have increased ability to respond to antigen and localise more rapidly to sites of ongoing immune response that express B7 ligands. It is also possible that changes in morphology and the arrest in cell movement can influence and facilitate reprogramming of differentiation pathways. Moreover, a link between induction of tolerance and remodelling of the actin filaments and changes in cell shape has yet to be investigated. It is still not clear what direct or indirect role the filopodia might play in inhibiting the TCR response. It is possible that the recruitment of CD3 to localised endings of the filopodia may disrupt the immunological synapse (Dustin and Cooper, 2000). In addition, these morphological changes may be necessary for efficient transduction of signals ***in vivo***, due to the complex environmental architecture of lymphoid tissues. Thus, I propose that, in contrast to CD28 which is

regarded as an amplifier of TCR signals, the function of CTLA-4 should be reconsidered as a modulator of the TCR signalling pathway, providing evidence for mechanism that implicates a molecular switch between proliferation, cytokine production and adhesion, decreased motility and morphological changes (Figure 11).

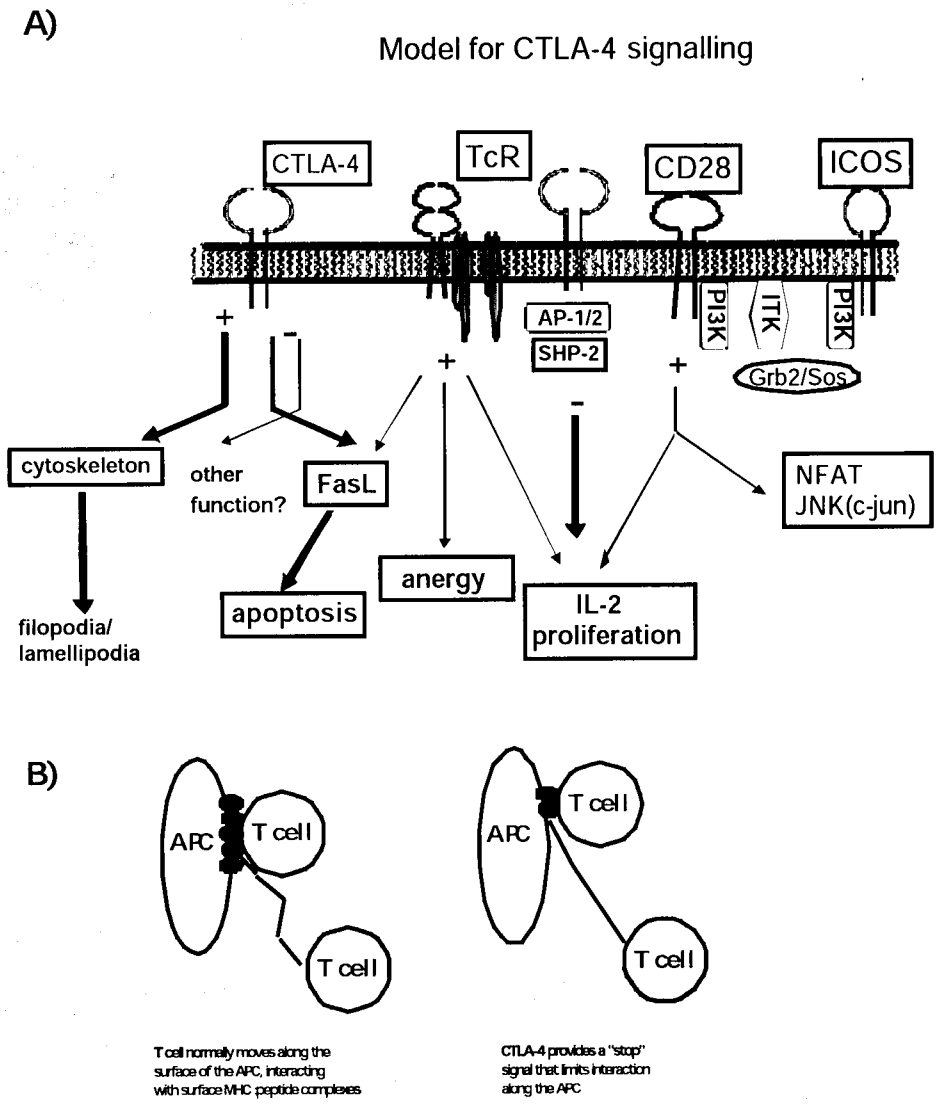



Figure 11: Model for CTLA-4 mediated signalling. A) Based on the results presented in this chapter, CTLA-4 may act at the level of inhibition of IL-2 and induction of lamellipodia and filopodia formation. B) Model for CTLA-4 induction of shape changes. CTLA-4 may prevent movement along the surface of the APC and restrict the number of MHC interactions. This event may allow the cell to re-direct signalling pathway towards differentiation or induce tolerance.

# Chapter 3.
# The Role of CTLA-4 in Antigen Induced Cell Death

"CTLA-4 blockade of antigen-induced cell death"
**Blood, 15 February 2001, vol 97, n4: 1134-1137.**

Experiments in T cell clones and activated murine T cells showed that CTLA-4 could induce cell death, suggesting that CTLA-4 may be responsible of inhibitory signalling via a mechanism involving apoptosis. The purpose of this study was to pinpoint the stage at which CTLA-4 inhibited TCR mediated signals and to determine whether crosslinking of CTLA-4 could actively generate negative signals.

Brief report

# CTLA-4 blockade of antigen-induced cell death

Silvy da Rocha Dias and Christopher E. Rudd

**While cytotoxic T lymphocyte antigen–4 (CTLA-4) negatively regulates T-cell receptor (TCR)–driven interleukin (IL)–2 production and proliferation, little is known regarding whether the coreceptor has the capacity to inhibit other events, such as Fas ligand (FasL) expression and antigen-induced cell death (AICD). In this study, it is shown that CTLA-4 expressed in a T-cell hybridoma can elicit a potent block of FasL expression and AICD. Inhibition occurred independently of CTLA-4 blockage of IL-2 production and was partially reversed by a single mutation in the cytoplasmic YVKM motif. These findings indicate that CTLA-4 can block TCR signaling prior to bifurcation of signals leading to IL-2 production and apoptosis.(Blood. 2001;97:1134-1137)**



## Introduction

Cytotoxic T lymphocyte antigen-4 (CTLA-4) negatively regulates T-cell activation initiated by ligation of T-cell receptor (TCR)ζ/CD3 and CD28.[1-3] Blocking with soluble anti–CTLA-4 Fab fragments can augment T-cell responses,[4,5] while co–cross-linking of CTLA-4 with either TCR or TCR × CD28 potently inhibits proliferation and interleukin (IL)-2 production.[6-8] Consistent with this, CTLA-4$^{-/-}$ mice show extensive lymphadenopathy with T-cell infiltration of various tissues.[9-13] This event requires CD28 since CTLA-4Ig blockage of CD28 binding to CD80/86 reverses the phenotype.[11] Isolated CTLA-4$^{-/-}$ T-cells also exhibit a greatly enhanced response to antigen,[14-16] leading to the notion that the lowering of the signaling threshold to self-antigen and/or weakly antigenic foreign antigen is responsible for the lymphadenopathy. In this context, TCR transgenic mice on a Rag$^{-/-}$ × CTLA-4$^{-/-}$ background fail to develop disease.[15,16] CTLA-4 has also been linked to anergy induction,[17,18] while its absence enhances the development of $T_H2$ cells.[12]

Despite its immunologic importance, the molecular basis for CTLA-4 function has yet to be established. Multiple intracellular proteins, including phosphatidylinositol 3-kinase (PI3-K),[19,20] the tyrosine phosphatase SHP-2,[21,22] as well as the clathrin-associated adaptor protein complexes AP-2[23-26] and AP-1[27] interact with the cytoplasmic domain. PI3-K, AP-1 and AP-2 bind directly to the GVYVKM motif, while the SHP-2 association appears to be indirect, possibly mediated through another protein such as PI3-K.[28] PI3-K binds a tyrosyl-phosphorylated version of the motif,[19] while AP-1/AP-2 complexes bind to an extended version of the nonphosphorylated sequence that includes additional N-terminal residues.[23-26] The AP-2 tetramer regulates trafficking and endocytosis of the coreceptor from the cell surface.[24,26] AP-1 acts to ensure steady-state levels of intracellular CTLA-4 by shuttling the receptor to lysosomes for degradation.[27] Further downstream, CTLA-4 engagement has been reported to block extracellular signal-regulated kinase and Jun NH2-terminal kinase activity[29] and the induction of IL-2 transcription.[30-32]

TCR/CD3 ligation induces antigen-induced cell death (AICD) as mediated by Fas (CD95/APO-1) and Fas ligand (CD95L) interactions and the activation of caspases, a process that ensures the elimination of self-reactive thymocytes and peripheral blood lymphocytes.[33-36] Although AICD operates in CTLA-4$^{-/-}$ T cells,[14] little is known regarding whether CTLA-4 has the capacity to block TCR-mediated signals leading to AICD. In this regard, T-cell hybridomas have served as a model for the study of AICD[33,34]; in particular, the hybridoma DC27.10 has been previously studied for the role of costimulation in the induction of apoptosis.[37] In this report, we demonstrate that CTLA-4 can block AICD in transfectants of the murine hybridoma DC27.10 via inhibition of Fas ligand (FasL) surface expression. These findings support a model in which CTLA-4 interferes with an early TCR-signaling event linked to both IL-2 production and FasL expression.

## Study design

### Cells, reagents, and antibodies

Murine T-cell hybridoma DC27.10 (gift from Dr R. Zamoyska, Medical Research Council, London, United Kingdom) was cultured in RPMI-1640 containing fetal bovine serum 5% (vol/vol) (Sigma, St Louis, MO), L-glutamine (2 mM), penicillin (100 U/mL), and streptomycin (100 μg/mL) (Life Technologies, Grand Island, NY) at 37°C, and 5% $CO_2$. DC27.10 transfectants carrying different transfected forms of human CTLA-4 (wild-type human CTLA-4, Y201-2F) were generated as described.[38] Hamster anti–murine CD3 (145-2C11) was purchased from American Type Culture Collection (Manassas, VA), mouse anti–human CTLA-4 (BNI3.1) was purchased from Immunotech (Marseilles, France). Mouse immunoglobulin G2a (specific for 2,4,6-trinitrophenol [TNP]),

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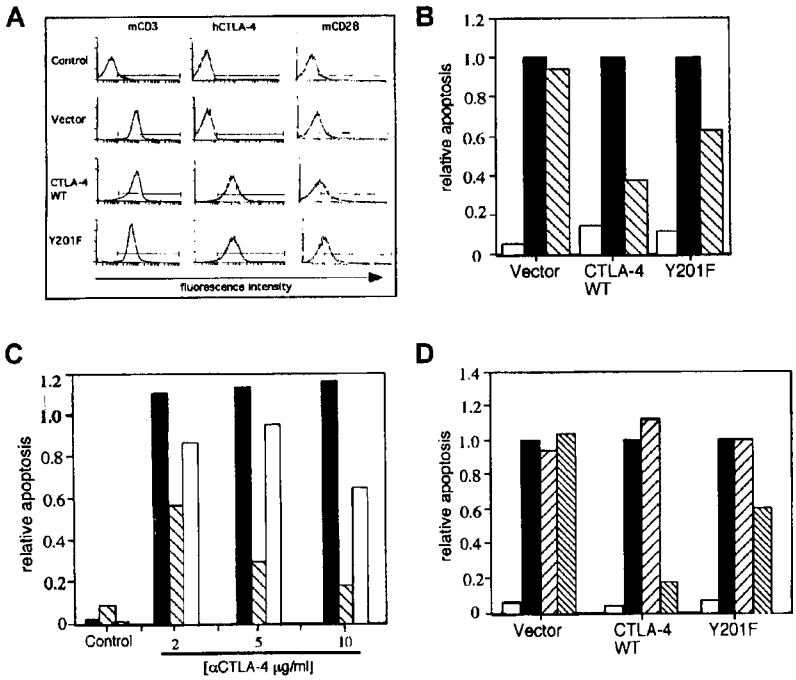


**Figure 1. CTLA-4 inhibition of AICD in T-cell hybridoma transfected with human CTLA-4.** (A) Fluorescent activated cell sorter (FACS) profile of TCRζ/CD3, human CTLA-4, and murine CD28 (mCD28) surface expression on transfectants. DC27.10 cells were stably transfected with a vector (denoted Vector), human CTLA-4 (denoted CTLA-4 WT), and CTLA-4 mutant (denoted Y201-F). (B) CTLA-4 inhibits AICD in CTLA-4 transfectants. Cells were stimulated on a 96-well plate coated with anti-CD3 (145-2C11) at a concentration of 0.5 μg/mL and with anti–CTLA-4 (10 μg/mL). Anti-TNP control antibody was added to keep a final concentration of 10.5 μg/mL with anti-CD3 alone. AICD was determined by PI as described in "Study design." □ indicates media; ■, αCD3; and ▧, αCD3 + αCTLA-4. (C) CTLA-4 inhibits AICD in a concentration-dependent manner. Anti-CD3 was used at a concentration of 0.5 μg/mL, while anti–CTLA-4 was used at concentrations of 2, 5, and 10 μg/mL. Anti-TNP control antibody was added to keep the final concentrations equivalent. ■ indicates vector; ▧, CTLA-4 WT; and □, Y201F. (D) CTLA-4 inhibits AICD induced by anti-CD3 in conjunction with anti-CD28. Anti-CD3 was used at 0.5 μg/mL; anti-CD28 at 5 μg/mL; and anti–CTLA-4 at 5 μg/mL. Anti-TNP control antibody was added to keep the final concentrations equivalent. □ indicates media; ■, αCD3; ▨, αCD3 + αCD28; ▧, αCD3 + αCD28 + αCTLA-4.

hamster anti–mouse CD28 (37.10) purified rat anti–mouse IL-2 (JES6-1A12), biotinylated rat anti–mouse IL-2 (JES6-5H4), and biotinylated anti-FasL were purchased from Pharmingen (San Diego, CA). Avidin-peroxidase was purchased from Sigma. ABTS tablets and recombinant mouse IL-2 were purchased from Boehringer Mannheim (Indianapolis, IN). Mouse anti-hamster was purchased from Sigma. Phycoerythrin-labeled streptavidin was a gift from the laboratory of Dr Lee Nadler, Dana-Farber Cancer Institute (Boston, MA).

### Stimulation assay

For all experiments, stimulation of T-cell hybridoma was performed by means of antibodies bound to plates. The antibodies were added at their designated concentration in a 96-well microtiter plate and left to adsorb overnight at 4°C. The wells were washed twice with complete media, followed by the addition of cells at a density of $1.5 \times 10^5$ cells per well and incubation for 24 hours at 37°C. The cells were then harvested and analyzed for apoptosis. The supernatant was frozen for later measurements of IL-2 by enzyme-linked immunosorbent assay (ELISA) (Pharmingen).

### Apoptosis measurements

The samples were fixed with propidium iodide (PI) staining buffer (sodium citrate 0.1%, sodium dodecyl sulfate 1%, 50 μg/mL PI) overnight at 4°C. Apoptosis was analyzed on an Epics XL flow cytometry system (Coulter, Hialeah, FL) by gating on the subdiploid population below the G1 peak.

## Results and discussion

Stable transfectants of the T-cell hybridoma DC27.10 were initially generated that express either wild-type human CTLA-4 (CTLA-4 WT), or a mutant form with a substituted cytoplasmic tyrosine at position 201 in the YVKM motif (Y201-F).[27] Both CTLA-4 WT and Y201-F express the coreceptor at moderate levels with stable expression of TCR/CD3 and CD28 (Figure 1A). To assess whether CTLA-4 could influence the ability of the TCRζ/CD3 to induce apoptosis, anti-CD3 was immobilized on plates at a concentration of 0.5 μg/mL in the presence or absence of anti–CTLA-4 at a concentration of 10 μg/mL. Cell death was assessed 24 hours after stimulation by examining the DNA content by PI

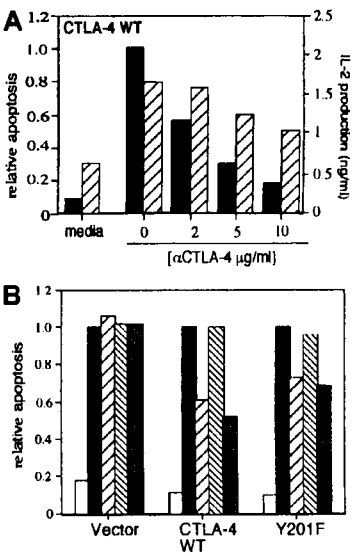


**Figure 2. CTLA-4 inhibition of AICD independent of a CTLA-4 effect on IL-2 production.** (A) CTLA-4 inhibits AICD and IL-2 production in CTLA-4 transfectants. Cells were stimulated with anti-CD3 at a concentration of 0.5 μg/mL and a range of anti–CTLA-4 concentrations from 2 to 10 μg/mL. Supernatants were collected 24 hours after stimulation, and IL-2 levels were determined by ELISA. Anti-TNP control antibody was added to keep the final concentrations equivalent. ▨ indicates IL-2 production; ■, relative apoptosis. (B) Addition of exogenous IL-2 does not reverse anti–CTLA-4 blockage of AICD. Cells were stimulated with 0.5 μg/mL of anti-CD3 and 10 μg/mL of anti–CTLA-4 with or without recombinant mouse IL-2 (20 U/mL). □ indicates media; ■, αCD3; ▨, αCD3 + αCTLA-4; ▧, αCD3 + rIL-2; ■, αCD3 + αCTLA-4 + rIL-2.

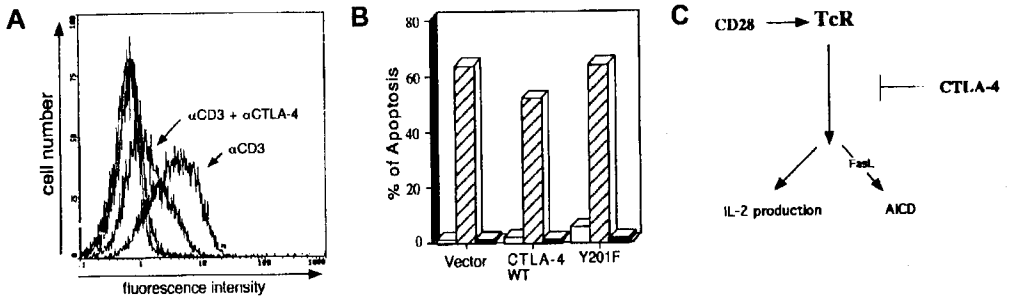


**Figure 3. Engagement of CTLA-4 accompanying reduced FasL surface expression.** (A) Anti–CTLA-4 reduced expression of FasL. Cells transfected with CTLA-4 WT were stimulated with the indicated antibodies bound to plates: anti–CTLA-4 (10 μg/mL), anti-TNP (10 μg/mL) control, and anti-CD3 (0.5 μg/mL). The cells were harvested after 6 hours, stained with biotinylated anti-FasL antibody followed by fluorescein isothiocyanate-labeled avidin. Subsequently, the cells were gated and analyzed by flow cytometry. A histogram of FasL staining on FL-1 is displayed. (B) Anti-FasL blocks anti-CD3–induced apoptosis in DC27.10 transfectants. CTLA-4 WT transfectants were stimulated with the indicated antibodies bound to plates: anti–CTLA-4 (10 μg/mL), anti-TNP (10 μg/mL) control, and anti-CD3 (0.5 μg/mL) with or without soluble blocking anti-FasL antibody (10 μg/mL). The cells were harvested 24 hours later and stained with PI. Apoptosis was measured as described in "Study design." □ indicates control; ▨, αCD3; ■, αCD3 + αFasL. (C) Model of CTLA-4 blockage of IL-2 production and AICD. The coreceptor acts to dampen TCR/CD3 signaling at an early stage in T-cell activation prior to the bifurcation of signals leading to IL-2 production and FasL expression and AICD.

staining as described in "Study design." The results were confirmed by DNA laddering techniques (data not shown). Anti-CD3 induced AICD in transfected cells in the range of 50% to 65% as observed in 7 experiments (data not shown).[33,34] Results were expressed as values relative to the anti-CD3 control. Coligation with anti–CTLA-4 caused a 60% reduction in the percentage of cells undergoing apoptosis in cells transfected with CTLA-4 WT (Figure 1B). In 7 experiments, anti–CTLA-4 blocked AICD within a range of 60% to 80%. No effect of anti–CTLA-4 was evident in vector-transfected control cells (Figure 1B). The efficacy of the AICD blocking was proportional to the concentration of anti–CTLA-4 in the range of 2 to 10 μg/mL (Figure 1C). Inhibition by CTLA-4 was also observed when anti-CD28 was coligated with anti-CD3 (Figure 1D). These findings indicate that coligation of CTLA-4 can attenuate anti-CD3–induced AICD.

The cytoplasmic tail of CTLA-4 has a tyrosine-based motif YVKM that binds several intracellular signaling proteins.[19-22] Mutation of the tyrosine within the YVKM sequence renders the motif unable to bind to these proteins. Disruption of the YVKM motif rendered CTLA-4 significantly less effective in blocking AICD (Figure 1B-C). A titration of anti–CTLA-4 showed that this partial blockage in Y201-F was observed only at the highest concentration of anti–CTLA-4 (Figure 1C). This partial inhibition was also observed with anti-CD3 × CD28 cross-linking (Figure 1D). These observations indicate that the optimal blockage of AICD by CTLA-4 is dependent on an intact YVKM motif.

CTLA-4 inhibition of IL-2 production is a hallmark of the blockage effects on T-cell activation.[6,7] Indeed, IL-2 production was inhibited by anti-CTLA-4 in a dose-dependent manner of 2 to 10 μg/ml of antibody on the CTLA-4 WT cells, albeit slightly less than observed for AICD (Figure 2A). To control for the possibility that the loss of IL-2 contributed to AICD, exogenous recombinant IL-2 was added in excess to cultures with anti-CD3 plus anti–CTLA-4 (Figure 2B). Under these conditions, IL-2 did not restore the induction of cell death, indicating that CTLA-4 blockage of AICD was not due to its blockage of IL-2.

AICD in T-cell hybridomas is mediated by the expression of Fas (CD95/APO-1), the expression of FasL (CD95L), and the activation of caspases.[33,34] To investigate whether CTLA-4 blocks AICD by means of an inhibitory effect on FasL expression, cells were incubated with anti-CD3 in the presence or absence of anti–CTLA-4 followed by staining for FasL surface expression. Under these conditions, anti–CTLA-4 coligation caused a 4- to 5-fold reduction in the level of FasL expression (Figure 3A). This level of inhibition correlated well with the overall reduction of AICD (Figure 1A-D). Fas expression was also reduced, but to a lesser extent and less reproducibly (data not shown). The blockage of FasL expression was observed reproducibly in 4 experiments. To confirm that the AICD in our hybridomas was mediated by Fas/FasL binding, blocking anti-FasL antibodies were added to cultures and found to completely inhibit cell death (Figure 3B). CTLA-4 therefore impinges on TCR-induced apoptosis by blocking the expression of FasL.

Our findings provide the first evidence that CTLA-4 has the capacity to block TCR-signaling events that lead to FasL expression and AICD. Scheipers and Reiser[44], however, have reported a role of CTLA-4 in the promotion of AICD. In their study, concanavalin A (ConA) is used as a stimulating agent instead of direct TCR cross-linking. It may be that stimulating with ConA and anti–CTLA-4 antibodies delivers different signals than using antibodies to directly cross-link the TCR with CTLA-4. Our study was not intended to address the physiological relevance of CTLA-4 on antigen-induced cell death, but rather to serve as a model to examine the point of blockage by CTLA-4 in the signaling cascade. With this perspective, our findings suggest that the coreceptor can block TCR signaling prior to the bifurcation of signals leading to FasL and IL-2 production (Figure 3C). Further CTLA-4 can block TCR signaling events that result in both positive (eg, IL-2 production) and negative outcomes (ie, apoptosis). Although the nature of signals that distinguish these events is unknown, $p56^{lck}$ and NF-AT have been reported to regulate both FasL and IL-2 expression.[39] Other transcription factors, c-Myc, Egr 2, and Egr 3, have been implicated in FasL expression.[40-42] In our system, the inhibitory effect of FasL expression and AICD must be an active process since it depends on the integrity of the cytoplasmic tail of the receptor (ie, YVKM motif). This contrasts with previous studies that demonstrated anti–CTLA-4 rescue of thymocytes from cell death by blockage of negative signaling.[43] In this context, our cellular assay could be used as an assay to distinguish between antibodies that operate by actively generating negative signals vs those that simply block engagement of the receptor with CD80/CD86. Further analysis will also be needed to define which of these associated proteins regulates the blockage of AICD and IL-2 production.

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# Chapter 4.
# The Role of the YVKM motif in CTLA-4 negative signalling

## "A regulatory role for cytoplasmic YVKM motif in CTLA-4 inhibition of TCR signaling", European Journal of Immunology, 2001 vol 31:2042-2050.

Subsequent to finding that CTLA-4 could actively generate a negative signal, several groups published that the cytoplasmic tail of CTLA-4 was not necessary for negative signalling. This is a significant discrepancy since it had been shown that the YVKM motif could become phosphorylated after activation and could bind to PI 3-kinase and SHP-2 signalling molecules. This study was undertaken to confirm the role of the YVKM motif and identify signalling pathways that may participate in CTLA-4 negative signalling.

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# A regulatory role for cytoplasmic YVKM motif in CTLA-4 inhibition of TCR signaling

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CTLA-4 negatively regulates TCR signaling, although the molecular basis for this effect has yet to be elucidated. The cytoplasmic YVKM motif, while binding to phosphatidylinositol 3-kinase, SHP-2 and the AP-1/AP-2 clathrin adaptor complexes, has been reported to play no role in CTLA-4 function. In contrast, in this study, we demonstrate that, although not essential, the YVKM motif contributes to optimal CTLA-4 blockage of TCRζ or combined TCRζ/CD28 signaling. Significantly, dependency on the YVKM motif varied with the mode of anti-receptor presentation, where soluble antibody ligation was more dependent on the presence of the motif than immobilized antibody. Previous studies have mainly relied on the use of immobilized antibody. Neither SHP-2 binding, alterations in TCRζ chain phosphorylation, nor ZAP-70 recruitment was involved in CTLA-4 wild-type or mutant inhibition. Overall, our findings clearly implicate the YVKM motif in optimal CTLA-4 function.

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**Key words:** CTLA-4 / YVKM motif / T cell signaling / Phosphatidylinositol 3-kinase / AP-2

## 1 Introduction

CTLA-4 signaling is well documented in its ability to down-regulate T cell proliferation and cytokine production [1–3]. *In vitro* studies using CTLA-4 antibodies have revealed that CTLA-4 can block TCRζ/CD28-mediated proliferation and IL-2 production in T cells [4–6]. Events such as NFAT translocation into the nucleus, the production of cell cycle proteins and the degradation of the cell cycle inhibitor p27 are inhibited by CTLA-4 [7]. Consistent with this, CTLA-4 knockout mice show extensive lymphadenopathy and cellular infiltration of tissues [8, 9]. In contrast to these negative effects, TGF-β production and polarization of cells into Th1 subtype have been reported to be dependent on proper CTLA-4 function [10].

Despite its central role in attenuating activation, little is known regarding the mechanism(s) responsible for CTLA-4 function. The cytoplasmic tail of CTLA-4 possesses two tyrosine residues at position Y201 and Y218 that are located in the motifs YVKM and YFIP, respectively. As originally shown with CD28 [11], src kinases $p56^{lck}$, $p59^{fyn}$ [12–14] and the Tec kinase Rlk [15] can phosphorylate CTLA-4 cytoplasmic tyrosines. Once phosphorylated, the CTLA-4 YVKM motif binds to the SH2 domains of the p85 subunit of phosphatidylinositol (PI) 3-kinase [16], and the tyrosine phosphatase SHP-2 [17–20]. The non-phosphorylated version of the same motif can bind to the clathrin adaptor complexes AP-1 and AP-2 [12, 13, 21–23]. In fact, phosphorylation of this residue prevents binding of AP-1/AP-2 to CTLA-4 and the internalization of the receptor [12–14, 22].

Several models have been proposed to explain CTLA-4 negative signaling. One model proposes that CTLA-4 binding to SHP-2 can mediate dephosphorylation of key constituents needed for TCR signaling [18–20]. However, SHP-2 is well established as a positive regulator of cell growth [24–26]. Further, several groups have found that negative signaling can occur independently of tyrosine phosphorylation and the YVKM motif [27–29].

Given the number of important intracellular proteins that bind to the YVKM motif, the finding that the YVKM motif plays no role in CTLA-4 function seemed quite unusual. In this study, we demonstrate that, although not essential, the YVKM motif does contribute to optimal CTLA-4 blockage of TCRζ/CD3 or combined TCRζ/CD3×CD28

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The first two authors contributed equally to this work.

**Abbreviations: PI 3-kinase**: Phosphatidylinositol 3-kinase **WT**: Wild type

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signaling. However, dependency on the motif varied with the mode of anti-receptor presentation. This was more evident with soluble antibody than with immobilized antibody, as used in other studies. Neither SHP-2 binding, alterations in TCRζ chain phosphorylation, nor ZAP-70 recruitment was involved in CTLA-4 wild-type (CTLA-4 WT) or mutant inhibition. Further, we found that inhibition of PI 3-kinase activity did not alter negative signaling, suggesting a possible role for the AP-2 complex.

