

Impact of combined mycophenolate mofetil and low
dose calcineurin inhibitor therapy on T cell function

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Impact of combined mycophenolate mofetil and low dose calcineurin inhibitor therapy on T cell function

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1. Introduction

1.1 Transplant immunology and mechanisms of allograft rejection

1.1.1 Central role of T lymphocytes

Although many cells can participate in the process of transplant rejection, only T lymphocytes appear to be absolutely required (Krensky et al., 1990). Mice with genetic or induced T-cell deficiency cannot reject grafts because they lack the cellular mechanisms to recognize an antigen as foreign (Sayegh and Turka, 1998).

Nature T cells express either the CD4 or the CD8 glycoprotein on their surface. The CD4 T cell, so-called helper T cell (Th cell), plays a critical role in both the initiation and the coordination of the rejection response (Krieger et al., 1996). The recipient mounts a rejection response following CD4 T cell recognition of foreign antigens derived from the allograft. These antigens are encoded predominantly by highly polymorphic loci within the major histocompatibility complex (MHC), which in human is known as the human leucocyte antigen (HLA) complex, located on the short arm of chromosome 6 (Krensky, 2000). Foreign MHC molecules are presented by both the direct and the indirect pathways to host T cells (Fig.1.1) (Denton et al., 1999). In the direct pathway, recipient T cells recognize intact allogeneic MHC (foreign MHC) molecules expressed on donor cells. In the indirect pathway, recipient antigen-presenting cells (APCs) process donor antigens intracellularly and present donor MHC-derived peptides on recipient MHC class II molecules to T cells, initiating the alloimmune response. The direct pathway may be responsible for the vigorous immune response in acute rejection, whereas indirect allorecognition may have the dominant role in chronic rejection.

A novel 'third pathway' of antigen presentation has been proposed, namely direct presentation of intact donor MHC by host APCs (Vongwiwatana et al., 2003). In this pathway, host APCs acquire membrane fragments from donor cells containing intact donor MHC molecules. These may then be presented to host T cells and/or B cells to generate an immune response. This pathway could provide the explanation for how recipient APCs can prime cytotoxic T lymphocytes (CTL) to react against intact donor MHC molecules (e.g. on epithelium), as these cells cannot be primed by the indirect

pathway.

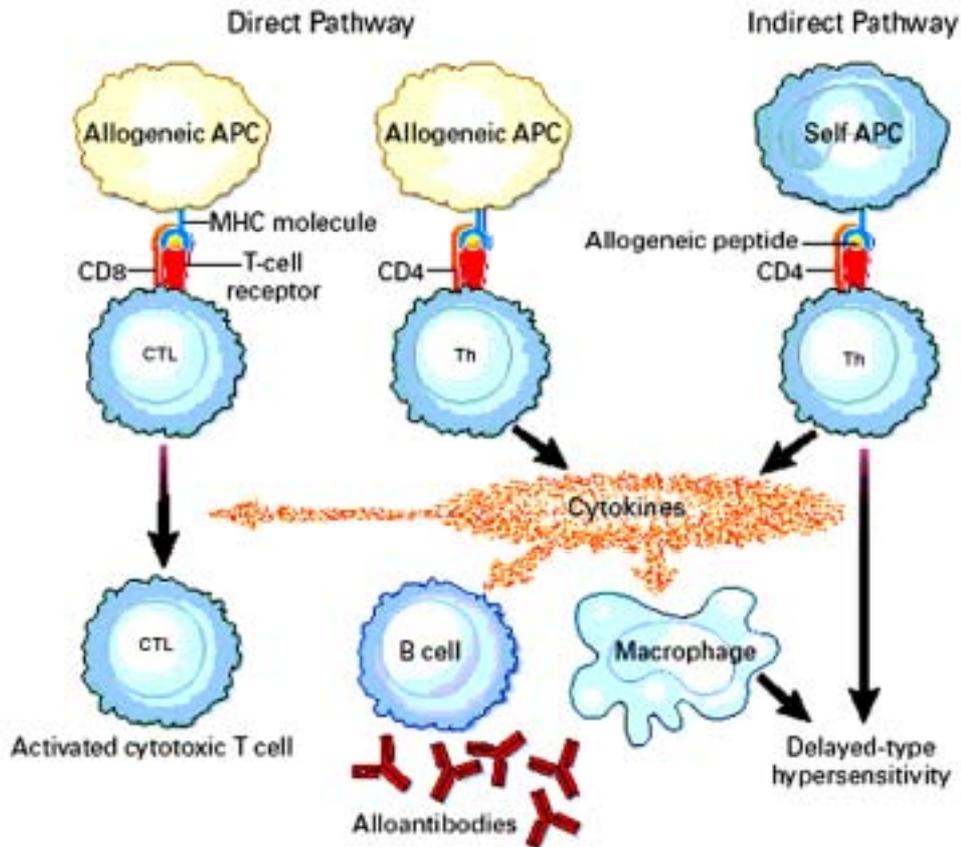


Figure 1.1 Pathways of Recognition of Allogeneic MHC Molecules and Mechanisms of Graft Rejection. Graft rejection is usually initiated by CD4 Th cell that bind peptides in complexes with MHC class II molecules on antigen-presenting cells. In the direct pathway of recognition, an MHC molecule on a foreign (allogeneic) cell, such as an antigen-presenting cell (allogeneic APCs), binds to the Th cell. In the indirect pathway, the foreign MHC molecule is processed into peptides that are presented to the Th cell by one of the body's own antigen-presenting cells (self APCs). In either case, activated CD4 Th cells proliferate and secrete a variety of cytokines that serve as growth and activation factors for CD8 T cells (CTL), B cells, and macrophages, which cause destruction of the graft by direct lysis of target cells, antibody production, and delayed-type hypersensitivity mechanisms, respectively.

Full activation of T cells requires two distinct but synergistic signals (Janeway, Jr. and Bottomly, 1994). Antigen recognition alone is insufficient to activate fully the CD4 T cell; a second (costimulatory) signal must be provided by cognate ligands on the antigen-presenting cell. When both signals are provided, the T cell secretes optimum concentrations of interleukin-2 (IL-2) and other cytokines, thus permitting the development of an expanded population of mature effector cells (T cells, B cells,

and macrophages) capable of mediating a sustained immune response. In the absence of the second signal, the CD4 T cell becomes unresponsive to further exposure to antigen, fails to secrete cytokines, and may undergo programmed cell death (apoptosis). The best-characterized co-stimulatory signal is provided by ligation of CD28 on the surface of the CD4 T cell with a member of the B7 family of molecules (B7-1 or B7-2) on the APCs (Krensky, 2000). CD40-CD154 interactions provide another important co-stimulatory pathway between activated T cells, B cells, APCs and endothelial cells (Figure 1.2). CD40-CD154 interaction plays an important role not only in acute graft rejection but also in chronic rejection (Kishimoto et al., 2000). The combined blockade of the CD40-CD154 and CD28-B7 pathways has been shown to be particularly effective in allo- (Larsen et al., 1996) as well as xeno-transplant models (Elwood et al., 1998). Moreover, this combination therapy induced long term rejection free renal allograft survival in non-human primates (Kirk et al., 1997). Several 'novel' co-stimulatory molecules were described recently (Sharpe and Freeman, 2002; Coyle and Gutierrez-Ramos, 2001; Yamada et al., 2002; Salama and Sayegh, 2003). Structurally, the novel co-stimulatory molecules are related either to CD28-B7 or to CD40-CD154. They differ in the timing and distribution of their expression and in delivery of positive or negative signals to T cell and B cells; they have both overlapping and distinct roles. The novel co-stimulatory molecules differentially affect proliferation, differentiation, survival, and functional repertoire. Thus, the initial notion that co-stimulatory molecules play a stimulatory role required for prevention of anergy has had to undergo revision. Indeed, it is the integration of positive and negative co-stimulatory signals that ultimately determines the outcome of the T-cell response (Rothstein and Sayegh, 2003).

Alloactivated CD4 T cells subsequently interact with effector cells of the rejection response via direct cell-cell contact and cytokine secretion. By an increase in the activation and function of B cells, CTL and monocyte/macrophages, alloactivated CD4 T cells promote alloantibody production, antigen-specific cell lysis, and delayed type hypersensitivity responses, respectively. These effector mechanisms ultimately result in graft destruction.

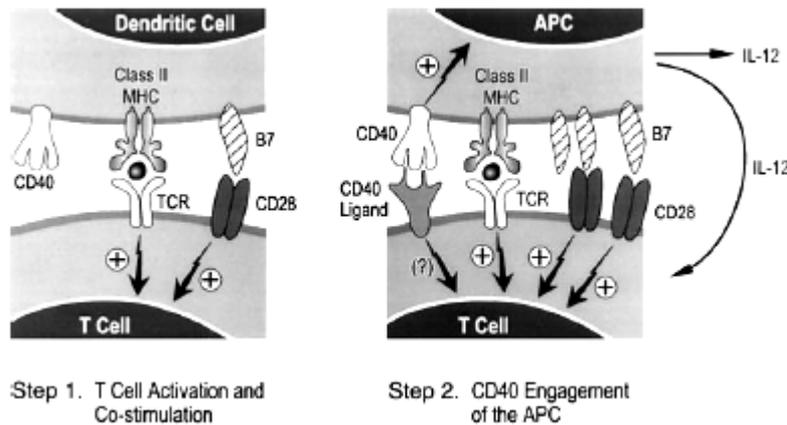


Figure 1.2 Costimulatory pathways for T-cell activation. Dendritic cells constitutively express B7 and CD40 cell surface molecules. Specific triggering of the T-cell receptor (TCR) on naive T cells occurs in the context of co-stimulatory signals via B7→CD28 engagement leads to T-cell activation. In a second step, activated T cells express CD154 (CD40 ligand, CD40L) on their cell surface. Interaction of CD40 on the APCs and CD154 on the T cell activates IL-12 production by the APCs.

Following the interaction of TCR and co-stimulatory molecules with their respective ligands, T-cell activation begins. Various kinases are activated at or near the cell surface and intracellular calcium levels rise. These events then activate signal transduction cascades which result in the transcription of new genes in the nucleus. As new genes are expressed, the T cell divides (proliferates) and differentiates. In parallel, a calcium-dependent pathway involving the phosphatase calcineurin provides access for cytoplasmic components of the nuclear factor of activated T cells, NFAT, to enter the nucleus. These factors bind in a combinatorial fashion to promoters/enhancers of T lymphocytes and result in transcription of new gene products. Transcription of IL-2, a T-cell growth factor, is well characterized and is important in understanding the mechanism of action of the widely used immunosuppressive drugs such as cyclosporine (CsA) and tacrolimus (Tac).

Extensive data from animal studies indicate that alloantigen-activated T cells play a key role in initiating the process of chronic rejection. It is hypothesized that activated CD4 T cells produce key cytokines which activate macrophages and B cells. Chemoattractants further recruit circulating mononuclear cells to the graft, which

along with endothelial cells, secrete growth factors that cause smooth muscle cell proliferation leading to vascular intimal occlusion and interstitial fibrosis (Womer et al., 2000)

1.1.2 B lymphocytes

B-cell responses against membrane MHC antigens begin with MHC binding to the B-cell receptor (BCR) of alloreactive B Cells, leading to activation as well as antigen uptake and processing. MHC molecules are generally found on the membrane of donor cells, but they can also be released as a soluble protein or on membrane vesicles. B-cell activation will not lead, however, to an effective antibody response unless help is provided by Th cells (Vongwiwatana et al., 2003).

As well as initiating signal transduction cascades, BCR crosslinking will also lead to internalization of the antigen. Antigen bound by B cells is internalized and processed into peptides to be presented on MHC class II molecules on the surface. MHC class II is expressed on the surface along with co-stimulatory molecules important in the function of B cells as APCs. Recognition of the presented peptide by a Th cell leads to a conjugate between the two cells (Figure 1.2). Cytokines are released from the Th cell, and interactions between co-stimulatory molecules allow for clonal expansion, diversification, and affinity maturation of the responding B cells. Following affinity maturation, B cells expressing antibodies with high affinity for the specified antigen can become memory B cells or antibody-producing plasma cells.

When a T cell finds an antigen-specific B-cell match, it will provide the required cytokines and co-stimulatory signals for antibody production. Antigen-activated B cells express B7 and CD40 and activated T cells express CD154. CD40-CD154 interactions are required for effective antibody responses (Figure 1.2).

1.2 Immunosuppressive therapy in solid organ transplantation

1.2.1 Mechanisms of action of immunosuppressive drugs

During the past 50 years, many immunosuppressive drugs have been described. These drugs now used fall into five groups with different mechanisms of action (Allison, 2000). a) Regulation of gene expression. The classical examples are the

glucocorticoids, which directly or indirectly regulate the expression of many genes. b) Alkylation agents. An example is cyclophosphamide. c) Kinase and phosphatase inhibitors. CsA and Tac not only inhibit the phosphatase activity of calcineurin (they are also considered calcineurin inhibitors) but also the JNK and p38 cascades linked to the T-cell receptor (Matsuda and Koyasu, 2000). Rapamycin inhibits a kinase cascade. d) Inhibition of de novo purine synthesis. The first generation included azathioprine (AZA), which effects are broad rather than selective (Allison, 2000). The second generation included mycophenolate mofetil (MMF). e) Inhibition of *de novo* pyrimidine synthesis. For example, leflunomide can suppress lymphocyte proliferation by inhibition of dihydrorotate dehydrogenase.

CsA, Tac and MMF are discussed in detail here.

1.2.1.1 Mechanism of action of cyclosporine (CsA)

CsA, a neutral lipophilic cyclic undecapeptide isolated from the fungus *Hypocladium inflatum gams*, has been widely used for the treatment of allograft rejection since Borel et al (Borel et al., 1976) reported its immunosuppressive activity. In the cytoplasm, CsA binds to its immunophilin, cyclophilin (CpN), forming a complex between cyclosporine and CpN. The cyclosporine–CpN complex binds and blocks the function of the enzyme calcineurin (CaN), which has a serine-threonine phosphatase activity. Inhibition of the action of calcineurin results in a complete block in the translocation of the cytosolic component of the NFAT, resulting in a failure to activate the genes regulated by the NFAT transcription factor (Figure 1.3). These genes include those necessary for T-cell proliferation such as IL-2, for the activation of macrophages such as interferon gamma (IFN- γ), as well as those required for B-cell help such as IL-4 and CD-154 (Ho et al., 1996).

1.2.1.2 Mechanism of action of tacrolimus (Tac)

Tac, the macrolide product of the bacterium *Streptomyces tsukubaensis*, inhibits IL-2 production by T cells in a fashion that is similar to that of CsA. After Tac forms a complex with FK-binding proteins (FKBP), the complex inhibits the phosphatase

activity of calcineurin (Figure 1.3), thereby blocking the activity of the cytoplasmic component of NFAT (Fung, 2004).

