Dissecting the molecular mechanisms regulating proliferation, antigen responsiveness, and differentiation of CD4 T lymphocytes : a central role for the mammalian target of Rapamycin (mTor)

Thesis

Sara Colombetti

Dissecting the molecular mechanisms regulating proliferation, antigen responsiveness, and differentiation of $CD4^+$ T lymphocytes: a central role for the mammalian target of Rapamycin (mTor)

Thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy in Molecular and Cellular Biology

April 2004

Sponsoring establishment:
Open University, London

Director of Study
Dr. Anna Mondino

External Supervisor
Prof. Jonathon Pines

Collaborating establishment:
DIBIT, Department of Biological and Technological Research
San Raffaele Scientific Institute
Milan, Italy

DATE OF SUBMISSION 30 MARCH 2004
DATE OF AWARD 16 SEPTEMBER 20

To Giacomo and my family,
always by my side

Acknowledgements

To acknowledge all the people who helped me and were by my side during the past years, both inside and outside the lab, I would probably need a whole book. So, if I will forget to mention someone, I am very sorry, but I will not do it intentionally.

First of all, I want to thank Dr. Anna Mondino, the best supervisor anyone could ever wish for, and without whom this thesis would never have been written. Thanks to her vast scientific knowledge, Anna introduced me to the secrets of the huge and fascinating world of Immunology. She has always been a constant and careful guide, an essential help for all my work. I don't remember a single day when I needed her advice or help, and she couldn't find the time to listen to me, and discuss with me. I really thank Anna for her patience and for never giving up when things didn't go our way, but always helping to figure out a new strategy to go on. Anna has passed me her enthusiasm, the curiosity, the trembling for a new result. She taught me to work with honesty and devotion. Thanks to Anna, maybe I will be able to continue this job, which I love, in an autonomous and valuable manner.

I would like to thank Prof. Doreen Cantrell, and Prof. Ruggero Pardi for critically reading the manuscript and acting as co-examiners in the jury. I acknowledge especially Prof. Pardi for the great scientific discussions, and for having always appreciated my work.

Together with Anna, I want to thank the fantastic "Mondino's", who have been more then good colleagues for me. They have been real friends, my companions during one of the most important and also difficult periods of my life. I think that the experience we all lived in that group was really unique. So, I thank especially Annina, Valérie, Stefano, and also Daniela, Laura and Fabio.
But my biggest thanks goes to Veronica, who shared with me the life in the lab and, most importantly, outside the lab. Veronica, my best friend, helped me with her strength and determination to overcome the hardest moments of

discouragement, which are unavoidable in the research work. With her continuous help, she made my work much easier, and with her great abilities, also more prolific. With her friendship, she supported me every single day and she always made me feel at home. I thank her for being always by my side.

I want to thank also Prof. Francesco Blasi, who followed me and advised me during all my Ph.D., and for his "nasty", but always stimulating questions.

A very big thank to all the CIGTP, for having sustained me all these years. In particular, thanks to my tutors, Dr. Pia Protti and Dr. Paolo Dellabona.
Among my colleagues, and more importantly my friends, thanks to Calzino and BJ, for the precious help when I had troubles in molecular biology, thanks to Sarina, Sabry, the "Bellone's", Dany, Raffa, Fausto, Annalisa, Zulma, Erika.

Thanks to the "Blasi's", a big family, of which I have always felt part. Thanks especially to Dr. Daniela Talarico who has always been ready to help me and advise me since my very first day at DIBIT. Thanks also to Resnik, Davide, Luis, Roby.
A very special thank to Elena, who shared with me the job and day life. Thanks for being part, together with Veronica and me, of the fabulous "Trio". And I don't forget a special thank for her wonderful cooking, that sustained me in the hardest hours.

Thanks to my Ph.D. colleagues, with whom I lived the fantastic retreats.

Thanks to Alessandra and Roberto, for the help with the quantitative PCR, thanks to Giovanni, for the time at the Phosphor Imager, and thanks to Antonio and Massimo for the never-ending analysis of the protein spots.

I thank all my friends, who were close to me during all these years, for having beard my chronic delays, and for having always waited for me, when I had to do the last incubation.

Thanks to Teresa, for the affection she has always demonstrated to me.
Thanks to Massimo, who shared with me the movie passion, thanks to Andrea, Gian, Max, and to the "Cobra".
A special thank to Arianna, my oldest friend, who has always been there.

I thank from the deepest of my heart my family, which is my root, my origin Thanks for their unfailing encouragement and support, and most importantly, thanks for giving me all the love that I need, and that has always been our big strength. Thanks to mamma, babbo, nonna, Ny and to the wonderful Viola e Davidino, who love me, and which I love so much. Grazie!!

My most special thank goes to Giacomo, my love, the companion of all my days, the support, the help, and most importantly, my most precious joy. He has always believed in me, giving me the strength to carry on. With his love and patience, he has been by my side in all the moments of our life together, so that I never feel alone. Without him, everything would have been much harder, and surely, not so beautiful.

Index

Declaration

This thesis has been composed by myself and has not been used in any previous application for a degree.
The data reported in the thesis were generated by myself with the exception of the 2-D electrophoresis analyses, and the RNase Protection assays, which were respectively performed in collaboration with Dr. M. Alessio and Dr. G. Sitia at DIBIT, San Raffaele Scientific Institute (Milan).
The results that I have presented and discussed in this thesis are contained in the following articles:

1) Colombetti S., F. Benigni, V. Basso and A. Mondino. "Clonal anergy is maintained independently of T cell proliferation". *J Immunol.*, 2002 Dec 1; 169(11):6178-86.

2) Colombetti S., V.Basso, G. Sitia and A. Mondino. "Rapamycin prevents differentiation, but not proliferation of T lymphocytes". *Manuscript in preparation.*

3) Colombetti S., V. Basso, S. Caserta, M. Alessio, D. Mueller and A. Mondino. "$p70^{S6k}$ and anergy? CD3/CD28 stimulation fails to activate $p70^{S6k}$ in anergic T cells. *Manuscript in preparation.*

4) Colombetti S. and A. Mondino. "IL-2 mediates a Rapamycin-resistant, PI3K-dependent T cell proliferation". *Manuscript in preparation.*

Summary

A proper control of T cell activation, proliferation and differentiation, and of antigen responsiveness is central to the development of protective immunity. Activation of $CD4^+$ T cells requires that the T Cell Receptor (TCR) and a costimulatory receptor, such as CD28, are simultaneously engaged. This initiates a number of intracellular events that lead to cell cycle entry and to the production of the autocrine growth factor IL-2. Following IL-2/IL-2R interaction, the cells proliferate and acquire effector capabilities. Several studies have indicated that in the absence of costimulation, and/or cytokine-initiated signals, T cells fail to proliferate and to differentiate. In particular, the engagement of the T cell receptor in the absence of proliferation has been shown to mediate the induction of clonal anergy, which has been proposed as one of the mechanisms by which peripheral tolerance to self-antigens is maintained.

Following antigen re-encounter anergic T cells fail to transcribe the IL-2 gene, and to proliferate and are specifically arrested in the G1 phase of the cell cycle. It was suggested that the failure of these cells to respond to optimal stimulation could be attributed to the presence of a putative anergic factor, uniquely expressed by the engagement of the TCR in the absence of proliferation. This model predicted that forcing proliferation, or G1 to S transition of anergic T cells would lead to dilution/degradation of the putative anergic factor, and by that restore the ability of the cells to properly respond to antigenic re-challenge. In the hope to restore antigen responsiveness, proliferation of anergic cells was forced by anti-CD3 and anti-CD28 mAbs. In contrast to antigenic stimulation, which failed to

bypass the G1 cell cycle arrest, CD3/CD28 mAbs-mediated activation of anergic T cells resulted in T cell proliferation. Indeed, optimal engagement of CD3 and CD28 elicited up-regulation of cyclin D3, of $p21^{Cip}$, hyperphosphorylation of Rb, downregulation of the cell cycle inhibitor $p27^{Kip}$ and cell proliferation. However by following the fate of individual T cells, we showed that, in spite of several rounds of cell division, clonal anergy was maintained. In contrast, anergic T cells allowed to proliferate in response to exogenous IL-2 re-acquired the ability to further proliferate upon antigenic rechallenge. Interestingly, while Rapamycin, an inhibitor of the Serine/Threonine kinase mTor (mammalian target of Rapamycin), only partially blocked IL-2-mediated proliferation, it completely abolished the ability of IL-2 to restore antigen responsiveness. This indicated that clonal anergy could be maintained in spite of both G1-S transition and cell proliferation, and thus that antigen responsiveness and cell cycle progression are independently regulated, and revealed a critical role for mTor. Indeed, mTor proved not only to be involved in the cell growth response, but also to be critically needed for proper regulation of antigen responsiveness.

While both CD3/CD28 and IL-2/IL-2R activate mTor-dependent events (namely, phosphorylation of the $p70^{S6k}$) in normal T cells, only IL-2 was capable of the Rapamycin-sensitive anergy reversal. Most likely the failure of CD3/CD28 to restore antigen responsiveness could be attributed to the inability of this stimulation to properly activate mTor-dependent pathways in anergic cells. Indeed, while CD3/CD28 and IL-2-driven T cell stimulation of control cells elicited Rapamycin-sensitive $p70^{S6k}$ phosphorylation in several residues, only IL-2, but not CD3/CD28 stimulation, induced optimal activation of this mTor substrate in anergic cells.

These findings indicated that antigen responsiveness is uniquely regulated by an IL-2/IL-2R-induced signalling event, which is delivered independently of IL-2-driven cell proliferation, and relies on intact mTor signalling.

To further investigate the role of mTor, we exploited a model of *in vitro* antigen driven T cell differentiation, and analyzed the effect of mTor blockade by Rapamycin. In our model, antigen driven $CD4^+$ T cell activation resulted in both T cell proliferation and differentiation of type 1 (IFN-γ-producing), or type 2 (IL-4-producing) effector Th cells. The addition of Rapamycin at the time of T cell activation allowed comparable T cell expansion, but completely prevented polarization of effector cells. Indeed, T cells activated in the presence of Rapamycin produced IL-2 and proliferated as effector cells upon re-stimulation, but failed to produce either IFN-γ or IL-4. Moreover, Rapamycin completely impaired differentiation of antigen-specific $CD8^+$ T cells towards IFN-γ-producing cytotoxic T cells, without preventing their expansion.

Together our results indicate that the intracellular events that dictate T cell proliferation are distinct from the intracellular signals that modulate the functional phenotype of activated T lymphocytes and suggest that, while mTor-dependent signalling is dispensable for T cell proliferation, it is primarily involved in the acquisition of proper T cell effector functions, and in the maintenance of functional responsiveness.

Abstract

The intracellular events that regulate lymphocyte proliferation upon antigen encounter and the ability of the cells to respond to subsequent stimulation and to differentiate into effector cells remain largely to be understood. Several studies have linked T cell proliferation with the maintenance of antigen responsiveness and with the ability of the cells to differentiate and to acquire proper effector functions.

The aim of my Ph.D. research project was to investigate the role of the TCR, CD28, and IL-2 generated intracellular events dictating $CD4^+$ T lymphocyte proliferation and differentiation.

In a model of CD3-induced clonal anergy, we have shown that antigen responsiveness was uniquely regulated by an IL-2/IL-2R-induced signalling event, which was delivered independently of IL-2-driven cell proliferation, and which was Rapamycin-sensitive. This indicates that proliferation and antigen responsiveness are independently regulated and that the latter specifically requires intact signalling through mTor, the mammalian target of Rapamycin.

Moreover, we have shown that proper activation of $p70^{S6k}$, one the known target of mTor, might play a crucial role in the maintenance of T lymphocyte responsiveness.

We have also investigated the role of mTor in a model of *in vitro* antigen driven naïve T cell differentiation. In this model, blocking mTor activity by the addition of Rapamycin during T cell activation, allowed comparable T cell expansion, but completely prevented polarization of effector cells.

Together our results indicate that the intracellular events that dictate T cell proliferation are distinct from the intracellular signals that modulate the functional

phenotype of activated T lymphocytes and suggest that, while mTor-dependent signalling is dispensable for T cell proliferation, it is primarily involved in the acquisition of proper T cell effector functions.

1. Introduction

1.1 The development of an immune response: general features

The immune system is a multiparameter and multicellular complex system whose physiologic function is to protect the organism against foreign (non-self) aggressions, such as bacteria, viruses, parasites, while not reacting harmfully to individual's own (self) structures. The balancing between effective immunity and tolerance is a crucial feature of the immune system, and a number of regulatory mechanisms are in place to maintain this equilibrium.

Upon encounter with an infectious agent or a foreign body, several coordinated events occur with the final aim of eliminating the pathogen. Schematically, one can distinguish between the early reaction of the so-called innate immunity, which represents the first line of defense of the organism, and the later response of the adaptive immunity. These two types of responses are very different in term of cellular components, anatomical organization, specificity and timing of development, but are strictly coordinated, regulating each other at different levels. Indeed, the innate immune response to pathogens stimulates the adaptive immune response and influences its nature, while, in turn, the adaptive immune response uses many of the effector mechanisms of the innate immunity to eliminate pathogens, and often amplifies its response.

The principal components of innate immunity are: 1) the physical and chemical barriers, such as epithelia and antimicrobial substances produced at the epithelial

surfaces, 2) phagocytic cells (neutrophils, macrophages), and natural killer (NK) cells, 3) blood proteins, including members of the complement system, and other mediators of inflammation, such as cytokines and chemokines that mediate and coordinate many cellular functions.

The innate immune response is rapid, does not distinguish fine differences between foreign substances, and reacts in essentially the same way to most infectious agents and to repeated infections.

In contrast, the adaptive immune response represents a more evolved defense mechanism characterized by high specificity and the ability to develop a memory of the antigenic encounter, which ensures that the system can perform a more rapid and effective response at subsequent challenges with the same antigen. The major players of the adaptive immune response are T and B lymphocytes, regulating cell-mediated and humoral immunity, respectively. These cells express unique surface receptors able to recognize a given antigen (Ag) (the T cell receptor, TCR, for T cells, and the B cell receptor, BCR, for B cells), which confer the cells a single specificity.

Lymphocytes originate in the bone marrow from hematopoietic stem cells precursors. During maturation, a tightly regulated program of sequential gene expression leads to changes in the phenotype of the developing cells, to the generation of a diverse repertoire, to the acquisition of functional competence, and to selection events, which ensure that most of the lymphocytes that enter peripheral tissues are useful, in that they respond to foreign antigens, but not to many self antigens. The lymphocyte repertoire is extremely diverse, indeed it is estimated that it can discriminate 10^9 to 10^{11} distinct antigenic determinants. The frequency of

lymphocytes specific for any given antigen is extremely low (≅ 1/100,000), and for this reason the immune system has evolved to allow rapid clonal expansion of antigen-specific effector cells (see below). Once matured, lymphocytes leave the primary lymphoid organs and recirculate between blood and lymph with intermediate stops in secondary lymphoid tissues, such as the lymph nodes and the spleen, where the initial encounter with the specific foreign antigen occurs and where the adaptive immune response initially develops.

At the time of infection, the breaking of natural physical barrier, such as skin and mucosal surfaces, by pathogen entry represents the first danger signal for the immune system. Indeed, this event elicits the activation of cells of the innate immune response, such as neutrophils and macrophages, and of dendritic cells (DCs). This event leads to secretion of soluble factors, such as pro-inflammatory mediators (interleukin (IL)-1, and tumor necrosis factor (TNF)-α), cytokines and chemokines that mediate the recruitment of circulating cells toward the site of inflammation. Among the cells recruited are natural killer (NK) cells, which are able to lyse infected cells. At the site of infection, antigens are captured and internalized by resident antigen presenting cells (APCs), such as macrophages, dendritic cells, and B lymphocytes. Among these, DCs are the most effective, and commonly referred to as professional APCs. Upon Ag internalization, DCs are activated, mature and migrate through the lymph and the blood towards secondary lymphoid organs, where they get in contact with T lymphocytes. Antigen and Ag-bearing APCs draining to the secondary lymphoid tissues increase the possibility that any given antigen is recognized by the small numbers of specific T and B lymphocytes, and thus that an adaptive immune response is initiated. Internalized

Ags are degraded by different enzymatic or chemical processes, that lead to the generation of peptides able to be displayed on the surface of APCs in association with class I or class II major histocompatibility complex (MHC) molecules. Antigenic peptides presented in the contest of MHC molecules are recognized by T lymphocytes expressing an appropriate T cell receptor (TCR), and either the CD8 coreceptor ($CD8^+$ T lymphocytes) in case of Ag/MHC class I molecule, or the CD4 coreceptor ($CD4^+$ T lymphocytes, or helper T lymphocytes) in case of Ag/MHC class II complexes.

Ag recognition by T cells expressing the appropriate TCR, elicits T cell activation and proliferation. The selective clonal expansion of the small number of T cells that share specificity, but express unique TCRs, is a critical feature in the development of the adaptive immune response. During Ag-driven T cell proliferation in the peripheral lymphoid tissues, T cells also differentiate and acquire effector function capabilities. $CD4^+$ T cells acquire the ability to secrete a number of soluble factors and up-regulate surface molecules critical for their helper function. $CD8^+$ T cells also acquire the ability to produce and secrete several soluble factors, such as interferon (IFN)-γ and TNF– α, or perforin and granzyme B through which they exert their cytolytic function.

Ag recognition by B cells, via their specific Immunoglobulin B Cell Receptor, and the interaction of the B cells with Ag-specific $CD4^+$ T cells cause the B cells to proliferate and differentiate into memory B cells and plasmacells. At the stage of plasmacells, B cells secrete immunoglobulins that disseminate into the biological fluids (blood and lymph) of the organisms allowing pathogen neutralization at long distances.

Following proliferation and differentiation, T and B lymphocytes acquire the ability to migrate out from the lymphoid organs and to reach the peripheral tissues where they then recruit and interact with components of the innate response, thus allowing the complete development of protective immune responses.

1.2 The central role of $CD4^+$ T lymphocytes

$CD4^+$ T lymphocytes are crucial for the development of both adaptive and innate immunity. Indeed, by direct cell-cell interaction and by secreting soluble factors, they can amplify and modulate the activity of distinct cell types of the immune system. For instance, during the development of adaptive immune responses, $CD4^+$ T cells can mediate APC activation (APC licensing), which results in the upregulation of costimulatory ligands and in the production of inflammatory cytokines (1, 2). This facilitates the activation of $CD8^+$ T cells that get in contact with the activated APCs. Furthermore, $CD4^+$ T cells, by direct interaction with $CD8^+$ T cells, can favor their proliferation and differentiation, participating to cytotoxic and inflammatory reactions (3). Moreover, interaction of $CD4^+$ T cells with B lymphocytes is necessary for immunoglobulin secretion by B cells, and for the generation of an effective humoral immune response (4). Finally, through ligand/receptor interaction and through secretion of chemokines and effector cytokines, $CD4^+$ T lymphocytes are also able to interact with and regulate the activity of the cells of the innate immunity, among which macrophages, NK cells, eosinophils, and mast cells (5).

Given such a crucial role played by $CD4^+$ T lymphocytes in coordinating the immune response, understanding the molecular mechanisms that regulate their activation and differentiation is critical to clarify how adaptive immunity develops, and to design new immunotherapeutic approaches for clinical conditions, such as organ transplantation, autoimmunity, cancer.

1.3 $CD4^+$ T cell activation

1.3.1 The "two-signal" model

$CD4^+$ T cell activation is a dynamic process, involving the interaction of several surface proteins, expressed by T cells, with their specific ligands expressed on the surface of APCs. These interactions initiate integrated intracellular signalling cascades, collectively defined as the commitment phase, which eventually induce the expression of an orderly sequence of genes whose products are necessary for cell-cell interaction, proliferation, immune functions, cell death and memory.

The critical requirement for $CD4^+$ T cell activation is the engagement of the TCR by the specific antigenic peptide bound to MHC class II molecules expressed on the surface of professional APCs (6). Studies initiated by Schwartz and co-workers provided, however, the initial evidence that TCR engagement alone (signal 1) was not sufficient to induce proper T cell activation, and that the simultaneous engagement of another cell surface receptor, defined as costimulatory receptor (signal 2), was required for proper T cell activation. These studies originated the "two-signal" model of T cell activation (7).

The two-signal concept of lymphocyte activation was originally proposed to explain discrimination between self and non-self, and has proven to explain in several instances either T cell activation, or T cell tolerance.

1.3.2 T cell costimulation: the central role of CD28

Over the past decade, a number of T cell costimulatory proteins and their ligands on the surface of APCs have been identified. These costimulatory receptors can be divided in two groups on the basis of ligand specificity and structural similarities. The first group includes members of the immunoglobulin superfamily such as CD28, the T cell inducible costimulator (ICOS), and PD-1 (8, 9). The counter-receptors for these costimulatory molecules are members of the B7 protein superfamily, which are expressed on the surface of APCs: B7.1 (CD80) and B7-2 (CD86) are ligands for CD28, B7-H2 is the ligand for ICOS, and B7-H1 for PD-1 (8, 9) (Fig. I). While CD28 is constitutively expressed by naïve T cells, ICOS is rapidly induced upon T cell activation. Thus, while CD28 might be critically needed for naïve T cells, ICOS might provide co-stimulatory signals to activated T cells (9). Similarly to ICOS, also PD-1 is not expressed by naïve T cells, but it is up-regulated following T cell activation (9).

The second group of costimulatory molecules includes members of the TNF receptor family: CD40L, 4-1BB, OX-40, CD30, CD27 and HVEM (herpes-virus entry mediator) (10). Their ligands CD40, 4-1BBL, OX-40L, CD30L, CD27L, and LIGHT are found to be constitutively expressed, or up-regulated following activation by professional APC (Fig. II). While CD27 and HVEM are expressed at

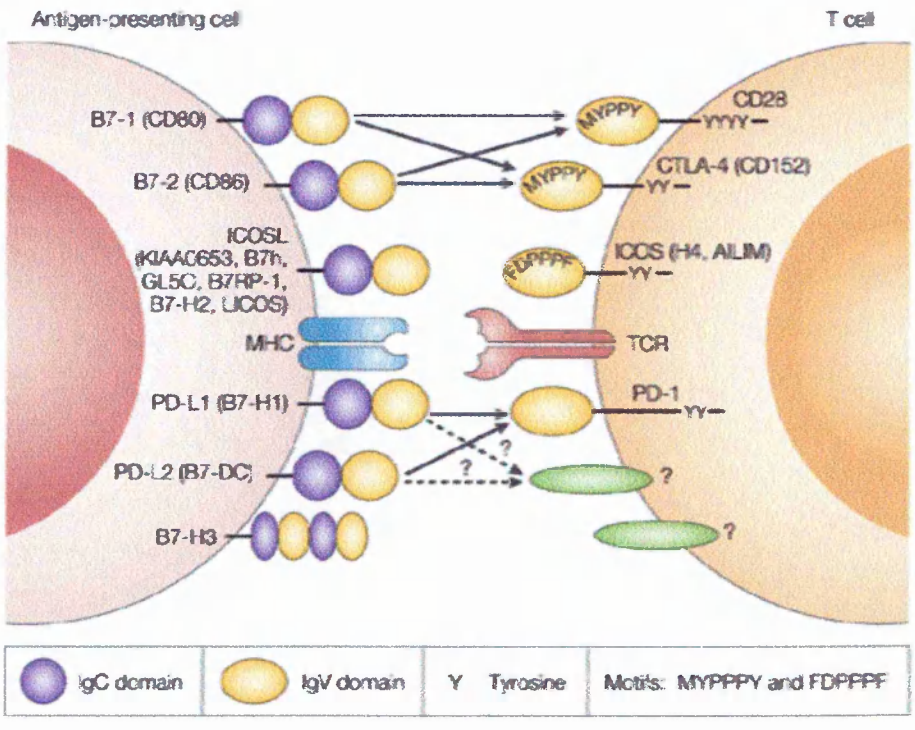


Figure I. T cell costimulation. Schematic representation of the costimulatory receptors of the immunoglobulin superfamily, expressed on the surface of the T cells, and their ligands expressed on the surface of the APCs.
Obtained from Sharpe and Freeman, 2002 (9).

Antigen-presenting cell

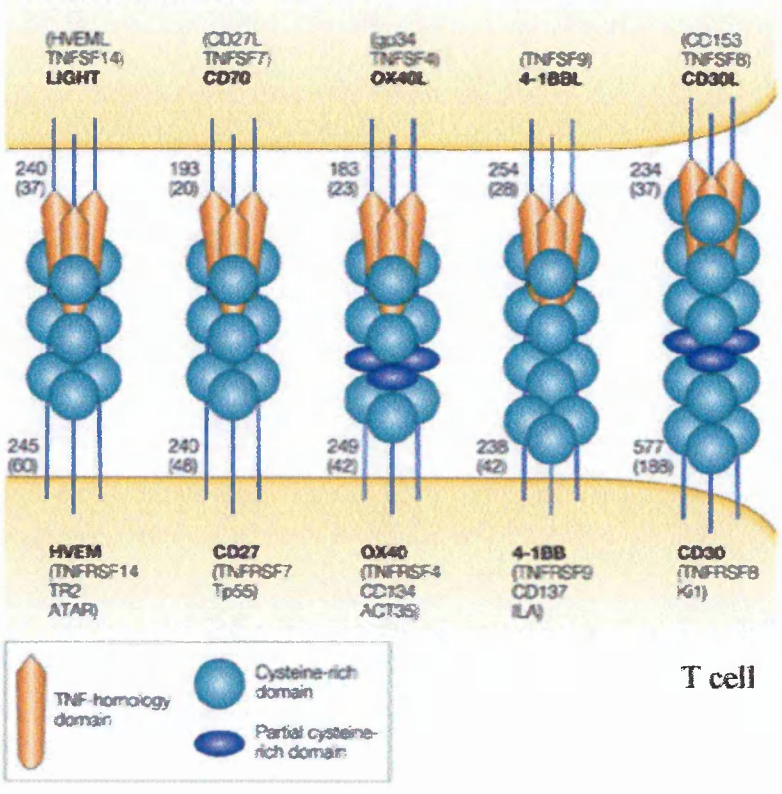


Figure II. T cell costimulation. Schematic representation of the members of the TNF receptor family of costimulatory receptors expressed on the surface of the T cells, and their ligands expressed on the surface of the APCs.
Adapted from Croft, 2003 (10).

low levels in naïve T cell, CD40L, OX-40, 4-1BB, and CD30 are up-regulated following both TCR and CD28 engagement (10).

In addition to these factors, several molecules, known to regulate cell adhesion, have been proposed to have costimulatory activity. For instance, LFA-1 (CD11a/CD18) and LFA-2 (CD2) integrins, which respectively bind to ICAM-1 (CD54) and to LFA-3 (CD58), are expressed on the surface of both hematopoietic and non hematopoietic cells, and rapidly and transiently promote cell adhesion upon TCR engagement (11-15).

Among the above mentioned costimulatory molecules, the best characterized is CD28, a disulphide-linked homodimeric glycoprotein, constitutively expressed on the surface of naïve T cells, and able to bind, as previously mentioned, to B7-1 and B7-2 membrane proteins on the surface of APCs.

The engagement of CD28 does not elicit a physiological response in the absence of TCR signalling, but it is critically needed to enhance and sustain TCR-initiated T cell responses. For instance, it has been shown that CD28 can synergize with the TCR to mediate the upregulation of several growth factor receptors, including IL-2 receptor (IL-2R) α, β, and γ chains (16, 17), to increase the transcription and the stability of cytokine mRNAs, in particular those encoding for IL-2 and IL-4 (18-20), and to enhance T cell proliferation (8, 21, 22). Moreover, CD28 signalling is capable of preventing apoptosis in activated T cells. Indeed, it has been reported that CD28 costimulation upregulates the expression of anti-apoptotic gene products, such as Bcl-x_L, able of protecting lymphocytes from Fas-induced cell death (23). TCR/CD28 stimulation also induces the expression of CTLA-4 (Cytotoxic T lymphocyte-associated antigen-4), a molecule highly homologous to CD28, which

binds to B7-1 and B7-2 with much higher affinity than CD28, and which inhibits further T cell activation (24). This suggests that CD28 might not only positively regulate T cell activation, by promoting T cell expansion and survival, but might also play a critical role for the termination of the T cell response.

The crucial role of CD28-mediated costimulation in T cell priming *in vivo* is demonstrated by the observation that $CD4^+$ T cell responses are greatly impaired in $CD28^{-/-}$ mice (25, 26), or when B7-CD28 interactions are prevented by the use of monoclonal antibodies, or of a CTLA-4-Ig fusion protein (8, 27, 28). For instance, blocking B7 *in vivo* prevented autoimmunity and graft rejection (27, 29-31), while triggering of CD28 greatly enhanced anti-tumor immune responses (32-34).

In addition to the *in vivo* generated evidences, a number of other studies have underlined a critical role for CD28-induced signals. Indeed, the engagement of the TCR *in vitro*, in the absence of CD28 costimulation, rather than T cell activation, induces a long-lasting state of T cell unresponsiveness (clonal anergy) (7, 35, 36) (see section 1.6). Moreover, it was shown that, while T cells required a very high TCR occupancy and a prolonged stimulation in the absence of CD28 engagement, they responded more rapidly to lower levels of TCR occupancy when costimulated through CD28 (37, 38). These studies indicated that CD28 costimulation could be critically needed when the level of antigenic stimulation is limiting, such at low antigen doses, or in the presence of low affinity TCR ligands. Consistent with this possibility is the finding that only repeated injections with high doses of antigen elicited a T cell response in CD28-deficient mice (25, 26, 39). Altogether the existing data support the possibility that CD28 costimulation might lower the

threshold needed for T cell activation, thus providing a quantitative contribution to increase the intensity and/or duration of TCR-mediated signals.

Lanzavecchia and coworkers first analyzed the contribution of CD28 to the kinesis and stoichiometry of the TCR-peptide-MHC interaction, and to the lowering of T cell activation threshold. By measuring intracellular calcium elevations to monitor sustained TCR signal (see section 1.4.1), and TCR downregulation as reliable readout of the number of TCRs triggered, these authors discovered a dynamic type of interaction between the T cell and the APC. Indeed, at the level of T/APC contact site, they showed that a large number of TCRs could be serially engaged, triggered and downregulated by a few specific peptide-MHC complexes (40). This process of serial TCR triggering resulted in the generation and maintenance of a sustained level of second messengers that was required to initiate and maintain transcription at specific loci. Sustained signal for optimal T cell activation required continuous engagement of TCRs by peptide/MHC complexes on APC, demonstrating that the signal emanating from triggered TCRs was short lived (41, 42). These authors calculated that, irrespective of the nature of the triggered ligand, T cells count the number of triggered TCRs, and respond when a threshold of approximately 8000 TCRs is reached (37). Costimulation by CD28 lowered the activation threshold to approximately 1500 TCRs, thus making T cells more sensitive to antigen stimulation (37). In addition, in these studies, CD28 costimulation also decreased the time required to have full T cell commitment (43).

From all these experiments it is believed that CD28 costimulation, by sustaining the signal inside the T cell and by increasing response longevity, allows a

lower number of triggered TCR and a shorter duration of T cell/APC contact to be effective for full T cell activation.

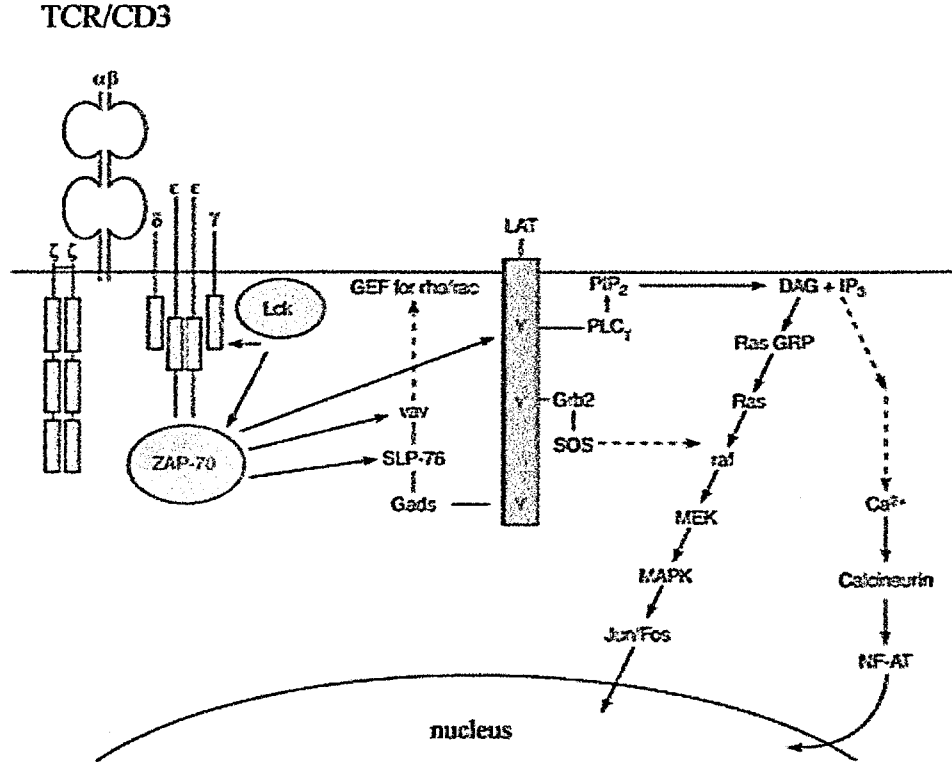
1.4 $CD4^+$ T cell commitment

The simultaneous engagement of the TCR and of CD28 initiates a complex network of intracellular signals, which render the cell competent to proliferate and eventually to differentiate. This initial phase of T cell activation has been defined as "the commitment phase".

A number of studies, mostly performed *in vitro* on transformed T cells and long-term T cell clones, and identified by the use of agonistic anti-receptor antibodies (Abs), have generated important information of the intracellular signalling pathways activated upon TCR and CD28 stimulations. Much of this information has now been validated *in vivo* by analyses performed on primary T cells, and on genetically modified mice.

1.4.1 TCR-induced signalling

The engagement of the TCR elicits an intracellular signal transduction cascade, which can be schematically divided in an early phase, that involved sequential tyrosine phosphorylation events, and a later phase of a complex network of downstream events, such as the activation of small GTPases and of serine/threonine kinases (44, 45) (Fig. III).



immunoreceptor tyrosine-based activation motifs (ITAMs)

Figure III. TCR-induced signalling. Schematic representation of the main intracellular signalling pathways activated upon TCR/CD3 crosslinking.
Adapted from Pitcher et al., 2003 (47).

The early tyrosine kinases cascade

The T cell antigen receptor complex comprises the polymorphic TCR α and β subunits, that recognize peptide/MHC complexes, and the tightly associated γ, δ, ε, and ζ signal transduction subunits, which form the CD3-ζ complex.

The capacity of the TCR to transduce signals across the T cell membrane relies on the cytoplasmic tails of the CD3-ζ subunits that contain common motifs, called immunoreceptor tyrosine-based activation motifs (ITAMs) that are phosphorylated upon TCR triggering (45-47). Even though phosphorylated ITAMs pre-exist in resting T cells, ITAMs phosphorylation is the earliest known event in T cell signalling, and is regulated by a pool of active Src protein tyrosine kinases (PTKs) (mostly Lck), and by protein tyrosine phosphatases (PTPs), such as CD45 (45, 46). It has been suggested that the binding of TCR with peptide-MHC complex favours ITAM phosphorylation not only by recruiting the kinases to the receptor, but also by mediating the exclusion, on a size-base, of phosphatase molecules away from the TCR-ligand complex (48).

ITAMs phosphorylation initiates the early PTK signalling cascade. Indeed phosphorylated ITAMs recruits ZAP-70, a Syk family PTK, which binds to the ITAMs via its tandem Src-homology 2 (SH2) domains (49). ITAM-bound ZAP-70 is then tyrosine phosphorylated and bound by the SH2 domains of Lck (50). It has been shown that the physical interaction between Lck and ZAP-70 and the Lck-mediated phosphorylation of ZAP-70 greatly increases ZAP-70 catalytic activity (51, 52). The Lck/ZAP-70 complex is critical for TCR-induced activation (53). Indeed it amplifies TCR-induced signalling by promoting continued ITAMs

phosphorylation with subsequent additional recruitment of ZAP-70 and other Lck substrates such as the Tec PTK Itk (54).

Stable ITAM phosphorylation is only led to completion when the Ag/MHC-TCR interaction has a sufficient half-life. This is achieved by strong TCR-ligand interactions or by the help of CD4 coreceptor (55). It has been shown that Lck binds a cysteine motif present in the tail of the CD4 coreceptor (56), and by that it stabilizes the association of Lck with ZAP-70, with consequent amplification of phosphorylation reactions that create a positive feedback loop between the TCR and the intracellular signalling pathways (44).

Downstream signalling pathways: Ras/Rho, PLCγ1, and PI3K

Upon the early tyrosine kinase reactions, a cascade of downstream signalling events is induced. This is initiated by phosphorylation of adapter molecules, such as LAT (linker of activated T cells) and SLP-76 (SH-2 domain containing lymphocyte protein of 76 kDa) (57, 58), mediated by ITAM-bound ZAP-70. Tyrosine phosphorylation of these adaptors represents the link between antigen receptors and downstream signalling pathways. Indeed, upon phosphorylation in tyrosine residues, adaptors act as scaffolding proteins, which recruit to the plasma membrane important signalling molecules, and assemble them with their regulatory partners. This leads to the activation of different pathways that disseminate TCR signals from the plasma membrane to the cytosol and the nucleus (44).

The key pathways assembled and activated by phosphorylated LAT and SLP76 are the Ras-and Rho-, and the inositol lipid-dependent networks. The induction of the latter requires activation of two key enzymes, the phospholipase C gammal

(PLCγ1) and the phosphatidyl inositide-3 kinase (PI3K), which in turn regulate the intracellular calcium concentration, and the activity of several serine-threonine kinases

Ras plays a crucial role in T cell activation, regulating both cytokine gene transcription and cellular proliferation (59). Ras is a guanine nucleotide binding protein with GTPase activity, which cycles between an active GTP-bound and an inactive GDP-bound form (60). The guanine nucleotide binding cycle of Ras is controlled by guanine nucleotide exchange proteins (GEFs), that promote transition from GDP-bound to GTP-bound form, and thus activate Ras, and by GTPase activating proteins (GAPs), that stimulate the intrinsic GTPase activity of Ras, and lead to Ras inhibition. GTP-bound form of Ras accumulates in T cells upon activation (44). One important GEF for Ras is Sos, the mammalian homologue of the Drosophila "Son of Sevenless" protein. Sos forms a complex with the adaptor Grb2, which is able to bind, through its SH2 domain, to phosphorylated tyrosines in the LAT tail, thus recruiting Sos to the plasma membrane. This leads to the recruitment and the activation of Ras at this site (60). Activated GTP-bound Ras in turn regulates the mitogen-activated protein kinase (MAP kinase) pathway, a kinase cascade that transmits signals from the membrane to the nucleus, activating several transcription factors involved in cytokine gene induction (60, 61). The first kinase recruited to the plasma membrane and activated by GTP-bound Ras is the serine/threonine kinase Raf-1, a MAP kinase kinase kinase (MKKK). Activated Raf-1 in turn phosphorylates and activates MAP kinase kinases (MEK 1, 2), which then directly activate the two MAP kinases, Erk1 and Erk2 (extracellular signal-regulated kinase 1 and 2) (62). One of the known functions of Erks is the

phosphorylation of the transcription factor Elk-1, which in turn regulates the transcription of the *Fos* gene (63). Fos transcription factors are members of the AP-1 (Activator Protein-1) complex and are required for *IL-2* gene expression (see section 1.4.3).

TCR-mediated PLCγ1 activation initiates a cascade of lipid metabolism, generating several phosphatidylinositols endowed with signalling capabilities. Indeed, PLCγ1 allows the hydrolysis of phosphatidylinositol (4,5) biphosphate ($PI(4,5)P_2$), and controls the production of inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). PLCγ1 activation by the TCR requires the PTKs activated during the early phases of T cell activation (Lck, ZAP-70 and Tec kinases, such as Rlk, and Itk), and the LAT and SLP76 adaptors (64-67). It has been proposed that LAT, which binds to SLP76, acts as a scaffold for the assembly of the PLCγ1 signalling complex with SLP76, thus allowing activation of the phospholipase by a Tec family kinase that is associated with SLP76 (44, 57)].

The products of PLCγ1 activity, IP3 and DAG, play key roles in T cell activation because they mediate an increase in the intracellular calcium concentration and activate serine/threonine kinases of the PKC family, respectively. The rapid and sustained IP3-dependent elevation of intracellular calcium concentration, that occurs within few seconds upon TCR triggering, is absolutely critical during the initial phases of T cell activation (46, 68). Indeed, calcium regulates PKCs activity, the activity of the phosphatase calcineurin, required for proper activation of the Nuclear Factor of Activated T cells (NFAT) transcription factor (see section 1.4.3), and the stabilization of the T cell/APC contact zone required for the formation of the immunological synapse, which then allows proper

signalling (69, 70). The production of DAG is critical for the activation of the different PKC isoforms expressed by T cells and activated upon TCR engagement. The PKC isoforms can be divided into two groups, one composed by the classical PKCs (α, βI, βII and γ), which are regulated by calcium, DAG and phospholipids, and novel PKCs (δ, ε, η and θ), which are Ca^{2+}-independent and are regulated by DAG and phospholipids (71). Even though different members of the PKC protein family can activate different target molecules and hence regulate distinct biological functions, the contribution of the different isoforms to TCR-mediated activation events is only partially known. In T cells the relevant PKC appears to be PKCθ (72, 73). This protein is the only known member of the PKC family to localize in the T-cell supramolecular activation complex (SMAC), a region of membrane and cytoplasmic polarization formed at the contact site between a T cell and an APC during recognition of cognate antigen (73). Correct localization of PKCθ is critically needed for T cell activation (74). PKCθ cooperates with Ca^{2+} and MAPK-dependent signals for the activation of NFAT, AP-1 and NF-kB transcription factors, required for *IL-2* gene induction (see section 1.4.3). PKCθ activation is Ca^{2+} independent, and is regulated by binding to DAG. In addition, a tyrosine kinase-stimulated Vav/Rac pathway (see below) also mediates selective membrane translocation and activation of PKCθ (75). In addition to mediate PKC activation, PLCγ1-generated DAG links the lipid metabolism to the Ras pathway. Indeed, DAG can bind to RasGRP, a GEF for Ras, and localize it at the plasma membrane, where it can activate Ras (76).

In addition to PLCγ1, TCR-dependent signalling activates the PI3K and thus stimulates the inositol lipid turnover. PI3K phosphorylates PI(4,5)P2 on the D-3

position of the inositol ring to produce PI(3,4,5)P3 (77). Through binding to different target molecules, PIP3 regulate many cellular events, such as cell growth and survival, and cytoskeletal remodelling (PI3K-dependent signalling will be also discussed in section 1.5.2).

There are multiple isoforms of PI3K, but the form biochemically linked to T cell activation comprises a regulatory 85-kDa (p85) subunit and a catalytic 110-kDa (p110) subunit (78). While the stimulatory effect of the TCR alone on PI3K activity is small, the combined triggering of the TCR and CD28 optimally activates the enzyme (79). It is believed that the p85 regulatory subunit, via its SH2 domains, binds to tyrosine phosphorylated LAT (80), and brings the enzyme to the plasma membrane. PI3K-generated PI(3,4,5)P3, in turn, serve as plasma membrane-associated docking sites for pleckstrin homology (PH) domain-containing proteins, and favour their relocalization to the plasma membrane where they can be activated (81). Among the PH domain containing proteins recruited following PI3K activation, are members of the Tec tyrosine kinases (82), two serine/threonine kinases, the phosphoinositide-dependent protein kinase (PDK) 1, and protein kinase B (PKB), also known as Akt (83, 84), and the GEFs for Rac, Rho and Cdc42 (85).

PDK1 regulates the activity of many serine/threonine kinases, including members of the PKC family, the p70 ribosomal protein S6 kinase, $p70^{S6k}$, and PKB (84, 86), which play very important roles in T cell activation. $p70^{S6k}$, for example, catalyzes the phosphorylation of the 40S ribosomal subunit protein S6, and regulates protein synthesis and cell growth (87, 88).

PKB, also known as Akt, controls cell cycle progression by activating E2F transcription factors, and promotes cell survival (89-92). It exerts its functions by

shuttling between the plasma membrane, where it gets activated, the cytosol and the nucleus, where it gets in contact with its substrates. In addition to its role in cell proliferation and survival, PKB plays also a role in the glycogen metabolism. Indeed it phosphorylates and inactivates glycogen synthase kinase-3 (GSK3) (93). Furthermore, PKB activity has also been shown to regulate, through GSK3, the nuclear export of NFAT transcription factor (94).

The best characterized GEF for the Rho family of GTPases in lymphocytes is Vav-1, that binds via its PH domain to PIP3, and is recruited to the plasma membrane where it is activated by TCR induced tyrosine kinases, such as Lck (95). Rho GTPases regulate several T cell functions by interacting with multiple effectors. One of the key events regulated by this class of GTPases upon TCR engagement is the dynamic reorganization of actin cytoskeleton (44). This is a crucial event that occurs during the formation of T cell/APC conjugate, required for the formation of a stable contact, which is needed for sustained T cell signalling and proper T cell activation.

1.4.2 CD28-induced signalling

A number of intracellular signalling pathways activated upon CD28 cross-linking, and in some instances distinct from signals generated by TCR/CD3 engagement, have been defined (96). These pathways integrate and enhance TCR signals both during the early phases of tyrosine phosphorylations and also downstream, at the level of gene expression (Fig. IV). For instance CD28-mediated costimulation increases the phosphorylation of the ζ chain of the TCR and of ZAP-

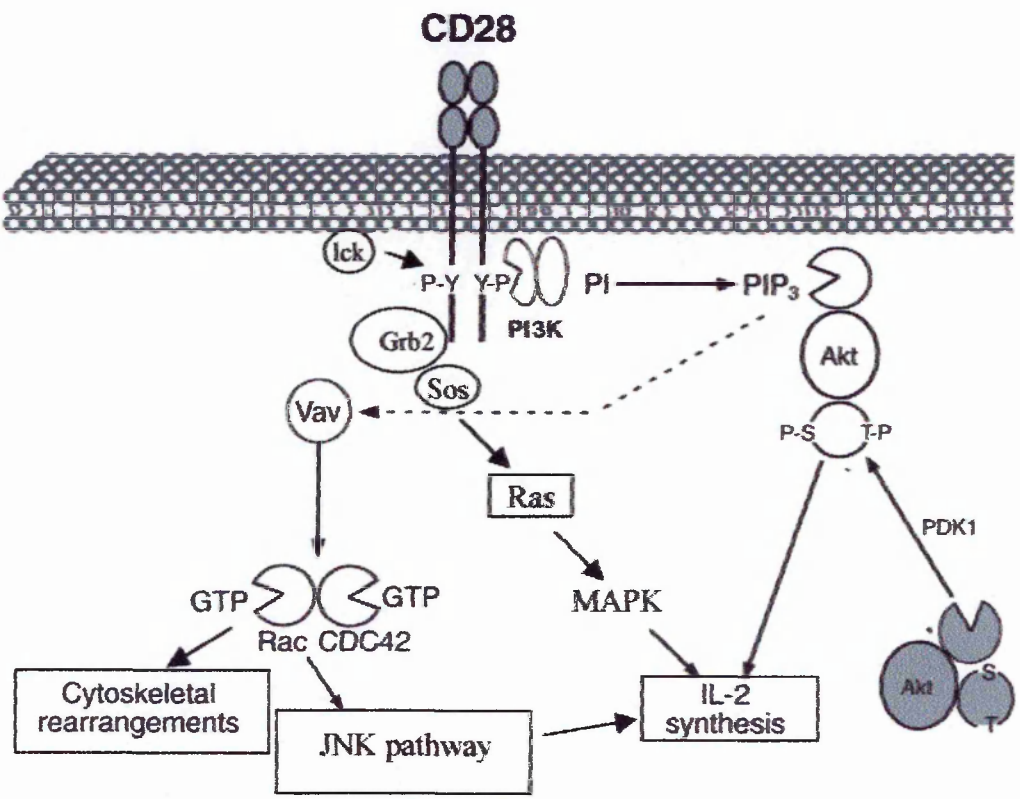


Figure IV. CD28-induced signalling. Schematic representation of the main intracellular signalling pathways activated upon crosslinking of the costimulatory receptor CD28.
Adapted from Frauwirth et al., 2002 (96).

70, and by that it possibly lowers the TCR activation threshold (97). Moreover, CD28 signalling promotes T cell activation by facilitating the recruitment of intracellular lipid rafts that contain kinases and adapter molecules to the site of TCR engagement (98, 99).

Even though CD28 contains a 41 amino-acid–residue cytoplasmic domain, which lacks an obvious catalytic capacity, upon ligation by specific Abs or by its natural ligands, it becomes phosphorylated on tyrosines by Src family kinases such as Lck and Fyn, and by Tec family kinases such as Itk, which can be found associated with the receptor at a very low stoichiometry (100, 101). Lck and Fyn mediate phosphorylation of a specific tyrosine that is found within a single YMNM motif in the cytoplasmic domain of CD28 (102). Phosphorylation of the YMNM motif permits recruitment of the signalling proteins PI3K and Grb2, which bind to the receptor via their SH2 domains (103). PI3K plays a central role in CD28-induced intracellular signalling (104, 105). Its binding to the cytoplasmic tail of CD28 anchors the enzyme to the inner face of the plasma membrane, and by that it facilitates its interaction with its specific substrates. Overexpression of the PI3K target PKB/Akt in T cell lines mimics CD28 costimulatory signal and increases transcription of the *IL-2* gene (106). Moreover, CD28-dependent signalling directly elicits phosphorylation of $p70^{S6k}$ and 4EBP-1, through Rapamycin-dependent pathways (107, 108). This has been correlated with the role of CD28 costimulation in increasing IL-2 mRNA stability and IL-2 production (108). It remains to be determined whether these signalling pathways induced by CD28 are downstream to PI3K activation.

The recruitment of Grb2 to the phosphorylated YMNM motif of CD28, allows the recruitment of Sos, of p85, and of Vav, thus connecting CD28 to a number of downstream signalling events (102, 103).

Upon binding to CD28, Vav-1 is rapidly phosphorylated in tyrosine residues. This can occur in the absence of simultaneous ligation of the TCR, and leads to the activation of Rho family members, such as Rac-1 (102). CD28-dependent Rac-1 activation might also be mediated by the CD28-dependent phosphorylation and inhibition of the Rac-inhibitor $p62^{dok}$ (109). Vav-1/Rac-1 activation links CD28 to actin remodelling events and contributes to the tightening and stabilization of the T cell/APC conjugate (44). In addition to cytoskeleton rearrangements, Rac-1-dependent signalling also regulates activation of the stress-related Jun N-terminal kinase (JNK) signalling pathway (110). This provides the link between CD28 costimulation and gene expression during T cell activation (102). Indeed, through activation of the MAP kinase kinases MKK4, and MKK7, JNK is activated, and in turn it phosphorylates the transcription factor c-Jun. This leads to an increase of AP-1 transcriptional activity (111, 112), thus regulating *IL-2* gene expression. As in the case of PI3K, also activation of the JNK kinases is optimal only upon simultaneous TCR and CD28 engagement, thus representing a point of convergence for TCR and CD28 signalling pathways.

CD28-mediated costimulation might not only favor T cell activation, but also participates in the shut down of the T cell response. Indeed, CD28-mediated recruitment of, and phosphorylation by Itk, at a site which is distinct from the YMNM motif, negatively regulates the amplitude of the CD28-induced proliferative

responses, thus suggesting that Itk could provide a means to modulate the strength and potentially the outcome of T cell activation (113).