## 2 Results

To establish a model for examining the function of CTLA-4, we generated stable transfectants of the mouse T cell hybridoma DC27.10 expressing human CTLA-4 (hCTLA-4). The same cells have been used in studies to examine the role of CD28 in T cell function [30]. Fig. 1A is representative of the surface expression of endogenous mouse TCRζ/CD3 (mCD3), mCD28 and hCTLA-4 for various clones. Although hCTLA-4 was over-expressed, the expression level was still lower than the level of mTCRζ/CD3 complex expression (Fig. 1A). Slight variations in the relative expression of mTCRζ/CD3 and mCD28 between the CTLA-4 WT and different Y201F clones were noted (Fig. 1A and D, left panel). However, this did not alter the functional trend outlined in this report.

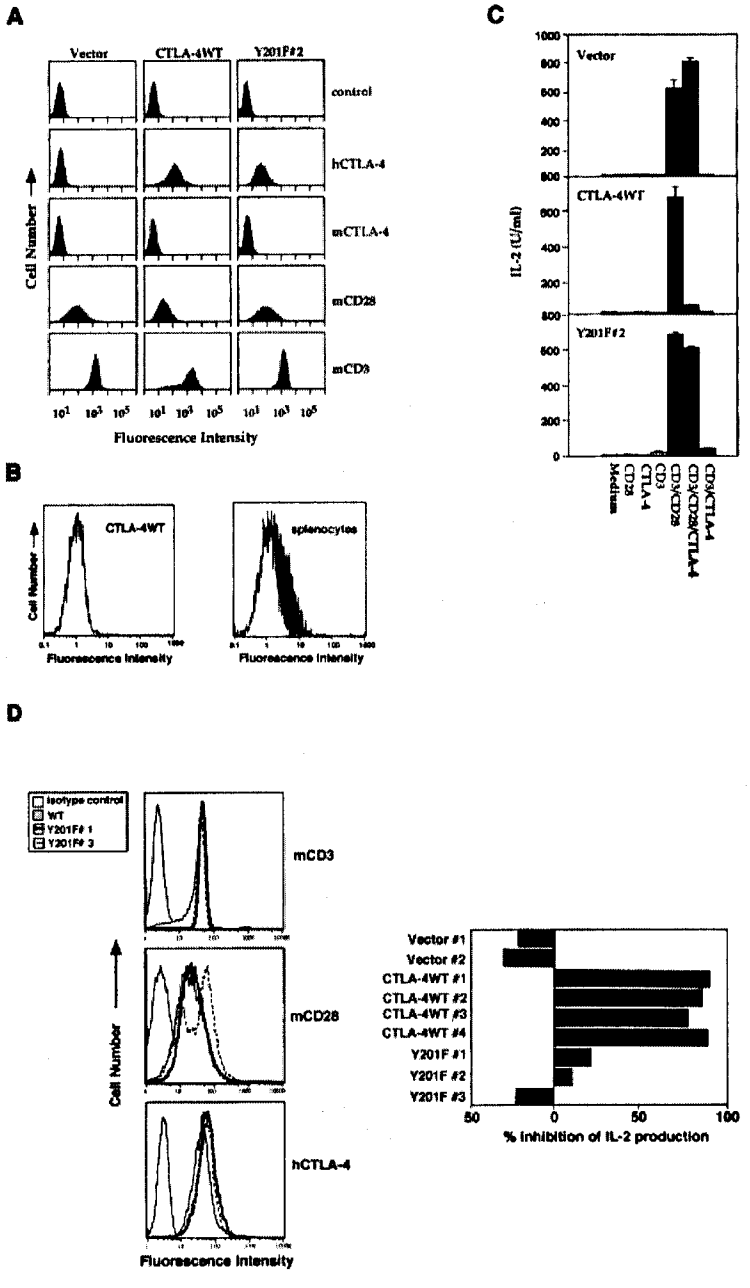
We next assessed whether cells expressed endogenous CTLA-4, since the antigen could alter the effects of transfected CTLA-4 in the generation of signals. Con A-stimulated hybridoma cells (2.5 μg/ml for 72 h) were analyzed for the presence of total CTLA-4. As shown in Fig. 1B (left panel), stimulated cells lack endogenous CTLA-4. As a positive control, Con A-stimulated splenocytes showed an induction of endogenous CTLA-4 (right panel).

Given this background, we next examined the effects of soluble antibody-induced cross-linking of CTLA-4 with CD28 and TCRζ/CD3 on IL-2 production, as described by others [31, 32]. As previously reported [30], induction of IL-2 production by soluble antibody in our hybridomas required co-ligation of TCRζ/CD3 and CD28. Anti-CD3 alone induced little if any IL-2 production. Co-ligation of CTLA-4 with TCRζ/CD3×CD28 markedly inhibited lymphokine production (Fig. 1C, D) [4, 5]. These data confirm that CTLA-4 WT can block TCRζ/CD3×CD28 induced IL-2 production in our transfectants.

CTLA-4 possesses two tyrosine residues (positions Y201 and Y218) in the motifs YVKM and YFIP, respectively. To assess whether the YVKM motif was required for CTLA-4 negative signaling, we assessed whether co-cross-linking of the mutant could block IL-2 production. As shown in Fig. 1C, disruption of the YVKM motif (*i.e.* Y201F) resulted in 80–90% reduction of soluble antibody-induced CTLA-4 inhibition of IL-2 production. This observation was not limited to the use of a single transfectant since an analysis of three other Y201F clones showed defective CTLA-4 negative signaling (Fig. 1D, right panel). These data indicate that the YVKM motif plays an important role in ensuring optimal negative signaling by CTLA-4.

Given the difference between our results and those from other studies [27–29], we were next interested in determining whether the relevance of the YVKM site varied with the mode of antibody presentation. Previous studies employed immobilized antibodies as a component in their presentation system [27–29]. Transfectants were stimulated with antibodies immobilized on plates. As demonstrated in Fig. 2A, plate-bound anti-CD3 was able to induce IL-2 production in the absence of anti-CD28 in all of the transfectants. Co-cross-linking of TCRζ/CD3×CTLA-4 induced a dose-dependent inhibition of IL-2 production in the CTLA-4 WT (Fig. 2A, from 25% to 70% of inhibition). In addition, co-cross-linking of the Y201F mutant with immobilized antibody was able to block IL-2 production by approximately 30–40% (Fig. 2A), in contrast to soluble antibody cross-linking (Fig. 1C, D). The level of inhibition in both CTLA-4 WT and mutant Y201F varied with the concentration of anti-CD3 mAb (Fig. 2B). Similar overall results were obtained with Y201F mutant and CTLA-4 WT in the blockage of TCRζ/CD3×CD28 mediated stimulation (Fig. 2C). Taken together, these data indicate that the YVKM motif can facilitate negative signaling, but is not essential. Further, the importance of the YVKM site varied with the mode of antibody presentation.

Given the importance of this motif, we next analyzed whether it was possible to distinguish between the different YVKM binding proteins in negative signaling. As shown in Fig. 3A, anti-CTLA-4 readily precipitated both the AP-1 and AP-2 complexes from whole cell lysates as detected using an antisera against the β-chains (lane 4, lower panel) as well as PI 3-kinase (lane 4, upper panel). The Y201F mutation abrogated AP-1 binding, which resulted in the release of CTLA-4 from the trans-Golgi network [21]. The same mutation resulted in the loss of AP-2 complex and PI 3-kinase binding (lane 3) [21]. CD28 also failed to bind to AP-1 and AP-2 (lane 2, lower panel) [13, 21]. To test for PI 3-kinase activity, the involvement of anti-CTLA-4 blockage of IL-2 production was conducted in the presence of various concentrations of the PI 3-kinase inhibitor LY294002 (Fig. 3B). To distinguish PI 3-kinase signals derived from CTLA-4 (and not CD28), we used plate-bound anti-CD3 mAb, which provides a potent signal and induces IL-2 production on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Vector
CTLA-4WT
Y201F#2
control
hCTLA-4
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mCD28
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B
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splenocytes
Cell Number
Fluorescence Intensity
Fluorescence Intensity
C
Vector
CTLA-4WT
Y201F#2
IL-2 (U/ml)
Medium
CD28
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CD3
CD3/CD28
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WT
Y201F# 1
Y201F# 3
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hCTLA-4
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Vector #1
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CTLA-4WT #1
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CTLA-4WT #4
Y201F #1
Y201F #2
Y201F #3
% inhibition of IL-2 production

*Fig. 1.* Negative signaling mediated by CTLA-4 in transfectants. (A) Histogram of CTLA-4 transfectants. DC27.10 cells transfected with vector, hCTLA-4 and mutant Y201F were analyzed for the expression of hCTLA-4, endogenous CD3, CD28 and CTLA-4 using mAb 11D4, 145–2C11, 37.51 and 4F10–11, respectively. The control corresponds to cell staining with secondary antibody alone (FITC-conjugated goat-anti-mouse mAb). (B) Expression of endogenous mCTLA-4. hCTLA-4 transfectants (left panel) and splenocytes (right panel) were activated with Con A (2.5 μg/ml) for 72 h. Cells were permeabilized with 0.3% saponin and incubated with mCTLA-4 (4F10–11) and goat anti-hamster FITC (black curve). Staining with goat anti-hamster FITC alone served as a negative control (white curve). (C) Negative signaling mediated by co-ligation of CTLA-4 with TCRζ/CD3 and CD28. Vector, hCTLA-4 and mutant Y201F (clone 2) transfectants were stimulated with soluble 145–2C11 (CD3; 0.1 μg/ml), 37.51 (CD28; 0.6 μg/ml), 11D4 (hCTLA-4; 6 μg/ml) and 10 μg/ml rabbit anti-hamster antibodies for 24 h. The supernatants derived from cell cultures were analyzed for IL-2 in a CTLL-2 proliferation assay as described [30]. (D) Negative signaling in various CTLA-4 transfectants (right panel). Various transfectants were subjected to antibody induced cross-linking [as described in (C)] followed by an assessment of IL-2 production. Left panel: Histogram of different Y201F mutant clones.

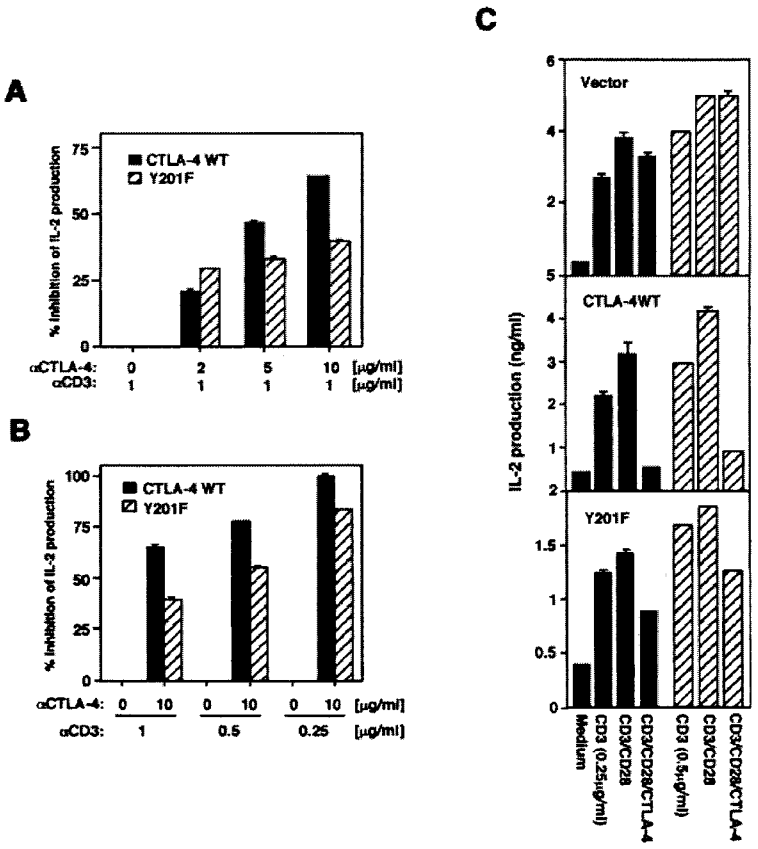


*Fig. 2.* Effect of various amounts of immobilized anti-CD3 and CTLA-4 on IL-2 production. (A) Effect on IL-2 production of increasing amounts of CTLA-4 mAb on CTLA-4 WT and mutant Y201F transfectants. CTLA-4 WT (black bars) and Y201F mutant (clone 2) (hatched bars) transfectants were incubated with plate-bound anti-CD3 (1 μg/ml) and anti-CTLA-4 (0, 2, 5, 10 μg/ml) mAb. Antibody concentration was adjusted by adding anti-TNP as an isotype specific mAb. After 24 h, the supernatants were taken and IL-2 production was measured by ELISA. (B) Effect on IL-2 production of decreasing amounts of CD3 mAb on WTCTLA-4 and mutant Y201F transfectants. CTLA-4 WT (black bars) and Y201F mutant (clone 2) (hatched bars) transfectants were incubated with plate-bound anti-CD3 (1, 0.5, 0.25 μg/ml) and anti-CTLA-4 (0, 10 μg/ml) mAb. Adjustment of antibody concentration and IL-2 measurement as described in (A). (C) Effect of cross-linking CTLA-4 with TCR/CD3×CD28 on CTLA-4 WT and mutant Y201F IL-2 production. Vector, hCTLA-4 WT and Y201F mutant (clone 2) transfectants were incubated with plate-bound anti-CD3 (0.25 μg/ml, black bars; 0.5 μg/ml, hatched bars), anti-CD28 (5 μg/ml) and anti-CTLA-4 (10 μg/ml) mAb. Adjustment of antibody concentration and IL-2 measurement as described in (A).

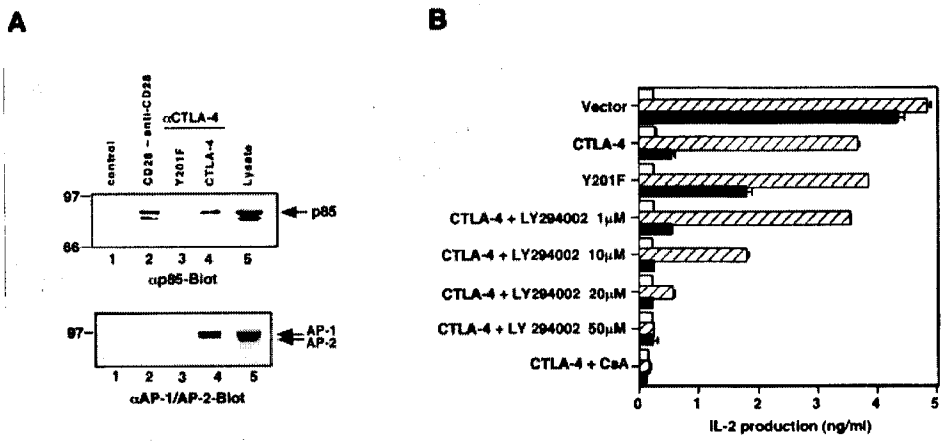


*Fig. 3.* Binding of CTLA-4 to p85 and AP-2 complexes. (A) CTLA-4 binding to p85 of PI 3-kinase and AP-2 complexes. Vector (lane 1), human CD28 (lane 2), Y201F (clone 2, lane 3) and human WTCTLA-4 transfectants (lane 4) were immunoprecipitated with anti-CTLA-4 (lanes 1,3,4) or anti-CD28 mAb (lane 2) and subjected to immunoblotting with anti-p85 Ab (upper panel) and with anti-β-chain antisera cross-reactive with AP-1 and AP-2 (lower panel).The presence of p85 and AP-1/AP-2 in whole cell lysate is shown in lane 5. (B) PI 3-kinase inhibitor LY294002 does not reverse CTLA-4 function. Vector, mutant Y201F (clone 2) and hCTLA-4 transfectants were either left unstimulated (white bars) or stimulated with plate-bound CD3 and TNP mAb (hatched bars) or with plate-bound CD3 and CTLA-4 mAb (black bars). After 24 h, IL-2 production was measured by ELISA. LY294002 (1, 10, 20, 50 μM) or cyclosporin (100 ng/ml) were added to the cells 1 h prior to stimulation.

its own. While increasing concentrations of LY294002 (1–50 μM) caused a gradual inhibition of anti-CD3-induced IL-2 production, the ability of CTLA-4 to inhibit IL-2 production was not reversed by this compound. LY294002 (1–50 μM) did not alter CTLA-4 surface expression (data not shown). As a control, cyclosporin A markedly blocked anti-CD3-induced IL-2 production. These data suggest that PI 3-kinase activity itself is not required for CTLA-4 negative signaling, a result consistent with its association with positive signaling receptors such as CD28 and ICOS [33–35].

Since SHP-2 has been reported to bind to the CTLA-4 YVKM motif [17–20], it was therefore of interest to assess whether this interaction could be detected in our transfectants. Anti-CTLA-4 precipitations from CTLA-4 transfectants were analyzed for the presence of SHP-2. As shown in Fig. 4A (upper panel), anti-CTLA-4 failed to precipitate detectable SHP-2 from either unligated (lane 2), or anti-CTLA-4 ligated cells (lanes 5, 10). Co-ligation of CTLA-4 and TCRζ/CD3 also failed to promote the association of the phosphatase (lane 11). At the same time, SHP-2 was detected in anti-SHP-2 precipitates (lanes 7, 12), or in cell lysates (lanes 8, 13) by immunoblotting. As a positive control, CTLA-4 precipitated PI 3-kinase as detected by anti-p85 immunoblotting (lower panel). Although we could not detect binding of SHP-2 to CTLA-4 in our transfectants, we could confirm the finding that anti-CTLA-4 can precipitate SHP-2 from detergent lysates of activated spleen/lymph node cells [36]. To further address this issue of whether SHP-2 or another phosphatase could play a role in negative signaling, we analyzed anti-CD3 induced TCRζ phosphorylation and recruitment of the tyrosine kinase ZAP-70 in vector, CTLA-4 WT and mutant Y201F (clone 2) transfectants. mAb to TCRζ/CD3 alone (Fig. 4B, lanes 2, 7, 12), or TCRζ/CD3×CD28 (lanes 3, 8, 13) induced the marked tyrosine phosphorylation of TCRζ and CD3 subunits as detected by anti-phosphotyrosine immunoblotting. Both pp21 and pp23 forms of TCRζ could be visualized. No phosphorylation was induced in non-activated cells (lanes 1, 6, 11). Significantly, co-ligation of CTLA-4 in the context of TCRζ/CD3 or TCRζ/CD3×CD28 did not lead to dephosphorylation of TCRζ/CD3 chains when compared with TCRζ/CD3 or TCRζ/CD3×CD28 alone (lanes 10, 9 *vs.* lanes 7, 8; and 15, 14 *vs.* 12, 13). Consistent with this, cross-linking of TCRζ/CD3 or TCRζ/CD3×CD28 induced the recruitment and phosphorylation of ZAP-70 in vector (Fig. 4B, lanes 2, 3), CTLA-4 WT (lanes 7, 8) and Y201F mutant (lanes 12, 13). Co-cross-linking of CTLA-4 had no apparent effect on the recruitment or phosphorylation of ZAP-70 (lanes 9, 10, 14, 15). These data demonstrate that under conditions where IL-2 production is inhibited, co-ligation of CTLA-4 with TCRζ/CD3×CD28 failed to dephosphorylate TCRζ or CD3 subunits, or to interfere with ZAP-70 recruitment/phosphorylation.

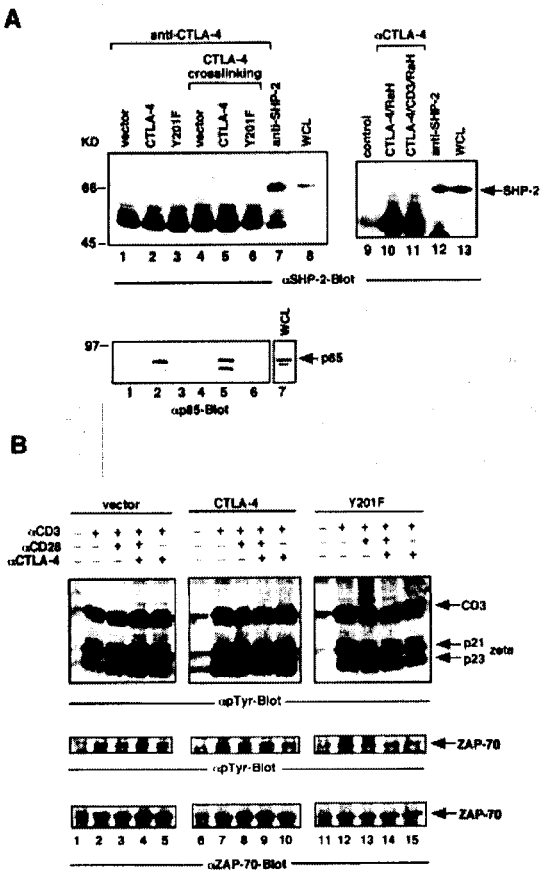


*Fig. 4.* SHP-2 phosphatase does not associate with CTLA-4 expressed in DC27.10. (A) Upper panel: Vector, hCTLA-4 and mutant Y201F (clone 2) transfectants were either left untreated (lanes 1–3) or stimulated with CTLA-4 mAb (lanes 4–6,10) or a combination of CTLA-4/CD3 mAb (lane 11). The cells were lysed, immunoprecipitated with anti-CTLA-4 mAb (lanes 1–6 and 10,11) and subjected to immunoblotting with anti-SHP-2 mAb. Lanes 7 and 12 show an anti-SHP-2 precipitate. The presence of SHP-2 in whole cell lysate is shown in lanes 8 and 13. As a negative control, lysates from CTLA-4 transfectants were subjected to precipitation with rabbit anti-hamster antibody (lane 9). Lower panel: Anti-CTLA-4 immunoprecipitates were analyzed by anti-p85 Ab. (B) Coligation of CTLA-4 with TCR/CD3 and CD28 results in normal TCRζ and ZAP-70 phosphorylation. Upper panel: Vector (lanes 1–5), hCTLA-4 (lanes 6–10) and Y201F mutant (clone 2) (lanes 11–15) transfectants were subjected to antibody induced cross-linking of CTLA-4 with TCR/CD3 and CD28 for 10 min, followed by lysis and precipitation with anti-CD3 mAb (lanes 1–15). Precipitates were analyzed by anti-pTyr immunoblotting. Middle Panel: Anti-CD3 immunoprecipitates were analyzed by anti-pTyr blotting. Lower Panel: Anti-CD3 immunoprecipitates were analyzed by anti-ZAP-70 immunoblotting.

## 3 Discussion

While CTLA-4 is well documented as a negative regulator of cytokine production and T cell proliferation [1, 2], the underlying molecular basis of signaling is poorly understood. The cytoplasmic tail carries two tyrosine-based motifs, YVKM and YFIP, of which the former has been the subject of recent debate. Several groups have reported that negative signaling can occur independently of tyrosine phosphorylation of this motif [27–29]. Given the number of important intracellular proteins that bind to the YVKM motif (*i.e.* PI3-K, SHP-2, AP-1/AP-2), it seemed quite remarkable that this motif has not been found to play a role in CTLA-4 function. To assess this further, we examined the ability of the YVKM mutant to support negative signaling under different conditions of receptor cross-linking. Our findings clearly demonstrate that, while not obligatory, the YVKM motif plays a role in ensuring optimal suppression of TCR signaling by CTLA-4. The importance of the motif varied with the mode of anti-receptor presentation where dependency on the motif was more evident with soluble rather than immobilized antibody. Previous studies claiming that negative signaling can occur independently of tyrosine phosphorylation and the YVKM motif have used immobilized antibody in some part of their assay [27–29].

The effect of soluble anti-CD3/CD28/CTLA-4 antibodies was found to be remarkably dependent on the YVKM motif (Fig. 1C, D). Mutation of the tyrosine Y201 reduced CTLA-4 effects on TCR signaling by 80–90 % (Fig. 1C). This result was observed in five independently derived transfectants, and was, therefore, not the property of one unusual transfectant. In contrast, the same mutation reduced CTLA-4 function by only 10–30% when using immobilized antibodies (Fig. 2). This difference between the sensitivity to soluble *vs.* immobilized antibody presentation was observed in multiple experiments, and should help resolve the discrepancy of results in the literature [27, 28]. Our findings indicate that the YVKM motif plays an accessory role in ensuring optimal CTLA-4 effects on TCR signaling. A recent study has reported that the reconstitution of the CTLA-4Y201F mutant was able to reconstitute CTLA-4 function [37]. In this case, the expression of the mutant was much higher than normally observed physiologically due to the ability of the YVKM motif to facilitate higher levels of surface expression [38]. As stated by Masteller et al. [37], the high levels of expression in these mice might compensate for the absence of the YVKM motif. This might be analogous to the ability of high levels of CD4-p56$^{lck}$ binding mutant to reconstitute normal CD4 function in the thymus [39]. To avoid this complication, the different transfectants in our study were selected to express similar levels of WT or

YVKM mutant CTLA-4. Slight variations in CTLA-4 expression in various clones were not great enough to influence the overall functional phenotype of the cells.

In summary, while the YVKM motif facilitated optimal CTLA-4 function, our results also showed that some negative signaling could occur in its absence, especially when immobilized antibodies were used. From this, we conclude that the YVKM motif plays an accessory, rather than a direct role in inducing negative signals. The involvement of the motif occurred without the association of the tyrosine phosphatase SHP-2 or dephosphorylation of TCR-associated subunits. This agrees with other studies claiming that SHP-2 binding is not needed for CTLA-4 function [27–29]. Further, inhibition of PI 3-kinase activity by Ly294002 had no effect on CTLA-4 signaling except at higher concentrations where the drug also inhibited TCRζ/CD3 signaling (Fig. 3B) [40, 41]. The absence of a role for PI 3-K is consistent with the fact that it is associated with positive co-receptors such as CD28 and ICOS [33–35]. These data suggest a role for the AP-2 complex or another unknown protein. AP-2 is needed for efficient receptor aggregation and endocytosis [42, 43]. Further studies will be needed to elucidate the possible role for AP-2/CTLA-4 complex in ensuring optimal CTLA-4 function.

## 4 Materials and methods

### 4.1 Cells, reagents and antibodies

The murine T cell hybridoma DC27.10 was cultured in RPMI 1640 medium, supplemented with 5% FCS and 50 μM 2-ME. DC27.10 cells were stably transfected with human CTLA-4 as described [21]. Anti-hCTLA-4 (11D4) has been described [44]. Anti-mCTLA-4 (4F10–11) was a kind gift from Dr. J. Bluestone (University of Chicago, Chicago, IL). Anti-mCD28 (37.51) was purchased from PharMingen (San Diego, CA). Anti-pTyr mAb was a kind gift from Dr. T. Roberts (Dana-Farber Cancer Institute, Boston, MA). ZAP-70 and SHP-2 mAb were bought from Transduction Laboratories (Lexington, KY), anti-TNP mAb from PharMingen. Anti-murine CD3 (145–2C11) was purchased from American Type Culture Collection (Manassas, VA). LY294002 and cyclosporin A were from Calbiochem (San Diego, CA). The specific anti-β-chain antiserum was a kind gift from Dr. T. Kirchhausen (Center for Blood Research, Boston, MA), the anti-p85 Ab was kindly provided by Dr. M. White (Joslin Diabetes Center, Boston, MA).

### 4.2 Flow cytometry

For analyzing endogenous CTLA-4 expression, splenocytes and hCTLA-4 transfectants were activated with Con A (2.5 μg/ml). After 72 h, cells were permeabilized with 0.3% saponin and stained for surface and intracellular localized CTLA-4 with mCTLA-4 mAb (4F10–11) and goat anti-hamster FITC as secondary antibody. Cells were analyzed on an Epic Scan flow cytometer (Coulter).

### 4.3 Immunoprecipitation and immunoblotting

For immunoprecipitation, cells were lysed in ice-cold lysis buffer containing 1% TritonX-100 in 20 mM Tris-HCl, pH 8.3, 150 mM NaCl. The lysis buffer contained protease and phosphatase inhibitors. Postnuclear lysates were incubated for 1 hour with the indicated antibody. Protein A-Sepharose beads (30 μl, Pharmacia) were added and incubated for 1 h at 4°C. The eluted proteins were separated by SDS-PAGE and transferred to nitrocellulose for immunoblotting. The membranes were blocked with 5% milk in TBS (10 mM Tris-HCl, pH 7.6, 150 mM NaCl) and incubated with the indicated antibody. Bound antibody was revealed with the appropriate secondary antibody, and protein was visualized by enhanced chemiluminescence (ECL, Amersham).

### 4.4 IL-2 assay

CTLA-4 transfectants ($1\times10^5$/200 μl) were stimulated either with soluble anti-CD3 (0.2 μg/ml), anti-mCD28 (0.6 μg/ml), anti-hCTLA-4 (6 μg/ml) and rabbit anti-hamster (10 μg/ml) antibodies or with plate-bound anti-CD3 (1, 0.5, 0.25 μg/ml), anti-mCD28 (5 μg/ml) and anti-CTLA-4 (2, 5, 10 μg/ml) mAb. Antibody concentration was adjusted by adding anti-TNP as an isotype-specific mAb. In some experiments, cells were preincubated with either LY294002 (1, 10, 20, 50 μM) or cyclosporin A (100 ng/ml) for 1 h prior to stimulation. After 24 h, supernatants were either added to CTLL-2 cells and IL-2 was determined as described [30] or IL-2 production was measured by ELISA (PharMingen).