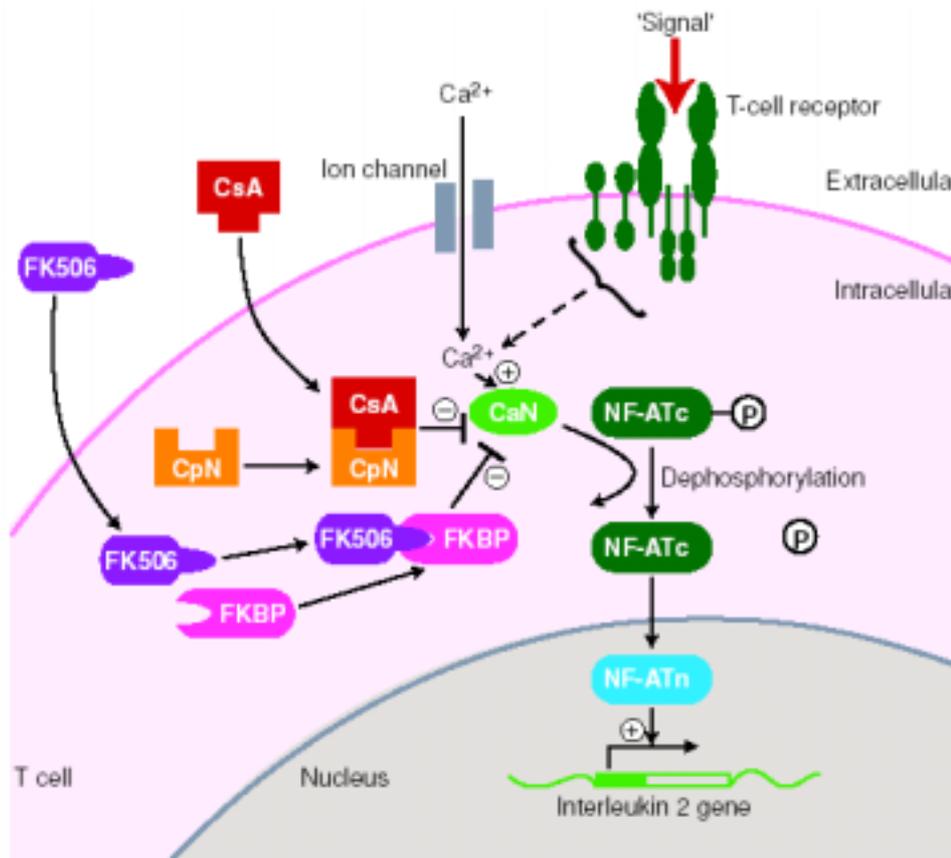


Figure 1.3 Mechanism of action of CsA or Tac (FK506)

1.2.1.3 Mechanism of action of Mycophenolate mofetil (MMF)

MMF is a prodrug of mycophenolic acid (MPA), an inhibitor of inosine monophosphate dehydrogenase (IMPDH). This is the rate-limiting enzyme in *de novo* synthesis of guanosine nucleotides (Figure 1.4). T- and B-lymphocytes are more dependent on this pathway than other cell types. Moreover, MPA is a fivefold more potent inhibitor of the type II isoform of IMPDH, which is expressed in activated lymphocytes, than of the type I isoform of IMPDH, which is expressed in most cell types. MPA has therefore a more potent cytostatic effect on lymphocytes than on other cell types. IMPDH type II enzyme knocked out mouse demonstrate significantly decreased lymphocyte responsiveness to stimulation with anti-CD3 and anti-CD28 antibodies indicating that a moderate decrease in the ability of murine lymphocytes to synthesize guanine nucleotides during stimulation results in significant impairment in

T-cell activation and function (Gu et al., 2000). In liver, MMF is converted by ester hydrolysis to its active metabolite, MPA, which non-competitively and reversibly inhibits the activity of IMPDH during DNA synthesis in the S phase of the cell cycle. This is the principal mechanism by which MPA exerts immunosuppressive effects (Allison and Eugui, 2000). MPA can also induce apoptosis of activated T-lymphocytes, which may eliminate clones of cells responding to antigenic stimulation.

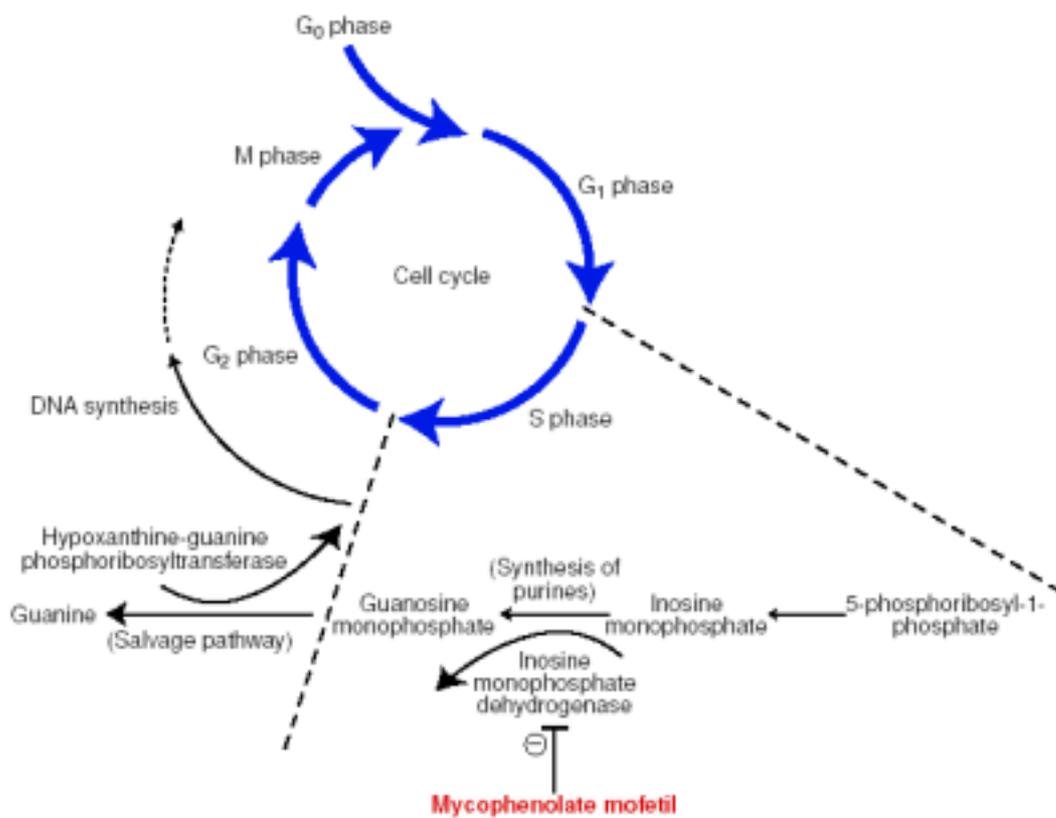


Figure 1.4. Mechanism of action of mycophenolate mofetil

1.2.2 Immunosuppressive agents in clinical transplantation

The goal of immunosuppression in solid organ transplantation is to blunt the immune response of the patient to the allograft, while maintaining sufficient resistance to avoid opportunistic infections and malignancy. Despite progress in this field, rejection processes, particularly of the chronic form, remain an important cause of morbidity and graft loss (Kilic and Kahan, 2000).

Because most of new immunosuppressants differ in their mechanisms of action, and because the toxicities are mechanism-based, the wide array of new drugs offers the opportunity to use combinations that block different pathways of immune activation while at the same time selecting combinations with non-overlapping toxicity profiles so that doses of each drug can be reduced below toxic levels (Gummert et al., 1999b).

1.2.2.1 Application of calcineurin inhibitors (CNI) in clinical transplantation and their side effects

CNI are currently the most of used immunosuppressive regimens in clinical organ transplantation. CNI prevents IL-2 gene transcription, thereby inhibiting T cell IL-2 production. The introduction of CsA proved an important advance in immunosuppressive therapy and increased 1-year graft survival for cadaveric kidney allografts by 10%. In maintenance immunosuppression therapy, CsA was the most-used CNI in solid organ transplantation until the introduction of Tac around 1994 (Kaufman et al., 2004). A switch from CsA to Tac occurred for transplants performed in 1995 for liver and in 2001 for kidney. The reasons for this conversion are most likely related to multicenter trial data that have suggested lower rates of acute rejection and lower rates of steroid resistant rejection associated with Tac (Johnson et al., 2000; Pirsch et al., 1997). Tac is widely used in liver transplantation, in which its greater water solubility and lesser dependence on bile salt absorption results in improved bioavailability over CsA (Denton et al., 1999). Both drugs can cause multiple non-immunological adverse events. The most common side-effect of CNI is nephrotoxicity (Klintmalm and Gonwa, 1995; Morales et al., 2001; Mor et al., 1994) although the progression of renal insufficiency after liver transplantation is slow (Braun et al., 2003). The incidence of end-stage renal disease requiring renal replacement therapy ranges between 0.8% and 9.5% after liver transplantation (Gonwa et al., 1991; Platz et al., 1994; Gonwa et al., 2001). Another study shows that by 13 years after liver transplantation, patients with end-stage renal disease had a 28.2% survival rate compared with 54.6% of the recipients that did not develop

end-stage renal disease (Gonwa et al., 2001). The other side effects include neurological disorders, arterial hypertension and metabolic disorders (Rabkin et al., 2002). Recent studies have also increased the understanding of mechanisms by which the nephrotoxicity and other side effects of CsA are mediated. Hemodynamic effects of CsA include reversible, dose-related renal vasoconstriction with reduced glomerular filtration rate and systemic hypertension. Endothelin-1 (ET-1) has been implicated in these effects. CsA has been shown to augment the action of endothelin-converting enzyme, and the production of ET-1, by endothelial cells. ET-1 is also believed to contribute to transplant vasculopathy and chronic rejection. CsA treatment can also lead to tubular interstitial fibrosis in the kidney, an effect associated with increased expression of transforming growth factor- β (TGF- β) (Han et al., 1995). CsA can increase the expression of pro-apoptotic genes in the kidney (*p53*, *Bax*, *Fal-L*) and decrease the expression of the survival gene *Bcl-2*. This is one mechanism leading to drop-out of tubular cells. Tac has shown efficacy similar to that of CsA, but also the nephrotoxicity and some other side effects of CsA (Allison, 2000).

For these undesirable effects immunosuppressive alternatives to CNI drugs have begun to emerge during the past few years (Flechner, 2003). Minimizing CNI drugs has been considered at various time points post-transplant, and can be described in several ways. The recent introduction of the potent immunosuppressive agent such as MMF represents an opportunity to develop a regimen designed to minimize drug-induced nephrotoxicity (Flechner, 2003).

1.2.2.2 MMF and immunosuppressive drug combinations in solid transplantation

As an immunosuppressive agent, MMF was introduced in 1982 and now approved for use in many countries to prevent allograft rejection and to treat ongoing rejection (Allison and Eugui, 2000; Mele and Halloran, 2000). MMF has become an important therapeutic tool in the transplant field. Randomized clinical trials have shown that MMF, when used with CsA and steroids, reduces the frequency and severity of acute rejection episodes in kidney and heart transplants, improves patient and graft survival in heart allograft recipients and increases renal allograft survival at 3 years (Mele and

Halloran, 2000). It has also been effective in reversing acute and resistant rejection episodes in heart, kidney and liver recipients. The ability of MMF to facilitate sparing of other immunosuppressive agents, particularly in CsA-related nephrotoxicity, is also promising. By permitting reduction in CsA dose, MMF may stabilize or improve renal graft function in patients with CsA-related nephrotoxicity or chronic allograft nephropathy. MMF also shows more immunosuppressive potency than AZA, another purine synthesis inhibitor used in clinic (Fischer et al., 2000; Taylor et al., 1999; Vincenti, 2004).

In renal transplantation, CsA withdrawal was associated with a significant risk of acute rejection and subsequent chronic rejection (Offermann, 2004; Smak Gregoor et al., 2002). Evidence has accumulated that MMF, administered to human organ graft recipients alone or with a low dose of CNI, can decrease the incidence of acute rejection (Triemer et al., 2000) and improve renal function (Houde et al., 2000; Francois et al., 2003). MMF may prevent chronic rejection by decreasing the recruitment of lymphocytes and monocytes into grafted organs and suppressing the proliferation of arterial smooth muscle cells (Allison, 2002). Addition of MMF to a CsA-based immunosuppressive regimen also significantly increased the safety of steroid withdrawal in clinical study (Hocker et al., 2004). The combination of Tac and MMF may be superior to Tac and sirolimus in terms of improved renal function and lower risk of hypertension and hyperlipidemia (Vincenti, 2004).

In liver transplantation, introduction of MMF as monotherapy resulted in an improvement in renal function, even when introduced several years after transplantation (Moreno et al., 2003b; Papatheodoridis et al., 1999; Raimondo et al., 2003; Moreno et al., 2003a). However, some reports demonstrated that the risk of acute rejection may increase with an incidence ranging between 18% and 20% (Schlitt et al., 2001; Stewart et al., 2001). Only in some selected patients MMF monotherapy seems to be safe and allows a significant improvement in renal function (Papatheodoridis et al., 1999).

Recently, the effectiveness and safety of a regimen with combination of minimal CsA and standard MMF doses in long-term liver transplant recipients was evaluated

(Cantarovich et al., 2003; Koch et al., 2004; Pfitzmann et al., 2002b; Bunchman et al., 2001) and the results are encouraging (Pfitzmann et al., 2003; Hodge et al., 2002). Similar findings were noticed in heart transplant recipients (Baryalei et al., 2003). MMF also was found to be effective in controlling ongoing chronic rejection in liver allografts under CNI based immunosuppression (Daly et al., 2002; Pfitzmann et al., 2002a). However, the benefit in renal function still has to be balanced with a risk of acute rejection in some patients, although low dose of CNI were remained. Therefore, careful follow-up of these patients is mandatory. Future studies should determine whether the risk of acute rejection could be minimized by therapeutic drug monitoring of immunosuppressive drugs, by other immunosuppressive combinations (Cantarovich et al., 2003) or by monitoring the immune status of recipients.

1.3 Immunologic monitoring of human T lymphocyte function

T lymphocytes play a critical role in the regulation of immune responses, and are responsible for mediating many of the effector mechanisms of the immune system. For this reason, there has always been a need for assays to measure accurately the activity of populations of T cells. Assessing the quantitative and qualitative nature of a T cell response following immunosuppressive therapy can provide valuable information about the efficacy of a treatment, in place of a clinical endpoint. The measurement of blood concentrations of immunosuppressants provides only an indirect evaluation of the degree of immunosuppression attained in an individual patient (Yatscoff et al., 1998; Dambrin et al., 2000). When combined treatments are used the biological impact may be higher than that predicted by the individual blood concentrations of each immunosuppressant. Thus the measurement of several markers has been proposed to assess the impact of immunosuppressants on the immune systems of individual patients (Millan et al., 2003).

1.3.1 Lymphocyte proliferation assays using carboxfluorescein diacetate succinimidyl ester (CFSE)

The measurement of the proliferation of lymphocytes that occurs following stimuli is a fundamental technique for assaying T-cell responses. Over 30 years, antigen-specific lymphocyte proliferation has been measured by the incorporation of ³H-thymidine into the DNA of cells in S phase by pulsing for 6-18 h at the end of an *in vitro* culture period (Piguet and Vassalli, 1973). The level of ³H-thymidine incorporation correlates directly with the number of cells that synthesis DNA during the pulse period. Although incorporation of ³H-thymidine provides a good correlated of T-cell division, the ³H-thymidine assay does not provide any information on the phenotype of the proliferating cells in heterogeneous cell populations such as PBMC (Mannering et al., 2003). Furthermore, because ³H-thymidine incorporation measures DNA synthesis during a short pulse at the end of a 3-7-day culture period, the fate of cells that have proliferated cannot be determined. It is well known that proliferation in response to antigen is followed by a wave of apoptosis (Renno et al., 1999; Mannering et al., 2002; Marin et al., 2003). T cells that exhibit a vigorous response by ³H-thymidine incorporation may be destined to die by apoptosis a few days later. Hence, the response measured by ³H-thymidine may overestimate the magnitude of the T-cell response, by assaying at the peak of DNA synthesis (Mannering et al., 2003).

The technique for analyzing cell division using serial dilution of the fluorescein-based dye CFSE was introduced in 1994 by Lyons and Parish (Lyons, 1999) and has become widely used around the world for investigating cell division-linked differentiation of lymphocytes and for the investigation of kinetics of proliferation during immune responses (Lyons, 1999). CFSE consists of a fluorescein molecule containing two acetate moieties and a succinimidyl ester functional group. In this form, it is membrane permeant and non-fluorescent. After diffusion into the intracellular environment, endogenous esterases remove the acetate groups, rendering the molecule highly fluorescent and non-permeant to the cell membrane. Cell division can be tracked by detecting the progressive halving of the

fluorescence intensity of the dye in cells after each division (Parish, 1999). It can be used both *in vitro* and *in vivo*, allowing 8 to 10 successive divisions to be resolved by flow cytometry. One of the major advantages of the CFSE division technique is that dividing cells can be identified in complex mixtures by immunophenotyping with appropriately conjugated monoclonal antibodies (Lyons, 2000).

1.3.2 Analysis of expression of activation surface markers of T cells by flow cytometry

Analysis of the expression of activation markers on the surface of T cells by flow cytometry has also been investigated as a method for evaluating T cell proliferation and function. Cell surface markers (molecules) that have been used include: CD25, CD69, CD71, CD154 and HLA-DR (a subtype of human MHC class II molecule), all of which are upregulated following T cell activation.

1.3.3 Measurement of cytokine production by T cells

Th cells can be subdivided into Th1 and Th2 populations, based on the types of cytokines that are produced following antigen-induced activation. Th1 cells and the pathway they dominate are heavily reliant on IFN- γ , and to a lesser extent IL-2. Th2 cells are most heavily reliant on IL-4 and sometimes IL-5. It can be valuable to know the relative proportions of these two populations of cells that are circulating *in vivo* during immunosuppressive therapy. Cytokine levels in body fluids can be measured directly; alternatively, populations of T cells can be stimulated and cultured *in vitro*, and the quantities of cytokines that are produced can be determined.

1.3.3.1 Assessment of cytokine production by flow cytometry

The most simply means to estimate Th1 and Th2 T-cell responses at the level of total cell population are to culture PBMCs or T cells *in vitro* in the presence of the appropriate antigen or stimulus. After a specified period of time, the supernatant from these cultures is removed for testing and assayed for the presence of cytokines, that are indicative of the Th1 and Th2 subsets respectively, using an immunoassay such as

enzyme-linked immunosorbent assay (ELISA). The cytometric Bead Array (CBA) from BD Pharmingen is a flow cytometric-based assay for the detection of cytokines from cell supernatants or serum samples. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The advantage of using the CBA assay is that it is an accurate and sensitive method for measuring 6 to 8 kinds of cytokine levels at one time. In addition, it requires small volumes for analysis.