1.4.3 *IL-2* gene transcription

The simultaneous deployment of the signalling cascades described to be activated by TCR/CD3 and CD28-mediated stimulations results in the transcription of genes encoding for cytokines and cytokine receptors, which promote proliferation and differentiation of mature T lymphocytes (114, 115). One of the first and most important cytokine-encoding gene regulated by the simultaneous activation of the TCR and of CD28, is the *IL-2* gene. The expression of the *IL-2* gene cannot be induced by single intracellular signalling pathways, but requires that the critical transcription factors are simultaneously induced and assembled at the 5' promoter/enhancer region of the *IL-2* gene, and that stable activation complexes are formed. Among the required transcription factors are AP-1, NFAT, NF-kB, and Oct-1 (116, 117).

The AP-1 complex is composed by members of the Fos (c-Fos, FosB, Fra-1, Fra-2), and Jun (c-Jun, JunB, JunD) families of bZip dimeric nuclear factors. These factors bind specific DNA sequences either alone (as dimers at AP-1 site) or complexed with another nuclear factor, such as NFAT (at the NFAT site) or octamer proteins (at OCT/OAP site), and enhance transcription (118). The activity of the AP-1 complex is regulated by Ras-, JNK-, and PKCθ-dependent pathways both at the level of gene transcription, and by post-translational modifications of its gene products (111, 119, 120). Ras, for instance, elicits Erk-dependent

phosphorylation of the ternary complex factor Elk-1, which in turn can bind and transactivate the *c-Fos* promoter, thus regulating *c-Fos* transcription (121, 122). Similarly, Ras-dependent signals also regulate the *JunB* promoter through Erk (123).

Similarly to Erk, also JNK participates to AP-1 activation. Indeed, CD28-dependent JNK activation, mediates phosphorylation of c-Jun augmenting its transcriptional activity (124). Moreover, JNK increased activity also augments Elk-1 dependent transcription at the *c-Fos* gene (125). Interestingly, the JNK pathway has also been implicated in the enhancement of IL-2 mRNA stability mediated by CD28 costimulation (126).

Finally, PKCθ, regulates AP-1 activation probably at the level of *c-Fos* or *c-Jun* transcription (120).

NFAT proteins are cytosolic in quiescent T cells, but they translocate into the nucleus in TCR-activated cells, where they bind to AP-1 family members to form a functional transcriptional complex at the *IL-2* enhancer region (127). Cooperation of NFAT with AP-1 is required for *IL-2* gene transcription, as apparent from studies using a mutant NFAT protein unable to interact with Fos/Jun heterodimers (128). The *IL-2* gene promoter contains at least four NFAT binding sites, of which two are composite NFAT/AP-1 binding elements (129). Each NFAT/AP-1 composite binding site is needed for *IL-2* gene transcription. Binding sites for NFAT are found in the enhancer of a number of cytokine genes including IL-2, IL-4 and TNF-α (127), demonstrating a key role of those transcription factors in regulating several T cell responses.

Nuclear translocation of NFAT factors is mediated by a calcium-dependent signalling pathway, induced by TCR/CD3-mediated PLCγ activation, and involves the activation of the phosphatase calcineurin (130). Upon calcium-dependent targeting of calcineurin to the regulatory domain of NFAT proteins, the phosphatase dephosphorylates NFAT proteins mediating their nuclear translocation (129). Because of the presence of constitutively active intracellular kinases that re-phosphorylate and inactivate NFAT, calcineurin, and sustainded calcium signal are continuously needed to maintain NFAT proteins in an active state. Both nuclear translocation of NFAT and its activation of gene transcription are reversed by treatment of T cells with the calcineurin inhibitors Cyclosporin A (a cyclical undecapeptide), and FK506 (a macrolide antibiotic) (129). Once translocated into the nucleus, NFAT proteins bind to AP-1 family members and to the *IL-2* enhancer region. Thus, NFAT-dependent transactivation is not only regulated by Ca^{2+} signals, but also by Ras activation (105, 131). NFAT transcriptional activity is also increased by PKC activation (132).

Thus, during optimal T cell stimulation through TCR/CD3 and CD28, the coordinate activation of calcium and MAP kinase/PKC signalling pathways leads to the formation of the transcriptionally active NFAT/AP-1 complexes, which then bind to the *IL-2* promoter and regulate *IL-2* gene transcription.

NF-kB (nuclear factor-kB) transcription factors are regulated by PKCθ (133), and, like NFATs, they can associate with AP-1 complexes at specific composite binding sites within the 5' regulatory regions of the *IL-2* gene. In resting cells, NF-kB factors reside in the cytoplasm as dimeric proteins, associated with specific inhibitors, known as IkBs, that retain NF-kB in an inactive state in the cytoplasm

(134). It has been proposed that, upon TCR cross-linking, PKCθ is activated and induces IKK (IkB kinase) complex-mediated serine phosphorylation of IkBs. Phosphorylation of IkBs targets the protein to the proteasome for degradation (133). Once released from IkB inhibitors, NF-kB transcription factors can enter into the nucleus and bind their specific target sequences, such as those within the *IL-2* promoter. Vav-dependent signals can synergize with PKCθ for NF-kB activation (135). CD28 costimulation seems to be required for NF-kB activation, since it can mediate binding of this transcription factor to the CD28 response elements (CD28RE) found in the *IL-2* promoter (136).

Finally, in peripheral T lymphocytes and in several T cell lines, both the ubiquitous Octamer family factor Oct-1, and the lymphocyte-specific factor Oct-2 are expressed and bind to the *IL-2* promoter. Prominent octamer binding sites of *IL-2* promoter act as inducible enhancers in T cells, and their induction is inhibited by the immunosuppressant Cyclosporin A (CsA). The binding of AP-1 and octamer factors to the *IL-2* promoter, and the tight association and functional cooperation of octamer with AP-1 factors is of crucial importance for the inducible transcription of the *IL-2* gene. Moreover, also NFAT and Oct factors, by binding simultaneously to the *IL-2* promoter, and by tightly associating with AP-1 factors, enhance the activity of the *IL-2* promoter (137, 138).

1.5 $CD4^+$ T cell proliferation

One ultimate effect of TCR/CD28 and IL-2 generated signals is to initiate cell proliferation, and influence the rate at which T lymphocytes divide. Stimulation of

resting T lymphocytes by TCR/CD28 initiates cell cycle entry (139), and renders the cells competent for subsequent proliferation by inducing IL-2 production and the expression of the high affinity IL-2 receptor (IL-2R) (140). Binding of IL-2 with its newly expressed IL-2R mediates the coordinated activation of several intracellular signalling events, and eventually induces cell division (141). Because of this, IL-2 can be considered as the third signal that, in addition to TCR and CD28, is required for T cell proliferation.

1.5.1 Cell cycle regulation

The sequence of events that occur during the cell cycle have been divided into four phases, designated G1 (gap 1), S (synthesis of DNA), G2 (gap 2), and M (mitosis). Cell cycle regulation takes place mainly at the transition points, or boundaries, between the different phases, which serve as checkpoints. In mammals, most variation in cell cycle timing is due to variation in the length of G1, and the G1/S transition is tightly regulated. There are three main classes of proteins directly involved in checkpoint regulation: the cyclins, whose levels rise and fall in specific phases of the cell cycle; the cyclin dependent kinases (Cdks), a class of serine-threonine kinases that are activated by the cyclins, and the Cdk inhibitors (CKIs), which inactivate Cdks. The sequential formation, activation and subsequent inactivation of a series of cyclin-Cdk complexes govern cell cycle entry and progression. The main cyclin-Cdk complexes are cyclin D-Cdk4 (and cyclin D-Cdk6), Cyclin E-Cdk2, cyclin A-Cdk2, and cyclin B1-Cdc2 (now renamed Cdk1), acting in G1, at G1/S, in S, and at G2/M, respectively (Fig. V). Formation and

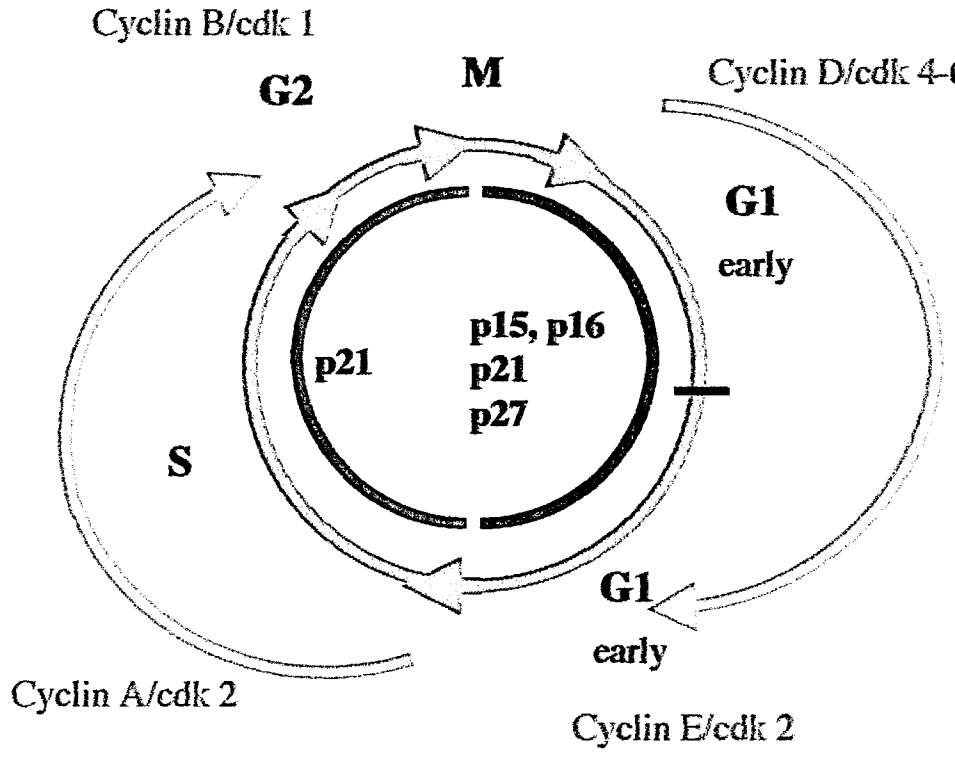


Figure V. Cell cycle regulation. Schematic representation of cell cycle progression in mammalian cells. Cyclin-Cdk complexes (in red), and cell cycle inhibitors (in blue) regulating progression through the different phases of the cell cycle are indicated.

activation of these complexes depend on cell cycle-regulated expression and assembling of cyclins with pre-existing Cdks (142-144). Cyclin-Cdk activity is both positively and negatively regulated by phosphorylation. Activation of cyclin-Cdk complexes requires phosphorylation of threonine residues of Cdks by constitutively expressed cyclin-activating kinases (CAKs). Cdks can also be negatively regulated by interaction with a group of proteins collectively termed Cdk inhibitors (CKIs). CKI levels, like cyclin levels, vary during the cell cycle and thus contribute to the periodicity of Cdk activation (145). Two classes of CKIs have been described: the INK proteins (p15, p16, p18, and p19), which associate with Cdk4 and Cdk6, and the Cip/Kip proteins (p21 and p27), which bind to cyclins D, E and A, and Cdks 4/6/2 containing complexes (146, 147).

Progression through the G1 phase of the cell cycle is also marked by the accumulation of specific transcription factors, including the proteins Fos, Jun and c-myc, which act on genes required for DNA replication (148, 149). A critical balance between cyclin/Cdk complexes and transcription factors is required for proper G1-to- S progression. If this balance is not achieved, the cells stop dividing, and either enter the G0 resting state, or die by apoptosis. In some instances, G0 resting phase can also be induced by high levels of CDKIs.

A number of other factors can also inhibit progression through the G1/S boundary (150). One of the most important is DNA damage. When the chromosomal DNA integrity is compromised, the CDKI $p21^{Cip}$ is induced via pathways depending on tumour suppressor protein p53, and this blocks G1/S progression until the damage is repaired (151). Moreover, there are pathways that ensure that DNA replication is complete before mitosis begins (152), and others that

ensure that the mitotic apparatus has been properly assembled before the cells attempts the completion of mitosis (153, 154).

Entry and progression into the cell cycle by resting cells mostly depend upon extracellular growth-controlling stimuli, such as mitogens. In most eukaryotic cells, these extracellular signals have their most profound effects on the transition from the G1 to the S phase, an event also referred to as "restriction point" (139, 155). Beyond G1/S restriction point, the cell cycle is largely insensitive to external factors (140).

Regulation of G1/S transition

Addition of mitogens to G0/G1 quiescent cells induces the expression of the D-type cyclins (cyclins D1, D2, and D3) which assemble with their partners Cdk4 and Cdk6. Mitogenic stimulation also mediates downregulation of $p27^{Kip1}$, a Cip/Kip protein that accumulates in quiescent cells, and that interacts with and inhibits the function of cyclin D/E-Cdk complexes (156-159). D-type cyclin complexes are activated in response to growth factors, and mediate phosphorylation of the anti-oncogene product Retinoblastoma (Rb). Rb, p107, and p130, the so-called pocket proteins, interact with various E2F family members and repress their transcriptional activity in G0/G1 quiescent cells (160). Hyperphosphorylation of pocket proteins, initially mediated by cyclin D-Cdk4/6, disrupts their association with E2F transcription factors, which are then able to induce the transcription of a series of genes whose activities are necessary for DNA synthesis, and for cell cycle regulation, such as cyclin E (161-164). As levels of cyclin D rise, it is thought that $p27^{Kip1}$ is titrated away from cyclin E-Cdk2 complexes. This allows cyclin E-Cdk2

to phosphorylate $p27^{Kip1}$, leading to its ubiquitin-targeted degradation (165, 166). As molecules of $p27^{Kip1}$ are degraded, increasing numbers of cyclin E-Cdk2 complexes are released, and further phosphorylate Rb in late G1 (167), thus creating a positive feedback autoregulatory loop.

Thus, the hyperphosphorylation of pocket proteins and the subsequent E2F activation represent a key step for transition through the G1/S restriction point.

1.5.2 G1/S transition in T cells

In T lymphocytes cell cycle entry is initiated by TCR-generated signals. TCR ligation induces the expression of cyclin D2 and D3 (T cells do not express cyclin D1), of Cdk4 and Cdk4 kinase activity (168). This occurs independently of IL-2 and mediates entry into early/mid G1 phase of the cell cycle. TCR-induced signals are however insufficient to promote exit from the G1 phase of the cell cycle. This is due to the fact that, while inducing expression of G1 cyclin-Cdk complexes, these signals fail to mediate their activation, because of the inability to inactivate the CKI $p27^{Kip}$, which is found expressed at high levels in quiescent lymphocytes. Studies performed on T cell lines and on primary T cells have shown that IL-2-mediated signalling elicits the further upregulation of cyclin D2, D3, the expression of cyclin E and, importantly, the downregulation of the CKI, $p27^{Kip}$. This allows the activation of cyclin D/E-Cdk complexes, the phosphorylation of critical Cdk substrates and progression through the S phase of the cell cycle (140, 156, 168-170). Among the Cdk substrates associated with cell cycle progression are pRb and

p130, whose hyperphosphorylation allows transcriptional activation of E2F factors, which regulate IL-2-driven S phase entry (89, 90).

Although IL-2 plays a critical role in controlling T lymphocytes proliferation, the intracellular signals responsible for IL-2-induced proliferation are still not fully understood. In addition, several evidences for IL-2 independent T cell proliferation have recently been provided.

IL-2R-dependent G1/S transition

The IL-2 receptor is comprised of three distinct transmembrane subunits, α, β, and γ chains (171). The high affinity IL-2R contains all three chains, and, as mentioned above, it is up-regulated following TCR/CD28-mediated T cell activation (172). IL-2Rα chain contributes to IL-2 binding affinity but not to the recruitment of signalling molecules, and expression and heterodimerization of the β and γ chains (which represent the intermediate affinity IL-2R) is sufficient to mediate IL-2 signalling (141, 171) (Fig. VI).

IL-2 binding to the IL-2R induces the heterodimerization of the cytoplasmic domains of the IL-2Rβ and γ_c, and initiates an intracellular signal transduction cascade. Indeed, heterodimerization of the IL-2R activates the preassociated tyrosine kinases, Janus kinase (Jak) 1 and 3, which phosphorylate key tyrosines on IL-2Rβ. These sites serve as docking sites for downstream signalling molecules, including Stat5 (signal transducer and activator of transcription 5) (Stat5a and Stat5b) and the adapter Shc, which coordinately induce the expression of a variety of genes, including *c-myc*, *bcl-2*, and *bcl-x*, critical for cell proliferation and survival (173-176). Upon binding to the IL-2R, Stat5 is phosphorylated by activated

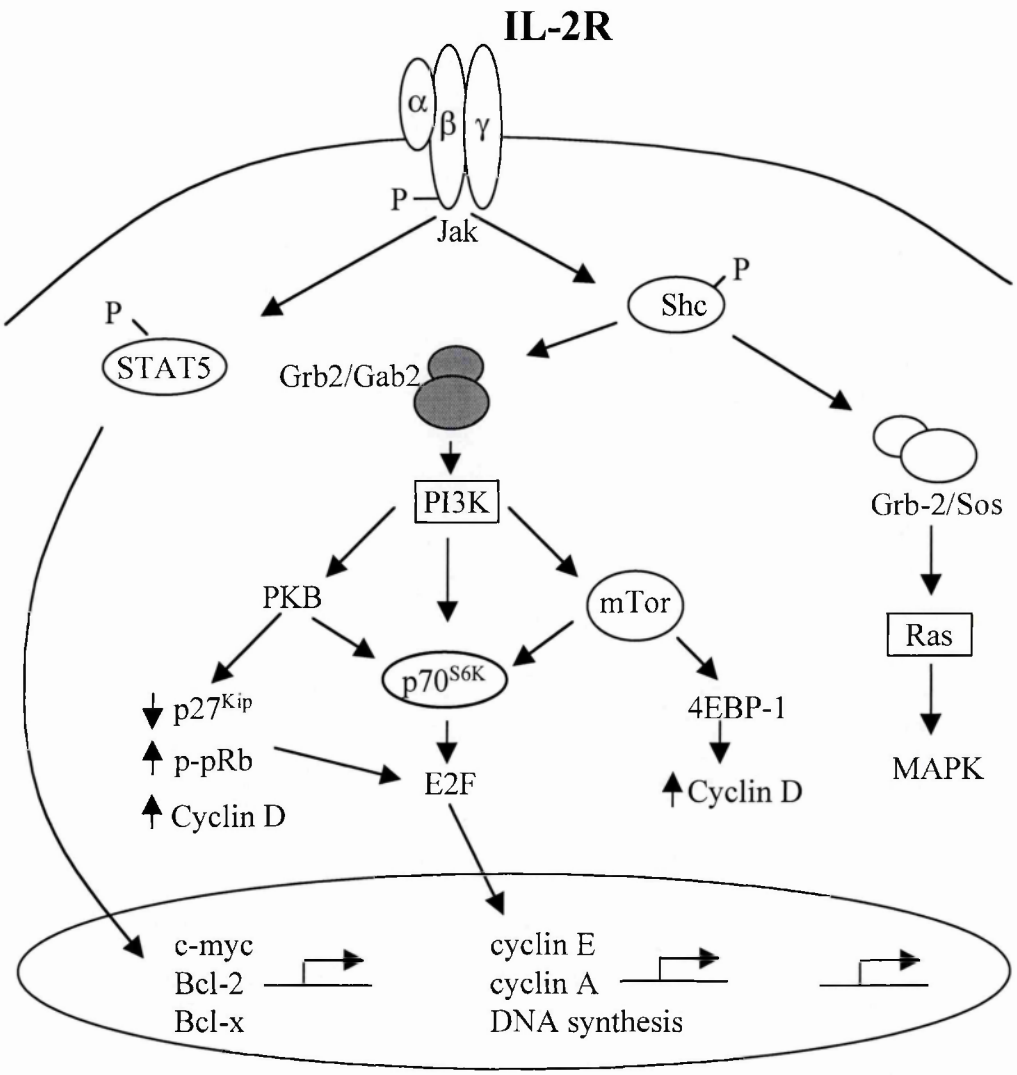


Figure VI. IL-2-dependent G1/S transition. Schematic representation of the IL-2-induced intracellular signalling pathways involved in T cell proliferation.

JAKs (177). The phosphotyrosines of Stat preferentially bind to Stat SH2 domains (178), causing Stat molecules to dimerize, release from the receptor, and entry into the nucleus. There, Stat5 dimers directly regulate transcription of target genes (176, 177). A critical role for Stat5 in IL-2-mediated mitogenesis has been suggested by the observation that TCR-stimulated T cells from mice lacking both the Stat5a and Stat5b genes have impaired proliferation in response to IL-2 (179). Moreover IL-2 activation of Stat5 has been suggested to be important for IL-2 induction of DNA synthesis (180).

Similarly to Stat 5, also the recruitment of the adapter Shc initiates a series of intracellular events. Thus, once recruited to phosphorylated IL-2Rβ chain, Shc recruits two important protein complexes: Grb2/Sos, which activates the Ras/Erk pathway, and Grb2/Gab-2, which activates the PI3K pathway (175, 181-183). Although the role of Ras/Erk signalling in the regulation of cell cycle progression is well established in non-lymphoid cells (184), its role in IL-2R-mediated proliferative response remains largely to be clarified. In contrast, PI3K-induced signalling have been largely characterized and shown to play a pivotal role in IL-2-dependent regulation of cell growth and proliferation (89). To exert these important functions, PI3K-mediated signals integrate with nutrients-sensitive pathways, and result in a tightly coordinated series of both transcriptional and translational activation events. Indeed, it has been shown that PI3K-dependent signals are required for the IL-2-induced upregulation of cyclin D3, for the downregulation of $p27^{Kip}$, and for the hyperphosphorylation of Rb and p130 (89). Moreover, PI3K activity has been shown to be necessary and sufficient for E2F transcriptional activity (89). Interestingly, while PI3K is sufficient for the induction of E2F

activation, it is not sufficient for DNA synthesis, and so for cell cycle progression into the S phase. This supports the idea that several signalling networks cooperate for cell cycle progression in T cells (89).

Following IL-2-dependent PI3K activation, the enzyme activates two serine/threonine kinases, PKB and the $p70^{S6k}$ (185). In addition, IL-2-induced PI3K signalling has also been shown to directly regulate the MAP kinase pathway, probably at the level of MEK (186).

Activation of both PKB and $p70^{S6k}$ is initiated by PI3K-induced translocation of these proteins to the plasma membrane, where they can get phosphorylated and activated by Ser/Thr kinases, such as PDK1 and PDK2 (187-190). Overexpression of a constitutive active form of PKB in T cell lines, is sufficient to drive E2F transcriptional activity, thus providing a link for PI3K to E2F activation (89). In addition, PKB directly phosphorylates Forkhead factors on several sites, causing their retention in the cytoplasm (191). These factors regulate transcription of the cyclin inhibitor $p27^{Kip}$ and of the pocket protein p130 (191, 192). PKB-dependent phosphorylation and thus retention in the cytoplasm, reduces the transcription of these inhibitors and promotes progression through the cell cycle. Finally, it has been shown that PKB can also directly regulate $p70^{S6k}$ activation (185).

$p70^{S6k}$ is one of the two isoforms encoded by the *S6K* gene, the other being $p85^{S6k}$. $p70^{S6k}$, the shorter isoform, is largely cytoplasmic and it is the most well studied, while $p85^{S6k}$, the larger isoform, appears to be exclusively nuclear, having an additional 23-aminoacid sequence residing at its amino terminus, which has been shown to function as a nuclear localization signal (NLS) (193-196). With the identification of a highly homologous gene product, S6K2, $p70^{S6k}$/$p85^{S6k}$ have been

collectively termed S6K1 (197). Activation of $p70^{S6k}$, results in the phosphorylation of the 40S ribosomal protein S6, and elicits the translation of 5' TOP (terminal oligopyrimidin tract) mRNAs (198). These mRNAs constitute a small family of abundant transcripts (up to 20% of cellular mRNA) that encode primarily ribosomal proteins and components of the translational apparatus (199). These are critically needed to satisfy the increased anabolic demand that occurs during the growth response (200). Since it has been demonstrated that the expression of many of the components of the protein synthetic apparatus is regulated at the translational level, as occurs in the case of 5' TOP mRNAs (201, 202), $p70^{S6k}$ happens to play a crucial role during cell growth and proliferation.

Activation of $p70^{S6k}$ not only depends on PI3K/PKB-induced signalling, but also on signalling induced by the serine/threonine kinase mTor (the mammalian target of Rapamycin), which, similarly to PI3K, plays a critical role in IL-2-driven T cell proliferation. Tor proteins were first identified in *Saccharomyces cerevisiae* through mutants that confer resistance to growth inhibition induced by Rapamycin (203). Yeast Tor proteins (Tor1/2) control a variety of processes contributing to cell growth, in response to nitrogen availability, including translational initiation and early G1 progression as well as the regulation of transcription, amino acid uptake, cytoskeletal organization, and protein degradation through autophagy (204).

mTor, the mammalian ortholog of Tor, is a member of the PIK (phosphoinositide kinase-related) family of kinases, and has homology to protein and lipid kinases (205). mTor is considered to be a central controller of cell growth, it is conserved throughout evolution, and regulates cellular responses to nutrient levels (particularly those of aminoacids) and growth factors (206). Although several

factors have been described to modulate mTor activity, the precise molecular mechanism responsible for the activation of this enzyme remains largely to be defined. For instance, mTor function seems to be regulated by the glucose, aminoacid and ATP levels (207, 208). Indeed, when aminoacids are limiting, uncharged tRNA pools accumulate in the cell and inhibit mTor signalling (207). Recently, two members of the nutrient sensing machinery, the tumour suppressor proteins of the tuberous sclerosis complex (TSC1 and TSC2) were identified as potential upstream negative regulators of mTor (209). Growth factor-dependent PI3K/PKB mediates mTor activation, probably through phosphorylation and inactivation of TSC2 (210). Similarly to TSCs proteins, Raptor (regulatory associated protein of mTor), a recently discovered 150 kDa protein containing HEAT and WD40 repeats, forms a nutrient-dependent complex with mTor, and regulates its enzymatic activity positively and negatively, perhaps depending on nutrients levels (211, 212).

Also the tumour suppressor protein p53 has been shown to inhibit mTor-dependent signalling, by a still unknown mechanism of action (213).

Finally, recent data indicate that, following growth factor stimulation, the p85 regulatory subunit of PI3K forms a ternary complex with mTor and $p70^{S6k}$, and by that it mediates $p70^{S6k}$ activation, providing another potential pathway for growth factor-dependent mTor signalling (214).

In addition to phosphorylation of the $p70^{S6k}$, mTor activation also elicits phosphorylation of the initiation factor 4E binding protein 1 (4EBP1) (215). While $p70^{S6k}$ regulates translation of Top mRNAs (see above), 4EBP1 mainly controls the translation of cap-containing mRNAs (206). Indeed, 4EBP1 is a translational

inhibitor that acts by binding and repressing the activity of the eukaryotic initiation factor 4E (eIF4E). mTor-dependent phosphorylation of 4EBP1 mediates its release from eIF4E, thus allowing the initiation factor to mediate translation of cap-containing mRNAs (206). eIF4E function is also important in the translation of mRNAs containing a highly structured 5' untraslated region (UTR), such as the transcripts encoding for the c-myc oncogene (216). This demonstrates that also 4EBP1 could play a very important role in the regulation of cell proliferation.

mTor is the only known target of the immunosuppressive drug Rapamycin. Rapamycin acts by associating intracellularly with the immunophilin FKBP12, and this complex negatively regulates mTor activity (217, 218). The use of Rapamycin has allowed to comparing the signalling initiated by nutrients, to the one initiated by growth factors. Indeed, Rapamycin blocks growth factors-dependent $p70^{S6k}$ activation to extents that are similar to the one found in limiting nutrient conditions (219).

In T cells, Rapamycin blocks IL-2-induced G1/S transition, and inhibits IL-2-dependent kinase activation of Cdk2 and Cdc2 (169), and downregulation of $p27^{Kip}$ (170). Moreover, as mentioned above, Rapamycin completely blocks IL-2-induced activation of $p70^{S6k}$ (185). Importantly, Brennan et al. (90) observed that the IL-2-dependent PI3K/PKB- pathway, which regulates E2F activity, was Rapamycin-sensitive, and was rendered Rapamycin-insensitive by the expression of a $p70^{S6k}$ Rapamycin-resistant mutant (90). Together these data indicate that, in T cells, $p70^{S6k}$ represents a link between IL-2, PI3K/PKB, and mTor signals for the regulation of E2F transcriptional activity, and that indeed $p70^{S6k}$ regulates a G1 checkpoint in T lymphocytes. However, expression of a $p70^{S6k}$ Rapamycin-resistant

mutant was not able to rescue Rapamycin inhibition of T cell cycle entry (90). This demonstrates that other pathways, controlled by Rapamycin, and independent from $p70^{S6k}$ activity (such as, for example, 4EBP1), could be involved in IL-2-mediated cell cycle regulation.

IL-2-independent G1/S transition

As mentioned above, TCR-dependent signalling is not sufficient to promote exit from the G1 phase of the cell cycle, an event that was historically attributed to IL-2-dependent signalling. However, in the past years, evidences for IL-2-independent T cell proliferation were obtained both *in vivo* and *in vitro*, and a new picture on the role of CD28-mediated costimulation in cell cycle regulation has emerged. Thus, several studies have indicated that CD28 costimulation, particularly at suboptimal levels of TCR engagement, increases the frequency of cells entering the proliferative pool and the number of mitotic events that responding cells undergo (220). This was previously attributed to the ability of CD28 to enhance IL-2 production. However, the observation that T cells from IL-2-deficient mice have reduced, but significant, proliferative T cell responses (221) support the existence of IL-2 independent T cell proliferation. In contrast to IL-2-deficient T cells, CD28-deficient T cells have dramatically impaired proliferative response. Moreover, while proliferation of the former can be fully restored by the addition of exogenous recombinant IL-2 (rIL-2) (222), proliferation of the latter is only partially increased by rIL-2 (25). This indicates that the failure of CD28-deficient cells to proliferate might not simply be due to the lack of IL-2 production, but rather to the absence of a CD28-dependent mitogenic signal. Accordingly, Ag/LPS-induced *in vivo*

expansion of Ag-specific T cells did not require IL-2, but CD28 expression on T cells (221). These findings, together with the observation that the massive T cell expansion observed in CTLA-4-deficient mice could not be correlated with increased IL-2 production (223), but rather to unlimited CD28-mediated costimulation, indicate that CD28-dependent signals might drive T cell proliferation directly and independently from IL-2.

Quite recently several reports have demonstrated a direct role of CD28 in cell cycle progression (224-227). Boussiotis and co-workers (226, 227) have shown that, *in vitro*, CD28 costimulation could induce downregulation of the CKI $p27^{Kip}$ via PI3K/PKB-dependent pathways in the absence of IL-2-mediated signalling. This CD28-mediated $p27^{Kip}$-downregulation resulted in the activation of cyclin D2-associated Cdk4/Cdk6 and cyclin E-associated Cdk2, and subsequent progression into the S phase. Thus, in the absence of IL-2, CD28 mediates initial entry into the cell cycle rendering the cells competent for progression into the S phase (226). Whether this would be sufficient *in vivo,* or require the synergy with cytokines other than IL-2 remains to be established.

1.6 $CD4^+$ T cell anergy

1.6.1 The establishment of T cell anergy: the role of costimulation

Antigen encounter in the presence of costimulation allows T lymphocytes to undergo intense and prolonged proliferation, ensuring that the initial small number of Ag-specific T cells is increased, and that the immune response is initiated. In contrast, antigen encounter in the absence of costimulatory signals, leads to T cell

activation, but not to T cell proliferation and induces T cells to enter a state of long-term antigen-specific unresponsiveness, defined as clonal anergy. This is characterized by the inability of the cells to produce IL-2, and to proliferate upon antigen restimulation (7), thus resulting in antigen-specific T cell tolerance.

Generally, tolerance to self-antigens is first established in the thymus (primary lymphoid organ) through negative selection of autoreactive T cell precursors (228, 229). However, some antigens are not represented in the thymus (developmentally regulated antigens), or do not reach the concentration required to effectively delete reactive T cells. In these instances, several peripheral mechanisms, such as ignorance, peripheral deletion, clonal anergy and suppression have evolved to regulate, and possibly inhibit, the activation of mature T cells potentially able to recognize self-antigens (and thus auto-reactive) (230). Among these mechanisms, antigen-specific clonal anergy has been proposed to play a major role in controlling peripheral tolerance to self antigens, and its disregulation has been involved in auto-immune manifestation, graft rejection and, in some instances, in tumour escape. Thus, many efforts were and are being made to understand the molecular mechanisms regulating the induction of clonal anergy to either induce it, or overcome it in the many different clinical conditions (auto-immunity, organ transplantation, tumours).

The term "clonal anergy" was originally created to describe the functional phenotype of B cells derived from mice that were immunized with a soluble antigen, which were shown to be unable of responding to antigen restimulation (231). Antigen-induced T cell clonal anergy was first demonstrated *in vitro*. Indeed,

Feldmann and co-workers (232) showed that the stimulation of T helper clones with low amount of the specific antigenic peptide, in the absence of autologous APCs, hampered the ability of the cells to further respond to APCs pulsed with the same antigen. These results suggested for the first time that professional APCs possessed special characteristics necessary for the productive presentation of antigen that T cells themselves lacked. It was indeed hypothesized that a second signal, distinct from the one generated by TCR-MHC/peptide interaction, was transduced from APC to the T cell, and the "two-signal" model of lymphocyte activation was proposed (7). The possibility that TCR-generated signals were not sufficient to drive optimal T cell activation was later supported by the observation that stimulation of $CD4^+$ T cells with purified MHC/peptide complexes immobilized in artificial lipid bilayers, (233), or with chemically fixed Ag-pulsed APCs (234), induced unresponsiveness rather than T cell activation. Moreover, it was shown that the addition of untreated syngeneic APCs to these cultures restored optimal T cell activation, thus supporting the existence of a second signal molecularly distinct from that mediated by TCR-MHC interaction (235).

Similar results were also obtained by stimulating $CD4^+$ T cell clones with immobilized anti-TCR, or anti-CD3 mAbs (236). In these cases, T cells were activated, but failed to produce IL-2 and to proliferate. Again, these cells resulted to be incapable of producing IL-2 upon optimal stimulation, mediated by either Ag-bearing APC or by anti-CD3 and anti-CD28 mAbs.

In contrast to IL-2, the production of IFN-γ or IL-4 seemed to be unaltered in anergic T cell clones. Moreover, anergic T cells remained viable for extended period of time and showed to be capable of proliferating to exogenous IL-2 (237),

thus suggesting that anergic cells failed to proliferate upon rechallenge because of a highly specific defect in IL-2 production.

Even though clonal anergy was first described *in vitro*, several evidences are now in place to support the idea that Ag stimulation of T lymphocytes, in the absence of costimulation, results in anergy induction also *in vivo*. For instance, the injection of bacterial superantigens (BSAgs), which bind to the lateral surfaces of TCRβ polypeptides and MHC glycoproteins, and is believed to be insensitive to CD28 costimulation, was shown to lead to potent activation and proliferation of a large proportion of T cells, but also to cause their rapid cell death, and the persistence of unresponsive T cells. Indeed, when optimally restimulated *in vitro*, these cells were unable to respond to antigenic rechallenge resembling the cells anergized *via* CD3-mediated stimulation (238-240). The establishment of clonal anergy has also been demonstrated in mice adoptively transferred with a monoclonal population of TCR transgenic antigen-specific $CD4^+$ T cells, and immunized with the specific Ag in the absence of adjuvant, which is required for the upregulation of costimulatory ligands on the surface of APC (241). In this model, intravenous injection of soluble antigen induced a transient accumulation and loss of adoptively transferred TCR transgenic T cells, among which some survived and persisted for several months but remained profoundly unresponsive to antigenic restimulation (221, 241-243).

1.6.2 Molecular features of anergic $CD4^+$ T cells

Several efforts have been made to characterize the molecular mechanisms that are in place in anergic T cells, and that prevent their activation upon optimal restimulation. The observation that anergic T cells seem to be selectively unable of producing IL-2 has generated intense investigation on the molecular mechanisms responsible for this functional defect. Data obtained in different laboratories throughout the years, and mostly regarding anergic T cell clones, have altogether demonstrated that anergic cells are unable to transcribe the mRNA coding for IL-2, because of molecular defects at different levels along the TCR-induced intracellular signal transduction pathways.

Kang et al. (244) first demonstrated that the failure to transcribe the *IL-2* gene in anergic T cell clones was associated with defective AP-1-dependent DNA binding and transactivation. This was later shown to be due to the defective induction of the AP-1 family members c-Fos, FosB, and JunB proteins, which impaired DNA-protein interaction and transactivation at both AP-1 and NFAT DNA enhancer elements (245). Defective TCR and CD28 induced AP-1 protein expression correlated with defective activation of the Ras pathway in these cells (246), and with reduced phosphorylation and catalytic activity of members of the MAPK family, such as Erk1, Erk2 and JNK (247). In addition to AP-1, the altered expression of NF-kB transcription factors was also reported in anergic T cells *in vivo* (248). Furthermore, data against a loss-of-function signalling defect as the sole basis for clonal anergy induction were generated, which demonstrated the presence of a dominant-acting repressor molecule that inhibits signal transduction to the *IL-2*

gene within viable anergic T cells (249-251). Indeed, Powell et al. (250) showed that the DNA binding activity of the cAMP-response element-binding protein/cAMP-response element modulator (CREB/CREM) transcriptional inhibitory complex to enhancer elements in the *IL-2* gene was increased in anergic T cells. Moreover, Telander et al. (251) demonstrated that the fusion of anergic murine T cells to human Jurkat T leukaemia cells, and formation of heterokaryons failed to result in a complementation of the signalling defect of anergic cells and restoration of murine IL-2 mRNA inducibility. Instead, signal transduction to the human *IL-2* gene became disrupted, demonstrating the existence of a negative regulatory factor in anergic cells.

In support of this, it has been shown that anergy induction is inhibited by the addition, at the time of the anergizing stimulus, of Cyclosporin A and cycloheximide, demonstrating that the establishment of anergy is an active mechanism, which requires TCR-induced signalling, and new protein synthesis (233, 236, 252).

Defective *IL-2* gene transcription might be due to altered upstream TCR-induced signalling events, which have been reported in anergic T cells. Gajewski et al. (253) have revealed increased basal levels of intracellular free calcium and phosphatidylinositol 1,4,5-triphosphate (IP3) in anergic T cells compared to control cells, and showed that these levels failed to increase significantly upon subsequent restimulation. These authors also reported an alteration in net tyrosine kinase activity in anergic cells, with preferred activation of the Src-family PTK Fyn (253). Quill et al. (254) also found a constitutively reduced amount of Lck, and constitutively elevated levels of Fyn in anergic T cell clones. These data might

suggest that an unbalanced TCR-linked Lck and Fyn kinases activation might determine the fate of T cell activation. Thus, while Lck might be the predominant kinase involved in productively activated cells, Fyn might dominate the response in anergic T cells (255).

Selective TCR-induced intracellular events have not only been described when comparing responsive and unresponsive (anergic) T cells, but also when analyzing the intracellular signalling of optimally (TCR/CD28) or suboptimally (TCR, ionomycin, altered peptide ligands) stimulated cells. For instance, stimulation of T cells by altered peptide ligands resulted in suboptimal phosphorylation of the TCRζ chain, and lack of association with ZAP-70 (256, 257). Moreover, while TCR engagement in the absence of costimulation was shown to result in preferential association of the TCR/CD3 complex with Fyn, co-ligation of the TCR and CD28 resulted in the recruitment of Lck (258).

These data, together with the observation that the establishment of the anergic phenotype requires TCR-generated signalling and new protein synthesis, suggest that anergy induction could represent an alternative type of activation. This could be initiated by selective signalling pathways resulting in a unique gene expression profile, which eventually accounts for the maintenance of the anergic phenotype.

Recently, Macián and co-workers (259) have compared the gene expression profile of control and ionomycin-induced anergic T cells. They found that, during ionomycin stimulation (anergy induction), a limited set of genes, distinct from those activated following productive stimulation, was induced. In particular, these authors demonstrated that ionomycin-induced anergy is mediated primarily by NFAT-

dependent expression of a new set of genes, which are independent of NFAT/AP-1 cooperation, and which include genes encoding phosphatases, proteases, and transcriptional repressors (259). They defined these genes as anergy associated genes, and further proved their unique expression also upon *in vivo* anergy induction. Similarly, Soares and co-workers (260), examined early changes in gene expression after TCR-induced signalling in the presence (activation) or absence (anergy) of B7-mediated costimulation, and found that some genes were uniquely expressed in anergizing stimulation. Among these, for instance, is the ubiquitin E3 ligase GRAIL (gene related to anergy in lymphocytes), whose activity correlated with the inhibition of cytokine gene expression, and which was uniquely expressed in T cells activated in the absence of costimulation (260).

In addition to *IL-2* gene transcription, some reports have indicated that the posttranscriptional regulation of IL-2 is differentially regulated by anergizing stimuli. Indeed, Garcia-Sanz et al. (261) have shown that, following ionomycin stimulation, IL-2 mRNA was transcribed to somehow detectable levels but failed to associate with active ribosomes, and thus failed to be translated. These authors also showed that in anergic cells, IL-2 mRNA remained detectable but failed to be loaded on ribosomes upon restimulation (261).

Thus, many evidences indicate that the absence of T cell costimulation, at the time of T cell activation, might be responsible for the induction of selective intracellular signalling events dictating the establishment of peculiar genetic programs that later regulate antigen responsiveness.

1.6.3 Anergy and proliferation

Several *in vitro* and *in vivo* evidences suggest that, in some instances, T cell anergy can also be induced in the presence of costimulation. It was shown that clonal anergy could be induced in the presence of both TCR- and CD28-mediated T cell activation, by preventing IL-2/IL-2 receptor interaction (262), or IL-2-dependent T cell proliferation (263). Sloan-Lancaster et al. (256) similarly reported that partial agonists caused T cell activation, but failed to elicit proliferation, in spite of the presence of CD28 costimulation, and induced clonal anergy. Similarly, TCR/CD28 optimally stimulated T cells became unresponsive when *n*-butyrate (264) or Rapamycin (265), which block G1-to-S transition, were provided at the time of stimulation. In all these conditions, the addition of exogenous IL-2 induced extensive T cell proliferation and resulted in either prevention or reversal of clonal anergy (263, 266).

These data supported the possibility that negative regulatory factors could be normally produced, but also degraded during IL-2-driven G1-to-S phase transition. In the case that IL-2 is not produced (suboptimal T cell stimulation), or IL-2 driven proliferation is prevented, these anergic factors might accumulate inside the cells and inhibit subsequent T cell responses (263, 267). The addition of exogenous IL-2 could drive extensive T cell proliferation, dilution or degradation of the anergic factors, and by that it could restore antigen responsiveness. A number of reports have indicated the G1 cyclin-dependent kinase inhibitor $p27^{Kip}$ as a putative anergic factor, since its deregulated expression directly correlated with T cell unresponsiveness (268, 269). Boussiotis and co-workers (268) showed that sub-

optimal stimulation with costimulatory deficient APC, which results in T cell anergy, failed to down-regulate $p27^{Kip}$, and elicited cell cycle arrest in early G1. Moreover, over-expression of $p27^{Kip}$ not only prevented G1-to-S transition upon optimal stimulation (269), but also inhibited *IL-2* transcription (268). Thus, deregulated expression of $p27^{Kip}$, or more generally the failure to progress from the G1 to the S phase of the cell cycle, might result in the establishment and maintenance of the anergic phenotype.

1.7 Aim of the project

Anergic T cells fail to transcribe the *IL-2* gene, and to proliferate, and are specifically arrested in the G1 phase of the cell cycle. It has been suggested that the failure of these cells to respond to optimal stimulation could be attributed to the presence of a putative anergic factor, uniquely expressed by the engagement of the TCR in the absence of proliferation. This model predicted that forcing proliferation, or G1 to S transition of anergic T cells would lead to dilution/degradation of the putative anergic factor, and by that it would restore the ability of the cells to properly respond to antigenic rechallenge.

Several reports have recently indicated that optimal engagement of the TCR and of CD28 drives IL-2 independent cyclin/Cdks activation, $p27^{Kip}$ degradation, and thus G1-to-S phase transition.

We thus set out to investigate whether optimal engagement of the TCR and of CD28 on the surface of anergic T cells, could force cell cycle entry, and T cell

proliferation. This would allow the dilution/degradation of the putative anergic factor and the reversal of clonal anergy.

2. Material and Methods

2.1 Mice and cells

Mice

B10.BR (H-2^k) mice were purchased from Harlan Laboratories (Milan, Italy) and used as donors of splenic APCs for the maintenance of the A.E7 Th clone. BALB/c mice (H-2^d) (Charles River Breeding Laboratories) were used as donors of splenic APCs for the maintenance of DO11.10 T cell lines, and for the stimulation of DO11.10 primary T cells. C57BL/6 mice (H-2^b) (Charles River Breeding Laboratories) were used as donors of splenic APCs for the stimulation of OT-1 primary T cells.

DO11.10 mice (H-2^d) were obtained from Dr. Luciano Adorini (Bioxell, Milan, Italy). These mice express a transgenic (Tg) αβ TCR specific for the CD4-restricted chicken ovalbumin (OVA) derived peptide ($OVA_{323-339}$.) on the majority of the $CD4^+$ T cells (270). OT-1 mice (H-2^b) were generously provided by Dr. Matteo Bellone (DIBIT, Milan). These mice express a Tg αβ TCR specific for the CD8 restricted OVA peptide ($OVA_{257-264}$) on the majority of the $CD8^+$ T cells (271). The transgenic mice were bred and maintained in the SPF animal facility according to the Institutional Guidelines. The animal usage was approved by the Ethical Committee of the San Raffaele Scientific Institute.

Cells

The A.E7 T cell clone is a H-2^K-restricted Th1 clone, specific for the pigeon cytochrome *c* derived 81-104 peptide (PCC_{81-104}) (272). A.E7 cells ($0.5x10^6$ T cells/well/24 well plates) were maintained in RPMI medium supplemented with 2 mM L-glutamine, 100 U/ml streptomycin, 100 U/ml penicillin, 20 mg/ml gentamicin, and 50 μM β-mercaptoethanol (β-ME) (Invitrogen Life technologies, Milan, Italy), and 5% heat-inactivated fetal bovine serum (FBS, Euroclone, Milan, Italy) (complete RPMI medium) at 37°C in a 5% CO_2 atmosphere, by periodic stimulation with irradiated syngeneic splenocytes ($3.5x10^6$ cells/well) pulsed with PCC_{81-104} (0.5μM) (Primm, Milan, Italy). Forty-eight hours (h) after antigenic stimulation, exogenous recombinant IL-2 (rIL-2; 10 IU/ml) (Roche, Milan, Italy) was added to the cultures.

The OVA specific DO11.10 and IL-$2^{-/-}$ DO11.10 T cell lines were generated by stimulating LN cells, respectively derived from DO11.10 and DO11.10/IL-$2^{-/-}$ mice (221), with irradiated syngeneic splenocytes and $OVA_{323-339}$ (0.5 μM) (Primm). The cells ($0.5x10^6$ T cells/well/24 well plates) were maintained in complete RPMI medium, and stimulated weekly with Ag and BALB/c splenocytes ($3.5x10^6$ cells/well), and rIL-2 (10 IU/ml) . The peptides used to stimulate the cells were commonly referred to as "antigen" (Ag) in the text. All the experiments were performed at least 10 days from the last antigen exposure. At this time, the cells appeared to be in the G0/G1 stage of the cell cycle.

Primary T cell cultures

Single cell suspensions were derived from peripheral LNs of 6-8 weeks-old DO11.10 and OT-I TCR Tg mice, by smeshing the organs on sterile nylon filters (Falcon, Milan, Italy).

In selected experiments, $CD4^+$ T cells were purified from the DO11.10 LN suspension. Purification was performed by negative selection (Dynal Biotech LTD., UK). Briefly, LN cells ($10x10^6$ cells/100µl) were incubated 1h at 4°C, with anti-B220 (clone TIB 146), anti-CD8 (clone 53.6.72), anti-I-A/I-E (clone 2G9), and anti-MAC-1 (clone M1/70) rat Abs (final concentration of each Ab, 10 µg/ml), in RPMI medium containing 2% FBS, and 5 mM EDTA (Sigma, Milan, Italy). The cells were then washed and resuspended in complete RPMI medium in the presence of goat anti-rat-coated magnetic beads ($20x10^6$ beads/$10x10^6$ cells), and incubated under rocking 20 min 4°C. Beads-coated cells were then removed using the Dynal magnet (Dynal Biotech LTD.). The purity of the preparation was analyzed by flow-cytometry after staining of the cells with anti-CD4 mAb (BD Pharmingen, Milan, Italy). In all the experiments $CD4^+$ cells were selected to a purity of >95%.

2.2 Anergy induction

Where indicated A.E7 cells were cultured for 16 hours on plate bound anti-CD3-ε mAb (clone 145-2C11, 4µg/ml) (273) ($25\text{-}30x10^6$ cells in 150 mm dishes). The cells were then removed from the Ab, and rested for additional 5 days in fresh medium (referred to as "anergic" T cells) (262). At the same time a similar number of A.E7 cells was harvested and rested in fresh medium (referred to as "control" T

cells). Both control and anergic cells appeared to be in the G0/G1 phase of the cell cycle. Thereafter, viable cells were separated on a Lympholyte-M (Cederlane, Hornby, Ontario, Canada) density gradient and restimulated as indicated in the different experiments.

2.3 T cell activation

Where indicated control and anergic A.E7 cells were stimulated with Ag (0.5 μM) and irradiated syngeneic splenocytes (1:4, T:APC ratio), with immobilized anti-CD3 mAb (2 μg/ml) and anti-CD28 mAb (clone 37.51) (5 μg/ml) (274), or with rIL-2 (10 IU/ml), in the absence or in the presence of Rapamycin (200 nM, Calbiochem, Merck, Milan, Italy), LY294002 (20 μM, SIGMA, Milan, Italy), or Cyclosporin A (0.5 μg/ml, Sandimmun; Novartis, Milan, Italy). Thereafter the cells were removed from the Abs, rested in fresh medium for 10 days, and then re-stimulated with Ag/APC, or with rIL-2. At the time indicated in each experiment, the cells were either left untreated or restimulated as indicated in the figures, and analyzed by flow cytometry or by Western Blot analyses.

Wild type and IL-$2^{-/-}$ DO11.10 T cells were stimulated for the indicated periods of time with immobilized anti-CD3 mAb (0.1 μg/ml) and anti-CD28 mAb (5 μg/ml), or with immobilized anti-CD3 mAb (0.01 μg/ml) and rIL-2 (10 IU/ml), and then analyzed by flow cytometry, or by Western blot (see below).

2.4 T cell differentiation

DO11.10 and OT-1 LN cells ($0.5x10^6$ $CD4^+$ or $CD8^+$ TCR Tg T cells/well/24 well plates) were cultured for 3-7 days in RPMI complete medium in the presence of irradiated syngeneic splenocytes ($3.5x10^6$/well) and either the CD4-restricted or the CD8-restricted peptide ($OVA_{323-339}$ and $OVA_{257-264}$, respectively, 0.5 μM) (referred to as Ag in the text). Rapamycin (100 nM) was provided to some of the wells at the time of Ag stimulation.

In some experiments, purified DO11.10 $CD4^+$ T cells were stimulated for 7 days with latex beads (diameter around 5 μM) (Interfacial Dynamic Corporation, Oregon) coated with anti-CD3 (0.05 μg/ml) and anti-CD28 (5μg/ml) mAbs ($2.5x10^6$ beads/1 $x10^6$ cells). Coated beads were prepared as follows: beads were incubated with the antibodies in PBS (pH 7.4-7.6; Euroclone, UK) at a concentration of $20x10^6$ beads/ml, for 20 min at 4°C, under rocking. The beads were then washed twice in PBS containing 2% FCS, and resuspended in RPMI complete medium containing 5% FCS, under rocking for 30 min at room temperature (RT). The beads were eventually pelleted and added to the cells.