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# Chapter 5.
# Discussion and Summary

As an overview, the enclosed thesis has attempted to uncover the molecular basis of CTLA-4 function in T cells. Previous studies had clearly demonstrated that CTLA-4 alters dramatically T cell function resulting on a higher threshold needed for IL-2 production and proliferation (Azuma et al., 1993). The current hypothesis at this point was that CTLA-4 actively generated negative signals that could block TCR signals [Blair, 1998 #220; Chambers and Allison, 1997c; Chen et al., 1994; Coyle et al., 2000; Damle et al., 1994; Hathcock et al., 1993; Krummel and Allison, 1995; Krummel and Allison, 1996; Lenschow et al., 1993; Linsley et al., 1992; Walunas et al., 1994). Various molecular mechanisms including SHP-2, PP2A binding were proposed (Chuang et al., 2000; Lee et al., 1998). The basis of my thesis was to argue that CTLA-4 may utilize another mechanism or pathway in its ability to alter T cell function. One proposed hypothesis was that CTLA-4 might redirect the T cell such that it re-prioritized its cellular machinery towards other cellular events such as migration, actin reorganization and away from the classic events such as IL-2 production.

Initially, my interest was whether CTLA-4 acted on an early step of TCR signals and whether it could affect other events such as apoptosis. In order to study such events, we adapted the system used in previous models to study the contribution of CD28 to TCR costimulation. As the Jurkat cells used in the previous systems may be defective in PTEN activity (Shan et al., 2001) and taken the fact that both CD28 and CTLA-4 bind to PI3-kinase (August and Dupont, 1994; Schneider et al., 1995b), we decided to use a T cell hybridoma system which has been extensively studied for the molecular mechanism of receptor mediated signalling (Cai et al., 1995; Prasad et al., 1994; Raab et al., 1995; Schneider et al., 1995a; Schneider et al., 1999) and has shown to have a normal PI3-kinase activity (Hu et al., 2001; Kang et al., 2002). I found that CTLA-4 was able to inhibit both apoptosis and IL-2 production, in a dose dependent manner. Higher concentrations of CTLA-4 cross-linking was capable of inhibiting IL-2 response as well as AICD. In addition, I also showed that CTLA-4 was able to inhibit CD3 responses independently of CD28 costimulation. Moreover, it was found that CTLA-4 is able to act at an early stage of CD3 signalling and that the inhibitory function of CTLA-4 was a direct consequence of signalling through its cytoplasmic tail. The inhibition of AICD was not a direct consequence of lack of production of the growth factor IL-2 or competition for B7 ligands but was due to decreased upregulation of FasL. The functional significance of this finding is still unclear. Different in vivo models have shown that CTLA-4 may not participate directly in regulating AICD (Blair et al., 1998; Cilio et al., 1998; Scheipers and Reiser,

1998). However, a model using a mixed chimera of lymphocytes (CTLA-4 +/+ and CTLA-4 -/-) showed a reduction in the number of CTLA-4 deficient cells after infection with pathogen although proliferation occurred as in the controls (Bachmann et al., 2001). In accordance with our results, this data would suggest that CTLA-4 may control CTLA-4 homeostasis by regulating apoptotic signals.

Having found that blocking of CD3 signals was not simply a matter of competitive binding for ligand but was the effect of generation of blocking signals by CTLA-4, I then tested the hypothesis of whether the YVKM motif was important for CTLA-4 function. I showed using invitro assays that, although the YVKM motif was important for efficient blockage, it was not absolutely essential. In fact, the YVKM motif was dispensable at high concentration of CTLA-4 crosslinking. These results were based on the mode of antibody presentation, which influenced greatly the strength of the stimulus. Platebound compared to soluble anti-CD3 delivered a more powerful signal. Whereas low concentration of platebound anti-CD3, which delivered a weak signal, was easily inhibited by CTLA-4, higher concentrations of CD3 required a more robust CTLA-4 co-crosslinking. It is worth mentioning that simultaneous anti-CD3 and anti-CTLA-4 needed to be presented to inhibit IL-2 production. Separate stimulation of the receptors (i.e. using platebound anti-CD3 with soluble anti-CTLA-4 or vice-versa) did not work properly. These results suggest that close proximity of CTLA-4 with the TCR is required for CTLA-4 inhibitory function. Thus, a "bystander" costimulation of CTLA-4 by APCs in ***trans*** may not be mechanistically possible for CTLA-4.

In addition, I also showed that PI 3-kinase activity was not involved in CTLA-4 blockage of signals. Since PI 3-kinase also binds to CD28, these results suggest that PI 3-kinase bound to CTLA-4 may have a different function other than negative signalling. It may be involved, for example, in guarding the integrity of the intracellular pool of CTLA-4 and ensuring proper protein trafficking. It may also have other unknown functions relating to cell growth and differentiation. Another group (Ellen Chang personal communication) has recently confirmed these results in vivo.

Considering that the cytoskeleton is important for TCR mediated signals, I hypothesised that CTLA-4 may negatively influence actin assembly. Although actin polymerisation is an important step in TCR signalling (Anton van der Merwe et al., 2000), little is known about the effect of costimulation on actin reorganisation. Using Jurkat T cells as a model, it has been described that TCR specific antibodies were able to induce cytoskeletal changes and spreading of T cells (Parsey and Lewis, 1993). T cell spreading

was restricted to the observation of cells flattening and lamellipodia extending in a concentric ring that was moving outward. These changes were later observed in an assay that implicated direct TCR ITAM involvement in signalling but remained independent of integrin involvement in the process (Borroto et al., 2000). Moreover, it has been proposed that these morphological changes induced by TCR ligation are dependent on LAT phosphorylation and subsequent recruitment of protein kinases to the plasma membrane and increases in cytoplasmic calcium (Bunnell et al., 2001). Using the CTLA-4 transfectants, I have shown that CTLA-4 may paradoxically promote actin assembly while inhibiting cytokine production. While CD3 and CD28 stimulation induced focal points of actin rich clusters, CTLA-4 costimulation was able to induce shape changes characterised by lamellipodia and filopodia formation. Interestingly, these shape changes were associated with an increased adhesion of cells to the substratum and decreased cell motility. The Rho family members Rac and cdc42 were activated following CTLA-4 cross-linking. As to my knowledge, this is the first evidence that shows CTLA-4 having a function other than blockage of signals. CTLA-4 was also found to cooperate with PMA and Ionomycin in inducing these shape changes without the need for TCR involvement and TCR phosphorylation. These findings confirm the role of CTLA-4 at regulating early signalling event independent of upstream signalling molecules.

Therefore a model emerges where CD28 may be an amplifier of CD3 signals whereas CTLA-4, in turn, may be a modifier of CD3 generated signals. While CTLA-4 may be able to block production of cytokines that serve as signalling factors for other cells and block proliferation of T cells, it may concomitantly promote the recruitment and localisation of signalling components that are involved in membrane reorganisation. CTLA-4 may provide a "stop" signal that would limit and restrict the movement of T cells along the surface of an APC. The reorganisation of the membrane structure into filopodia and lamellipodia might restrict the number of MHC:TCR interactions and facilitate the process of differentiation of naive T cells into helper, memory, cytotoxic or suppressor T cells. T lymphocytes from the CTLA-4 knockout mice have the inherent capacity to infiltrate non-haematopoetic tissues under normal conditions. It is possible that, based on my results, one can hypothesise that these cells are perhaps unable to stop when encountering an APC, adhere tightly and form membrane protrusions necessary for further differentiative re-programming. Consequently, one may also speculate that T lymphocytes from the CTLA-4$^{-/-}$ mice may have a deficiency in homing and localisation to specific lymphoid and non-lymphoid sites. This new function for CTLA-4 may be quite important for the establishment of long-term memory cells and may also play a role in the induction of tolerance. It is noteworthy to mention that memory cells have a high intracellular pool of

CTLA-4, which is recycled from the intracellular stores back to the membrane. One can then envisage that CTLA-4 expression and release to the site of TCR engagement would permit faster adherence, localisation and differentiation of T cells and thus provide for a more rapid and efficient immune response.

Lastly, attempts were made to isolate novel proteins associated to CTLA-4 that may function to negatively regulate T cell activation (see appendix 2). The use of immunoaffinity chromatography to isolate and characterize proteins proved to be informative, since several associated proteins were detected. CTLA-4 immunoprecipitation was found to coprecipitate along with several other proteins (approximate molecular weights): CAP1 (128 kDa), Cap-2 (72 kDa), CAP-3 (66 kDa), CAP-4 (48 kDa), CAP-5 (39 kDa), CAP-6 (36kDa). A 68 kDa protein was detected by protein sequencing and found to be ribophorin I. The mammalian oligosaccharyltransferase (OST) is an oligomeric complex composed of four membrane proteins of the endoplasmic reticulum: ribophorin I (RI), ribophorin II (RII), OST48 and DAD1. OST is responsible for the co-translational N-glycosylation (transfer of GlcNAc2-Man9-Glc3 from Dol-PP onto specific asparagine residues) in the nascent polypeptide (Schweizer A et la., 1991; Kumar V et al., 1998). Ribophorin has also been found to be selectively expressed on the surface of immature thymocytes although its role is unclear (Wiest et al., 1997). Since CTLA-4 is highly glycosylated, the association with ribophorin to CTLA-4 was not so surprising. Other such highly glycosylated proteins have also been found to be associated with ribophorin when immunoprecipitated (Santhamma and Sen, 2000). While this protein may regulate CTLA-4 expression to some extent, by controlling modification of the sugar residues, it is highly improbable that it may be involved in TCR/CTLA-4 proximal signalling. A peptide sequence analysis demonstrated as well that peptides corresponding to the human lymphocyte adaptor LNK (63.1 kDa) (#9) and the human/mouse receptor protein kinase ryk (67.8 kDa) (#46) might be present within the molecular weight range of the expected of protein. The structure of LNK adaptor indicates the presence of an N-terminal proline-rich region, a pleckstrin homology domain (PH), and a Src homology 2 domain. LNK was found to be associated with phosphorylated CD3 ζ chain after cotransfection with $p56^{lck}$ through its Src homology 2 domain. The overexpression of Lnk in Jurkat cells has lead to inhibition of anti-CD3 mediated NF-AT-Luc activation (Li et al., 2000). Thus LNK might be a good candidate for negative signalling via CTLA-4. The RYK receptor tyrosine kinase, receptor related to tyrosine kinases (RYK), is unique amongst the catalytically inactive RTKs (Gough et al., 1995; Halford et al., 1999). Recent analyses of deficient RYK mice and its Drosophila orthologue have defined a role for this novel subfamily of receptors in the control of craniofacial development and

neuronal pathway selection, respectively (Dura et al., 1995; Halford et al., 2000). Biochemical data involving RYK signal transduction pathways has lead to the hypothesis that it may act at a junctional spot for crosstalk and scaffold assembly with Eph receptors. Since RYK is also expressed in lymphoid tissues (Simmoneaux DK, 1995), it is possible that RYK may be a target for CTLA-4 negative signalling. However, uncertainty about the validity of these proteins remains and is highlighted by several caveats. Both LNK and RYK proteins were present at very low levels and only one peptide was found for each. Only 16% for LNK and 60% for RYK of experimental ions matched with the theoretical ions for the peptide listed in the database. In addition, the human peptide but not the mouse peptide of LNK was found which might suggest some type of cross-contamination of the sample. The peptide found for LNK (RRAPGPDGPDRVL) encompassed the PH domain of the protein. One might have expected that this particular sequence should have been reserved through evolution. Surprisingly, however, this peptide sequence is not found in the mouse LNK. This result puts into question the validity of the "hit". One plausible possibility may be that this protein may be an orthologue of the human form, which has not yet been described in the mouse system. Further analysis of the peptides need to be performed to accurately assess the significance of this finding.

In conclusion, recent advances in understanding the mechanisms that potentially differentiates T cell activation and T cell anergy have highlighted the properties of the CD28/CTLA-4:B7 signalling pathway. Mouse models were generated to study the differential effect of B7-1 or B7-2 ligand blockage, CD28 costimulation and CTLA-4 negative regulation of the response against various peptides. These pioneering studies have been extremely important at estimating the potential clinical application of the costimulatory pathways and the usefulness of the intervention at various stages of the disease. In cases where potent immune response is desired, such as in generating tumour immunity, suppression of the CTLA-4 pathway is actively sought. In instances where a break in tolerance has transpired, as in the case of autoimmune diseases and graft-vs-host disease, the negative feedback provided by CTLA-4 has helped in reducing the inflammatory response. Therefore, the complexity of the disease dictates a defined coordination in the nature of costimulatory blockade and modulation in the response that distinguishes self from non-self. The nature of CTLA-4 negative signalling pathways that leads to the immune regulation of diseases is still unknown. As new and exciting data emerge on the molecular basis for CTLA-4 mediated signalling properties, new questions arise concerning the function of CTLA-4 as an inhibitory coreceptor: how does activation of the Rho family of GTPases prevent cytokine production and cell cycle arrest? Does activation of the Rho GTPases occur during agonist as well as antagonist peptide

presentation? How is the activity of small GTPases regulated during tolerance induction? Are there adaptor proteins, such as FYB, Gads or SKAP55, which are able to modify CD3 mediated signals in conjunction with CTLA-4 costimulation? Further experiments will be needed to clarify the role of membrane reorganisation on T cell differentiation and its implication on blockage of CD3 signalling.

# Chapter 6.
# Discussão dos Resultados

Este resumo, sumariza o trabalho laboratorial realizado no âmbito da minha tese de doutoramento. A análise do receptor CTLA-4 no contexto da produção de citoquinas e indução de AICD (morte celular induzida por antigénio) foi realizada assumindo que este receptor era capaz de agir numa fase precoce nas vias de transdução de sinal. Verificou-se que o receptor CTLA-4 era capaz de inibir os dois mecanismos atrás mencionados num processo dependente da concentracao de anticorpo utilizado para estimulação das celulas. Deste modo, uma concentração mais elevada de anti-CTLA-4 inibe a resposta de IL-2 assim como a AICD. Por outro lado, também se demonstrou que o receptor CTLA-4 inibe respostas CD3 independentemente da coestimulação pelo CD28. A inibição da AICD não foi uma consequência directa da falta de produção da IL-2, mas deveu-se à diminuição no aumento de expressão do FasL. Os resultados obtidos evidenciaram acima de tudo, que o receptor CTLA-4 era capaz de actuar numa fase precoce nas vias de transdução de sinal iniciadas pelo CD3 e que a função inibidora do CTLA-4 era uma consequência directa de signalling através da sua cauda citoplasmática.

Verificou-se que o bloqueio dos sinais CD3 não era apenas resultado de uma ligação competitiva ao ligando, mas devia-se ao efeito da geração de sinais bloqueadores pelo CTLA-4. Testou-se então a hipótese do motivo YVKM ser relevante para a função do receptor CTLA-4. Ensaios in vitro confirmaram que o motivo YVKM era necessário para um bloqueio eficiente, apesar de não ser essencial. Estes resultados basearam-se no modo de apresentação do anticorpo, que influencia, de modo geral, o poder do estímulo pelo receptor. O sinal iniciado pelo anti-CD3 solúvel era mais potente do que o sinal iniciado pelo anti-CD3 ligado a uma matriz. Uma baixa concentração de anti-CD3 desencadeava um sinal fraco, sendo facilmente inibido pelo receptor CTLA-4, enquanto que concentrações mais elevadas de anti-CD3 necessitavam de uma ligação do CTLA-4 mais forte. É de salientar que tanto o anti-CD3 assim como o anti-CTLA-4 necessitavam estar presentes em simultâneo na mesma matriz para inibir a produção de IL-2. Esta inibição não ocorria caso um dos anticorpos estivesse presente na sua forma soluvel. Também se demonstrou que, a actividade de PI 3-quinase não estava envolvida no bloqueio dos sinais iniciados pelo CTLA-4. Recentemente estes resultados foram confirmados por outro grupo de investigação em exepriências in vivo (Ellen Chang, comunicação pessoal).

Considerando que o citoesqueleto se associa à cadeia ζ do complexo CD3, e por isso, é importante no processo de sinalização mediado pelo receptor das cellulas T (RCT), pô-se a hipótese do receptor CTLA-4 influenciar negativamente a polimerização da actina. Utilizando linhas celulares transfectadas com CTLA-4, verificou-se que, apesar de inibir a produção de citoquinas, o receptor CTLA-4 pode paradoxalmente promover a polimerização da actina. Estes resultados demonstraram pela primeira vez que o receptor CTLA-4 esta associado a outra função para além do bloqueio de sinais. Estes dados também confirmam o papel do CTLA-4 como regulador dos mecâmismos associados a transduçao de signais. Como consequência, foi proposto um modelo onde o receptor CD28 pode ser um amplificador dos sinais mediados pelo CD3 ao passo que o receptor CTLA-4 poderá ser um modificador dos sinais gerados pelo receptor CD3. O receptor CTLA-4 pode promover o recrutamento e localização dos componentes de signalling envolvidos na reorganização das membranas, para além de poder bloquear a produção de citoquinas que funcionam como factores de sinalização para outras células e bloqueam a proliferação de células T. A reorganização da estrutura das membranas em filopodia e lamelipodia pode facilitar o processo de diferenciação das células T naif em células T de memória, citotóxicas ou supressoras. Em condições normais os linfócitos T dos ratinhos CTLA-4 knockout têm a capacidade inerente de infiltrar tecidos não hematopoiéticos. Estes resultados sugerem que estas células talvez não sejam capazes de aderir e formar protusões membranárias. Assim também se poderá presumir que os linfócitos T podem apresentar uma deficiência na migração e na localização dos seus destinos específicos. Esta nova função para o receptor CTLA-4 pode ser bastante importante para o desenvolvimento de células mémoria. Convém mencionar que as células memória possuem um depósito elevado de CTLA-4 intracelular. Pode considerar-se que a expressão do receptor CTLA-4 permitiria uma adesão mais rápida assim como a localização e diferenciação das células T conduzindo a uma resposta imune mais rápida e eficiente.

Com o objetivo de identificar proteínas associadas com o receptor CTLA-4, foram isoladas por cromatografia proteínas que se revelaram importantes. Em resultado da imunoprecipitação observou-se que o receptor CTLA-4 coprecipitou várias proteínas (peso molecular aproximado): CAP1 (128 kDa), Cap-2 (72 kDa), CAP-3 (66 kDa), CAP-4 (48 kDa), CAP-5 (39 kDa), CAP-6 (36kDa). Uma proteína de 68 kDa foi detectada por sequenciação proteica e verificou-se ser a riboporina I. A oligosacariltransferase mamífera (OST) é um complexo oligomérico composto por quatro proteínas

membranárias do retículo endoplasmático: : riboporina I (RI), riboporina II (RII), OST48 e DAD1. A OST é responsável pela glicosilação (transferência de GlcNAc2-Man9-Glc3 a partir de Dol-PP para os resíduos específicos de asparagina) no polipéptido em síntese. Apesar da função da riboporina ainda não ser completamente conhecida, verificou-se a sua expressão selectiva na superfície dos timócitos imaturos. Dado que o receptor CTLA-4 é altamente glicosilado, a associação da riboporina com o CTLA-4 não foi inesperada. Também se tem observado a associação de outras proteínas fortemente glicosiladas à riboporina quando imunoprecipitadas. Se bem que esta proteína pode regular a expressão do receptor CTLA-4 até algum limite é pouco provável que possa estar envolvida na sinalização pelo RCT/CTLA-4. A análise da sequência de péptidos demonstrou que o adaptador linfocitário humano LNK assim como o receptor quinase ryk de origem humana e/o murina correspondem dentro do limite ao peso molecular da proteína esperada. A estrutura do adaptador LNK indica a presença de uma região N-terminal rica em prolina, um domínio homólogo à pleqstrina (PH) e um domínio homólogo a Src 2. Verificou-se que o LNK através do seu domínio homólogo Src 2 estava associado com a cadeia ζ fosforilada após cotransfecção com $p56^{LcK}$. A sobreexpressão de Lnk em células Jurkat conduziu à inibição da activação por anti-CD3 mediada por NF-AT-Luc. Deste modo LNK poderá ser um bom candidato para transmissão de sinais negativos através do receptor CTLA-4. A tirosina quinase do receptor RYK (receptor relacionado com tirosina quinase), é único entre os RTKs cataliticamente inactivos. Resultados experimentais recentes em ratinhos RYK-/- e no seu orthologo na Drosophila revelaram uma nova função para esta nova subfamília de receptores no controlo do desenvolvimento cranio-facial e na selecção de vias neurais respectivamente. Dados bioquímicos relativos às vias de transdução envolvendo sinal do receptor RYK conduziram à hipótese deste receptor poder funcionar como ponto de comunicação cruzada com os receptores Eph. Considerando que o receptor RYK também é expresso nos tecidos linfóides, é possível que este seja um alvo para a regulação de sinais negativos. No entanto, a incerteza relativa a estas proteínas é posta em evidência por vários factos. Tanto as proteínas RYK bem como LNK estavam presentes em níveis muito baixos e apenas um péptido para cada uma foi encontrado. Apenas 16% de LNK e 60% de RYK dos iões experimentais condiziam com os iões teoricamente esperados para o péptido de acordo com uma base de dados. Por outro lado, encontrou-se o péptido humano do LNK, mas não o murino, o que pode indicar algum tipo de contaminação cruzada na amostra. O péptido encontrado para o LNK inclui o domínio PH da proteína. Seria de esperar que esta sequência particular tivesse sido conservada ao longo da evolução. No entanto, esta sequência peptídica não se encontra no LNK murino. Este resultado experimental põe em causa a nossa hipótese. Uma explicação credível

relaciona-se com o facto desta proteína poder ser um orthologue da forma humana, não tendo ainda sido descrita no sistema murino. Análises adicionais destes péptidos necessitam ser efectuadas para averiguar com rigor o significado deste resultado.

O aparecimento de dados recentes e estimulantes referentes à base molecular das caracteristicas da transmissão de sinal mediada pelo receptor CTLA-4 aponta para novas questões relativas a função inibidora do CTLA-4. Como é que a activação das GTPases da família Rho previne a produção de citoquinas e a interrupção do ciclo celular? Será que a activação das GTPases Rho ocorre durante a apresentação de péptidos agonistas e antagonistas? Existirão proteínas adaptadoras como a FYB, Gads ou SKAP que serão capazes de modificar os sinais mediados pelo receptor CD3 em conjunto com o co-estímulo pelo CTLA-4? Experiências suplementares serão necessárias para esclarecer o papel da reorganização membranar na diferenciação das células T e a sua implicação no bloqueio do sinalização mediada pelo receptor CD3.

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# Appendices

# Appendix 1. Δ18CT Contruct

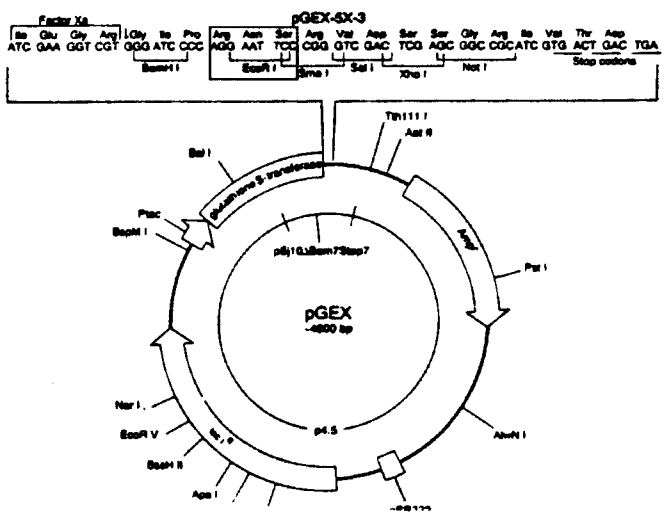
human CTLA-4 cDNA sequence

extracellular

transmembrane

cytoplasmic

1/1 31/11
atg gct tgc ctt gga ttt cag cgg cac aag gct cag ctg aac ctg gct gcc agg acc tgg
Met ala cys leu gly phe gln arg his lys ala gln leu asn leu ala ala arg thr trp
61/21 91/31
ccc tgc act ctc ctg ttt ttt ctt ctc ttc atc cct gtc ttc tgc aaa gca atg cac gtg
pro cys thr leu leu phe phe leu leu phe ile pro val phe cys lys ala met his val
121/41 151/51
gcc cag cct gct gtg gta ctg gcc agc agc cga ggc atc gcc agc ttt gtg tgt gag t
ala gln pro ala val val leu ala ser ser arg gly ile ala ser phe val cys glu tyr
181/61 211/71
gca tct cca ggc aaa gcc act gag gtc cgg gtg aca gtg ctt cgg cag gct gac agc cag
ala ser pro gly lys ala thr glu val arg val thr val leu arg gln ala asp ser gln
241/81 271/91
gaa gtc tgt gcg gca acc tac atg acg ggg aat gag ttg acc ttc cta
u val cys ala ala thr tyr met thr gly asn glu leu thr phe leu asp asp
301/101 331/111
tcc atc tgc acg ggc acc tcc agt gga aat caa gtg aac ctc act atc caa gga ctg agg
ser ile cys thr gly thr ser ser gly asn gln val asn leu thr ile gln gly leu arg
361/121 391/131
cc atg gac acg gga ctc tac atc tgc aag gtg gag ctc atg tac cca ccg cca tac t
a met asp thr gly leu tyr ile cys lys val glu leu met tyr pro pro pro tyr tyr
421/141 451/151
ata ggc aac gga acc cag att tat gta att gat cca gaa ccg tgc cca gat tct
e gly asn gly thr gln ile tyr val ile asp pro glu pro cys pro asp ser
481/161 BamHI 511/171
gac ttc ctc ctc tgg atc ctt gca gca gtt agt tcg ggg ttg ttt ttt tat agc ttt ctc
asp phe leu leu trp ile leu ala ala val ser ser gly leu phe phe tyr ser phe leu
541/181 571/191
ctc aca gct gtt tct ttg agc aaa atg cta aag aaa aga agc cct ctt aca aca ggg gtc
leu thr ala val ser leu ser lys met leu lys lys arg ser pro leu thr thr gly val
601/201 631/211
tat gtg aaa atg ccc cca aca gag cca gaa tgt gaa aag caa ttt cag cct tat ttt att
tyr val lys met pro pro thr glu pro glu cys glu lys gln phe gln pro tyr phe ile
661/221
ccc atc aat tga CTT AAG
pro ile asn OPA
EcoRI



## *Appendix 2. Isolation of CTLA-4 Associated Proteins*

# 2.1. Introduction

## 2.1.1. Background

While CTLA-4 has been regarded to bind to proteins as diverse as PI3-kinase(Chuang et al., 1999; Cilio et al., 1998; Lee et al., 1998; Marengere et al., 1996; Schneider et al., 1995a), SHP-2 (Chambers and Allison, 1996; Cilio et al., 1998; Frearson and Alexander, 1998; Lee et al., 1998; Marengere et al., 1996), and AP-1/AP-2 (Bradshaw et al., 1997; Chuang et al., 1997; Schneider et al., 1999; Shiratori et al., 1997; Zhang and Allison, 1997), great uncertainty still exists in our understanding of the mechanism by which CTLA-4 alters T cell function. To address the hypothesis of whether novel proteins bind to CTLA-4 and transduce signals in a negative pathway, several attempts had been made in the past to isolate associated proteins to CTLA-4 using techniques such as the yeast 2-hybrid and 3-hybrid screen. The yeast 2-hybrid system was able to identify the association of CTLA-4 with the clathrin adaptor complex AP-50 (Bradshaw et al., 1997; Chuang et al., 1997; Zhang and Allison, 1997). However, both techniques failed to demonstrate the association of a signalling partner with either kinase or phosphatase activity. The association of CTLA-4 with SHP-2, PI-3 kinase as well as with Jak2 were discovered using immunoprecipitation (Chikuma et al., 2000; Schneider et al., 1995b). Thus, as far as effectiveness in isolating associated proteins is concerned, antibody based purification methods were clearly the most successful. Therefore, I decided to use affinity chromatography to isolate potentially associated protein followed by silver staining as a means for detection and mass spectrometry for identification of the associated protein.

## 2.1.2. Affinity Chromatography

For Immunoaffinity purification, antibodies specific for proteins that we wish to isolate. The antibodies are coupled to sepharose (large pore chromatography matrix) that is bound to protein A (from *S. aureus*) followed by cross-linking of the complex by the chemical crosslinker dimethyl pimelimidate (DMP). DMP is a bifunctional crosslinker that reacts with primary amines groups without affecting the ionic charge of proteins. This covalently bound matrix allows optimal orientation of the antibody with the Fc portion of the antibody bound to protein A and the Fab portion free to bind its antigen. The TritonX-100 cell lysate was passed through the column at a slow rate flow to optimise binding (see materials and methods). The column is then washed to remove non-specific material. The antibody-antigen interaction is destabilised using high pH buffers and eluded of the column (Coligan et al., 2002; Schneider et al., 1982) and aliquots were collected.