1.3.3.2 Measurement of cytokine mRNA expression by real-time RT PCR

Assaying cytokine production at the level of the total cell population has the advantage that the cytokines are produced in measurable amounts. However, the amount of cytokine measured might not accurately reflect the total quantity of cytokine produced, because some of the cytokine might have bound to, and been 'used up' by cells that are present in the culture. One solution to this problem is to measure cytokine messenger RNA (mRNA) levels, which can be done quantitatively using competitive polymerase chain reaction (PCR) assays. The interest in a quantitative detection of cytokine mRNA profiles has increased substantially in recent years (Hartel et al., 2001).

Real-time quantitative reverse transcriptase PCR (RT-PCR), which is the latest innovation in the field of PCR technology, is becoming widely used and provides a sensitive, reproducible, and accurate method for determining mRNA cytokine levels in tissues or cells (Giulietti et al., 2001; Overbergh et al., 2003). This method is based on the detection of a fluorescent signal produced and monitored during the amplification process, without the need for post-PCR processing.

1.4 Aim of this work

The increasing number of more selective immunosuppressive agents available for organ transplantation has the potential to be of great benefit. Typically, newer agents are used as adjuncts in a regimen based on corticosteroids and either CsA or Tac. The initial trials of agents such as MMF and sirolimus showed clinical efficacy when used

as adjuncts. However, limited data are available about the changes of immunological function after alternative treatment strategy. It is difficult to optimize the regimens in general or for specific subgroups using large clinical trials with outcome endpoints for each of the many possible combinations. In particular, it is unclear whether it is preferable to maximize the dose of one agent (e.g. CNI), or to add a second agent (e.g. MMF) to a moderate dose of the first agent to optimize immunosuppression. Lower doses of multiple agents may minimize side effects associated with any single agent. A surrogate method of measuring immunosuppressive effects in patients and *in vitro* is desirable to determine the response to new agents and to prevent the complications of over- or under-immunosuppression (Ogawa et al., 2001).

Therefore, in this study, we investigated the impact of combined MMF and low dose of CNI therapy on T cell function *in vivo* and *in vitro*.

2. Materials and methods

2.1 Materials

Immunosuppressive agents

Mycophenolic acid (MPA) (Roche Bioscience, USA)

CsA (LC Laboratories, USA, Cat. No.C-6000)

Tac (FK506) (LC Laboratories, USA, Cat. No.F-4900)

Reagents for PBMC isolation and culture

Ficoll Separating Solution (Biochrom AG, Germany, Cat.No. L6115)

RPMI 1640 (PAA Laboratories, Austria, Cat. No. E15-039)

L-glutamine (PAA Laboratories, Austria, Cat. No.M11-004)

Penicillin/Streptomycin 100 x (PAA Laboratories, Austria, Cat. No. 011-010)

Fetal bovine serum (FBS) (PAA Laboratories, Austria, A15-043)

Dulbecco's Phosphate-buffered saline (DPBS) (PAA Laboratories, Austria, Cat.
No. H15-002)

Bovine Serum Albumin Fraction V (Roche Diagnostics GmbH, Germany, Cat.
No. 735 078)

Antibodies for T cell stimulation

anti-human CD3 (clone HIT3 a, BD Pharmingen, 555336)

anti-human CD28 (clone CD28.2, BD Pharmingen, 555725),

Reagents and kits for cell proliferation and cell cycle assay

CFSE (Molecular Probes, USA, Cat. No. C-1157).

Dimethylsulfoxide (DMSO) (Sigma Aldrich, Germany, Cat.No. D2650)

FITC BrdU Flow Kits (BD Pharmingen, Cat. No.559619)

Cytokine assay kit

BD™ Cytometric Bead Array (CBA) Human Th1/Th2 Cytokine Kit (BD
Pharmingen, 550749)

Antibodies for surface antigen analysis

Specificity	Clone	Conjugation	Source	Cat. No
CD45RA	HI100	FITC	BD pharMingen	555488
CD25	M-A251	FITC	BD pharMingen	555431
CD30	BerH8	FITC	BD pharMingen	555829
CD62L	Dreg 56	FITC	BD pharMingen	555543
CD71	M-A712	FITC	BD pharMingen	555536
CD4	RPA-T4	FITC	BD pharMingen	555346
CD14	M5E2	FITC	BD pharMingen	555397
Anti-TCR	B1	FITC	BD pharMingen	559878
HLA-DR,DP,DQ	TU39	FITC	BD pharMingen	555558
CD3	SK7	FITC	BD Biosciences	345764
CD45RO	UCHL1	PE	BD pharMingen	555493
CD28	CD28.2	PE	BD pharMingen	555729
CD38	HIT2	PE	BD pharMingen	555460
CD69	FN50	PE	BD pharMingen	555531
CD154	TRAP1	PE	BD pharMingen	555700
CD80	L307.4	PE	BD pharMingen	557227
CD103	Ber-ACT8	PE	BD pharMingen	550260
CD134	ACT35	PE	BD pharMingen	555838
CD27	M-T271	PE	BD pharMingen	555441
CD56	B159	PE	BD pharMingen	555516
CD122	Mik-b2	PE	BD pharMingen	554522
CD3	UCHT1	PerCP-Cy5.5	BD pharMingen	555334
CD56	B159	PerCP-Cy5.6	BD pharMingen	555517
CD8	RPA-T8	APC	BD pharMingen	555369
CD4	RPA-T4	APC	BD pharMingen	555349
CD45	HI30	APC	BD pharMingen	555485
CD95	B159	APC	BD pharMingen	558814
CD19	HIB19	FITC	BD pharMingen	555412

Chemicals and kit for real time RT-PCR

Isopropyl alcohol (Sigma Aldrich, Germany, Cat. No I-9516)

Chloroform (Sigma Aldrich, Germany, Cat. No. C-2432)

DEPC-treated water (Ambion, Germany, Cat. No.9920)

Ethanol (SERVA, Germany, Cat. No.11903)

TRIzol Reagent (Invitrogen, USA, Cat. No.115596-018)

TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems, USA,
Cat.No. 4309169)

Stock solution

10mM CFSE stock solution

CFSE	25 mg
DMSO	4.5 mL

Aliquot and store at -20° C

1mg/mL Anti-human CD3 antibody stock solution

Anti-human CD3 antibody	0.5 mg
Sterile PBS	0.5 mL

Store at 4° C.

1mg/mL Anti-human CD28 antibody stock solution

Anti-human CD28 antibody	0.5 mg
Sterile PBS	0.5 mL

Store at 4°C.

10mg/mL MPA stock solution

Mycophenolic acid	10 mg
DMSO	1.0 mL

Store at 4 °C

50mg/mL CsA stock solution

CsA	100 mg
DMSO	2 mL

Store at -20 °C

10mg/mL Tac stock solution

Tac	10 mg
DMSO	1 mL

Store at -20 °C

PBS containing 5% (v/v) heat-inactivated FBS

DPBS	475 mL
Heat-inactivated FBS	25 mL

Culture Medium

RPMI-1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin , 100 µg/mL streptomycin and 2 mM L-glutamine.

RPMI-1640 medium	450 mL
Heat-inactivated FBS	50 mL
200mM L-glutamine	5 mL
100 x Penicillin/streptomycin cocktail	5 mL

Buffers

Staining/wash buffer:

PBS	500 mL
BSA	0.5 g
10% sodium azide	5 mL
Store at 4°C	

Fixation buffer for FACS analysis

PBS	500 mL
Paraformaldehyde	10 g
Store at 4°C	

2.2 Monitoring of surface markers of PBMC in liver transplant patients

2.2.1 Patients

32 liver transplant recipients (22 male/10 female) under CNI-based immunosuppression were enrolled in this prospective study. The patients were divided into two groups. In MMF/CNI reduction group (n=22), MMF dose was increased from 2 x 500 mg/day to 2 x 1 g/day; CNI dose was progressively tapered to achieve target trough levels of 2-4 ng/mL for Tac and 25-50 ng/mL for CsA. In the control group (n=10), patients maintained their CNI dose.

Four months after conversion to MMF regimen, blood samples were collected for PBMC isolation.

2.2.2 PBMC isolation

Blood samples were collected from patients in the presence of sodium citrate. PBMC were isolated by density separation over Ficoll separating solution (1700 rpm/30 min). Mononuclear cells at the interface were collected and washed twice with PBS.

2.2.3 Surface marker staining and flow cytometric analysis

The PBMCs were resuspended at 1×10^6 /mL in staining buffer and labeled with antibodies (Table 2.1) for 30 min on ice. Then the cells were washed twice and fixed with 2% paraformaldehyde in PBS. The expression of each surface marker was measured by FACSCalibur and CellQuest Pro software (BD Biosciences, USA). Forward and sideward scatter were used to identify the lymphocyte and monocyte gate, and subsequent analysis was performed on this gated set of cells; 100,000 cells per sample were counted. Results were analyzed by WinMDI version 2.8 software (Joseph Trotter, Scripps Research Institute, La Jolla, CA).

2.3 T cell function study *in vitro*

2.3.1 Isolation of PBMC

Blood samples were collected from healthy volunteers and the PBMCs were isolated as described in 2.2.2.

2.3.2 Pre-treatment of cells with immunosuppressive drugs

PBMCs were resuspended in RPMI medium (1×10^6 cells/mL) and divided into 12 groups. The stock solutions of immunosuppressive drugs were firstly diluted in RPMI to have a 310 μ M working solution of MPA, 100 μ g/mL working solution of CsA and 10 μ g/mL working solution of Tac separately. These working solutions were added to each group of PBMCs according Table 2.2. Drugs were allowed to equilibrate at 37 °C for 30 min to ensure homogeneous distribution of all cells.

Table 2.1 Combinations of antibodies

No.	FITC	PE	CY	APC
1	Iso	Iso	Iso	Iso
2	CD45RA	CD45RO	CD3	CD8
3	CD25	CD28	CD3	CD8
4	CD30	CD38	CD3	CD8
5	CD62L	CD69	CD3	CD8
6	CD71	CD154	CD3	CD8
7	CD4	CD80	CD3	CD8
8	CD14	CD103	CD3	CD8
9	Anti-TCR (γ/δ)	CD134	CD3	CD8
10	CD45RA	CD27	CD3	CD4
11	HLA-DR,DP,DQ	CD154	CD3	CD4
12	CD25	CD28	CD3	CD4
13	CD19	CD56	CD3	CD45
14	CD3	CD122	CD56	CD95

2.3.3 Cell stimulation and culture

For T-cell activation, a human antibody directed against the CD3 molecular complex was used in combination with a co-stimulatory human anti-CD28 antibody.

2.3.3.1 Preparation of anti-CD3 coating plates

One mg/mL stock solution of anti-human CD3 antibody was diluted with PBS to a 10 $\mu\text{g/mL}$ working solution. Fifty μL of the diluted anti-human CD3 antibody working solution was distributed into each well of 96-well round-bottom plates. For the control wells (non-stimulated group), 50 μL of sterile PBS was added instead of anti-human CD3 antibody. The plate was covered and incubated for 90 min at 37 $^{\circ}\text{C}$. Excess antibody was then removed and the plate was washed twice with cold PBS 200 $\mu\text{L/well}$.

2.3.3.2 Cell culture

Pre-treated cells from step 2.3.2 were distributed into each well of the anti-CD3 coated 96-well plate (see 2.3.3.1) for culture (200 $\mu\text{L/well}$, containing 2×10^5

pre-treated cells, 1×10^6 cells/mL). Anti-human CD28 stock solution (1 mg/mL) was added to each well for a final concentration of 2.5 μ g/mL. The plate was placed in a humidified 37 °C, 5% CO₂ incubator for 48 hours (for BrdU staining) or 72 hours (for CFSE and cell surface marker analysis).

Table 2.2 Combinations of immunosuppressive drugs

Group	Final Concentration		
	MPA (μ M)	CsA (ng/mL)	Tac (ng/mL)
1	0	0	0
2	0	0	0
3	1	0	0
4	10	0	0
5	0	25	0
6	0	100	0
7	0	0	2
8	0	0	10
9	1	25	0
10	1	0	2
11	10	25	0
12	10	0	2

2.3.4 CFSE labeling and cell proliferation study

Healthy PBMC were resuspended (1×10^7 /mL in PBS) with fluorescent dye CFSE added to a final concentration of 5 μ M, and the suspension was then incubated at room temperature for 5 min. At the end of this incubation, the cells were immediately washed twice with 10 volumes of PBS containing 5% FBS. The stained PBMC were resuspended with RPMI medium (1×10^6 cells/mL), treated with immunosuppressive drugs and cultured as indicated in 2.3.2 and 2.3.3. After 72 hours, cells were harvested, incubated with anti-CD4 (PerCP-Cy5.5) and anti-CD8 (APC) antibody for

30min in an ice bath. The cells were subsequently washed and resuspended with staining buffer. CFSE staining (FL1-height) was measured by flow cytometry using a FACSCalibur and CellQuest Pro software. Lymphocyte, CD4 and CD8 positive cells were gated and data was analyzed by WinMDI 2.8 and Weasel 2.1 software (F. Battye, The Walter and Eliza Hall Institute of Medical Research).

2.3.5 BrdU staining and cell cycle study

After 48 hours of cultured (2.3.3.2), the PBMCs were labeled for 30 min at 37°C with 10uM BrdU in cell culture medium. Then the cells were harvested, fixed, permeabilized and stained with anti-BrdU and 7-AAD following the instructions of the BrdU Flow Kits. G0/G1, S and G2/M fractions were quantified by WinMDI 2.8. Cells that were not BrdU-labeled were used as negative staining control.

2.3.6 Cells surface antigen study

PBMCs were treated with immunosuppressive drugs, stimulated and cultured according to 2.3.2 and 2.3.3. After 72 hours culture, cells were resuspended in staining buffer and labeled with antibodies (Table 2.3) for 30 min on ice. Then the cells were washed with staining/wash buffer twice and fixed with 2% paraformaldehyde in PBS. The expression of each surface marker was measured by FACSCalibur and CellQuest Pro software. Forward scatter signal was collected in liner mode; sideward scatter and the four fluorescence signals were analyzed on a logarithmic scale. Electronic compensation was adjusted to eliminate spectral overlap between FITC/PE, PE/Cy-Chrom and Cy-Chrom/APC channels, respectively. Non-stimulated PBMC were used as negative controls. Cells stained with the respective isotype served as control. Forward and sideward scatter was used to identify the lymphocyte gate, and subsequent analysis was performed on this gated set of cells; 10,000 cells per sample were counted.

Table 2.3 Combinations of antibodies for *in vitro* study

FITC	PE	CY	APC
CD25	CD69	CD4	CD8
CD71	CD154	CD4	CD8

2.3.7 Cytokine Study.

Healthy PBMC were treated with immunosuppressive drugs, stimulated and cultured in anti-human CD3-coated 12-well plate for 24 hours as description in 2.3.2 and 2.3.3. For preparation of the plate, 300 μ L of anti-human CD3 (10 μ g/mL) antibody were added to each well instead of 50 μ L as described in 2.3.3.1. Each well contained 2 mL medium (2×10^6 cells) and the final concentration of anti-human CD28 is 2.5 μ g/mL. The groups of high concentration of MPA (10 μ M) were omitted in this study. After 24 hours culture, the supernatant of each well was harvested and incubated with human cytokine capture bead following the instruction manual of the “Human Th1/Th2 Cytokine CBA” kit. The samples and serially diluted cytokine standard dilutions were analyzed by FACSCalibur and CellQuest Pro software. The threshold to FSC was set at 650 and other settings following the instructions of the kit. The raw data were analyzed by BD CBA Software (BD Biosciences, USA) to obtain standard curves. The levels of cytokines in supernatants were calculated automatically. Using this method, the levels of 6 kinds of cytokine proteins including IL-2, IL-4, IL-5, IL-10, TNF- α and IFN- γ can be quantitatively measured in a single sample.

2.3.8 Cytokine mRNA expression by real-time RT PCR

Healthy PBMC were treated with immunosuppressive drugs, stimulated and cultured for 4 hours as description in 2.3.7. After the time of culture, cells were harvested and the total RNA was isolated by TRIzol reagent (Invitrogen) according to manufactures protocol. Briefly, $5-10 \times 10^6$ pellet cells were lysed in 1 mL TRIzol reagent. The homogenized samples were incubated for 5 minutes at room temperature and 0.2 mL of chloroform was added. The samples were then centrifuged at 11,500

× g for 15 minutes at 4°C. The aqueous phase of sample was isolated and 0.5 mL of isopropyl alcohol was used to precipitate the RNA. The RNA precipitates were centrifuged, washed with 1 mL 75% ethanol and redissolved in DEPC-treated water. The concentration of RNA samples were assessed by spectrophotometer and the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

Sequence-specific PCR primers and fluorochrome-labeled internal oligonucleotide probes for IL-2, IFN- γ and TNF- α were designed. In order to rule out amplification of contaminating DNA, the primer sequences were designed to span exon-intron junction and checked in Genebank. Forward and reverse primers were synthesized by Carl Roth GmbH; the FAM-TAMRA labeled TaqMan probes were synthesized by QIAGEN (Table 2.4).