Where indicated purified $CD4^+$ DO11.10 T cells cells were cultured with anti-CD3/CD28 coated beads, in Th1 or Th2 polarizing conditions. Briefly, rIL-12 (0.5 ng/ml, Bender MedSystem, Milan, Italy), and neutralizing anti-IL-4 mAb (5 μg/ml, BD Pharmingen), or rIL-4 (1 ng/ml, Bender MedSystem), and neutralizing anti-IL-12 mAb (5 μg/ml, BD Pharmingen) were provided to the cultures at the time of stimulation.

2.5 Western blot analysis

Analysis of cell cycle proteins

Control and anergic A.E7 cells were stimulated with anti-CD3/CD28 mAbs (2 mg/ml and 5 mg/ml respectively), or with rIL-2 (10 IU/ml) for 30 min. The cells were then harvested, washed twice with ice cold PBS, and lysed in Lysis buffer 1, containing 50 mM Tris-HCl pH 7.4, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin (Sigma, Milan, Italy). Samples were incubated in the lysis buffer for 20 min on ice. The soluble fraction was separated by centrifugation at 13,000 rpm for 15 min at 4°C and the protein content was determined by the Bradford assay (Bio-Rad, Milan, Italy). Samples containing an equal amount of proteins (10-20 mg) were mixed with an equal volume of 2X Laemmli buffer (275), boiled, and separated on a standard SDS-PAGE. Proteins were then transferred onto nitrocellulose membranes (Protran, Schleicher & Schuell, Milan, Italy). Non-specific binding sites were blocked by incubating the membranes in PBS containing 5% non-fat dry milk and 0.05% Tween-20. Immunodetection was accomplished by incubating the membranes with primary antibodies (1 μg/ml) first, and then with horseradish peroxidase-conjugated secondary antibodies directed against mouse IgG or rabbit IgG (1:5000 dilution) (Amersham Biosciences, Milan, Italy) diluted in PBS pH 7.4, containing 1% dry non fat milk, 1% bovine serum albumine (Sigma) and 0.1% Tween-20. The immunocomplexes were then detected by chemiluminescence (SuperSignal, West Dura Extended Duration Substrate, Pierce, Milan, Italy). Expression of cyclin D3, $p27^{Kip}$, $p21^{Cip}$ was analyzed by 12% SDS-PAGE (acrylamide:bis-acrylamide 29:1;

Euroclone, UK). Expression and phosphorylation of Rb were analyzed by 6% SDS-PAGE (acrylamide:bis-acrylamide 29:1) as described (268). The following antibodies were used: cyclin D3 (C-16), $p27^{Kip}$ (C-19), $p21^{Cip}$ (F-5), and Rb (C15) antibody (Santa Cruz Biotechnology, Milan, Italy).

Analysis of $p70^{S6k}$ phosphorylation

Control and anergic A.E7 cells were stimulated with anti-CD3/CD28 mAbs (2 μg/ml and 5 μg/ml respectively), or with rIL-2 (10 IU/ml) for 30 min. Where indicated the cells were pre-treated with Rapamycin (200 nM) for 30 min at 37°C, and then stimulated in the presence of the drug. The cells were then harvested, washed twice with ice cold PBS, and lysed for 20 minutes on ice in Lysis buffer 2, containing 50 mM Tris-HCl pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 1 μg/ml PMSF, 1 μg/ml aprotinin, 5 μg/ml leupeptin, 1mM NaF, 1 mM $NaOv_3$ (Sigma). Protein extracts were quantified by the Bradford assay. Where indicated, the samples were treated for 1h at 37°C with alkaline phosphatase (2 U enzyme /25 μg of extract) (Sigma). Samples containing an equal amount of proteins (20-40 μg) were mixed with an equal volume of 2X Laemmli buffer, boiled, and separated on a standard 10% SDS-PAGE (acrylamide:bis-acrylamide 10:0.16) (Sigma), as previously described (90). Western Blot analysis was performed as described above, by using an anti-$p70^{S6k}$ (C-18) Ab (0.5 μg/ml) (Santa Cruz Biotechnology).

For the analysis of phospho-Thr^{389}-$p70^{S6k}$, control and stimulated T cells were harvested, washed twice with ice cold PBS, and lysed in SDS sample buffer, containing 62.5 mM Tris-HCl pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT,

0.01% w/v bromophenol blue (Sigma). The extracts were sonicated for 10-15 seconds to shear DNA and reduce sample viscosity, and then boiled for 5 minutes. Proteins from $2x10^6$ cells were separated on a standard 10% SDS-PAGE (Acrylamide:bis-acrylamide 10:0.16), transferred onto nitrocellulose membranes, and analysed by Western blot with an overnight incubation at 4°C with the primary anti-phospho-$p70^{S6k}$ (Thr^{389}) Ab (1:1000) (Cell Signaling Technology, Milan, Italy).

Where indicated, protein bands were quantified by densitometric analysis using the Image Quant analysis software (Amersham Biosciences).

2.6 2-D electrophoresis (2DE)

Cells were washed extensively with ice cold PBS, and lysed in Lysis buffer 2. 400 μg of total proteins were resolved onto preparative 10% SDS-PAGE (acrylamide:bis-acrylamide 10:0.16). The portion of the gel corresponding to the $p70^{S6k}$ (revealed by Western blot analysis of an electrotransferred lane from the same gel) was excised, and the contained proteins were eluted by an overnight incubation with 0.1% SDS. Proteins were then precipitated using 80% acetone. Where indicated, eluted proteins were concentrated by Vivaspin Concentrator (30000 MWCO) (Sartorius, Germany), treated for 1h at 37°C with alkaline phosphatase (2 U/25 μg protein), and then heated for 15 minutes at 65°C. Eventually, samples were acetone precipitated. The pellet was resuspended in 2DE buffer (9 M Urea, 10 mM Tris, 4% CHAPS, 65 mM DTT, 2% IPG buffer ampholine pH 3-10, protease inhibitor cocktail; Sigma), and samples were applied to 7 cm IPGstrips pH 3-10NL (Amersham Biosciences) by in-gel rehydration at

20°C. Focusing was performed with an IPGphor system (Amersham Biosciences) at 50 μA max per IPG strip with a gradient voltage (5000 V max) for a total of 25 KVh. Strips were equilibrated for 15 min in 50 mM Tris-HCl buffer pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS and 2% DTT, then for 15 min in the same buffer replacing DTT by 2.5% iodoacetamide. The strips were then transferred onto 12% acrylamide SDS-PAGE gels for the second dimension separation. 2-D gels were transferred onto nitrocellulose membranes and analyzed by Western blot with the anti-$p70^{S6k}$ Ab (see above). Densitometric analyses of protein spots were performed by using the Image Master 2D Elite analysis software (Amersham Biosciences).

2.7 Analysis of T cell proliferation

[^{3}H]-Thymidine incorporation assay

Control and anergic A.E7 T cells were stimulated in flat-bottomed 96-well plates ($2x10^4$ cells/well) with increasing amounts of the PCC-derived peptide and irradiated syngeneic splenocytes ($5x10^5$ cells/well), with immobilized anti-CD3 mAb (2 μg/ml) and anti-CD28 mAb (5μg/ml), or with rIL-2 (10 IU/ml), until the total time of culture reached 48 h. Then, [^{3}H]-Thymidine (1 mCi/well) was added, and the plates were harvested and counted 16 hr later.

Propidium iodide staining

T cells were stimulated as indicated in the figures, washed with PBS, counted and fixed overnight at 4°C in 70% ethanol. Cells were then pelleted,

resuspended at a concentration of $1x10^6$ cells/ml in DNA staining buffer containing 0.1% sodium citrate, 0.05% Nonidet P-40, 50 μg/ml propidium iodide, and 50 μg/ml Rnase A (Sigma), and incubated for at least 1 h at room temperature. PI contents were assessed by flow cytometry (FACScan, Becton Dickinson, Milan, Italy) using standard Cell Quest™ acquisition/analysis software.

Analysis of CFSE dilution

T cells were washed twice with PBS, and resuspended at a density of $2x10^7$ cells per ml in PBS. An equal volume of a PBS solution containing 1.25 μM of the fluorescent dye 5-(and 6-) carboxy-fluorescein diacetate, succinimidyl ester (CFDASE; Molecular Probes, Inc., Milan, Italy) was added, and the cells were gently mixed for 8 min at room temperature. In the case of A.E7 T cells, the final concentration of CFDASE used was 2.5 μM. Unbound CFDASE, or the deacetylated form, CFSE, was quenched by the addition of an equal volume of FBS. The labelled cells were washed twice in complete medium, and stimulated as indicated in the figures. When indicated, at the time of harvest, CFSE-labelled cells were washed twice in PBS, and the vital dye TO-PRO-3 (Molecular Probes, Inc.) was added to each sample (1 nM final concentration) before acquisition to distinguish live and dead cells. Cell division analysis was performed on a Becton Dickinson System (Milan, Italy) FACSCalibur® dual-laser cytometer using standard Cell Quest™ acquisition/analysis software. CFSE-labelling remained stable for up to three weeks in our cell cultures.

2.8 Analysis of cytokine production

ELISA assay

The presence of IL-2 in culture supernatants of activated T cells was determined 24 h after stimulation by standard capture ELISA assay (BD Pharmingen). Functional assay was performed in 96-well plates using $1x10^5$ cells per well. The ELISA standard curve was generated using recombinant murine IL-2 (BD Pharmingen), and the level of detection was 1 ng/ml.

Intracellular cytokine staining

The cells ($0.5x10^6$ cells/ml) were either left untreated or stimulated for 4h with PMA (Phorbol 12-myristate 13-acetate) (50 ng/ml) and Ionomycin (1μg/ml) (Sigma). Following surface staining with anti-CD4 or anti-CD8 mAb, the cells were fixed in 2% formaldehyde (Sigma) for 20 min at RT, then permeabilized by washing them in saponin buffer, containing PBS, 2% FBS, 0.2% sodium azide, 2% rat serum, and 0.5% saponin (Sigma). After fixation the cells were incubated for 20 min in saponin buffer either with isotype control, or with anti-IL-2, anti-IFN-γ, or anti-IL-4 mAbs (BD Pharmingen). The cells were then washed twice with saponin buffer, and twice with staining buffer, and analyzed by flow cytometry.

2.9 *In vitro* cytotoxic assay

OT-1 cells were tested for cytolytic activity in a standard 4 h ^{51}Cr release assay (276). RMA cells, a H-2^b Rausher virus-induced thymoma (277), were used

as target cells. Target cells were labelled with ^{51}Cr, and left unpulsed or pulsed with 1 μM $OVA_{257-264}$ for 30' in ice-cold RPMI complete medium. After extensive washing, target cells (1000 target/well) were seeded with OT-1 cells at effector to target ratios ranging from 6:1 to 50:1. The percentage specific ^{51}Cr release of triplicates was calculated as [(average experimental cpm - average spontaneous cpm)/(average maximum cpm-average spontaneous cpm)] x 100. ^{51}Cr release of target cells alone (spontaneous release) was always < 25% of maximal ^{51}Cr release (target cells in 0.25 M HCl).

2.10 Analysis of surface markers expression

At the indicated time, the cells were harvested and stained with anti-CD4 mAb (Pharmingen). In the indicated experiments T cells were also stained with anti-CD69, CD25, CD44, CD62L, and CD132 (common γ_c) mAbs (Pharmingen). The cells were incubated in staining buffer (PBS, 2% FBS, 0.2% sodium azide) with the Ab for 15 minutes on ice. The cells were then analyzed by flow cytometry (FACScan, Becton Dickinson) using standard Cell Quest™ acquisition/analysis software.

2.11 RNase Protection assay (RPA)

DO11.10 LN cells were stimulated for a week with Ag and irradiated splenocytes in the absence or in the presence of Rapamycin (see section 2.4). Thereafter the cells were harvested and either left untreated or restimulated with

PMA (50 ng/ml) and Ionomycin (1 mg/ml) for 1-4 h. Total RNA was then obtained using the Total RNA Isolation Reagent (TRIZOL, Life Technologies, Milan, Italy). Briefly, $10x10^6$ cells were lysed in 500 μl TRIZOL, for 5 minutes at RT. Total RNA was extracted by phase separation with chloroform, precipitated with isopropyl alcohol, rinsed in ethanol and then solubilized in water. The presence of the messages for different cytokines in the cells was analyzed using the multi-probe RNase protection assay (RPA) as previously described (278-280). Briefly, probe synthesis was driven by T7 bacteriophage RNA polymerase with -[^{32}P]UTP as the labelling nucleotide. The subsequent steps of probe purification, RNA-probe hybridization, Rnase treatment, purification of protected RNA duplexes, and resolution of protected probes by denaturing PAGE were performed as described (278). Probe bands were visualized by autoradiography, and were quantified by phosphor imaging analysis, using the Image Quant analysis software (Amersham Biosciences). The relative abundance of mRNA species was obtained dividing the values of each specificity by the relative L32 value of the same sample, in order to obtain a relative expression value for each individual mRNA species. The expression of IL-2, IFN-γ and IL-4 mRNAs in each group of cells and in the different stimulation conditions was displayed as fold induction over the unstimulated condition (set as 1).

3. Results

3.1 mTor signalling regulates $CD4^+$ T cell antigen responsiveness

3.1.1 Optimal engagement of CD3/CD28 bypasses G1 cell cycle block and drives proliferation of anergic T cell

The engagement of the TCR in the absence of either costimulation or cell proliferation, elicits a state of T cell unresponsiveness defined as clonal anergy. Upon optimal restimulation anergic T cells fail to produce IL-2 and to proliferate, and appear to be selectively blocked in the G1 phase of the cell cycle (7, 35, 36, 268, 269).

Starting from the observation that TCR- and CD28-generated signals mediate G1-to-S phase transition via IL-2-independent mechanisms (224, 226, 227), we set out to investigate whether a combination of anti-CD3 and anti-CD28 mAbs would overcome the G1 cell cycle block reported for anergic T cells, and by that would cause reversal of clonal anergy.

T cell anergy was induced in cloned $CD4^+$ T helper (Th) lymphocytes by chronic TCR engagement in the absence of costimulation as previously described (262). Non-proliferating A.E7 T cells were harvested and rested in fresh medium (referred to as "control" T cells), or cultured for 16 hours on plate bound anti-CD3 mAb, removed from the Ab and rested for additional 5 days in fresh medium (referred to as "anergic" T cells) (Fig. 1A). At the end of the resting time control and anergic T cells appeared to be in the G0/G1 phase of the cell cycle (refer to Fig.

Figure 1. Chronic TCR engagement results in the establishment of T cell clonal anergy. **A)** Schematic representation of anergy induction. **B)** Control (CT) and CD3-treated (anergic, AN) A.E7 T cells were challenged with irradiated splenic APC and the indicated amount of the pigeon cytochrome c derived peptide (PCC) (Ag/APC), with immobilized anti-CD3/anti-CD28 mAbs (2 and 5 μg/ml, respectively) (CD3/CD28), or with recombinant IL-2 (rIL-2) (10 IU/ml). After 48h, [^{3}H]-Thymidine was added to each well, and the cells were harvested and assayed for proliferation after an additional 16h of culture. **C)** IL-2 production was measured in the supernatants of CD3/CD28-stimulated T cells after 24 hours of culture by capture ELISA. The standard deviations of triplicate cultures are reported. The figure shows one of several (>5) independent similar experiments.

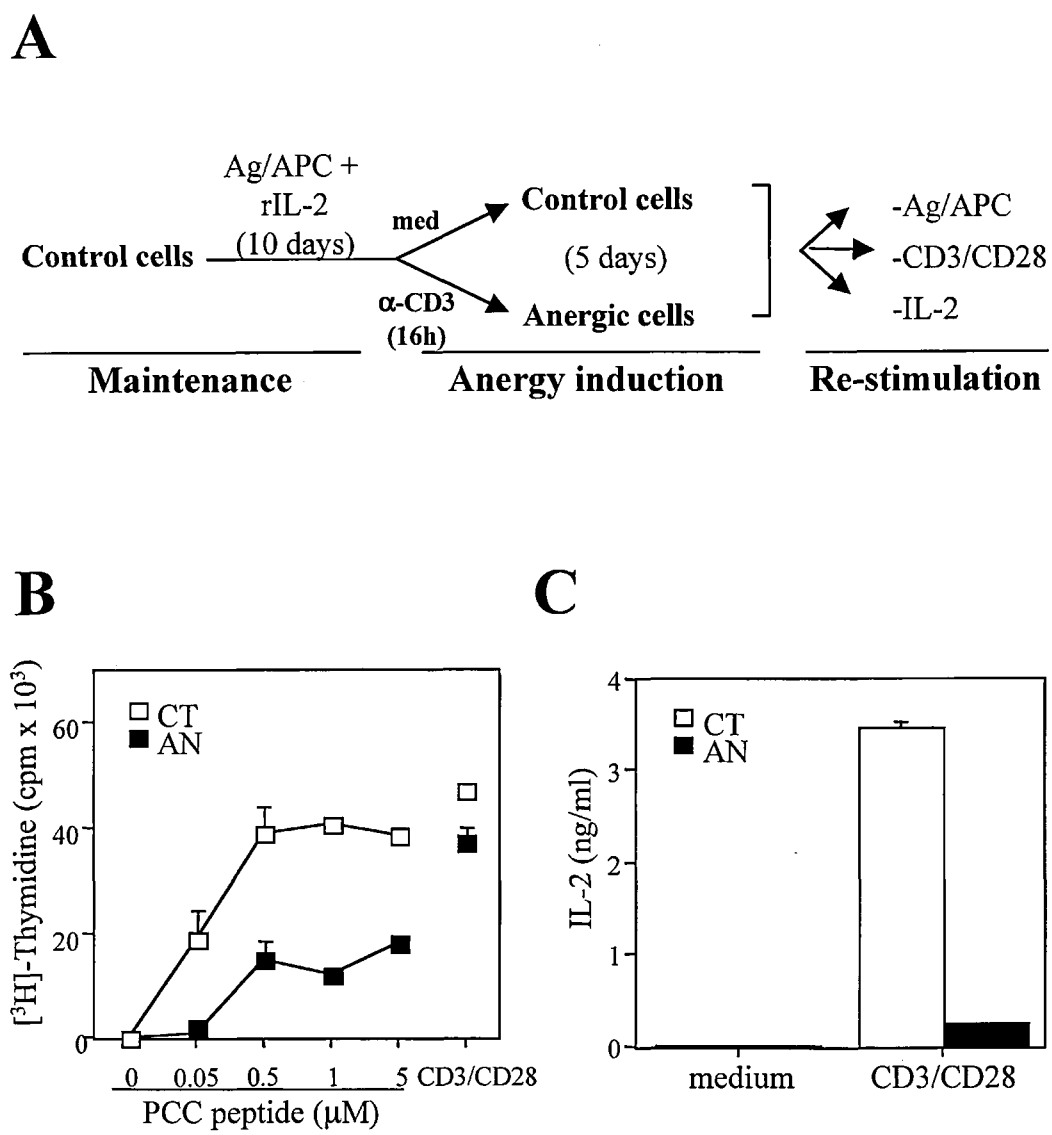

A
Ag/APC +
rIL-2
(10 days)
Control cells
med
α-CD3
(16h)
Control cells
(5 days)
Anergic cells
-Ag/APC
-CD3/CD28
-IL-2
Maintenance
Anergy induction
Re-stimulation
B
[3H]-Thymidine (cpm x 10³)
CT
AN
0
20
40
60
0 0.05 0.5 1 5 CD3/CD28
PCC peptide (μM)
C
IL-2 (ng/ml)
CT
AN
0
1
2
3
4
medium
CD3/CD28

Figure 1

2 and 4). In addition, control and anergic cells expressed similar surface levels of the TCR (Vβ3, MFI: 41.66±0,34 vs. 41.35±0,12), of CD3 (MFI: 27.01±1.39 vs. 23.83±3.66, see also (245)) and of CD25 (MFI: 23.87±0.07 vs. 26.54±0.17). To investigate the functional capabilities of these cells, control and anergic T cells were restimulated with either antigen-pulsed APCs (Ag/APC), or with a combination of anti-CD3 and anti-CD28 mAbs. Proliferation and IL-2 secretion were then measured by [^{3}H]-Thymidine incorporation and by capture ELISA, respectively. Upon antigen rechallenge, anergic A.E7 T cells proliferated to much lower extents when compared to control T cells (Fig. 1B), and showed defective IL-2 production even in response to optimal TCR and CD28 engagement (Fig. 1C), as previously described (245, 262). Interestingly, despite the failure to produce IL-2, control and anergic T cells produced comparable amounts of IFN-γ (not shown). Most importantly, anergic cells proliferated to similar extents to control cells in response to immobilized anti-CD3/CD28 mAbs (Fig. 1B).

The observation that anergic cells proliferated to larger extents when stimulated with CD3/CD28, than when stimulated with Ag/APC, prompted us to investigate whether these types of stimulation could differentially regulate G1-to-S transition in control and anergic T cells.

We first analyzed the expression of G1 cell cycle proteins by Western blot (Fig. 2). While antigen-dependent stimulation of control cells resulted in the up-regulation of cyclin D3, and of $p21^{Cip}$, in the down-regulation of $p27^{Kip}$ levels, and in the hyperphosphorylation of Rb (Fig. 2A), it failed to induce G1-related events in anergic T cells. Thus, as also reported elsewhere (264, 269), anergic A.E7 T cells

Figure 2. CD3/CD28-, but not Ag/APC-mediated stimulation, drives anergic cells to exit from the G1 phase of the cell cycle. T cell anergy was induced as described in Fig. 1. Control (CT) and anergic (AN) T cells were stimulated with either Ag-pulsed irradiated syngeneic splenocytes (Ag/APC) **(A)** or with anti-CD3/CD28 mAbs (CD3/CD28) **(B)** for 24 hours. As a positive control the cells were stimulated for 24 hours also with rIL-2. Thereafter, the cells were lysed and protein expression was analyzed by SDS-PAGE and Western blot analysis with the antibodies indicated in the figure. $p27^{Kip}$ and $p21^{Cip}$ were detected on the same membrane filter by sequential hybridization. The relative mobility of the proteins is indicated in the figure by the arrows.

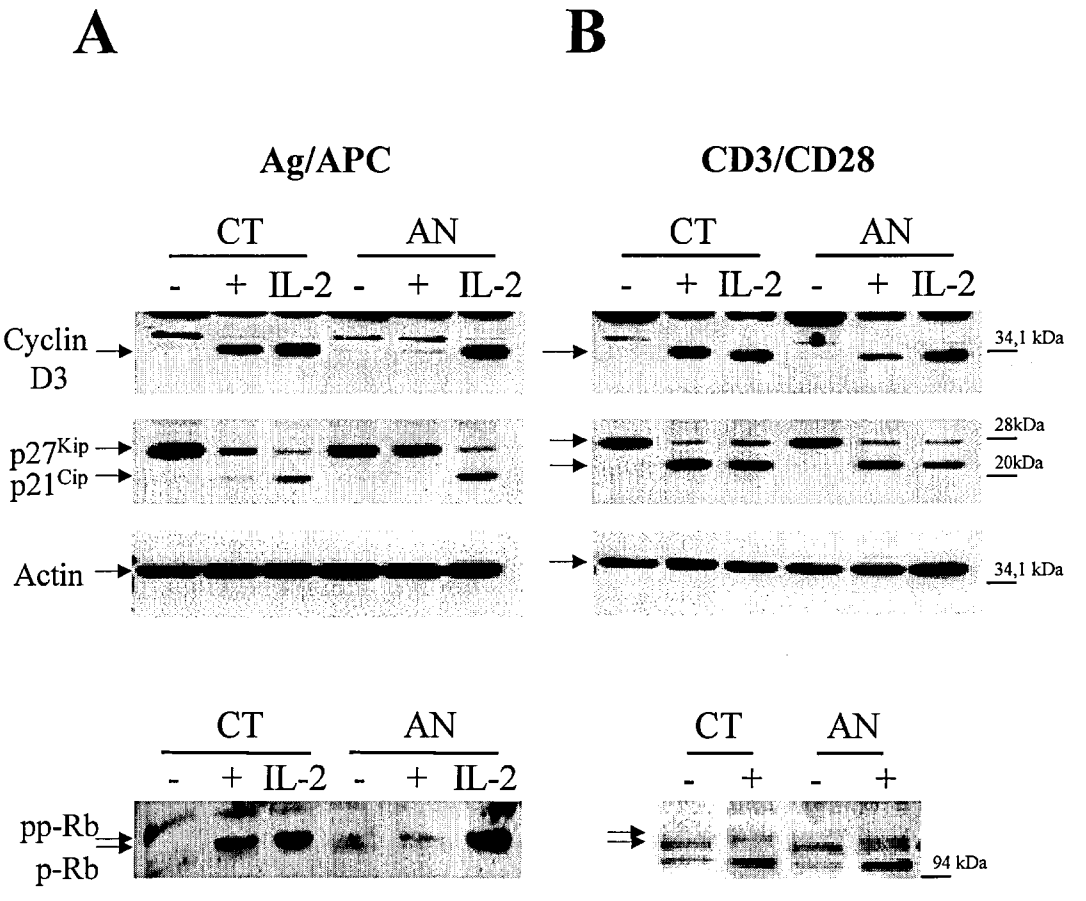

A
B
Ag/APC
CD3/CD28
CT
AN
- + IL-2 - + IL-2
Cyclin D3
34,1 kDa
p27Kip
p21Cip
28kDa
20kDa
Actin
34,1 kDa
pp-Rb
p-Rb
94 kDa

Figure 2

were unable to progress through the G1 cell cycle checkpoint upon Ag/APC encounter.

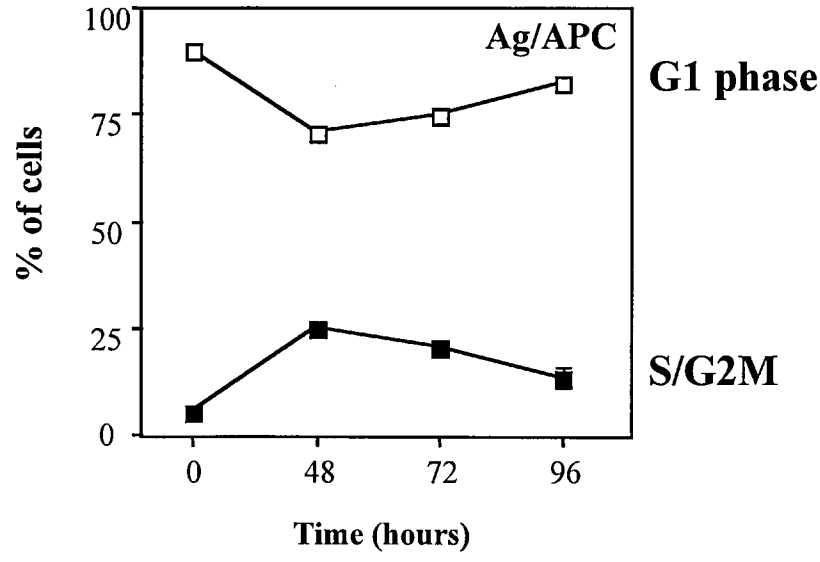
In contrast, stimulation with anti-CD3/CD28 mAbs elicited comparable up-regulation of cyclin D3 and of $p21^{Cip}$, down-regulation of $p27^{Kip}$ expression, and hyperphosphorylation of Rb (Fig. 2B), in control and anergic T cells. Moreover, this stimulation also induced up-regulation of cyclin D2 and cyclin E levels in both groups of cells (not shown). These results indicate that optimal engagement of TCR and CD28 drives the transition through the G1/S restriction point in anergic T cells.

To determine the fraction of anergic T cells able to proceed from G1 to S, and then to G2/M phases of the cell cycle upon CD3/CD28-mediated stimulation, we measured DNA content by Propidium Iodide (PI) staining. This technique allows to discriminate cells in G0/G1 phase, recognized by a typical DNA content equal to 1N, from cells in S/G2M phases which have a DNA content > 1N (2N for cells in the M phase). Preliminary experiments indicated that by 48h of both Ag/APC and CD3/CD28-mediated stimulations, 20-40% of control A.E7 T cells had entered the S/G2M phase of the cell cycle (Fig. 3).

Control and anergic T cells were therefore stimulated with either Ag/APC or immobilized anti-CD3/CD28 mAbs for 48 hours, fixed, stained with PI, and analyzed by flow-cytometry (Fig 4). As expected, while control cells increased their DNA content upon Ag/APC-stimulation, anergic T cells failed to do so (Fig. 4A). In contrast, upon CD3/CD28 stimulation, both control and anergic cells had a sizable fraction of cells with a DNA content >1N, and thus in S/G2M phases (23.83%±3.92% and 34.89%±5.68% respectively, Fig. 4B). After 48 hours of stimulation, no differences were detected in the total number of live cells or in the

Figure 3. Analysis of cell cycle entry in control cells. Control T cells were stimulated with either Ag/APC **(A)** or with anti-CD3/CD28 mAbs **(B)** for the indicated times. Thereafter the cells were counted, fixed, stained with propidium iodide, and analyzed by flow cytometry. The percentage of cells in G1 (DNA content =1N) or S/G2M (DNA content >1N) phase of the cell cycle is reported.

A


Ag/APC
G1 phase
S/G2M
% of cells
100
75
50
25
0
0
48
72
96
Time (hours)

B

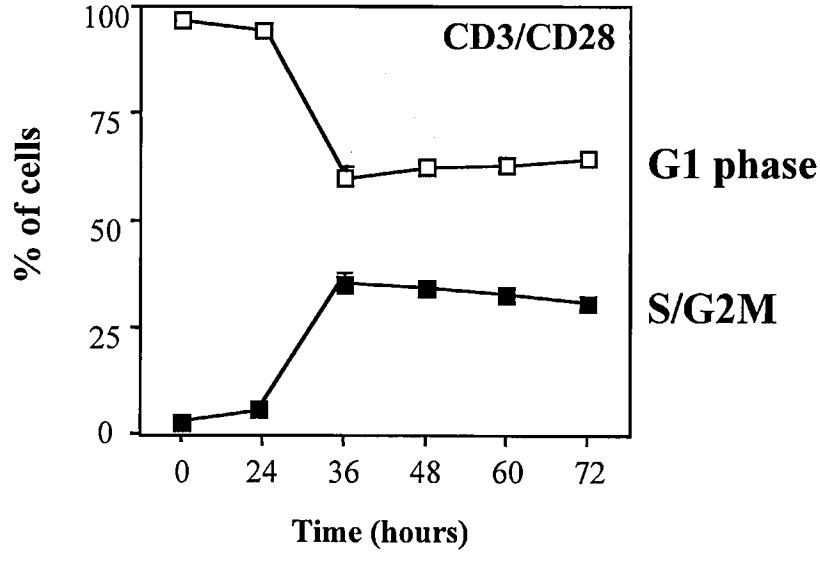

CD3/CD28
G1 phase
S/G2M
% of cells
100
75
50
25
0
0
24
36
48
60
72
Time (hours)

Figure 3

Figure 4. CD3/CD28-mediated stimulation of anergic T cells bypasses G1 cell cycle block and results in G1-to-S phase transition. T cell anergy was induced as described in Fig. 1. Control (CT) and anergic (AN) T cells were then stimulated with either Ag/APC **(A)** or with anti-CD3/CD28 mAbs **(B)** for 48 hours. Thereafter the cells were counted fixed, stained with propidium iodide, and analyzed by flow cytometry. Representative FACS histograms of the DNA content of unstimulated (untreated or stimulated with APC only), Ag/APC-stimulated and CD3/CD28-stimulated control and anergic T cells are reported. **C)** The graph reports the percentage of cells in the S/G2M phase (DNA content >1N) in resting (untr.) and stimulated (Ag/APC, CD3/CD28) control (empty bars) and anergic (filled bars) T cells calculated in four independent experiments (mean ± SD).

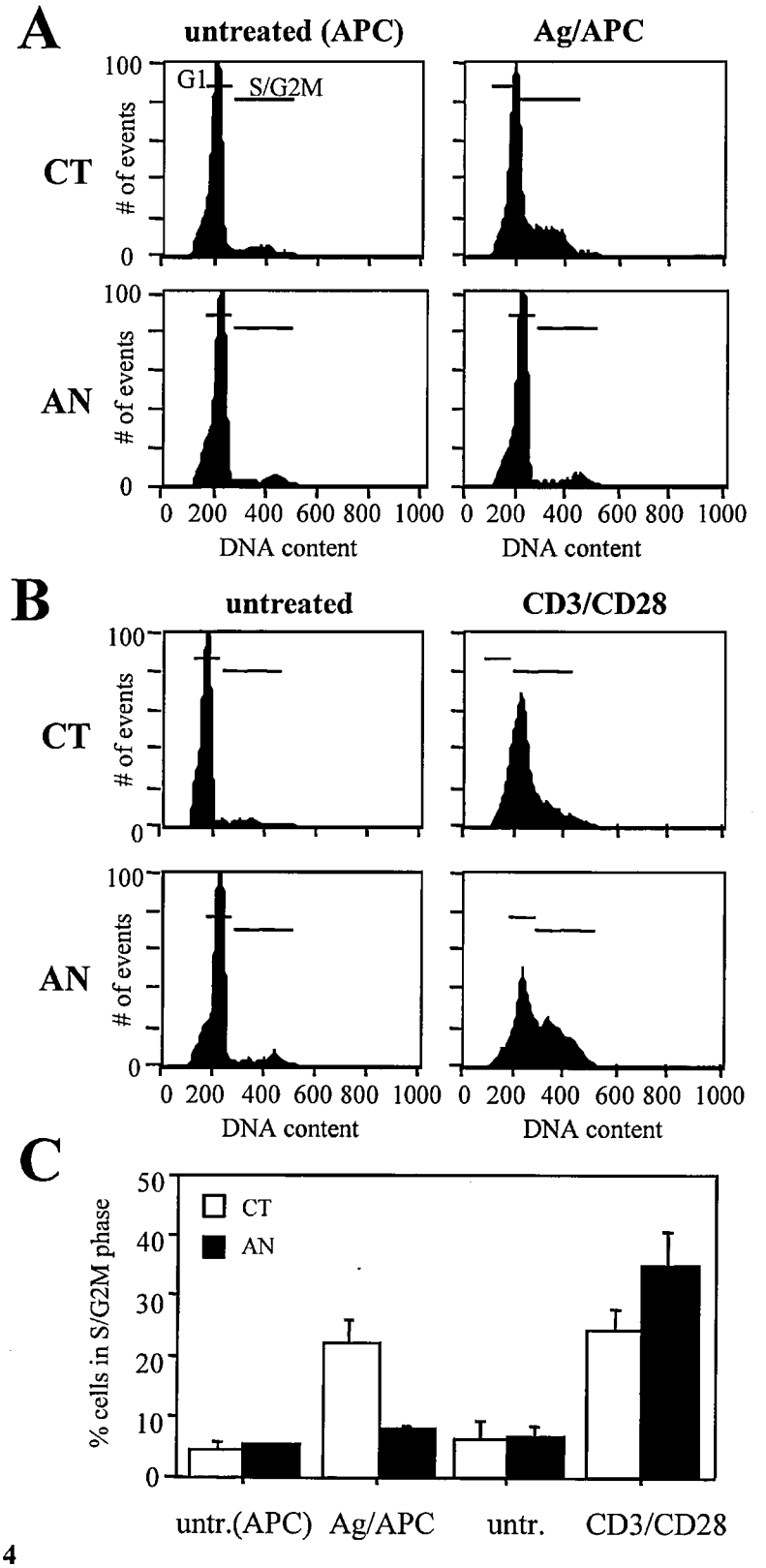

A
untreated (APC)
Ag/APC
G1
S/G2M
CT
AN
# of events
100
0
0 200 400 600 800 1000
DNA content
B
untreated
CD3/CD28
C
CT
AN
% cells in S/G2M phase
50
40
30
20
10
0
untr.(APC)
Ag/APC
untr.
CD3/CD28

Figure 4

fraction of the cells with DNA content <1N (sub-G1, apoptotic cells), suggesting that anergic T cells were not preferentially dying in our cell culture conditions (see below).

To demonstrate that CD3/CD28-mediated stimulation could promote cell division, we labelled the cells with the fluorescent dye carboxyfluorescein succinimidyl ester (CFSE). This dye passively diffuses into the cells, it is retained at the cell surface, and segregates equally between daughter cells upon cell division, resulting in the sequential halving of cellular fluorescence intensity with each successive generation, and allowing one to trace the fate of single cells by flow-cytometry (281). Thus, control and anergic T cells were labelled with CFSE, stimulated with Ag/APC or with anti-CD3/CD28 mAbs, and analyzed five days later by flow-cytometry. While only control cells divided upon Ag/APC-mediated stimulation, both control and anergic T cells completed several cycles of cell division upon CD3/CD28-mediated stimulation (Fig. 5). Interestingly, a comparable fraction of control and anergic cells proliferated on immobilized anti-CD3/CD28 mAbs, despite IL-2 was undetectable in culture supernatants of anergic T cells (refer to Fig.1).

Altogether these results suggest that optimal cross-linking of the TCR and of the CD28 costimulatory receptor is able to overcome the G1 cell cycle block, and to drive anergic T cell proliferation, in an IL-2-independent manner.

Figure 5. CD3/CD28-mediated stimulation drives comparable proliferation of control and anergic T cells. T cell anergy was induced as described in Fig. 1. Control (CT) and anergic (AN) T cells were then labeled with the fluorescent dye CFSE, and left untreated or stimulated with Ag/APC, or with anti-CD3/CD28 mAbs for 5 days, and then analyzed by flow cytometry. **A)** Representative FACS histograms reporting the degree of CFSE dilution are shown. **B)** The graph reports the percentage of control and anergic T cells which divided more than once (and thus falling in the indicated M2 gate) calculated in four independent experiments (mean ± SD).

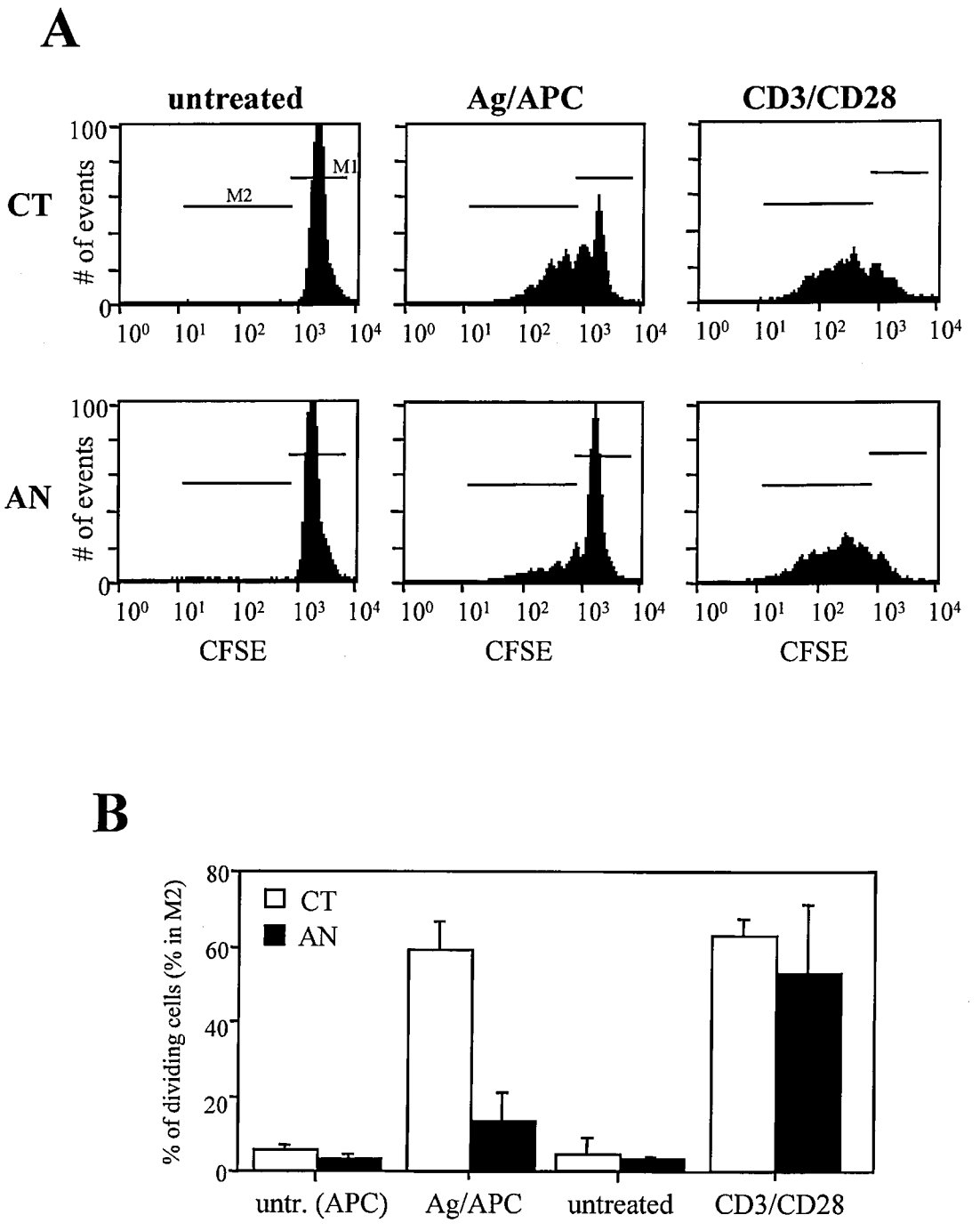

A
untreated
Ag/APC
CD3/CD28
CT
AN
# of events
M1
M2
100
0
10^0 10^1 10^2 10^3 10^4
CFSE
B
% of dividing cells (% in M2)
80
60
40
20
0
CT
AN
untr. (APC)
Ag/APC
untreated
CD3/CD28

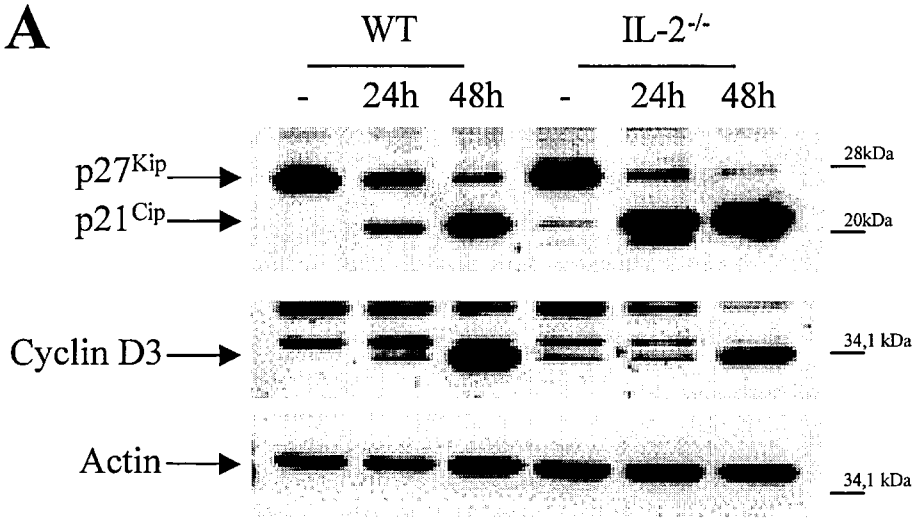
Figure 5

3.1.2 CD3/CD28-induced signals drive IL-2 independent cell proliferation

To better define whether anti-CD3/CD28 mAbs could drive G1-to-S transition and cell proliferation in the absence of IL-2, we used a T cell line derived from DO11.10 TCR transgenic IL-2 deficient mice (221), and analyzed the ability of these cells to respond to CD3/CD28 stimulation. Wild type and IL-$2^{-/-}$ T cells were stimulated with immobilized anti-CD3 and anti-CD28 mAbs for 48 h. Thereafter, the cells were harvested and lysed, and protein extracts were analyzed by Western blot. Following stimulation, up-regulation of cyclin D3 and of $p21^{Cip}$, and down-regulation of $p27^{Kip}$ were detected in both wild type and IL-$2^{-/-}$ T cells (Fig. 6A), suggesting that optimal TCR/CD28 engagement can normally regulate G1 cell cycle proteins in the absence of autocrine IL-2. Interestingly, cyclin D3 up-regulation was less pronounced in both anergic (Fig. 2B) and IL-$2^{-/-}$ (Fig. 6A) T cells when compared to the control and wild type populations. This could be due to the fact that activation of both anergic and IL-$2^{-/-}$ T cells occurs in the absence of IL-2, which has been shown to elicit and sustain cyclin D3 expression (140, 282).

We next asked whether anti-CD3/CD28 mAbs could drive complete cell cycle progression and could elicit cell division in the absence of IL-2. To this aim, wild type and IL-$2^{-/-}$ T cells were labelled with CFSE, stimulated with immobilized anti-CD3/CD28 mAbs, and analyzed by flow cytometry 5 days later (Fig. 6B). As control, T cells were stimulated with rIL-2 normally used to propagate this clone in culture. Results show that similarly to wild type T cells (not shown), IL-$2^{-/-}$ DO11.10 T cells completed several rounds of cell division as detected by serial CFSE dilution upon either CD3/CD28- and IL-2-mediated stimulation.

Figure 6. CD3/CD28 engagement drives exit from the G1 phase of the cell cycle, and elicits proliferation of IL-2 $^{-/-}$ T cells. **A)** Wild-type and IL-2$^{-/-}$ DO11.10 T cells were either left untreated (-) or stimulated with immobilized anti-CD3 and anti-CD28 mAbs (0.1 and 5 μg/ml, respectively) for the indicated times. Western blot analysis of total cell lysates were then performed with the Abs indicated in the figure. $p27^{Kip}$ and $p21^{Cip}$ were detected on the same membrane filter by sequential hybridization. **B)** IL-2$^{-/-}$ DO11.10 T cells were labeled with the fluorescent dye CFSE, and either left untreated (thin line) or stimulated with anti-CD3/CD28 mAbs (CD3/CD28) or sub-optimal anti-CD3 mAb (0.01 μg/ml) and rIL-2 (IL-2) for 5 days and then analyzed by flow cytometry.


A
WT
IL-2-/-
- 24h 48h - 24h 48h
p27Kip
p21Cip
Cyclin D3
Actin
28kDa
20kDa
34,1 kDa
34,1 kDa

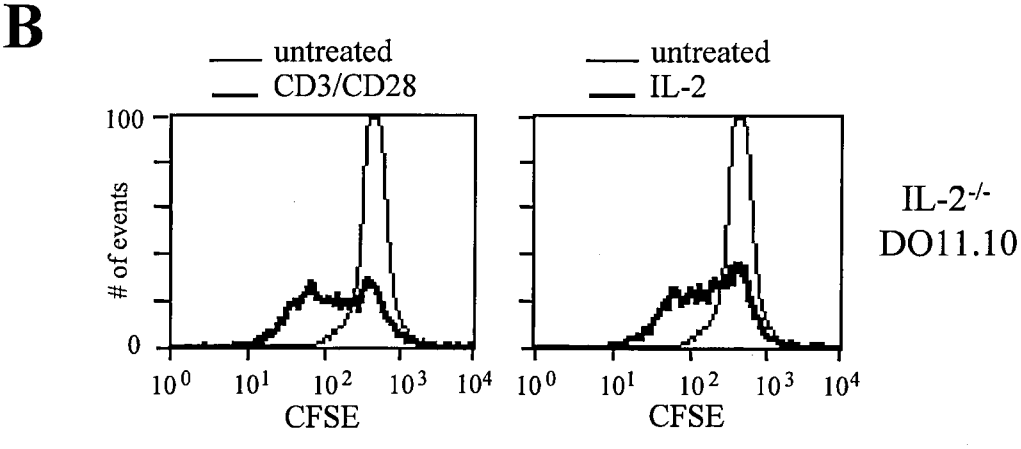

B
untreated
CD3/CD28
untreated
IL-2
100
0
# of events
10 0 10 1 10 2 10 3 10 4
CFSE
10 0 10 1 10 2 10 3 10 4
CFSE
IL-2-/-
DO11.10

Figure 6

Together these results support the idea that CD3/CD28 can regulate cell cycle related events and induce T cell proliferation via IL-2 independent mechanisms.

3.1.3 CD3/CD28-induced T cell proliferation does not restore Ag responsiveness

Since IL-2-dependent G1-to-S transition was previously shown to prevent and reverse clonal anergy (263, 266), we next investigated whether proliferation induced by anti-CD3/CD28 mAbs could restore antigen responsiveness in anergic T cells.

Control and anergic T cells were first allowed to proliferate in response to immobilized anti-CD3 and anti-CD28 mAbs or to rIL-2 ("First stimulation", refer to Fig. 7A for a schematic representation of the experiment). Proliferation during this time was monitored by CFSE dilution in separate samples (not shown). Thereafter, the cells were rested for 10 days, labelled with CFSE and restimulated with Ag/APC or rIL-2. CFSE dilution in response to Ag/APC rechallenge was then used to compare antigen responsiveness in untreated, CD3/CD28- and IL-2-treated cells. As expected, only untreated control cells proliferated in response to Ag/APC, and completed up to four rounds of cell division, while untreated anergic T cells did not divide in response to Ag/APC (Fig. 7B, left panels). Surprisingly, similar results were obtained also with cells allowed to proliferate in response to immobilized anti-CD3/CD28 mAbs (Fig. 7B, middle panels). Indeed, only CD3/CD28-stimulated control T cells, and not CD3/CD28-stimulated anergic cells, divided in response to Ag/APC restimulation (Fig. 7B, middle panels, and Fig. 7C). This indicates that, in

Figure 7. Proliferation of anergic T cells induced by optimal CD3/CD28 engagement does not restore antigen-responsiveness. **A)** Schematic representation of the experimental design. T cell anergy was induced as described in Fig. 1. Control (CT) and anergic (AN) T cells were then allowed to proliferate in response to immobilized anti-CD3/CD28 mAbs for 5 days as described in Fig. 4, and rested for the following 10 days. In parallel, the cells were also stimulated with rIL-2. Thereafter control and anergic T cells were labeled with CFSE, and either left untreated, or stimulated with Ag/APC or with rIL-2 (IL-2), and analyzed by flow cytometry 5 days later. **B)** Representative FACS histograms reporting the degree of CFSE dilution in the absence (thin line) and in the presence (thick line) of stimulation are depicted. **C)** The graph reports the percentage (mean ± SD of three independent experiments) of control and anergic cells pretreated as indicated in the figure (First stimulation), dividing to secondary rechallenge with Ag/APC (Second stimulation).