### 2.1.3. Silver Staining

There are several methods for analysing proteins. The detection and characterisation of proteins is done using either a one dimensional (1D) SDS-PAGE gel, which allows the separation and detection of a single band or 2-dimensional (2D) gel electrophoresis, which yields single spots. The 1D gels are used to separate proteins based on their molecular weights. There is no discrimination of proteins that have the same molecular weights. 2D gels are used to initially separate proteins based on their respective charge and then separate them based on molecular weight. To analyse the subunit composition of proteins, other types of gel composition may be used. Under non-denaturing/non-reducing conditions (without the presence of 0.1% SDS and 2-ME), the proteins are separated depending on their size, shape and charge (Cleveland et al., 1977). To visualise the proteins on the gel, several methods can be used. The gels can either be transferred onto a nitrocellulose membrane or (polyvinyl difluoride) PVDF membrane, or the gel itself can be stained. In the case of staining the gel, Coomassie blue can be used to detect proteins on a gel. Coomassie blue staining depends on its non-specific binding of a dye to proteins and thus can be used for quantitative measurements of amount of proteins. However, its detection limit is low, between 0.5-1 micrograms of protein. For a more sensitive method, silver staining is the best choice. Detection limit of protein bands is around 5ng of protein. Detection of proteins by silver staining depends on binding of silver to sulfhydryl and carboxyl moeities in proteins. As Figure 1 shows, a sensitizer is used (DTT or sodium thiosulphate) to enhance the contrast between stained protein bands and background. As the intensity of the bands depend directly on the amount of sulfhydryl or carboxyl groups present in the protein, this method can only be used for roughly estimating the amount of protein on the gel. Thus, a weak signal on silver stained gel does not necessarily mean small amount of protein.

### 2.1.4. Mass Spectrometry

Analysis and identification of proteins is an area that has recently been revived with the application of mass spectrometry (MS) and bioinformatics. Mass Spectrometry (MS) is a powerful tool that is used for the analysis of all kinds of chemicals, ranging from environmental analysis to biological products. MS works is by producing charged particles from substances which then uses electrical and magnetic fields to measure the weight of the charged particle. The relative abundance of masses in a mass spectrum can then determine the structure and elemental composition of a molecule. There are many kinds of

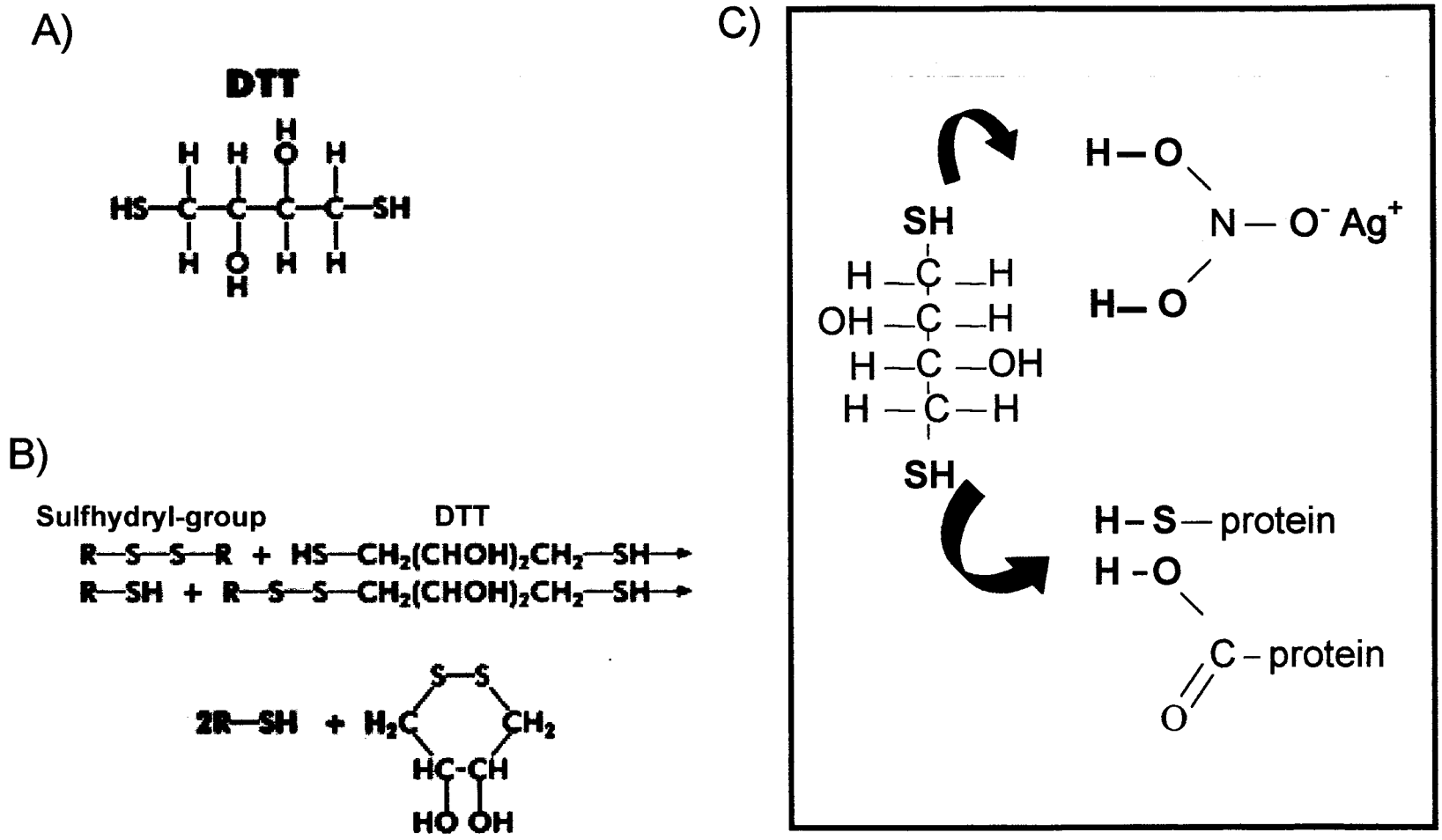
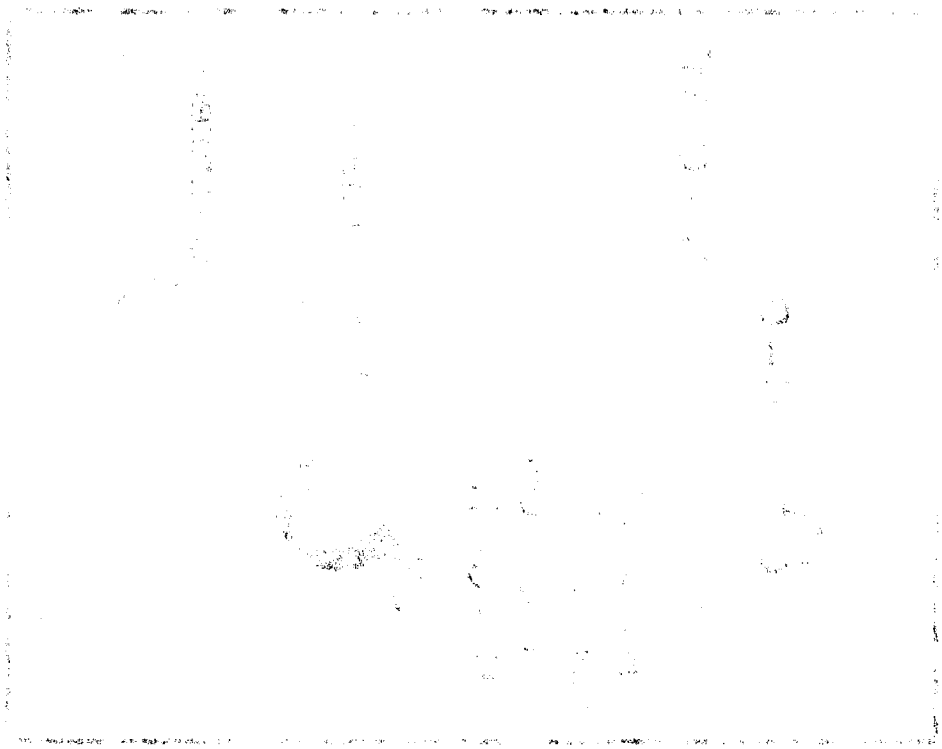



Figure 1: Chemical reaction of DTT. A) Chemical structure of αDithiothreitol. B) Reaction of DTT with disulfhydryl group. An intermediary compound is formed where DTT is covently attached to the protein via the one sulfhydryl group. DTT is released in a cyclic form by reduction of the second sulfhydryl group. C) Formation of a stable complex between proteins and silver nitrate by DTT reduction of sulfhydryl or carboxyl groups.



mass spectrometers but all are based on the same principle of separating the charged particles by magnetic/electric fields and all have the same three sections:

1. a source where the ions from the molecule to be analysed are produced
2. an analyser which separate ions according to their mass
3. a detector which captures and produces a signal

There are several ways to form charged particles: electron impact (EI), chemical ionization (CI) fast atom bombardment (FAB), field desorption (FD), electrospray ionization (ESI) and laser desorption (LD). The choice in the ionisation method depends greatly on the sample to be analysed. For biological material such as proteins or DNA fragments, ionization is difficult without vaporisation causing direct degradation of the specimen. Thus, the ionisation method of choice for these substances is laser desorption. Direct laser desorption relies on the principle that rapid heating of a sample vaporises the molecules so quickly that it does not have time to decompose. Matrix-assisted laser desorption ionization (MALDI) uses a solution which includes a chromophore that absorbs the laser of a specific wavelength. A small amount of this solution is applied to the sample in question to create a solid matrix. The matrix is then hit by the laser and absorbs the energy. The plasma produced vaporises and ionises the sample without causing destruction to the biological material.

Thus, the goal of this study section was to isolate and characterise novel CTLA-4 associated proteins, which may account for CTLA-4 function.

## 2.2 Materials and Methods

### 2.2.1 Cell lines, antibodies and reagents

The murine T cell hybridoma DC27.10 was cultured in RPMI 1640 medium, supplemented with 5% FCS and 50 uM 2-ME. DC27.10 cells were stably transfected with either vector control or human CTLA-4 as described (Hu et al., 2001). Anti-CD28 (37.51) was purchased from Pharmingen (San Diego, CA). Anti-pTyr mAb was a kind gift from Dr. T. Roberts (Dana Farber Cancer Institute, Boston, MA). MIgG technical grade (#I8765) and IgG1 (clone MOPC-21, #M7894) were purchased from Sigma. Anti-CTLA-4 antibodies clone GI#25, GI#26B and GI#35 were a kind gift from Dr. M. Collins from Genetics Institute, Boston, MA, BNI.3 was purchased from Coulter Immunotech, Marseille, France. The hybridoma BNI.4, BNI.8, BNI.9 clones producing anti-CTLA-4 antibody were a generous gift from Dr. Barbara Bröker, Bernhard-Nocht-Institute for Tropical Medicine,Hamburg,Germany. Dimethyl pimelimidate was purchased from Pierce (#21666). αDTT (D-5545), AgNO3 and sodium carbonate were purchased from Sigma. Protein A-Sepharose was purchased from Pharmacia Biotech. Econocolumns were purchased from Bio-Rad.

### 2.2.2. Preparation of the immunoaffinity column

Protein A-Sepharose CL-4B was mixed with antibody BNI.9 in 0.1M borate buffer, pH 8.2 for 30 mins at room temperature with gentle shaking, after which the sepharose beads were washed with excess borate buffer. The sepharose was washed with 0.2M triethanolamine, pH 8.2, and then resuspended in 20 volumes of dimethyl pimelimidate freshly made in 0.2M triethanolamine with the pH reajusted to pH 8.2. The mixture was agitated gently at room temperature for 45 min and the reaction was stopped by centrifuging the beads and resuspending in an equal volume of ethanolamine, pH 8.2, of the same molarity as the dimethyl pimelimidate dihydrochloride. After 5 min, the crosslinked beads were washed three times with 0.1M borate buffer, pH 8.2, supplemented with 0.02% sodium azide.

### 2.2.3. Affinity Chromatography

Cell lysates prepared as described below, either freshly prepared or stored at –70°C, were pre-absorbed to remove non-specifically binding components by incubation with 1/50 volume of a 10% suspension of ***Staphylococcus aureus*** (Pansorbin A, Calbiochem,# 507858). The cell lysate was applied in the precolumn and allowed to flow through the precolumn and specific column linked in series. The columns were washed with 5 column volumes of wash buffer (0.01 M Tris HCL, pH 8.0, 0.14M NaCl, 0.025% $NaN_3$, 0.5% TritonX-100, 0.5% sodium deoxycholate). The immunoaffinity column was then washed with 5 column volume of Tris buffer pH 8.0 (50 mM Tris HCl pH 8.0, 0.1% TritonX-100, 0.5M NaCl). The column was washed with 5 column volumes of Tris buffer pH 9.0. CTLA-4 was then eluted with 5 column volume of triethanolamine solution (50 mM triethanolamine pH 11.5, 0.1% TritonX-100, 0.15M NaCl). Collection of fractions into 1 column volume into tubes containing 0.2 vol of 1M Tris HCl, pH 6.7 to neutralize the fractions. The column was then washed and stored with TSA (0.002M Tris CL pH 8.0, 0.14M NaCl, 0.025% $NaN_3$). A 100 µl aliquots of the fraction was analysed by SDS-PAGE and silver staining.

### 2.2.4. Immunoprecipitation and Immunoblotting

For immunoprecipitation, cells were lysed in ice-cold lysis buffer containing 1% TritonX-100 in 20 mM Tris-HCL, pH 8.2, 150 mM NaCl. The lysis buffer contained phosphatase inhibitor 1mM sodium vanadate and the protease inhibitors 1mM PMSF, 1 mM sodium fluoride. Postnuclear lysates were incubated for 1 hour with the indicated antibody. Protein A-Sepharose beads (40 µl Pharmacia) were added and incubated for 1 hour at 4°C. The eluted proteins were separated by SDS-PAGE and either transferred to nitrocellulose for immunoblotting or silver stained. For immunoblotting, the membranes were blocked with 5% milk or 2% gelatine in TBS (10 mM Tris-HCL, pH 7.6, 150 mM NaCl) and incubated with the indicated antibody. Bound antibody was revealed with the appropriate secondary antibody, and protein was visualized by enhanced chemiluminescence (ECL, Amersham).

### 2.2.5. Stimulation of cells with PTP'ase inhibitor

Cells were resuspended in 1ml of supplemented RPMI. Stock Vanadate (100mM) was diluted 1:10 in sterile water and 1:1000 (30%) $H_2O_2$ was added to make a 100X stimulating solution. The solution was incubated at room temperature for 20 min until colour turned yellow.

## 2.2.6. Silver staining

After the proteins were separated by SDS-PAGE, the gel was fixed in 50% methanol, twice. The gel was washed once with 5% methanol for 10 min and rinsed quickly once with water. The gel was then incubated with a solution of 10μM of DTT (Dithiothreitol) for 20 min. The gel was washed quickly twice with water and incubated with chilled 0.1% (w/v) $AgNO_3$ for 20 min. The gel was washed quickly twice before addition of 2% sodium carbonate. The gel was incubated with intensive shaking until developing solution turned yellow. The solution was discarded and fresh developing solution was added until desired band intensity is achieved. Developing was terminated by addition of solid citric acid (10g/ 200ml of developer solution) and soaked for 10 min. The gel was finally washed with 5% acetic acid. The gel was stored in 1% acetic acid at 4°C until analysed.

## 2.3. Results

For the purposes of my study, I decided to use an antibody-based technique for isolation of associated proteins. Successful isolation of proteins is dependent on many factors but primarily, the type of detergent used for extracting membrane proteins as well as the quality and specificity of the antibody are of the utmost importance. There is a wide range of detergents that are available for solubilising biological membranes. Table A lists some of the detergents that are commonly used for the purpose of protein solubilisation. Detergents can be classified by their overall chemical structure. Type A detergents have flexible hydrophobic tails and hydrophilic head groups, while type B detergents are cholesterol based structures with amphiphilic properties. Furthermore, detergents can be subdivided according to their electrical charge. Non-ionic detergents do not affect the charge of the protein. Zwitterionic detergents, which are bipolar ionic detergent and thus electronically neutral, can stabilise highly charged molecules in an hydrophobic environment. Electrostatic and hydrogen bonding interaction between proteins and the detergent polar groups can affect the activity and binding affinity of proteins to one another. Thus, TritonX-100, the choice of a non-ionic detergent used for this study, was based on previous experience using the DC27.10 cells in coprecipitation experiments. For this study, I used as a control the vector transfected cell line V5 and the CTLA-4WT transfectant, which is stably transfected with human CTLA-4, as the main source for CTLA-4 protein isolation. As shown in Figure 2A, immunoprecipitation of CTLA-4 using the CTLA-4WT cell line was clearly visible with a buffer composition of 1% TritonX-100 (lane 3, 4, 5 and 6). In these preliminary experiments, 4 different antibodies were used to immunoprecipitate CTLA-4. The BNI.3 (IgG2a) is a commercially available antibody while the GI#25 (IgG1), GI#26B (IgG1) and GI#35 (IgG1) were acquired through collaboration with Genetics Institute. These antibodies are all monoclonal and derived from mouse species. Western blotting of the immunoprecipitated protein showed that all 4 of the anti-CTLA-4 antibodies were able to precipitate CTLA-4 with comparably equal amounts, BNI.3 and GI#26B being slightly more efficient. A mouse IgG (low technical grade mIgG) control as well as the monoclonal antibody 37.10 against mouse CD28 was used as controls (lane 1 and 2). The 37.10 antibody does not show a light chain band since the antibody was derived from a syrian hamster strain which does not crossreact with anti-mouse antibodies. Due to high glycosylation levels and post-translational modification of CTLA-4, it has been shown that CTLA-4 may display a smear-like band migrating between 29 and 35 kDa in western blots. This is the reason why, in general, single bands for CTLA-4 cannot be detected. The BNI.3 antibody was used to test whether CTLA-4 could be detected by

| | Detergent A | Detergent B |
|---|---|---|
| Non-ionic | Trition-X<br>NP-40<br>Octylglucoside<br>Lubrol PX | Digitonin |
| Zwittergent | Zwittergent 3-14 | Chaps |
| Ionic | | Sodium cholate |

**Table A: Commonly used detergents for solubilization of proteins.**

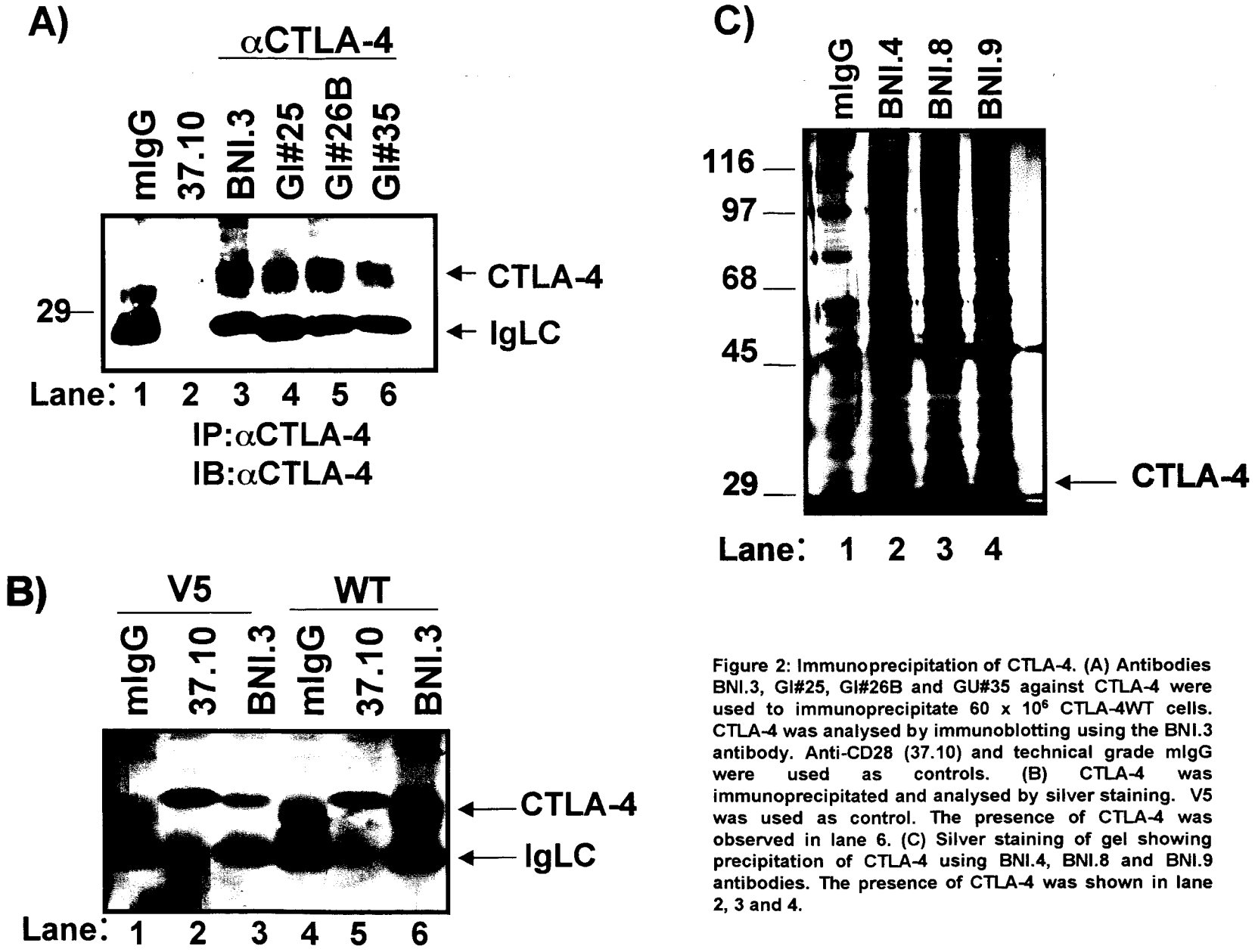



**Figure 2: Immunoprecipitation of CTLA-4. (A) Antibodies BNI.3, GI#25, GI#26B and GU#35 against CTLA-4 were used to immunoprecipitate 60 x $10^6$ CTLA-4WT cells. CTLA-4 was analysed by immunoblotting using the BNI.3 antibody. Anti-CD28 (37.10) and technical grade mIgG were used as controls. (B) CTLA-4 was immunoprecipitated and analysed by silver staining. V5 was used as control. The presence of CTLA-4 was observed in lane 6. (C) Silver staining of gel showing precipitation of CTLA-4 using BNI.4, BNI.8 and BNI.9 antibodies. The presence of CTLA-4 was shown in lane 2, 3 and 4.**

silver staining. As Figure 2B shows, a dark smeary shadow above the immunoglobulin light chain appears at the position where CTLA-4 is expected to migrate (lane 6). This band does not appear in the Vector control (V5) (lane 3) or with the 37.10 antibody (lane 2 and 5). A non-specific band can be detected with the control mIgG at approximately 32 kDa (lane 1 and 4), which may in certain experiments blur detection of CTLA-4. Other antibodies were used to test for detection of CTLA-4 using silver staining. Supernatant from hybridoma BNI.4, BNI.8 and BNI.9 were used to immunoprecipitate CTLA-4 from the CTLA-4WT cells. As shown in Figure 2C, BNI.4, BNI.8 and BNI.9 were able to immunoprecipitate CTLA-4 with the same binding efficiency (lane 2, 3 and 4).

Protein interactions, which are usually reversible, are mediated by 3 types of bond: electrostatic bonds, hydrogen bond and van der Waals bonds. These weak, non-covalent interactions combine to create highly specific binding. The efficiency of the antibody's binding sites to its specific antigen is dependent on many factors. One critical determinant, however is the effect of pH on disruption of protein binding and protein folding. This basic principle is the foundation on which affinity chromatography is based. Having either a basic or acidic environment can adversely affect the charge of critical amino group side chains necessary for stabilisation of the protein-antibody complex. Thus, two different buffers with slight differences of pH but still within the physiological range were tested to determine which pH would be favourable for better CTLA-4 binding to the antibody and to potentially associated proteins. As shown in Figure 3, using a neutral pH of 7.4 and a slightly more basic buffer of pH 8.2, BNI.3 antibody was more efficient at binding CTLA-4 within the range of pH 8.2. Moreover, silver staining of proteins associated with CTLA-4 showed a reduced background of proteins associating with the CTLA-4 complex (Figure 4). This was not dependent on the cell type since both GI#26B and BNI.3 showed the same pattern in the control V5 as well as in CTLA-4WT cells. Interestingly, two distinct proteins were reported to bind in lane 7 (approximate molecular weight of A)66 and B)50 kDa). This interaction was lost when using the buffer pH7.4 (lane 13), suggesting that the interaction may be specific. CTLA-4 detection is shown in the white box (lane 7 and 13). Thus for the remainder of the experiments, I selected a buffer of pH 8.2 to carry out the CTLA-4 immunoprecipitation.

In the first instance, it was of interest to investigate whether phosphorylation of CTLA-4 might recruit proteins to its cytoplasmic tail. As CTLA-4 is phosphorylated after T cell activation, it may be possible that proteins are recruited to the plasma membrane. To this end, pervanadate, a phosphatase inhibitor, was used to induce

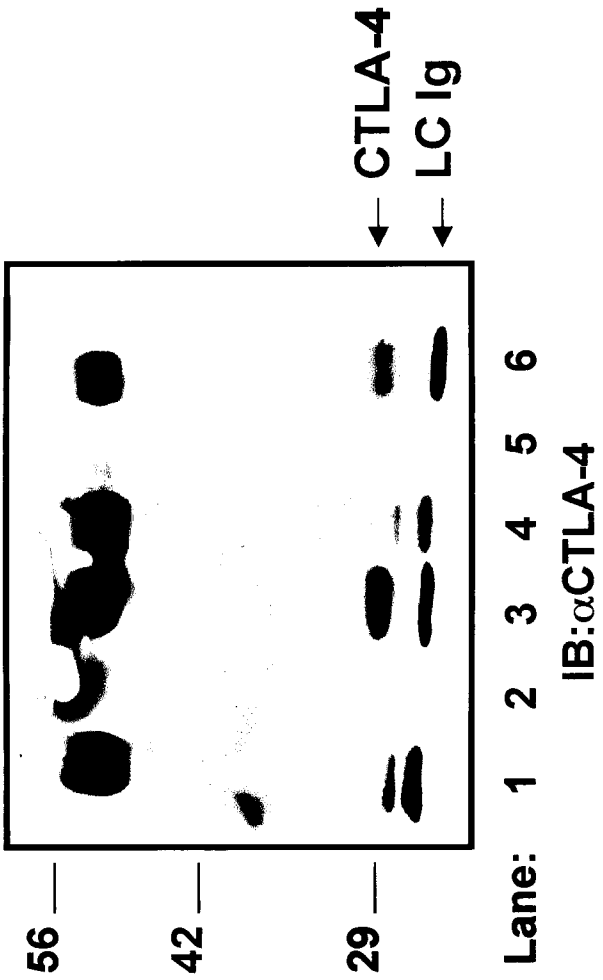



Figure 3: The effect of buffer pH on CTLA-4 precipitation. Antibody BNI.3 was used to immunoprecipitate CTLA-4 from 60 x $10^6$ CTLA-4WT cells. The cells were lysed in lysis buffer of either pH8.2 or 7.4. Immunoprecipitated CTLA-4 was subjected to Western blotting analysis with BNI.3 anti-CTLA-4. Anti-CD28 (37.10) and technical grade mIgG were used as controls.