Table 2.4 Primers and TaqMan probes for real-time RT PCR

Primer Name	Sequence (5'-3')	Length (bp)	Genebank Accession No	Tm (°C)
IL2-F	ACT CAC CAG GAT GCT CAC AT	193	GI:28178860	61.5
IL2-R	AGA TCC CTT TAG TTC CAG AAC			59.4
IL2-TP	TTT TAC ATG CCC AAG AAG GCC ACA GAA CT			71.5
IFN γ -F	TCA GCT CTG CAT CGT TTT G	166	GI:32679	62.2
IFN γ -R	TCT GTC ACT CTC CTC TTT CCA			61.5
IFN γ -TP	TTG GCT GTT ACT GCC AGG ACC CAT ATG T			71.6
TNF α -F	CAG GCA GTC AGA TCA TCT TC	165	GI:339737	59.3
TNF α -R	CTC TGA TGG CAC CAC CAG			61.1
TNF α -TP	TAG CCC ATG TTG TAG CAA ACC CTC AAG CT			71.6
Actin β -F	ACC GAG GCC CCC CTG	62	GI:5016088	63.1
Actin β -R	GGT CTC AAA CAT GAT CTG GGT CA			63.4
Actin β -TP	ACC CCA AGG CCA ACC GCG A			72.5

F: forward primer; R: reverse primer; TP: TaqMan probe.

Quantitative RT-PCR was carried out on a 7700 Sequece Detector Systems and Sequence Detection system (SDS) Software (Applied Biosystems, Foster City, CA, USA) using the $\Delta\Delta C_t$ method of calculating the relative expression (Simon et al., 2003). Briefly, gene expression was determined from measurements of the increase in fluorescence, corresponding with exponential amplification and degradation of the bi-fluorescent probe during the PCR reaction. The cycle in which fluorescence

exceeds the manually set threshold level of detection is termed the ‘threshold cycle’ (Ct value). Greater amounts of initial specific template add to the reaction will result in threshold-exceeding fluorescence during earlier cycles (lower Ct value). Thus, the Ct value is inversely proportional to the log of the input target specific mRNA (IL-2, IFN- γ and TNF- α , in this study). All Ct value measurements were obtained for each sample (target gene and β -actin endogenous control). The Ct value of the endogenous control gene was used to calculate normalized target gene expression, referred to as ‘delta Ct (Δ Ct), in order to correct differences between samples; Δ Ct values for each target gene in individual samples were calculated as follows:

$$\Delta\text{Ct (target gene)} = \text{Ct (target gene)} - \text{Ct (\beta-actin mRNA)}$$

Calculation of relative gene expression was then performed, which allowed for comparison of the gene expression in one sample relative to another. The sample on which the comparison is based is termed the ‘calibrator’ sample and the sample that is being compared is referred to as the ‘target’ sample. For this study, we presented calibrated gene expressions from samples stimulated by anti-CD3/anti-CD28 antibody and treated by immunosuppressants relative to non-stimulated sample. This calculation is accomplished by the use of two formulas. First, the normalized Δ Ct values for calibrator and target sample are substituted into the delta-delta C_T ($\Delta\Delta$ Ct) equation:

$$\Delta\Delta\text{Ct} = \text{target sample } \Delta\text{Ct} - \text{Calibrator sample } \Delta\text{Ct}$$

The relative expressions then calculated according to the formula:

$$\text{Relative expression} = 2^{-\Delta\Delta\text{Ct}}$$

Calibrator expression is always considered one. The relative amounts of target IL-2, IFN- γ and TNF- α mRNA of other groups were obtained by comparing them to the calibrator. All PCR reactions were carried out in duplicate on each one-step RT-PCR and the average $\Delta\Delta$ Ct was used to calculate relative expression.

Using TaqMan One-Step RT-PCR Master Mix Reagents Kit, the RT and PCR amplification occur at one step without interruption. All reactions were performed

with 96-well microtitre plates and each reaction volume was 25 μ L. The One-Step RT PCR conditions were 30 min at 48 $^{\circ}$ C for RT, 10 min at 95 $^{\circ}$ C for DNA-polymerase activation, followed by 40 cycles of 15s at 95 $^{\circ}$ C for denaturation and 1 min 30s at 60 $^{\circ}$ C for annealing/extension with a final 25 $^{\circ}$ C hold.

Table 2.5 RT-PCR Reaction

Rx component	Volume per Rx (μ L)	Volume for 100 Rx (μ L)	Final Value
2 x Master Mix	12.5	1250	1 x
40 x MultiScribe and Rnase Inhibitor Mix	0.625	62.5	0.25U/ μ L 0.4U/ μ L
10 μ M Forward Primer	1	100	400 nM
10 μ M Reverse Primer	1	100	400 nM
5 μ M TaqMan probe	0.5	50	100nM
RNA sample	2		0.2ng to 20ng
Water	7.375	737.5	
Total	25	2300	

2.4 Statistical Analysis

All data are expressed as mean \pm SD. For statistical analysis, SPSS version 8.0 (SPSS Corp., Birmingham, AL) was used. Nonparametric test was used and a value of $P < 0.05$ was considered to be significant. The one-way ANOVA with the Student-Newman-Keuls (SNK) post hoc test was used to estimate the levels of significance for the differences between more than two groups.

3. Results

3.1 Monitoring of surface markers of PBMC in liver transplant patients

After 4 months of conversion to combined MMF/CNI reduction therapy (MMF 2 g/day), the mean trough levels of Tac and CsA in the MMF/CNI reduction group were 2.2 ± 0.5 ng/mL and 36 ± 9.1 ng/mL respectively. For controls, the mean trough levels of CsA and Tac were 116 ± 19.4 ng/mL and 5.6 ± 1.3 ng/mL respectively.

In the MMF/CNI reduction group, the percentage of CD8⁺ T cells (CTL) was significantly lower than the control group ($P < 0.05$; Table 3.1, Figure 3.1). The percentages of CD3⁺CD56⁺ T cells (NK T cells) ($P < 0.005$; Table 3.1, Figure 3.2) and CD4⁺CD45RO⁺ T cells (memory T cells) ($P < 0.01$; Table 3.1, Figure 3.3) also decreased significantly in the MMF/CNI reduction group.

Table 3.1 Percentage of CD8⁺ T cells, CD3⁺CD56⁺ NK T cell and CD4⁺CD45RO⁺ T cells in the MMF/CNI reduction group and control group

	MMF/CNI reduction group (%)	Control Group (%)
CD8 ⁺ T cells	20.34 ± 8.14	29.09 ± 9.66
CD3 ⁺ CD56 ⁺ NK T cells	5.71 ± 1.66	9.68 ± 3.08
CD4 ⁺ CD45RO ⁺ T cells	57.99 ± 11.98	72.88 ± 8.3

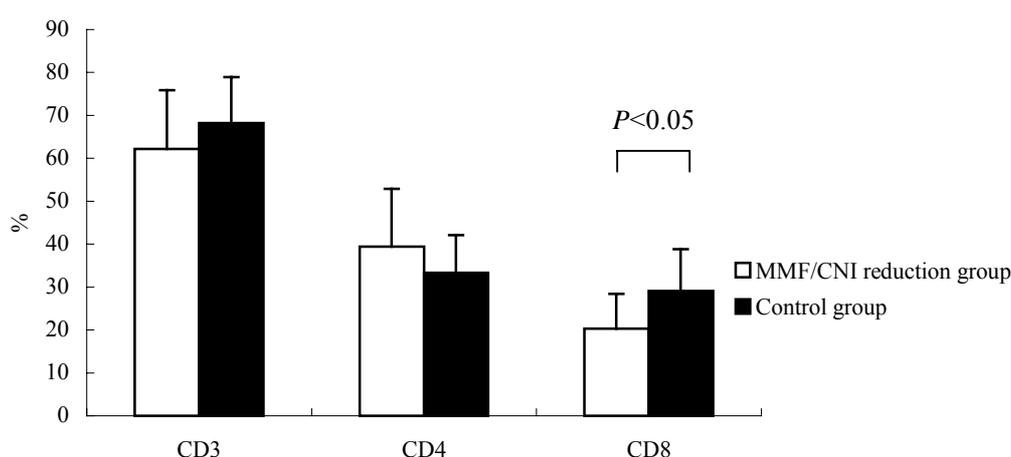


Figure 3.1 Percentage of cells expressing CD3 (T cells), CD4 (Th), CD8 (CTL) in the MMF/CNI reduction group versus control group. Values are expressed as mean \pm SD.

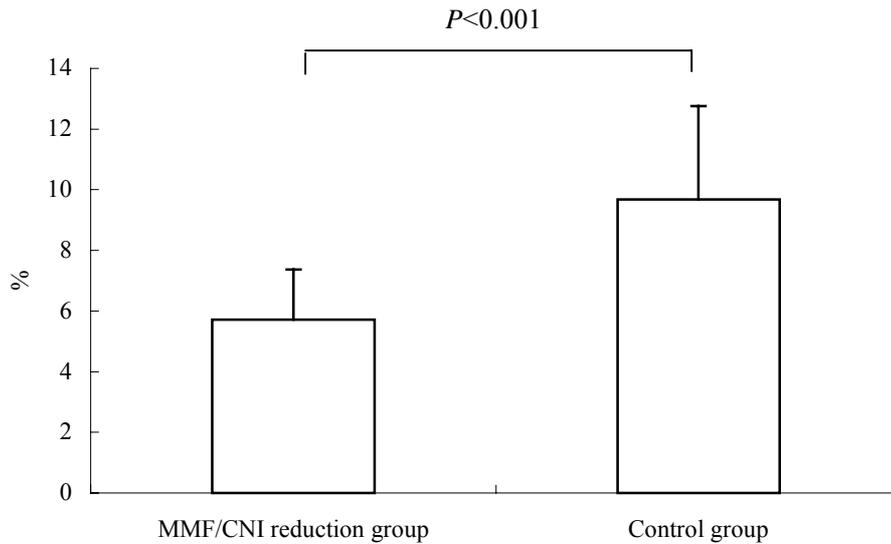


Figure 3.2 Percentage of CD3⁺CD56⁺ NK cells in the MMF/CNI reduction group versus control group. Values are expressed as mean \pm SD.

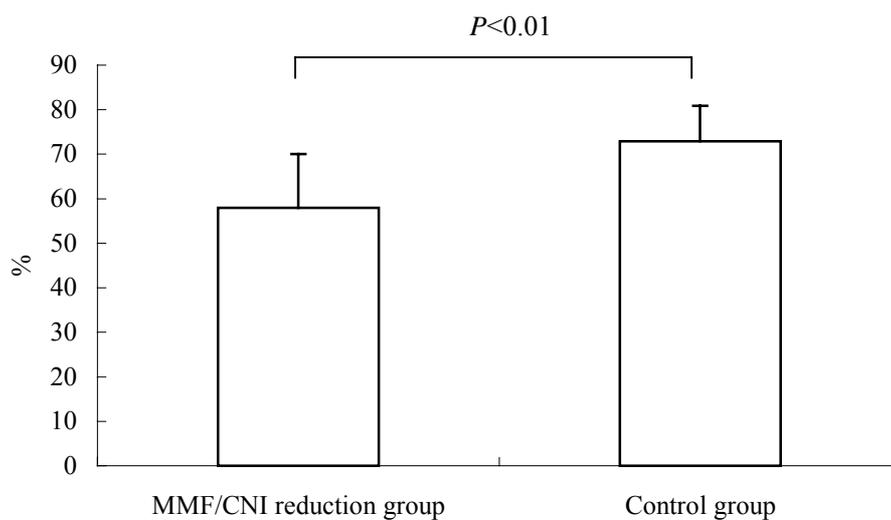


Figure 3.3 Percentage of CD4⁺CD45RO⁺ T cells (memory T cells) in the MMF/CNI reduction group versus control group. Values are expressed as mean \pm SD.

To assess the function of lymphocytes, the expression of activation markers (CD25, CD69, CD71 and CD154) was investigated (Table 3.2). The expression of CD71 on CD4 cells was significantly lower in the MMF/CNI reduction group ($P < 0.05$; Figure 3.4). In the MMF/CNI reduction group, there was a trend towards lower CD154 expression on CD4 T cells ($P = 0.08$; Figure 3.4)

We also found that the percentage of $\gamma\delta$ TCR⁺ T cells was significantly lower in the

MMF/CNI reduction group than in the control group (20.06% ± 8.64% vs 29.49% ± 8.90%, $P < 0.05$; Figure 3.5).

Table 3.2 Expression of activation surface markers on CD4 T cells in the MMF/CNI reduction group and control group

	MMF/CNI reduction group (%)	Control Group (%)
CD25	7.88 ± 10.39	6.59 ± 2.72
CD69	12.10 ± 8.12	20.45 ± 11.34
CD71	6.54 ± 2.93	11.36 ± 5.90
CD154	6.73 ± 3.10	11.76 ± 7.80

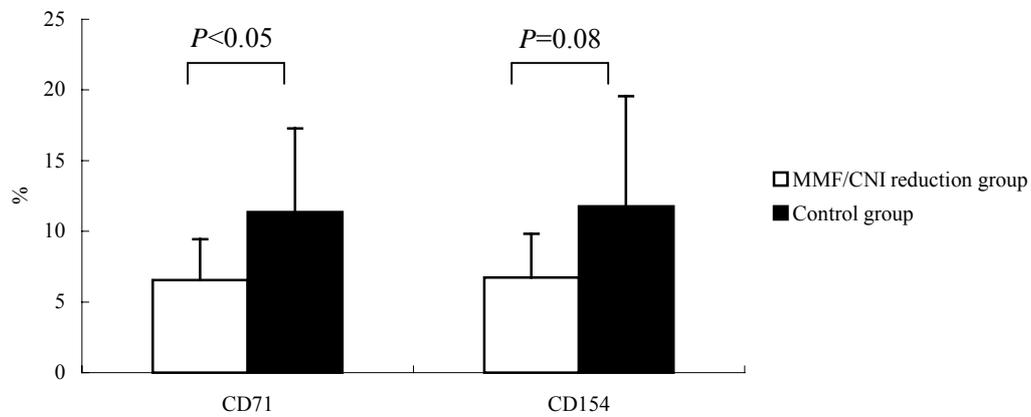


Figure 3.4 Expression of CD71 and CD154 on CD4⁺ T cells in the MMF/CNI reduction group versus control group. Values are expressed as mean ± SD.

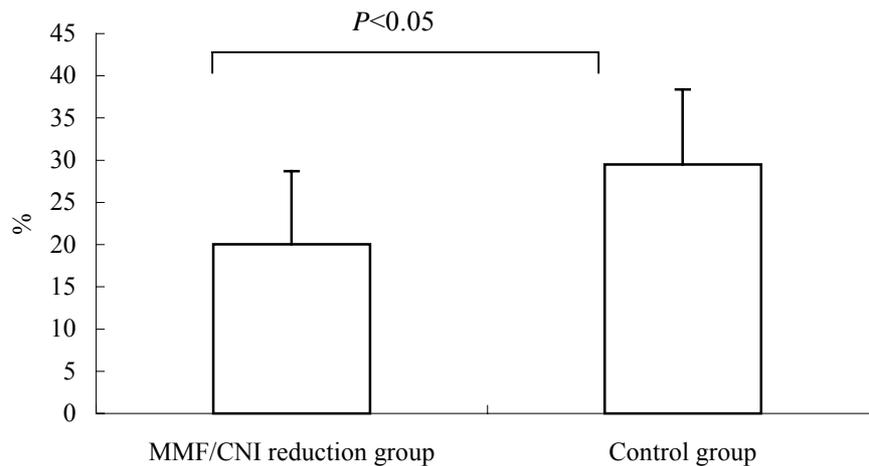


Figure 3.5 Percentage of $\gamma\delta$ TCR on T cells in the MMF/CNI reduction group versus control group. Values are expressed as mean ± SD.