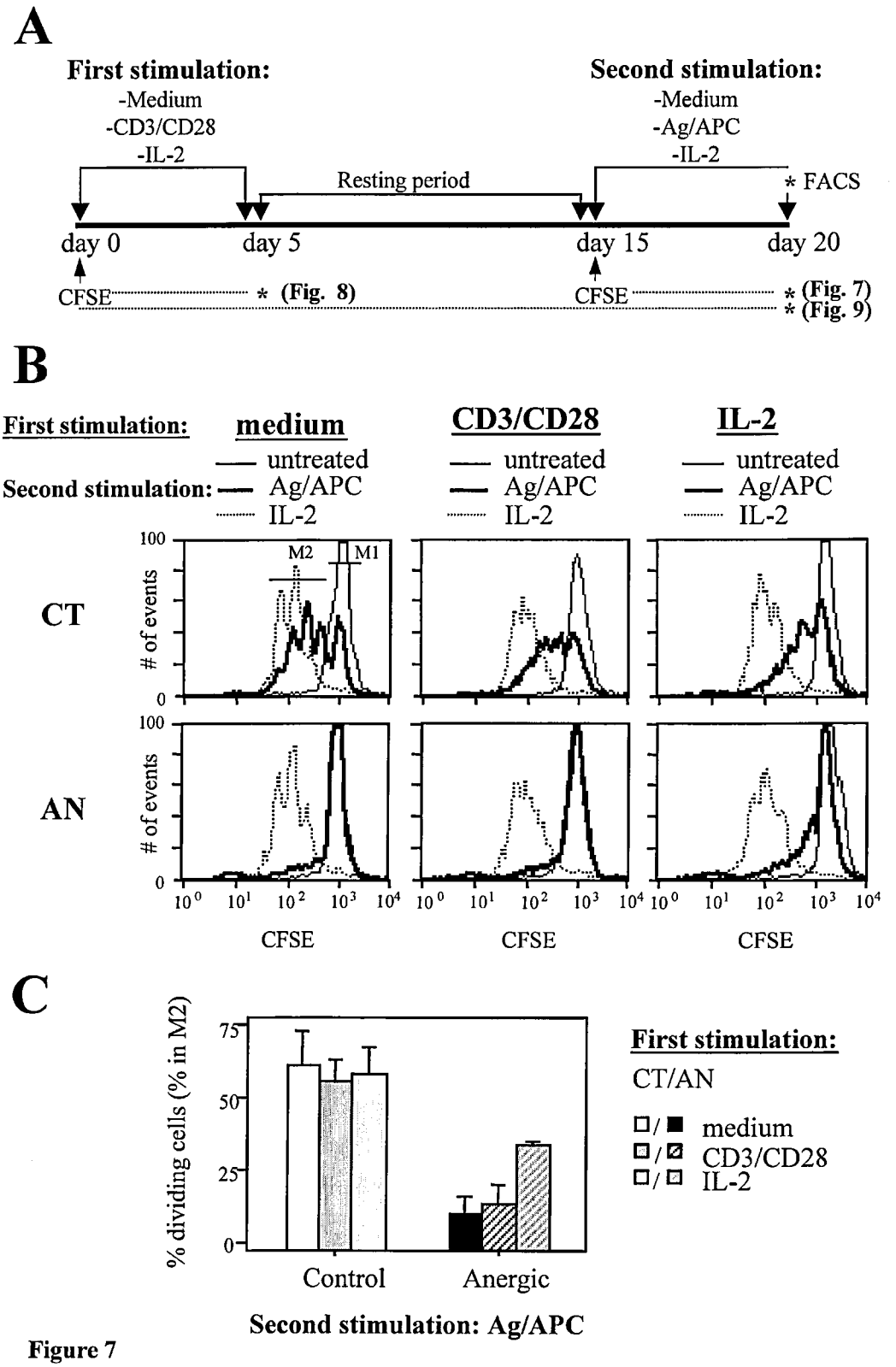

A
First stimulation:
-Medium
-CD3/CD28
-IL-2
Second stimulation:
-Medium
-Ag/APC
-IL-2
Resting period
* FACS
day 0
day 5
day 15
day 20
CFSE
* (Fig. 8)
CFSE
* (Fig. 7)
* (Fig. 9)
B
First stimulation:
medium
CD3/CD28
IL-2
Second stimulation:
untreated
Ag/APC
IL-2
CT
AN
M2
M1
# of events
CFSE
C
% dividing cells (% in M2)
Control
Anergic
First stimulation:
CT/AN
medium
CD3/CD28
IL-2
Second stimulation: Ag/APC

Figure 7

spite of completing several rounds of cell division, anergic T cells still fail to respond to antigenic rechallenge.

In contrast to anergic T cells which had divided in response to immobilized mAbs, anergic T cells allowed to proliferate in response to rIL-2, re-gained, at least in part, the ability to respond to Ag/APC (Fig. 7B, right panels and Fig. 7C). The IL-2-dependent anergy reversal was detected, although to variable extents, in several independent experiments (the average proliferative response to Ag/APC rechallenge of rIL-2-treated anergic T cells was 77.81%±36.42% of control cells).

IL-2-driven proliferation not only restored the ability of anergic T cells to proliferate in response to an antigen rechallenge, but also to produce IL-2 (data not shown). Thus, while the IL-2 produced by CD3/CD28-stimulated anergic cells represented only 2.5% of the IL-2 produced by control cells, the amount of IL-2 produced by anergic T cells allowed to proliferate in response to rIL-2 was 87.5%±22.5% of the amount produced by control T cells. By contrast, IL-2 was never detected upon restimulation of anergic T cells allowed to proliferate on immobilized anti-CD3/CD28 mAbs (not shown).

Finally, all the cells proliferated to similar extents in response to rIL-2 (dotted lines in Fig. 7B) indicating that untreated and CD3/CD28-stimulated anergic cells were viable in our cultures, but specifically unable to respond to Ag/APC.

In spite of several rounds of cell division, the total number of viable cells recovered from the cultures of CD3/CD28-stimulated control and anergic T cells was only 100-200% of the one recovered from the untreated population. To exclude the possibility that CD3/CD28-activated anergic T cells could die shortly after activation, and that an unresponsive population could be selected by this

stimulation, cell survival was monitored by flow cytometry over time in two independent sets of experiments.

In the first set of experiments, control and anergic T cells were labelled with CFSE, stimulated with anti-CD3/CD28 mAbs or rIL-2, and then stained with the fluorescent dye TO-PRO-3 at the time of the flow cytometric analysis (Fig. 8). This dye has been previously used to identify viable and dead cells in each pool of divided cells (283). Although a higher fraction of TO-PRO-3^+ cells was detected in CD3/CD28 stimulated cells when compared to IL-2-stimulated cells (~30% and ~2% respectively), the fraction of TO-PRO-3^+ cells was similar in CD3/CD28-stimulated control and anergic cells (30.52% and 20.24%, respectively). This indicated that the majority of the cells that proliferated in response to immobilized mAbs were viable at the end of the culture, and that there was no preferential loss of proliferating cells over non-responding cells.

In a second set of experiments, control and anergic T cells were labelled with CFSE, rested in fresh medium or allowed to proliferate on immobilized anti-CD3/CD28 mAbs, and then restimulated with either Ag/APC or with rIL-2 (Fig. 9). A further dilution of the CFSE content during the second stimulation would measure antigen responsiveness of cells that proliferated during the first stimulation. As shown above (Fig. 7), while control cells proliferated to Ag/APC and to rIL-2, anergic T cells failed to respond to Ag/APC and instead proliferated to rIL-2 (Fig. 9, left panels). As also shown above, while both control and anergic T cells proliferated in response to CD3/CD28 during the first stimulation (Fig. 9, right panels, thin lines), only control cells, and not anergic cells further diluted their CFSE contents upon Ag/APC restimulation (right panels, thick lines). By contrast

Figure 8. Survival of control and anergic T cells undergoing CD3/CD28-induced stimulation. T cell anergy was induced as described in Fig. 1. Thereafter control (CT) and anergic (AN) cells were labeled with CFSE, and then, either left untreated, or allowed to proliferate in response to immobilized anti-CD3/CD28 mAbs (CD3/CD28) or rIL-2 (IL-2) for 5 days. Right before the analysis, TO-PRO-3 was added to each sample. Representative dot plots reporting the degree of CFSE dilution (proliferation) and TO-PRO-3 staining (cell death) are depicted. The percentage of TO-PRO3^{+} cells are reported.

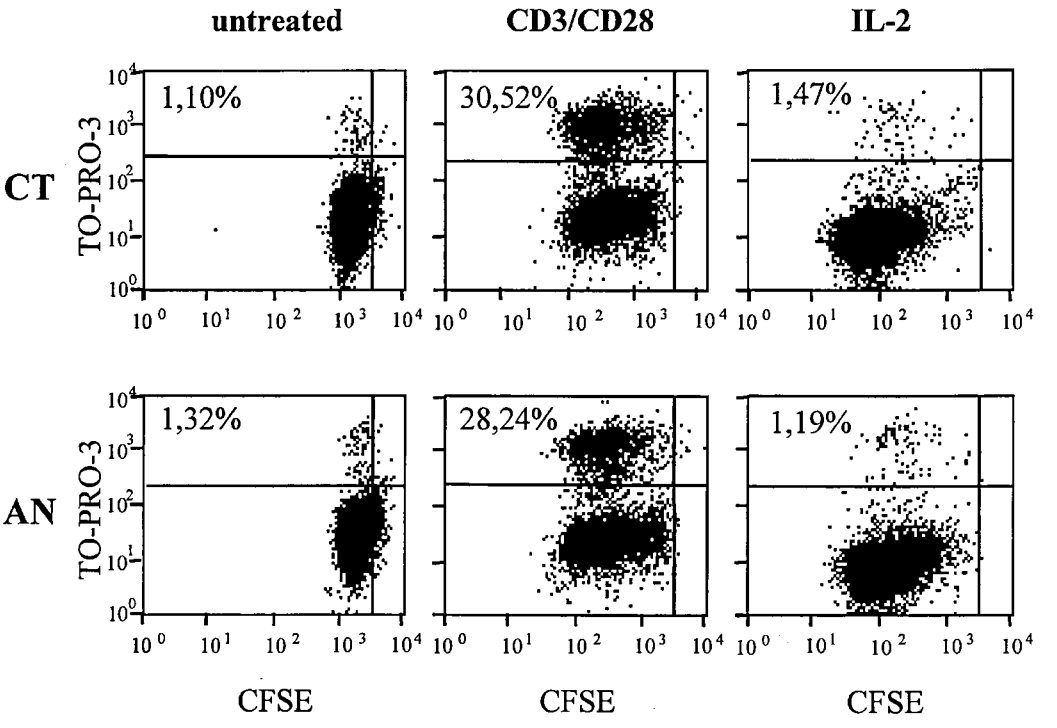

untreated
CD3/CD28
IL-2
CT
AN
TO-PRO-3
1,10%
30,52%
1,47%
1,32%
28,24%
1,19%
CFSE
CFSE
CFSE

Figure 8

Figure 9. Control, but not anergic T cells expanded to CD3/CD28, further proliferate upon Ag re-encounter. T cell anergy was induced as described in Fig. 1. Control (CT) and anergic (AN) T cells were labeled with CFSE, and cultured for 5 days in fresh medium (left panels), or on anti-CD3/CD28 mAbs-coated plates (CD3/CD28, right panels). Thereafter the cells were rested for 10 days, and either left untreated (thin lines), re-stimulated with Ag/APC (thick lines) or with rIL-2 (dotted lines). Representative FACS histograms of one out of two similar experiments (performed with duplicates) are reported in the figure. The overlays demonstrate that the cells, which proliferated during the first stimulation, are still able to divide upon rechallenge, as indicated by further dilution of their CFSE content.

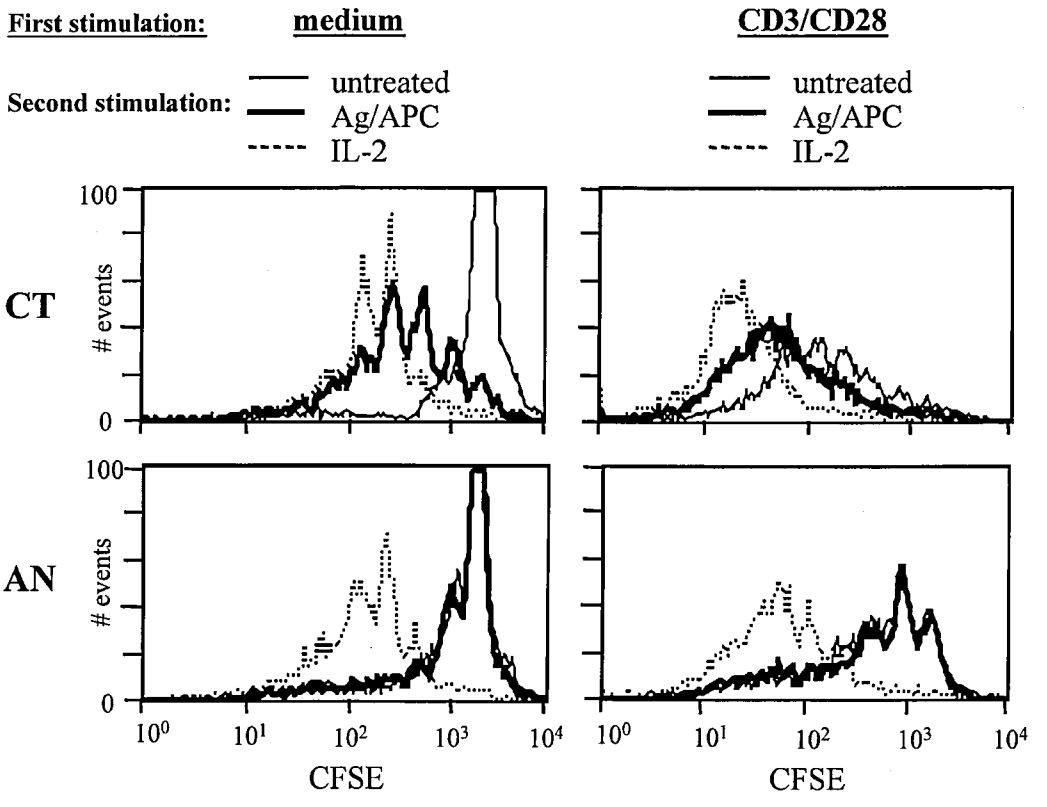

First stimulation:
medium
CD3/CD28
Second stimulation:
untreated
Ag/APC
IL-2
untreated
Ag/APC
IL-2
CT
AN
events
100
0
100
0
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 9

all the cells further diluted their CFSE content in response to rIL-2 (right panels, dashed lines), supporting the idea that anergic T cells, which proliferated to CD3/CD28, remain viable and able to respond to rIL-2, but incapable of responding to Ag/APC.

Together these results indicate that antigen unresponsiveness can be maintained in spite of several rounds of cell division, demonstrating that proliferation *per se* is not sufficient to reverse clonal anergy, and that instead anergy reversal specifically requires an IL-2-generated signal.

3.1.4 IL-2-induced mTor-dependent signalling, and not IL-2-induced T cell proliferation, regulates $CD4^+$ T cell antigen responsiveness

Previous findings and our own data indicate that IL-2-generated signals are important in preventing the induction of T cell anergy (263, 266, 284), and in restoring antigen responsiveness in already anergized T cells (Fig. 7). It has been shown that the ability of IL-2 to prevent the induction of T cell anergy was completely prevented by the addition of the immunosuppressive agent Rapamycin (265). This drug blocks the function of the serine/threonine kinase mTor (the mammalian target of Rapamycin), which is considered a central controller of cell growth since it regulates translational activity of the cell in response to nutrients and mitogens levels (206). We therefore investigated whether Rapamycin, beside its role in anergy induction, would also prevent the ability of IL-2 to reverse clonal anergy. Control and anergic T cells were thus labelled with CFSE, and either left untreated or stimulated with IL-2, in the absence or in the presence of Rapamycin

(200 nM) for 15 days (refer to Fig.10A for the experimental plan). Thereafter the cells were collected, washed and either left untreated or restimulated for 5 days with Ag/APCs. We first analyzed the ability of Rapamycin to block IL-2-driven proliferation. While Rapamycin partially blocked control T cell division in the first 5 days of culture (Fig. 10B, left panels), no differences between IL-2- and IL-2+Rapamycin-treated cells could be observed after 20 days of culture (Fig. 10B, right panels). The same result was obtained with anergic T cells (Fig. 10B). The failure of Rapamycin to block T cell proliferation was not due to drug inactivation, since the same effect was observed when Rapamycin was added to the culture every day (data not shown). Even though the inefficacy of Rapamycin to prevent cell cycle progression was initially surprising, this result is consistent with the possibility that Rapamycin does not completely prevent, but only delays, entry into the cell cycle (285).

We then compared the ability of untreated, IL-2 and IL-2+Rapamycin-treated cells to respond to antigen rechallenge. As shown above (Fig. 7), while the vast majority of untreated control T cells proliferated in response to Ag/APC, only a fraction of the anergic T cell population responded to the antigenic stimulation (Fig. 10C, left panels). As also shown above (Fig. 7), antigen unresponsiveness was lost upon IL-2-driven cell proliferation. Indeed, both control and anergic T cells stimulated for 20 days in IL-2 responded to a similar extent to Ag/APC (Fig. 10C, middle panels). By contrast, anergic T cells stimulated with IL-2 in the presence of Rapamycin failed to further dilute their CFSE content upon Ag/APC rechallenge (Fig. 10C, right panels). This indicates that, while Rapamycin does not block T cell proliferation, it completely prevents IL-2 mediated reversal of clonal anergy. Thus,

Figure 10. Rapamycin prevents IL-2-mediated reversal of clonal anergy, and not IL-2 driven proliferation. T cell anergy was induced as described in Fig. 1. **A)** Schematic representation of the experimental design. **B)** CFSE-labeled control (CT) and anergic (AN) T cells were either left untreated (thin lines) or stimulated with rIL-2 in the absence (IL-2, thick lines) or in the presence of Rapamycin (200 nM) (IL-2+Rap, dotted line). Cell proliferation was determined by flow cytometry after 5 (left panels) and 20 days (right panels). Representative histograms are shown. **C)** CFSE-labeled untreated (medium), and IL-2 or IL-2+Rapamycin-treated cells were left unstimulated (untreated; thin line), stimulated with Ag/APC (thick line), or with rIL-2 (dotted line) for 5 days. Representative FACS histograms reporting CFSE content are shown (top, middle panel: the CFSE profile obtained with rIL-2 is underneath the profile elicited by Ag/APC). The overlays demonstrate further dilution of CFSE content upon secondary stimulation. A representative experiment (performed with duplicates) out of three independent experiments is shown.

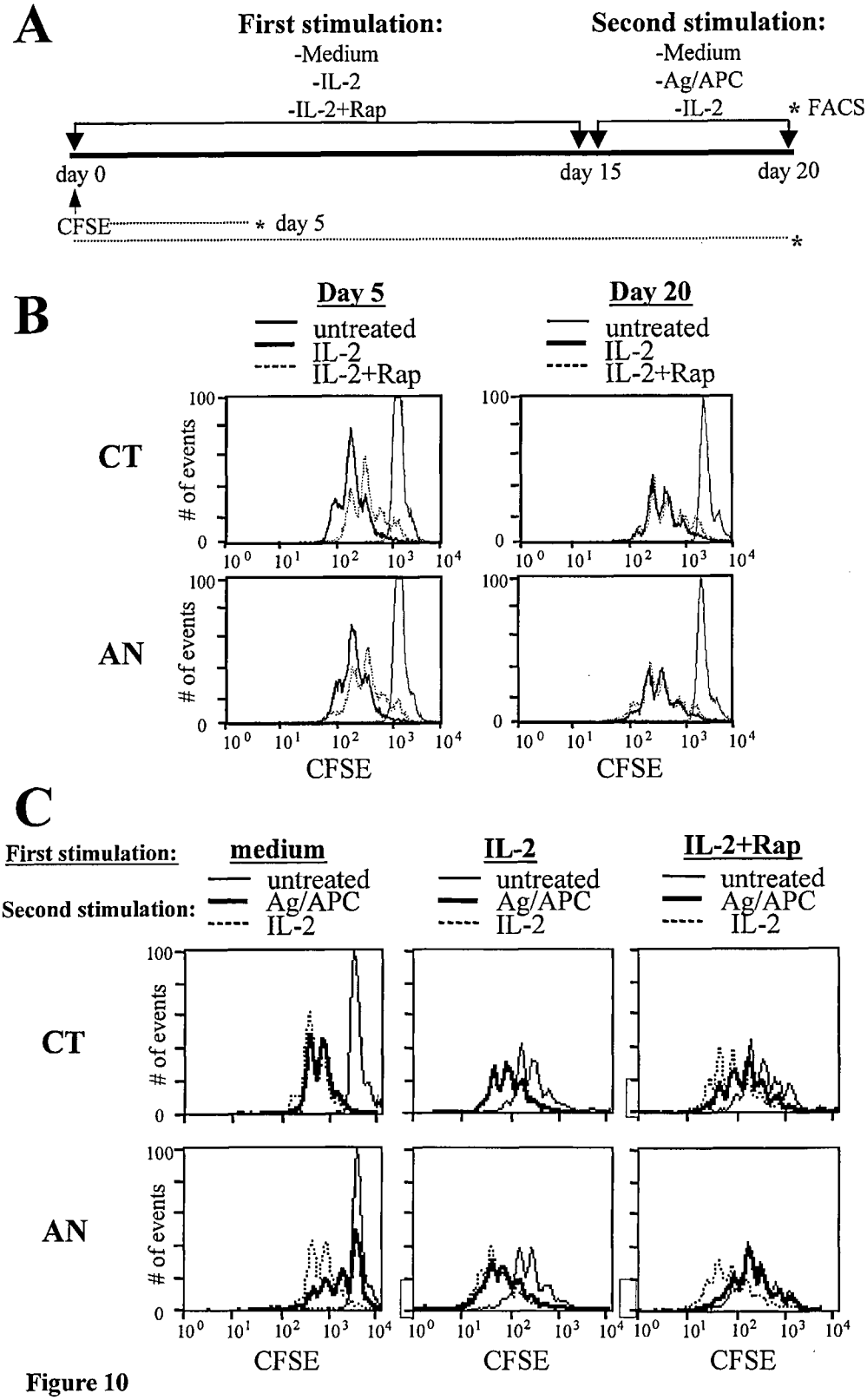

A
First stimulation:
-Medium
-IL-2
-IL-2+Rap
Second stimulation:
-Medium
-Ag/APC
-IL-2
* FACS
day 0
day 15
day 20
CFSE
* day 5
*
B
Day 5
Day 20
untreated
IL-2
IL-2+Rap
CT
AN
# of events
CFSE
C
First stimulation:
Second stimulation:
medium
IL-2
IL-2+Rap
untreated
Ag/APC
IL-2
CT
AN
# of events
CFSE

Figure 10

altogether our results indicate that IL-2-induced mTor signalling, independently of IL-2-driven proliferation, is absolutely required to reverse clonal anergy and thus to regulate lymphocyte responsiveness.

3.1.5 CD3/CD28-induced T cell proliferation involves mTor-dependent signalling

In addition to IL-2, also CD28 elicits mTor-dependent signalling pathways. Indeed, it has been shown that Rapamycin blocks CD28-dependent phosphorylation of $p70^{S6k}$ (107) and 4EBP-1 (108). Thus we sought of investigating whether CD3/CD28 could activate mTor in anergic T cells, and whether functional differences between CD3/CD28- and IL-2/IL-2R-generated signals might explain the different functional outcome elicited by these stimuli.

First we investigated whether CD3/CD28-induced T cell proliferation was sensitive to Rapamycin inhibition. To this aim, CFSE-labelled control and anergic A.E7 T cells were stimulated for 5 days with immobilized anti-CD3 and anti-CD28 mAbs, in the absence (Fig. 11, left panels) or in the presence (Fig. 11, right panels) of Rapamycin. Proliferation of both control and anergic T cells was inhibited by the presence of Rapamycin. Even though control cells produced IL-2, the amounts were not sufficient to drive Rapamycin-insensitive proliferation (see section 3.3). This supports the idea that CD3/CD28 can induce IL-2 independent cell proliferation, and that to do so it requires proper mTor-dependent signalling.

To further investigate this possibility, similar experiments were performed with wild type DO11.10 and IL-$2^{-/-}$ T cell clones. To this aim the cells were labelled

Figure 11. CD3/CD28 stimulation relies on mTor activity to drive IL-2-independent T cell proliferation. **A)** Control (CT) and anergic (AN) A.E7 cells, DO11.10 IL-2$^{-/-}$, and DO11.10 wt cells were labeled with CFSE, and either left untreated or stimulated with anti-CD3/CD28 mAbs in the absence or in the presence of Rapamycin (Rap) (200 nM) for 5 days, and then analyzed by flow cytometry. Representative FACS histograms reporting the degree of CFSE dilution are shown.

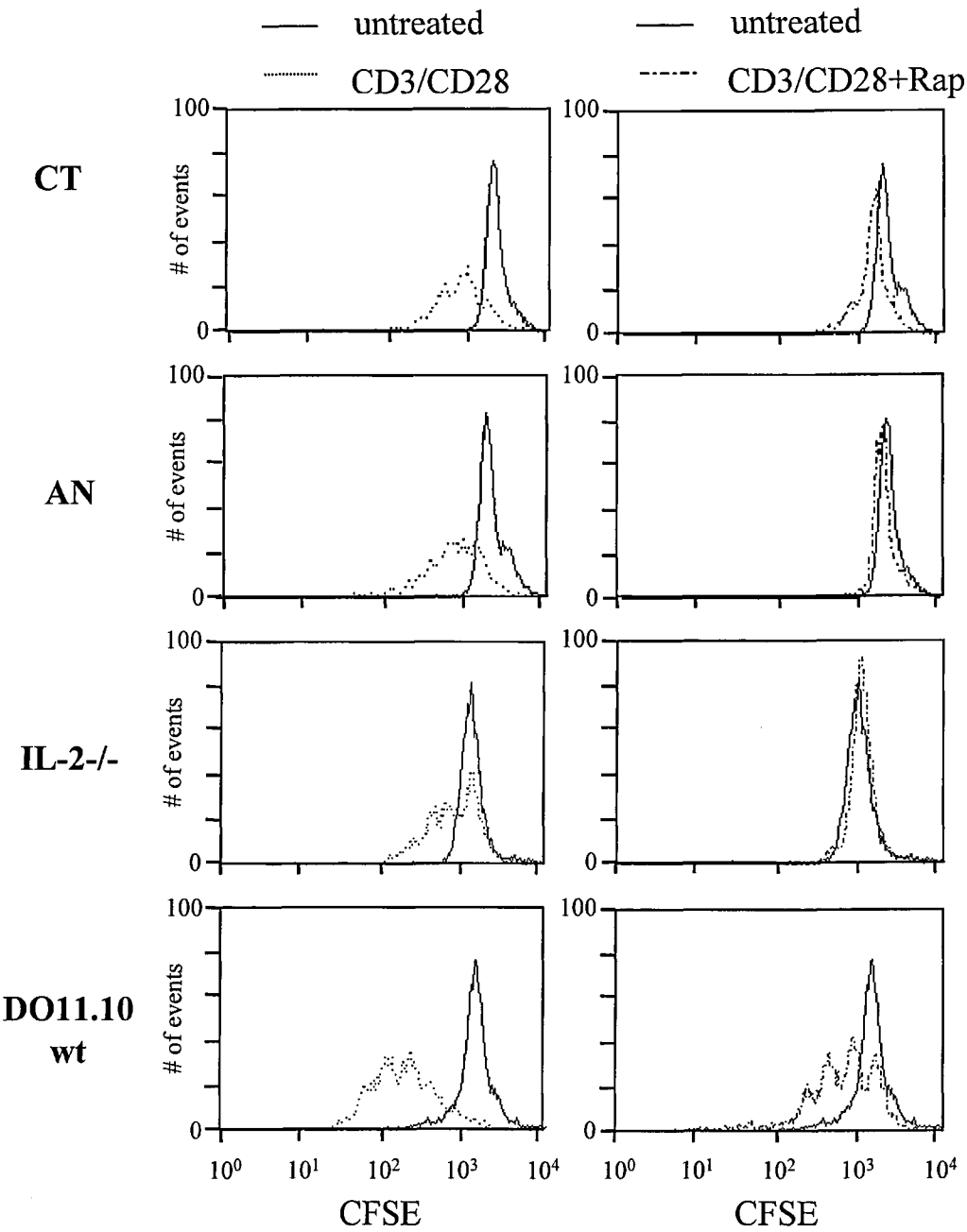

untreated
CD3/CD28
untreated
CD3/CD28+Rap
CT
AN
IL-2-/-
DO11.10
wt
# of events
100
0
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 11

with CFSE, and stimulated for 5 days with immobilized anti-CD3 and anti-CD28 mAbs, in the absence (Fig. 11, left panels) or in the presence (Fig. 11, right panels) of Rapamycin. While Rapamycin-insensitive CD3-CD28-initiated proliferation was observed in IL-2 sufficient DO11.10, it was completely absent in IL-2 deficient cells.

Together these results indicate that CD3/CD28-mediated stimulation, in functionally (anergic) or genetically (IL-$2^{-/-}$) IL-2-deficient T cells, drives T cell proliferation in a Rapamycin sensitive manner, thus involving mTor in TCR/CD28-induced intracellular signalling pathways

3.1.6 CD3/CD28-induced signals fail to restore Ag responsiveness even in the presence of Cyclosporin A

The observation that CD3/CD28-mediated proliferation was sensitive to Rapamycin, indicated that either the TCR or CD28 elicited mTor activation. However, CD3/CD28 stimulation failed to restore Ag responsiveness. Thus, while IL-2-induced mTor activation restored Ag responsiveness, CD3/CD28-induced mTor activation did not. Thus, we investigated the reasons for the failure of CD3/CD28-mediated stimulation to reverse clonal anergy.

First we investigated the possibility that the failure of CD3/CD28-mediated stimulation to reverse clonal anergy could be attributed to chronic engagement of the TCR by the immobilized anti-CD3 mAb. Indeed, this was previously hypothesized to induce the continuous production of a putative anergic factor, possibly able to maintain the anergic phenotype. Since the addition of Cyclosporin

A, at the time of TCR stimulation, can inhibit CD3-induced T cell anergy (233, 236, 252), we asked whether the addition of Cyclosporin A to CD3/CD28-stimulated anergic cells would allow anergy reversal. In a preliminary experiment we verified the ability of Cyclosporin A to prevent CD3-induced T cell anergy (Fig. 12). To this aim, we stimulated A.E7 T cells with immobilized anti-CD3 mAb for 16 hours (see "anergy induction" in Fig. 1), in the absence or in the presence of Cyclosporin A (0.5 μg/ml). Thereafter the cells were rested in fresh medium for 5 days, labelled with CFSE, and restimulated for 5 days with Ag/APC or with rIL-2. A fraction of the cells was similarly treated with the exception of the initial CD3 stimulation. At the end of the restimulation, the CFSE content was determined by flow cytometry. As expected, while control cells proliferated in response to antigen stimulation (Fig.12, upper panel), CD3-treated cells failed to do so (Fig.12, middle panel). In contrast, cells stimulated with anti-CD3 mAb in the presence of Cyclosporin A, did proliferate to the antigen (Fig.12, lower panel), indicating that, also in our hands, Cyclosporin A actively prevents the induction of clonal anergy.

We thus asked whether addition of Cyclosporin A during CD3/CD28-mediated restimulation of anergic cells, would allow the reversal of clonal anergy (Fig. 13). Control and anergic A.E7 T cells were either left untreated (medium in the figure), or stimulated for 5 days with immobilized anti-CD3/CD28 mAbs, rIL-2, in the absence or in the presence of Cyclosporin A, or Rapamycin. The cells were then rested for 10 days, labelled with CFSE, and restimulated with Ag/APC, or with rIL-2 to determine their functional responsiveness. While untreated and CD3/CD28-stimulated control cells proliferated in response to the antigen, untreated and CD3/CD28-stimulated anergic cells failed to do so (Fig. 13, upper panels), as also

Figure 12. Cyclosporin A prevents CD3-induced T cell anergy. A.E7 cells were either left untreated or stimulated with anti-CD3 mAb (α-CD3), in the absence or in the presence of Cyclosporin A (CsA) (0.5 μg/ml). After 16h the cells were removed from the Ab, and rested for an additional 5 days. Thereafter, the cells were labeled with CFSE, restimulated with Ag/APC or rIL-2, as indicated at the bottom of the figure, and analyzed by flow cytometry. Representative FACS histograms reporting the degree of CFSE dilution are shown.

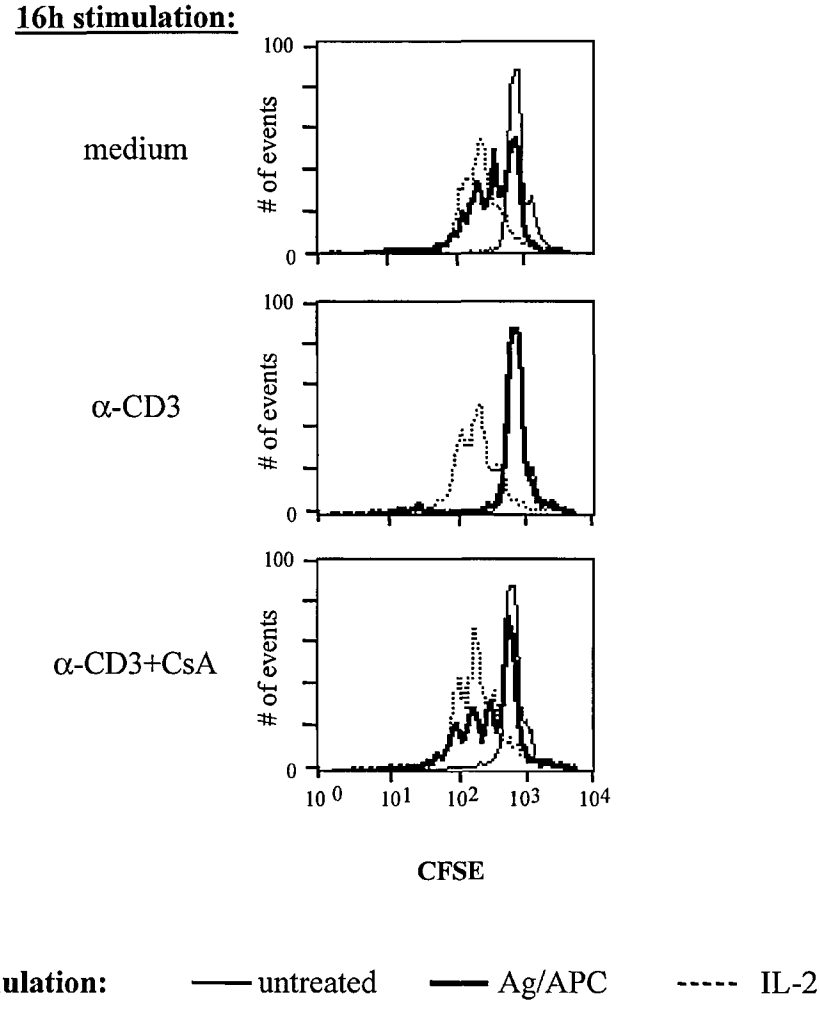

16h stimulation:
medium
α-CD3
α-CD3+CsA
100
0
# of events
10 0
10 1
10 2
10 3
10 4
CFSE
Restimulation:
untreated
Ag/APC
IL-2

Figure 12

Figure 13. CD3/CD28 stimulation fails to restore Ag responsiveness even in the presence of Cyclosporin A. A.E7 cells were either left untreated (CT) or induced into anergy (AN) as described in Fig.1. The cells were cultured for an additional 5 days with medium only, with anti-CD3/CD28 mAbs, or with rIL-2, in the absence or in the presence of either CsA (0.5μg/ml), or Rapamycin (Rap) (200nM) (First stimulation). Thereafter, the cells were rested for 10 days in fresh medium, then labeled with CFSE and re-stimulated with Ag/APC, or with rIL-2 (Second stimulation). A representative experiment out of three independent experiments is shown.

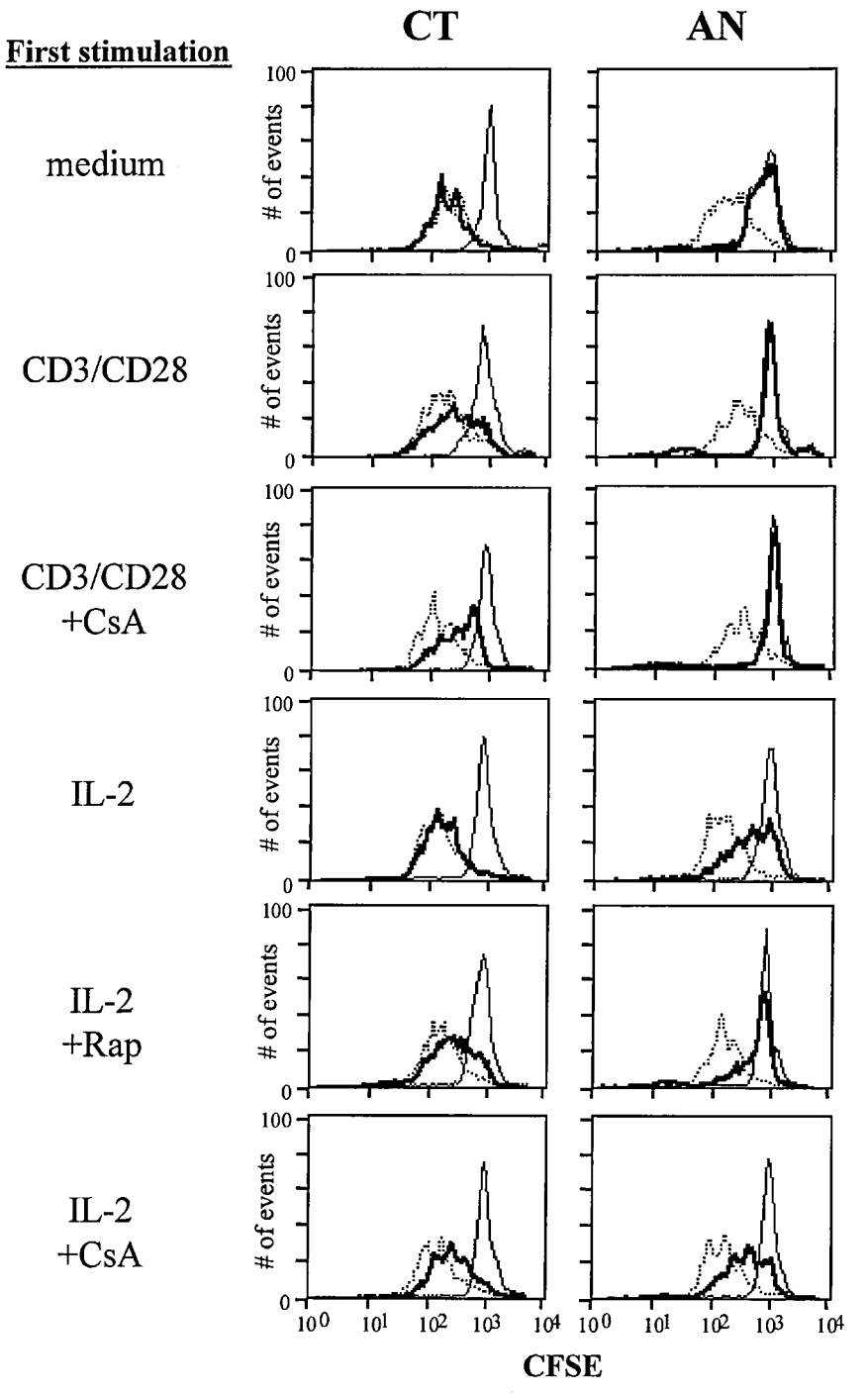

First stimulation
CT
AN
medium
CD3/CD28
CD3/CD28 +CsA
IL-2
IL-2 +Rap
IL-2 +CsA
# of events
100
0
10^0 10^1 10^2 10^3 10^4
CFSE
Second stimulation: untreated Ag/APC IL-2

Figure 13

shown above (Fig. 7). Similarly to CD3/CD28-stimulated anergic cells, also the cells stimulated with CD3/CD28 in the presence of Cyclosporin A failed to respond to Ag restimulation, suggesting that the presence of Cyclosporin A did not allow CD3/CD28-dependent signalling to restore antigen responsiveness. As expected, IL-2 stimulated anergic T cells re-acquired the ability to proliferate in response to Ag stimulation, and this was insensitive to the presence of Cyclosporin A, but sensitive to Rapamycin (Fig. 13, last panels).

Altogether these results demonstrate that mTor activation by CD3/CD28-mediated signals is not sufficient to induce reversal of clonal anergy, and that this event specifically requires IL-2/IL-2R-induced mTor activation.

3.1.7 The engagement of CD3/CD28 elicits Rapamycin-sensitive $p70^{S6k}$ phosphorylation in control, but not in anergic T cells

Starting from the observation that both CD3/CD28 and IL-2 stimulations induced a Rapamycin sensitive proliferation, but only IL-2 restored antigen responsiveness, we asked whether TCR/CD28 and IL-2R could differentially regulate mTor functions. To address this question, we investigated the phosphorylation/activation status of the mTor target $p70^{S6k}$.

A.E7 cells were either left untreated or stimulated with immobilized anti-CD3/CD28 mAbs or with rIL-2 for 30 min (Fig. 14). Thereafter the cells were lysed, and equal protein amounts were separated on SDS-PAGE, and analyzed by Western blot using an anti-$p70^{S6k}$ Ab. Both CD3/CD28 and IL-2 stimulations induced a shift in the molecular weight of $p70^{S6k}$, revealed by a retarded

Figure 14. CD3/CD28 and IL-2-mediated stimulations induce $p70^{S6k}$ phosphorylation. A.E7 cells were either left untreated (-) or stimulated with anti-CD3/CD28 mAbs (3/28) or rIL-2 (IL-2) for 30 min. The cells were then lysed, and the protein extracts were analyzed by Western blot with anti-$p70^{S6k}$ Ab. Where indicated, the extracts were treated with alkalin phosphatase (AP) before the electrophoretic separation.

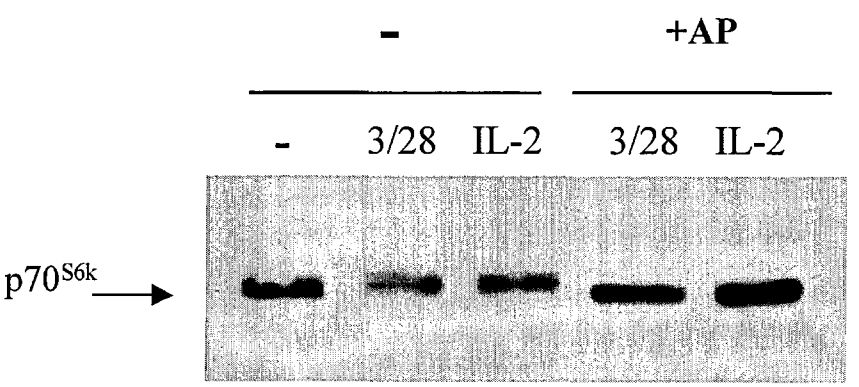


Figure 14

electrophoretic mobility of the protein. This shift was previously reported to correlate with $p70^{S6k}$ phosphorylation in multiple Ser and Thr sites (286). Accordingly, when the extracts were treated with alkalin phosphatase (AP), the mobility of the protein was comparable in untreated and stimulated cells. Thus, as previously reported (107), also in A.E7 cells CD3/CD28 engagement results in $p70^{S6k}$ phosphorylation.

We next investigated whether, also in anergic T cells, CD3/CD28 and IL-2/IL-2R could drive $p70^{S6k}$ phosphorylation. To this aim, control and anergic T cells were stimulated, and $p70^{S6k}$ phosphorylation was analyzed by Western blot (Fig. 15A). As described before, while both CD3/CD28 and IL-2 stimulations induced a shift in the electrophoretic mobility of $p70^{S6k}$ in control cells, only IL-2, and not CD3/CD28, did so in anergic T cells. Indeed, in these cells, CD3/CD28 stimulation induced only a partial shift in the electrophoretic mobility of the protein. As expected, in all stimulation conditions, the addition of Rapamycin at the time of stimulation reduced the electrophoretic mobility of $p70^{S6k}$ to the one observed in unstimulated cells (Fig. 15B). This indicates that mTor signalling is required in both CD3/CD28- and IL-2- driven $p70^{S6k}$ phosphorylation.

3.1.8 CD3/CD28 stimulation fails to elicit proper Thr^{389} phosphorylation and post-translational modifications of $p70^{S6k}$ in anergic cells

Phosphorylation of the threonine in position 389 was previously shown to be absolutely required for proper activation of $p70^{S6k}$ (287). Thus, we analyzed protein extracts derived from untreated and stimulated control and anergic cells by Western

Figure 15. CD3/CD28 and IL-2 stimulations differentially regulate $p70^{S6k}$ in anergic cells. Control (CT) and anergic (AN) cells were either left untreated, or stimulated for 30 min with anti-CD3/CD28 mAbs (3/28), or with rIL-2, in the absence **(A)** or in the presence **(B)** or Rapamycin (+Rap) (1μM). The cells were then lysed, and the protein extracts were analyzed by Western blot with anti-$p70^{S6k}$ Ab.

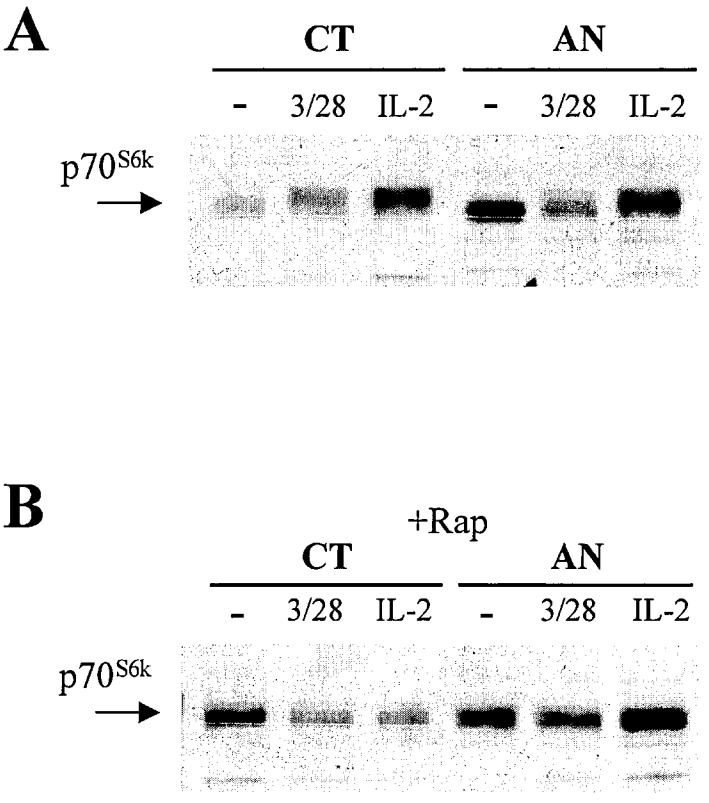
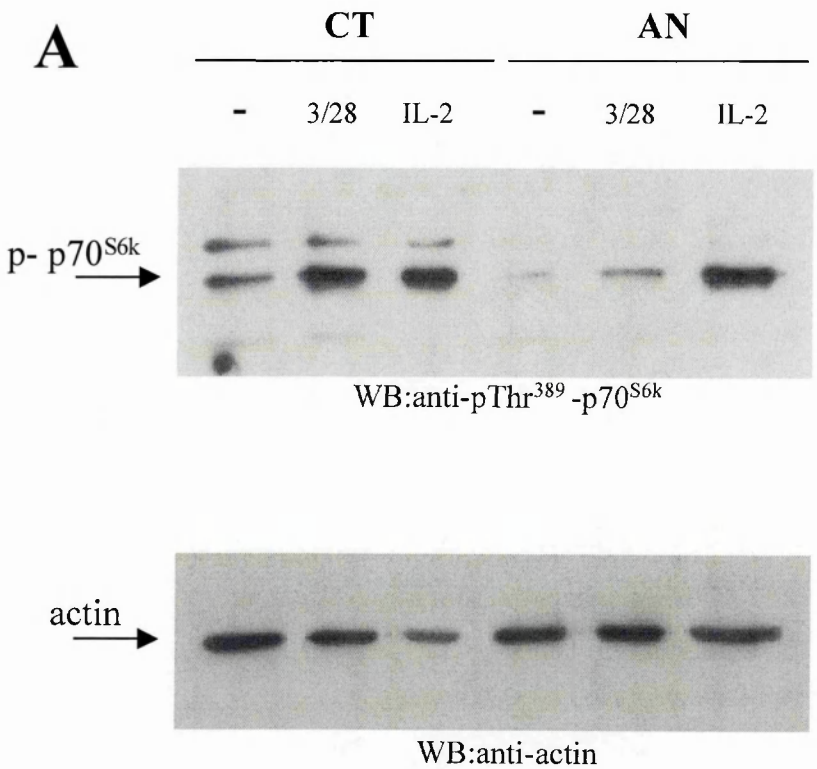


Figure 15

blot with an Ab specific for the phospho-Thr^{389}(pThr^{389})-p70^{S6k} (Fig. 16). The results obtained show that, while both CD3/CD28 and IL-2 elicited the appearance of pThr^{389}-p70^{S6k} in control cells, only IL-2 did so in anergic cells. Indeed, CD3/CD28 stimulation of anergic cells failed to elicit significant phosphorylation of this residue. As expected from previous findings (288), in all stimulation conditions, the addition of Rapamycin completely abrogated Thr^{389} phosphorylation (data not shown).

In addition to Thr^{389}, optimal activation of p70^{S6k} involves the ordered phosphorylation of several residues, which are positioned within distinct intramolecular regulatory domains of the protein. To study in further details the nature of p70^{S6k} post-translational modifications under different stimulation conditions, we analyzed protein extracts of control and anergic T cells by two-dimensional electrophoresis (2DE) (Fig. 17-20). This technique allows to resolve proteins in a gel on the basis of both their isoelectric points (pI) and their molecular weight (MW), and by that it allows the discrimination of protein variants, deriving from different post-translational modifications, such as phosphorylation (289). Briefly, untreated and stimulated cells were lysed, and the proteins (400 μg) were separated on SDS-PAGE. Proteins with an apparent molecular weight of 60-80 kDa were then eluted from the gel, acetone precipitated and resolved by 2DE (see Materials and Methods). The proteins were then transferred to nitrocellulose membrane, which were developed with an anti-p70^{S6k} Ab, and analyzed by Image Master 2D Elite analysis software. First we analyzed protein extracts from unstimulated control A.E7 cells (Fig. 17). The signal corresponding to p70^{S6k} appeared as a train of spots (pI ranging between 5.67 and 6.06 pH values), each of

Figure 16. CD3/CD28 stimulation fails to elicit $p70^{S6k}$-Thr^{389} phosphorylation in anergic cells. **A)** Control (CT) and anergic (AN) cells were either left untreated, or stimulated for 30 min with anti-CD3/CD28 mAbs (3/28), or with rIL-2. The cells were then lysed, and protein extracts were analyzed by Western blot with anti-phospho Thr^{389}-$p70^{S6k}$ Ab (p- $p70^{S6k}$). The filter was then stripped and re-probed with anti-actin Ab. Densitometric analysis was performed and the graph in panel B reports the relative optical density (OD) of phosphoThr^{389}-$p70^{S6k}$ specific bands normalized to the OD of actin on the same lanes.


A
CT
AN
-
3/28
IL-2
-
3/28
IL-2
p- p70S6k
WB:anti-pThr389 -p70S6k
actin
WB:anti-actin

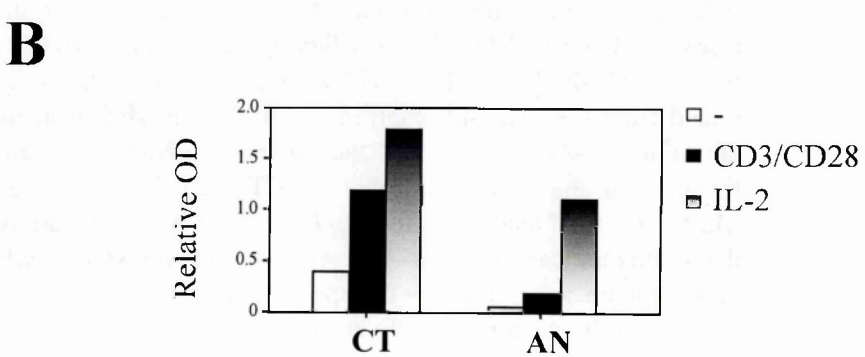

B
Relative OD
2.0
1.5
1.0
0.5
0
CT
AN
-
CD3/CD28
IL-2

Figure 16

Figure 17. Rapamycin-sensitive phosphorylation of $p70^{S6k}$ in resting A.E7 T cells. A.E7 T cells were either left untreated (CT), or treated for 30 min with Rapamycin (1μM) (CT+Rap). The cells were then lysed, and the protein extracts were separated by SDS-PAGE. The proteins with an apparent molecular weight of 60-80 kDa were eluted from the gel, and resolved by 2D-PAGE, shown in the figure, by using an anti-$p70^{S6k}$ Ab (see Materials and Methods). Where indicated, cell extracts were treated with the alkalin phosphatase (CT+AP). 2D maps were analyzed by Image Master 2D Elite analysis software. Densitometric graphs are reported: the values on the X axis indicate the relative pixel position, expressed in arbitrary units, while the values on the Y axis indicate the optical density (OD) units. The pI range of the spots is indicated by the arrows. IEF: isoelectrofocusing.