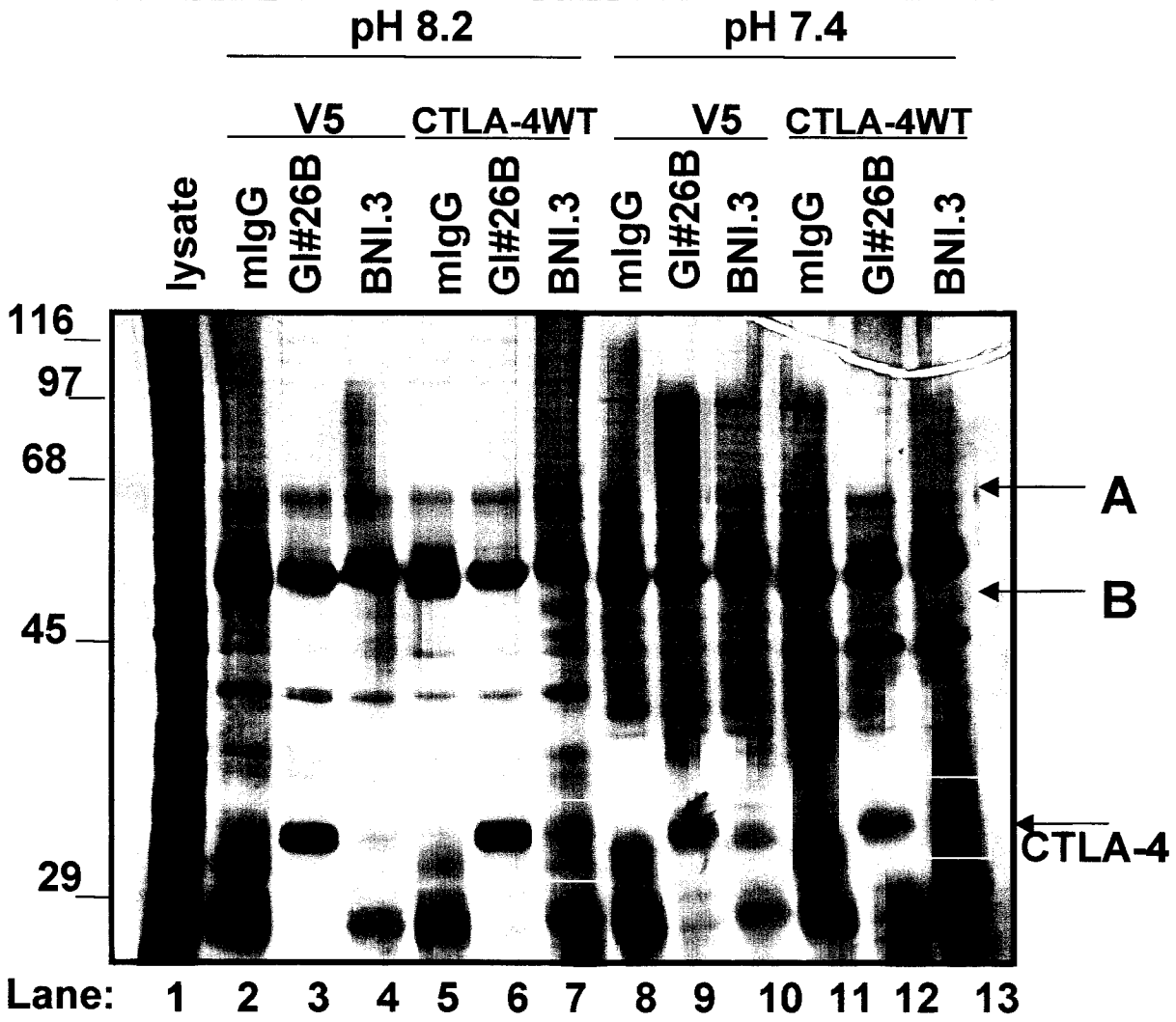



Figure 4: The effect of buffer pH on precipitation of CTLA-4 associated proteins. V5 control cell and CTLA-4WT cells were subjected to lysis buffer of either pH8.2 or 7.4. CTLA-4 was immunoprecipitated using GI#26B and BNI.3 anti-CTLA-4 antibodies. Technical grade mIgG was used as control. Silver staining of the gel shows the presence of immunoprecipitated CTLA-4 in lane 7 and 13. In addition two proteins designated as A and B were lost when lysis buffer pH 7.4 was used compared to lysis buffer pH8.2. Conversely, less overall background proteins are found to be associated with CTLA-4 immunoprecipitation in lysis buffer pH 8.2 than in lysis buffer pH7.4.

non-specific phosphorylation of proteins. As seen in Figure 5A, incubation of pervanadate for a time period of 20 min gradually increased the total cellular levels of phosphorylation. Thus to examine the effects of phosphorylation on protein binding to the cytoplasmic tail of CTLA-4, the cells were treated for 20 min with pervanadate before lysing and immunoprecipitated with the antibodies BNI.3 and GI#26B. As Figure 5B shows, pervanadate treatment of the CTLA-4WT cells induced protein phosphorylation as seen in the total lysate (lane 1 and 2). Furthermore, GI#26 and BNI.3 immunoprecipitated a band corresponding to CTLA-4, which appeared highly phosphorylated (lane 10, 11). This band did not show up in the control mIgG and mIgG1 (lane 8 and 9) or in the non-treated cells (lane 5 and 6). Unfortunately, no other phosphorylated band was precipitated with CTLA-4. A GST fusion protein encompassing the cytoplasmic tail of CTLA-4 was used to try to pull down phosphorylated proteins that may associate to the non-phosphorylated tail of CTLA-4 (see appendix 1 for construct). However, the non-phosphorylated tail of CTLA-4 failed to bind any other protein (lane 7 and 12). Thus, I can conclude from these results that unphosphorylated CTLA-4 may not associate with phosphorylated proteins.

These results do not necessarily infer that other proteins, which are not phosphorylated, may not associate with CTLA-4. As Figure 4 suggests, non-phosphorylated CTLA-4 may associate with non-phosphorylated proteins. Conversely, CTLA-4 phosphorylation may relive binding of certain proteins as in the case with AP-1. To determine whether CTLA-4 could associate to non-phosphorylated proteins, CTLA-4 immunoprecipitates were analysed by silver staining. Initially, the antibodies GI#26 and GI#35 were used for precipitation due to their high affinity binding to CTLA-4. As shown in Figure 6, there are 6 proteins, which could be detected with the anti-CTLA-4 antibodies that did not precipitate with the control mIgG, mIgG1, and 37.10 (lane 1, 2 and 3). Figure 6 right panel, shows a detailed view of the associated proteins (lane 4 and 5 region A, B, C and D). These proteins were designated as CAP (CTLA-4 Associated Proteins) and their approximate molecular weights were calculated as follows: CAP1 (128 kDa), Cap-2 (72 kDa), CAP-3 (66 kDa), CAP-4 (48 kDa), CAP-5 (39 kDa), CAP-6 (36kDa). Based on a titration curve for BSA, one can deduce a relatively broad approximation of the amount of protein present. It is estimated to be between 10 and 50 ng. Next, it was of interest to compare stimulated and non-stimulated cells in terms of protein binding detected by silver staining. CTLA-4WT cells were stimulated with pervanadate, and immunoprecipitation of CTLA-4 was detected by silver staining. As shown in Figure 7, precipitation of CTLA-4 in unstimulated cells had a comparable protein profile (band pattern) as in the stimulated cells. In this gel, CD28 and CTLA-4 were immunoprecipitated with 37.10 (lane 4, 7 and 9) and BNI.3 (lane 5, 8 and 11) antibodies, respectively. No differences between the CD28 and control mIgG were observed. However, anti-CTLA-4 was revealed to coprecipitate only CAP-3 and CAP-4 in this experiment. There was no evidence of decreased binding of

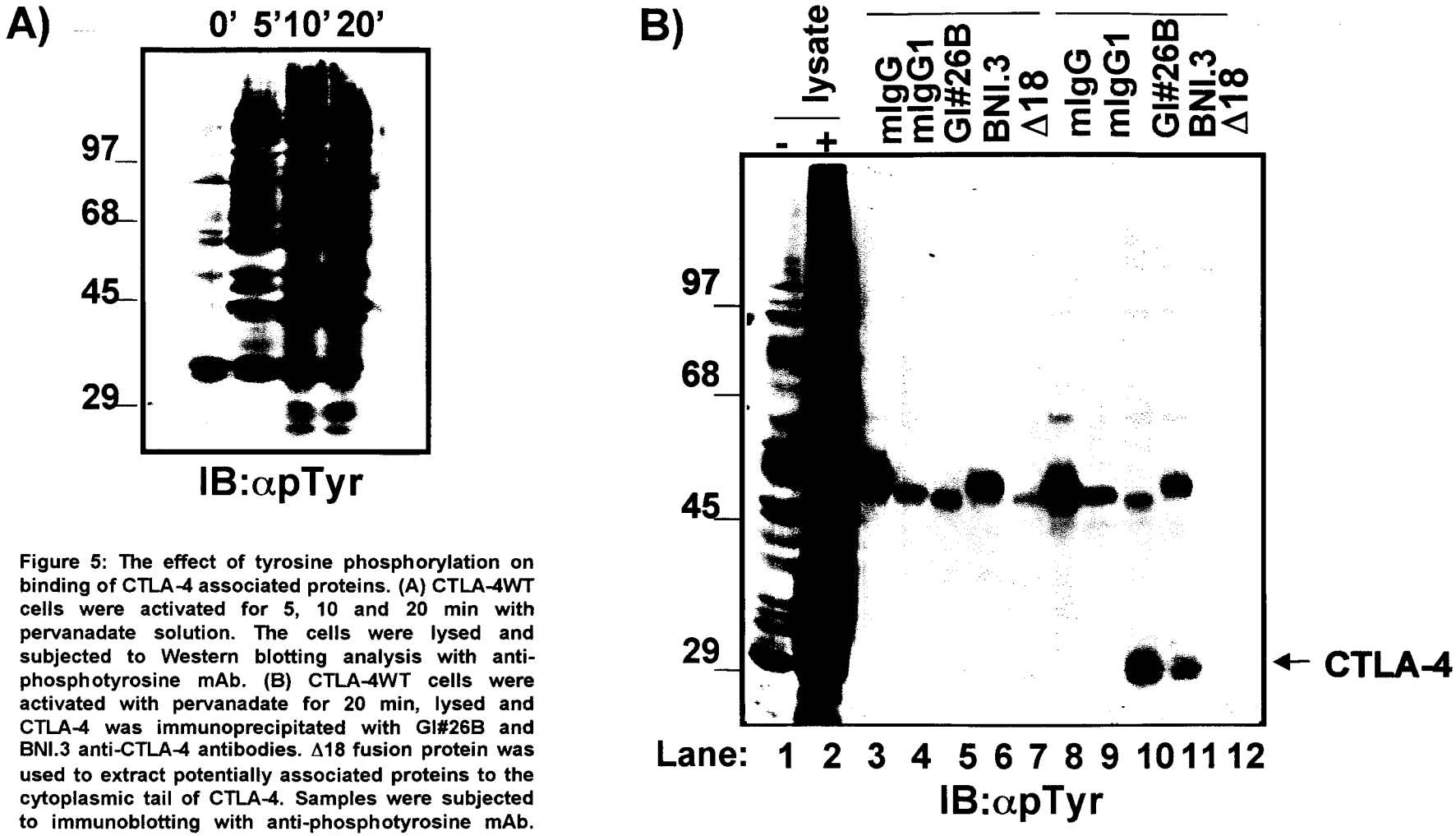



**Figure 5: The effect of tyrosine phosphorylation on binding of CTLA-4 associated proteins. (A) CTLA-4WT cells were activated for 5, 10 and 20 min with pervanadate solution. The cells were lysed and subjected to Western blotting analysis with anti-phosphotyrosine mAb. (B) CTLA-4WT cells were activated with pervanadate for 20 min, lysed and CTLA-4 was immunoprecipitated with GI#26B and BNI.3 anti-CTLA-4 antibodies. Δ18 fusion protein was used to extract potentially associated proteins to the cytoplasmic tail of CTLA-4. Samples were subjected to immunoblotting with anti-phosphotyrosine mAb. Phosphorylated CTLA-4 was observed in lanes 10 and 11. However no other phosphorylated protein was observed associated with CTLA-4 (lane 5, 6, 10, 11).**

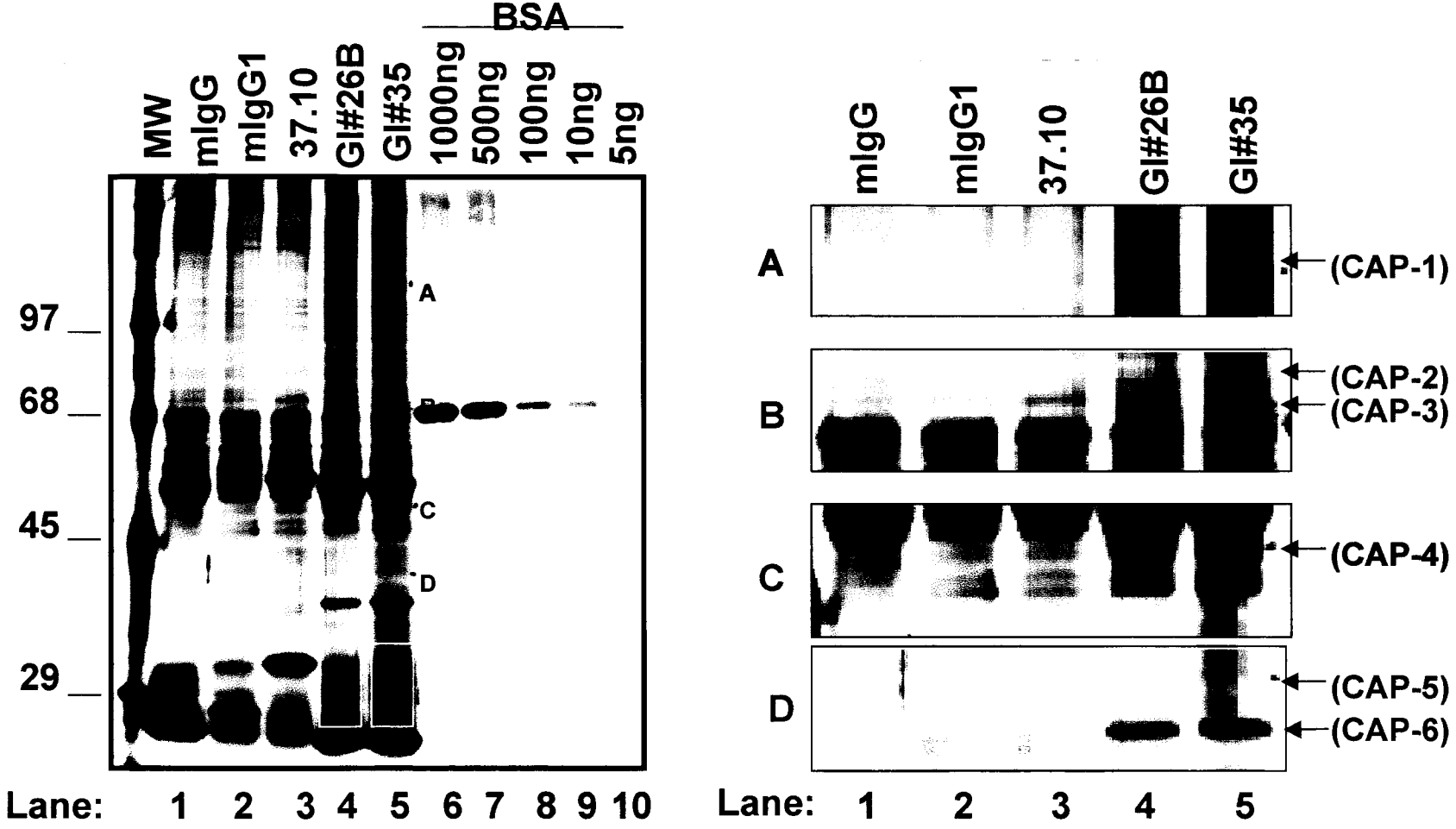



**Figure 6: Immunoprecipitation of CD28 and CTLA-4 in nonstimulated CTLA-4WT cells. 60 x $10^6$ CTLA-4WT cells were used to immunoprecipitate CTLA-4 using GI#26 and GI#35 anti-CTLA-4 antibody. Technical grade mIgG, IgG1 and anti-CD28 (37.10) antibodies were used as control immunoprecipitates. The samples were subjected to silver staining. The gel shows the presence of immunoprecipitated CTLA-4 in lane 4 and 5. In addition, associated proteins to CTLA-4 were detected in region A (CAP-1), region B (CAP-2 and 3), region C (CAP-4) and region D (CAP-5 and 6). BSA was used for quantification purposes.**

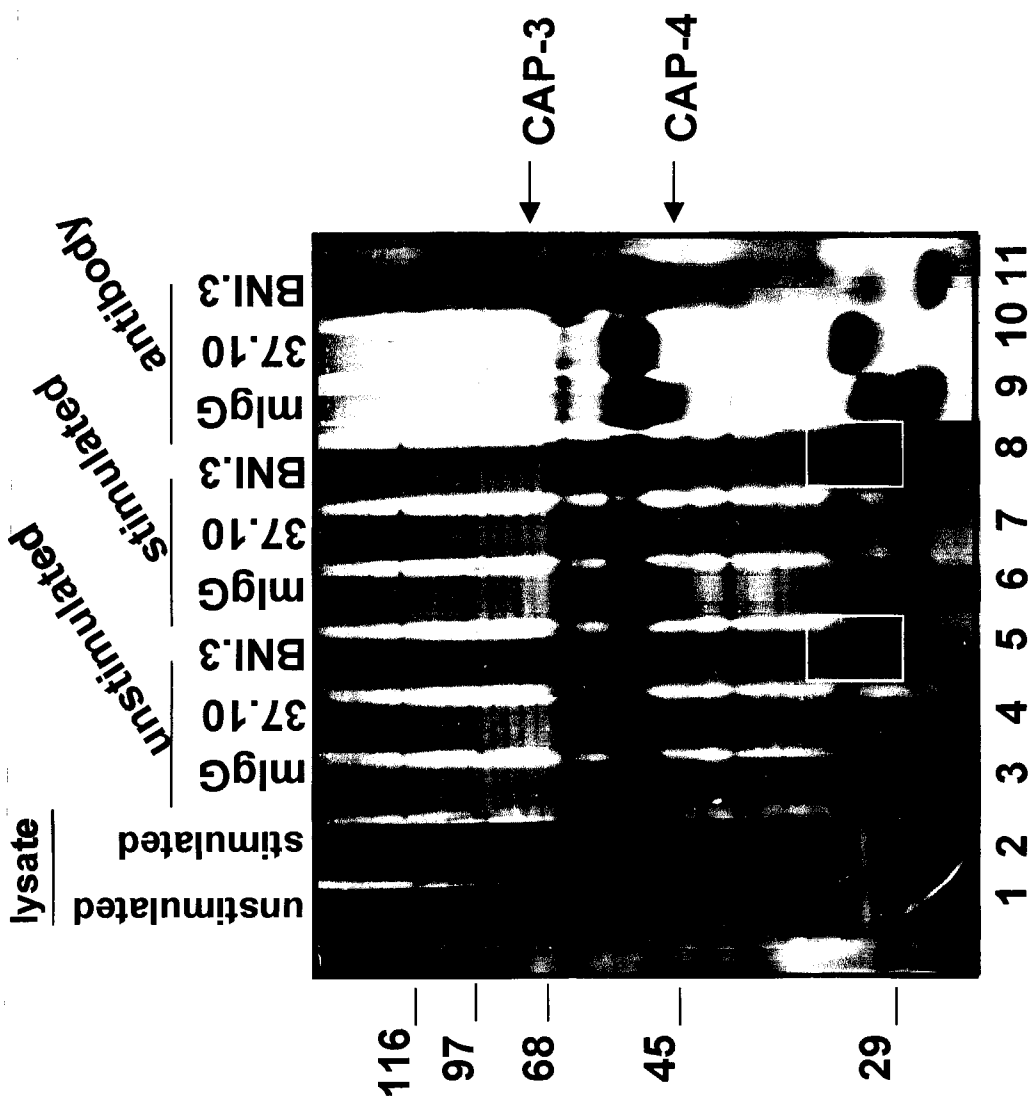



Figure 7: Immunoprecipitation of phosphorylated CTLA-4. CTLA-4WT cells were treated with pervanadate for 20 min, lysed and subjected to CTLA-4 immunoprecipitatiom using BNI.3 antibody. Technical grade mIgG and anti-CD28 (37.10) antibodies were used as control immunoprecipitates. The samples were subjected to silver staining. The gel shows the presence of immunoprecipitated CTLA-4 in lane 5 and 8. In addition, associated proteins CAP-3 and CAP-4 were shown to co-precipitate with CTLA-4. However, no differences in protein binding between stimulated and unstimulated CTLA-4 immunoprecipitates were detected.

other proteins. To determine whether binding of CAP proteins was specific, V5 control cells were included in the next experiment. As shown in Figure 8, anti-CTLA-4 antibodies GI#26B and BNI.3 were able to precipitate several proteins. The right panels of Figure 8 show a detailed view of the gel. Several proteins, designated A, B, C, and D corresponded approximately to CAP-1, 2, 3, and 4, respectively (lane 5, 6, 11 and 12). These proteins were not observed using the V5 control (lane 2, 3, 8, and 9). CTLA-4 (in white boxes) was also detected above the Ig light chain. Thus, one can conclude from these results that CTLA-4 may possibly associate with the same proteins whether it is in its phosphorylated form or not. It is still possible that a few proteins get recruited to the phosphorylated form of CTLA-4 in a low stoichiometric ratio. This scenario is quite unlikely since it is known that PI3- kinase can be recruited by CTLA-4 after TCR ligation as well as other proteins (Chikuma et al., 2000; Chuang et al., 1999; Lee et al., 1998; Schneider and Rudd, 2000; Schneider et al., 1998; Zhang and Allison, 1997). It is conceivable that silver staining method is not sensitive enough to detect small fluctuations of protein binding which may be recruited after stimulation or phosphorylation. In addition, the presence of low numbers of carboxyl and sulfhydryl groups in the composition of the associated proteins may impede their detection by silver staining. Thus, based on these results, I may not conclusively determine whether there is a difference in associated proteins between phosphorylated and non-phosphorylated states. However, proteins that associate with non-phosphorylated CTLA-4 and detected by silver staining seem to be specific since they do not appear in the vector control or in antibody controls.

In view of the fact that it is possible that the quantity of protein isolated by immunoprecipitation was not sufficient to detect changes in protein concentration on silver staining, affinity chromatography was used as a means to purify large amounts of protein. As described in materials and methods, two chromatographic columns were set up to isolate specific CTLA-4 associated proteins. A precolumn was packed and bound to non-specific antibody of the IgG1 subclass. A specific -CTLA-4 column was packed and bound with specific anti-CTLA-4 antibody from the hybridoma producing BNI.9 antibody. I purified 10 mg of antibody and covalently attached it to sepharose beads with the crosslinker dimethyl pimelimidate. IgG1 and technical grade mIgG antibody was used to make the precolumn. As shown in Figure 9, antibody that was incubated with sepharose beads but not treated with dimethyl pimelimidate were easily discharged into solution after boiling (lanes 3, 5, 9 and 11). The covalently bound antibody did not detach from the beads after boiling (lane 6, 10 and 12). There was some leakage observed with the technical grade antibody due to the low quality grade of the antibody preparation (lane 4). This result serves as a quality control over the crosslinking method and the performance of the columns. Having tested the precolumn and CTLA-4 column for leakage, both columns were washed and lysates from V5 control cells and CTLA-4-WT cells were passaged through

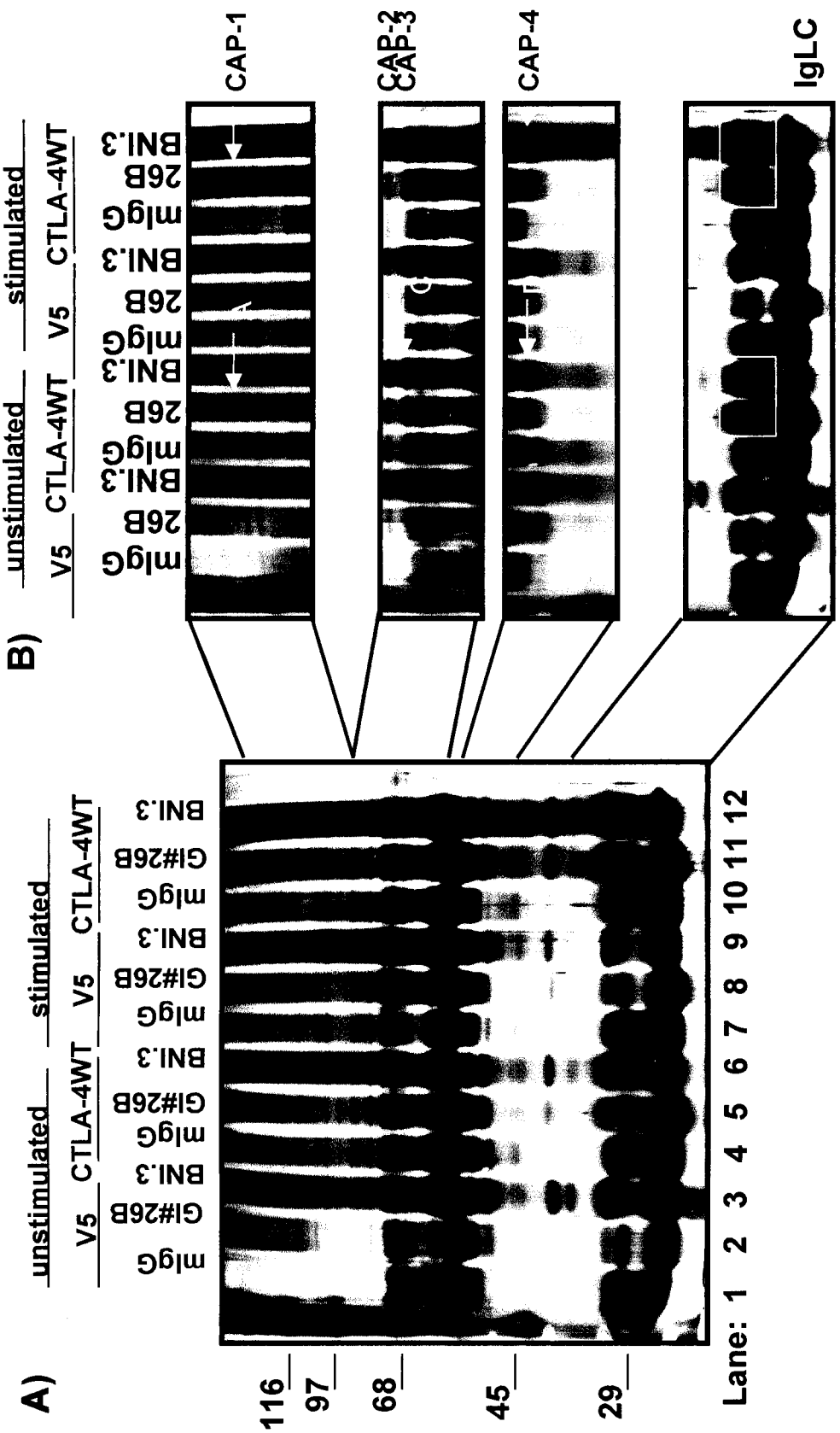



**Figure 8: Immunoprecipitation of CTLA-4 in CTLA-4WT cells reveals specific associated proteins. V5 control cells and CLA-4WT cells were either left untreated or were stimulated with pervanadate for 20 min. Immunoprecipitation of CTLA-4 was performed using GI#26 and BNI.3 anti-CTLA-4 antibody. Technical grade mIgG antibody was used as control. The samples were subjected to silver staining. The gel shows the presence of immunoprecipitated CTLA-4 in lane 5, 6, 11 and 12. In addition, associated proteins to CTLA-4 were detected in region A (CAP-1), region B (CAP-2), region C (CAP-3) and region D (CAP-4). These proteins were not observed in the vector control lane 2, 3, 8 and 9. In addition, no differential display of proteins was observed between unstimulated and stimulated cells.**

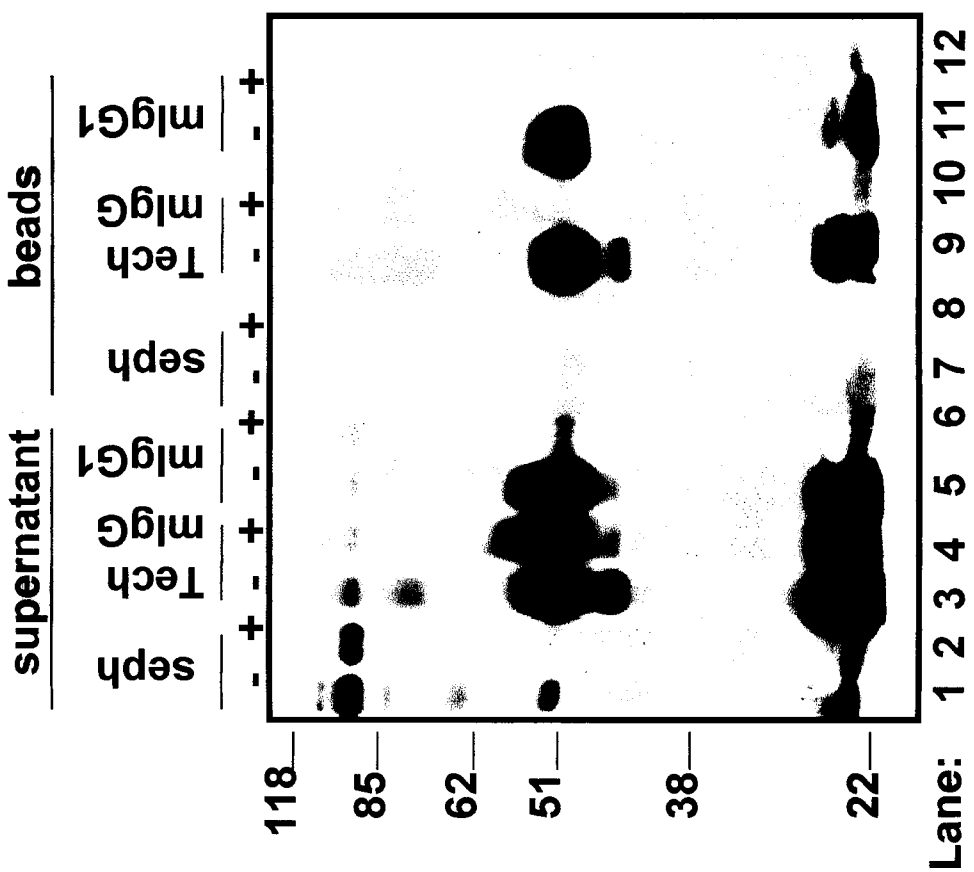



Figure 9: Chemical crosslinking of antibody to sepharose beads prevents leakage of antibody. A pool of technical grade mouse IgG and specific IgG1 antibodies were used to prepare a precolumn for antibody linked affinity matrix. The antibodies were crosslinked as described in materials and methods. A small aliquot of 100 µl of each sample was boiled in sample buffer. The supernantant and sepharose beads were separated, run on an SDS-PAGE and subjected to immunoblotting with secondary anti-mouse HRPO.

the precolumn twice and then through the anti-CTLA-4 column at a very slow rate. Once the lysate had been passed through the column, the column was washed twice and eluded using basic pH buffers. Different fractions were collected and subsequently run on a gel to determine whether CTLA-4 could coprecipitate along with associated proteins. Figure 10 shows a silver stain gel of the different fractions collected with V5 control lysate over the BNI.9 anti-CTLA-4 specific column. The gel showed a high amount of non-specific protein binding to the BNI.9 column. There was a slight increase in proteins eluted with the triethanolamine buffer pH11.5 which suggests that the antibody might be binding to distinct but non-specific proteins. Figure 11 shows passage of CTLA-4WT lysate through the BNI.9 column. Fractions 10 to 15 show an increase of protein dissociation from the column that demonstrates adequate stabilization and dissociation of proteins from the column. Having set the parameters and knowing in which fraction the proteins had the highest rate of dissociation, I next compared the fractions derived from the precolumn with the BNI.9 column from V5 and CTLA-4WT lysates. Figure 12 shows a representative experiment using the control and the BNI.9 column. CTLA-4 was not detected in the basic fractions collected in the BNI.9 column (Figure 12B, lane 7 to 12). The same profile of proteins was observed in the vector control (Figure 12A, lane 7 to 12). Several intense bands were collected with both V5 and CTLA-4WT cells in the BNI.9 column but were absent in the precolumn (Figure 12A, B lanes 1 to 6). To elucidate the origin of these bands, the same fractions from Figure 12B were analysed by western blot. As Figure 13 shows, CTLA-4 could not be detected in these collected fractions by western blot. The bands observed by silver staining appeared also in the western blot against the CTLA-4 antibody, suggesting that the antibody might recognise unspecific proteins. Thus, it is possible that the anti-CTLA-4 BNI.9 antibody lost its activity during the process of crosslinking. That hypothesis is highly unlikely since the same chemical treatment of the IgG control antibody with this agent did not produce the same effect in the precolumn. Subsequent analysis of the BNI.9 hybridoma derived antibody revealed that contamination with an unspecified antibody producing clone may have occurred.