3.2 *In vitro* study of lymphocyte function

3.2.1 MPA and CNi inhibit T cell proliferation

To assess how MPA and CNi interfere with the expansion of activated lymphocytes, healthy human PBMC were activated with anti-CD3 antibody and anti-CD28 antibody in the presence of MPA and CNi (Table 2.2). By labeling PBMC before cell division with fluorescent dye CFSE, cell division can be monitored by the decrease in dye content of the cells as the dye is divided between daughter cells. After 3 days in the presence of anti-CD3 antibody and anti-CD28 antibody, 65.74% of lymphocytes had undergone one to four divisions, and only 34.26% of the cells remained undivided. Addition of 1 μ M MPA at the onset of the culture inhibited cell division completely. Neither CsA nor Tac alone blocked T cells going into daughter division. However, both of them reduced the number of cells in each division generation ($P<0.001$ vs stimulation group) and this effect was stronger in high dose CsA group (100 ng/mL) than that in lower concentration CsA group (25 ng/mL) ($P<0.001$). Combined MMF and CsA or Tac inhibited almost all of lymphocytes division (Table 3.3, Figure 3.6). MPA blocked proliferation of both CD4 and CD8 subpopulations (Figure 3.7)

Table 3.3 Percentage of division 0 in the presence of immunosuppressive drugs

Groups	Percentage of cells of Division 0
Non-stimulated PBMC	95.50 \pm 3.10
Stimulated PBMC	34.26 \pm 6.73
MPA 1 μ M	99.58 \pm 0.56 *
MPA 10 μ M	97.68 \pm 1.92 *
CsA 25 ng/mL	40.43 \pm 3.99 *
CsA 100 ng/mL	48.89 \pm 2.91 * §
Tac 2 ng/mL	54.86 \pm 4.56 * §
Tac 10 ng/mL	54.56 \pm 3.00 * §
MPA 1 μ M+ CsA 25 ng/mL	97.41 \pm 5.22 *
MPA 1 μ M+ Tac 2 ng/mL	99.74 \pm 0.19 *
MPA 10 μ M+ CsA 25 ng/mL	99.97 \pm 0.04 *
MPA 10 μ M+ Tac 2 ng/mL	99.98 \pm 0.02 *

* vs stimulation group, $P<0.001$

§ vs CsA 25ng/mL group, MPA alone and other combined MPA /CNi groups, $P<0.001$

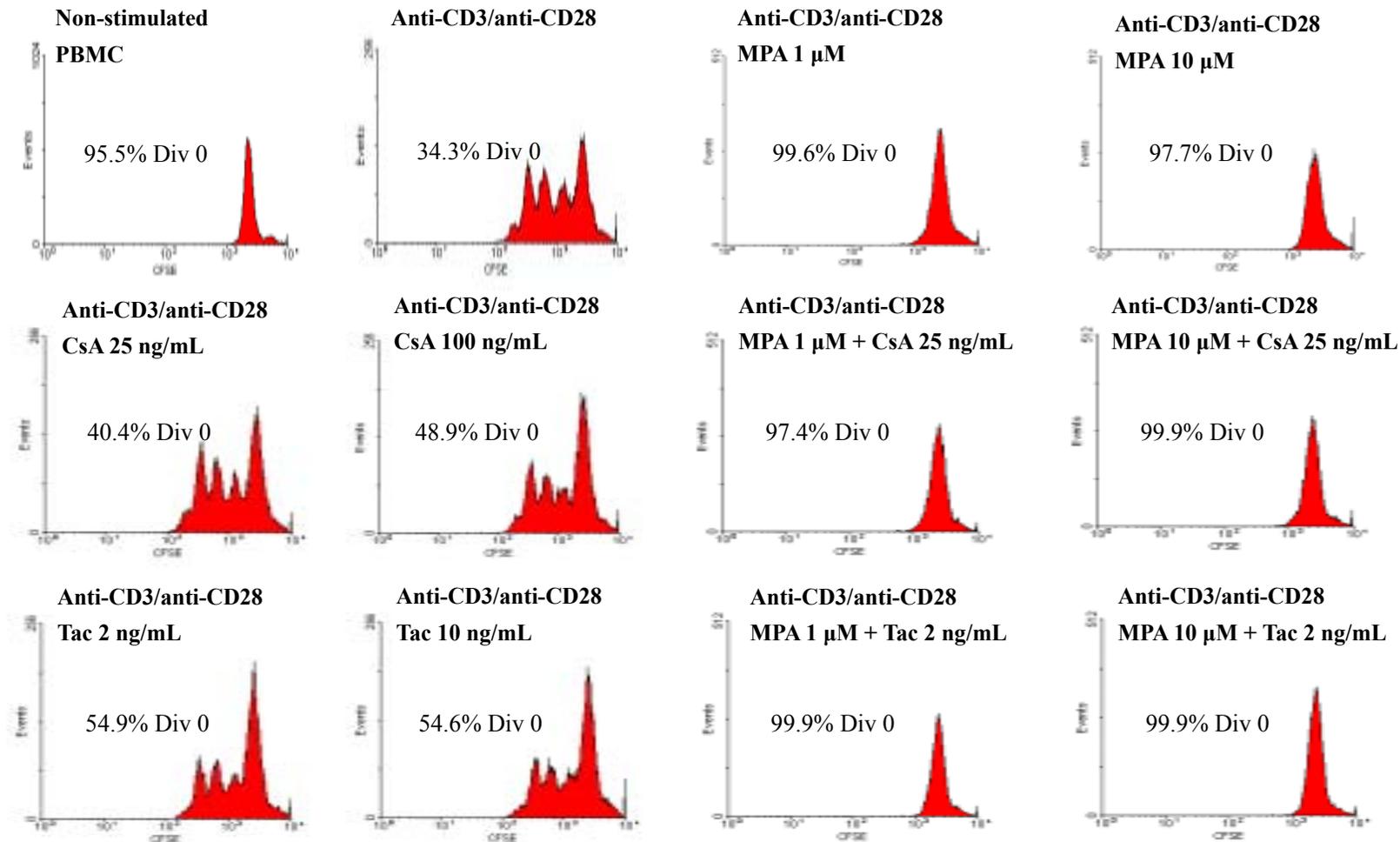


Figure 3.6 Antiproliferative effects of MPA and CNI in primary T lymphocytes. PBMC were labeled by CFSE as described in Materials and Methods, and activated by anti-CD3 and anti-CD28 antibodies in the presence of immunosuppressive drugs. After 3 days of culture, cells were harvested and cell divisions were analyzed by FACS. Percentage of cells, that did not divide, is indicated in the figures. Results are from one experiment among four individual experiments showing similar results.

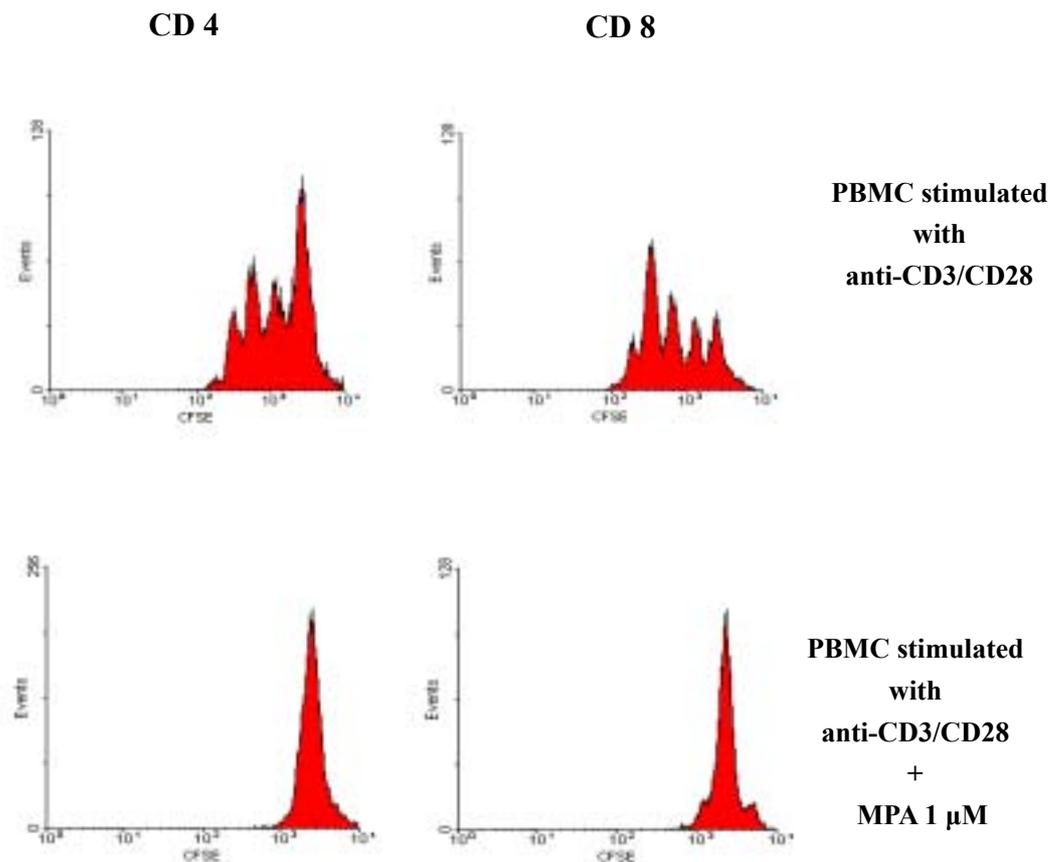


Figure 3.7 Inhibitions of CD4 and CD8 T cells by MPA. After staining with PerCP-Cy-conjugated anti-CD3 and APC-conjugated anti-CD8 antibodies, cells were gated on CD4⁺ or CD8⁺ T cells and the expression of CFSE was examined. Histograms are representative of one experiment among four independent experiments showing similar results.

3.2.2 MPA inhibits PBMC from G0/G1 transition to S phase of the cell cycle

To determine whether MPA or CN1 induced a specific blockade in the cell cycle, we analyzed the cellular DNA content in parallel with BrdU staining which reveals cells in the S phase of the cell cycle. As shown in Figure 3.8, about 94% of cells cultured in medium alone were in G0/G1. After 48h of activation with anti-CD3/anti-CD28 antibody, 31.5% of cells enter into the S phase of the cell cycle. In the presence of MPA, most of cells accumulated in the G0/G1 phase. CsA or Tac alone did not block cells into S phase but reduced the percentage of cells into S phase (Table 3.4). Combined MPA and low dose CN1 enhanced their effects.

Table 3.4 Percentage of PBMC in each phase of cell cycle

Groups	G0/G1 Phase	S Phase	G2+M Phase
Non-stimulated	94.07±0.97	0.47±0.12	5.50±0.92
Stimulated	60.30±0.66 *	31.50±0.62	8.20±0.26
MPA 1 µM	74.77±0.75	12.73±0.25	12.57±0.91
MPA 10 µM	83.17±2.40	5.63±0.64	11.13±1.80
CsA 25 ng/mL	78.23±1.42	13.6±1.40	8.27±0.70
CsA 100 ng/mL	78.63±2.38	12.17±2.50	9.17±1.00
Tac 2 ng/mL	81.57±1.89	10.00±2.21	8.13±0.35
Tac 10 ng/mL	83.1±1.40	8.87±0.25	8.07±1.10
MPA 1 µM+ CsA 25 ng/mL	79.67±0.47	9.37±1.34	10.97±1.15
MPA 1 µM+ Tac 2 ng/mL	87.07±0.85 **	4.57±0.51	8.37±0.84
MPA 10 µM+ CsA 25 ng/mL	85.83±1.91 §	3.80±0.90	10.40±1.85
MPA 10 µM+ Tac 2 ng/mL	90.57±0.92 **	2.70±0.60	6.73±0.47

* vs non-stimulated and immunosuppressant groups, $P<0.001$

** vs MPA or CNI alone groups, $P<0.001$

§ vs MPA 1 µM, CsA alone, Tac 2 ng/mL and MPA 1 µM + CsA 25 ng/mL groups, $P<0.001$.

3.2.3 MPA and CNI inhibited blastogenesis and the expressions of activation markers

Progression from quiescent state (G0) into and through the G1 phase of the cell cycle is characterized by RNA synthesis, decondensation of the chromatin, cellular enlargement, and synthesis of new proteins. Cellular enlargement as well as up-regulation of the activation marker CD69, the IL-2R α -chain CD25, CD154, and the transferrin receptor CD71, were analyzed by FACS (Figure.3.9). As expected, anti-CD3/anti-CD28 antibody stimulation induced an increase in the forward scatter of the cells and triggered CD69, CD25, CD154, and CD71 expression (Figure 3.9, Table 3.5, 3.6). At day 3, up-regulation of CD25 and CD71 expression were blocked by MPA. CNI also partially blocked the expression of CD25, CD69 and CD71. Both MPA and CNI alone strongly inhibited the up-regulation of CD154 expression. Combined MPA and low concentration of CNI inhibited the expression of CD25, CD71 and CD154 as well as the development of lymphoblasts characterized by an increase in size. In this study, we didn't find that MPA or MPA plus CNI inhibited the expression of CD69 after three days of stimulation.

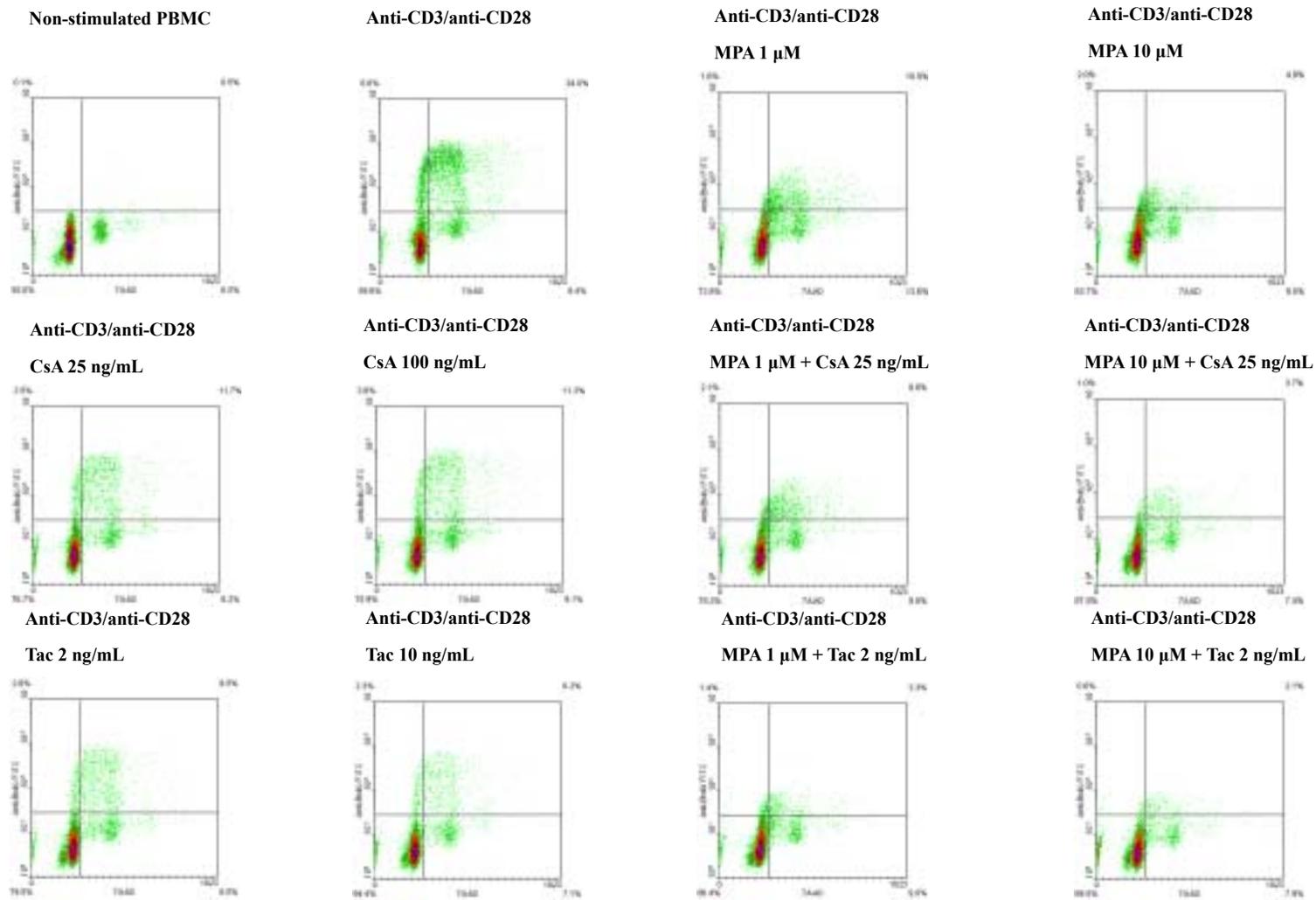


Figure 3.8. Effect of MPA and CNI inhibition of PBMC from G0/G1 transition to S phase of the cell cycle. PBMC were activated by anti-CD3/anti-CD28. After 48h of incubation, cell were incubated with BrdU for 30 min. The fraction of cells engaged in G0/G1 and S phases were evaluated. BrdU-labeled cells were stained with FITC conjugated anti-BrdU antibody and 7AAD. Percentages of cells in G0/G1 (lower left quadrant), S (upper left and right quadrant) and G2/M (lower right quadrant) phases of the cell cycle are indicated in each dot plot. Results are representative of one among three independent experiments and show similar results.