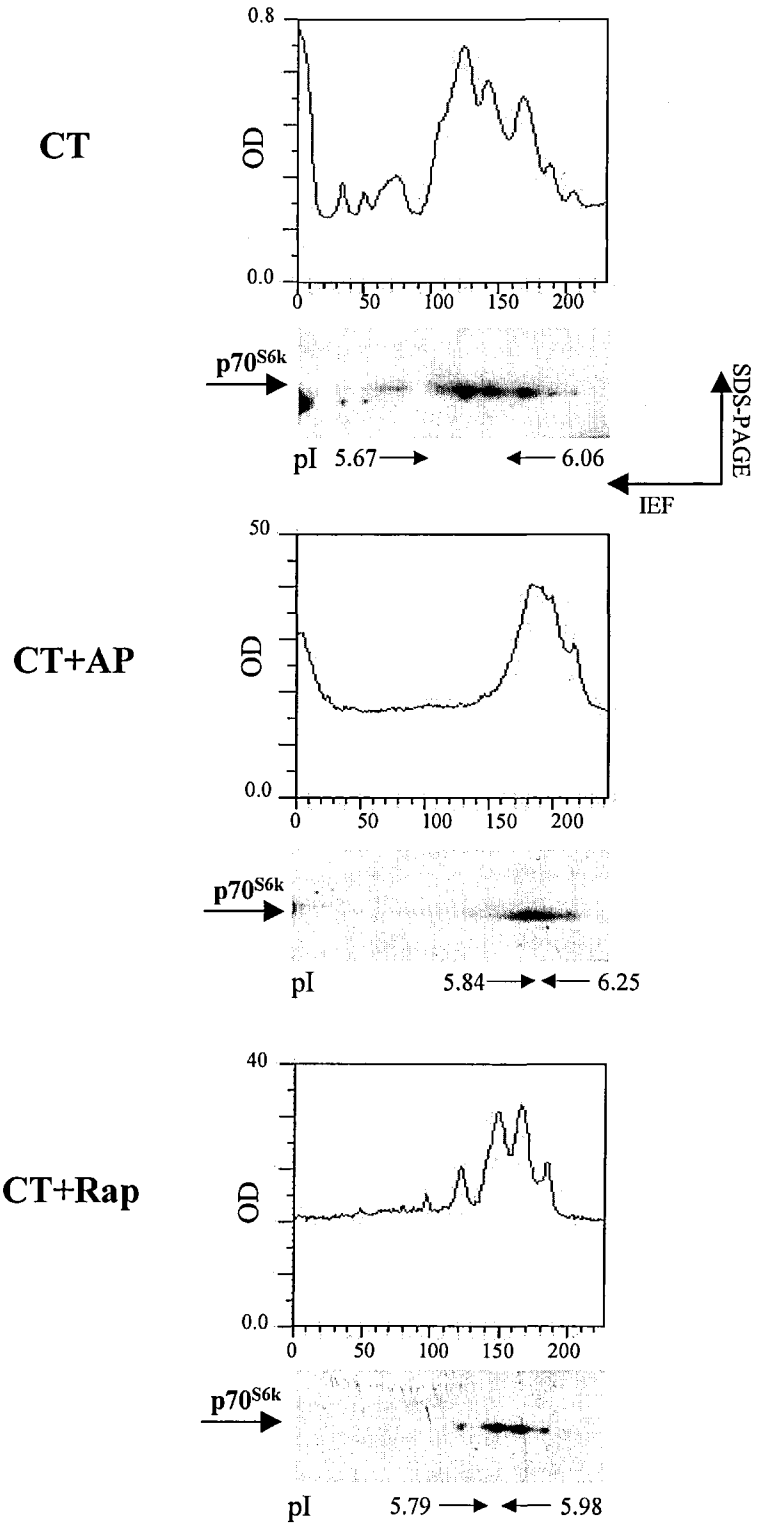

CT
0.8
OD
0.0
0
50
100
150
200
p70S6k
pI 5.67
6.06
SDS-PAGE
IEF
CT+AP
50
OD
0.0
0
50
100
150
200
p70S6k
pI
5.84
6.25
CT+Rap
40
OD
0.0
0
50
100
150
200
p70S6k
pI
5.79
5.98

Figure 17

them corresponding to a differentially modified protein variant (Fig. 17, upper panel). Treatment of the extracts with AP reduced the number of $p70^{S6k}$ specific spots and revealed a more basic pI (pI range: 5.84-6.25). A similar picture was obtained when proteins were derived from control cells treated for 30 min with Rapamycin. When compared to the two-dimensional (2-D) maps obtained from control extracts, 2-D maps obtained from Rapamycin-treated cells showed a reduced number of $p70^{S6k}$ specific spots with a lower pI (pI range: 5.79-5.98) (Fig. 17, lower panel). These data demonstrate that, in the absence of stimulation, the electrophoretic profile of $p70^{S6k}$ is consistent with constitutive phosphorylation of the protein (responsible for its acidification), which is controlled, at least in part, by mTor-dependent activity.

We thus compared, by 2-D analysis, protein extracts derived from control and anergic cell. The cells were either left untreated, or stimulated with CD3/CD28 or rIL-2, lysed and analyzed as described above (Fig. 18). When compared to untreated cells (CT), both CD3/CD28 and IL-2 stimulations resulted in an acidic pI shift of $p70^{S6k}$ specific spots in control cells (Fig. 18, upper panels). Even though the $p70^{S6k}$ specific spots pI range was comparable in CD3/CD28 and IL-2–stimulated extracts (pI range for CD3/CD28: 5.72-5.87; pI range for IL-2: 5.64-5.89), densitometric analyses revealed that IL-2 stimulation induced a preferential accumulation of $p70^{S6k}$ specific spots towards the more acidic pH values (refer to the densitometric graphs on top of each 2-D map). The addition of Rapamycin at the time of stimulation inhibited acidification of $p70^{S6k}$, and instead rendered the protein even more basic (pI range: 5.80-5.98) (Fig. 19). These data indicates that both CD3/CD28 and IL-2 stimulations of control cells elicit an overall acidification

Figure 18. 2D analysis of post-translational modifications of $p70^{S6k}$. Control (CT) and anergic (AN) cells were either left untreated, or stimulated with anti-CD3/CD28 mAbs (CD3/CD28) or with rIL-2 for 30 min. Thereafter protein extracts were prepared as described in Fig. 17, and 2D-PAGE analysis was performed by using an anti-$p70^{S6k}$ Ab. 2D maps were analyzed by Image Master 2D Elite analysis software. Densitometric graphs and pI range of the spots are reported.

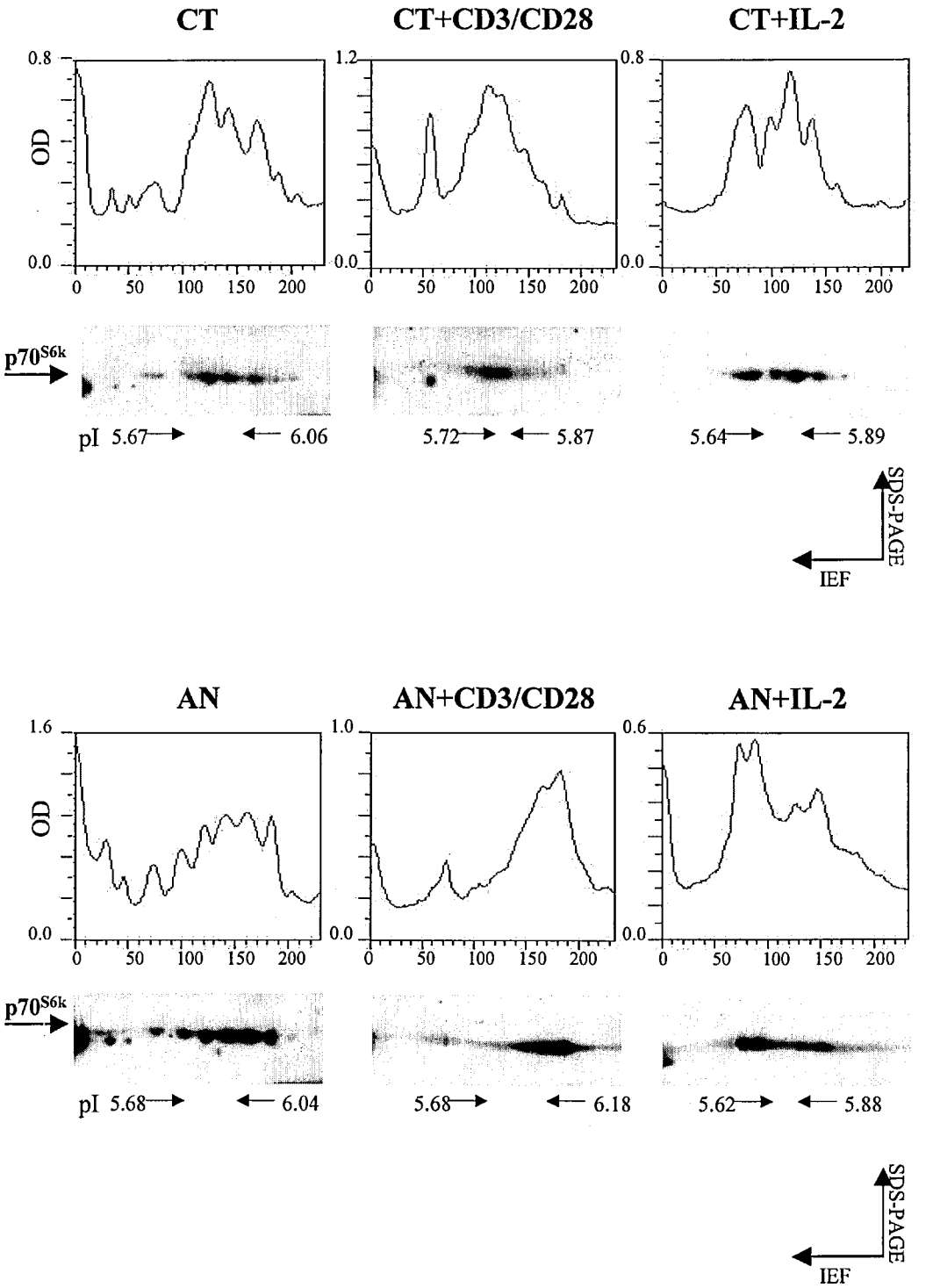

CT
CT+CD3/CD28
CT+IL-2
OD
p70S6k
pI 5.67
6.06
5.72
5.87
5.64
5.89
SDS-PAGE
IEF
AN
AN+CD3/CD28
AN+IL-2
OD
p70S6k
pI 5.68
6.04
5.68
6.18
5.62
5.88
SDS-PAGE
IEF

Figure 18

Figure 19. Rapamycin prevents post-translational modifications of $p70^{S6k}$ in control T cells. Control (CT) cells were either left untreated, or stimulated with anti-CD3/CD28 mAbs (CD3/CD28), in the absence or in the presence of Rapamycin (1 μM) (+Rap) for 30 min. Thereafter protein extracts were prepared as described in Fig. 17, and 2D-PAGE analysis was performed by using an anti-$p70^{S6k}$ Ab. 2D maps were analyzed by Image Master 2D Elite analysis software. Densitometric graphs and pI range of the spots are reported.

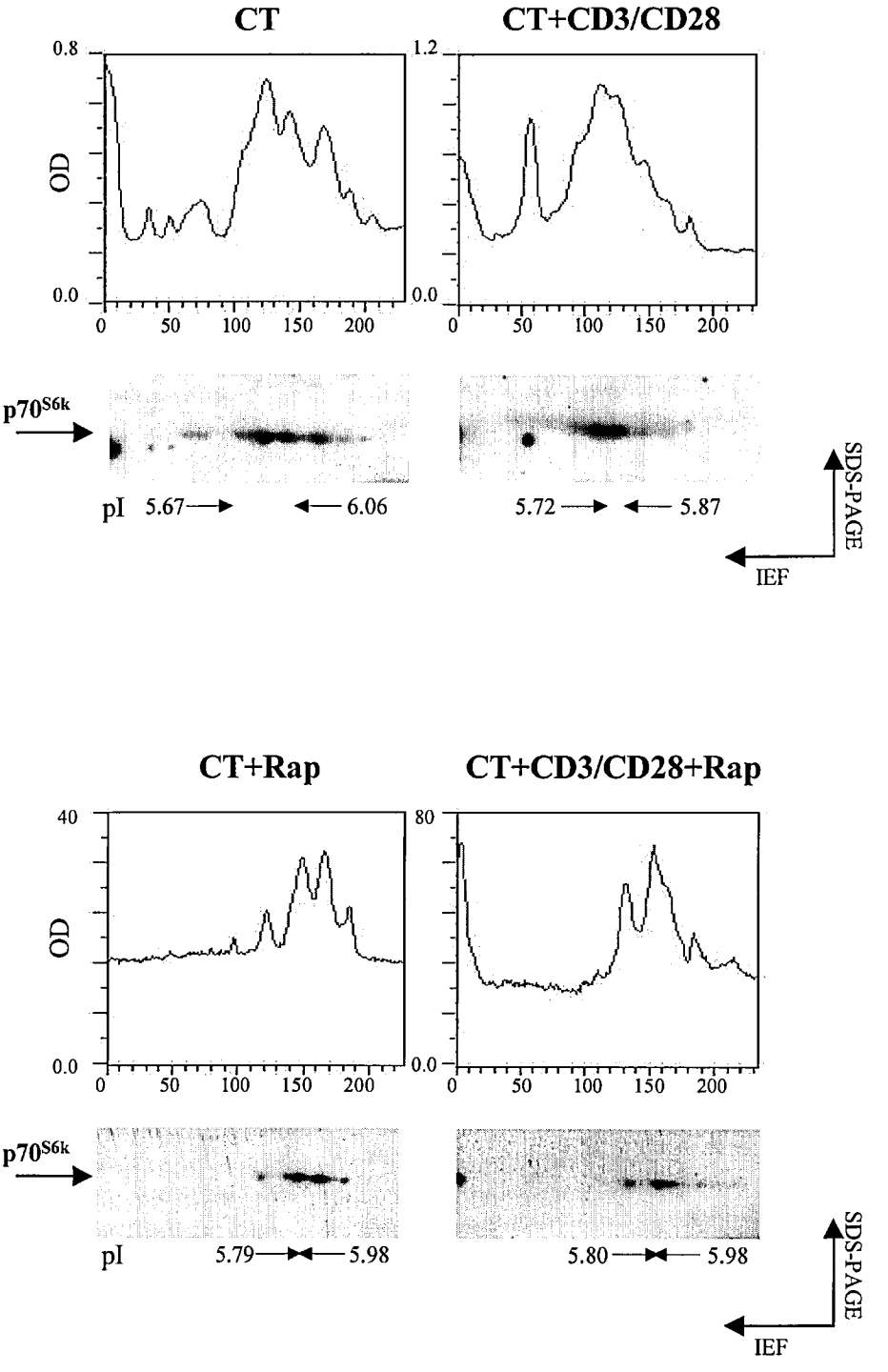

CT
CT+CD3/CD28
0.8
0.0
1.2
0.0
OD
0
50
100
150
200
p70S6k
pI 5.67
6.06
5.72
5.87
SDS-PAGE
IEF
CT+Rap
CT+CD3/CD28+Rap
40
0.0
80
0.0
OD
0
50
100
150
200
p70S6k
pI 5.79
5.98
5.80
5.98
SDS-PAGE
IEF

Figure 19

of $p70^{S6k}$, which most likely reflect a number of phosphorylation events (see below), which require intact mTor- signalling capabilities.

We next analyzed $p70^{S6k}$ electrophoretic profile in anergic cells (Fig. 18, lower panels). The distribution pattern of $p70^{S6k}$ specific spots in unstimulated anergic cells (pI range: 5.68-6.04) was similar to that obtained in untreated control cells (pI range: 5.67-6.06). In anergic cells, the more basic spots were more abundant than in control cells, suggesting that in these cells $p70^{S6k}$ may be hypophosphorylated. Upon CD3/CD28 stimulation, no significant acidification of $p70^{S6k}$ was detected in anergic cells (pI range: 5.68-6.18), and instead some of the more acidic spots detectable in the unstimulated counterparts were lost. Thus, while CD3/CD28 elicited $p70^{S6k}$ acidification in control cells, it failed to do so in anergic cells. In these cells, instead, IL-2 stimulation induced a clear acidic pI shift of $p70^{S6k}$ specific spots (pI range: 5.62 –5.88), which resulted comparable to the one obtained in control cells. Addition of Rapamycin to anergic cells at the time of stimulation (Fig. 20), similarly to control cells, induced a basic shift in the $p70^{S6k}$ pI (pI range: 5.79-6.05), compared to untreated cells. Together these data support the possibility that, while IL-2-mediated signals drive acidification, probably due to phosphorylation, of $p70^{S6k}$, CD3/CD28-mediated signals fail to properly induce this kind of post-translational modification in the protein.

Altogether the results reported in sections 3.1 and 3.2 indicate that mTor-signals are involved in both T cell proliferation and in T cell antigen responsiveness, and that the inability of CD3/CD28 to reverse clonal anergy, in spite of driving mTor-dependent T cell proliferation, could be correlated with its inability to properly activate $p70^{S6k}$.

Figure 20. Rapamycin prevents post-translational modifications of $p70^{S6k}$ in anergic T cells. Anergic (AN) cells were either left untreated, or stimulated with anti-CD3/CD28 mAbs (CD3/CD28), in the absence or in the presence of Rapamycin (+Rap) (1 μM) for 30 min. Thereafter protein extracts were prepared as described in Fig. 17, and 2D-PAGE analysis was performed by using an anti-$p70^{S6k}$ Ab. 2D maps were analyzed by Image Master 2D Elite analysis software. Densitometric graphs and pI range of the spots are reported.

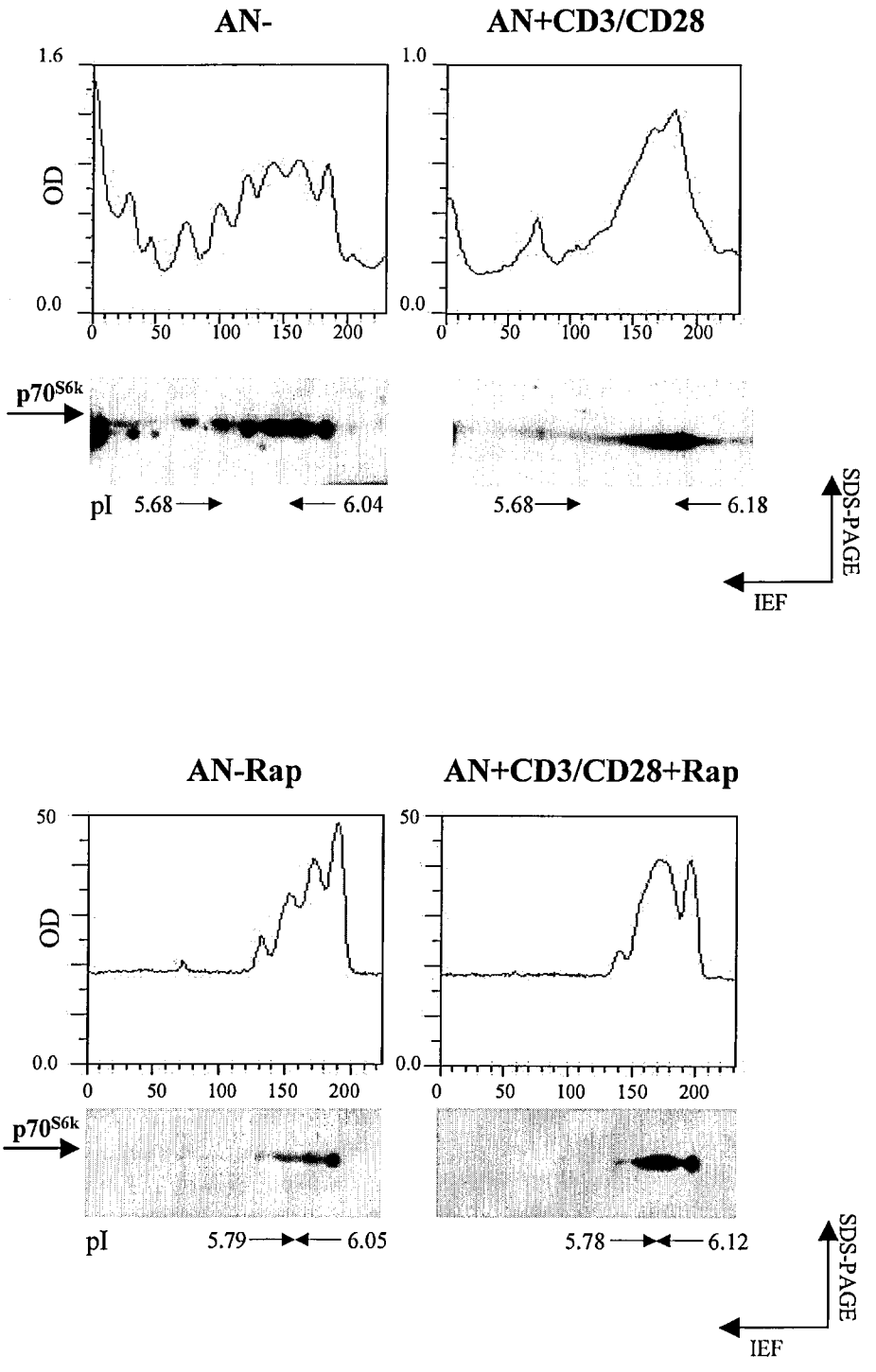

AN-
AN+CD3/CD28
1.6
1.0
OD
0.0
0.0
0
50
100
150
200
p70S6k
pI 5.68
6.04
5.68
6.18
SDS-PAGE
IEF
AN-Rap
AN+CD3/CD28+Rap
50
50
OD
0.0
0.0
0
50
100
150
200
p70S6k
pI 5.79
6.05
5.78
6.12
SDS-PAGE
IEF

Figure 20

3.2 mTor and PI3K-dependent signals synergize to drive $CD4^+$ T cell proliferation

3.2.1 IL-2-driven T cell proliferation is only delayed, and not inhibited, by Rapamycin

The observation that T cell expansion was still detected in the presence of Rapamycin challenged the original idea that Rapamycin inhibits T cell responses by blocking T cell proliferation. To better investigate the ability of Rapamycin to regulate cell cycle progression in T cells, we labelled A.E7 T cells with CFSE and analyzed IL-2-induced T cell proliferation in the presence or in the absence of Rapamycin at different times (Fig. 21). After 3 days of stimulation, control cells had already performed two-three division cycles. In contrast, in the presence of Rapamycin, the cells failed to divide at this time (Fig. 21, upper panels). After 4 days of culture, control cells had completed another 2 rounds of cell division. At this time, the cells stimulated in the presence of Rapamycin started to divide (Fig. 21, middle panels) and by day 10 they showed a CFSE profile indistinguishable from that of control cells (Fig. 21, lower panels).

Increasing the concentration of Rapamycin, or providing the drug to the cells every day, did not prevent T cell expansion (data not shown), meaning that the failure of the drug to block cell cycle progression was not due to drug inactivation. These results suggest that, rather than inhibiting T cell proliferation, Rapamycin might only delay cell cycle entry. Once performed the first G1/S transition, Rapamycin-

Figure 21. Rapamycin delays, but does not inhibit, T cell division. Control A.E7 cells were labeled with the fluorescent dye CFSE, and either left untreated, or stimulated with rIL-2 (10 IU/ml) in the absence (IL-2; left panels) or in presence of Rapamycin (200 nM) (IL-2+Rap; right panels) for the indicated days and then analyzed by flow cytometry. Representative FACS histograms reporting the degree of CFSE dilution are shown.

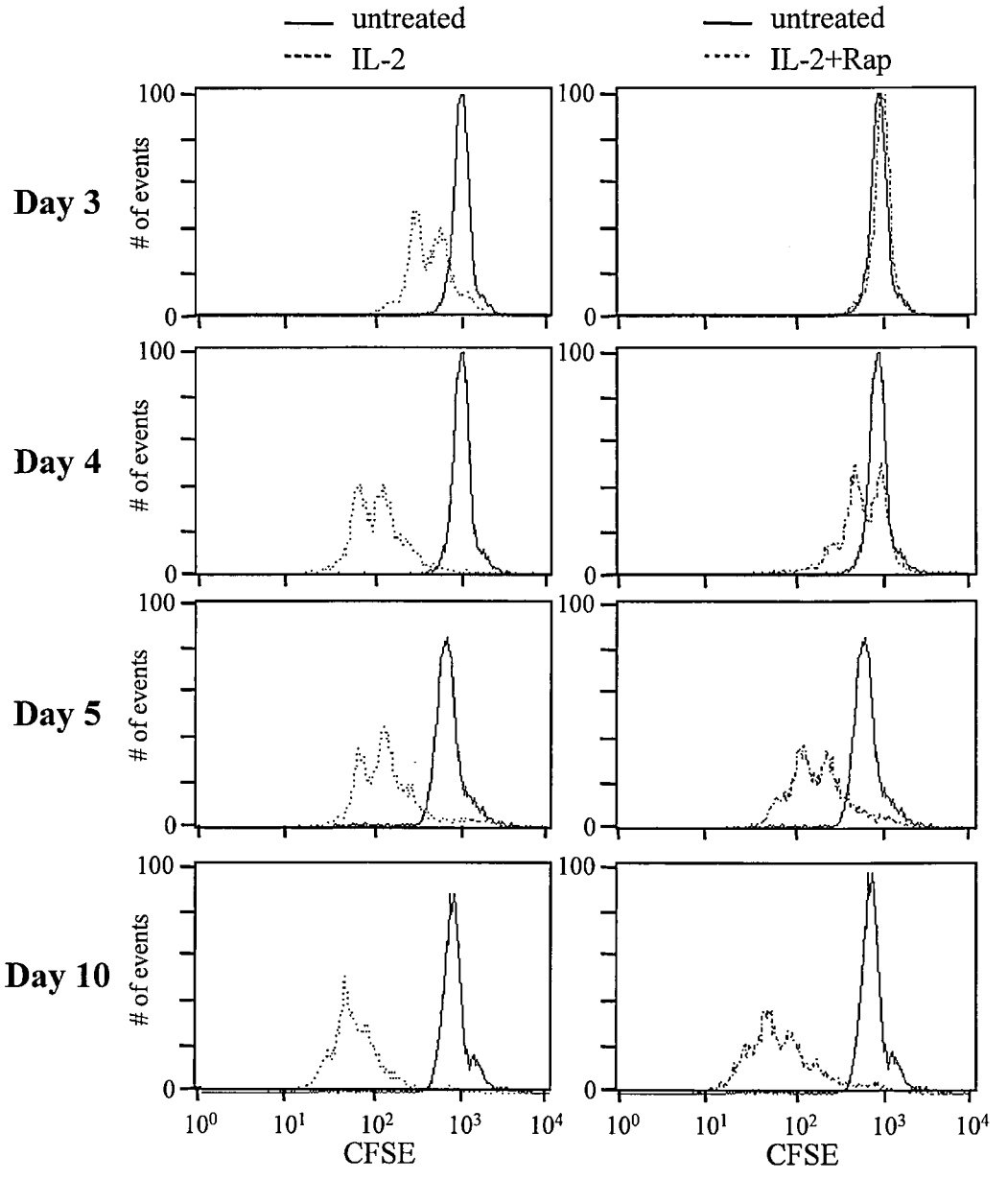

untreated
IL-2
untreated
IL-2+Rap
Day 3
Day 4
Day 5
Day 10
# of events
100
0
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 21

treated cells could then be able to progress into the cell cycle and to divide as much as untreated cells. These findings are consistent with previously published observations demonstrating that Rapamycin induced a prolongation of the G1 phase of the cell cycle in mitogen stimulated T lymphocytes (285).

3.2.2 mTor-dependent signalling is required when IL-2 is limiting

We next investigated whether Rapamycin insensitive T cell expansion could be observed also in the presence of limiting amounts of IL-2. To this aim, CFSE-labelled A.E7 cells were stimulated for 5 days with different doses of IL-2 in the absence or in the presence of Rapamycin (Fig. 22). The percentage of dividing cells and the number of cell cycles performed by the T cell population varied depending on the dose of IL-2 in culture (Fig. 22, left panels). At the lowest dose of IL-2 tested (0.4 IU/ml), the percentage of dividing cells was about 20%, and increased to 70% and almost to 100% in the presence of 2 IU/ml and 10 IU/ml of IL-2, respectively. In addition, while at the lowest dose of IL-2 the cells performed only one division cycle, in the presence of 2 IU/ml and 10 IU/ml of IL-2, the cells performed two and more than four cell cycles, respectively.

In the presence of Rapamycin (Fig. 22, right panels), no cell division was observed at 0.4 IU/ml of IL-2. At 2 IU/ml, 50% of the cells failed to enter the cell cycle and the remaining 50% completed only one division cycle. At 10 IU/ml of IL-2, only 10% of the cells were still undivided, and the dividing cells had performed a number of cell cycles comparable to the one observed in cells stimulated in the absence of Rapamycin.

Figure 22. Rapamycin inhibits IL-2-mediated proliferation only at low doses of IL-2. Control A.E7 cells were labeled with the fluorescent dye CFSE, and either left untreated or stimulated with the indicated amounts of rIL-2 (indicated beside the panels) in the absence (IL-2; left panels) or in presence of Rapamycin (200 nM) (IL-2+Rap; right panels) for 5 days, and then analyzed by flow cytometry. Representative FACS histograms reporting the degree of CFSE dilution are shown. The percentages of dividing cells is reported.

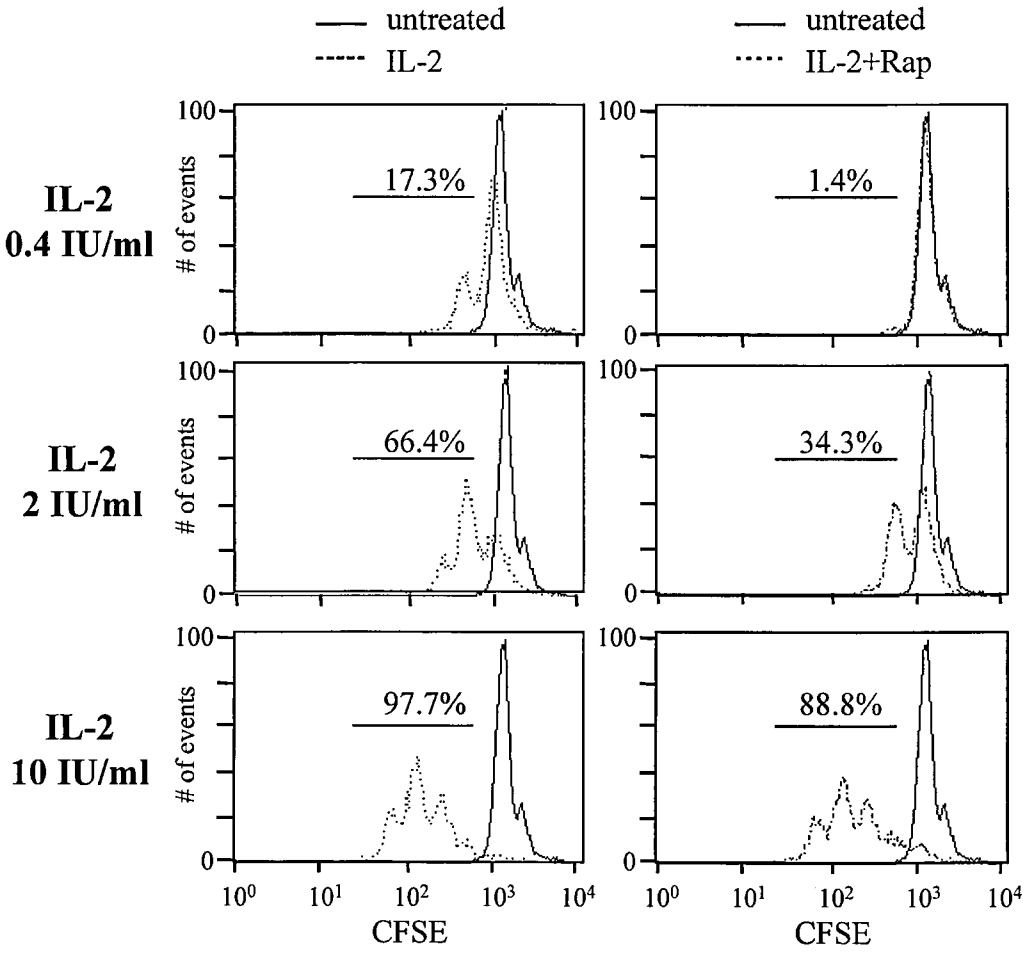

untreated
IL-2
untreated
IL-2+Rap
IL-2
0.4 IU/ml
IL-2
2 IU/ml
IL-2
10 IU/ml
# of events
100
0
17.3%
1.4%
66.4%
34.3%
97.7%
88.8%
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 22

These results indicate that, while mTor-induced signalling is required in the presence of limiting amounts of IL-2, it is dispensable at saturating concentration of IL-2. In this latter situation, mTor-independent IL-2-dependent signalling pathways driving cell cycle progression are induced, and allow Rapamycin insensitive T cell proliferation.

IL-2-driven T cell proliferation has been previously shown to involve PI3K activity (89). To investigate the role of PI3K in our model, we stimulated CFSE-labelled A.E7 T cells for 5 days with rIL-2 (10 IU/ml) in the absence or in the presence of the PI3K inhibitor LY 294002 (LY), Rapamycin, or a combination of the two drugs (Fig. 23). As shown above (Fig. 21, 22), the CFSE profiles of untreated and Rapamycin- treated cells after 5 days of culture were very similar. In contrast, inhibition of PI3K activity by LY treatment, almost completely inhibited IL-2-driven T cell proliferation. The residual proliferation observed with LY treatment was completely inhibited by the addition of Rapamycin (Fig. 23, last panel). These data indicate that, at high doses of IL-2, T cell proliferation is mostly driven by PI3K-dependent, mTor-independent signalling. The observation that the combination of Rapamycin and LY is more potent in blocking IL-2-mediated T cell proliferation, than either of the two drugs alone, suggests that an mTor-dependent signal, activated independently of PI3K, is also required for cell cycle progression.

Figure 23. PI3K and mTor signals synergize to drive optimal IL-2-induced T cell proliferation. Control A.E7 cells were labeled with the fluorescent dye CFSE, and either left untreated, or stimulated with rIL-2 (10 IU/ml), in the absence or in the presence of Rapamycin (200 nM) (IL-2+Rap), LY294002 (20 mM) (IL-2+LY), or the combination of the two drugs (IL-2+Rap+LY) for 5 days, and then analyzed by flow cytometry. Representative FACS histograms reporting the degree of CFSE dilution are shown.

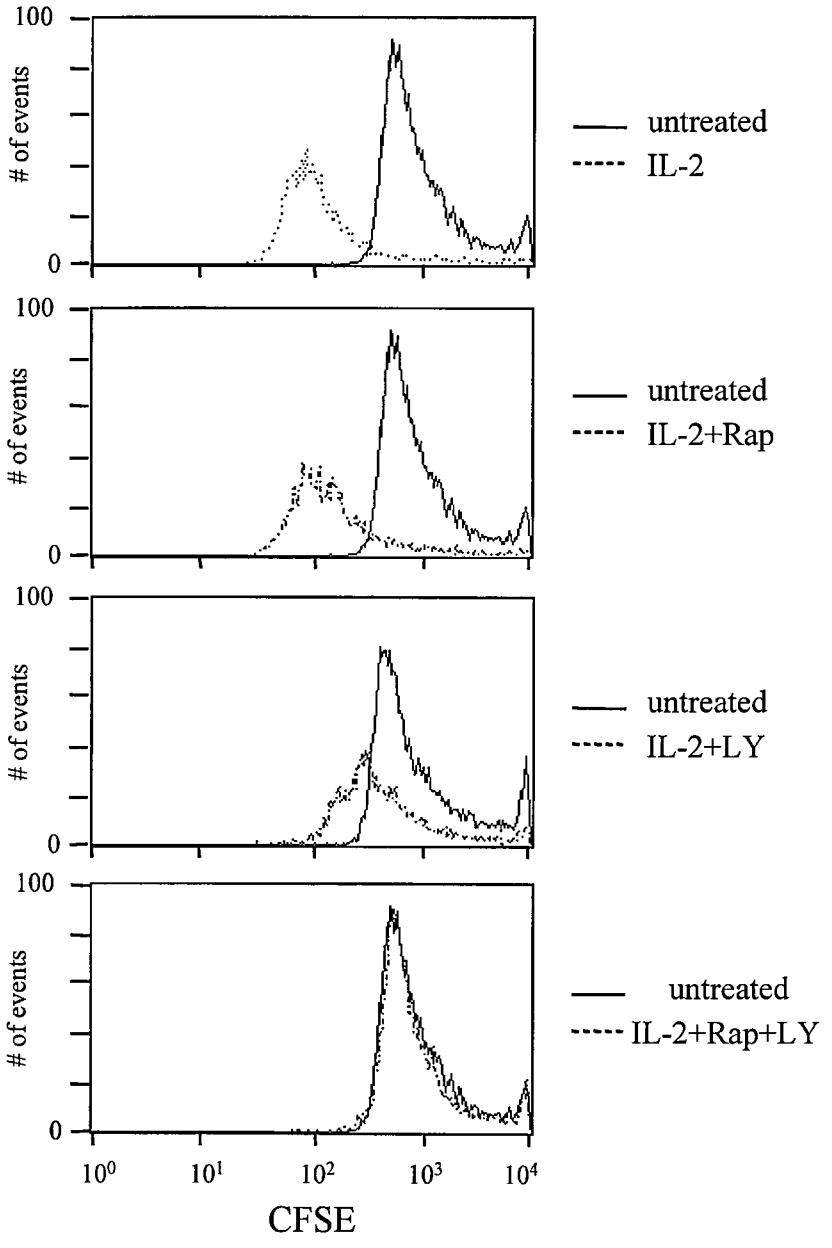

100
# of events
0
untreated
IL-2
100
# of events
0
untreated
IL-2+Rap
100
# of events
0
untreated
IL-2+LY
100
# of events
0
untreated
IL-2+Rap+LY
10^0
10^1
10^2
10^3
10^4
CFSE

Figure 23

3.3 mTor-dependent signals are required for T cell differentiation

The results obtained with A.E7 T cell clones indicated that mTor-dependent signals were critically required by CD3/CD28 to induce T cell proliferation, and by IL-2/IL-2R to maintain and restore antigen responsiveness.

To further analyze the role of mTor during T cell responses, we investigated the effect of Rapamycin on Ag-driven proliferation and differentiation of naïve $CD4^+$ and $CD8^+$ T cells. As model antigens, we used the chicken ovalbumin-derived class II and class I-restricted peptides, $OVAp_{323-339}$ and $OVAp_{257-264}$ (referred to as "Ag"), respectively recognized in the context of IA^d and K^b, by TCR Tg T cells of DO11.10, and OT-I mice (270, 271).

3.3.1 mTor signalling is dispensable for Ag-driven T cell proliferation

To analyze the effect of Rapamycin on naïve T cell proliferation, LN cells derived from DO11.10 TCR Tg mice were labelled with the fluorescent dye CFSE (281), stimulated with Ag and irradiated syngeneic splenocytes in the absence or in the presence of Rapamycin (200 nM), and analyzed by flow cytometry after 3 and 7 days of culture. By 72 hours of stimulation (day 3), most of the $CD4^+$ T cells acquired an activated phenotype ($CD69^{high}$, $CD44^{high}$) in both control and Rapamycin-treated cultures (not shown). At this time, however, while Ag-driven stimulation of $CD4^+$ T cells induced a fraction of the cells to complete up to three cell divisions, most of the cells derived from the Ag+Rapamycin treated cultures remained undivided (Fig. 24A, left panels). In contrast, by seven days of culture, the

Figure 24. Rapamycin delays, but does not inhibit antigen-driven naïve T cell proliferation. **A, C)** CFSE-labeled DO11.10 LN cells were cultured with Ag and irradiated syngeneic splenocytes, in the absence (Ag), or in the presence of Rapamycin (100 nM) (Ag+Rap), and in the presence of Rapamycin and rIL-2 (10 IU/ml) (Ag+Rap+IL-2). After 3 (A; left panel, and C) and 7 (A; right panel) days, the cultures were harvested, and analyzed by flow cytometry following staining with anti-CD4 mAb. The histograms in A and C were obtained after gating on $CD4^+/CFSE^+$ cells. **B)** DO11.10 LN cells were stimulated with Ag and irradiated syngeneic splenocytes (Ag), or with anti-CD3/CD28 mAbs (CD3/CD28), in the absence (-) or in the presence of Rapamycin (100 nM) (+Rap). Twenty-fours hours after stimulation, culture supernatants were recovered and IL-2 was measured by capture ELISA. Data ± SD of triplicate cultures are indicated.

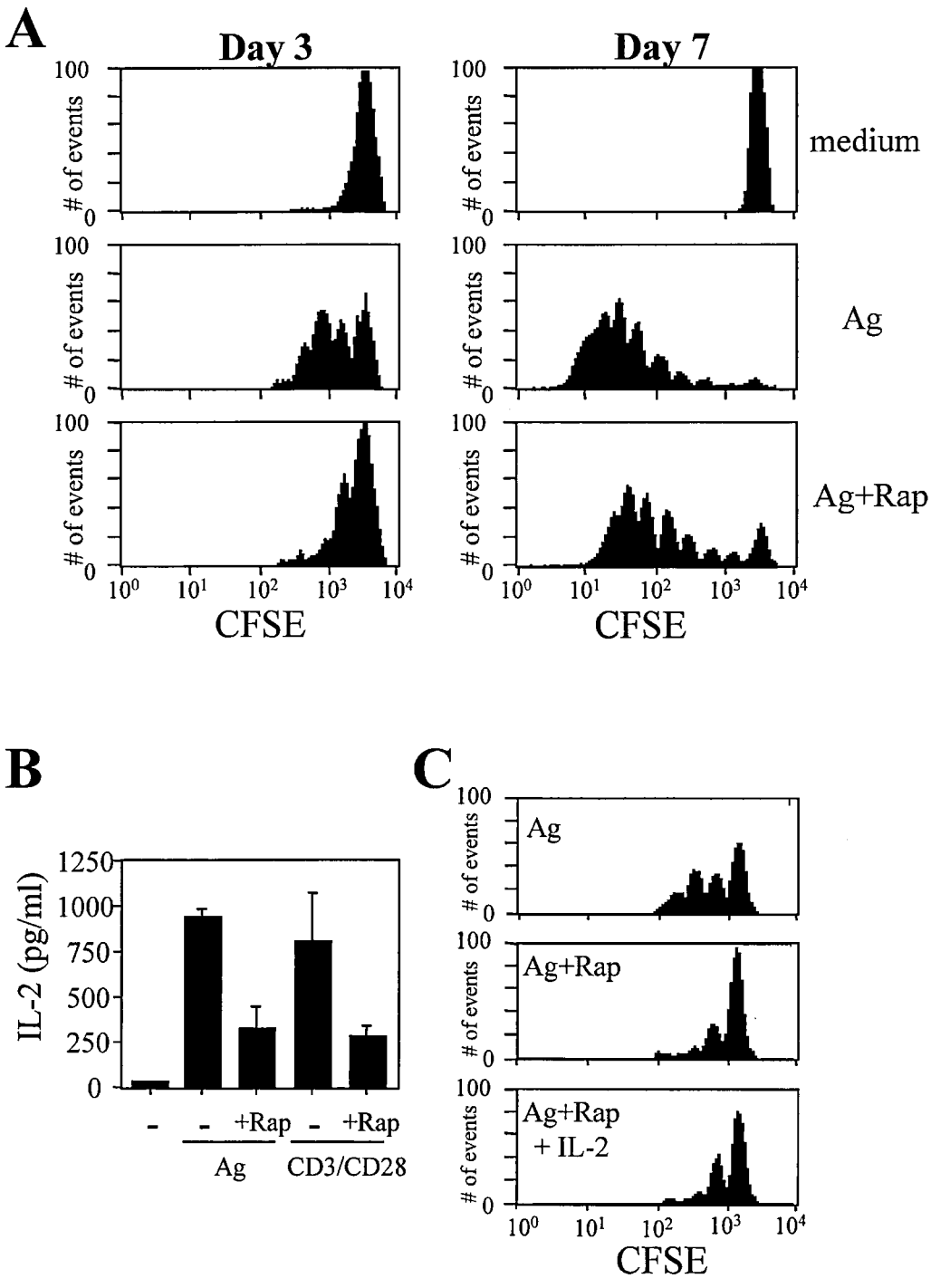

A
Day 3
Day 7
medium
Ag
Ag+Rap
# of events
CFSE
B
IL-2 (pg/ml)
1250
1000
750
500
250
0
- - +Rap - +Rap
Ag
CD3/CD28
C
Ag
Ag+Rap
Ag+Rap
+ IL-2

Figure 24

CFSE profile of control and Rapamycin-treated cells was mostly indistinguishable (the average number of cell divisions in the absence and in the presence of Rapamycin, calculated over 7 independent experiments, was 7.5 ± 1.1 and 5.9 ± 1.1, respectively) (Fig. 24A, right panels). Thus, Rapamycin failed to prevent Ag-driven T cell proliferation. Even though this finding seems to contradict previously reported results, it should be noted that most of the previous studies measured proliferation by [^{3}H]-Thymidine incorporation 72 hours following Ag stimulation. In our own experiments Rapamycin prevented T cell proliferation at this time by either CFSE-dilution (Fig. 24A), or by [^{3}H]-Thymidine incorporation (140,842 ± 17,077 cpm and 40,516 ± 6,498 cpm, control and Rapamycin-treated cultures, respectively), but failed to do so at later times (Fig. 24A). Repeated additions of Rapamycin did not prevent T cell division (not shown), suggesting that inactivation of the drug could not account for its failure to block T cell expansion. Moreover, even though Rapamycin reproducibly inhibited IL-2 secretion (Fig. 24B), the addition of exogenous rIL-2 failed to restore Ag-driven T cell expansion (Fig. 24C).

Thus, our results are consistent with the possibility that Rapamycin only delays cell cycle entry (285), and indicate that, as shown for T cell clones, also naïve CD4$^+$ T cells are capable of mTor-independent T cell expansion.

3.3.2 Rapamycin inhibits Ag-driven Th1 and Tc1 T cell polarization

Preliminary experiments indicated that Ag-driven T cell activation of DO11.10 T cells in our culture conditions favoured differentiation of the CD4$^+$ T

cell population into IFN-γ-producing cells (Th1). Thus, after a week in culture, a fraction of DO11.10 T cells showed to be able to produce IFN-γ, and in some experiments also IL-4, upon restimulation with PMA and Ionomycin. Thus, we set to investigate whether Rapamycin could have an effect on T cell differentiation (Fig. 25). To this aim, LN cells from DO11.10 mice were labelled with CFSE, stimulated with Ag and irradiated syngeneic splenocytes for a week in the absence (Ag) or in the presence of Rapamycin (Ag-Rap), and then either left untreated, or restimulated with PMA and Ionomycin (PMA/Iono). After 4 hours, intracellular cytokine release was assessed by flow cytometry. As shown above, by seven days of culture the CFSE profiles of control and Rapamycin treated cells were mostly indistinguishable (Fig. 25). Moreover, both control and Rapamycin-treated cultures were able to produce IL-2 upon restimulation. Thus up to 52.2% and 67.9% of $CD4^+$ T cells were $IL\text{-}2^+$ in control and Rapamycin treated culture, respectively (Fig. 25A and 25C). In contrast, while up to 65.3% of the cells found in control cultures secreted IFN-γ, only 12.0% of the cells in the Rapamycin-treated cultures were capable of doing so (Fig. 25B and 25C). The failure to produce IFN-γ was also revealed by measuring the presence of the cytokine in culture supernatants by capture ELISA. Indeed, while IFN-γ was detected between 24 and 72 hours in the culture supernatant of cells cultured in the absence of Rapamycin and restimulated with PMA and Ionomycin, this cytokine could never be detected in culture supernatant of restimulated Rapamycin-treated cells (data not shown). While IL-4 was detected in some experiments in the culture supernatants of restimulated control cells, neither IL-4 nor IL-10 could ever be found in culture supernatants of Rapamycin-treated cells (data not shown).

Figure 25. Rapamycin prevents antigen-driven Th1 cell differentiation, independently of cell proliferation. CFSE-labeled DO11.10 LN cells were cultured for 7 days with Ag and irradiated syngeneic splenocytes in the absence (Ag), or in the presence of Rapamycin (100 nM) (Ag-Rap). Thereafter, the cells were restimulated for 4 hours with medium only (-) or with PMA and Ionomycin (PMA/Iono), and analyzed following staining with anti-CD4 mAb, and either anti-IL-2 **(A)** or anti-IFN-γ mAbs **(B)**. Dot plots show events upon gating on $CD4^+/CFSE^+$ cells. The percentage of $IL\text{-}2^+$ or $IFN\text{-}\gamma^+$ cells is reported. The quadrants were defined based on the CFSE/cytokine content of undivided/unstimulated cells (not shown). **C)** The mean percentage ± SD of naïve D011.10 T cells, and of cells expanded for 7 days in Ag, Ag and Rapamycin (Ag-Rap), and Ag, Rapamycin and rIL-2 (Ag/IL-2-Rap) able to produce IL-2 or IFN-γ upon PMA/Iono restimulation, calculated over 5 independent experiments, is reported in the graphs.