Nevertheless, since associated proteins could be detected in the silver stained gels of co-immunoprecipitates of anti-CTLA-4, proteins were selected for sequencing by MALDI analysis. The most commonly observed protein being coprecipitated with CTLA-4 was the 66 kDa CAP-3 protein, estimated to be present in Figure 4, 6, 7 and 8. This protein band was dissected from the gel and sent to the sequencing facility at University of Massachussets Medical School directed by John Leszyk. The protein mixture was examined using MALDI LC/MS (iontrap) analysis. The resulting peptides were searched using SEQUEST Database analytical program (see appendix 2). The sequences of the protein obtained showed that the 66 kDa band was composed of a mixture of proteins. The two main peptide sequences found (Sp>500; RSp=1) were derived from cytokeratin (a

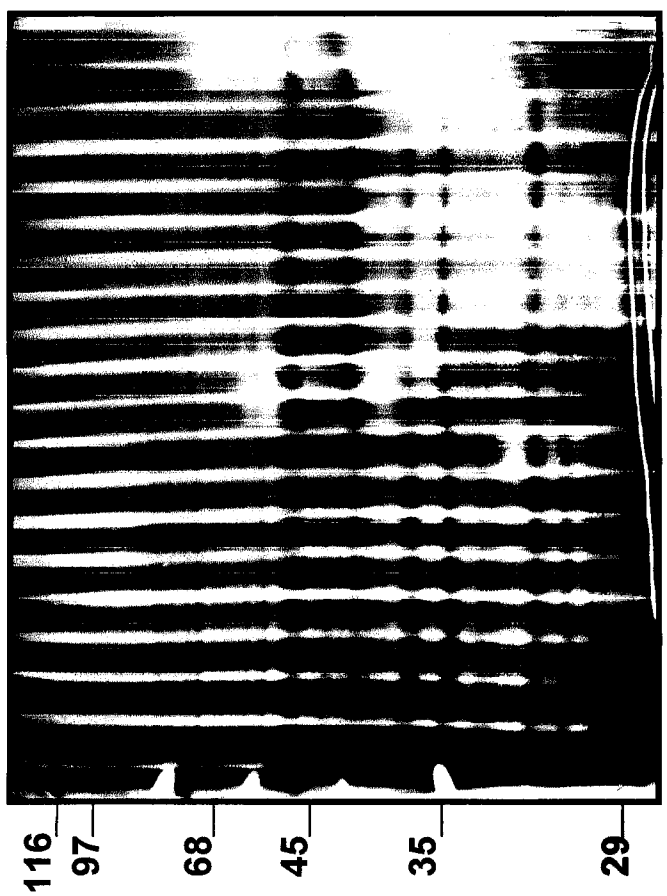



Figure 10: Elution of Vector control cell lysate from BNI.9 column. 1 x $10^9$ cells were lysed in 12 ml of 1% TritonX-100 lysis buffer. The supernatant was precleared with Pansorbin A and run through a non-specific IgG precolumn. The supernatant was applied to the BNI.9 column at a slow flow rate. The column was washed and eluted. Fractions of 1ml were collected and brought to neutrality.100µl from each fraction was analysed by silver staining.

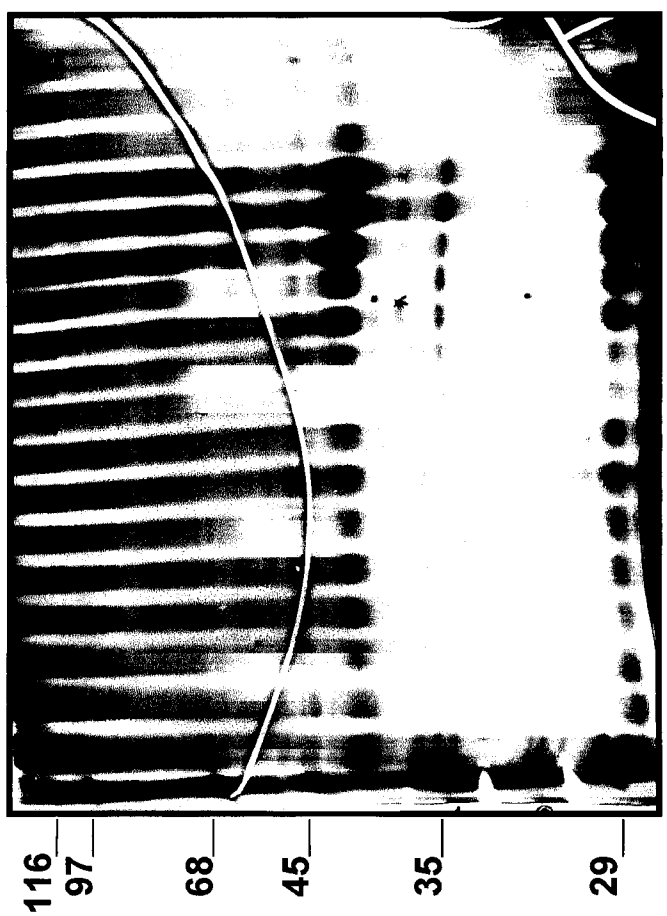



Figure 11: Elution of CTLA-4WT lysate from BNI.9 column. 1 x $10^9$ cells were lysed in 12 ml of 1% TritonX-100 lysis buffer. The supernatant was precleared with Pansorbin A and run through a non-specific IgG precolumn. The supernatant was applied to the BNI.9 column at a slow flow rate. The column was washed and eluted. Fractions were collected of 1ml and brought to neutrality.100µl from each fraction was analysed by silver staining.

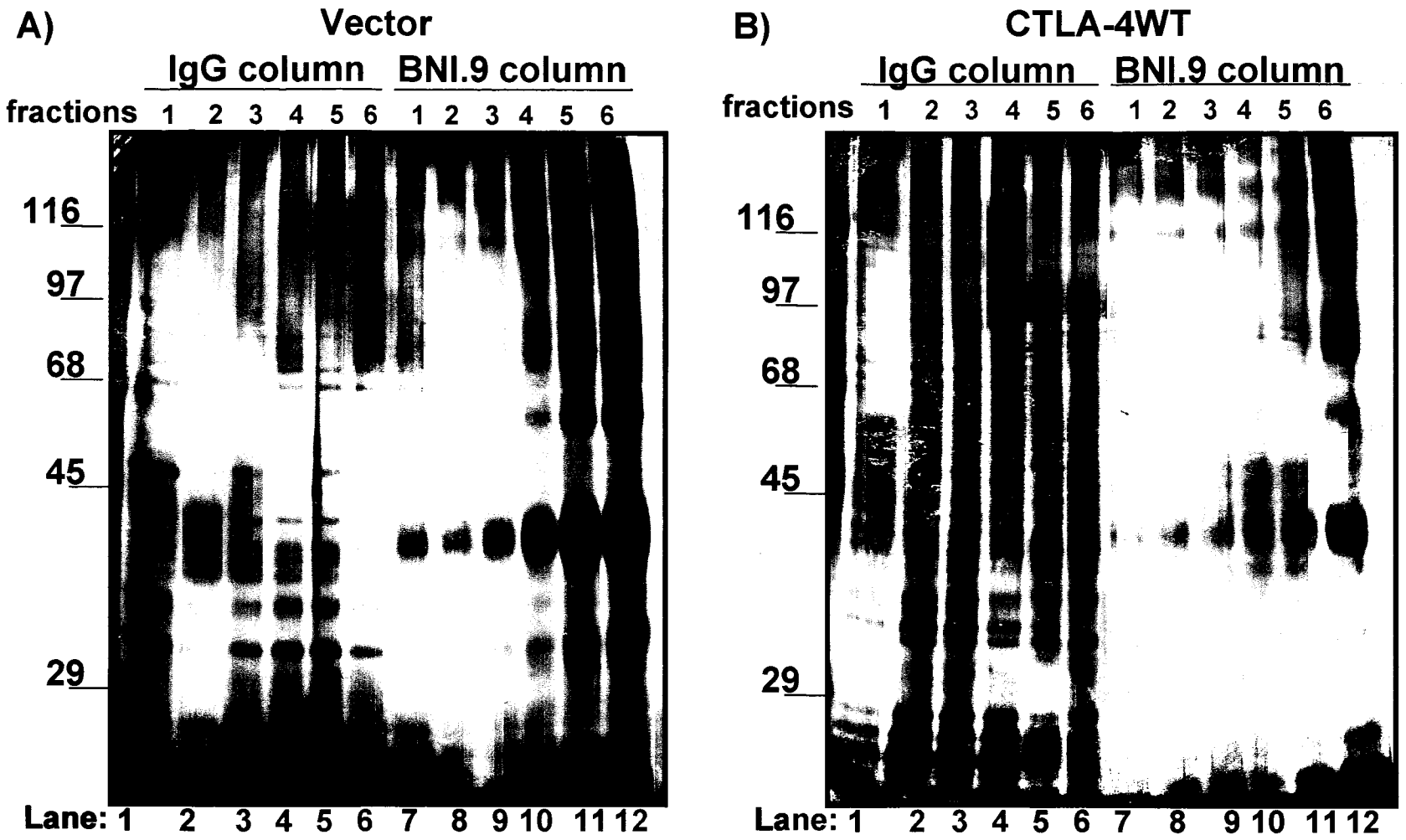



Figure 12: Eluate from V5 cells show same pattern of proteins as CTLA-4WT cells. 1 x $10^9$ V5 and CTLA-4WT cells were lysed in 12 ml of 1% TritonX-100 lysis buffer. The supernatant of each cell line was precleared with Pansorbin A and run through a non-specific IgG precolumn. Proteins bound to the non-specific IgG column were eluted. The supernatant was applied to the BNI.9 column at a constant slow flow rate. The column was washed and eluted. Fractions were collected of 1ml and brought to neutrality.100µl from fractions 6-13 eluted from the BNI.9 and IgG precolumn with triethanolamine buffer pH11.5 were analysed by silver staining.

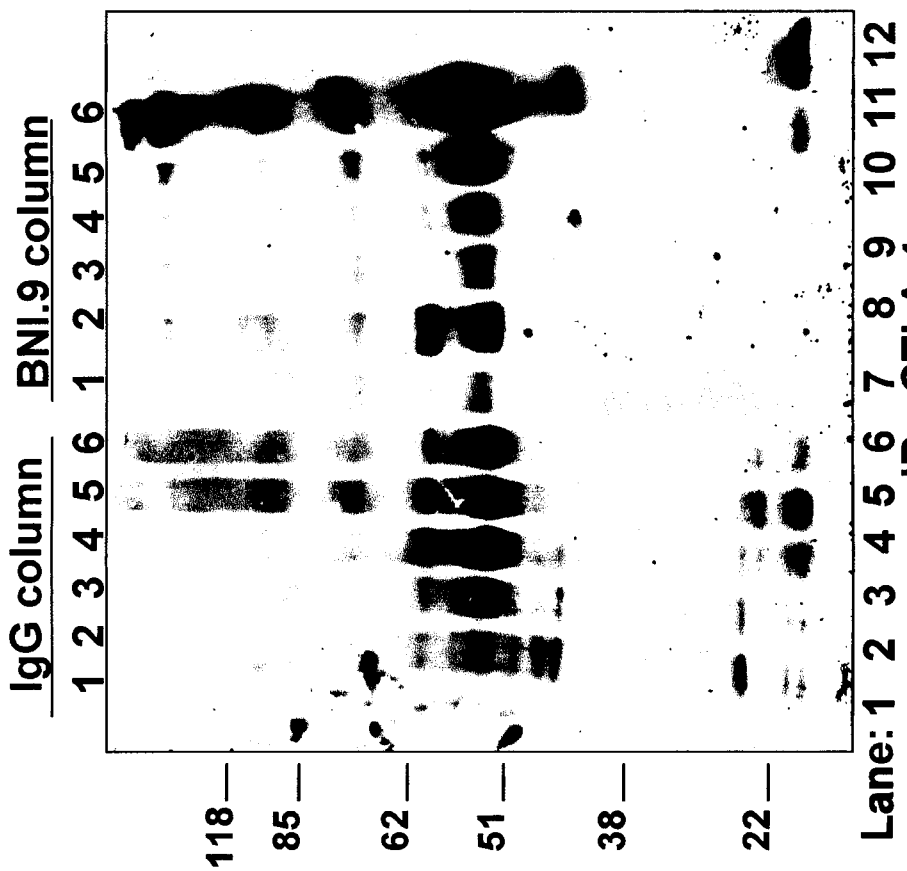



Figure 13: Specificity of CTLA-4WT eluate from BNI.9 column. 1 x $10^9$ CTLA-4WT cells were lysed in 12 ml of 1% TritonX-100 lysis buffer. The supernatant was precleared with Pansorbin A and run through a non-specific IgG precolumn. Proteins bound to the non-specific IgG column were eluted. The supernatant was applied to the BNI.9 column at a constant slow flow rate. The column was washed and eluted. Fractions were collected of 1ml and brought to neutrality.100µl from fractions 6-13 eluted from the BNI.9 and IgG precolumn with triethanolamine buffer pH11.5 were analysed by Western blotting with an anti-CTLA-4 (BNI.3) antibody.

contaminant that migrates at 65.8 kDa) (Johnson et al., 1985; Steinert et al., 1985) and ribophorin (a 68.5 kDa; #24,42,46) (see appendix 3 for further details on sequences of the peptides derived from the gel). Ribophorin is a mammalian glycosylsaccharyltransferase that is involved in post-translational modification of proteins. Since this protein is involved in maintenance of protein integrity and folding, it is highly unlikely that it may function as negative signalling molecule for early events of TCR signalling. In addition, two other peptide sequences were also isolated. A peptide sequence corresponding to the human lymphocyte adaptor LNK (63.1 kDa) (#9) and the human/mouse receptor protein kinase ryk (67.8 kDa) (#46). However both these proteins were present at low levels and only one peptide was found for each. This result puts into question the validity of the "hit". In addition, the human peptide but not the mouse peptide of LNK was found. It is possible that these proteins may be homologues of the human form, which have not yet been described in the mouse system. Further analysis of the peptides needs to be performed to accurately assess the significance of this finding.

## 2.4. Discussion

Since unknown proteins associated with CTLA-4 may be directly involved in the negative function of CTLA-4, I set out to investigate whether I could isolate and characterise proteins associated with CTLA-4 that may signal in a negative manner. To address this question, I aimed to purify associated proteins that could bind to non-phosphorylated CTLA-4 using affinity chromatography. Phosphorylation of CTLA-4 did not show any remarkable recruitment of associated proteins. To this end, 5 different anti-CTLA-4 antibodies from hybridoma clones (BNI.3, BNI.4, BNI.7, BNI.8 and BNI.9), which were kindly given to the lab by Dr. Barbara Bröker, and 3 different purified antibodies provided by Genetics Institute (GI#25, GI#26B, GI#35), were tested for their ability to immunoprecipitate CTLA-4. Each of the tested clones was analysed by silver staining and western blotting. Since affinity chromatography requires large amounts of purified antibody, antibody-producing hybridomas were required to generate such large amounts. From the hybridomas tested, BNI.9 appeared to be the most efficient at immunoprecipitating CTLA-4. Dr. Bröker generously provided the hybridoma and I purified 10 mg of antibody from this particular clone to be used for the preparation of the CTLA-4 affinity column. Unfortunately, after collecting and concentrating the eluate of the BNI.9 column and testing it against the control column, I found that the BNI.9 antibody had lost its activity given that both V5 and CTLA-4WT lysate precipitated the same non-specific proteins (Figure 12). Whether the antibody lost its activity before or after the purification step is still unclear. Nevertheless, using the antibody that was used in the bioassays, BN1.3, as well as the monoclonal antibody GI#26B, which precipitated the same protein profile, several associated proteins were detected. CTLA-4 immunoprecipitation was found to coprecipitate along with several other proteins (approximate molecular weights): CAP1 (128 kDa), Cap-2 (72 kDa), CAP-3 (66 kDa), CAP-4 (48 kDa), CAP-5 (39 kDa), CAP-6 (36kDa). One of the proteins, CAP3 was sent for Matrix Assisted Laser Deionization/Ionization (MALDI) sequencing. The sequencing revealed two major different proteins. One of the proteins was a cytokeratin contamination (66kD) (Johnson et al., 1985; Steinert et al., 1985) while the other appears to be specific and is called ribophorin (68kD). The mammalian oligosaccharyltransferase (OST) is an oligomeric complex composed of four membrane proteins of the endoplasmic reticulum: ribophorin I (RI), ribophorin II (RII), OST48 and DAD1. OST is responsible for the co-translational N-glycosylation (transfer of GlcNAc2-Man9-Glc3 from Dol-PP onto specific asparagine residues) in the nascent polypeptide (Kumar et al., 1998; Schweizer et al., 1991). Ribophorin has also been found to be selectively expressed on the surface of immature thymocytes although its role is unclear (Wiest et al., 1997). Since CTLA-4 is highly glycosylated, the association with

ribophorin to CTLA-4 was not so surprising. Other such highly glycosylated proteins have also been found to be associated with ribophorin when immunoprecipitated (Santhamma and Sen, 2000). While this protein may regulate CTLA-4 expression to some extent, by controlling modification of the sugar residues, it is highly improbable that it may be involved in TCR/CTLA-4 proximal signalling. A peptide sequence analysis demonstrated as well that peptides corresponding to the human lymphocyte adaptor LNK (63.1 kDa) (#9) and the human/mouse receptor protein kinase ryk (67.8 kDa) (#46) might be present. The LNK adaptor was found to be associated with phosphorylated CD3 ζ chain after cotransfection with $p56^{lck}$ through its Src homology 2 domain. The overexpression of Lnk in Jurkat cells has to lead inhibition of anti-CD3 mediated NF-AT-Luc activation (Li et al., 2000). Thus LNK might be a good candidate for negative signaling via CTLA-4. The RYK receptor (receptor related to tyrosine kinases) is unique amongst the catalytically inactive RTKs (Gough et al., 1995; Halford et al., 1999). Recent analyses of deficient RYK mice and its Drosophila orthologue have defined a role for this novel subfamily of receptors in the control of craniofacial development and neuronal pathway selection, respectively (Dura et al., 1995; Halford et al., 2000). Biochemical data involving RYK signal transduction pathways has lead to the hypothesis that it may act at a junction for crosstalk and scaffold assembly with Eph receptors. Since RYK is also expressed in lymphoid tissues (Simoneaux et al., 1995), it is possible that RYK may be a target for CTLA-4 negative signalling. However, both these proteins were present at low levels and only one peptide was found for each. In addition, the human peptide but not the mouse peptide of LNK was found which might suggest some type of contamination in the sample preparation. This result puts into question the validity of the "hit". However, one plausible possibility may be that these proteins may be homologues of the human form, which have not yet been described in the mouse system. Further analysis of the peptides need to be performed to accurately assess the significance of this finding. Perhaps some of the other sequences, for which the proteins are known but the function remains unassigned, may participate in signal transduction. Others may potentially pursue continuation of this project and sequencing for other proteins.

# Appendix 3. Mass Spectra Analysis of Identified Peptides

Protein analysis was performed using the program Sequest from ThermoFinnigan which correlates experimental LC-MS/MS spectra of peptides to theoretical ones derived from protein and nucleotide databases. The following definitions explain the parameters used to evaluate the SEQUEST search results and to use as indicators of "solid hits".

**DelCn** is the Delta Correlation value. When viewing the summary page, DelCn shows how different the first hit is from the second hit in your search results. A general rule of thumb is that a DelCn of 0.1 or greater is good. If searching a large database (nr.fasta or owl.fasta) you may find that your DelCn values are smaller than those obtained by searching a small database. This is due to the fact that the chance of sequence similarity is higher in the larger databases.

**Sp** is the preliminary score. SEQUEST performs two types of scoring. After finding all peptides that match within your mass tolerance, SEQUEST does a preliminary scoring of these candidates to weed the list down to 500 for the final correlation analysis. The scoring is based on the number of ions in the MS/MS spectrum that match with the experimental data. The higher the value of Sp the better. I like to see a value of at least 200. One thing to note is that larger peptides will have bigger Sp values. For good hits, a 20 residue peptide will usually have an Sp value of over 1000, while a 6 residue peptide will likely be below 500.

**RSp or Rank/Sp** shows the ranking of the particular match during the preliminary scoring (see Sp above). The preliminary scoring can be thought as "the compulsories". One should be wary of any search result that scores a #1 hit after scoring #450 in the compulsories. Ideally, the match should be ranked #1 in the preliminary scoring. But, it is not uncommon to see a peptide move up 10 or so notches from the preliminary ranking.

**Ions**. The Ions value shows how many of the experimental ions matched with the theoretical ions for the peptide listed. For example, 8/10 says that the MS/MS spectrum contained 8 of the 10 predicted ions for the peptide. It is rare to see 100% coverage of the predicted ions, but 70% or 80% coverage is good. You may drop off ions at either end of your spectrum due to scan range limitations or the low mass cutoff feature of the ion trap.

**XCorr**. The XCorr value is the cross-correlation value from the search. The #1 hit will always have the highest value of Xcorr, as Xcorr is used to produce the final ranking of the candidate peptides in the search. Usually, you'll see the top 10 or 12 ranked in your search results. XCorr values above 2.0 are usually indicative of a good correlation. However, as with Sp, XCorr values are usually higher for well-matched, large peptides, and lower for smaller peptides. It's not uncommon for a 20 residue peptide to have an Xcorr of 5, while a 6 residue peptide might be around 1.5.

References

http://www.enovatia.com/stories/storyReader$64
http://swehsc.pharmacy.arizona.edu/analysis/TurboSequest.pdf

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Start_scan=414; End_scan=1192; Precursor_mass_tol =1.40; Group_scan_tol =1; Min_group_count =2;
Min_ion_count =35; Low_mass_limit =300.00; High_mass_limit =4000.00; Use_charge_state =2; Subsequence =[];
peptide_tol=3.0000; Fragment_Ion_Tol=0.0000; B_ion=Yes; Y_ion=Yes;
Enzyme:Trypsin (2)