Table 3.5 Expression of activation markers on CD4 T cells

	CD25	CD69	CD71	CD154
1. Non-stimulated	12.10±2.16†	12.76±3.98†	18.80±5.34†	6.13±2.09
2. Stimulated	95.68±2.26*	55.38±20.98	94.20±1.97*	48.26±16.91*
3. MPA 1 µM	73.05±5.82	42.90±5.99	62.37±8.65	15.36±4.38
4. MPA 10 µM	74.50±2.73	56.45±5.50	60.88±9.65	7.52±4.00
5. CsA 25 ng/mL	91.01±1.70*	42.67±4.55	85.69±2.80*	32.90±12.66*§
6. CsA 100 ng/mL	83.22±4.33	35.37±5.45§	73.42±5.43	17.93±5.16
7. Tac 2 ng/mL	77.47±3.97	35.60±3.11§	63.67±6.39	11.70±5.46
8. Tac 10 ng/mL	78.42±4.24	32.37±6.23§	63.98±5.09	14.98±5.69
9. MPA 1 µM + CsA 25 ng/mL	76.70±5.75	47.47±1.35	66.80±10.27	5.17±1.16
10. MPA 1 µM + Tac 2 ng/mL	64.40±5.08**	39.50±7.96	44.88±9.50**	3.85±1.03
11. MPA 10 µM + CsA 25 ng/mL	69.95±2.84	63.30±2.84	57.15±6.65	8.32±3.38
12. MPA 10 µM + Tac 2 ng/mL	62.40±5.79**	41.73±5.85	40.23±7.62**	3.57±1.37

† vs groups 2-12, $P<0.001$

* vs group 1 and group 3,4,6-12, $P<0.001$

** vs group 3,4,6-9, $P<0.001$

§ vs group 2, $P<0.001$

Table 3.6 Expression of activation markers on CD8 T cells

	CD25	CD69	CD71	CD154
1. Non-stimulated	1.20±0.35†	5.08±2.34†	0.71±0.27†	0.04±0.05
2. Stimulated	93.79±1.89*	46.32±25.46	93.32±2.18‡	28.46±9.53*
3. MPA 1 µM	68.22±3.27	34.30±6.63	54.10±8.63	2.90±3.04
4. MPA 10 µM	66.55±3.96	47.10±6.18	51.30±10.18	0.89±0.97
5. CsA 25 ng/mL	86.22±3.02*	33.05±4.96	79.16±8.82	15.42±5.49*§
6. CsA 100 ng/mL	77.70±4.27	32.80±3.90	74.00±3.74	6.75±4.89
7. Tac 2 ng/mL	75.70±2.72	32.40±3.64	69.60±4.48	5.62±3.58
8. Tac 10 ng/mL	72.40±2.21	30.00±5.22	65.80±2.16	6.42±3.28
9. MPA 1 µM + CsA 25 ng/mL	67.50±3.36	41.80±2.52	61.05±4.54	0.58±0.33
10. MPA 1 µM + Tac 2 ng/mL	57.00±5.47**	34.40±7.86	45.97±5.93	0.53±0.26
11. MPA 10 µM + CsA 25 ng/mL	63.30±4.20	56.70±5.30	52.95±4.72	1.35±1.16
12. MPA 10 µM + Tac 2 ng/mL	49.90±4.07**	35.70±4.39	37.65±5.27	0.43±0.25

† vs groups 2-12, $P<0.001$

* vs group 1 and group 3,4,6-9,11, $P<0.001$

** vs group 3,4,6-9, $P<0.001$

§ vs group 2, $P<0.001$

‡ vs group 1,3-12, $P<0.001$

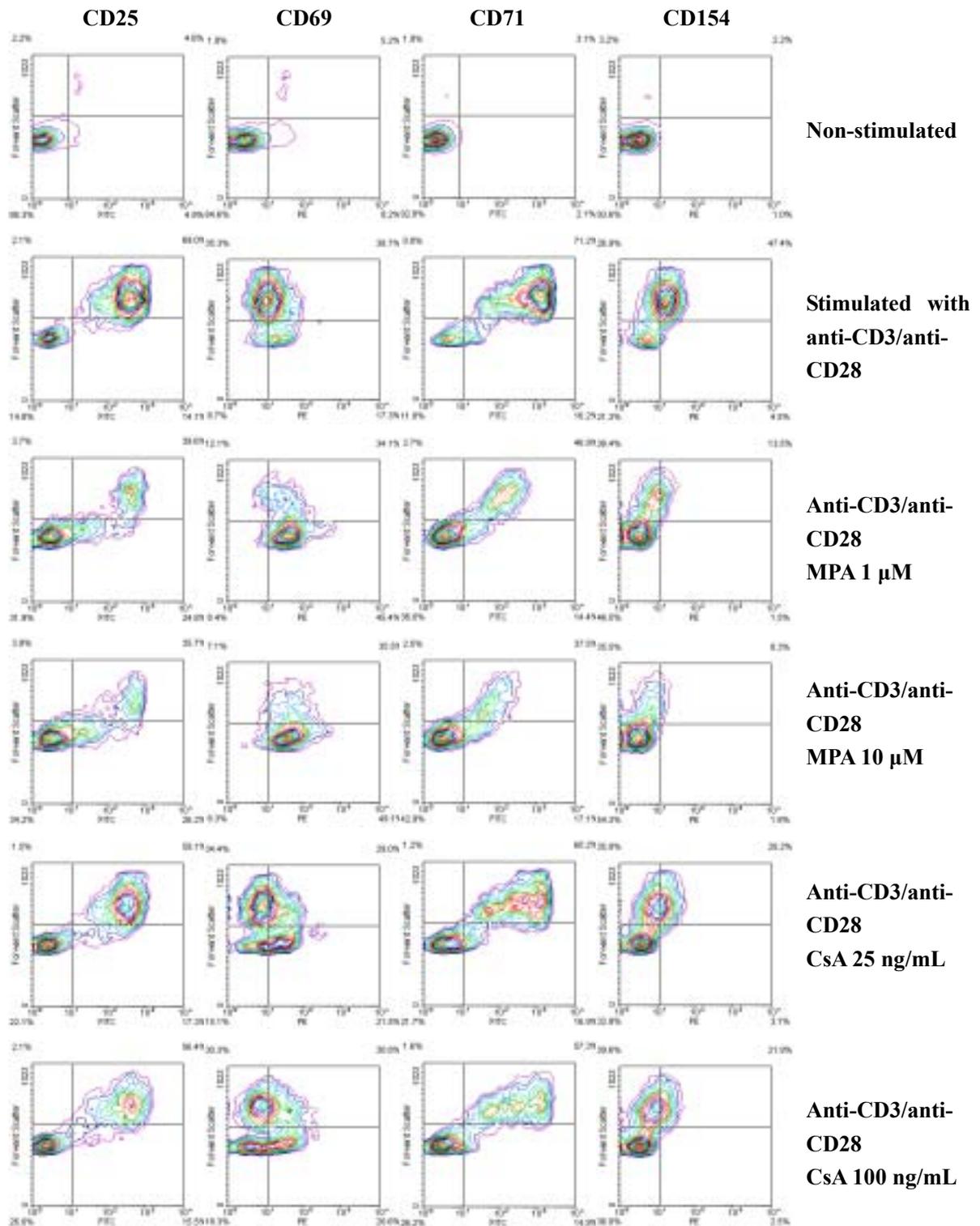


Figure 3.9 Effects of MPA and CNI on blastogenesis and the expression of activation markers. PBMC were stimulated with anti-CD3/anti-CD28 antibody alone or in the presence of MPA/CNI. After 72 hours, the expressions of activation markers (CD25, CD69, CD71 and CD154) were analyzed by FACS. Results are representative of one among three independent experiments and show similar results.

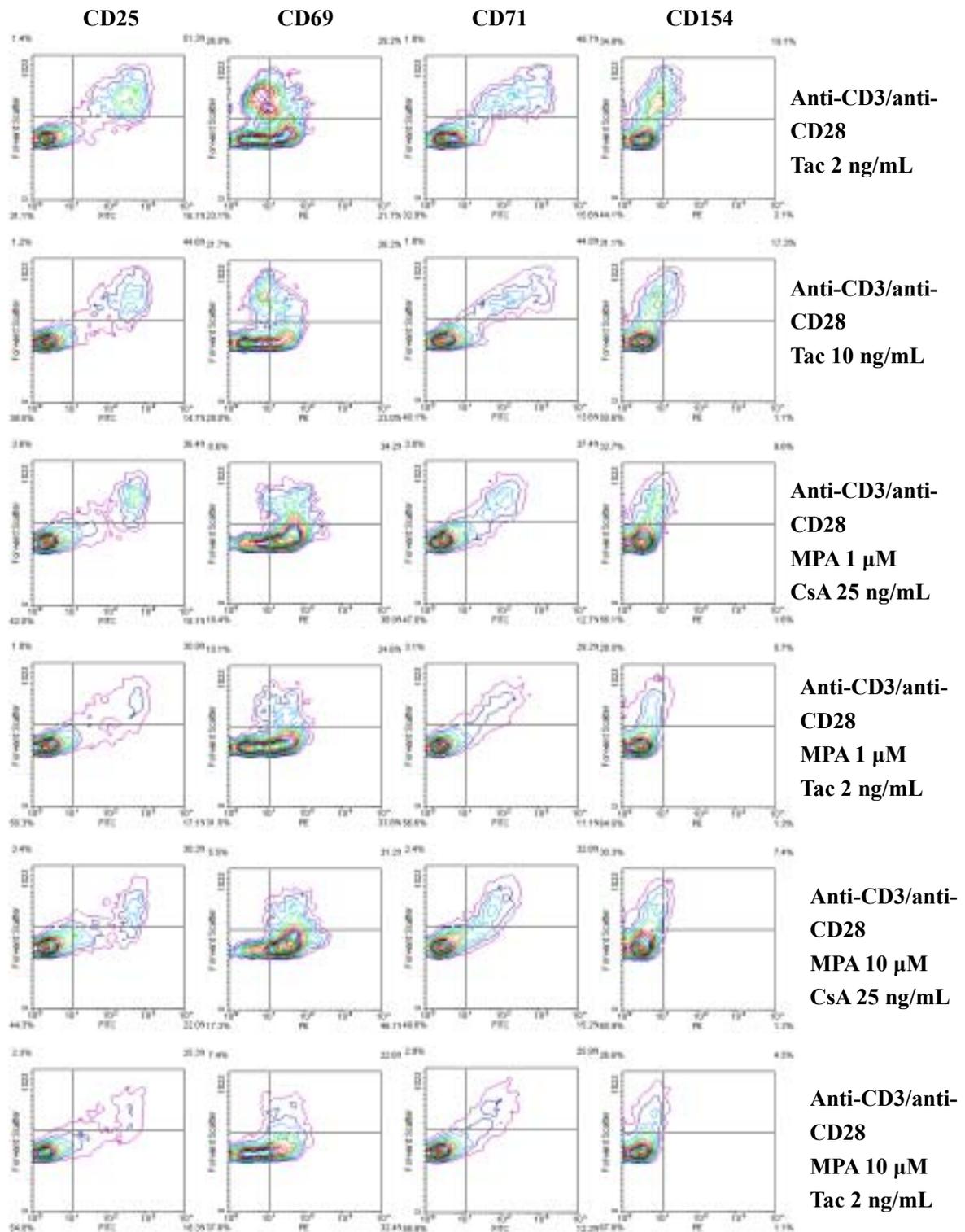


Figure 3.9 (Continue) Effects of MPA and CNI on blastogenesis and the expression of activation markers. PBMC were stimulated with anti-CD3/anti-CD28 antibody alone or in the presence of MPA/CNI. After 72 hours, the expressions of activation markers (CD25, CD69, CD71 and CD154) were analyzed by FACS. Results are representative of one among three independent experiments and show similar results.

3.2.4 Effects of MPA and CNI on the cytokine expression of lymphocytes

To assess the effects of MPA and CNI on the cytokine expression of lymphocytes, human PBMC were activated with anti-CD3/anti-CD28 antibody in the presence or absence of MPA and/or CNI. After 24 hours of stimulation, the supernatant of cell cultures were harvested and the cytokine levels were measured using the ‘Human Th1/Th2 Cytokine CBA kit’ for IL-2, IL-4, IL-5, IL-10, TNF- α and IFN- γ . The data was collected by flow cytometry and analyzed by BD CBA software. The standard curves of each cytokine were performed (Figure 3.10). The final levels of Th1 and Th2 cytokine of Th1 cells (Table 3.7, figure 3.11) and Th2 cells (Table 3.8, figure 3.12) were calculated.

Non-stimulated PBMC secreted cytokine proteins at very low levels. Anti-CD3/anti-CD28 antibody stimulation induced all six cytokines significantly. Addition of MPA did not change the up-regulated secretion of cytokines at all. However, low dose of CNI and Tac strongly inhibited IL-2, IL-4, IL-10 and IFN- γ secretion. Combined MPA and low dose of CNI did not show more effects than CNI alone.

Table 3.7 Cytokine levels of Th1 cells

Group	IL-2 (pg/mL)	IFN- γ (pg/mL)
1. Non-stimulated	0.00 \pm 0.00 *	0.00 \pm 0.00
2. Stimulated	3621.93 \pm 487.70 **	377.53 \pm 42.08.00 §
3. MPA 1 μ M	4490.77 \pm 882.02 **	363.40 \pm 28.64 §
4. CsA 25 ng/mL	972.90 \pm 133.48	0.00 \pm 0.00
5. CsA 100 ng/mL	836.27 \pm 207.29	0.00 \pm 0.00
6. Tac 2 ng/mL	413.03 \pm 84.13	0.00 \pm 0.00
7. Tac 10 ng/mL	408.10 \pm 88.31	0.00 \pm 0.00
8. MPA 1 μ M + CsA 25 ng/mL	1111.73 \pm 35.80	27.28 \pm 19.43
9. MPA 1 μ M + Tac 2 ng/mL	571.97 \pm 168.45	19.79 \pm 23.81