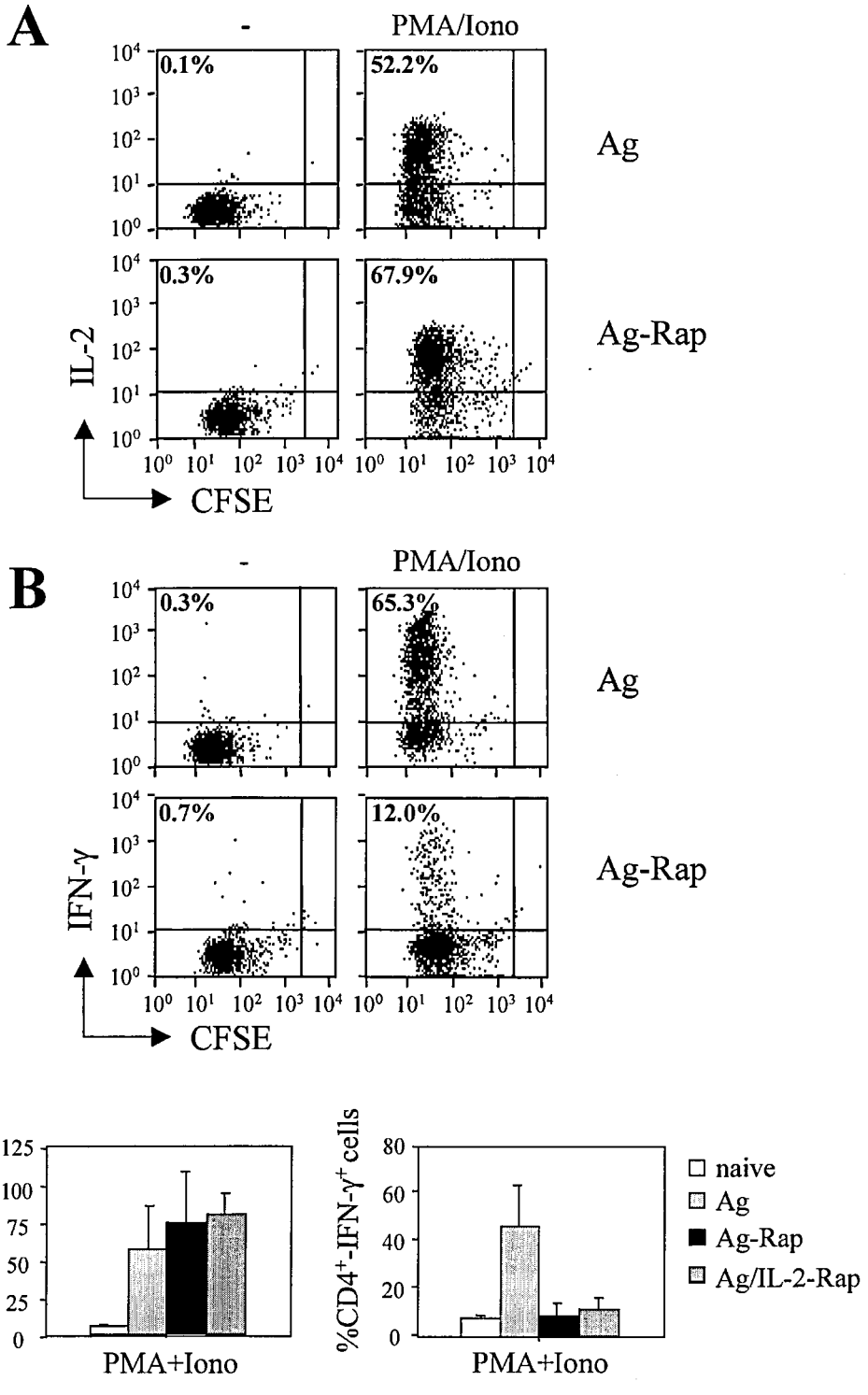

A
-
PMA/Iono
0.1%
52.2%
Ag
0.3%
67.9%
Ag-Rap
IL-2
CFSE
B
-
PMA/Iono
0.3%
65.3%
Ag
0.7%
12.0%
Ag-Rap
IFN-γ
CFSE
C
%CD4+-IL-2+ cells
125
100
75
50
25
0
PMA+Iono
%CD4+-IFN-γ+ cells
80
60
40
20
0
PMA+Iono
naive
Ag
Ag-Rap
Ag/IL-2-Rap

Figure 25

To verify that Rapamycin was inhibiting T cell polarization by blocking mTor signalling directly in T cells, and not in other cells present in the LN cultures, $CD4^+$ T cells were purified, labelled with CFSE and stimulated for a week with anti-CD3/CD28 mAbs immobilized on latex beads, in the absence or in the presence of Rapamycin (Fig. 26). As shown for Ag stimulation, also CD3/CD28-driven T cell expansion was insensitive to the presence of Rapamycin. Thus, all the cells diluted their CFSE content in response to CD3/CD28 and completed up to 9 cell division cycles. Moreover, as in the case of Ag stimulated cultures, CD3/CD28 cultured cells acquired the ability to secrete IFN-γ upon PMA/Iono restimulation, which was prevented by Rapamycin. Thus, while up to 22.4% of the cells derived from CD3/CD28 cultures produced IFN-γ upon rechallenge, only 8.7% of the cells derived from the Rapamycin-treated cultures did so. These results indicate that Rapamycin prevents a cell autonomous Th1 differentiation program, revealing a critical role for mTor in T cell activation.

Similarly to $CD4^+$ T cells, also OVA-specific $CD8^+$ T cells failed to acquire proper effector functions when activated in the presence of Rapamycin (Fig. 27). Thus, CFSE-labelled OT-I T cells, stimulated for a week with the specific Ag, proliferated to similar extent in the absence or in the presence of Rapamycin, and completed up to seven cell divisions (Fig. 27A). However while up to 83.0% of $CD8^+$ T cells in control OT-I cultures produced IFN-γ upon restimulation with PMA and Ionomycin, only 21.5% of the cells derived from Rapamycin-treated cultures did so (Fig. 27A). Furthermore, while OT-I T cells expanded in Ag were able to recognize and kill OVA-pulsed target cells, no specific lytic activity was detected in cells derived from Rapamycin-treated culture (Fig. 27B).

Figure 26. Rapamycin prevents cell-autonomous Th1 cell differentiation. **A)** $CD4^+$ DO11.10 T cells were purified, labeled with CFSE and cultured for 7 days with anti-CD3/CD28 mAbs coated latex beads, in the absence (CD3/CD28) or in the presence of Rapamycin (100 nM) (CD3/CD28-Rap). Thereafter the cells were restimulated for 4 hours with medium only (-) or with PMA and Ionomycin (PMA/Iono), and analyzed following staining with anti-IFN-γ mAb. Dot plots show events upon gating on viable cells. The percentage of IFN-γ^+ cells is reported. **B)** The graph reports the mean percentage ± SD of IFN-γ^+ cells calculated over 3 independent experiments. The significance of differences in cytokine production by T cells expanded in the absence or in the presence of Rapamycin was tested with a two-tailed Student's *t* test.

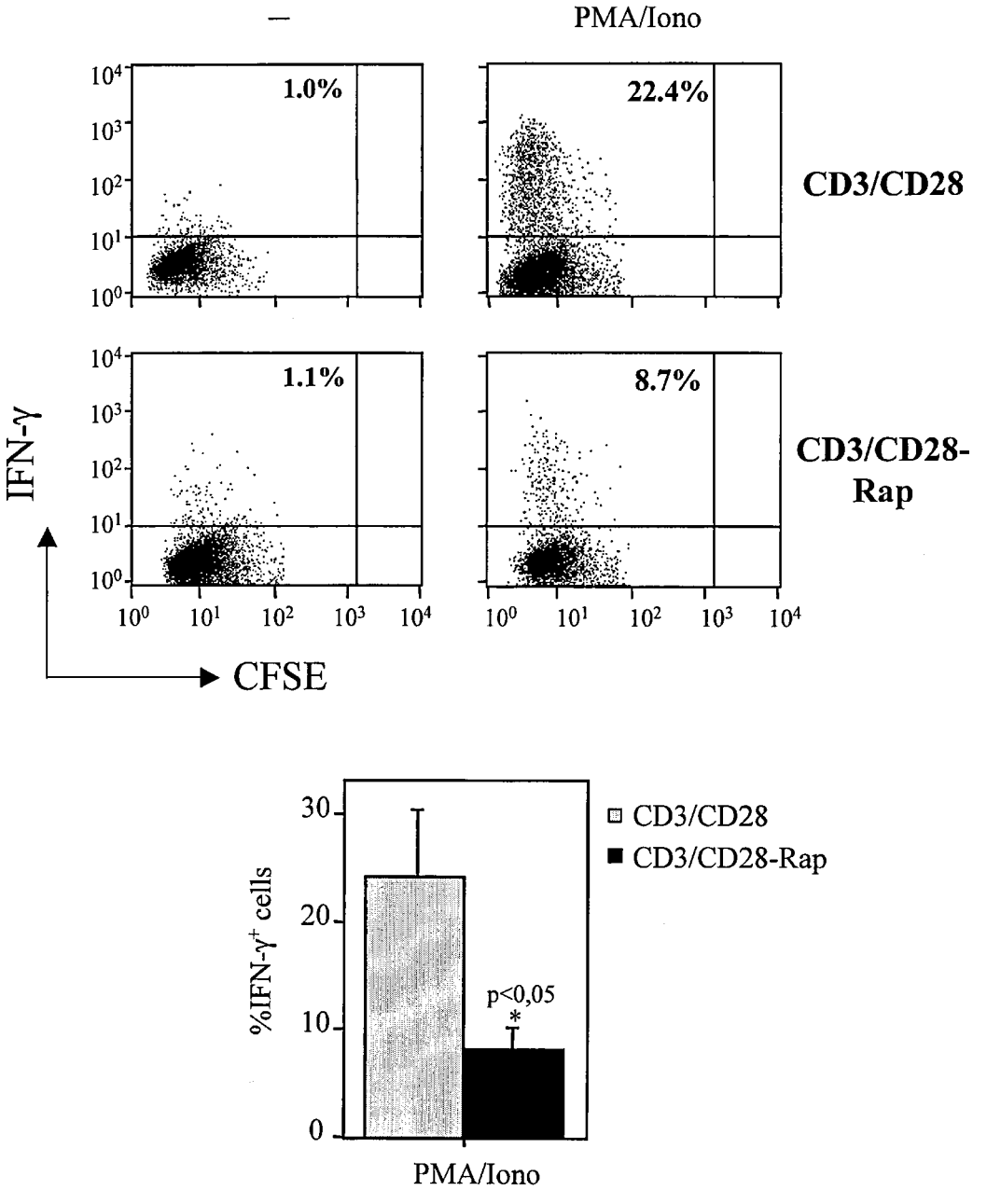
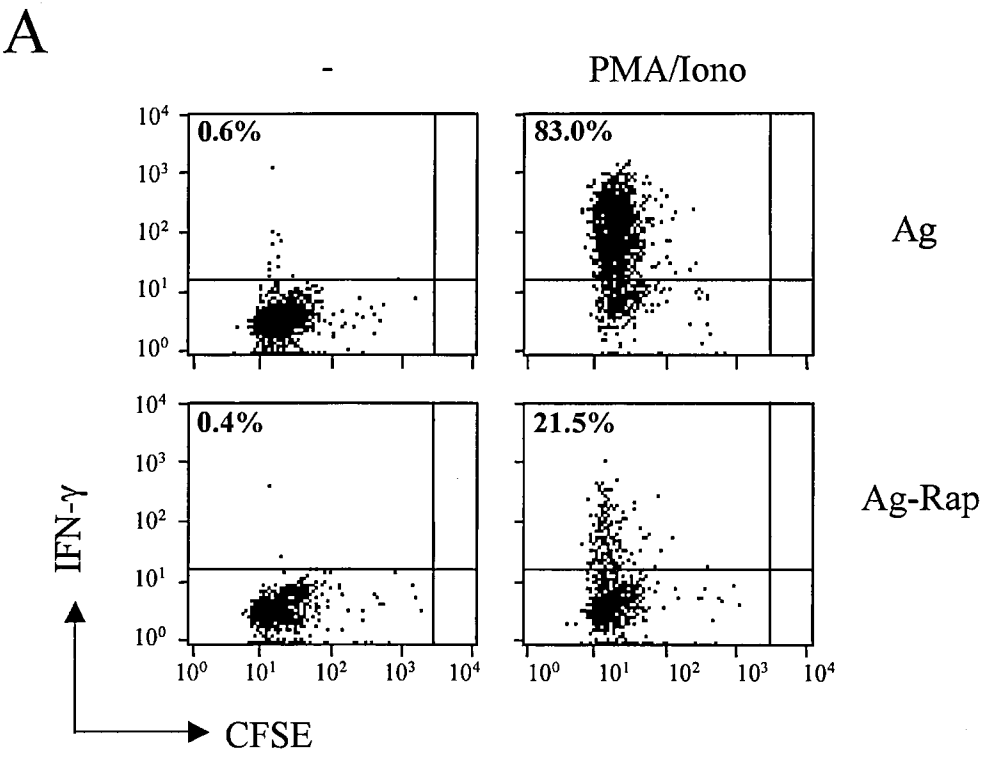

—
PMA/Iono
1.0%
22.4%
CD3/CD28
1.1%
8.7%
CD3/CD28-Rap
IFN-γ
CFSE
%IFN-γ+ cells
CD3/CD28
CD3/CD28-Rap
p<0,05
*
PMA/Iono

Figure 26

Figure 27. Rapamycin prevents $CD8^+$ T cell differentiation, independently of cell proliferation. **A)** CFSE-labeled OT-I LN cells were stimulated with Ag and irradiated syngeneic splenocytes in the absence (Ag) or in the presence of Rapamycin (100 nM). (Ag-Rap). After seven days of culture, the cells were harvested and analyzed for their ability to secrete IFN-γ upon restimulation **(A)**, or to kill OVA-pulsed target cells **(B)**. **A)** The cells were restimulated for 4 hours with medium only (-) or with PMA and Ionomycin (PMA/Iono), and analyzed following staining with anti-CD8 mAb, and anti-IFN-γ mAb. Representative dot plots of events gated on $CD8^+/CFSE^+$ T cells are shown in the figure. The percentage of IFN-γ producing cells is reported. **B)** Ag, and Ag-Rap cells were tested in a standard cytotoxicity assay against unpulsed (RMA) or OVA-pulsed (RMA+OVA) target cells. Data are means ± SD of triplicates of the percentages of specific ^{51}Cr release at the indicated E:T ratios.


A
-
PMA/Iono
0.6%
83.0%
Ag
0.4%
21.5%
Ag-Rap
IFN-γ
CFSE

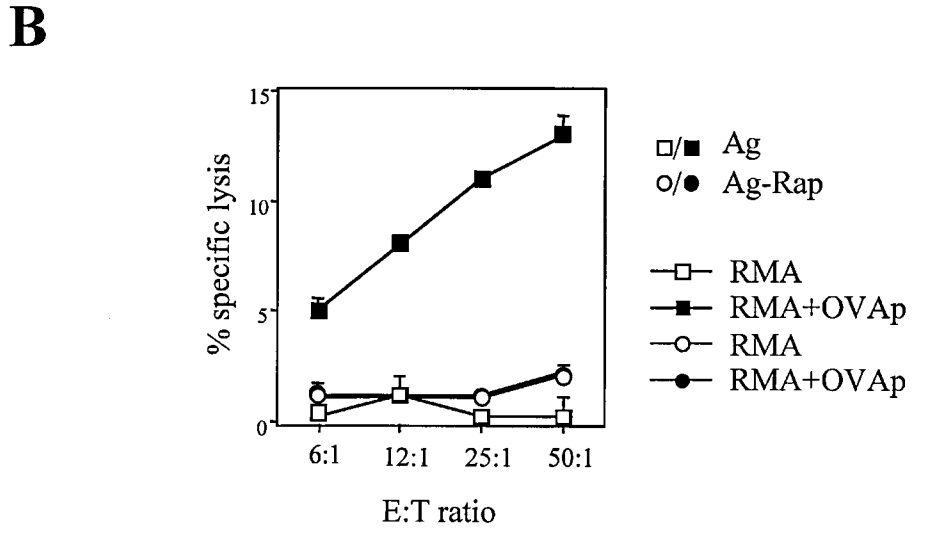

B
% specific lysis
E:T ratio
6:1
12:1
25:1
50:1
Ag
Ag-Rap
RMA
RMA+OVAp
RMA
RMA+OVAp

Figure 27

Altogether these results indicate that Rapamycin, rather than blocking T cell expansion, prevents effector T cell polarization.

3.3.3 Rapamycin fails to establish T cell unresponsiveness

Results derived from *in vitro* and *in vivo* studies suggested that Rapamycin, by preventing IL-2/IL-2R signalling and IL-2-driven T cell expansion, induced a state of T cell antigen unresponsiveness (265, 290). To investigate this possibility, we compared the surface and functional phenotype of DO11.10 T cells expanded in the absence or in the presence of Rapamycin. To this aim, control and Rapamycin-treated cells were analyzed by flow cytometry following staining with anti-$CD4^+$ and anti-CD44, CD25 and CD62L mAbs (Fig. 28). After a week in culture with the specific Ag, the vast majority of $CD4^+$ T cells in either control, and Rapamycin-treated cultures, had undergone more than five cell divisions and had up-regulated the surface expression of CD44 to similar extents (Fig. 28, upper panels). Similarly, in both control and Rapamycin-treated cultures, a comparable fraction of the cells showed increased surface expression of CD25 (Fig. 28, middle panels). However, Rapamycin-treated cells showed suboptimal CD25 expression, as revealed by the MFI of these cells, which was reduced by 50% when compared to the MFI derived from control cells. Furthermore, while up to 84.7% of control cells had downregulated surface expression of CD62L, only 42.4% of Rapamycin-treated cells expressed low levels of this LN homing molecule. These data support the possibility that, in the presence of Rapamycin, Ag-stimulated T cells do not fully differentiate.

Figure 28. Surface phenotype of Rapamycin-treated cells. CFSE-labeled DO11.10 LN cells were cultured for 7 days with Ag and irradiated splenocytes in the absence (Ag) or in the presence of Rapamycin (100 nM) (Ag+Rap). Thereafter, the cells were harvested and analyzed by flow cytometry following staining with anti-CD4, and either anti-CD44, -CD25 or -CD62L mAbs. The percentage of cells with increased expression of CD44, and CD25, or with downregulated expression of CD62L is reported in the plots.

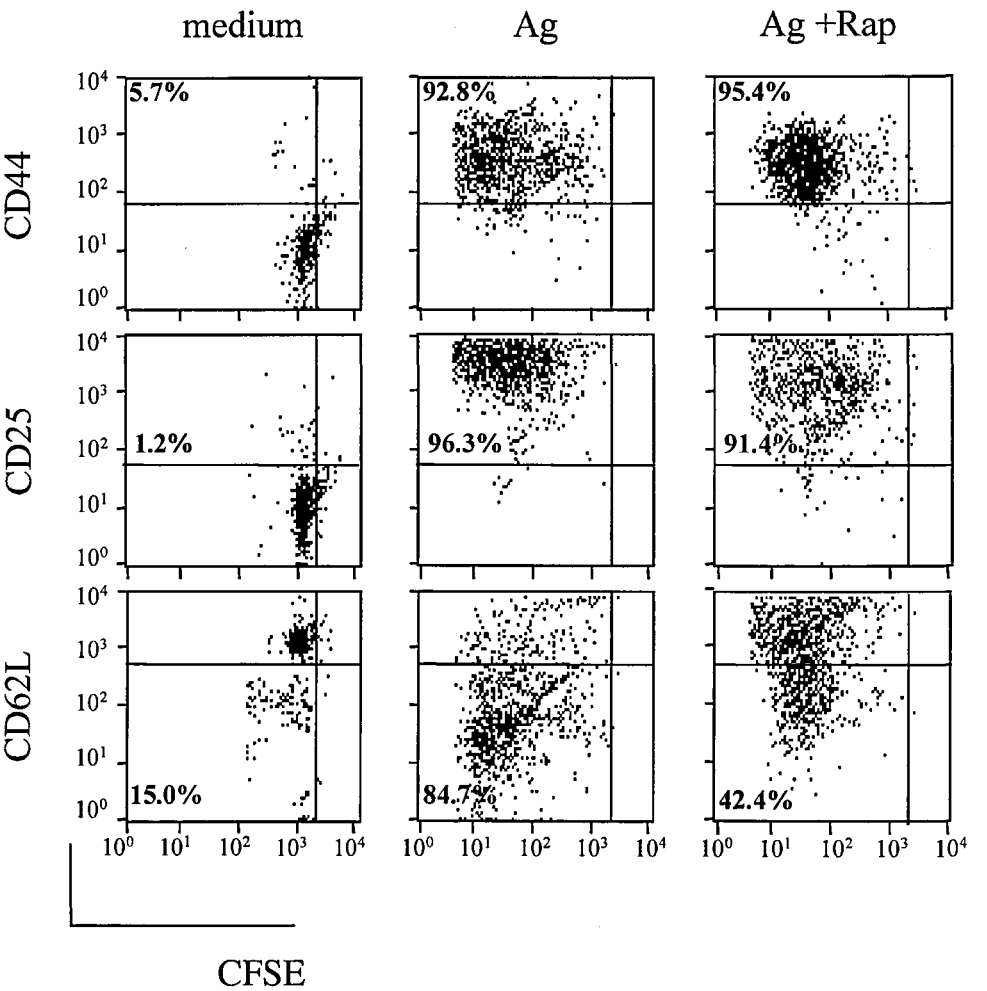
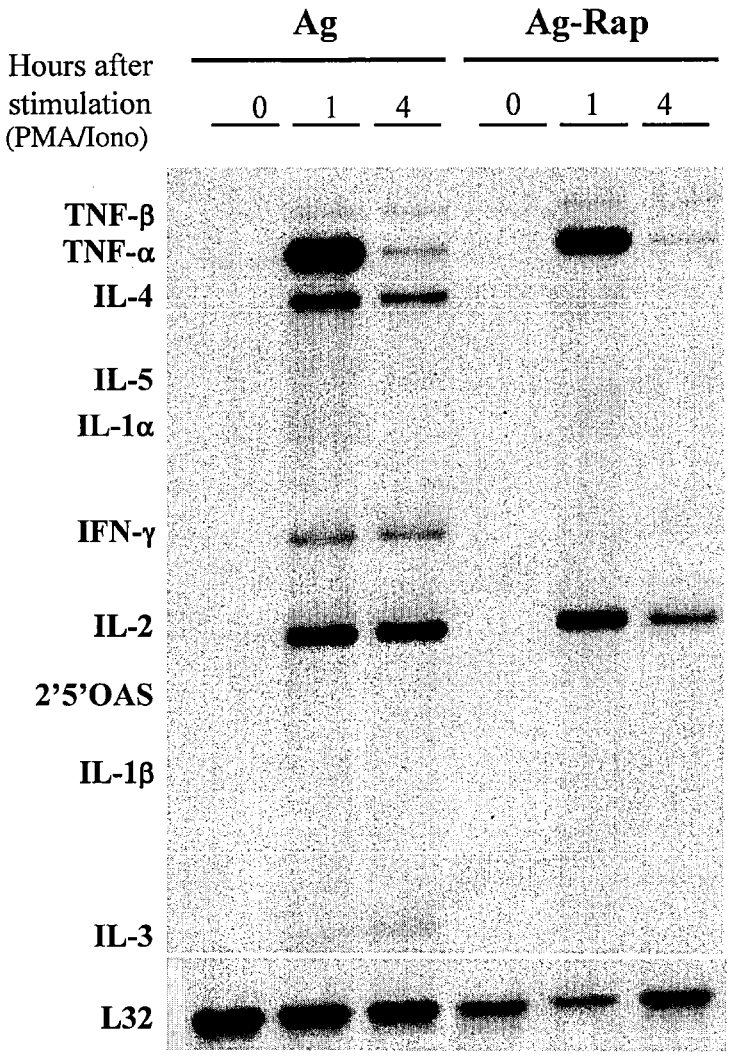

medium
Ag
Ag +Rap
CD44
CD25
CD62L
5.7%
92.8%
95.4%
1.2%
96.3%
91.4%
15.0%
84.7%
42.4%
CFSE

Figure 28

To better characterize the state of differentiation of cell expanded in the presence of Rapamycin, we analyzed the expression of several cytokine mRNAs. To this aim, DO11.10 cells cultured for a week with Ag, in the absence or in the presence of Rapamycin, were restimulated with PMA and Ionomycin for 0, 1 and 4 h. Total RNA was then obtained and analyzed by Ribonuclease protection assay (RPA) (Fig. 29) (see Materials and Methods). Interestingly, in the unstimulated condition (0h PMA/Iono), the level of expression of all mRNAs analyzed were very low in both groups of cells. Expression of the mRNAs for IL-2, TNF-α and β were induced to similar extents upon PMA/Iono restimulation, in control and Rapamycin-treated cells. In contrast, while control cells up-regulated the expression of the mRNAs for IFN-γ, IL-4 and IL-5 upon restimulation, these mRNAs failed to be expressed in Rapamycin-treated cells. This indicates that, while Ag driven differentiation confers the cells the ability to rapidly up-regulate mRNAs coding for effector cytokines (such as IFN-γ and IL-4), this fails to happen if Rapamycin is present at the time of activation. Thus, Rapamycin-treated cells appear to be Ag-experienced cells, lacking a fully differentiated effector phenotype.

We then investigated whether these cells could expand upon Ag-re-encounter. To this aim, DO11.10 T cells cultured for a week with Ag, in the absence or in the presence of Rapamycin, were labelled with CFSE, and restimulated with Ag and irradiated syngeneic splenocytes (Ag/APC) (Fig. 30). Dilution of the CFSE content was then analyzed after 7 days of culture by flow cytometry. As comparison, naïve T cells were also used. Interestingly, DO11.10 $CD4^+$ T cells stimulated for a week with Ag demonstrated higher proliferative potential when compared to naïve DO11.10 $CD4^+$ T cells, as demonstrated by the increased

Figure 29. Analysis of cytokine genes transcription upon Rapamycin treatment, by RNAse protection assay. **A)** DO11.10 LN cells were cultured for 7 days with Ag and irradiated splenocytes in the absence (Ag), or in the presence of Rapamycin (100 nM) (Ag-Rap). Thereafter, the cells were harvested, restimulated for 0, 1, and 4 hours with PMA and Ionomycin (PMA/Iono), and total RNA was obtained and analyzed by RNase protection assay (RPA) as described in Material and Methods, by using a probe set specific for the indicated cytokine RNAs. Protected RNAs were then visualized by autoradiography. **B)** The relative amounts of the indicated cytokine RNAs were quantified by phosphor imaging analysis and normalized to the amount of the housekeeping mRNA encoding the ribosomal protein L32. The graph reports the relative amount of the indicated RNAs expressed as fold induction over the unstimulated condition (0h = 1). The experiment is representative of two independent experiments.


Ag
Ag-Rap
Hours after stimulation (PMA/Iono)
0 1 4 0 1 4
TNF-β
TNF-α
IL-4
IL-5
IL-1α
IFN-γ
IL-2
2'5'OAS
IL-1β
IL-3
L32

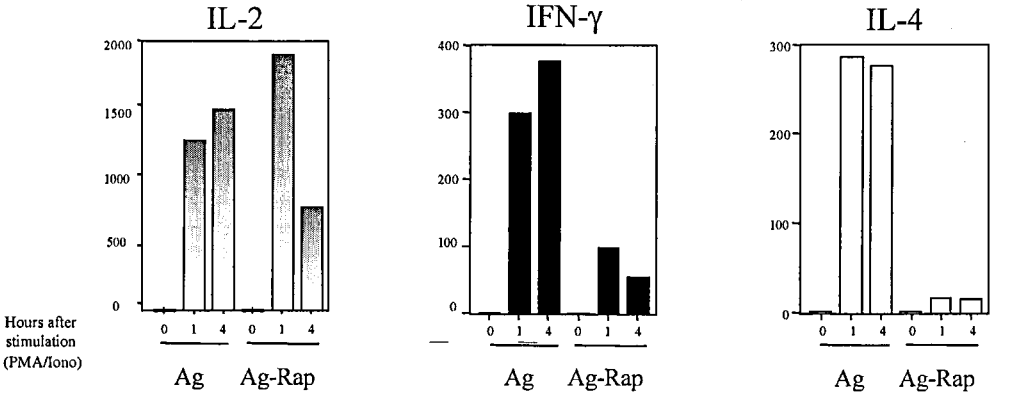

IL-2
IFN-γ
IL-4
2000
1500
1000
500
0
400
300
200
100
0
300
200
100
0
0 1 4 0 1 4
Hours after stimulation (PMA/Iono)
Ag Ag-Rap
Ag Ag-Rap
Ag Ag-Rap

Figure 29

Figure 30. Rapamycin-treated cells optimally expand upon Ag re-encounter. DO11.10 LN cells were cultured for 7 days with Ag and irradiated splenocytes in the absence (Ag) or in the presence of Rapamycin (100 nM) (Ag+Rap). Thereafter, the cells were harvested, and either left untreated (-), or restimulated for 5 days with Ag and irradiated splenocytes (Ag/APC). Naïve $CD4^+$ T cells were similarly stimulated as comparison. At the end of the culture, the cells were stained with anti-CD4 mAb, and analyzed by flow cytometry for their CFSE content. The histograms reports the CFSE content of $CD4^+/CFSE^+$ gated cells.

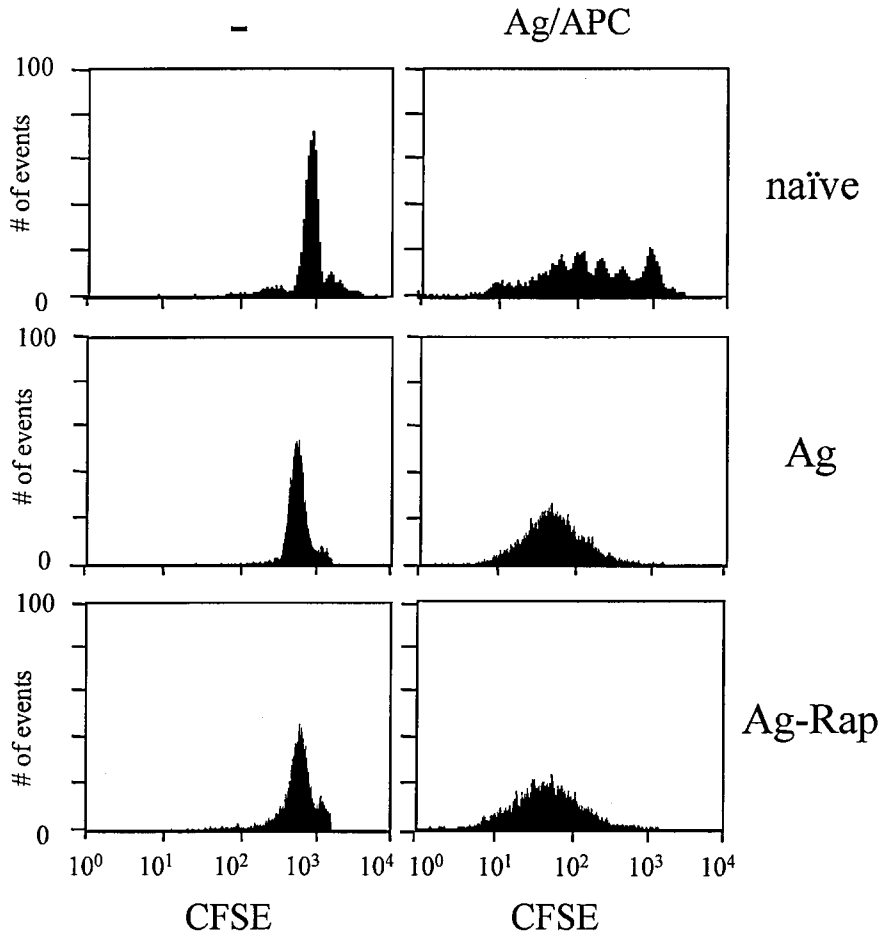


Figure 30

number of cell divisions. No differences in the proliferative capacity could be found after comparing $CD4^+$ T cells previously cultured with Ag, or with Ag and Rapamycin. This indicates that Ag stimulation in the presence of Rapamycin does not hamper the ability of the cells to further proliferate upon Ag re-encounter.

To further analyze the functional capabilities of T cells previously expanded in the presence of Rapamycin, we investigated whether the addition of exogenous polarizing cytokines at the time of Ag restimulation could drive these cells to differentiate into Th1 or Th2 effector cells. To this aim, DO11.10 T cells were first stimulated with Ag for a week in the presence of Rapamycin, and then restimulated for another week with Ag, with Ag and rIL-12 (and neutralizing anti-IL-4 mAb; Th1 polarizing condition), or with Ag and rIL-4 (and neutralizing anti-IL-12 mAb; Th2 polarizing condition) (Fig. 31). At the end of the second week, T cells were restimulated with PMA and Ionomycin, and IFN-γ and IL-4 release was measured by intracellular cytokine staining. A very small fraction of Rapamycin-treated $CD4^+$ T cells, restimulated during the second week only with Ag/APC, produced IFN-γ (4.3%) and almost no cells produced IL-4 (0.9%) after PMA/Iono restimulation. In contrast, stimulation of Rapamycin-treated cells during the second week with Ag/APC and rIL-12, or with Ag/APC and rIL-4, drove 17.3% and 10.3% of $CD4^+$ T cells to secrete detectable amounts of IFN-γ and IL-4, respectively.

Together these results indicate that Ag encounter in the presence of Rapamycin does not hamper the ability of $CD4^+$ T lymphocytes to either proliferate or to differentiate upon Ag re-encounter. Thus, rather than resulting in T cell unresponsiveness (265, 290), Rapamycin determines the accumulation of unpolarized Ag-experienced T cells.

Figure 31. Rapamycin-treated cells are capable of differentiating when cultured in Th1 or Th2 polarizing conditions. **A)** Schematic representation of the experiment. DO11.10 LN cells, cultured for 7 days in the presence of Ag/APC and Rapamycin (Ag+Rap) (First stimulation), were re-stimulated for an additional week with Ag/APC (Ag), Ag/APC and rIL-12 (and anti-IL-4 mAb) (Ag+IL-12), or with Ag/APC and rIL-4 (and anti-IL-12 mAb) (Ag+IL-4) (Second stimulation). Thereafter, the cells were harvested, re-stimulated for 4 hours with medium only (not shown) or with PMA and Ionomycin (PMA/Iono), and analyzed by flow cytometry after staining with anti-CD4, anti-IL-4, and anti-IFN-γ mAbs **(B)**. Dot plots represent events after gating on viable $CD4^+$ T cells. The percentage of cytokine producing cells is reported in the figure. In the absence of restimulation the % of cytokine producing cells was $\leq$ 2%. The experiment shown is representative of two independently performed experiments.

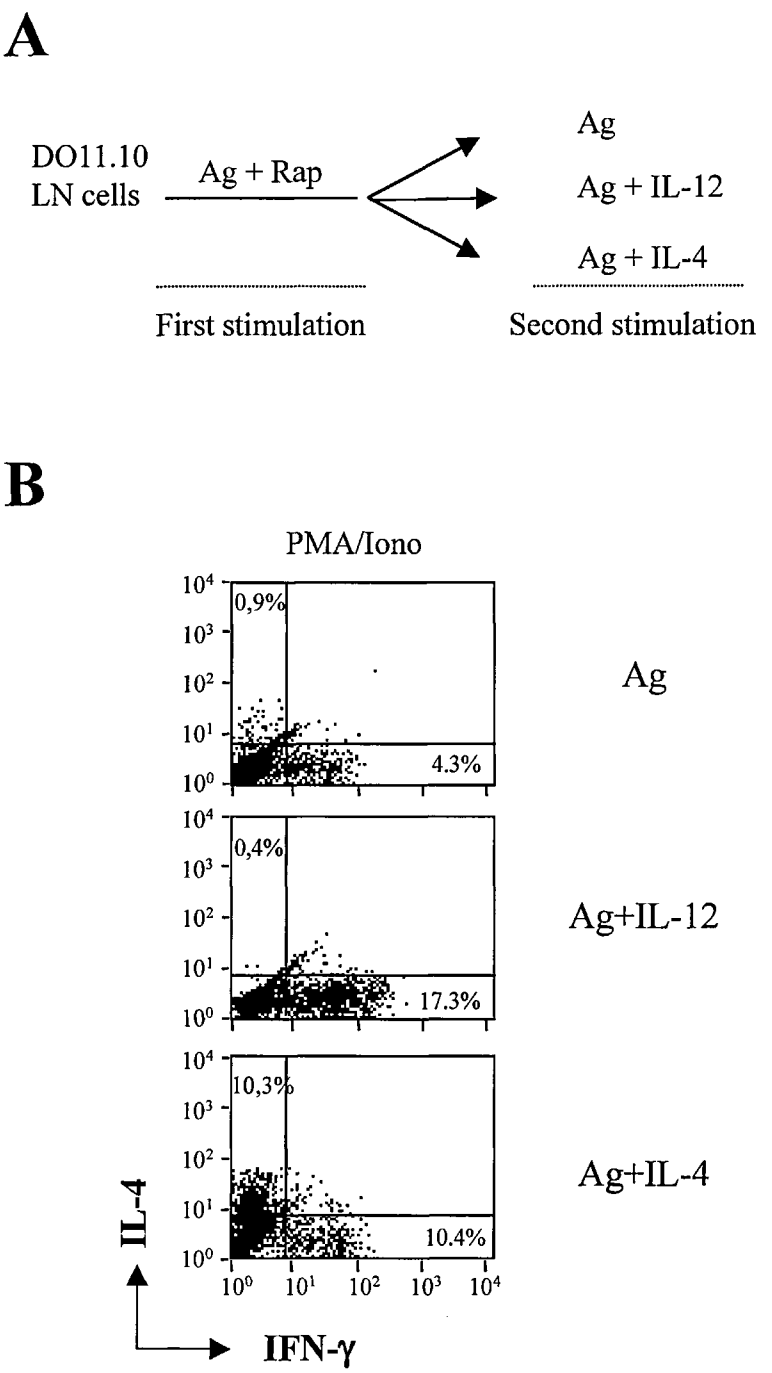

A
DO11.10
LN cells
Ag + Rap
Ag
Ag + IL-12
Ag + IL-4
First stimulation
Second stimulation
B
PMA/Iono
0,9%
4.3%
0,4%
17.3%
10,3%
10.4%
Ag
Ag+IL-12
Ag+IL-4
IL-4
IFN-γ

Figure 31

3.3.4 Rapamycin prevents IL-4-driven T cell differentiation: a role for the common γ chain

Polarization of Th1 and Th2 cells is normally initiated by TCR/IL-2 generated signals, and then sustained by polarizing cytokines, such as IL-12 and IL-4, respectively (291, 292). It was of interest to investigate whether Rapamycin could also prevent T cell differentiation in the presence of polarizing conditions. To this aim, CFSE-labelled DO11.10 purified $CD4^+$ T cells were stimulated with CD3/CD28 latex beads, in the presence of rIL-12 (and neutralizing anti-IL-4 mAb), or rIL-4 (and neutralizing anti-IL-12 mAb). After a week, the cells were harvested, restimulated with PMA and Ionomycin, and analyzed by flow cytometry for IFN-γ and IL-4 production, respectively (Fig. 32). The results reported in Fig. 32A and 32B indicate that, in the presence of Rapamycin, Th1 cell differentiation could occur if rIL-12 was added to the culture. In contrast, IL-4-driven Th2 cell differentiation was inhibited by Rapamycin treatment (Fig. 32C, D). Thus, while up to 45.5% of the cells expanded in Ag and rIL-4 differentiated into IL-4-producing cells, only 12.7% of the cells cultured in the presence of Rapamycin did so (Fig. 32C).

Interestingly, IL-2 and IL-4 are both required for optimal Th2 T cell differentiation (293-295), and IL-2 is required for proper Th1 differentiation (221, 282). These cytokines share the common γ_c (CD132) of the cytokine receptor to proper signal into the cells (296). We thus investigated whether Rapamycin could influence CD132 expression, and by that hamper cytokine-driven T cell

Figure 32. Rapamycin prevents IL-4-, but not IL-12-induced T cell polarization. $CD4^+$ T cells were purified from the LNs of DO11.10 mice, labeled with CFSE and cultured for 7 days with anti-CD3/CD28 mAbs-coated latex beads in Th1 (CD3/CD28/IL-12) or Th2 (CD3/CD28/IL-4) polarizing conditions in the absence (-) or in the presence of Rapamycin (100 nM) (Rap). Thereafter, the cells were harvested, restimulated for 4 hours with medium only (not shown) or with PMA and Ionomycin (PMA/Iono), and analyzed by flow cytometry after staining with anti-CD4, anti-IL-4, and anti-IFN-γ mAbs. **A, C)** Dot plots represent events after gating on viable cells. The percentage of cytokine producing cells is reported in the figure. **B, D)** The graphs report the mean percentage ± SD of cytokine producing cells calculated over 3 independent experiments. The significance of differences in cytokine production by T cells expanded in the absence or in the presence of Rapamycin was tested with a two-tailed Student's *t* test.

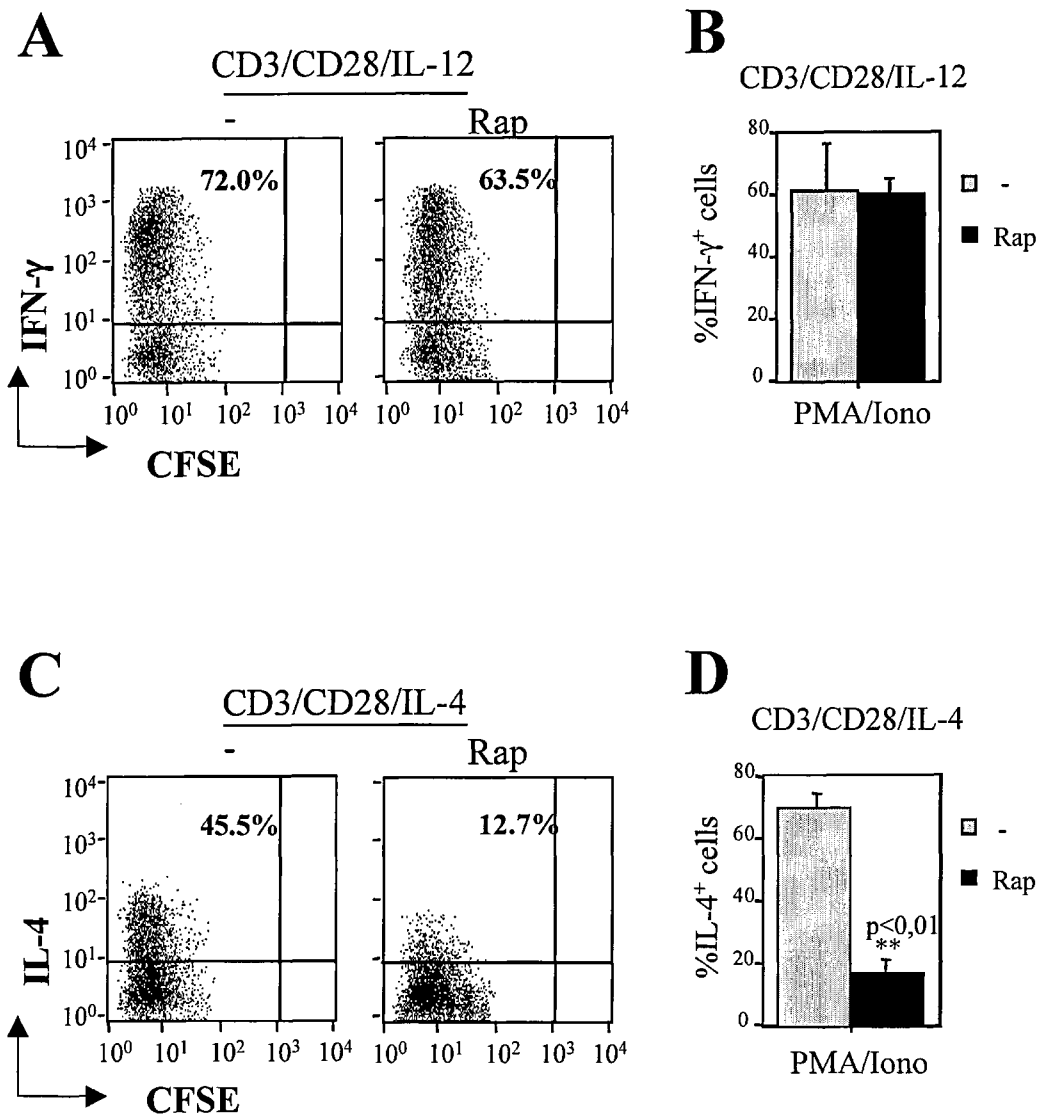

A
CD3/CD28/IL-12
-
Rap
72.0%
63.5%
IFN-γ
CFSE
B
CD3/CD28/IL-12
%IFN-γ+ cells
PMA/Iono
-
Rap
C
CD3/CD28/IL-4
45.5%
12.7%
IL-4
CFSE
D
CD3/CD28/IL-4
%IL-4+ cells
p<0,01
**
PMA/Iono

Figure 32

differentiation. CFSE labelled purified $CD4^+$ T cells were stimulated with CD3/CD28 latex beads, with or without rIL-4, in the absence or in the presence of Rapamycin, and analyzed by flow cytometry, either after 3 days or after 7 days of culture (Fig. 33). While stimulation of DO11.10 $CD4^+$ T cells was able to elicit optimal up-regulation of CD132 on the surface of the cells by day 3, suboptimal surface expression was detected in Rapamycin-treated cells. The same effect was observed with addition of rIL-4 to the culture. Indeed, as shown for CD25, also CD132 was expressed to levels that were only 50% of those observed on control cells (MFI control cell: 37.3, MFI Rapamycin-treated cells: 16.9). By day seven, both control and Rapamycin-treated cells expressed CD132 at the same level. Similar results were obtained in Th2 polarizing condition (MFI control cells + rIL-4: 42.0; MFI Rapamycin-treated cells + rIL-4: 24.7). These results suggest that the ability of Rapamycin to prevent optimal common γ_c up-regulation during the early phases of T cell activation, might explain its ability to impair T cell polarization.

Altogether the results reported in this thesis indicate that proper mTor signalling is required for T cell proliferation, Ag responsiveness, and differentiation. In the absence of proper mTor activation, T cells lack the ability to proper respond to either cytokine or Ag driven T cell activation.

Figure 33. Rapamycin inhibits optimal upregulation of the γ_c (CD132) expression. $CD4^+$ T cells were purified from the LNs of DO11.10 mice, labeled with CFSE and cultured for 3 and 7 days with anti-CD3/CD28 mAbs-coated latex beads (CD3/CD28) or anti-CD3/CD28 mAbs-coated latex beads and rIL-4 (CD3/CD28/IL-4), in the absence (-) or in the presence of Rapamycin (Rap) (100 nM). Thereafter, the cells were harvested, and analyzed, together with freshly isolated purified $CD4^+$ DO11.10 T cells (naïve), by flow cytometry after staining with anti-CD4 mAb, isotype control (not shown), and anti-CD132 mAb. Dot plots represent events after gating on viable cells. The Mean Fluorecence Intensity (MFI) of the total viable population is reported. The experiment is representative of three independently performed experiments.

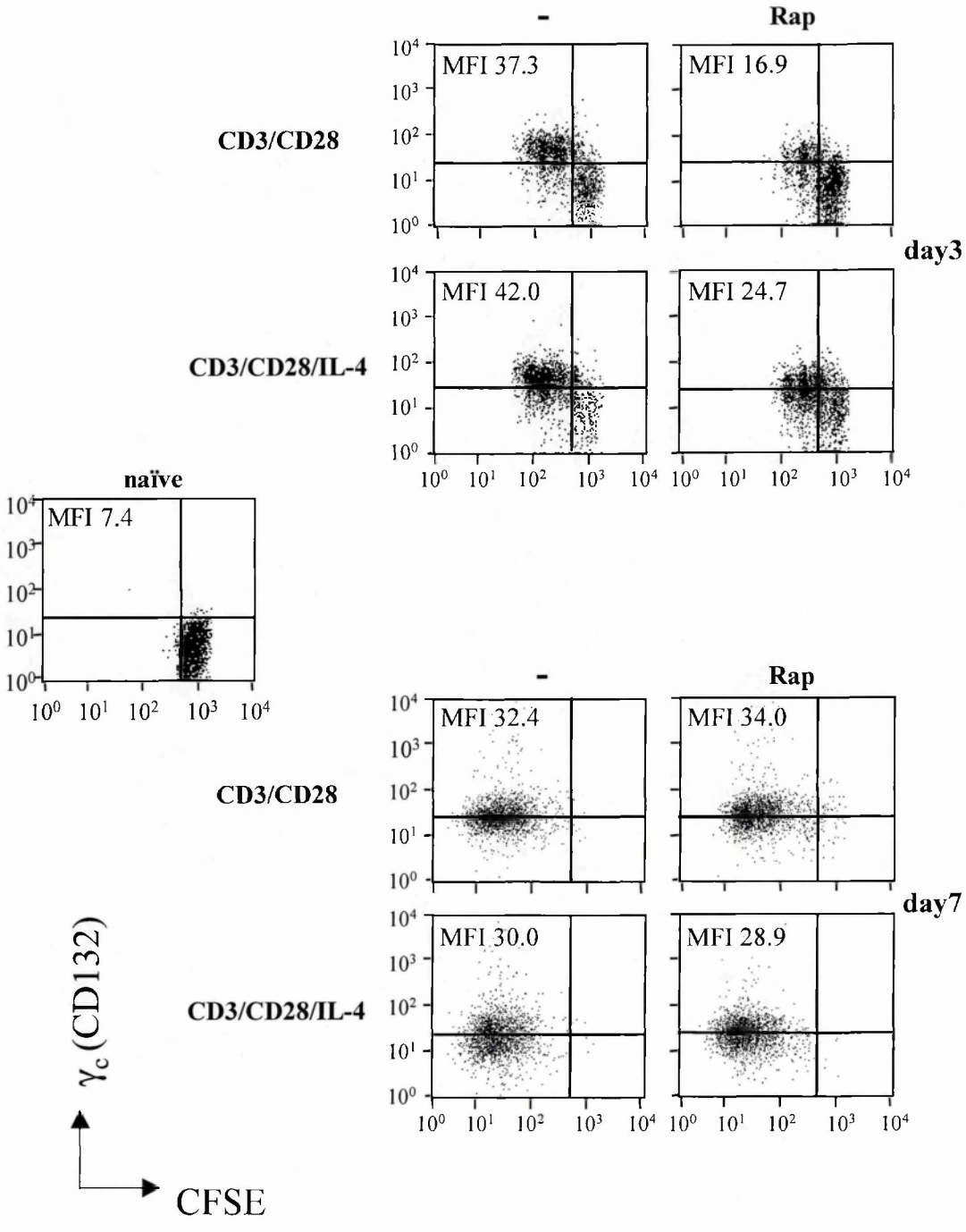

-
Rap
CD3/CD28
MFI 37.3
MFI 16.9
day3
CD3/CD28/IL-4
MFI 42.0
MFI 24.7
naïve
MFI 7.4
-
Rap
CD3/CD28
MFI 32.4
MFI 34.0
day7
CD3/CD28/IL-4
MFI 30.0
MFI 28.9
γc (CD132)
CFSE

Figure 33

4. Discussion

The results obtained during my thesis generated a number of new findings contributing to the current understanding of the molecular mechanisms dictating T cell activation, proliferation, and differentiation. Indeed these studies indicate that proper activation of the mammalian target of Rapamycin (mTor) is absolutely required for (1) IL-2 mediated signalling in the reversal of clonal anergy, (2) CD3/CD28-dependent, IL-2-independent T cell proliferation, and (3) CD3/CD28/IL-2 or IL-4-induced $CD4^+$ and $CD8^+$ T cell differentiation (Fig. VII). Thereafter I will discuss our results in light of the previously published data.

4.1 The role of mTor signalling in the regulation of T cell antigen responsiveness

While the simultaneous engagement of CD3 (TCR) and CD28 elicits T cell activation and proliferation, chronic TCR engagement in the absence of costimulation and proliferation results in the establishment of clonal anergy (7). Anergic T cells are characterized by the inability to transcribe and produce IL-2, and to proliferate upon Ag re-encounter, and are arrested in the G1 phase of the cell cycle (7, 268, 269).