| # | File* | MassA* | | Xcorr* | DelCn* | Sp | RSp | Ions | Reference* | | MW* | Sequence** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | diasp6801.0424.0427.2 | 1316.7 | (+0.9) | 1.2464 | 0.295 | 168.9 | 1 | 8/ 24 | gi\|9663153\|emb\|CAC01132.1\| | | 58148 | R.GPAAAAAMEPRE |
| 2 | diasp6801.0430.0433.2 | 1115.6 | (+3.0) | 1.4162 | 0.159 | 263.8 | 1 | 10/ 18 | gi\|5296000\|dbj\|BAA81890.1\| | +5 | 86047 | R.LDAELQLSAR.L |
| 3 | diasp6801.0445.0448.2 | 906.5 | (-0.1) | 1.7294 | 0.201 | 152.0 | 20 | 9/ 14 | gi\|2134852\|pir\|\|I53413 | +2 | 30866 | K.QNKFGQGK.K |
| 4 | diasp6801.0451.0454.2 | 1228.7 | (+2.1) | 0.9512 | 0.070 | 248.2 | 2 | 9/ 18 | gi\|3122367\|sp\|Q61211\|LIGA_MOUSE | | 62181 | K.RNRLVDADNR.N |
| 5 | diasp6801.0466.0469.2 | 1065.5 | (-0.4) | 3.1955 | 0.389 | 673.4 | 1 | 13/ 16 | gi\|386854\|gb\|AAA36153.1\| | | 52738 | K.AQYEDIAQK.S |
| 6 | diasp6801.0484.0487.2 | 1041.6 | (-0.4) | 2.3856 | 0.392 | 237.9 | 4 | 11/ 16 | gi\|4557703\|ref\|NP_000414.1\| | +1 | 65807 | K.VDPEIQNVK.A |
| 7 | diasp6801.0491.0494.2 | 954.5 | (+2.1) | 0.9237 | 0.001 | 64.1 | 132 | 7/ 16 | gi\|3413906\|dbj\|BAA32317.1\| | +1 | 41304 | R.QLEAGHSGR.L |
| 8 | diasp6801.0497.0500.2 | 1107.5 | (-0.5) | 3.4897 | 0.367 | 1123.2 | 1 | 15/ 16 | gi\|34069\|emb\|CAA24760.1\| | +9 | 39176 | K.AQYEEIAQR.S |
| 9 | diasp6801.0512.0515.2 | 2076.1 | (-2.7) | 0.9939 | 0.103 | 43.1 | 35 | 6/ 36 | gi\|4885455\|ref\|NP_005466.1\| | +1 | 63167 | R.RAPGPDGPDRVL |
| 10 | diasp6801.0518.0521.2 | 1254.6 | (-0.2) | 2.9797 | 0.387 | 991.0 | 1 | 17/ 26 | gi\|4557703\|ref\|NP_000414.1\| | +1 | 65807 | R.GFSSGSAVVSGG |
| 11 | diasp6801.0536.0539.2 | 807.4 | (-0.3) | 2.5306 | 0.463 | 496.5 | 1 | 10/ 12 | gi\|71528\|pir\|\|KRHUQ | +47 | 59473 | R.LAADDFR.L |
| 12 | diasp6801.0554.0557.2 | 1033.5 | (-0.6) | 2.5232 | 0.355 | 710.1 | 1 | 14/ 16 | gi\|5174491\|ref\|NP_006112.1\| | +1 | 65959 | R.TLLEGEESR.M |
| 13 | diasp6801.0575.0578.2 | 1154.6 | (-1.3) | 1.6299 | 0.186 | 299.3 | 3 | 10/ 20 | gi\|2135121\|pir\|\|A49674 | +2 | 144510 | K.ACSAIHAVNLR. |
| 14 | diasp6801.0598.0601.2 | 861.4 | (+1.8) | 0.6306 | 0.298 | 50.5 | 3 | 4/ 14 | gi\|4097089\|gb\|AAD10340.1\| | | 8522 | R.RLGSQVSD.- |
| 15 | diasp6801.0604.0607.2 | 828.4 | (+0.7) | 0.5702 | 0.072 | 57.4 | 175 | 4/ 12 | gi\|9664859\|gb\|AAF97239.1\|AF282730_1 | | 25738 | K.LETNSHK.Q |
| 16 | diasp6801.0622.0625.2 | 1265.6 | (-0.3) | 3.4486 | 0.521* | 1020.5 | 1 | 15/ 20 | gi\|5174491\|ref\|NP_006112.1\| | +2 | 65959 | R.TNAENEFVTIK. |
| 17 | diasp6801.0637.0640.2 | 949.5 | (+1.8) | 0.4551 | 0.004 | 55.6 | 3 | 4/ 14 | gi\|34228\|emb\|CAA27173.1\| | +2 | 79330 | R.KLESTESR.S |
| 18 | diasp6801.0643.0646.2 | 919.5 | (-2.3) | 0.5620 | 0.004 | 71.3 | 317 | 5/ 14 | gi\|8923398\|ref\|NP_060286.1\| | | 43588 | K.GYEGKTHK.G |
| 19 | diasp6801.0649.0652.2 | 1475.8 | (-0.3) | 4.3650 | 0.446* | 2383.8 | 1 | 20/ 22 | gi\|4557703\|ref\|NP_000414.1\| | +2 | 65807 | R.FLEQQNQVLQTK |
| 20 | diasp6801.0655.0658.2 | 822.5 | (+0.8) | 1.5611 | 0.137* | 290.4 | 62 | 9/ 10 | gi\|7705809\|ref\|NP_057121.1\| | | 30410 | K.KDYKLR.A |
| 21 | diasp6801.0664.0667.2 | 1665.9 | (-0.4) | 1.5036 | 0.121 | 125.3 | 79 | 9/ 28 | gi\|4504201\|ref\|NP_001508.1\| | +1 | 52135 | K.RLMVVTPAGHSD |
| 22 | diasp6801.0674.0677.2 | 1038.6 | (+0.8) | 0.4610 | 0.006 | 43.3 | 17 | 4/ 16 | gi\|5689427\|dbj\|BAA82997.1\| | | 47981 | R.HQAAKRGDR.D |
| 23 | diasp6801.0680.0683.2 | 1002.5 | (+2.5) | 0.7977 | 0.076 | 213.4 | 5 | 7/ 14 | gi\|4503727\|ref\|NP_002004.1\| | +2 | 25143 | R.AWTVEQLR.S |
| * 24 | diasp6801.0686.0689.2 | 1273.7 | (-0.6) | 3.3522 | 0.342 | 1022.5 | 1 | 14/ 22 | gi\|6981486\|ref\|NP_037199.1\| | | 68243 | K.AVTSEIAVLQSR |
| 25 | diasp6801.0699.0702.2 | 2063.2 | (-1.2) | 1.2359 | 0.061 | 168.3 | 31 | 9/ 34 | gi\|7661950\|ref\|NP_055448.1\| | +1 | 133401 | K.SNLKEVLNAVKE |
| 26 | diasp6801.0711.0714.2 | 1127.6 | (-0.1) | 0.7375 | 0.021 | 43.1 | 27 | 4/ 18 | gi\|7959325\|dbj\|BAA96053.1\| | | 194557 | R.HQSLDAFPGR.R |
| 27 | diasp6801.0717.0720.2 | 1091.6 | (+1.3) | 1.0866 | 0.234 | 246.4 | 7 | 9/ 18 | gi\|3413850\|dbj\|BAA32289.1\| | +1 | 110547 | K.APPSPEQLPR.E |
| 28 | diasp6801.0726.0729.2 | 1191.6 | (-1.3) | 1.8194 | 0.135 | 226.0 | 36 | 9/ 20 | gi\|6679479\|ref\|NP_032961.1\| | +1 | 96143 | K.NTLDSISSNIK. |
| 29 | diasp6801.0744.0747.2 | 1216.7 | (-1.3) | 0.6755 | 0.016 | 45.0 | 22 | 6/ 18 | gi\|8923771\|ref\|NP_061023.1\| | | 38878 | K.GILSNSWKRR.Y |
| 30 | diasp6801.0750.0753.2 | 1183.7 | (-2.7) | 1.1020 | 0.017 | 247.4 | 7 | 8/ 16 | gi\|6678834\|ref\|NP_032595.1\| | | 215930 | R.FRHLKMPVR.A |
| 31 | diasp6801.0777.0780.2 | 1301.7 | (+1.7) | 0.5522 | 0.068 | 34.3 | 14 | 4/ 20 | gi\|2134714\|pir\|\|S62786 | +2 | 64175 | R.HPRRSRPSTAH. |
| 32 | diasp6801.0783.0786.2 | 1821.0 | (+0.1) | 1.1064 | 0.031 | 129.1 | 7 | 10/ 28 | gi\|6754724\|ref\|NP_034947.1\| | +2 | 36499 | R.ERIVGWYHTGPK |
| 33 | diasp6801.0795.0798.2 | 1174.7 | (-0.4) | 3.2628 | 0.434* | 800.3 | 1 | 15/ 18 | gi\|6755891\|ref\|NP_035775.1\| | | 26148 | K.TLNNDIMLLK.L |
| 34 | diasp6801.0801.0804.2 | 1130.6 | (-0.2) | 2.2879 | 0.187 | 599.0 | 1 | 14/ 18 | gi\|5174739\|ref\|NP_006078.1\| | +16 | 49580 | R.FPGQLNADLR.K |
| 35 | diasp6801.0807.0810.2 | 1389.7 | (+1.9) | 0.7321 | 0.166 | 26.3 | 98 | 4/ 22 | gi\|9663936\|dbj\|BAB03600.1\| | | 392057 | K.SWPNILFQASAR |
| 36 | diasp6801.0833.0836.2 | 1475.7 | (-0.2) | 4.1993 | 0.609 | 1911.9 | 1 | 19/ 22 | gi\|5174491\|ref\|NP_006112.1\| | +1 | 65959 | K.WELLQQVDTSTR |
| 37 | diasp6801.0839.0842.2 | 1601.8 | (-0.1) | 3.7014 | 0.390 | 762.2 | 1 | 19/ 28 | gi\|5174739\|ref\|NP_006078.1\| | +3 | 49580 | R.AVLVDLEPGTMD |
| 38 | diasp6801.0845.0848.2 | 815.5 | (-2.4) | 1.0511 | 0.032* | 104.9 | 251 | 6/ 12 | gi\|123678\|sp\|P07900\|HS90A_HUMAN | +4 | 84602 | R.ALLFVPR.R |
| 39 | diasp6801.0860.0863.2 | 1097.6 | (+2.4) | 1.1826 | 0.134 | 320.4 | 1 | 12/ 18 | gi\|729919\|sp\|P38656\|LA_RAT | | 47729 | K.WKSKGGHAAR.R |
| 40 | diasp6801.0870.0873.2 | 1524.8 | (+1.1) | 1.2884 | 0.068 | 176.0 | 3 | 9/ 22 | gi\|6754478\|ref\|NP_034787.1\| | | 246750 | K.EQNRGYQTPYLR |
| 41 | diasp6801.0879.0882.2 | 1518.8 | (-2.7) | 1.3084 | 0.059 | 181.0 | 1 | 11/ 26 | gi\|6552299\|ref\|NP_009229.1\| | +1 | 207573 | K.NSLNDCSNQVIL |
| + 42 | diasp6801.0891.0894.2 | 909.5 | (-1.0) | 2.3153 | 0.355 | 678.2 | 1 | 12/ 14 | gi\|4506675\|ref\|NP_002941.1\| | +1 | 68508 | K.IQHILDAL.- |
| 43 | diasp6801.0899.0902.2 | 2273.2 | (-0.2) | 1.6293 | 0.009 | 149.9 | 3 | 11/ 40 | gi\|4504183\|ref\|NP_000843.1\| | +3 | 34898 | K.LKAFLASPEYVN |
| 44 | diasp6801.0918.0922.2 | 837.5 | (+1.4) | 0.6982 | 0.012 | 198.9 | 7 | 5/ 10 | gi\|7512744\|pir\|\|T12460 | +1 | 38174 | K.FKWTKK.E |
| 45 | diasp6801.0926.0929.2 | 807.4 | (-0.7) | 0.4892 | 0.071 | 44.2 | 46 | 4/ 14 | gi\|4079804\|gb\|AAC98782.1\| | | 5392 | R.APFALSSD.- |

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 46 | diasp6801.0937.0940.2 | 1647.9 | (-0.4) | 3.5962 | 0.497 | 876.0 | 1 | 17/ 28 | gi\|6981486\|ref\|NP_037199.1\| | | 68243 | R.ASSFVLALEPEL |
| 47 | diasp6801.0956.0959.2 | 1594.8 | (-0.3) | 1.0612 | 0.129 | 243.3 | 2 | 10/ 26 | gi\|780367\|gb\|AAB05844.1\| | | 23196 | R.LCPEGLYGIKCD |
| 48 | diasp6801.0962.0965.2 | 828.4 | (+2.7) | 0.8116 | 0.140 | 48.5 | 86 | 5/ 10 | gi\|2136058\|pir\|\|I37560 | +5 | 67815 | R.RKMCYK.K |
| 49 | diasp6801.0974.0977.2 | 1302.7 | (-0.4) | 4.1984 | 0.473 | 1962.7 | 1 | 19/ 22 | gi\|5174491\|ref\|NP_006112.1\| | +3 | 65959 | R.SLDLDSIIAEVK |
| 50 | diasp6801.0998.1001.2 | 2256.1 | (-2.5) | 1.0137 | 0.135 | 79.6 | 29 | 9/ 42 | gi\|398574\|gb\|AAA33010.1\| | | 15672 | K.GLEWVSGTGGRG |
| 51 | diasp6801.1007.1010.2 | 1073.6 | (-2.6) | 0.4294 | 0.007 | 58.1 | 40 | 4/ 16 | gi\|2911243\|gb\|AAC12787.1\| | +1 | 93831 | K.EKLSPKDTR.D |
| 52 | diasp6801.1028.1031.2 | 1156.6 | (+2.5) | 0.4831 | 0.003 | 119.5 | 3 | 8/ 22 | gi\|6706620\|emb\|CAB66088.1\| | | 43340 | K.AQKSSGPVPSAK |
| 53 | diasp6801.1040.1043.2 | 813.5 | (-0.5) | 0.7775 | 0.052 | 274.1 | 1 | 8/ 12 | gi\|7513070\|pir\|\|T00354 | | 87381 | R.DPVLTLR.A |
| 54 | diasp6801.1046.1049.2 | 1244.6 | (+2.7) | 0.8189 | 0.270 | 93.9 | 24 | 5/ 18 | gi\|2353298\|gb\|AAC52043.1\| | | 2390 | -.YYCARENGLR.G |
| 55 | diasp6801.1052.1055.2 | 2872.4 | (+0.8) | 4.9149 | 0.546 | 644.5 | 1 | 22/ 52 | gi\|71528\|pir\|\|KRHU0 | +2 | 59473 | R.NVSTGDVNVEMN |
| 56 | diasp6801.1058.1061.2 | 1006.5 | (+2.2) | 1.0265 | 0.026 | 210.4 | 1 | 8/ 14 | gi\|5042223\|emb\|CAB44705.1\| | | 60019 | K.QDPYDKLK.F |
| 57 | diasp6801.1067.1070.2 | 1336.7 | (-1.5) | 0.8125 | 0.087 | 54.5 | 153 | 5/ 22 | gi\|6005709\|ref\|NP_009134.1\| | +1 | 121978 | K.NAPSLPSRTCYK |
| 58 | diasp6801.1145.1148.2 | 985.6 | (+1.1) | 0.7351 | 0.024 | 46.4 | 102 | 4/ 18 | gi\|5901980\|ref\|NP_008962.1\| | +1 | 37602 | K.AILGGDKALK.F |

1. gi|5174491|ref|NP_006112.1| 53.5 (5,0,0,0,0) ( 12 16 19 36 49 ) Keratin
2. gi|4557703|ref|NP_000414.1| 42.3 (4,0,0,0,0) ( 6 8 10 19 ) Keratin
3. gi|386854|gb|AAA36153.1| 40.7 (4,0,0,0,0) ( 5 12 16 36 ) Keratin
4. gi|6981486|ref|NP_037199.1| 31.6 (3,0,0,0,0) ( 24 42 46 ) Ribophorin
5. gi|71528|pir||KRHU0 21.8 (2,0,0,0,0) ( 11 55 ) Keratin
6. gi|5174739|ref|NP_006078.1| 21.3 (2,0,0,0,0) ( 34 37 )
7. gi|5174735|ref|NP_006079.1| 20.0 (2,0,0,0,0) ( 34 37 )
8. gi|6678643|ref|NP_032499.1| 20.0 (2,0,0,0,0) ( 16 19 )
9. gi|106849|pir||PC1102 20.0 (2,0,0,0,0) ( 11 55 )
10. gi|1857526|gb|AAB48456.1| 20.0 (2,0,0,0,0) ( 34 37 )
11. gi|1805274|gb|AAB41497.1| 20.0 (2,0,0,0,0) ( 34 37 )
12. gi|398168|emb|CAA52708.1| 20.0 (2,0,0,0,0) ( 6 10 )
13. gi|4557697|ref|NP_000412.1| 20.0 (2,0,0,0,0) ( 11 55 )
14. gi|5031839|ref|NP_005545.1| 18.0 (1,1,0,0,0) ( 8 : 19 )
15. gi|4506145|ref|NP_002760.1| 18.0 (1,1,0,0,0) ( 33 : 3 )

| File# | Sequence | MW | Function |
|---|---|---|---|
| 1 | transport-secretion protein 2.2 (TTS-2.2) [Homo sapiens] gi\|9663153\|emb\|CAC01132.1 \|[9663153] | 58.148 | TTS-2, a novel protein implicated in vesicular transport of the cell surface receptor ICAM-3 |
| 2 | a-helix coiled-coil rod homologue [Homo sapiens] gi\|5296000\|dbj\|BAA81890.1\|[ 5296000] | 86.047 | pathogenic gene for psoriasis vulgaris is located within a reduced interval of 111 kb spanning 89-200 kb telomeric of the HLA-C gene. In addition to three known genes, POU5F1, TCF19 and S, this 111 kb fragment contains four new, expressed genes identified in the course of our genomic sequencing of the entire HLA class I region. |
| 3 | calcium sensor protein - human (fragment) gi\|2134852\|pir\|\|I53413[21348 52] | 30.866 | The 500-kDa protein belongs to the LDL-receptor superfamily of glycoproteins, claimed to function primarily as protein receptors and characterized by functionally important calcium-binding capacity. It is proposed that the currently identified protein constitutes part of a common structure for the sensing of extracellular calcium concentrations and influences calcium homeostasis in different organs. |
| 4 | Ligatin gi\|13638293\|sp\|Q61211\|LIG A_MOUSE[13638293] | 62.181 | Trafficking receptor for phosphoglycoproteins. Localizes phosphoglycoproteins within the endosomes and at the cell periphery where they participate specific metabolic processes as well as intercellular adhesion (by similiarity). Subcellular location peripheral membrane |
| 5 | type II keratin subunit protein [Homo sapiens] gi\|386854\|gb\|AAA36153.1\|[3 86854] | 52.738 | keratin |
| 6 | keratin 2a [Homo sapiens] gi\|4557703\|ref\|NP_000414.1\| [4557703] | 65.807 | keratin |
| 7 | KIAA0472 protein [Homo sapiens] gi\|3413906\|dbj\|BAA32317.1\|[ 3413906] | 41.304 | unknown |
| 8 | keratin [Homo sapiens] gi\|34069\|emb\|CAA24760.1\|[3 4069] | 39.176 | keratin |

| | | | |
|---|---|---|---|
| 9 | lymphocyte adaptor protein [Homo sapiens] gi\|4885455\|ref\|NP_005466.1\| [4885455] | 63.167 | human Lnk, an adaptor protein with pleckstrin homology and Src homology 2 domains that can inhibit T cell activation |
| 10 | keratin 2a [Homo sapiens] gi\|4557703\|ref\|NP_000414.1\| [4557703] | 65.807 | keratin |
| 11 | AAH02641. keratin 15 [Homo ...[gi:12803613] | 59.473 | keratin |
| 12 | keratin 1 (epidermolytic hyperkeratosis); Keratin-1 [Homo sapiens]. ACCESSION NP_006112 | 65.959 | keratin |
| 13 | flightless-I homolog - human (fragment) gi\|2135121\|pir\|\|A49674[2135 121] | 144.51 0 | The Drosophila melanogaster flightless-I gene involved in gastrulation and muscle degeneration encodes gelsolin-like and leucine-rich repeat domains and is conserved in Caenorhabditis elegans and humans |
| 14 | Tcte2 protein [Mus musculus] gi\|4097089\|gb\|AAD10340.1\|[ 4097089] | 8.522 | Identification of a male meiosis-specific gene, Tcte2, which is differentially spliced in species that form sterile hybrids with laboratory mice and deleted in t chromosomes showing meiotic drive |
| 15 | tissue inhibitor of metalloproteinases TIMP4 [Mus musculus] gi\|9664859\|gb\|AAF97239.1\|A F282730_1[9664859] | 25.738 | murine tissue inhibitor of metalloproteinase (TIMP) family, designated Timp-4. The nucleotide sequence predicts a protein of 22,609 Da that contains the characteristic 12 cysteine TIMP signature. |
| 16 | keratin 1 (epidermolytic hyperkeratosis); Keratin-1 [Homo sapiens]. ACCESSION NP_006112 | 65.959 | keratin |
| 17 | put. lamin A precursor (aa 1-702) [Homo sapiens] gi\|34228\|emb\|CAA27173.1\|[3 4228] | 79.330 | The A, B and C lamins are the major proteins of the nuclear envelope. The complete nucleotide sequence of the coding region of the A and C lamins shows that these proteins are identical except for their carboxy termini. The most prominent structural feature of both lamins is an alpha-helical region of repeating heptads of amino acids that shows striking homology with the entire family of cytoplasmic intermediate filament proteins. These features suggest that the nuclear envelope is made up of a network of coiled-coil polymers. |

| | | | |
|---|---|---|---|
| 18 | hypothetical protein FLJ20425 [Homo sapiens] gi\|8923398\|ref\|NP_060286.1\| [8923398] | 43.588 | unknown |
| 19 | keratin 2a [Homo sapiens] gi\|4557703\|ref\|NP_000414.1\| [4557703] | 65.807 | keratin |
| 20 | CGI-94 protein [Homo sapiens] gi\|7705809\|ref\|NP_057121.1\| [7705809] | 30.410 | unknown |
| 21 | general transcription factor IIH, polypeptide 4 (52kD subunit); General transcription factor IIH, polypeptide 4 [Homo sapiens] gi\|4504201\|ref\|NP_001508.1\| [4504201] | 52.134 | Cloning and characterization of p52, the fifth subunit of the core of the transcription/DNA repair factor TFIIH |
| 22 | KIAA1045 protein [Homo sapiens] gi\|5689427\|dbj\|BAA82997.1\|[ 5689427] | 47.981 | unknown |
| 23 | FK506-binding protein 3; FK506-binding protein 3 (25kD); rapamycin-selective 25 kDa immunophilin; peptidyl-prolyl cis-trans isomerase; rotamase; rapamycin binding protein; FK506-binding protein 25, T-cell [Homo sapiens] gi\|4503727\|ref\|NP_002004.1\| [4503727] | 25.143 | The protein encoded by this gene is a member of the immunophilin protein family, which play a role in immunoregulation and basic cellular processes involving protein folding and trafficking. This encoded protein is a cis-trans prolyl isomerase that binds the immunosuppressants FK506 and rapamycin. It has a higher affinity for rapamycin than for FK506 and thus may be an important target molecule for immunosuppression by rapamycin. |
| 24 | ribophorin I [Rattus norvegicus] gi\|6981486\|ref\|NP_037199.1\| [6981486] | 68.243 | Isolation and characterization of cDNA clones for rat ribophorin I: complete coding sequence and in vitro synthesis and insertion of the encoded product into endoplasmic reticulum membranes |
| 25 | KIAA0155 gene product; likely ortholog of mouse TPR-containing, SH2-binding phosphoprotein [Homo sapiens] gi\|7661950\|ref\|NP_055448.1\| [7661950] | 133.401 | p150TSP, a conserved nuclear phosphoprotein that contains multiple tetratricopeptide repeats and binds specifically to SH2 domains |
| 26 | KIAA1529 protein [Homo sapiens] gi\|7959325\|dbj\|BAA96053.1\|[ 7959325] | 194.557 | cell signaling/communication, nucleic acid management, cell structure/motility, protein management and metabolism |
| 27 | KIAA0444 protein [Homo | 110.54 | unknown |

| | | | |
|---|---|---|---|
| | sapiens] gi\|3413850\|dbj\|BAA32289.1\|[ 3413850] | 7 | |
| 28 | prominin [Mus musculus] gi\|6679479\|ref\|NP_032961.1\| [6679479] | 96.143 | prominin is preferentially localized to plasma membrane protrusions such as filopodia, lamellipodia, and microspikes. These observations imply that prominin contains information to be targeted to, and/or retained in, plasma membrane protrusions rather than the planar cell surface. Moreover, our results show that the mechanisms underlying targeting of membrane proteins to microvilli of epithelial cells and to plasma membrane protrusions of non-epithelial cells are highly related<br>AC133 antigen is also expressed on subsets of CD34+ leukemias, suggesting that it may be an important early marker for HSCs, as well as the first described member of a new class of TM receptors |
| 29 | ELG protein [Homo sapiens] gi\|8923771\|ref\|NP_061023.1\| [8923771] | 38.878 | Transcriptional regulation of the alpha E integrin gene |
| 30 | meiotic check point regulator [Mus musculus] gi\|6678834\|ref\|NP_032595.1\| [6678834] | 215.93 0 | A novel murine gene encoding a 216-kDa protein is related to a mitotic checkpoint regulator previously identified in Aspergillus nidulans |
| 31 | aggrecan - human (fragment) gi\|2134714\|pir\|\|S62786[2134 714] | 64.175 | polyglycan |
| 32 | proteasome (prosome, macropain) 26S subunit, non-ATPase, 7; Moloney leukemia virus 34 [Mus musculus] gi\|6754724\|ref\|NP_034947.1\| [6754724] | 36.499 | Mov-34 gene, the transcription unit disrupted by the proviral integration. This cDNA is predicted to encode a novel 321-amino acid, 36-kDa protein of unknown function |
| 33 | trypsin 3 [Mus musculus] gi\|6755891\|ref\|NP_035775.1\| [6755891] | 26.148 | trypsin |
| 34 | tubulin, beta, 5 [Homo sapiens] gi\|5174739\|ref\|NP_006078.1\| [5174739] | 49.580 | microtubules |
| 35 | zinc-finger homeodomain protein 4 [Mus musculus] gi\|9663936\|dbj\|BAB03600.1\|[ 9663936] | 392.05 7 | mouse zfh-4 cDNA which is 12 kb long and capable of encoding a 3,550-amino acid protein containing four homeodomains and 22 zinc fingers including two pseudo zinc finger motifs. |
| 36 | keratin 1 (epidermolytic | 65.959 | keratin |

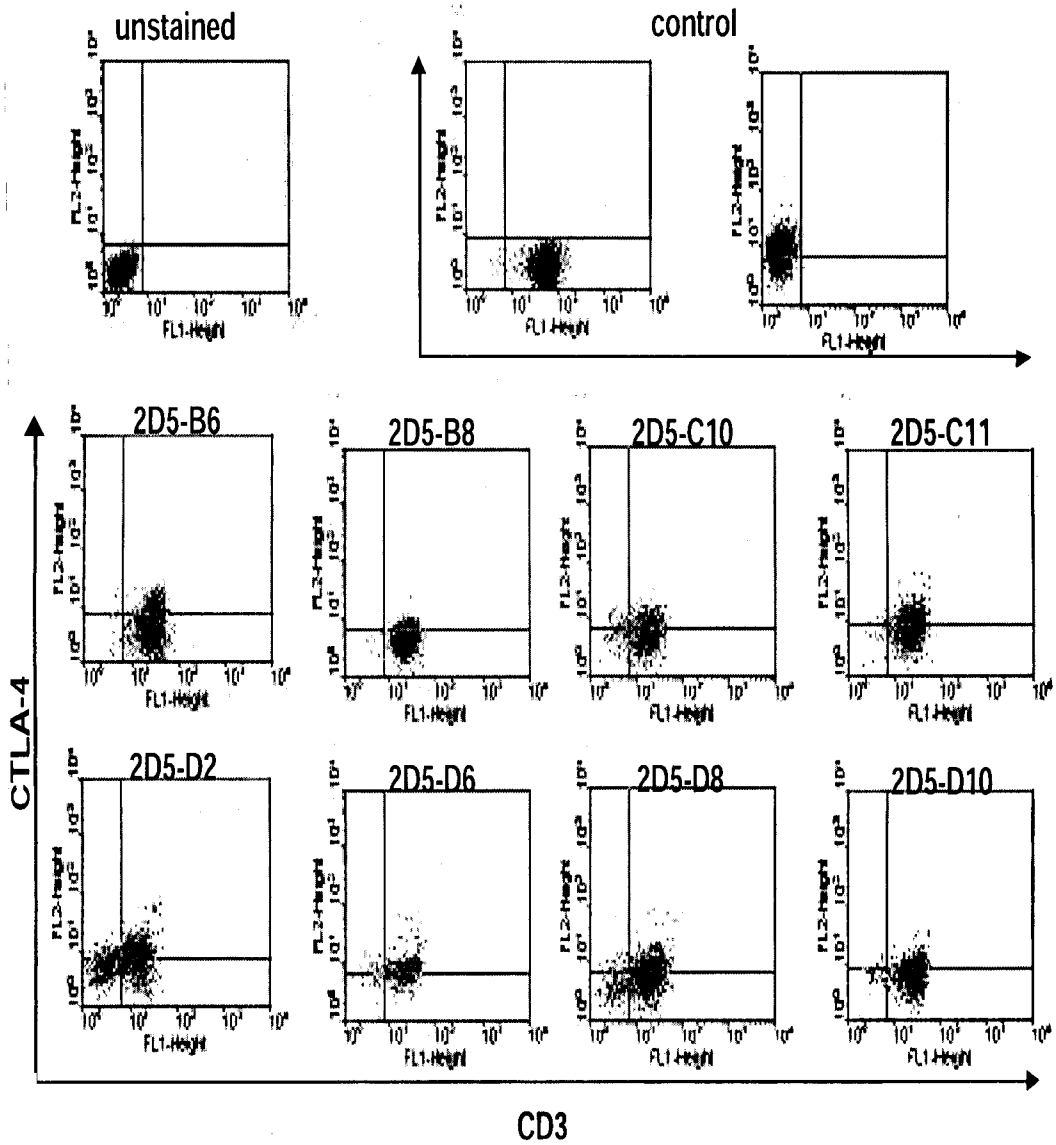
| | | | |
|---|---|---|---|
| | hyperkeratosis); Keratin-1 [Homo sapiens]. ACCESSION NP_006112 | | |
| 37 | tubulin, beta, 5 [Homo sapiens] gi\|5174739\|ref\|NP_006078.1\| [5174739] | 49.580 | microtubules |
| 38 | Heat shock protein HSP 90-alpha (HSP 86) gi\|123678\|sp\|P07900\|HS9A_HUMAN[123678] | 84.602 | Nucleotide sequence of a full-length cDNA for 90 kDa heat-shock protein from human peripheral blood lymphocytes |
| 39 | Lupus LA protein homolog (LA ribonucleoprotein) (LA autoantigen homolog) gi\|729919\|sp\|P38656\|LA_RAT[729919] | 47729 | LA protein plays a role in the transcription of RNA polymerase III. It is most probably a transcription termination factor. Binds to the 3' termini of virtually all nascent polymerase III transcripts. It is associated with precursor forms of RNA polymerase III trasncripts including tRNA and 4.5S, 5S, 7S, and 7-2 RNAs. subcellular location nuclear. |
| 40 | kappa B and Rss recognition component [Mus musculus] gi\|6754478\|ref\|NP_034787.1\| [6754478] | 246.750 | Sequence comparisons show that Rc belongs to a ZAS protein family that is involved in gene transcription and/or DNA recombination. The major histocompatibility complex class I gene enhancer binding proteins MBP1 and MBP2 are other representatives of this ZAS protein family. |
| 41 | breast cancer 1, early onset; breast-ovarian cancer, included [Homo sapiens] gi\|6552299\|ref\|NP_009225.1\| [6552299] | 207.573 | BRCA1, which functions as a tumor suppressor in human breast cancer cells, is a nuclear phosphoprotein which associates with RNA polymerase II holoenzyme. Mutations in BRCA1 are predicted to be responsible for approximately 45% of inherited breast cancer and more than 80% of inherited breast and ovarian cancer. BRCA1 may function as a transcriptional regulator, due to an amino terminal DNA-binding ring finger motif, nuclear localization signals, and an acidic carboxy terminal domain. BRCA1 is also a granin-like protein that functions as a secreted growth inhibitory protein. BRCA1 may normally serve as a negative regulator of mammary epithelial cell growth. |
| 42 | ribophorin I [Homo sapiens] gi\|4506675\|ref\|NP_002941.1\| [4506675] | 68.508 | Human ribophorins I and II: the primary structure and membrane topology of two highly conserved rough endoplasmic reticulum-specific glycoproteins |

| | | | |
|---|---|---|---|
| 43 | glutathione transferase; fatty acid ethyl ester synthase III; deafness, X-linked 7 [Homo sapiens] gi\|4504183\|ref\|NP_000843.1\| [4504183] | 34.898 | Glutathione S-transferases (GSTs) are a family of enzymes that play an important role in detoxification by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Based on their biochemical, immunologic, and structural properties, the soluble GSTs are categorized into 4 main classes: alpha, mu, pi, and theta. The glutathione S-transferase pi gene (GSTP1) is a polymorphic gene encoding active, functionally different GSTP1 variant proteins that are thought to function in xenobiotic metabolism and play a role in susceptibility to cancer, and other diseases |
| 44 | hypothetical protein DKFZp564G202.1 - human (fragment) gi\|7512744\|pir\|\|T12460[7512744] | 38.174 | unknown |
| 45 | glial derived neurotrophic factor [Homo sapiens] gi\|4079804\|gb\|AAC98782.1\|[4079804] | 5.392 | Glial cell line-derived neurotrophic factor (GDNF), a distant member of the TGF-beta superfamily, is a survival factor for various neurons, making it a potential therapeutic agent for neurodegenerative disorders. |
| 46 | ribophorin I [Rattus norvegicus] gi\|6981486\|ref\|NP_037199.1\| [6981486] | 68.243 | Isolation and characterization of cDNA clones for rat ribophorin I: complete coding sequence and in vitro synthesis and insertion of the encoded product into endoplasmic reticulum membranes |
| 47 | ORF [Rattus norvegicus] gi\|780367\|gb\|AAB05844.1\|[780367] | 23.196 | unknown |
| 48 | protein-tyrosine kinase (EC 2.7.1.112) ryk - human gi\|2136058\|pir\|\|I37560[2136058] | 67.815 | The human ryk cDNA sequence predicts a protein containing two putative transmembrane segments and a tyrosine kinase catalytic domain |
| 49 | ref\|NP_006112.1\|[5174491] | 65.959 | keratin 1 (epidermolytic hyperkeratosis); Keratin-1 [Homo sapiens] |
| 50 | immunoglobulin heavy chain [Homo sapiens] gi\|398574\|gb\|AAA53010.1\|[398574] | 15672 | Ig |
| 51 | dentin sialophosphoprotein precursor [Mus musculus] gi\|2911243\|gb\|AAC12787.1\|[2911243] | 93.831 | Two dentin proteins, dentin sialoprotein and dentin phosphoprotein, have been shown to be tooth-specific being expressed mostly by odontoblast cells. |

| | | | |
|---|---|---|---|
| 52 | Steerin-1 protein [Homo sapiens] gi\|6706620\|emb\|CAB66088.1 \|[6706620] | 43.340 | human Steerin-1 gene which is homologous to the C. elegans UNC53 gene |
| 53 | hypothetical protein KIAA0692 - human (fragment) gi\|7513070\|pir\|\|T00354[7513 070] | 87.381 | Prediction of the coding sequences of unidentified human genes. Unknown. |
| **54** | immunoglobulin heavy chain CDR3 [Homo sapiens] gi\|2353298\|gb\|AAC52043.1\|[ 2353298] | 2390 | Ig |
| 55 | P13645. Keratin, type I c...[gi:547749] | | Keratin, type I cytoskeletal 10 (Cytokeratin 10) (K10) (CK 10). |
| 56 | L1 protein [Homo sapiens] gi\|5042223\|emb\|CAB44705.1 \|[5042223] | 60.019 | Homo sapiens partial N-myc (exon 3), HPV45 L2, HPV45 L1, HPV45 E6, HPV45 E7 and HPV45 E1 genes isolated from IC4 cervical carcinoma cell line |
| **57** | A kinase (PRKA) anchor protein 2 [Homo sapiens] gi\|6005709\|ref\|NP_009134.1\| [6005709] | 121.97 8 | Prediction of the coding sequences of unidentified human genes; proteins relating to cell signaling/communication, cell structure/motility and nucleic acid management |
| 58 | heat shock transcription factor 2 binding protein; heat shock factor 2 binding protein [Homo sapiens] gi\|5901980\|ref\|NP_008962.1\| [5901980] | 37.602 | HSF2 binding protein (HSF2BP) associates with HSF2. The interaction occurs between the trimerization domain of HSF2 and the amino terminal hydrophilic region of HSF2BP that comprises two leucine zipper motifs. HSF2BP may therefore be involved in modulating HSF2 activation. |