* vs groups 2-9, $P < 0.001$

** vs groups 4-9, $P < 0.001$

§ vs groups 1, 4-9, $P < 0.001$

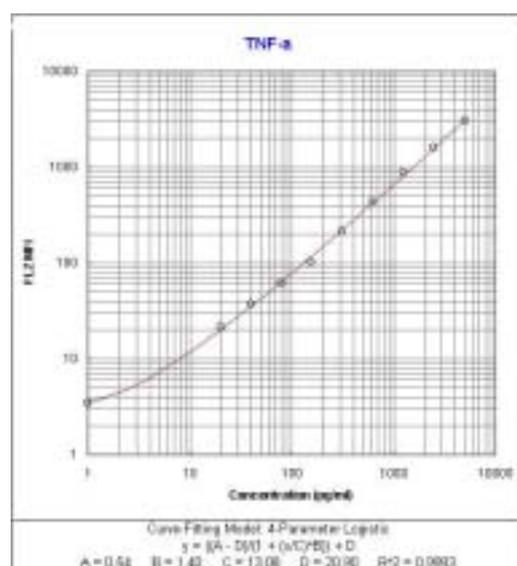
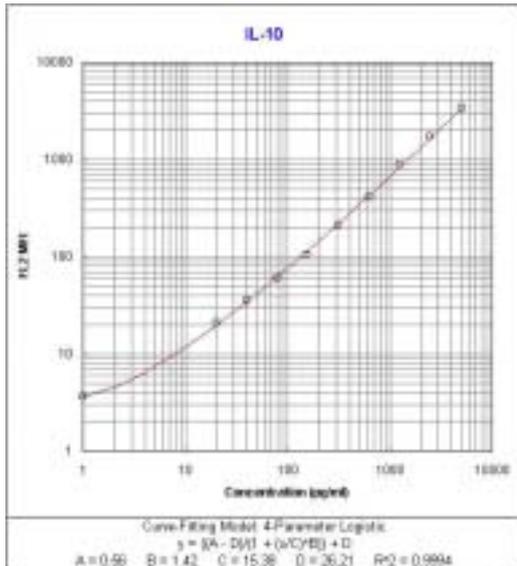
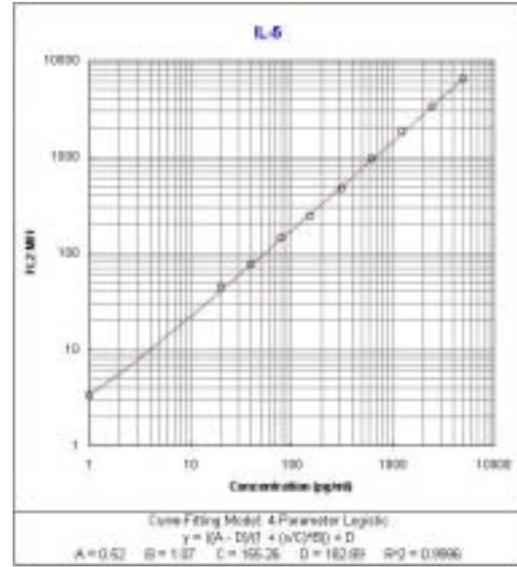
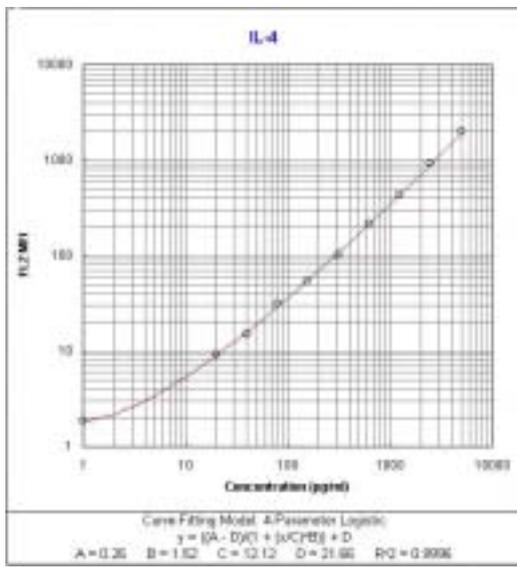
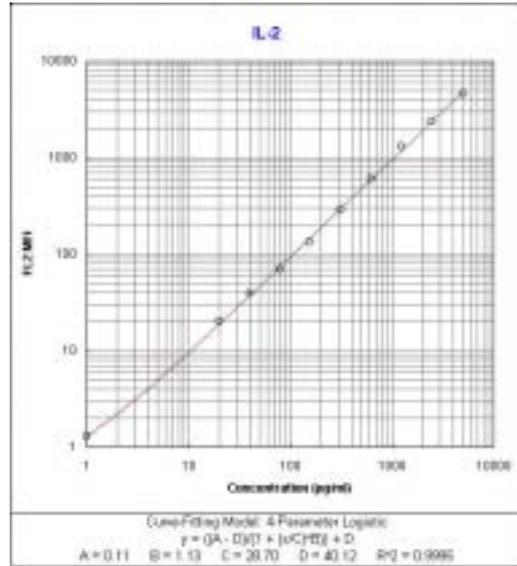
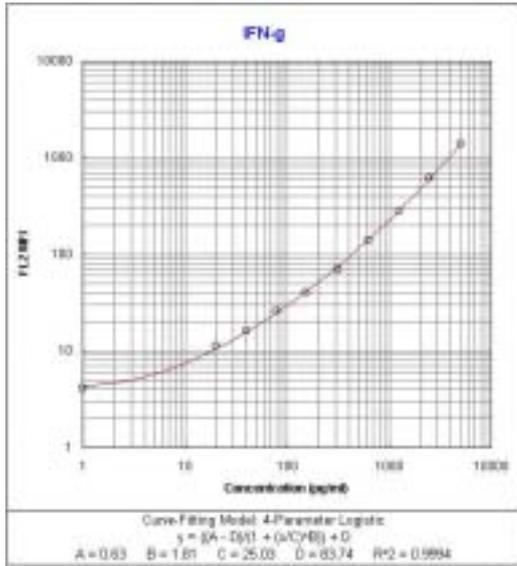


Figure 3.10 Standard curves of IFN- γ , IL-2, IL-4, IL-5, IL-10 and TNF- α

Table 3.8 Cytokine levels of Th2 cells

	IL-4 (pg/mL)	IL-5 (pg/mL)	IL-10 (pg/mL)	TNF- α (ng/mL)
1. Non-stimulated	0.00 \pm 0.00	0.00 \pm 0.00	0.93 \pm 1.62	0.024 \pm 0.003 *
2. Stimulated	169.27 \pm 17.16 **	271.87 \pm 32.76	103.90 \pm 16.49 **	11.27 \pm 1.01
3. MPA 1 μ M	216.60 \pm 60.31 **	353.47 \pm 115.28 **	110.30 \pm 44.60 **	34.06 \pm 8.52
4. CsA 25 ng/mL	51.27 \pm 7.10	260.67 \pm 34.18	30.00 \pm 3.87	24.96 \pm 6.18
5. CsA 100 ng/mL	42.47 \pm 9.07	222.80 \pm 48.30	32.80 \pm 7.86	26.15 \pm 2.36
6. Tac 2 ng/mL	28.40 \pm 1.15	159.37 \pm 18.20	45.40 \pm 6.36	23.05 \pm 11.43
7. Tac 10 ng/mL	27.23 \pm 8.21	154.13 \pm 28.81	45.80 \pm 10.57	16.82 \pm 7.29
8. MPA 1 μ M + CsA 25 ng/mL	52.03 \pm 3.27	229.57 \pm 20.75	26.90 \pm 1.68	22.05 \pm 4.50
9. MPA 1 μ M + Tac 2 ng/mL	32.73 \pm 7.95	169.93 \pm 32.26	52.90 \pm 12.52	19.19 \pm 10.01

* vs groups 2-9, $P < 0.001$

** vs groups 4-9, $P < 0.001$

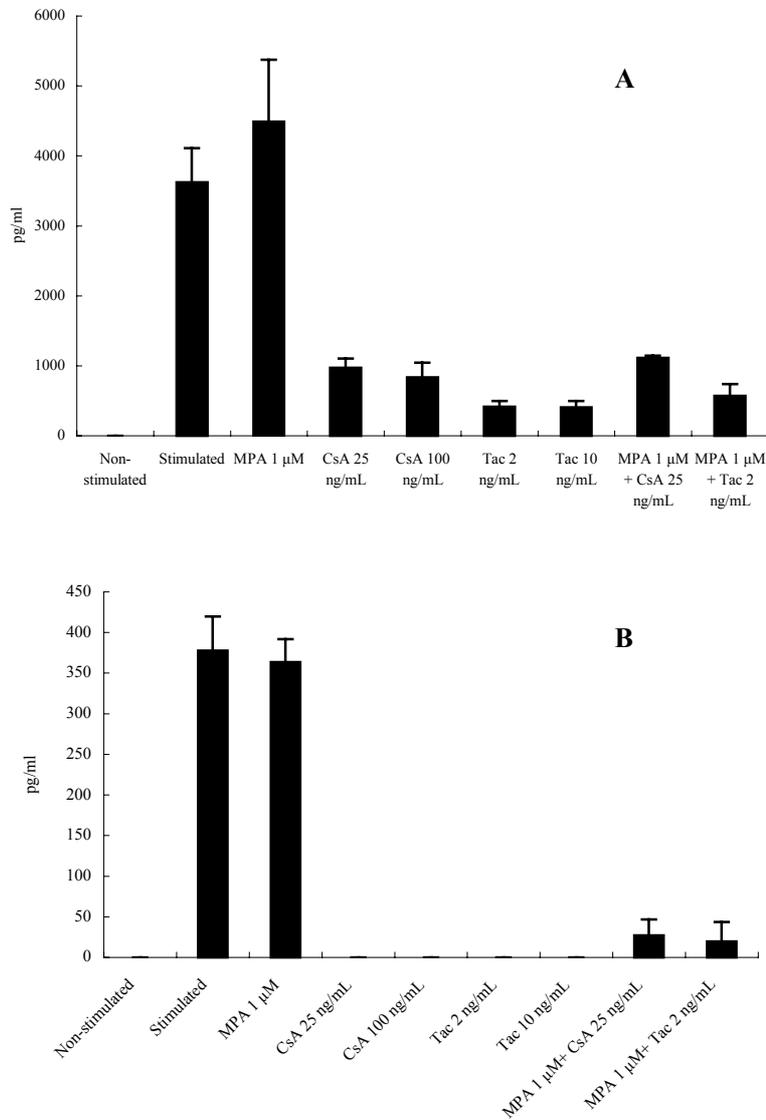


Figure 3.11 Th1 cytokine expression after 24h of stimulation. A: IL-2 levels. B: IFN- γ levels. Values are expressed as mean \pm SD from three independent experiments.

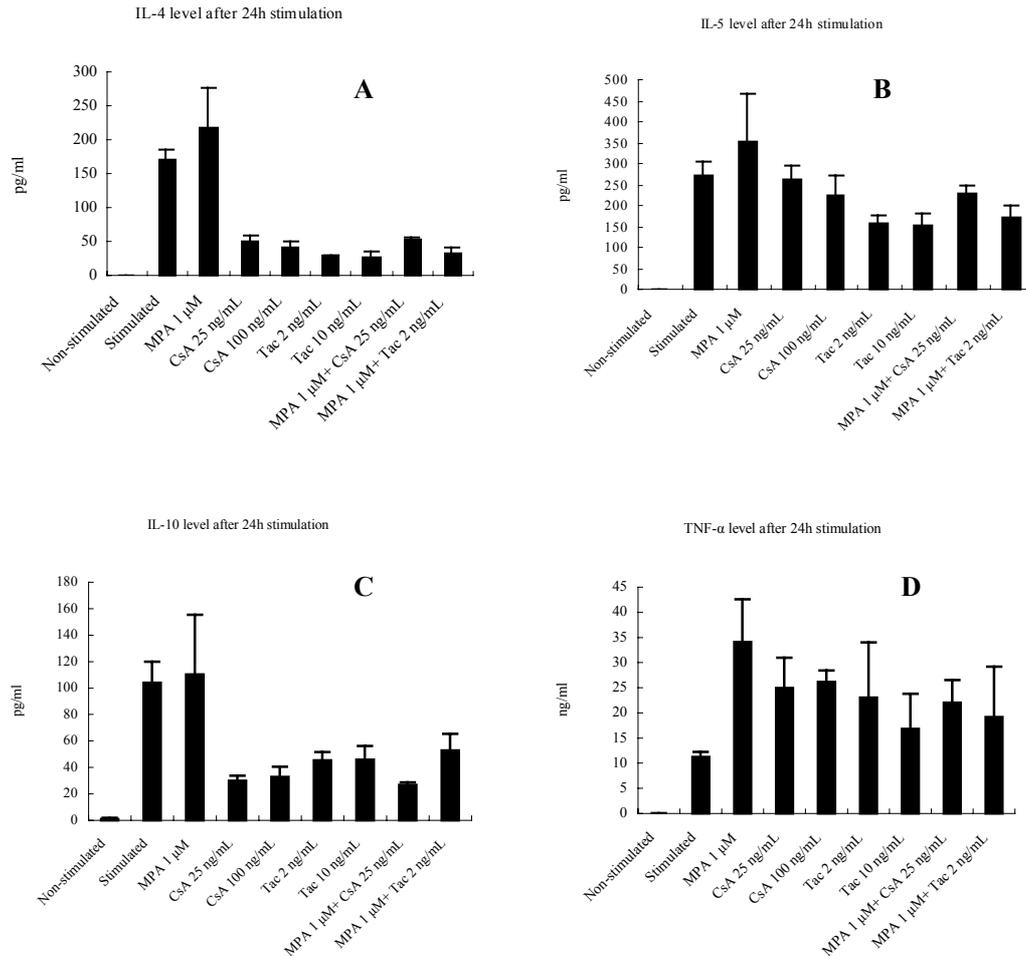


Figure 3.12 Th2 cytokine expression after 24h stimulation. A: IL-4 levels, B: IL-5 levels, C: IL-10, D: TNF- α . Values are expressed as mean \pm SD from three independent experiments.

3.2.5 CNI but not MPA inhibited NFAT-regulated cytokine mRNA expression of lymphocytes

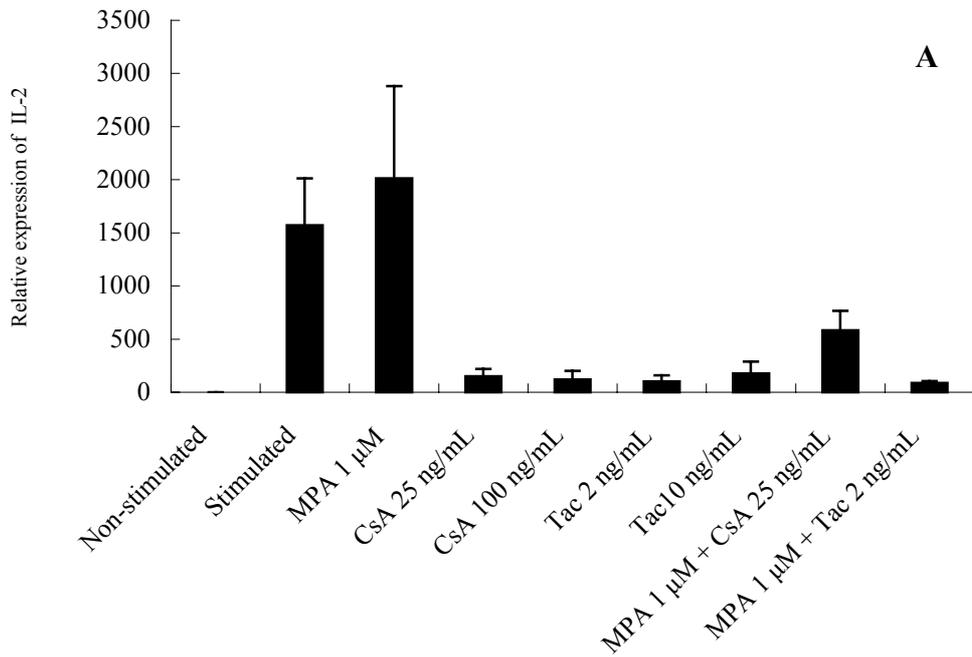
We next analyzed whether MPA or CNI affected NFAT-regulated cytokine (IL-2 and IFN- γ) mRNA or TNF- α mRNA expression (Table 3.9, Figure 3.13). Cytokine mRNA was quantified using a competitive quantitative real-time RT PCR method. The non-stimulated group served as calibrator. After anti-CD3/CD28 antibody stimulation, the expression of IL-2 mRNA was increased to 1500-fold as compared to the non-stimulated group and IFN- γ was increased 760-fold. Addition of MPA did not affect the up-regulation of mRNA expression of the two cytokines. Low dose of CNI or combined MMF/CNI inhibited IL-2 and IFN- γ mRNA expression significantly. However, we didn't find inhibited expression of TNF- α mRNA in this study. This

result is in line with the TNF- α level presented in Table 3.8.