It was suggested that defective anergic T cell responses could be caused by the presence of a putative anergic factor, induced at the time of TCR engagement and accumulated in the cells in the absence of proliferation. This model predicts that forcing proliferation or G1 to S transition of anergic T cells might lead to

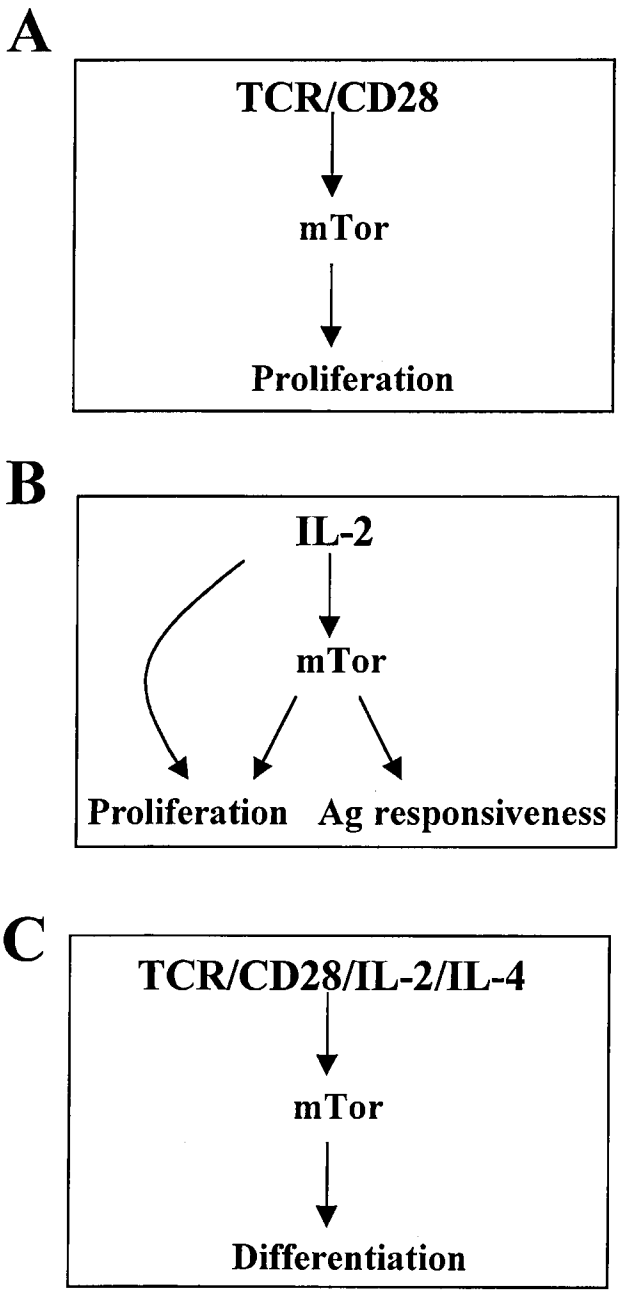


Figure VII. Model of mTor-dependent regulation of T cell proliferation, Ag responsiveness, and differentiation. A) mTor signalling is required for CD3/CD28-induced T cell proliferation. B) mTor signalling is involved in IL-2-mediated T cell proliferation, and it is necessary for IL-2-driven reversal of clonal anergy. C) mTor signalling is required for Ag/IL-2-driven and IL-4-driven Th1 and Th2 differentiation.

dilution/degradation of the putative anergic factor, and by that it might restore the ability of the cells to properly respond to antigenic rechallenge (263, 267). Since optimal engagement of TCR/CD28 was reported to elicit G1-to-S transition via IL-2 independent mechanisms (226, 227), we investigated whether CD3/CD28 stimulation of anergic T cells would elicit cell cycle progression or proliferation, and by that restore antigen responsiveness.

We show here that optimal occupancy of the TCR and of CD28 overcomes G1 cell cycle block and drives anergic cell proliferation, but fails to restore antigen responsiveness. Indeed, while antigenic stimulation of anergic T cells failed to properly regulate the G1-associated events required for S phase transition, optimal engagement of the TCR and of CD28 by plate-bound agonistic mAbs overcame G1 cell cycle arrest and elicited the up-regulation of the cyclins D2, D3 and E, the downregulation of the inhibitor $p27^{Kip}$, and the hyperphosphorylation of Rb to extents which were found to be similar in control and anergic T cells. Furthermore, comparable numbers of control and anergic T cells entered the S phase (as detected by DNA content) and completed several rounds of cell division (as detected by CFSE dilution) upon stimulation with plate-bound anti-CD3 and anti-CD28 mAbs. CD3/CD28-induced G1-to-S transition and proliferation occurred in the absence of IL-2. Indeed, plate-bound anti-CD3/CD28 mAbs drove proliferation of anergic T cells and of IL-2-deficient DO11.10 T cells, which are respectively functionally and genetically incapable of producing IL-2. Appleman et al. (226) recently found that ligation of CD28, in the presence of TCR engagement, resulted in $p27^{Kip}$ ubiquitination and degradation, and in subsequent activation of cyclin D2-Cdk4/6 and of cyclin E-Cdk2 complexes. In our hands CD3/CD28 ligation elicited

comparable down-regulation of $p27^{Kip}$ in IL-2 sufficient (control A.E7 cells, wild type DO11.10 T cells) and IL-2-deficient (anergic A.E7 and DO11.10 IL-$2^{-/-}$) T cells. In our cultures, down-regulation of $p27^{Kip}$ in CD3/CD28-activated DO11.10 IL-$2^{-/-}$ T cells, was accompanied by the up-regulation of cyclin D3 and by cell proliferation. In contrast to this finding, Powell et al. (297) reported that CD3/CD28 stimulation of IL-$2^{-/-}$ cells induced downregulation of $p27^{Kip}$, but failed to elicit T cell proliferation. It is possible that the discrepancy can be attributed to the fact that these authors used soluble and not plate-bound anti-CD3/CD28 mAbs, and only looked at $p27^{Kip}$ expression early after stimulation. Thus, either stronger receptor cross-linking, or longer stimulation might need to be provided to elicit complete $p27^{Kip}$ degradation and cell division. In spite of this discrepancy, our finding indicate that CD3/CD28 either directly, or through secretion of a yet to be defined cytokine, different from IL-2, directly control cell cycle progression, via an IL-2-independent mechanism.

The reason for the failure of Ag/APC to elicit cell cycle progression in anergic cells remains to be elucidated. It is possible that the establishment of T cell anergy lowers the sensitivity of the TCR or of CD28, or that only optimal receptor engagement provided by anti-CD3/CD28 mAbs elicits the intracellular signals required for IL-2 independent cell cycle progression. Even though these possibilities require further investigation, they seem unlikely since normal Ca^{2+}-dependent responses were detected following Concanavalin A and Ag stimulation of anergic T cells, thus indicating a normal TCR sensitivity ((245) and unpublished data). Furthermore IL-2 independent, CD28-dependent T cell proliferation was described during antigen recognition *in vivo*, indicating that, in addition to anti CD3/CD28 mAbs, also Ag

stimulation drives IL-2 independent T cell proliferation (221, 298). It is interesting that, while maximal TCR and CD28 ligation bypassed the G1 cell cycle block, it failed to elicit detectable IL-2 production in anergic T cells. Boussiotis et al. (268) previously showed that the over-expression of $p27^{Kip}$ sequestered JAB-1, a co-activator of Jun transcription factors (299), and by that inhibited AP-1-dependent *IL*-2 gene transcription. For this reason these authors suggested the augmented expression of $p27^{Kip}$ to be responsible for defective *IL-2* transcription in anergic T cells (268). Our results do not support a role for $p27^{Kip}$ in defective IL-2 production in anergic A.E7 T cells. Indeed, in spite of CD3/CD28-dependent efficient $p27^{Kip}$ downregulation ((297), and the results reported in Fig. 2), IL-2 was barely detectable in culture supernatants of anergic T cells (Fig. 1). This suggests that the signals involved in *IL-2* gene transcription and in the regulation of the G1 checkpoints might be of a different nature in our anergized Th clone.

Regardless of the final mechanism that dictated CD3/CD28-dependent anergic T cell proliferation, this was not sufficient to elicit reversal of clonal anergy. As mentioned above, the idea that proper cell cycle progression and proliferation upon activation was required to maintain lymphocyte responsiveness, was originally proposed by Jenkins and Schwartz (234, 237, 300). These authors showed that TCR engagement in the absence of costimulation elicited T cell activation, but not proliferation, and resulted in the establishment of clonal anergy. In this model, CD28 costimulation was proposed to promote escape from anergy by eliciting IL-2 production and cell division. It was, however, later shown that proliferation was not the critical event required for escaping anergy induction, but that G1-to-S transition had to occur to preserve lymphocyte responsiveness. Indeed, anergy was induced

only if the cells were blocked in early G1 ($G1_a$) at the time of stimulation, but not in G0, $G1_b$, or S/G2 phases (264, 265). Our results expand these original observations and further indicate that neither G1-to-S phase transition, nor cell proliferation, elicited by CD3/CD28, is sufficient to obtain anergy reversal.

The possibility that anergy reversal and proliferation are independently regulated is also supported by the finding that the addition of Rapamycin at the time of IL-2 stimulation failed to prevent T cell expansion, but completely prevented the ability of IL-2 to restore Ag responsiveness (Fig. 10). Altogether these data show that, rather than cell proliferation, an mTor driven signalling event seems to specifically regulates Ag responsiveness in T cells.

Rapamycin not only prevented IL-2 mediated anergy reversal, but also inhibited CD3/CD28 induced T cell proliferation (Fig. 11). This indicates that mTor is a common effector shared by either CD3/CD28 and IL-2R induced signalling. Indeed, we also found that, as previously reported (107), the engagement of CD3 and CD28 induced the phosphorylation of the $p70^{S6k}$, one of the known mTor substrates, in a Rapamycin sensitive way (further discussed below). In spite of this, CD3/CD28-induced signalling was not sufficient to restore Ag responsiveness.

We reasoned that this could be due to several reasons. For instance, it was possible that chronic TCR engagement provided by the immobilized anti-CD3 Ab, in the absence of IL-2, could result in the continuous production and in the accumulation of the putative anergic factor(s), and by that it could prevent mTor-dependent reversal of clonal anergy. Our results indicate that this does not seem to be the case. Indeed, the addition of Cyclosporin A, previously shown to inhibit anergy induction

in Th cells (233, 236, 252), did not allow CD3/CD28 stimulation to restore antigen responsiveness in anergic cells (Fig. 13).

It was also possible that CD3/CD28 and IL-2 could activate mTor, or mTor-dependent signalling, through different pathways. Our analysis of the mTor-dependent post-translational modifications of $p70^{S6k}$ indicate that this could be partially true (discussed below). However, more detailed analysis will have to be performed to correctly address this possibility.

Finally, it was also possible that, specifically in anergic T cells, only IL-2, and not CD3/CD28, could elicit optimal activation of mTor. To address this possibility we analyzed the mTor-dependent post-translational modifications of $p70^{S6k}$. The current model proposed for $p70^{S6k}$ activation has been derived from studies mostly performed on mitogen-activated cell lines, and involves an ordered sequence of phosphorylation events mediated by different kinase activities. $p70^{S6k}$ is comprised of at least three distinct functional domains, containing the crucial residues for its activation: a C-terminal autoinhibitory domain (or pseudosubstrate domain), a linker region, and an N-terminal catalytic domain (286, 288). Four proline-directed sites have been identified in the autoinhibitory domain (Ser^{411}, Ser^{418}, Thr^{421}, Ser^{424}) (301), they are rapidly phosphorylated upon mitogenic stimulation, possibly by a Ras/MAPK-dependent pathway (302), are not sufficient for $p70^{S6k}$ activation, but are required to modulate its kinase activity (303). Upon phosphorylation of the autoinhibitory domain, the catalytic domain is released, and this permits subsequent phosphorylation of Thr^{389} and Ser^{371}, which are located in the $p70^{S6k}$ linker region, and have to be phosphorylated for full $p70^{S6k}$ activation (286, 287, 303). Once Thr^{389} is phosphorylated, PDK1 directly phosphorylates

Thr^{229}, lying within the catalytic domain (188, 189). Phosphorylation of Thr^{389} and Thr^{229} is induced by growth factors and is prevented in the presence of Wortmannin (a PI3K inhibitor) and Rapamycin (the inhibitor for mTor) (303, 304). Even though it remains controversial whether Thr^{389} phosphorylation is directly mediated by mTor (287, 305, 306), or by the NEK6/7 kinases, which belong to the NIMA (never-in-mitosis-*Aspergillus)*-like family kinases (307), phosphorylation of this residue correlates with $p70^{S6k}$ activation (287). The activity of $p70^{S6k}$ is also modulated by the activity of phosphatases, such as PP2A, which directly binds and dephosphorylates $p70^{S6k}$ (308). It has been shown that this event is inhibited by an mTor-dependent signalling, suggesting that mTor could allow $p70^{S6k}$ phosphorylation by protecting the protein from PP2A activity (308).

In our unstimulated A.E7 cells, $p70^{S6k}$ was found to be constitutively phosphorylated, as demonstrated by the observation that, either the treatment of the cells with Rapamycin, or the treatment of the cell extracts with alkalin phosphatase, reduced the mobility of the protein in both mono and bidimensional SDS-PAGE analyses. This indicates that, as reported for other cell types (309), also A.E7 T cells have a constitutive level of mTor activity, possibly linked to normal nutritional conditions. CD3/CD28 and IL-2-driven T cell stimulation elicited Rapamycin-sensitive $p70^{S6k}$ phosphorylation in Thr^{389} and in several additional residues, as detected by either mono and bi-dimensional analyses (Fig. 14-19). This finding confirms that, as reported by others (286), also in our control cells, mTor is involved in stimulation-dependent $p70^{S6k}$ phosphorylation. Interestingly, IL-2 elicited the appearance of several spots with a more acidic PI (possibly indicative of additional phosphorylations), compared to the ones found upon CD3/CD28

stimulation, suggesting that IL-2 and CD3/CD28 might activate $p70^{S6k}$ through different pathways.

In contrast to control cells, only IL-2 stimulation induced Rapamycin-sensitive $p70^{S6k}$ phosphorylation in anergic cells. Indeed CD3/CD28 stimulation of anergic cells failed to elicit either Thr^{389} phosphorylation, or general acidification of $p70^{S6k}$, and instead it induced the accumulation of the more basic variants of the proteins (Fig. 15, 16, 18, 20). Thus, together these results indicate that the failure of CD3/CD28 to restore Ag responsiveness could be attributed to the failure of this stimulation to optimally activate an mTor-dependent event involved in anergy reversal.

The failure of CD3/CD28-mediated stimulation to induce proper phosphorylation of $p70^{S6k}$ could be due to several reasons. First, it is possible that the previously reported defect in the Ras/MAPK dependent pathway in anergic cells (247) hampers the MAPK-dependent phosphorylation of the $p70^{S6k}$ catalytic domain. Furthermore, it is possible that, in anergic cells, CD3/CD28-induced signalling fails to optimally activate the kinase activity of mTor, which is required for initial $p70^{S6k}$ phosphorylation. Similarly, CD3/CD28 might also fail to activate the NEK6/7 kinases, and thus fail to elicit phosphorylation of Thr^{389}. Since mTor and the NEK6/7 kinases can be both regulated by PI3K activity (90, 210, 307), it is also possible that anergic cells have defective PI3K-dependent signalling. Finally, it is possible that anergic cells have a deregulated phosphatase activity. A possible candidate could be a member of the PP2A phosphatase family, which has been shown to bind to the cytoplasmic domain of CD28 (310). PP2A association to CD28 is inhibited by Lck-dependent CD28 phosphorylation (310). Since it has been

reported that Lck activity is reduced in anergic T cells, in favour of Fyn activity (253, 254), it is conceivable that, in these cells, the CD28/PP2A complex might be more represented, thus impairing proper $p70^{S6k}$ phosphorylation. Interestingly, we have preliminary results indicating that, in anergic cells, the addition of okadaic acid, an inhibitor of PP2A, at the time of CD3/CD28 stimulation, increased Thr^{389} phosphorylation.

Regardless of the reasons for the failure of CD3/CD28 stimulation to induce proper phosphorylation of $p70^{S6k}$ in anergic cells, this correlates with the inability of CD3/CD28 stimulation to reverse clonal anergy. Even though, the proof that $p70^{S6k}$ might directly control T cell anergy is still missing, given its crucial role in the regulation of the translational machinery, it is conceivable that its defective activation might account for the peculiar phenotype of anergic cells. Thus, the future characterization of the mTor-dependent $p70^{S6k}$ activation will shed some light on the molecular mechanisms regulating antigen responsiveness.

4.2 The role of mTor signalling in the regulation of T cell proliferation

Proliferation of $CD4^+$ T lymphocytes is controlled by both TCR/CD28 and IL-2/IL-2R-initiated signals. It was believed that the stimulation of resting T lymphocytes by TCR/CD28 initiated cell cycle entry (139), and rendered the cells competent for proliferation by eliciting the production of the T cell growth factor IL-2, and the expression of its high affinity receptor. The binding of IL-2 to its receptor would then mediate the coordinated activation of several intracellular signalling events, and would eventually induce cell division (140, 141). A number

of studies, however, recently indicated that the engagement of the TCR and of costimulatory receptors results in the activation and in proliferation of T cells, independently of IL-2 (221, 222, 224-227, 282, 311). Here we showed that, in addition to IL-2, also CD3/CD28 could elicit G1 to S transition by directly regulating the critical cell cycle proteins such as cyclin D3, Rb, $p21^{Cip}$, and $p27^{Kip}$ (Fig. 2, 6).

While both TCR/CD28 and IL-2/IL-2R-driven proliferation were similarly sensitive to the PI3K inhibitor LY294002 (LY), only TCR/CD28 driven T cell proliferation was completely abrogated by the presence of Rapamycin. Given the crucial role attributed in the past to mTor signalling in the regulation of IL-2-mediated T cell proliferation, it was surprising to discover that IL-2-induced proliferation of A.E7 cells could occur also in the presence of Rapamycin (Fig. 10). Indeed, we found that Rapamycin blocked cell division up to the third day of culture (Fig. 21). After this time Rapamycin-treated cells started to proliferate and by the end of the culture they have performed the same number of cell divisions of their untreated counterparts. These findings are not consistent with the previous idea that Rapamycin inhibits G1-to S transition and T cell expansion (312). However, it should be noted that the previously reported ability of Rapamycin to prevent T cell proliferation mostly derived from studies detecting proliferation by standard [^{3}H]-thymidine incorporation assays, which measure cell proliferation after 48-72 hours of culture. Instead, by following single cell history over time, we found that, as previously suggested at the molecular levels (285), also at the functional level, T cells are capable to proliferate in the presence of Rapamycin.

The finding that Rapamycin only delays, and does not inhibit cell division supports the idea that Rapamycin prolongs IL-2-induced G1-to-S phase transition. Indeed Terada et al. (285) showed that Rapamycin induced a prolongation of the G1 phase of the cell cycle, and attributed this effect to the ability of the drug to inhibit the translation of ribosomal protein mRNAs, and thus the synthesis of total cellular proteins, and to delay the cell growth response. These authors also showed that Rapamycin-treated cells initiated DNA synthesis and entered the cell cycle as soon as they reached the size of the untreated counterparts (285), supporting a role for mTor in the cell growth response, rather than in the regulation of cell cycle proteins. Accordingly, we found that Rapamycin prevented proliferation at day 3 after stimulation, but failed to do so at later times, when cells stimulated in the absence or in the presence of Rapamycin had performed the same number of cell divisions.

The peculiar sensitivity of either CD3/CD28 and IL-2/IL-2R-induced proliferation to Rapamycin, might either indicate that these receptors use different intracellular signalling for the regulation of cell cycle progression, or that, in the presence of saturating amounts of IL-2, mTor-independent pathways are induced. Our results partially support both these possibilities. Indeed, while the downregulation of $p27^{Kip}$ induced by CD3/CD28 was Rapamycin-insensitive, IL-2-induced $p27^{Kip}$ downregulation was prevented by this drug (data not shown), thus suggesting that, at least for $p27^{Kip}$, the intracellular signals induced following CD3/CD28 and IL-2/IL-2R are distinct. Moreover, while IL-2 driven T cell proliferation was Rapamycin-insensitive in the presence of saturating IL-2 concentration, it was Rapamycin-sensitive in the presence of limiting amounts of

IL-2 (Fig. 22). These findings indicate that Rapamycin-insensitive pathways might be uniquely induced upon maximal IL-2R occupancy.

The IL-2-induced mTor-independent T cell proliferation was still sensitive to the PI3K inhibitor LY. Indeed, the addition of LY prevented IL-2-dependent proliferation either early (three days) (not shown) or late (seven days) (Fig. 23) after stimulation. The fact that both LY and Rapamycin were sufficient to prevent T cell proliferation at day 3, could either indicate that PI3K regulates mTor function as proposed elsewhere (90), or that both PI3K and mTor-generated signals are required to enter the cell cycle. Upon cell cycle entry, mTor could then become dispensable (Rapamycin-resistant proliferation was detected by day 7, and cell cycle progression was previously observed in spite of Rapamycin-dependent inactivation of the $p70^{S6k}$ (313), while PI3K-dependent signals might still be required to sustain IL-2 mediated T cell proliferation. In support of this, Slavik et al. (314) recently reported that freshly isolated $CD8^+$ T cells and $CD8^+$ T cell clones were capable of a Rapamycin-resistant proliferation, which was inhibited by the PI3K inhibitor LY. Even though IL-2R β and γ chain-generated signals, involving Bcl-2, c-Myc, and JAK3/STAT5-dependent transcription, have been shown to contribute to T cell proliferation (175, 180), no residual proliferation was observed in A.E7 T cells stimulated in the presence of both Rapamycin and LY, suggesting that proliferation of these cells critically requires PI3K-PKB-mTor pathways. It is interesting that in lower eukaryotes (such as *Cenorabditis elegans*), mTor and PI3K pathways are distinct and regulate in parallel the response to nutrients and growth factors, respectively. In higher eukaryotes, such as mammals, these two pathways seem to have converged at the level of PI3K, being mTor a downstream effector target of

this lipid kinase (90, 210), but still might regulate only partially overlapping intracellular signalling pathways. Indeed, our results showed that, in the presence of LY, a residual proliferation was observed, which was completely inhibited by the concomitant treatment with Rapamycin. Even though much more work needs to be done, the available data indicate that both PI3K and mTor play a crucial role in T cell proliferation, but that, while the former is absolutely required for T cell expansion, the latter might be dispensable.

Altogether, these findings contribute to the current understanding of the molecular mechanisms that regulate TCR/CD28 and IL-2/IL-2R-induced proliferation, and underline the importance of performing additional investigation on the immunosuppressive activity of Rapamycin. Indeed, the finding that Rapamycin was unable to block T cell expansion is in contrast to the previously reported immunosuppressive function of this drug, which was historically attributed to its ability to prevent the expansion of the pathogenic T cells. Our data instead indicate that the immunosuppressive activity of Rapamycin could not be explained by inhibition of proliferation, but rather by its ability to prevent the mTor-induced signalling involved in the regulation of T cell antigen responsiveness (section 4.1), and T cell polarization (discussed in the following section).

4.3 The role of mTor signalling in antigen-driven T cell polarization

Following antigen stimulation, naïve T cells not only proliferate, but, provided that several conditions are in place, they also undergo a program of differentiation that commits the cells to a specific effector function (292, 315). Our

data indicate that, in addition to control T cell antigen responsiveness, and to some extent T cell proliferation, mTor signalling also controls T cell differentiation. Indeed, by using an *in vitro* model of antigen driven T cell differentiation, we showed that, in the presence of Rapamycin, T cells proliferated, but fail to differentiate into cytokine-producing effector T cells.

$CD4^+$ T lymphocytes most commonly differentiate into Th1 and Th2 effector cells (292, 315, 316). Th1 cells are mainly capable of producing IL-2, IFN-γ, TNFβ and lymphotoxin, they orchestrate cell-mediated immune responses, and can protect against intracellular pathogens. Th2 cells are mainly capable of producing IL-4, IL-5, IL-6, IL-10, and IL-13, and sustain both humoral immune responses and protection against extracellular infections (317-319). Several factors have been described to influence the fate of $CD4^+$ T cell differentiation at the time of antigen encounter. Among these are: the dose of antigen, the strength of TCR stimulation, the presence of pro-inflammatory cytokines, and, in some instances, the ability of the cells to proliferate (294, 320-322). For instance, while high antigen doses drive the development of Th1-like cells producing increased amounts of IFN-γ, low antigen doses induce T cells to switch to Th2-like cells mainly capable of producing IL-4 (323, 324). Moreover the presence of IL-12 at the time of Ag recognition elicits Th1 T cell differentiation, a mechanism that requires the induction of the Th1-specific transcription factor T-bet. Similarly, the presence of IL-4 favours Th2 T cell differentiation, and the expression of the Th2-specific transcription factor GATA-3 (291, 292, 294, 325, 326). Finally, also cell proliferation plays a major role in regulating T cell differentiation (321, 322). Indeed, Bird et al. (321) demonstrated that, while the expression of IL-2 by antigen activated T cells was cell

cycle independent, the expression of effector cytokines was acquired as a function of the number of cell divisions. In particular, IL-4 production occurred only in cells that had undergone at least three cell divisions, while IFN-γ production required only one round of cell division.

In our cultures Ag-driven T cell expansion induced Th1 T cell differentiation. Indeed, DO11.10 ovalbumin-specific $CD4^+$ T cell cultured for a week with Ag and irradiated syngeneic splenocytes, were mainly capable of producing IFN-γ upon restimulation. Similar results were obtained by using purified $CD4^+$ T cells optimally stimulated by CD3/CD28-coated beads, suggesting that, in the presence of optimal TCR/CD28 engagement, ovalbumin-specific $CD4^+$ T cells naturally differentiated to IFN-γ producing cells through a cell autonomous mechanism. In some experiments, a small fraction of the cells also spontaneously polarized to IL-4 producing cells (data not shown).

The addition of Rapamycin at the time of stimulation only delayed and did not inhibit either Ag/APC or CD3/CD28-induced T cell proliferation, as in the case of IL-2-stimulated A.E7 T cell clones, but drastically prevented T cell polarization. Indeed, when cells stimulated in the presence of Rapamycin were optimally re-stimulated, they failed to secrete IFN-γ (and IL-4 whenever this was detected in control culture). Similar results were obtained with $CD8^+$ T cells. Indeed, while Ag-specific $CD8^+$ T cells proliferated in response to antigenic challenge and acquired both the ability to produce IFN-γ and to lyse target cells upon restimulation, cells cultured in the presence of Rapamycin optimally expanded but lacked effector capabilities. These results indicate that, in the absence of proper mTor signalling, T cells fail to acquire effector function capabilities.

It is now recognized that T cell differentiation occurs through different phases. Initially, the engagement of the TCR activates an immediate and global chromatin derepression program, which results in a low, but readily detectable transcription of both *IL-4* and *IFN-γ* genes (327). This transcriptional activity is associated with increased histone acetylation in both the *IL-4* and *IFN-γ* loci (328-330). As differentiation proceeds, cytokine-induced signals specifically determine the final commitment to one of the two Th subsets. This is determined by the silencing of the *IL-4* gene in Th1 cells, and of the *IFN-γ* gene in Th2 cells, and by the maintenance of histone acetylation, and thus transcriptional competence, of the specific cytokine genes (*IL-4* gene for Th2 cells, and *IFN-γ* gene for Th1 cells) (292, 294, 325, 326). It is interesting that Rapamycin-treated cells not only failed to express the cytokine proteins, but they were also unable to transcribe the mRNA for IFN-γ and IL-4. In contrast to IFN-γ and IL-4 mRNAs, the mRNA for IL-2 and TNF-α were normally induced, suggesting that Rapamycin-treated cells have a specific defect in the up-regulation of effector cytokine mRNAs. Moreover, in addition to IL-4, also the mRNA for IL-5 failed to be induced in the Rapamycin-treated cells. Since the *IL-5* and *IL-4* genes are located in the same gene cluster, it is tempting to speculate that activation in the presence of Rapamycin prevents the establishment of transcriptional competence for entire genomic regions. Even though Rapamycin prevented T cell polarization, it did not result in T cell unresponsiveness. Indeed, $CD4^+$ T cell activated and expanded in the presence of the drug, showed to be able to produce IL-2, and to proliferate upon Ag restimulation, and a fraction of the cells differentiated towards a Th1 or a Th2 phenotype, when restimulated in the presence of rIL-12 or rIL-4, respectively.

When compared to naïve T cells, cells expanded in Rapamycin showed an increased surface expression of CD44, of CD25, and reduced surface levels of CD62L. Moreover, cells expanded in the presence of Rapamycin showed a faster kinetic of TNF-α and IL-2 production (data not shown), and of proliferation (Fig. 30) when compared to naïve cells, suggesting that the cells are no longer naïve, but express a surface and functional phenotype consistent with Ag-experienced unpolarized T cells.

Several reasons might account for the ability of Rapamycin to inhibit T cell differentiation. In our cell cultures mTor-dependent signalling could interfere with either TCR/CD28-induced early differentiation (as proven by the fact that both of these receptors elicit phosphorylation of the $p70^{S6k}$ via an mTor-dependent pathway), or at the level of the cytokine-induced signalling. The ability of Rapamycin to interfere with TCR/CD28-induced signals is supported by the observation that the drug inhibited both the production of IL-2, as also reported elsewhere (108), and the proper up-regulation of the α and γ chain of the high affinity IL-2R. It is also conceivable that Rapamycin might interfere with the poorly defined initial phase of TCR-induced chromatin remodelling events, which is required for rendering effector cytokine genes accessible for subsequent transcription (327, 328). This possibility is supported by the recent finding that the Tor pathway is linked to Esa1 histone acetylase pathway, which leads to transcriptional activation of ribosomal protein (RP) in response to nutrient levels (331). Along the same line is the possibility that Rapamycin might inhibit TCR/CD28-mediated expression of the essential lineage specific transcription factors, such as T-bet and GATA-3, which in turn regulate chromatin remodelling

events on cytokine loci (292). Indeed, it was shown that T-bet is readily induced in naïve T cells by TCR triggering and by IFN-γ-induced STAT1 signalling (332, 333). T-bet expression leads to chromatin remodelling of the *IFN-γ* gene, to the stabilization of its own expression (332, 334), and to suppression of IL-4 and IL-5 production (335, 336). In addition to T-bet, also GATA-3 is rapidly induced by TCR triggering and by IL-4-induced STAT6 signalling (337). Once expressed, GATA-3 leads to chromatin remodelling of the *IL-4* gene, establishing transcriptional competence (338-340), it stabilizes its own expression (338, 339), and suppress Th1 polarization (341). Our preliminary findings indicate that the addition of Rapamycin at the time of Ag-stimulation reduces, albeit to different extents, the transcription of both T-bet and GATA-3 mRNAs (data not shown), suggesting that proper mTor-signalling might indeed be required for the expression of the polarizing transcription factors. The reduced transcription of T-bet and GATA-3 might also be due to the reduced up-regulation of the IL-2R and IL-4R common γ_c on the surface of the T cells at day 3, which support the possibility that Rapamycin prevents T cell differentiation by inhibiting the final cytokine-mediated T cell commitment. Indeed, even though Rapamycin prevented IL-2 secretion, neither the addition of recombinant IL-2, nor the addition of recombinant IL-4 restored the cell autonomous Th1 T cell differentiation, and the IL-4 driven Th2 differentiation. In contrast, the suboptimal IL-2R common γ_c expression directly correlates with the functional impairment of the cells. Indeed, in the absence of the high affinity IL-2R, T cells might fail to receive the IL-2 driven signals enabling them to respond to cytokine-dependent expansion and polarization. Indeed, several data support the role for IL-2/IL-2R in T cell polarization. For instance, in the

absence of IL-2, or IL-2Rβ-generated signalling, T cells fail to differentiate into IFN-γ producing cells and to acquire CTL capabilities, both *in vivo* and *in vitro* (221, 282). Moreover, IL-2Rβ$^{-/-}$ T cells failed to respond to several recombinant cytokine *in vitro*, supporting the idea that IL-2 generated signals are required for proper T cell growth and maturation into functional effector T cells (282). Similarly, IL-2 has been shown to be crucial also for Th2 differentiation (293, 342). Furthermore, activation of STAT5A, a downstream target of the IL-2R, was linked to Th2 differentiation, via IL-4 and GATA-3 independent pathways (295). Since STAT5 and GATA-3 seem to favour Th2 polarization by distinct mechanisms (295), it is possible that Rapamycin, by inhibiting the early expression of the high affinity IL-2R, might prevent STAT5 activation, and by that might hamper T cell polarization. Consistent with the possibility that Rapamycin prevents T cell polarization by inhibiting γ_c up-regulation is the finding that Rapamycin failed to control IL-12-induced Th1 differentiation, which occurs through a common γ_c-independent (343), but STAT4–dependent pathway (344).

Finally, it is possible that Rapamycin, by delaying cell proliferation might prevent terminal differentiation. To this respect, Reiner and co-worker (321) reported that, to induce proper Th2 T cell differentiation, T cells had to complete at least three cell divisions. Our results dissociate cell cycle progression from differentiation. Indeed, in spite of completing the same number of cell divisions, cells expanded in the presence of Rapamycin still failed to produce either IFN-γ or IL-4. It is however possible that, by delaying cell cycle entry and the up-regulation of the common γ_c, Rapamycin might uncouple TCR and cytokine generated signals, which have to be delivered in close proximity to elicit proper T cell differentiation (345).

Altogether these results indicate that progression through the cell cycle might be necessary, but not sufficient to drive T cell polarization, and that an mTor-dependent signal is critically required to elicit T cell polarization.

4.4 Concluding remarks

The results reported in this thesis demonstrate that mTor signalling plays a central role in T cell proliferation, antigen responsiveness, and differentiation, which are tightly regulated events during the development of protective immunity. Moreover, by tracing the fate of single cells activated in the presence of the mTor inhibitor Rapamycin, we revealed a new mechanism at the base of the clinical activity of this drug. Indeed, even though additional studies will have to be performed *in vivo* to validate these *in vitro* generated findings, it is possible that the immunosuppressive activity of Rapamycin relies on the ability of the drug to modulate, rather than suppress, adaptive immune responses.

Bibliography

1. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J Exp Med 180:1263.*
2. Koch, F., U. Stanzl, P. Jennewein, K. Janke, C. Heufler, E. Kampgen, N. Romani, and G. Schuler. 1996. High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med 184:741.*
3. Bourgeois, C., B. Rocha, and C. Tanchot. 2002. A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory. *Science 297:2060.*
4. Kawabe, T., T. Naka, K. Yoshida, T. Tanaka, H. Fujiwara, S. Suematsu, N. Yoshida, T. Kishimoto, and H. Kikutani. 1994. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. *Immunity 1:167.*
5. Janeway, C. A., Jr. 2001. How the immune system works to protect the host from infection: a personal view. *Proc Natl Acad Sci U S A 98:7461.*
6. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature 334:395.*
7. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol 7:445.*
8. Lenschow, D. J., T. L. Walunas, and J. A. Bluestone. 1996. CD28/B7 system of T cell costimulation. *Annu Rev Immunol 14:233.*
9. Sharpe, A. H., and G. J. Freeman. 2002. The B7-CD28 superfamily. *Nat Rev Immunol 2:116.*
10. Croft, M. 2003. Co-stimulatory members of the TNFR family: keys to effective T-cell immunity? *Nat Rev Immunol 3:609.*
11. Watts, T. H., and M. A. DeBenedette. 1999. T cell co-stimulatory molecules other than CD28. *Curr Opin Immunol 11:286.*
12. Deeths, M. J., and M. F. Mescher. 1999. ICAM-1 and B7-1 provide similar but distinct costimulation for CD8+ T cells, while CD4+ T cells are poorly costimulated by ICAM-1. *Eur J Immunol 29:45.*
13. Geginat, J., B. Clissi, M. Moro, P. Dellabona, J. R. Bender, and R. Pardi. 2000. CD28 and LFA-1 contribute to cyclosporin A-resistant T cell growth by stabilizing the IL-2 mRNA through distinct signaling pathways. *Eur J Immunol 30:1136.*
14. Ni, H. T., M. J. Deeths, and M. F. Mescher. 2001. LFA-1-mediated costimulation of CD8+ T cell proliferation requires phosphatidylinositol 3-kinase activity. *J Immunol 166:6523.*
15. Sims, T. N., and M. L. Dustin. 2002. The immunological synapse: integrins take the stage. *Immunol Rev 186:100.*

16. Cerdan, C., Y. Martin, M. Courcoul, H. Brailly, C. Mawas, F. Birg, and D. Olive. 1992. Prolonged IL-2 receptor alpha/CD25 expression after T cell activation via the adhesion molecules CD2 and CD28. Demonstration of combined transcriptional and post-transcriptional regulation. *J Immunol 149:2255.*
17. Cerdan, C., Y. Martin, M. Courcoul, C. Mawas, F. Birg, and D. Olive. 1995. CD28 costimulation regulates long-term expression of the three genes (alpha, beta, gamma) encoding the high-affinity IL2 receptor. *Res Immunol 146:164.*
18. Lindstein, T., C. H. June, J. A. Ledbetter, G. Stella, and C. B. Thompson. 1989. Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science 244:339.*
19. Thompson, C. B., T. Lindsten, J. A. Ledbetter, S. L. Kunkel, H. A. Young, S. G. Emerson, J. M. Leiden, and C. H. June. 1989. CD28 activation pathway regulates the production of multiple T-cell-derived lymphokines/cytokines. *Proc Natl Acad Sci U S A 86:1333.*
20. Fraser, J. D., B. A. Irving, G. R. Crabtree, and A. Weiss. 1991. Regulation of interleukin-2 gene enhancer activity by the T cell accessory molecule CD28. *Science 251:313.*
21. Gimmi, C. D., G. J. Freeman, J. G. Gribben, K. Sugita, A. S. Freedman, C. Morimoto, and L. M. Nadler. 1991. B-cell surface antigen B7 provides a costimulatory signal that induces T cells to proliferate and secrete interleukin 2. *Proc Natl Acad Sci U S A 88:6575.*
22. Linsley, P. S., W. Brady, L. Grosmaire, A. Aruffo, N. K. Damle, and J. A. Ledbetter. 1991. Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med 173:721.*
23. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-XL. *Immunity 3:87.*
24. Chambers, C. A., and J. P. Allison. 1999. Costimulatory regulation of T cell function. *Curr Opin Cell Biol 11:203.*
25. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science 261:609.*
26. Kundig, T. M., A. Shahinian, K. Kawai, H. W. Mittrucker, E. Sebzda, M. F. Bachmann, T. W. Mak, and P. S. Ohashi. 1996. Duration of TCR stimulation determines costimulatory requirement of T cells. *Immunity 5:41.*
27. Lin, H., S. F. Bolling, P. S. Linsley, R. Q. Wei, D. Gordon, C. B. Thompson, and L. A. Turka. 1993. Long-term acceptance of major histocompatibility complex mismatched cardiac allografts induced by CTLA4Ig plus donor-specific transfusion. *J Exp Med 178:1801.*
28. Linsley, P. S., and J. A. Ledbetter. 1993. The role of the CD28 receptor during T cell responses to antigen. *Annu Rev Immunol 11:191.*
29. Lenschow, D. J., Y. Zeng, J. R. Thistlethwaite, A. Montag, W. Brady, M. G. Gibson, P. S. Linsley, and J. A. Bluestone. 1992. Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4lg. *Science 257:789.*

30. Linsley, P. S., P. M. Wallace, J. Johnson, M. G. Gibson, J. L. Greene, J. A. Ledbetter, C. Singh, and M. A. Tepper. 1992. Immunosuppression in vivo by a soluble form of the CTLA-4 T cell activation molecule. *Science 257:792.*
31. Finck, B. K., P. S. Linsley, and D. Wofsy. 1994. Treatment of murine lupus with CTLA4Ig. *Science 265:1225.*
32. Chen, L., S. Ashe, W. A. Brady, I. Hellstrom, K. E. Hellstrom, J. A. Ledbetter, P. McGowan, and P. S. Linsley. 1992. Costimulation of antitumor immunity by the B7 counterreceptor for the T lymphocyte molecules CD28 and CTLA-4. *Cell 71:1093.*
33. Townsend, S. E., and J. P. Allison. 1993. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science 259:368.*
34. Moro, M., A. M. Gasparri, S. Pagano, M. Bellone, P. Tornaghi, F. Veglia, A. Corti, G. Casorati, and P. Dellabona. 1999. Induction of therapeutic T-cell immunity by tumor targeting with soluble recombinant B7-immunoglobulin costimulatory molecules. *Cancer Res 59:2650.*
35. Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science 248:1349.*
36. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature 356:607.*
37. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science 273:104.*
38. Bachmann, M. F., K. McKall-Faienza, R. Schmits, D. Bouchard, J. Beach, D. E. Speiser, T. W. Mak, and P. S. Ohashi. 1997. Distinct roles for LFA-1 and CD28 during activation of naive T cells: adhesion versus costimulation. *Immunity 7:549.*
39. Ronchese, F., B. Hausmann, S. Hubele, and P. Lane. 1994. Mice transgenic for a soluble form of murine CTLA-4 show enhanced expansion of antigen-specific CD4+ T cells and defective antibody production in vivo. *J Exp Med 179:809.*
40. Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia. 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature 375:148.*
41. Valitutti, S., M. Dessing, K. Aktories, H. Gallati, and A. Lanzavecchia. 1995. Sustained signaling leading to T cell activation results from prolonged T cell receptor occupancy. Role of T cell actin cytoskeleton. *J Exp Med 181:577.*
42. Beeson, C., J. Rabinowitz, K. Tate, I. Gutgemann, Y. H. Chien, P. P. Jones, M. M. Davis, and H. M. McConnell. 1996. Early biochemical signals arise from low affinity TCR-ligand reactions at the cell-cell interface. *J Exp Med 184:777.*
43. Iezzi, G., K. Karjalainen, and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity 8:89.*
44. Acuto, O., and D. Cantrell. 2000. T cell activation and the cytoskeleton. *Annu Rev Immunol 18:165.*
45. Cantrell, D. A. 2002. T-cell antigen receptor signal transduction. *Immunology 105:369.*

46. Weiss, A., and D. R. Littman. 1994. Signal transduction by lymphocyte antigen receptors. *Cell 76:263.*
47. Pitcher, L. A., and N. S. van Oers. 2003. T-cell receptor signal transmission: who gives an ITAM? *Trends Immunol 24:554.*
48. Davis, S. J., and P. A. van der Merwe. 2001. The immunological synapse: required for T cell receptor signalling or directing T cell effector function? *Curr Biol 11:R289.*
49. Chu, D. H., C. T. Morita, and A. Weiss. 1998. The Syk family of protein tyrosine kinases in T-cell activation and development. *Immunol Rev 165:167.*
50. Duplay, P., M. Thome, F. Herve, and O. Acuto. 1994. p56lck interacts via its src homology 2 domain with the ZAP-70 kinase. *J Exp Med 179:1163.*
51. Iwashima, M., B. A. Irving, N. S. van Oers, A. C. Chan, and A. Weiss. 1994. Sequential interactions of the TCR with two distinct cytoplasmic tyrosine kinases. *Science 263:1136.*
52. Chan, A. C., M. Dalton, R. Johnson, G. H. Kong, T. Wang, R. Thoma, and T. Kurosaki. 1995. Activation of ZAP-70 kinase activity by phosphorylation of tyrosine 493 is required for lymphocyte antigen receptor function. *Embo J 14:2499.*
53. Qian, D., M. N. Mollenauer, and A. Weiss. 1996. Dominant-negative zeta-associated protein 70 inhibits T cell antigen receptor signaling. *J Exp Med 183:611.*
54. Heyeck, S. D., H. M. Wilcox, S. C. Bunnell, and L. J. Berg. 1997. Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity. *J Biol Chem 272:25401.*
55. Madrenas, J., L. A. Chau, J. Smith, J. A. Bluestone, and R. N. Germain. 1997. The efficiency of CD4 recruitment to ligand-engaged TCR controls the agonist/partial agonist properties of peptide-MHC molecule ligands. *J Exp Med 185:219.*
56. Turner, J. M., M. H. Brodsky, B. A. Irving, S. D. Levin, R. M. Perlmutter, and D. R. Littman. 1990. Interaction of the unique N-terminal region of tyrosine kinase p56lck with cytoplasmic domains of CD4 and CD8 is mediated by cysteine motifs. *Cell 60:755.*
57. Myung, P. S., N. J. Boerthe, and G. A. Koretzky. 2000. Adapter proteins in lymphocyte antigen-receptor signaling. *Curr Opin Immunol 12:256.*
58. Zhang, W., and L. E. Samelson. 2000. The role of membrane-associated adaptors in T cell receptor signalling. *Semin Immunol 12:35.*
59. Genot, E., and D. A. Cantrell. 2000. Ras regulation and function in lymphocytes. *Curr Opin Immunol 12:289.*
60. Cantrell, D. A. 2003. GTPases and T cell activation. *Immunol Rev 192:122.*
61. Cooper, J. A. 1994. MAP kinase pathways. Straight and narrow or tortuous and intersecting? *Curr Biol 4:1118.*
62. Marshall, C. J. 1994. MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr Opin Genet Dev 4:82.*
63. Hunter, T., and M. Karin. 1992. The regulation of transcription by phosphorylation. *Cell 70:375.*
64. Scharenberg, A. M., O. El-Hillal, D. A. Fruman, L. O. Beitz, Z. Li, S. Lin, I. Gout, L. C. Cantley, D. J. Rawlings, and J. P. Kinet. 1998. Phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3)/Tec kinase-

dependent calcium signaling pathway: a target for SHIP-mediated inhibitory signals. *Embo J 17:1961.*

65. Schaeffer, E. M., J. Debnath, G. Yap, D. McVicar, X. C. Liao, D. R. Littman, A. Sher, H. E. Varmus, M. J. Lenardo, and P. L. Schwartzberg. 1999. Requirement for Tec kinases Rlk and Itk in T cell receptor signaling and immunity. *Science 284:638.*
66. Finco, T. S., T. Kadlecek, W. Zhang, L. E. Samelson, and A. Weiss. 1998. LAT is required for TCR-mediated activation of PLCgamma1 and the Ras pathway. *Immunity 9:617.*
67. Clements, J. L., B. Yang, S. E. Ross-Barta, S. L. Eliason, R. F. Hrstka, R. A. Williamson, and G. A. Koretzky. 1998. Requirement for the leukocyte-specific adapter protein SLP-76 for normal T cell development. *Science 281:416.*
68. Crabtree, G. R. 1999. Generic signals and specific outcomes: signaling through Ca2+, calcineurin, and NF-AT. *Cell 96:611.*
69. Negulescu, P. A., T. B. Krasieva, A. Khan, H. H. Kerschbaum, and M. D. Cahalan. 1996. Polarity of T cell shape, motility, and sensitivity to antigen. *Immunity 4:421.*
70. Shaw, A. S., and M. L. Dustin. 1997. Making the T cell receptor go the distance: a topological view of T cell activation. *Immunity 6:361.*
71. Mellor, H., and P. J. Parker. 1998. The extended protein kinase C superfamily. *Biochem J 332 (Pt 2):281.*
72. Altman, A., N. Isakov, and G. Baier. 2000. Protein kinase Ctheta: a new essential superstar on the T-cell stage. *Immunol Today 21:567.*
73. Arendt, C. W., B. Albrecht, T. J. Soos, and D. R. Littman. 2002. Protein kinase C-theta;: signaling from the center of the T-cell synapse. *Curr Opin Immunol 14:323.*
74. Bi, K., Y. Tanaka, N. Coudronniere, K. Sugie, S. Hong, M. J. van Stipdonk, and A. Altman. 2001. Antigen-induced translocation of PKC-theta to membrane rafts is required for T cell activation. *Nat Immunol 2:556.*
75. Villalba, M., N. Coudronniere, M. Deckert, E. Teixeiro, P. Mas, and A. Altman. 2000. A novel functional interaction between Vav and PKCtheta is required for TCR-induced T cell activation. *Immunity 12:151.*
76. Ebinu, J. O., S. L. Stang, C. Teixeira, D. A. Bottorff, J. Hooton, P. M. Blumberg, M. Barry, R. C. Bleakley, H. L. Ostergaard, and J. C. Stone. 2000. RasGRP links T-cell receptor signaling to Ras. *Blood 95:3199.*
77. Rameh, L. E., and L. C. Cantley. 1999. The role of phosphoinositide 3-kinase lipid products in cell function. *J Biol Chem 274:8347.*
78. Ward, S. G., and D. A. Cantrell. 2001. Phosphoinositide 3-kinases in T lymphocyte activation. *Curr Opin Immunol 13:332.*
79. Cantrell, D. 1996. T cell antigen receptor signal transduction pathways. *Annu Rev Immunol 14:259.*
80. Zhang, W., J. Sloan-Lancaster, J. Kitchen, R. P. Trible, and L. E. Samelson. 1998. LAT: the ZAP-70 tyrosine kinase substrate that links T cell receptor to cellular activation. *Cell 92:83.*
81. Rameh, L. E., A. Arvidsson, K. L. Carraway, 3rd, A. D. Couvillon, G. Rathbun, A. Crompton, B. VanRenterghem, M. P. Czech, K. S. Ravichandran, S. J. Burakoff, D. S. Wang, C. S. Chen, and L. C. Cantley.