## Appendix 4. Characterisation of T8.1 CTLA-4WT

| Clone# | Cell type | CTLA-4 Expression level |
|---|---|---|
| 2D5-B6 | T8.1- CTLA-4 wild type | Low |
| 2D5-B8 | T8.1- CTLA-4 wild type | Low |
| 2D5-C10 | T8.1- CTLA-4 wild type | Medium |
| 2D5-C11 | T8.1- CTLA-4 wild type | Medium |
| 2D5-D2 | T8.1- CTLA-4 wild type | Medium |
| 2D5-D6 | T8.1- CTLA-4 wild type | Medium |
| 2D5-D8 | T8.1- CTLA-4 wild type | Low |
| 2D5-D10 | T8.1- CTLA-4 wild type | Low |
| 2D5-E3 | T8.1- CTLA-4 wild type | Low |
| 2D5-F2 | T8.1- CTLA-4 wild type | Medium |
| 2D5-F3 | T8.1- CTLA-4 wild type | High |
| 2D6-E3 | T8.1- CTLA-4 wild type | Medium |
| 2-D6-E6 | T8.1- CTLA-4 wild type | Medium |
| 2D6-F4 | T8.1- CTLA-4 wild type | Low |
| 2B4-D7 | T8.1- CTLA-4 wild type | Very low |
| 2B4-D8 | T8.1- CTLA-4 wild type | Medium |
| 2B4-G3 | T8.1- CTLA-4 wild type | Very high |
| 2D2-B7 | T8.1- CTLA-4 wild type | Very low |
| 2D2-C4 | T8.1- CTLA-4 wild type | Low |
| 2D2-D6 | T8.1- CTLA-4 wild type | Very low |
| 2D2-C6 | T8.1- CTLA-4 wild type | Low |
| 2D2-D11 | T8.1- CTLA-4 wild type | High |
| 2D2-E3 | T8.1- CTLA-4 wild type | Medium |
| 2D2-E7 | T8.1- CTLA-4 wild type | Medium |
| 2D2-F6 | T8.1- CTLA-4 wild type | Low |
| 2D2-F10 | T8.1- CTLA-4 wild type | Medium |
| 2D5-G7 | T8.1- CTLA-4 wild type | Medium |
| 2D5-G10 | T8.1- CTLA-4 wild type | High |
| 2B1-B3 | T8.1- CTLA-4 wild type | Very low |
| 2B4-F10 | T8.1- CTLA-4 wild type | Very low |
| 2B4-F3 | T8.1- CTLA-4 wild type | Very low |
| 2D2-E2 | T8.1- CTLA-4 wild type | Very low |
| 2F1-C3 | T8.1- vector pBABE | none |
| 2F1-D10 | T8.1- vector pBABE | None |
| 2F3-C4 | T8.1- vector pBABE | None |
| 2F3-E10 | T8.1- vector pBABE | None |
| 2F5-F5 | T8.1- vector pBABE | None |
| 2F5-E8 | T8.1- vector pBABE | None |
| 2F2-B7 | T8.1- vector pBABE | None |
| 2F2-F11 | T8.1- vector pBABE | None |
| 2F4-F4 | T8.1- vector pBABE | None |
| 2F4-B10 | T8.1- vector pBABE | None |
| 2F6-H4 | T8.1- vector pBABE | None |
| 2F6-C10 | T8.1- vector pBABE | None |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2D5-B8
2D5-C10
2D5-C11
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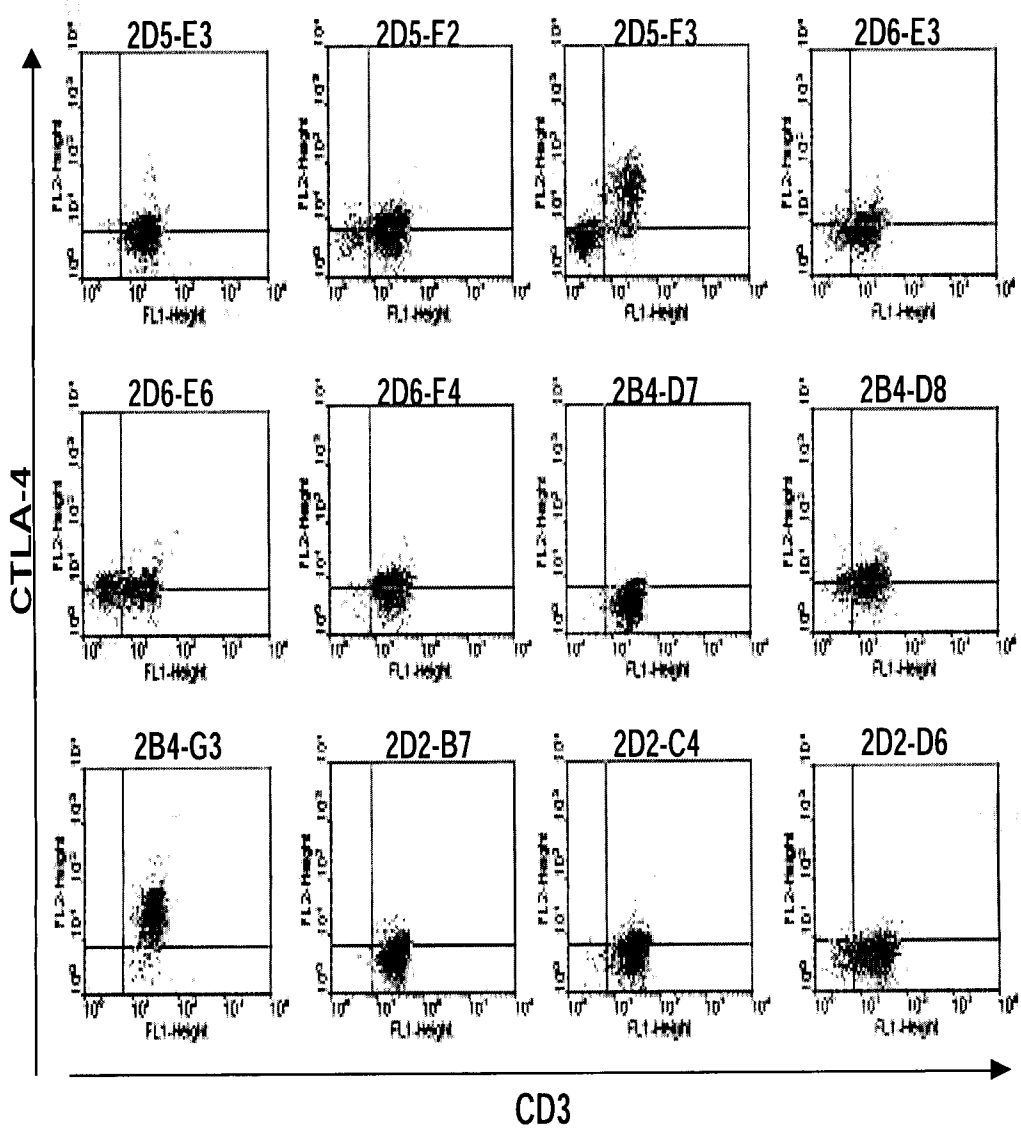
2D5-D6
2D5-D8
2D5-D10
CTLA-4
CD3
FL1-Height
FL2-Height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2D5-F2
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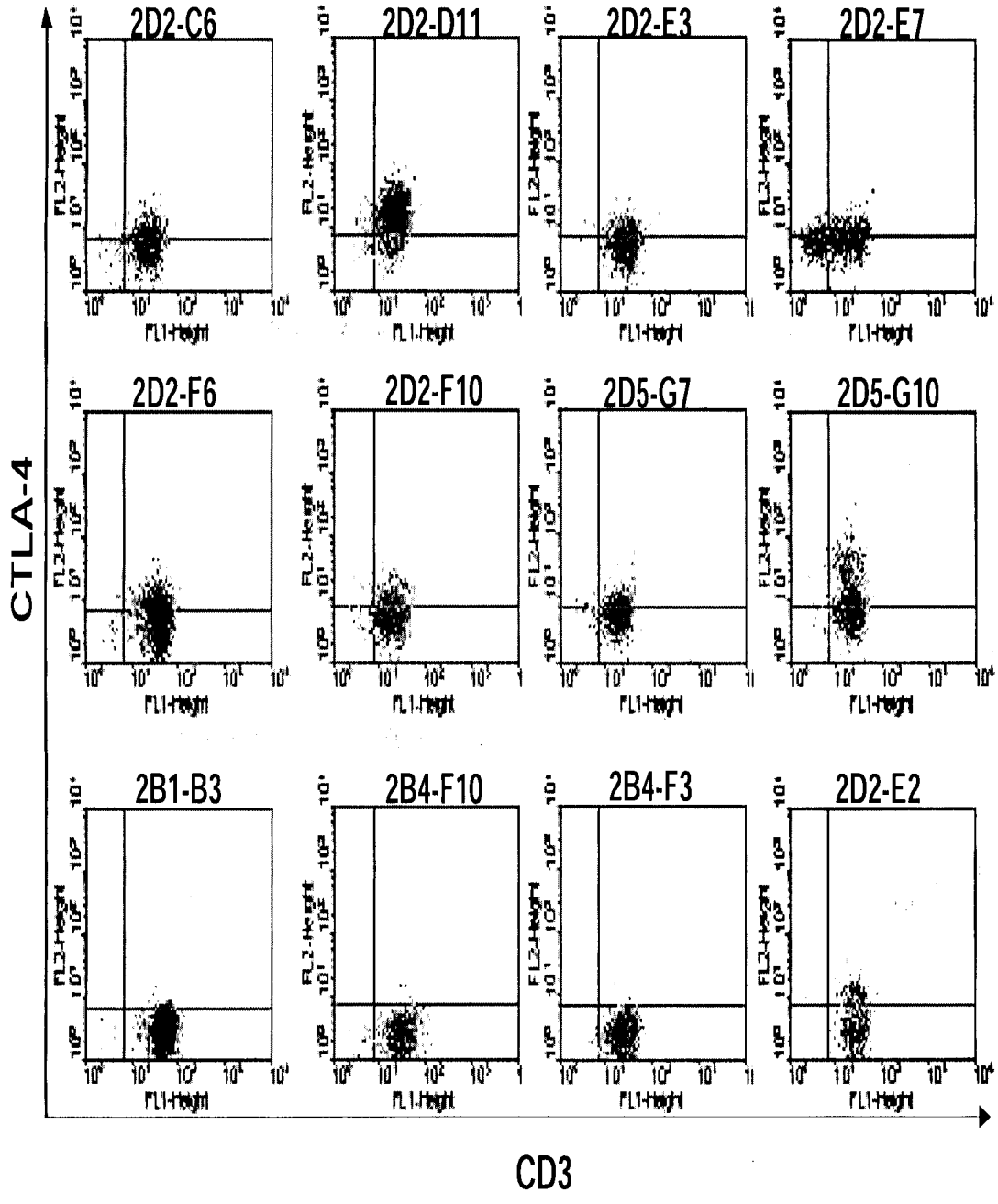
2D2-C4
2D2-D6
CTLA-4
CD3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2D2-D11
2D2-E3
2D2-E7
2D2-F6
2D2-F10
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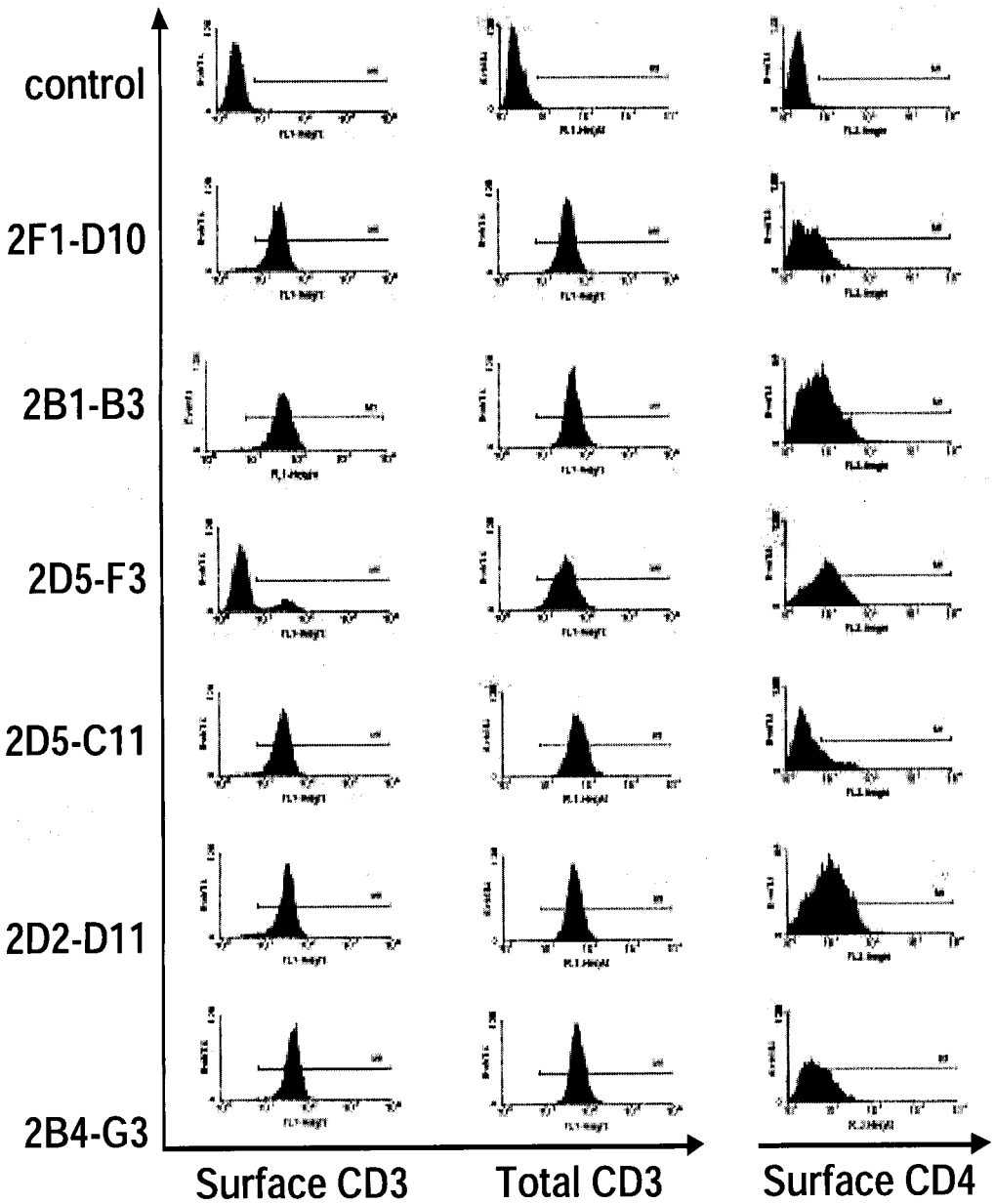
2D2-E2
CTLA-4
CD3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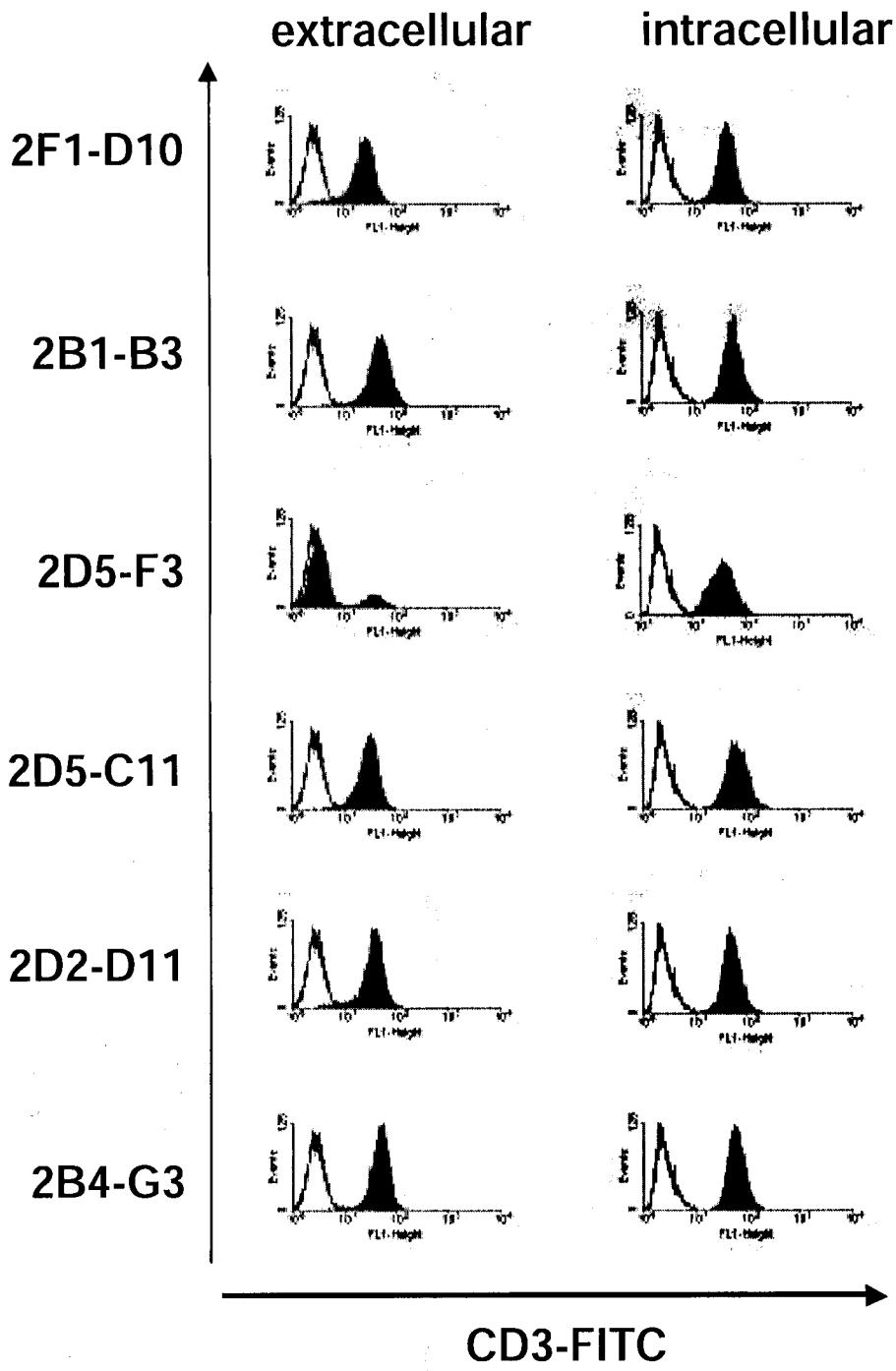
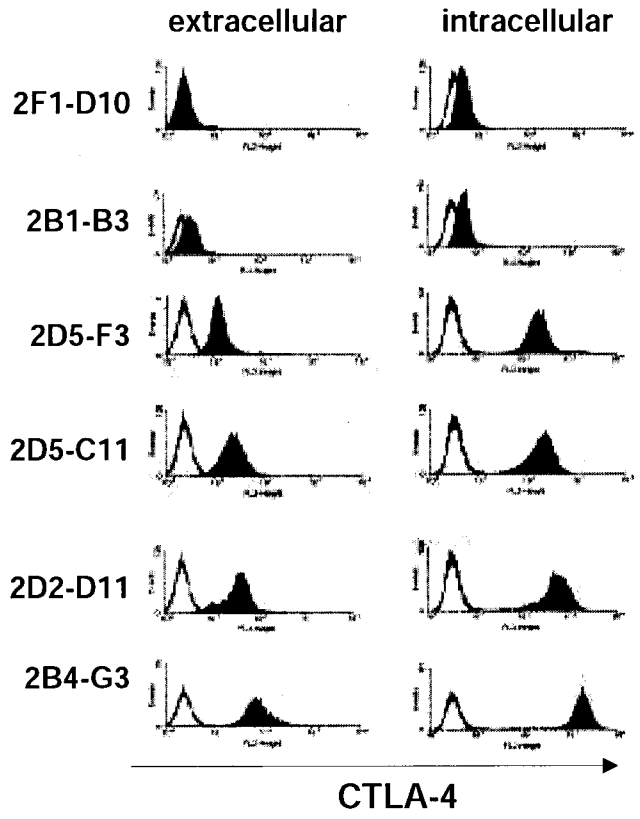
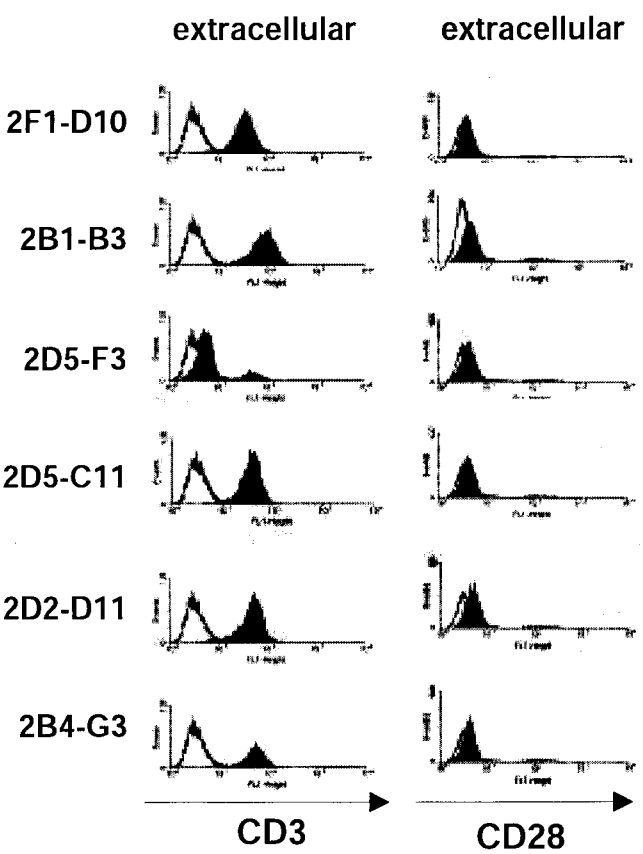
2D5-F3
2D5-C11
2D2-D11
2B4-G3
Surface CD3
Total CD3
Surface CD4


extracellular
intracellular
2F1-D10
2B1-B3
2D5-F3
2D5-C11
2D2-D11
2B4-G3
CD3-FITC


extracellular
intracellular
2F1-D10
2B1-B3
2D5-F3
2D5-C11
2D2-D11
2B4-G3
CTLA-4


extracellular
extracellular
2F1-D10
2B1-B3
2D5-F3
2D5-C11
2D2-D11
2B4-G3
CD3
CD28

# *CURRICULUM VITAE*

**Name:** Silvy da Rocha Dias
**Place and Date of birth:** Montreal, 1971/08/09
**Nationality:** Portuguese
**Home address:** Entre Ambos-os-Rios, S. Miguel, Lugar Igreja, 4980 Ponte da Barca, Portugal
**Institutional address:** Instituto de Ciências Biomédicas de Abel Salazar
Largo Prof. Abel Salazar, 2
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**Academic degrees:**

University of Oporto/Portugal **PhD** **12/96-present**
Dana Farber Cancer Institute, Boston,USA
Imperial College of ScienceTechnology and Medicine,London, UK
Thesis: "The mechanism of CTLA-4 mediated negative signaling in T cells"
Supervisor: Prof. Christopher E. Rudd
Co-supervisor: Prof. Maria de Sousa

University of Oporto/Portugal **MSc** **09/94-09/96**
Netherlands Cancer Institute, Amsterdam, Netherlands
Thesis: "The role of CD45 in the regulation of the RAG1 and pTα mRNA"
Supervisor: Dr. Hergen Spits
Co-supervisor: Prof. Maria de Sousa

McGill University/Montreal, Canada **BSc** **09/90-06/93**
Major: Microbiology and Immunology
Advisor: Dr. Michael Julius

**Fields of study:**

-Investigation of CTLA-4 negative signalling in T cells: examining cellular and molecular aspects which defined inhibitory signaling by CTLA-4. I have an interest in studying specifically the relationship between TcR and CTLA-4 signaling in the context of induction of apoptosis, production of cytokines, changes in cell morphology and delineation of potential intracellular pathway utilized.

-Interest in the role of CD45 in the downregulation of the recombinase activation gene RAG1 and pTα during positive selection of thymocytes. In addition, analysing the role of dominant negative tyrosine kinase ZAP-70 during the process of positive selection of thymocytes using Jurkat cells as a model: establishing an *in vitro* model to study retroviral mediated transduction of stem cells.

**Previous Positions:**

Research Assistant 09/2000-present
Hammersmith Hospital/Imperial College of Science, Technology and Medicine

Associate Research in the Department of Pathology 12/96-06/2002
Dana Farber Cancer Institute/Harvard Medical School

Graduate research assistant 09/95-09/96
Netherlands Cancer Institute

**Awards**

1994-1996 fellowship from the Junta Nacional de Investigação para a Ciência e Tecnologia
1996-2000 fellowship from the Fundação para a Ciência e Tecnologia

**Publications**

1. da Rocha Dias S, Rudd CE.CTLA-4 blockade of antigen induced cell death. Blood 2001 Feb 15;97(4):1134-7
2. Schneider H, da Rocha Dias S, Hu H, Rudd CE.* A regulatory role for cytoplasmic YVKM motif in CTLA-4 inhibition of TCR signaling. Eur J Immunol. 2001 Jul;31(7):2042-50. (*first two authors contributed equally)
3. da Rocha Dias S, Rudd CE. Mechanism for CTLA-4 regulation of negative signals. *Manuscript in preparation 2002*

**Poster Presentations**

1. da Rocha Dias S, Schneider H, Hu H, Rudd CE. **CTLA-4 negatively regulates apoptotic signals generated from the TCR.** Presented at the Federation of American Societies of Experimental Medicine meeting, Washington, DC, April 17-21, 1999.
2. da Rocha Dias S, Schneider H, Hu H, Rudd CE. **The role of cytoplasmic YVKM motif in CTLA-4 inhibition of TCR signaling.** Presented at the American Society of Hematology meeting, Orlando Florida, December 7-11 2001.

**Scientific Meetings**

Sociedade Portuguesa de Immunologia, Lisboa 1995
ENII Conference, European Immunology Meeting, Les Embiez, France 1995
Immunology Symposium, Bergen aan Zee, The Netherlands, 1996
Annual Retreat, Department of Pathology, Havard Medical School, North Falmouth, MA, 1998
Annual Retreat, Department of Pathology, Havard Medical School, North Falmouth, MA, 1999
Cell signaling meeting, Woods Hole, MA, 1999