1997. A comparative analysis of the phosphoinositide binding specificity of pleckstrin homology domains. *J Biol Chem 272:22059.*

82. Scharenberg, A. M., and J. P. Kinet. 1998. PtdIns-3,4,5-P3: a regulatory nexus between tyrosine kinases and sustained calcium signals. *Cell 94:5.*
83. Toker, A., and A. C. Newton. 2000. Cellular signaling: pivoting around PDK-1. *Cell 103:185.*
84. Vanhaesebroeck, B., and D. R. Alessi. 2000. The PI3K-PDK1 connection: more than just a road to PKB. *Biochem J 346 Pt 3:561.*
85. Tybulewicz, V. L., L. Ardouin, A. Prisco, and L. F. Reynolds. 2003. Vav1: a key signal transducer downstream of the TCR. *Immunol Rev 192:42.*
86. Williams, M. R., J. S. Arthur, A. Balendran, J. van der Kaay, V. Poli, P. Cohen, and D. R. Alessi. 2000. The role of 3-phosphoinositide-dependent protein kinase 1 in activating AGC kinases defined in embryonic stem cells. *Curr Biol 10:439.*
87. Montagne, J., M. J. Stewart, H. Stocker, E. Hafen, S. C. Kozma, and G. Thomas. 1999. Drosophila S6 kinase: a regulator of cell size. *Science 285:2126.*
88. Volarevic, S., M. J. Stewart, B. Ledermann, F. Zilberman, L. Terracciano, E. Montini, M. Grompe, S. C. Kozma, and G. Thomas. 2000. Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. *Science 288:2045.*
89. Brennan, P., J. W. Babbage, B. M. Burgering, B. Groner, K. Reif, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase couples the interleukin-2 receptor to the cell cycle regulator E2F. *Immunity 7:679.*
90. Brennan, P., J. W. Babbage, G. Thomas, and D. Cantrell. 1999. p70(s6k) integrates phosphatidylinositol 3-kinase and rapamycin-regulated signals for E2F regulation in T lymphocytes. *Mol Cell Biol 19:4729.*
91. Jones, R. G., M. Parsons, M. Bonnard, V. S. Chan, W. C. Yeh, J. R. Woodgett, and P. S. Ohashi. 2000. Protein kinase B regulates T lymphocyte survival, nuclear factor kappaB activation, and Bcl-X(L) levels in vivo. *J Exp Med 191:1721.*
92. Parsons, M. J., R. G. Jones, M. S. Tsao, B. Odermatt, P. S. Ohashi, and J. R. Woodgett. 2001. Expression of active protein kinase B in T cells perturbs both T and B cell homeostasis and promotes inflammation. *J Immunol 167:42.*
93. Cohen, P., D. R. Alessi, and D. A. Cross. 1997. PDK1, one of the missing links in insulin signal transduction? *FEBS Lett 410:3.*
94. Beals, C. R., C. M. Sheridan, C. W. Turck, P. Gardner, and G. R. Crabtree. 1997. Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science 275:1930.*
95. Han, J., K. Luby-Phelps, B. Das, X. Shu, Y. Xia, R. D. Mosteller, U. M. Krishna, J. R. Falck, M. A. White, and D. Broek. 1998. Role of substrates and products of PI 3-kinase in regulating activation of Rac-related guanosine triphosphatases by Vav. *Science 279:558.*
96. Frauwirth, K. A., and C. B. Thompson. 2002. Activation and inhibition of lymphocytes by costimulation. *J Clin Invest 109:295.*
97. Tuosto, L., and O. Acuto. 1998. CD28 affects the earliest signaling events generated by TCR engagement. *Eur J Immunol 28:2131.*

98. Wulfing, C., and M. M. Davis. 1998. A receptor/cytoskeletal movement triggered by costimulation during T cell activation. *Science 282:2266.*
99. Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science 283:680.*
100. Hutchcroft, J. E., and B. E. Bierer. 1994. Activation-dependent phosphorylation of the T-lymphocyte surface receptor CD28 and associated proteins. *Proc Natl Acad Sci U S A 91:3260.*
101. August, A., S. Gibson, Y. Kawakami, T. Kawakami, G. B. Mills, and B. Dupont. 1994. CD28 is associated with and induces the immediate tyrosine phosphorylation and activation of the Tec family kinase ITK/EMT in the human Jurkat leukemic T-cell line. *Proc Natl Acad Sci U S A 91:9347.*
102. Acuto, O., and F. Michel. 2003. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol 3:939.*
103. Rudd, C. E. 1996. Upstream-downstream: CD28 cosignaling pathways and T cell function. *Immunity 4:527.*
104. Ward, S. G., J. Westwick, N. D. Hall, and D. M. Sansom. 1993. Ligation of CD28 receptor by B7 induces formation of D-3 phosphoinositides in T lymphocytes independently of T cell receptor/CD3 activation. *Eur J Immunol 23:2572.*
105. Woodrow, M. A., S. Rayter, J. Downward, and D. A. Cantrell. 1993. p21ras function is important for T cell antigen receptor and protein kinase C regulation of nuclear factor of activated T cells. *J Immunol 150:3853.*
106. Kane, L. P., P. G. Andres, K. C. Howland, A. K. Abbas, and A. Weiss. 2001. Akt provides the CD28 costimulatory signal for up-regulation of IL-2 and IFN-gamma but not TH2 cytokines. *Nat Immunol 2:37.*
107. Pai, S. Y., V. Calvo, M. Wood, and B. E. Bierer. 1994. Cross-linking CD28 leads to activation of 70-kDa S6 kinase. *Eur J Immunol 24:2364.*
108. Ghosh, P., M. A. Buchholz, S. Yano, D. Taub, and D. L. Longo. 2002. Effect of rapamycin on the cyclosporin A-resistant CD28-mediated costimulatory pathway. *Blood 99:4517.*
109. Klasen, S., F. Pages, J. F. Peyron, D. A. Cantrell, and D. Olive. 1998. Two distinct regions of the CD28 intracytoplasmic domain are involved in the tyrosine phosphorylation of Vav and GTPase activating protein-associated p62 protein. *Int Immunol 10:481.*
110. Davis, R. J. 2000. Signal transduction by the JNK group of MAP kinases. *Cell 103:239.*
111. Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah. 1994. JNK is involved in signal integration during costimulation of T lymphocytes. *Cell 77:727.*
112. Rincon, M., and R. A. Flavell. 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. *Embo J 13:4370.*
113. Liao, X. C., S. Fournier, N. Killeen, A. Weiss, J. P. Allison, and D. R. Littman. 1997. Itk negatively regulates induction of T cell proliferation by CD28 costimulation. *J Exp Med 186:221.*
114. Crabtree, G. R. 1989. Contingent genetic regulatory events in T lymphocyte activation. *Science 243:355.*

115. Alberola-Ila, J., S. Takaki, J. D. Kerner, and R. M. Perlmutter. 1997. Differential signaling by lymphocyte antigen receptors. *Annu Rev Immunol 15:125.*
116. Ullman, K. S., J. P. Northrop, C. L. Verweij, and G. R. Crabtree. 1990. Transmission of signals from the T lymphocyte antigen receptor to the genes responsible for cell proliferation and immune function: the missing link. *Annu Rev Immunol 8:421.*
117. Ho, S., N. Clipstone, L. Timmermann, J. Northrop, I. Graef, D. Fiorentino, J. Nourse, and G. R. Crabtree. 1996. The mechanism of action of cyclosporin A and FK506. *Clin Immunol Immunopathol 80:S40.*
118. Karin, M., Z. Liu, and E. Zandi. 1997. AP-1 function and regulation. *Curr Opin Cell Biol 9:240.*
119. Hill, C. S., and R. Treisman. 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. *Cell 80:199.*
120. Isakov, N., and A. Altman. 2002. Protein kinase C(theta) in T cell activation. *Annu Rev Immunol 20:761.*
121. Janknecht, R., W. H. Ernst, V. Pingoud, and A. Nordheim. 1993. Activation of ternary complex factor Elk-1 by MAP kinases. *Embo J 12:5097.*
122. Kortenjann, M., O. Thomae, and P. E. Shaw. 1994. Inhibition of v-raf-dependent c-fos expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. *Mol Cell Biol 14:4815.*
123. Coffer, P., M. de Jonge, A. Mettouchi, B. Binetruy, J. Ghysdael, and W. Kruijer. 1994. junB promoter regulation: Ras mediated transactivation by c-Ets-1 and c-Ets-2. *Oncogene 9:911.*
124. Hibi, M., A. Lin, T. Smeal, A. Minden, and M. Karin. 1993. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev 7:2135.*
125. Li, W., C. D. Whaley, J. L. Bonnevier, A. Mondino, M. E. Martin, K. M. Aagaard-Tillery, and D. L. Mueller. 2001. CD28 signaling augments Elk-1-dependent transcription at the c-fos gene during antigen stimulation. *J Immunol 167:827.*
126. Chen, C. Y., F. Del Gatto-Konczak, Z. Wu, and M. Karin. 1998. Stabilization of interleukin-2 mRNA by the c-Jun NH2-terminal kinase pathway. *Science 280:1945.*
127. Rao, A. 1994. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. *Immunol Today 15:274.*
128. Macian, F., C. Garcia-Rodriguez, and A. Rao. 2000. Gene expression elicited by NFAT in the presence or absence of cooperative recruitment of Fos and Jun. *Embo J 19:4783.*
129. Macian, F., C. Lopez-Rodriguez, and A. Rao. 2001. Partners in transcription: NFAT and AP-1. *Oncogene 20:2476.*
130. Clipstone, N. A., and G. R. Crabtree. 1992. Identification of calcineurin as a key signalling enzyme in T-lymphocyte activation. *Nature 357:695.*
131. Rayter, S. I., M. Woodrow, S. C. Lucas, D. A. Cantrell, and J. Downward. 1992. p21ras mediates control of IL-2 gene promoter function in T cell activation. *Embo J 11:4549.*

132. Genot, E. M., P. J. Parker, and D. A. Cantrell. 1995. Analysis of the role of protein kinase C-alpha, -epsilon, and -zeta in T cell activation. *J Biol Chem 270:9833.*
133. Sun, Z., C. W. Arendt, W. Ellmeier, E. M. Schaeffer, M. J. Sunshine, L. Gandhi, J. Annes, D. Petrzilka, A. Kupfer, P. L. Schwartzberg, and D. R. Littman. 2000. PKC-theta is required for TCR-induced NF-kappaB activation in mature but not immature T lymphocytes. *Nature 404:402.*
134. Karin, M., and Y. Ben-Neriah. 2000. Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol 18:621.*
135. Costello, P. S., A. E. Walters, P. J. Mee, M. Turner, L. F. Reynolds, A. Prisco, N. Sarner, R. Zamoyska, and V. L. Tybulewicz. 1999. The Rho-family GTP exchange factor Vav is a critical transducer of T cell receptor signals to the calcium, ERK, and NF-kappaB pathways. *Proc Natl Acad Sci U S A 96:3035.*
136. Ghosh, P., T. H. Tan, N. R. Rice, A. Sica, and H. A. Young. 1993. The interleukin 2 CD28-responsive complex contains at least three members of the NF kappa B family: c-Rel, p50, and p65. *Proc Natl Acad Sci U S A 90:1696.*
137. Pfeuffer, I., S. Klein-Hessling, A. Heinfling, S. Chuvpilo, C. Escher, T. Brabletz, B. Hentsch, H. Schwarzenbach, P. Matthias, and E. Serfling. 1994. Octamer factors exert a dual effect on the IL-2 and IL-4 promoters. *J Immunol 153:5572.*
138. Kamps, M. P., L. Corcoran, J. H. LeBowitz, and D. Baltimore. 1990. The promoter of the human interleukin-2 gene contains two octamer-binding sites and is partially activated by the expression of Oct-2. *Mol Cell Biol 10:5464.*
139. Pardee, A. B. 1989. G1 events and regulation of cell proliferation. *Science 246:603.*
140. Turner, J. M. 1993. IL-2-dependent induction of G1 cyclins in primary T cells is not blocked by rapamycin or cyclosporin A. *Int Immunol 5:1199.*
141. Minami, Y., T. Kono, T. Miyazaki, and T. Taniguchi. 1993. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol 11:245.*
142. Reed, S. I. 1992. The role of p34 kinases in the G1 to S-phase transition. *Annu Rev Cell Biol 8:529.*
143. Pines, J. 1993. Cyclins and cyclin-dependent kinases: take your partners. *Trends Biochem Sci 18:195.*
144. Sherr, C. J. 1993. Mammalian G1 cyclins. *Cell 73:1059.*
145. Sherr, C. J., and J. M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev 13:1501.*
146. Ruas, M., and G. Peters. 1998. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta 1378:F115.*
147. Hengst, L., and S. I. Reed. 1998. Inhibitors of the Cip/Kip family. *Curr Top Microbiol Immunol 227:25.*
148. Shaulian, E., and M. Karin. 2001. AP-1 in cell proliferation and survival. *Oncogene 20:2390.*
149. Vermeulen, K., Z. N. Berneman, and D. R. Van Bockstaele. 2003. Cell cycle and apoptosis. *Cell Prolif 36:165.*

150. Hartwell, L. H., and T. A. Weinert. 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science 246:629.*
151. Weinert, T. A., and L. H. Hartwell. 1988. The RAD9 gene controls the cell cycle response to DNA damage in Saccharomyces cerevisiae. *Science 241:317.*
152. Dasso, M., and J. W. Newport. 1990. Completion of DNA replication is monitored by a feedback system that controls the initiation of mitosis in vitro: studies in Xenopus. *Cell 61:811.*
153. Hoyt, M. A., L. Totis, and B. T. Roberts. 1991. S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. *Cell 66:507.*
154. Li, R., and A. W. Murray. 1991. Feedback control of mitosis in budding yeast. *Cell 66:519.*
155. Pardee, A. B. 1974. A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A 71:1286.*
156. Firpo, E. J., A. Koff, M. J. Solomon, and J. M. Roberts. 1994. Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol 14:4889.*
157. Kato, J. Y., M. Matsuoka, K. Polyak, J. Massague, and C. J. Sherr. 1994. Cyclic AMP-induced G1 phase arrest mediated by an inhibitor (p27Kip1) of cyclin-dependent kinase 4 activation. *Cell 79:487.*
158. Agrawal, D., P. Hauser, F. McPherson, F. Dong, A. Garcia, and W. J. Pledger. 1996. Repression of p27kip1 synthesis by platelet-derived growth factor in BALB/c 3T3 cells. *Mol Cell Biol 16:4327.*
159. Winston, J., F. Dong, and W. J. Pledger. 1996. Differential modulation of G1 cyclins and the Cdk inhibitor p27kip1 by platelet-derived growth factor and plasma factors in density-arrested fibroblasts. *J Biol Chem 271:11253.*
160. Taya, Y. 1997. RB kinases and RB-binding proteins: new points of view. *Trends Biochem Sci 22:14.*
161. Chellappan, S. P., S. Hiebert, M. Mudryj, J. M. Horowitz, and J. R. Nevins. 1991. The E2F transcription factor is a cellular target for the RB protein. *Cell 65:1053.*
162. Weinberg, R. A. 1995. The retinoblastoma protein and cell cycle control. *Cell 81:323.*
163. DeGregori, J., T. Kowalik, and J. R. Nevins. 1995. Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol 15:4215.*
164. Botz, J., K. Zerfass-Thome, D. Spitkovsky, H. Delius, B. Vogt, M. Eilers, A. Hatzigeorgiou, and P. Jansen-Durr. 1996. Cell cycle regulation of the murine cyclin E gene depends on an E2F binding site in the promoter. *Mol Cell Biol 16:3401.*
165. Montagnoli, A., F. Fiore, E. Eytan, A. C. Carrano, G. F. Draetta, A. Hershko, and M. Pagano. 1999. Ubiquitination of p27 is regulated by Cdk-dependent phosphorylation and trimeric complex formation. *Genes Dev 13:1181.*
166. Tsvetkov, L. M., K. H. Yeh, S. J. Lee, H. Sun, and H. Zhang. 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol 9:661.*

167. Lundberg, A. S., and R. A. Weinberg. 1998. Functional inactivation of the retinoblastoma protein requires sequential modification by at least two distinct cyclin-cdk complexes. *Mol Cell Biol 18:753.*
168. Modiano, J. F., J. Domenico, A. Szepesi, J. J. Lucas, and E. W. Gelfand. 1994. Differential requirements for interleukin-2 distinguish the expression and activity of the cyclin-dependent kinases Cdk4 and Cdk2 in human T cells. *J Biol Chem 269:32972.*
169. Morice, W. G., G. Wiederrecht, G. J. Brunn, J. J. Siekierka, and R. T. Abraham. 1993. Rapamycin inhibition of interleukin-2-dependent p33cdk2 and p34cdc2 kinase activation in T lymphocytes. *J Biol Chem 268:22737.*
170. Nourse, J., E. Firpo, W. M. Flanagan, S. Coats, K. Polyak, M. H. Lee, J. Massague, G. R. Crabtree, and J. M. Roberts. 1994. Interleukin-2-mediated elimination of the p27Kip1 cyclin-dependent kinase inhibitor prevented by rapamycin. *Nature 372:570.*
171. Nelson, B. H., and D. M. Willerford. 1998. Biology of the interleukin-2 receptor. *Adv Immunol 70:1.*
172. Nakarai, T., M. J. Robertson, M. Streuli, Z. Wu, T. L. Ciardelli, K. A. Smith, and J. Ritz. 1994. Interleukin 2 receptor gamma chain expression on resting and activated lymphoid cells. *J Exp Med 180:241.*
173. Nelson, B. H., J. D. Lord, and P. D. Greenberg. 1994. Cytoplasmic domains of the interleukin-2 receptor beta and gamma chains mediate the signal for T-cell proliferation. *Nature 369:333.*
174. Karnitz, L. M., and R. T. Abraham. 1996. Interleukin-2 receptor signaling mechanisms. *Adv Immunol 61:147.*
175. Lord, J. D., B. C. McIntosh, P. D. Greenberg, and B. H. Nelson. 1998. The IL-2 receptor promotes proliferation, bcl-2 and bcl-x induction, but not cell viability through the adapter molecule Shc. *J Immunol 161:4627.*
176. Lord, J. D., B. C. McIntosh, P. D. Greenberg, and B. H. Nelson. 2000. The IL-2 receptor promotes lymphocyte proliferation and induction of the c-myc, bcl-2, and bcl-x genes through the trans-activation domain of Stat5. *J Immunol 164:2533.*
177. Leonard, W. J., and J. J. O'Shea. 1998. Jaks and STATs: biological implications. *Annu Rev Immunol 16:293.*
178. Greenlund, A. C., M. O. Morales, B. L. Viviano, H. Yan, J. Krolewski, and R. D. Schreiber. 1995. Stat recruitment by tyrosine-phosphorylated cytokine receptors: an ordered reversible affinity-driven process. *Immunity 2:677.*
179. Moriggl, R., D. J. Topham, S. Teglund, V. Sexl, C. McKay, D. Wang, A. Hoffmeyer, J. van Deursen, M. Y. Sangster, K. D. Bunting, G. C. Grosveld, and J. N. Ihle. 1999. Stat5 is required for IL-2-induced cell cycle progression of peripheral T cells. *Immunity 10:249.*
180. Friedmann, M. C., T. S. Migone, S. M. Russell, and W. J. Leonard. 1996. Different interleukin 2 receptor beta-chain tyrosines couple to at least two signaling pathways and synergistically mediate interleukin 2-induced proliferation. *Proc Natl Acad Sci U S A 93:2077.*
181. Izquierdo Pastor, M., K. Reif, and D. Cantrell. 1995. The regulation and function of p21ras during T-cell activation and growth. *Immunol Today 16:159.*

182. Gadina, M., C. Sudarshan, R. Visconti, Y. J. Zhou, H. Gu, B. G. Neel, and J. J. O'Shea. 2000. The docking molecule gab2 is induced by lymphocyte activation and is involved in signaling by interleukin-2 and interleukin-15 but not other common gamma chain-using cytokines. *J Biol Chem 275:26959.*
183. Gu, H., H. Maeda, J. J. Moon, J. D. Lord, M. Yoakim, B. H. Nelson, and B. G. Neel. 2000. New role for Shc in activation of the phosphatidylinositol 3-kinase/Akt pathway. *Mol Cell Biol 20:7109.*
184. Steelman, L. S., S. C. Pohnert, J. G. Shelton, R. A. Franklin, F. E. Bertrand, and J. A. McCubrey. 2004. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. *Leukemia 18:189.*
185. Reif, K., B. M. Burgering, and D. A. Cantrell. 1997. Phosphatidylinositol 3-kinase links the interleukin-2 receptor to protein kinase B and p70 S6 kinase. *J Biol Chem 272:14426.*
186. Karnitz, L. M., L. A. Burns, S. L. Sutor, J. Blenis, and R. T. Abraham. 1995. Interleukin-2 triggers a novel phosphatidylinositol 3-kinase-dependent MEK activation pathway. *Mol Cell Biol 15:3049.*
187. Stephens, L., K. Anderson, D. Stokoe, H. Erdjument-Bromage, G. F. Painter, A. B. Holmes, P. R. Gaffney, C. B. Reese, F. McCormick, P. Tempst, J. Coadwell, and P. T. Hawkins. 1998. Protein kinase B kinases that mediate phosphatidylinositol 3,4,5-trisphosphate-dependent activation of protein kinase B. *Science 279:710.*
188. Pullen, N., P. B. Dennis, M. Andjelkovic, A. Dufner, S. C. Kozma, B. A. Hemmings, and G. Thomas. 1998. Phosphorylation and activation of p70s6k by PDK1. *Science 279:707.*
189. Alessi, D. R., M. T. Kozlowski, Q. P. Weng, N. Morrice, and J. Avruch. 1998. 3-Phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates and activates the p70 S6 kinase in vivo and in vitro. *Curr Biol 8:69.*
190. Lim, M. A., C. K. Kikani, M. J. Wick, and L. Q. Dong. 2003. Nuclear translocation of 3'-phosphoinositide-dependent protein kinase 1 (PDK-1): a potential regulatory mechanism for PDK-1 function. *Proc Natl Acad Sci U S A 100:14006.*
191. Kops, G. J., and B. M. Burgering. 1999. Forkhead transcription factors: new insights into protein kinase B (c-akt) signaling. *J Mol Med 77:656.*
192. Dijkers, P. F., R. H. Medema, C. Pals, L. Banerji, N. S. Thomas, E. W. Lam, B. M. Burgering, J. A. Raaijmakers, J. W. Lammers, L. Koenderman, and P. J. Coffer. 2000. Forkhead transcription factor FKHR-L1 modulates cytokine-dependent transcriptional regulation of p27(KIP1). *Mol Cell Biol 20:9138.*
193. Banerjee, P., M. F. Ahmad, J. R. Grove, C. Kozlosky, D. J. Price, and J. Avruch. 1990. Molecular structure of a major insulin/mitogen-activated 70-kDa S6 protein kinase. *Proc Natl Acad Sci U S A 87:8550.*
194. Grove, J. R., P. Banerjee, A. Balasubramanyam, P. J. Coffer, D. J. Price, J. Avruch, and J. R. Woodgett. 1991. Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. *Mol Cell Biol 11:5541.*
195. Kozma, S. C., S. Ferrari, P. Bassand, M. Siegmann, N. Totty, and G. Thomas. 1990. Cloning of the mitogen-activated S6 kinase from rat liver

reveals an enzyme of the second messenger subfamily. *Proc Natl Acad Sci U S A 87:7365.*

196. Reinhard, C., G. Thomas, and S. C. Kozma. 1992. A single gene encodes two isoforms of the p70 S6 kinase: activation upon mitogenic stimulation. *Proc Natl Acad Sci U S A 89:4052.*
197. Lee-Fruman, K. K., C. J. Kuo, J. Lippincott, N. Terada, and J. Blenis. 1999. Characterization of S6K2, a novel kinase homologous to S6K1. *Oncogene 18:5108.*
198. Jefferies, H. B., S. Fumagalli, P. B. Dennis, C. Reinhard, R. B. Pearson, and G. Thomas. 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. *Embo J 16:3693.*
199. Thomas, G. 2002. The S6 kinase signaling pathway in the control of development and growth. *Biol Res 35:305.*
200. Nasmyth, K. 1996. Retinoblastoma protein. Another role rolls in. *Nature 382:28.*
201. Jefferies, H. B., and G. Thomas. 1994. Elongation factor-1 alpha mRNA is selectively translated following mitogenic stimulation. *J Biol Chem 269:4367.*
202. Terada, N., H. R. Patel, K. Takase, K. Kohno, A. C. Nairn, and E. W. Gelfand. 1994. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. *Proc Natl Acad Sci U S A 91:11477.*
203. Heitman, J., N. R. Movva, and M. N. Hall. 1991. Targets for cell cycle arrest by the immunosuppressant rapamycin in yeast. *Science 253:905.*
204. Raught, B., A. C. Gingras, and N. Sonenberg. 2001. The target of rapamycin (TOR) proteins. *Proc Natl Acad Sci U S A 98:7037.*
205. Keith, C. T., and S. L. Schreiber. 1995. PIK-related kinases: DNA repair, recombination, and cell cycle checkpoints. *Science 270:50.*
206. Schmelzle, T., and M. N. Hall. 2000. TOR, a central controller of cell growth. *Cell 103:253.*
207. Iiboshi, Y., P. J. Papst, H. Kawasome, H. Hosoi, R. T. Abraham, P. J. Houghton, and N. Terada. 1999. Amino acid-dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. *J Biol Chem 274:1092.*
208. Dennis, P. B., A. Jaeschke, M. Saitoh, B. Fowler, S. C. Kozma, and G. Thomas. 2001. Mammalian TOR: a homeostatic ATP sensor. *Science 294:1102.*
209. McManus, E. J., and D. R. Alessi. 2002. TSC1-TSC2: a complex tale of PKB-mediated S6K regulation. *Nat Cell Biol 4:E214.*
210. Krymskaya, V. P. 2003. Tumour suppressors hamartin and tuberin: intracellular signalling. *Cell Signal 15:729.*
211. Hara, K., Y. Maruki, X. Long, K. Yoshino, N. Oshiro, S. Hidayat, C. Tokunaga, J. Avruch, and K. Yonezawa. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell 110:177.*
212. Kim, D. H., D. D. Sarbassov, S. M. Ali, J. E. King, R. R. Latek, H. Erdjument-Bromage, P. Tempst, and D. M. Sabatini. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell 110:163.*

213. Horton, L. E., M. Bushell, D. Barth-Baus, V. J. Tilleray, M. J. Clemens, and J. O. Hensold. 2002. p53 activation results in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1, inhibition of ribosomal protein S6 kinase and inhibition of translation initiation. *Oncogene 21:5325.*
214. Gonzalez-Garcia, A., E. Garrido, C. Hernandez, B. Alvarez, C. Jimenez, D. A. Cantrell, N. Pullen, and A. C. Carrera. 2002. A new role for the p85-phosphatidylinositol 3-kinase regulatory subunit linking FRAP to p70 S6 kinase activation. *J Biol Chem 277:1500.*
215. Thomas, G., and M. N. Hall. 1997. TOR signalling and control of cell growth. *Curr Opin Cell Biol 9:782.*
216. Tan, A., P. Bitterman, N. Sonenberg, M. Peterson, and V. Polunovsky. 2000. Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. *Oncogene 19:1437.*
217. Brown, E. J., M. W. Albers, T. B. Shin, K. Ichikawa, C. T. Keith, W. S. Lane, and S. L. Schreiber. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. *Nature 369:756.*
218. Sabatini, D. M., H. Erdjument-Bromage, M. Lui, P. Tempst, and S. H. Snyder. 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell 78:35.*
219. Edinger, A. L., and C. B. Thompson. 2002. Akt maintains cell size and survival by increasing mTOR-dependent nutrient uptake. *Mol Biol Cell 13:2276.*
220. Wells, A. D., H. Gudmundsdottir, and L. A. Turka. 1997. Following the fate of individual T cells throughout activation and clonal expansion. Signals from T cell receptor and CD28 differentially regulate the induction and duration of a proliferative response. *J Clin Invest 100:3173.*
221. Khoruts, A., A. Mondino, K. A. Pape, S. L. Reiner, and M. K. Jenkins. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp Med 187:225.*
222. Schorle, H., T. Holtschke, T. Hunig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature 352:621.*
223. Tivol, E. A., F. Borriello, A. N. Schweitzer, W. P. Lynch, J. A. Bluestone, and A. H. Sharpe. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity 3:541.*
224. Boonen, G. J., A. M. van Dijk, L. F. Verdonck, R. A. van Lier, G. Rijksen, and R. H. Medema. 1999. CD28 induces cell cycle progression by IL-2-independent down-regulation of p27kip1 expression in human peripheral T lymphocytes. *Eur J Immunol 29:789.*
225. Boulougouris, G., J. D. McLeod, Y. I. Patel, C. N. Ellwood, L. S. Walker, and D. M. Sansom. 1999. IL-2-independent activation and proliferation in human T cells induced by CD28. *J Immunol 163:1809.*
226. Appleman, L. J., A. Berezovskaya, I. Grass, and V. A. Boussiotis. 2000. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. *J Immunol 164:144.*

227. Appleman, L. J., A. A. van Puijenbroek, K. M. Shu, L. M. Nadler, and V. A. Boussiotis. 2002. CD28 costimulation mediates down-regulation of p27kip1 and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J Immunol 168:2729.*
228. Houssaint, E., and M. Flajnik. 1990. The role of thymic epithelium in the acquisition of tolerance. *Immunol Today 11:357.*
229. Ikuta, K., N. Uchida, J. Friedman, and I. L. Weissman. 1992. Lymphocyte development from stem cells. *Annu Rev Immunol 10:759.*
230. Miller, C., J. A. Ragheb, and R. H. Schwartz. 1999. Anergy and cytokine-mediated suppression as distinct superantigen-induced tolerance mechanisms in vivo. *J Exp Med 190:53.*
231. Nossal, G. J., and B. L. Pike. 1980. Clonal anergy: persistence in tolerant mice of antigen-binding B lymphocytes incapable of responding to antigen or mitogen. *Proc Natl Acad Sci U S A 77:1602.*
232. Lamb, J. R., B. J. Skidmore, N. Green, J. M. Chiller, and M. Feldmann. 1983. Induction of tolerance in influenza virus-immune T lymphocyte clones with synthetic peptides of influenza hemagglutinin. *J Exp Med 157:1434.*
233. Quill, H., and R. H. Schwartz. 1987. Stimulation of normal inducer T cell clones with antigen presented by purified Ia molecules in planar lipid membranes: specific induction of a long-lived state of proliferative nonresponsiveness. *J Immunol 138:3704.*
234. Jenkins, M. K., and R. H. Schwartz. 1987. Antigen presentation by chemically modified splenocytes induces antigen- specific T cell unresponsiveness in vitro and in vivo. *J Exp Med 165:302.*
235. Mueller, D. L., M. K. Jenkins, and R. H. Schwartz. 1989. An accessory cell-derived costimulatory signal acts independently of protein kinase C activation to allow T cell proliferation and prevent the induction of unresponsiveness. *J Immunol 142:2617.*
236. Jenkins, M. K., C. A. Chen, G. Jung, D. L. Mueller, and R. H. Schwartz. 1990. Inhibition of antigen-specific proliferation of type 1 murine T cell clones after stimulation with immobilized anti-CD3 monoclonal antibody. *J Immunol 144:16.*
237. Jenkins, M. K., D. M. Pardoll, J. Mizuguchi, T. M. Chused, and R. H. Schwartz. 1987. Molecular events in the induction of a nonresponsive state in interleukin 2-producing helper T-lymphocyte clones. *Proc Natl Acad Sci U S A 84:5409.*
238. Rammensee, H. G., R. Kroschewski, and B. Frangoulis. 1989. Clonal anergy induced in mature V beta 6+ T lymphocytes on immunizing Mls-1b mice with Mls-1a expressing cells. *Nature 339:541.*
239. Kawabe, Y., and A. Ochi. 1990. Selective anergy of V beta 8+,CD4+ T cells in Staphylococcus enterotoxin B-primed mice. *J Exp Med 172:1065.*
240. Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Matis. 1990. In vivo induction of anergy in peripheral V beta 8+ T cells by staphylococcal enterotoxin B. *J Exp Med 172:1091.*
241. Kearney, E. R., K. A. Pape, D. Y. Loh, and M. K. Jenkins. 1994. Visualization of peptide-specific T cell immunity and peripheral tolerance induction in vivo. *Immunity 1:327.*

242. Pape, K. A., A. Khoruts, E. Ingulli, A. Mondino, R. Merica, and M. K. Jenkins. 1998. Antigen-specific CD4+ T cells that survive after the induction of peripheral tolerance possess an intrinsic lymphokine production defect. *Novartis Found Symp 215:103.*
243. Pape, K. A., R. Merica, A. Mondino, A. Khoruts, and M. K. Jenkins. 1998. Direct evidence that functionally impaired CD4+ T cells persist in vivo following induction of peripheral tolerance. *J Immunol 160:4719.*
244. Kang, S. M., B. Beverly, A. C. Tran, K. Brorson, R. H. Schwartz, and M. J. Lenardo. 1992. Transactivation by AP-1 is a molecular target of T cell clonal anergy. *Science 257:1134.*
245. Mondino, A., C. D. Whaley, D. R. DeSilva, W. Li, M. K. Jenkins, and D. L. Mueller. 1996. Defective transcription of the IL-2 gene is associated with impaired expression of c-Fos, FosB, and JunB in anergic T helper 1 cells. *J Immunol 157:2048.*
246. Fields, P. E., T. F. Gajewski, and F. W. Fitch. 1996. Blocked Ras activation in anergic CD4+ T cells. *Science 271:1276.*
247. Li, W., C. D. Whaley, A. Mondino, and D. L. Mueller. 1996. Blocked signal transduction to the ERK and JNK protein kinases in anergic CD4+ T cells. *Science 271:1272.*
248. Sundstedt, A., M. Sigvardsson, T. Leanderson, G. Hedlund, T. Kalland, and M. Dohlsten. 1996. In vivo anergized CD4+ T cells express perturbed AP-1 and NF-kappa B transcription factors. *Proc Natl Acad Sci U S A 93:979.*
249. Becker, J. C., T. Brabletz, T. Kirchner, C. T. Conrad, E. B. Brocker, and R. A. Reisfeld. 1995. Negative transcriptional regulation in anergic T cells. *Proc Natl Acad Sci U S A 92:2375.*
250. Powell, J. D., C. G. Lerner, G. R. Ewoldt, and R. H. Schwartz. 1999. The -180 site of the IL-2 promoter is the target of CREB/CREM binding in T cell anergy. *J Immunol 163:6631.*
251. Telander, D. G., E. N. Malvey, and D. L. Mueller. 1999. Evidence for repression of IL-2 gene activation in anergic T cells. *J Immunol 162:1460.*
252. Gajewski, T. F., P. Fields, and F. W. Fitch. 1995. Induction of the increased Fyn kinase activity in anergic T helper type 1 clones requires calcium and protein synthesis and is sensitive to cyclosporin A. *Eur J Immunol 25:1836.*
253. Gajewski, T. F., D. Qian, P. Fields, and F. W. Fitch. 1994. Anergic T-lymphocyte clones have altered inositol phosphate, calcium, and tyrosine kinase signaling pathways. *Proc Natl Acad Sci U S A 91:38.*
254. Quill, H., M. P. Riley, E. A. Cho, J. E. Casnellie, J. C. Reed, and T. Torigoe. 1992. Anergic Th1 cells express altered levels of the protein tyrosine kinases p56lck and p59fyn. *J Immunol 149:2887.*
255. Appleman, L. J., and V. A. Boussiotis. 2003. T cell anergy and costimulation. *Immunol Rev 192:161.*
256. Sloan-Lancaster, J., B. D. Evavold, and P. M. Allen. 1993. Induction of T-cell anergy by altered T-cell-receptor ligand on live antigen-presenting cells. *Nature 363:156.*
257. Madrenas, J., R. L. Wange, J. L. Wang, N. Isakov, L. E. Samelson, and R. N. Germain. 1995. Zeta phosphorylation without ZAP-70 activation induced by TCR antagonists or partial agonists. *Science 267:515.*

258. Boussiotis, V. A., D. L. Barber, B. J. Lee, J. G. Gribben, G. J. Freeman, and L. M. Nadler. 1996. Differential association of protein tyrosine kinases with the T cell receptor is linked to the induction of anergy and its prevention by B7 family-mediated costimulation. *J Exp Med 184:365.*
259. Macian, F., F. Garcia-Cozar, S. H. Im, H. F. Horton, M. C. Byrne, and A. Rao. 2002. Transcriptional mechanisms underlying lymphocyte tolerance. *Cell 109:719.*
260. Anandasabapathy, N., G. S. Ford, D. Bloom, C. Holness, V. Paragas, C. Seroogy, H. Skrenta, M. Hollenhorst, C. G. Fathman, and L. Soares. 2003. GRAIL: an E3 ubiquitin ligase that inhibits cytokine gene transcription is expressed in anergic CD4+ T cells. *Immunity 18:535.*
261. Garcia-Sanz, J. A., and D. Lenig. 1996. Translational control of interleukin 2 messenger RNA as a molecular mechanism of T cell anergy. *J Exp Med 184:159.*
262. DeSilva, D. R., K. B. Urdahl, and M. K. Jenkins. 1991. Clonal anergy is induced in vitro by T cell receptor occupancy in the absence of proliferation. *J Immunol 147:3261.*
263. Beverly, B., S. M. Kang, M. J. Lenardo, and R. H. Schwartz. 1992. Reversal of in vitro T cell clonal anergy by IL-2 stimulation. *Int Immunol 4:661.*
264. Gilbert, K. M., and W. O. Weigle. 1993. Th1 cell anergy and blockade in G1a phase of the cell cycle. *J Immunol 151:1245.*
265. Powell, J. D., C. G. Lerner, and R. H. Schwartz. 1999. Inhibition of cell cycle progression by rapamycin induces T cell clonal anergy even in the presence of costimulation. *J Immunol 162:2775.*
266. Essery, G., M. Feldmann, and J. R. Lamb. 1988. Interleukin-2 can prevent and reverse antigen-induced unresponsiveness in cloned human T lymphocytes. *Immunology 64:413.*
267. Jenkins, M. K. 1992. The role of cell division in the induction of clonal anergy. *Immunol Today 13:69.*
268. Boussiotis, V. A., G. J. Freeman, P. A. Taylor, A. Berezovskaya, I. Grass, B. R. Blazar, and L. M. Nadler. 2000. p27kip1 functions as an anergy factor inhibiting interleukin 2 transcription and clonal expansion of alloreactive human and mouse helper T lymphocytes. *Nat Med 6:290.*
269. Jackson, S. K., A. DeLoose, and K. M. Gilbert. 2001. Induction of anergy in Th1 cells associated with increased levels of cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1. *J Immunol 166:952.*
270. Murphy, K. M., A. B. Heimberger, and D. Y. Loh. 1990. Induction by antigen of intrathymic apoptosis of CD4+CD8+TCRlo thymocytes in vivo. *Science 250:1720.*
271. Hogquist, K. A., S. C. Jameson, W. R. Heath, J. L. Howard, M. J. Bevan, and F. R. Carbone. 1994. T cell receptor antagonist peptides induce positive selection. *Cell 76:17.*
272. Hecht, T. T., D. L. Longo, and L. A. Matis. 1983. The relationship between immune interferon production and proliferation in antigen-specific, MHC-restricted T cell lines and clones. *J Immunol 131:1049.*
273. Leo, O., M. Foo, D. H. Sachs, L. E. Samelson, and J. A. Bluestone. 1987. Identification of a monoclonal antibody specific for a murine T3 polypeptide. *Proc Natl Acad Sci U S A 84:1374.*

274. Gross, J. A., T. St John, and J. P. Allison. 1990. The murine homologue of the T lymphocyte antigen CD28. Molecular cloning and cell surface expression. *J Immunol 144:3201.*
275. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227:680.*
276. Bellone, M., D. Cantarella, P. Castiglioni, M. C. Crosti, A. Ronchetti, M. Moro, M. P. Garancini, G. Casorati, and P. Dellabona. 2000. Relevance of the tumor antigen in the validation of three vaccination strategies for melanoma. *J Immunol 165:2651.*
277. Karre, K., H. G. Ljunggren, G. Piontek, and R. Kiessling. 1986. Selective rejection of H-2-deficient lymphoma variants suggests alternative immune defence strategy. *Nature 319:675.*
278. Hobbs, M. V., W. O. Weigle, D. J. Noonan, B. E. Torbett, R. J. McEvilly, R. J. Koch, G. J. Cardenas, and D. N. Ernst. 1993. Patterns of cytokine gene expression by CD4+ T cells from young and old mice. *J Immunol 150:3602.*
279. Guidotti, L. G., and F. V. Chisari. 1999. Cytokine-induced viral purging--role in viral pathogenesis. *Curr Opin Microbiol 2:388.*
280. Sitia, G., M. Isogawa, K. Kakimi, S. F. Wieland, F. V. Chisari, and L. G. Guidotti. 2002. Depletion of neutrophils blocks the recruitment of antigen-nonspecific cells into the liver without affecting the antiviral activity of hepatitis B virus-specific cytotoxic T lymphocytes. *Proc Natl Acad Sci U S A 99:13717.*
281. Lyons, A. B., and C. R. Parish. 1994. Determination of lymphocyte division by flow cytometry. *J Immunol Methods 171:131.*
282. Malek, T. R., A. Yu, P. Scibelli, M. G. Lichtenheld, and E. K. Codias. 2001. Broad programming by IL-2 receptor signaling for extended growth to multiple cytokines and functional maturation of antigen-activated T cells. *J Immunol 166:1675.*
283. Wells, A. D., M. C. Walsh, D. Sankaran, and L. A. Turka. 2000. T cell effector function and anergy avoidance are quantitatively linked to cell division. *J Immunol 165:2432.*
284. Boussiotis, V. A., D. L. Barber, T. Nakarai, G. J. Freeman, J. G. Gribben, G. M. Bernstein, A. D. D'Andrea, J. Ritz, and L. M. Nadler. 1994. Prevention of T cell anergy by signaling through the gamma c chain of the IL-2 receptor. *Science 266:1039.*
285. Terada, N., K. Takase, P. Papst, A. C. Nairn, and E. W. Gelfand. 1995. Rapamycin inhibits ribosomal protein synthesis and induces G1 prolongation in mitogen-activated T lymphocytes. *J Immunol 155:3418.*
286. Pullen, N., and G. Thomas. 1997. The modular phosphorylation and activation of p70s6k. *FEBS Lett 410:78.*
287. Pearson, R. B., P. B. Dennis, J. W. Han, N. A. Williamson, S. C. Kozma, R. E. Wettenhall, and G. Thomas. 1995. The principal target of rapamycin-induced p70s6k inactivation is a novel phosphorylation site within a conserved hydrophobic domain. *Embo J 14:5279.*
288. Dufner, A., and G. Thomas. 1999. Ribosomal S6 kinase signaling and the control of translation. *Exp Cell Res 253:100.*

289. Guy, G. R., R. Philip, and Y. H. Tan. 1994. Analysis of cellular phosphoproteins by two-dimensional gel electrophoresis: applications for cell signaling in normal and cancer cells. *Electrophoresis 15:417.*
290. Vanasek, T. L., A. Khoruts, T. Zell, and D. L. Mueller. 2001. Antagonistic roles for CTLA-4 and the mammalian target of rapamycin in the regulation of clonal anergy: enhanced cell cycle progression promotes recall antigen responsiveness. *J Immunol 167:5636.*
291. Murphy, K. M., W. Ouyang, J. D. Farrar, J. Yang, S. Ranganath, H. Asnagli, M. Afkarian, and T. L. Murphy. 2000. Signaling and transcription in T helper development. *Annu Rev Immunol 18:451.*
292. Murphy, K. M., and S. L. Reiner. 2002. The lineage decisions of helper T cells. *Nat Rev Immunol 2:933.*
293. Le Gros, G., S. Z. Ben-Sasson, R. Seder, F. D. Finkelman, and W. E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med 172:921.*
294. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
295. Zhu, J., J. Cote-Sierra, L. Guo, and W. E. Paul. 2003. Stat5 activation plays a critical role in Th2 differentiation. *Immunity 19:739.*
296. Leonard, W. J., and J. X. Lin. 2000. Cytokine receptor signaling pathways. *J Allergy Clin Immunol 105:877.*
297. Powell, J. D., D. Bruniquel, and R. H. Schwartz. 2001. TCR engagement in the absence of cell cycle progression leads to T cell anergy independent of p27(Kip1). *Eur J Immunol 31:3737.*
298. Kneitz, B., T. Herrmann, S. Yonehara, and A. Schimpl. 1995. Normal clonal expansion but impaired Fas-mediated cell death and anergy induction in interleukin-2-deficient mice. *Eur J Immunol 25:2572.*
299. Claret, F. X., M. Hibi, S. Dhut, T. Toda, and M. Karin. 1996. A new group of conserved coactivators that increase the specificity of AP-1 transcription factors. *Nature 383:453.*
300. Jenkins, M. K., D. M. Pardoll, J. Mizuguchi, H. Quill, and R. H. Schwartz. 1987. T-cell unresponsiveness in vivo and in vitro: fine specificity of induction and molecular characterization of the unresponsive state. *Immunol Rev 95:113.*
301. Ferrari, S., W. Bannwarth, S. J. Morley, N. F. Totty, and G. Thomas. 1992. Activation of p70s6k is associated with phosphorylation of four clustered sites displaying Ser/Thr-Pro motifs. *Proc Natl Acad Sci U S A 89:7282.*
302. Mukhopadhyay, N. K., D. J. Price, J. M. Kyriakis, S. Pelech, J. Sanghera, and J. Avruch. 1992. An array of insulin-activated, proline-directed serine/threonine protein kinases phosphorylate the p70 S6 kinase. *J Biol Chem 267:3325.*
303. Han, J. W., R. B. Pearson, P. B. Dennis, and G. Thomas. 1995. Rapamycin, wortmannin, and the methylxanthine SQ20006 inactivate p70s6k by inducing dephosphorylation of the same subset of sites. *J Biol Chem 270:21396.*
304. Weng, Q. P., K. Andrabi, A. Klippel, M. T. Kozlowski, L. T. Williams, and J. Avruch. 1995. Phosphatidylinositol 3-kinase signals activation of p70 S6

kinase in situ through site-specific p70 phosphorylation. *Proc Natl Acad Sci U S A 92:5744.*

305. Burnett, P. E., R. K. Barrow, N. A. Cohen, S. H. Snyder, and D. M. Sabatini. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. *Proc Natl Acad Sci U S A 95:1432.*
306. Isotani, S., K. Hara, C. Tokunaga, H. Inoue, J. Avruch, and K. Yonezawa. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. *J Biol Chem 274:34493.*
307. Belham, C., M. J. Comb, and J. Avruch. 2001. Identification of the NIMA family kinases NEK6/7 as regulators of the p70 ribosomal S6 kinase. *Curr Biol 11:1155.*
308. Peterson, R. T., B. N. Desai, J. S. Hardwick, and S. L. Schreiber. 1999. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycinassociated protein. *Proc Natl Acad Sci U S A 96:4438.*
309. Gingras, A. C., S. P. Gygi, B. Raught, R. D. Polakiewicz, R. T. Abraham, M. F. Hoekstra, R. Aebersold, and N. Sonenberg. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev 13:1422.*
310. Chuang, E., T. S. Fisher, R. W. Morgan, M. D. Robbins, J. M. Duerr, M. G. Vander Heiden, J. P. Gardner, J. E. Hambor, M. J. Neveu, and C. B. Thompson. 2000. The CD28 and CTLA-4 receptors associate with the serine/threonine phosphatase PP2A. *Immunity 13:313.*
311. Razi-Wolf, Z., G. A. Hollander, and H. Reiser. 1996. Activation of CD4+ T lymphocytes form interleukin 2-deficient mice by costimulatory B7 molecules. *Proc Natl Acad Sci U S A 93:2903.*
312. Abraham, R. T., and G. J. Wiederrecht. 1996. Immunopharmacology of rapamycin. *Annu Rev Immunol 14:483.*
313. Terada, N., R. A. Franklin, J. J. Lucas, J. Blenis, and E. W. Gelfand. 1993. Failure of rapamycin to block proliferation once resting cells have entered the cell cycle despite inactivation of p70 S6 kinase. *J Biol Chem 268:12062.*
314. Slavik, J. M., D. G. Lim, S. J. Burakoff, and D. A. Hafler. 2004. Rapamycin-resistant proliferation of CD8+ T cells correlates with p27kip1 down-regulation and bcl-xL induction, and is prevented by an inhibitor of phosphoinositide 3-kinase activity. *J Biol Chem 279:910.*
315. Abbas, A. K., K. M. Murphy, and A. Sher. 1996. Functional diversity of helper T lymphocytes. *Nature 383:787.*
316. Ho, I. C., and L. H. Glimcher. 2002. Transcription: tantalizing times for T cells. *Cell 109 Suppl:S109.*
317. Mosmann, T. R., H. Cherwinski, M. W. Bond, M. A. Giedlin, and R. L. Coffman. 1986. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol 136:2348.*
318. Mosmann, T. R., and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol 7:145.*
319. Sher, A., and R. L. Coffman. 1992. Regulation of immunity to parasites by T cells and T cell-derived cytokines. *Annu Rev Immunol 10:385.*

320. Constant, S. L., and K. Bottomly. 1997. Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches. *Annu Rev Immunol 15:297.*
321. Bird, J. J., D. R. Brown, A. C. Mullen, N. H. Moskowitz, M. A. Mahowald, J. R. Sider, T. F. Gajewski, C. R. Wang, and S. L. Reiner. 1998. Helper T cell differentiation is controlled by the cell cycle. *Immunity 9:229.*
322. Gett, A. V., and P. D. Hodgkin. 1998. Cell division regulates the T cell cytokine repertoire, revealing a mechanism underlying immune class regulation. *Proc Natl Acad Sci U S A 95:9488.*
323. Constant, S., C. Pfeiffer, A. Woodard, T. Pasqualini, K. Bottomly, N. Schweitzer, J. West, P. Ranney, D. Leitenberg, D. D. Lu, B. R. Smith, D. Sant'Angelo, T. Taylor, D. Levin, and R. Flavell. 1995. Extent of T cell receptor ligation can determine the functional differentiation of naive CD4+ T cells
B lymphocytes can be competent antigen-presenting cells for priming CD4+ T cells to protein antigens in vivo
CD4 and CD45 regulate qualitatively distinct patterns of calcium mobilization in individual CD4+ T cells
Peptide and protein antigens require distinct antigen-presenting cell subsets for the priming of CD4+ T cells. *J Exp Med 182:1591.*
324. Hosken, N. A., K. Shibuya, A. W. Heath, K. M. Murphy, and A. O'Garra. 1995. The effect of antigen dose on CD4+ T helper cell phenotype development in a T cell receptor-alpha beta-transgenic model. *J Exp Med 182:1579.*
325. Avni, O., and A. Rao. 2000. T cell differentiation: a mechanistic view. *Curr Opin Immunol 12:654.*
326. Ansel, K. M., D. U. Lee, and A. Rao. 2003. An epigenetic view of helper T cell differentiation. *Nat Immunol 4:616.*
327. Grogan, J. L., M. Mohrs, B. Harmon, D. A. Lacy, J. W. Sedat, and R. M. Locksley. 2001. Early transcription and silencing of cytokine genes underlie polarization of T helper cell subsets. *Immunity 14:205.*
328. Agarwal, S., and A. Rao. 1998. Modulation of chromatin structure regulates cytokine gene expression during T cell differentiation. *Immunity 9:765.*
329. Agarwal, S., J. P. Viola, and A. Rao. 1999. Chromatin-based regulatory mechanisms governing cytokine gene transcription. *J Allergy Clin Immunol 103:990.*
330. Fields, P. E., S. T. Kim, and R. A. Flavell. 2002. Cutting edge: changes in histone acetylation at the IL-4 and IFN-gamma loci accompany Th1/Th2 differentiation. *J Immunol 169:647.*
331. Rohde, J. R., and M. E. Cardenas. 2003. The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. *Mol Cell Biol 23:629.*
332. Afkarian, M., J. R. Sedy, J. Yang, N. G. Jacobson, N. Cereb, S. Y. Yang, T. L. Murphy, and K. M. Murphy. 2002. T-bet is a STAT1-induced regulator of IL-12R expression in naive CD4+ T cells. *Nat Immunol 3:549.*
333. Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul, and J. J. O'Shea. 2001. T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A 98:15137.*

334. Mullen, A. C., F. A. High, A. S. Hutchins, H. W. Lee, A. V. Villarino, D. M. Livingston, A. L. Kung, N. Cereb, T. P. Yao, S. Y. Yang, and S. L. Reiner. 2001. Role of T-bet in commitment of TH1 cells before IL-12-dependent selection. *Science 292:1907.*
335. Szabo, S. J., S. T. Kim, G. L. Costa, X. Zhang, C. G. Fathman, and L. H. Glimcher. 2000. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell 100:655.*
336. Szabo, S. J., B. M. Sullivan, C. Stemmann, A. R. Satoskar, B. P. Sleckman, and L. H. Glimcher. 2002. Distinct effects of T-bet in TH1 lineage commitment and IFN-gamma production in CD4 and CD8 T cells. *Science 295:338.*
337. Ouyang, W., S. H. Ranganath, K. Weindel, D. Bhattacharya, T. L. Murphy, W. C. Sha, and K. M. Murphy. 1998. Inhibition of Th1 development mediated by GATA-3 through an IL-4-independent mechanism. *Immunity 9:745.*
338. Ouyang, W., M. Lohning, Z. Gao, M. Assenmacher, S. Ranganath, A. Radbruch, and K. M. Murphy. 2000. Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment. *Immunity 12:27.*
339. Lee, H. J., N. Takemoto, H. Kurata, Y. Kamogawa, S. Miyatake, A. O'Garra, and N. Arai. 2000. GATA-3 induces T helper cell type 2 (Th2) cytokine expression and chromatin remodeling in committed Th1 cells. *J Exp Med 192:105.*
340. Takemoto, N., Y. Kamogawa, H. Jun Lee, H. Kurata, K. I. Arai, A. O'Garra, N. Arai, and S. Miyatake. 2000. Cutting edge: chromatin remodeling at the IL-4/IL-13 intergenic regulatory region for Th2-specific cytokine gene cluster. *J Immunol 165:6687.*
341. Usui, T., R. Nishikomori, A. Kitani, and W. Strober. 2003. GATA-3 suppresses Th1 development by downregulation of Stat4 and not through effects on IL-12Rbeta2 chain or T-bet. *Immunity 18:415.*
342. Ben-Sasson, S. Z., G. Le Gros, D. H. Conrad, F. D. Finkelman, and W. E. Paul. 1990. IL-4 production by T cells from naive donors. IL-2 is required for IL-4 production. *J Immunol 145:1127.*
343. Presky, D. H., H. Yang, L. J. Minetti, A. O. Chua, N. Nabavi, C. Y. Wu, M. K. Gately, and U. Gubler. 1996. A functional interleukin 12 receptor complex is composed of two beta-type cytokine receptor subunits. *Proc Natl Acad Sci U S A 93:14002.*
344. Sinigaglia, F., D. D'Ambrosio, P. Panina-Bordignon, and L. Rogge. 1999. Regulation of the IL-12/IL-12R axis: a critical step in T-helper cell differentiation and effector function. *Immunol Rev 170:65.*
345. Richter, A., M. Lohning, and A. Radbruch. 1999. Instruction for cytokine expression in T helper lymphocytes in relation to proliferation and cell cycle progression. *J Exp Med 190:1439.*