Table 3.9 Relative expression of IL-2, IFN- γ and TNF- α mRNA

Group	IFN- γ	IL-2	TNF- α
1. Non-stimulated	1	1	1
2. Stimulated	761 *	1573 *	37
3. MPA 1 μ M	581 *	2012 *	38
4. CsA 25 ng/mL	32	150	30
5. CsA 100 ng/mL	16	119	39
6. Tac 2 ng/mL	10	102	81
7. Tac 10 ng/mL	7	178	86
8. MPA 1 μ M + CsA 25 ng/mL	59	582	98
9. MPA 1 μ M + Tac 2 ng/mL	10	88	49

* vs groups 1 and 4-9, $P < 0.001$



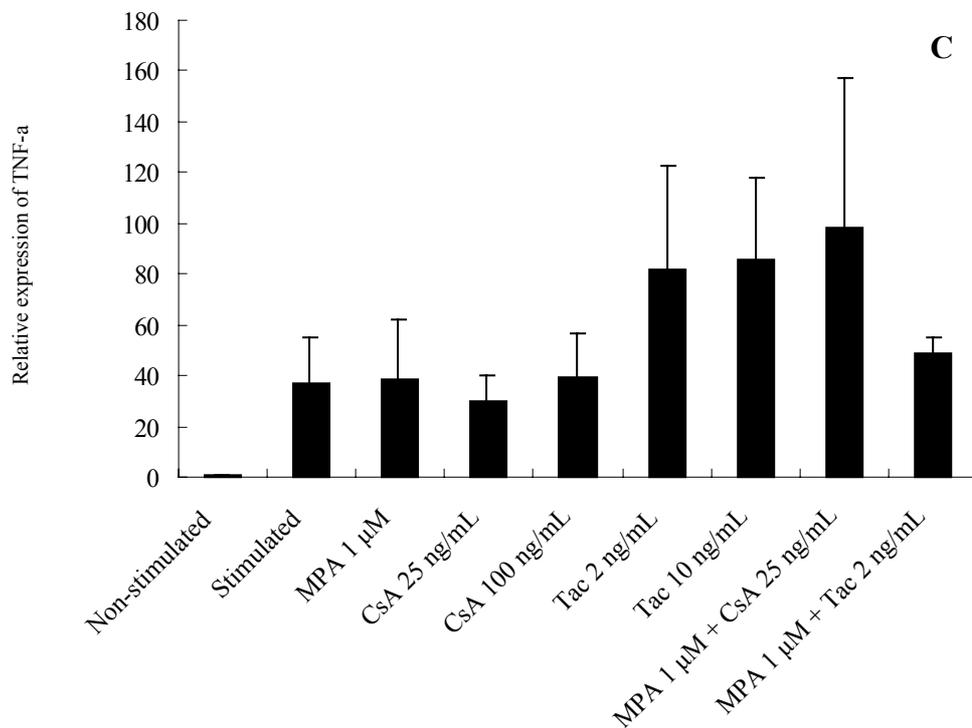
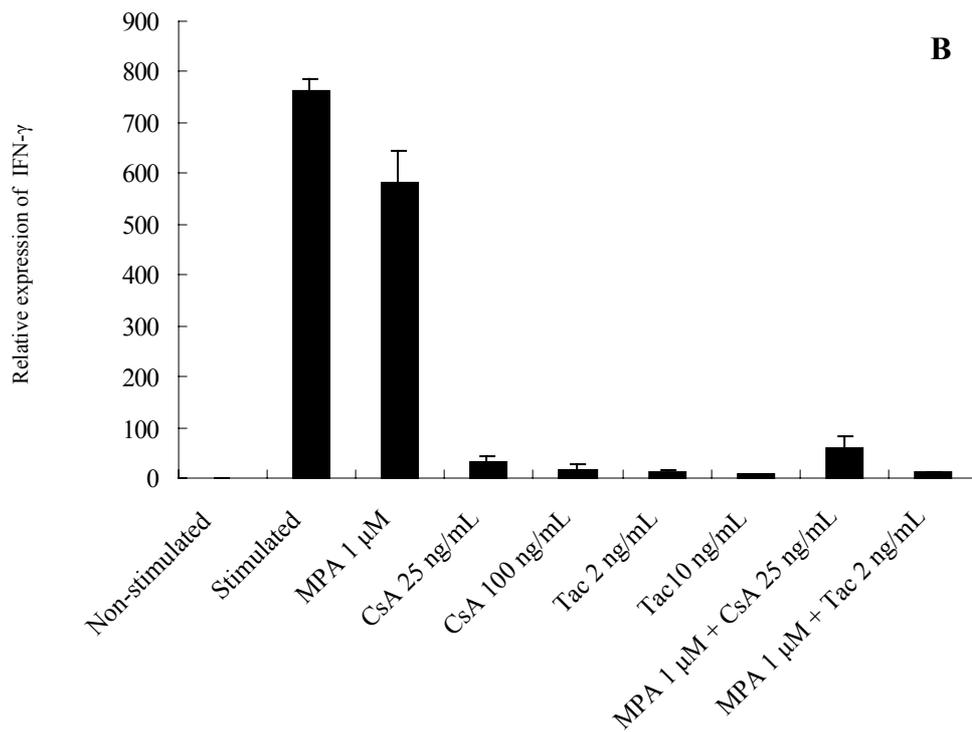


Figure 3.13 Effects of MPA and CNI on the expression of IL-2, IFN- γ and TNF- α mRNA. PBMC were activated with anti-CD3/anti-CD28 antibody in the presence or absence of MPA and/or CNI. The expression of IL-2 (A), IFN- γ (B) and TNF- α (C) mRNA were analyzed by real-time RT PCR. Values are expressed as mean \pm SD from three independent experiments.

4. Discussion

The major effect of immunosuppressive treatment is to prevent the activation-induced proliferation of T cells essential for the immune response (Nakamura et al., 2001). Immunosuppressive therapy in clinical transplantation has developed with the initial use of the triple drug regimen of CsA, prednisolone and AZA. Later Tac and MMF have been introduced immunosuppressants, which effectively prevent allograft rejection with a low incidence of side effects in the impaired natural host defenses. Immunosuppressive drugs are regularly used in distinct combinations, intended to maximize immunosuppression while reducing the adverse effects of each individual drug (Barten et al., 2003).

4.1 Potencies and efficacies of various single immunosuppressive drugs after anti-CD3/anti-CD28-activated lymphocyte function.

4.1.1 Inhibition of lymphocyte function by CNI

CsA and Tac differ in their structure, but show similar actions. CsA and Tac inhibit IL-2-dependent growth and differentiation of activated lymphocytes. CsA causes a different pattern of cell division blockage than MPA. The cells continue to divide, but in lower numbers in each division generation as CsA dosage is increased; MPA, on the other hand, is associated with blockage of progression to multiple division generations. This could be consistent with the major difference in action on activation *per se* (Nakamura et al., 2001).

In this study, neither therapeutic level of CNI (100 ng/mL CsA, 10 ng/mL Tac) nor sub-therapeutic level of CNI (25 ng/mL CsA, 2 ng/mL Tac) completely blocked lymphocyte cell division. However, they reduced the number in each division generation and kept more cells in division 0 (Figure 3.6). For CsA, this effect was dose dependent. Tac blocked 54.9% cells into division at 2 ng/mL concentration and this effect was not enhanced when increasing its concentration to 10 ng/mL.

Both CsA and Tac blocked cells at G0/G1 phase after anti-CD3/CD28-stimulation.

One of the key events during G1 phase is the synthesis of IL-2 by activated T lymphocytes. IL-2 production is required for progression of T cells through G1 into the S phase of the cell cycle (Quemeneur et al., 2002). We hypothesized that sub-therapeutic levels of CNI also inhibited IL-2 effectively and following experiments of NFAT-regulated gene expression confirmed it.

Calcineurin dephosphorylates NFAT family members, allowing them to translocate into the nucleus and activate gene expression through the *cis*-element named NFAT (Flanagan et al., 1991; Northrop et al., 1994; Shaw et al., 1995; Loh et al., 1996; Timmerman et al., 1996). Activated calcineurin also translocates into the nucleus together with NFAT family members, where it may maintain the sustained activation of NFAT proteins (Shibasaki et al., 1996). Among NFAT family members, NFAT1, NFAT2, and NFAT4 are involved in the transcriptional activation of genes encoding cytokines including IL-2, IL-4, CD154 (CD40L) (Ho et al., 1996; Rao et al., 1997) and IFN- γ (Giese et al., 2004). By preventing their calcineurin-mediated dephosphorylation, CNI inhibit the nuclear translocation of these NFAT family members and subsequent gene expression in activated T cells. Undoubtedly, inhibition of the calcineurin-NFAT pathway is one of the mechanisms of CsA/Tac-mediated immunosuppression (Matsuda and Koyasu, 2000). Sakuma et al (Sakuma et al., 2000) noted that anti-CD3/anti-CD28-costimulated IL-2 and IFN- γ mRNA expression in PBMC is significantly inhibited by the addition of low concentrations of Tac or CsA. Hartel et al (Hartel et al., 2004) also reported that the expression of IL-2 mRNA and TNF- α was inhibited by Tac. However, several studies demonstrated that IL-2 expression in purified T cells or peripheral blood lymphocytes, induced by the co-stimulatory B7/CD28 pathway, is resistant to inhibition by Tac *in vitro*, even with drug concentrations of 100 $\mu\text{g/L}$ (Lin et al., 1991; Anderson et al., 1999; Bierer et al., 1991).

In our study, either therapeutic or sub-therapeutic doses of CsA/Tac inhibited IL-2, IL-4, IL-10, IFN- γ expression and IL-2, IFN- γ mRNA expression significantly (Table 3.7, 3.8 and 3.9; Figure 3.11, 3.12 and 3.13). Increased concentrations of CNI did not

show more inhibitory effect on cytokine expression. Previous reports demonstrated that the effects of Tac were evident at concentrations 20-100 times lower than that required for CsA (Andersson et al., 1992). We also noticed the trend that the inhibition effect of Tac on IL-2, IL-4 expression (Figure 3.11 A, figure 3.12A) was stronger than that of CsA although the data were not statistically significant. In contrast with Sakuma (Sakuma et al., 2000) and Hartel (Hartel et al., 2004), we didn't find that CsA or Tac revealed inhibited expression of TNF- α and TNF- α mRNA in this study. Th1 cells preferentially produce IL-2 and IFN- γ , which promote cellular immune response against intracellular pathogens, whereas Th2 cells produce IL-4, IL-5, IL-6 and IL-10, which promote humoral immunity by aiding in B cell activation and differentiation, leading in particular to production of IgG1 and IgE (Viola and Rao, 1999). Our data and other studies showed that CNI do not only inhibit T cell function but also influence the synthesis of B cells by direct inhibition or via the inhibition of Th2 associated cytokines (Stark et al., 2002), even though at sub-therapeutic level.

To assess T cell function, the expression of activation surface markers were studied. CD25 is an IL-2 receptor and presents on a subset of peripheral blood lymphocytes. The density of CD25 increases on PHA, Con A and CD3-activated T lymphocytes. CD69 antigen is present on activated T, B, and NK lymphocytes. CD71 is a human transferrin receptor. The transferrin receptor is essential for iron transport into proliferating cells. CD71 antigen is expressed on all cells upon activation. CD154 (CD40 ligand, CD40L) antigen is a member of the tumor necrosis factor (TNF) receptor family and is transiently expressed on activated CD4 T lymphocytes. The expression of CD154 by activated T-helper cells triggers B-cell cyclin through binding to CD40. This co-receptor interaction is required for B-lymphocyte maturation, response to T-dependent ligands, and immunoglobulin class switching. T-lymphocyte proliferation and generation of CTL is associated with the presence of CD154 (Fuleihan et al., 1994; van Kooten et al., 1994; Banchereau et al., 1994; Nishioka and Lipsky, 1994). CD154-CD40 interaction is an essential step in the initiation of allograft rejection (Van Rijen et al., 2002).

We observed that both CsA and Tac inhibited expression of all activation surface markers *in vitro*, especially CD154. Higher concentrations of CsA further inhibited T-cell surface antigen expression such as CD25, CD71 and CD154. This finding is in accordance with other studies (Van Rijen et al., 2003; Van Rijen et al., 2002; Barten et al., 2001b). No difference was observed at both concentrations of Tac indicating that sub-therapeutic concentration of Tac also has enough inhibitory effect on expression of lymphocyte surface antigens.

Summary: CNI did not block lymphocyte cell division. However, they reduced the T-cell number in each division generation and kept more cells in division 0. Sub-therapeutic level of CNI effectively inhibited NFAT-regulated genes and their proteins expression such as IL-2, IL-4, IL-10 and IFN- γ . Both CsA and Tac inhibited the expression of activation surface markers. However, the inhibition effect of CsA was dose dependent under our experimental conditions.

4.1.2 Inhibition of lymphocyte function by MPA

MPA inhibits inosine monophosphate dehydrogenase (IMPDH), an enzyme of the *de novo* purine pathway. Both T and B cells are preferentially inhibited by the antimetabolic effect of the drug. MPA arrests the cell cycle at G1. Purines control both G1 to S phase transition and progression through the S phase. Purine nucleotides play critical roles in DNA and RNA synthesis as well as in membrane lipid biosynthesis and protein glycosylation. They are necessary for the development and survival of mature T lymphocytes. Activation of T lymphocytes is associated with an increase of purine pools (Quemeneur et al., 2003). Animal models and *in vitro* studies show that B cells are also target cells of MPA. Not only was the proliferation of lymphocytes reduced, the immunoglobulin and cytokine production were also decreased (Jonsson and Carlsten, 2003; Stark et al., 2002). Previous study showed that DNA synthesis in PHA-stimulated PBMC was suppressed completely by 1 μ M MPA (Allison and Eugui, 2000). In clinical studies, the trough levels of MPA were 6-12 μ M (Pou, 2001). In our *in vitro* experiments, we selected 1 μ M and 10 μ M MPA as study concentration.

In our study, the proliferation of lymphocytes was investigated using CFSE staining. The proliferation of lymphocytes induced by anti-CD3/anti-CD28 antibodies was completely inhibited in the presence of 1 μ M MPA (Figure 3.6). MPA affects both CD4 and CD8 in parallel (Figure 3.7). No difference was observed between 1 μ M MPA group and 10 μ M MPA group. Unlike CNI, which allows multiple cell generations but suppression of the numbers of cells in each generation, MPA blocked proliferation into subsequent generations completely. These findings corroborate earlier *in vitro* studies (Ogawa et al., 2001; Jonsson and Carlsten, 2001; Nakamura et al., 2001).

We found that in the presence of MPA, most of the cells did not incorporate BrdU and accumulated in the G0/G1 phase (Fig.3.3). The effect of 10 μ M MPA group was stronger than that of 1 μ M MPA group. Quemeneur et al (Quemeneur et al., 2003) demonstrated that inhibitors of *de novo* purine synthesis (MPA) prevent cell cycle progression and down regulated survival of activated T lymphocytes. Depletion of guanine nucleotide pools after inhibition of IMPDH by MPA potently inhibits cell cycle progression by arresting activated T cells in the G1 phase of the cell cycles. This effect was associated with inhibition of cyclin D3/CDK6 induction and down-regulation of the CDK inhibitor p27^{Kip1} following activation of PBMC (Laliberte et al., 1998; Quemeneur et al., 2002). Addition of purine synthesis inhibitors after activation did not allow further cell divisions in the presence of IL-2, suggesting that clonal expansion of T lymphocytes does not only require an initial purine pool expansion but rather a recurrent *de novo* synthesis of these pools for each division to proceed (Quemeneur et al., 2003). *De novo* purine synthesis does not only control the cell cycle, but also regulated survival of activated T lymphocytes. Inhibitors of *de novo* purine synthesis, like MPA, would prevent clonal expansion if given before and during antigenic stimulation or, alternatively, trigger apoptosis of cycling T cells (Quemeneur et al., 2003).

There was not only suppression of new DNA synthesis by MPA, but also suppression of the expression of several cell surface T cell activation markers (Barten

et al., 2001c; Jain et al., 2001; Van Rijen et al., 2003; Barten et al., 2001a). In our investigation, up-regulation of CD25, CD71 and CD154 induced by anti-CD3/anti-CD28 antibodies were inhibited by MPA (Table 3.5 and 3.6).

Previous studies of MPA's biochemical effects may explain the mechanisms of action by which MMF suppressed expression of specific T-cell surface activation antigens. MPA, which is a specific inhibitor of IMPDH, provides depletion of guanine nucleotides in lymphocytes. This may limit the synthesis of mRNA for these molecules. Because guanine nucleotides are necessary for glycosylation of lymphocyte proteins (Allison and Eugui, 2000; Gummert et al., 1999a) and because these surface antigen molecules are glycoproteins, MPA may alter their glycosylation pattern, resulting in a decrease of expression (Van Rijen et al., 2003). Proliferating lymphocytes depend on both the purine and the pyrimidine pathways to supply ribonucleotides necessary for DNA, RNA and protein synthesis as well as for ribonucleotide intermediates, which are required for proper lipid and protein glycosylation and membrane synthesis (Fairbanks et al., 1995; Ruckemann et al., 1998). In addition, studies in human cell lines showed that MPA suppresses the synthesis of RNA-primed DNA intermediates (Catapano et al., 1995). In other studies, MPA has been shown to suppress guanine nucleotide protein (G-protein) levels (Mustelin, 1987), which are required for activation of some T-cell surface antigens (Lub et al., 1995). MPA also blocks the glycosylation of adhesion molecules (Allison and Eugui, 1996; Laurent et al., 1996); necessary for proper folding and regulation of other T-cell surface antigens (Hayes et al., 1995; Springer, 1990). However, addition of MPA did not modify up-regulation of the early G1 markers CD69 after 72 hours stimulation. Previous studies showed that MPA did not interfere with initial activation events following TCR engagement such as blast transformation or CD69 expression (Quemeneur et al., 2002). Inhibition of lymphocyte antigen expression by MPA has been reported to highly correlating with MMF dose (Gummert et al., 1999a; Barten et al., 2002c). We also found that inhibition of CD154 expression induced by MPA is dose dependent (Table 3.5 and

3.6).

As we expected, MPA did not inhibit expression of NFAT-regulated genes and their proteins at all. Unlike CsA and Tac, which inhibit early stages of lymphocyte activation, including the production of IL-2 (Thomson et al., 1993), MPA concentrations up to 10uM had not detectable effect on production of IL-2, IL-4, IL-5, IFN- γ and GM-CSF (Allison and Eugui, 2000). These findings emphasize the major differences between effects of CNI and MPA on lymphocytes. Recently, Weimer et al (Weimer et al., 2003) showed that MMF therapy suppressed LPA stimulated IL-10 and TNF- α secretion in long-term renal transplant patients. IL-10 may have deleterious effects with respect to both acute and chronic rejection. IL-10 acts as a potent B-cell growth and differentiation factor and may augment humoral immune responses against the graft (Rousset et al., 1992; te Velde et al., 1992; Nonoyama et al., 1993; Llorente et al., 1995). MMF treatment may be expected to have beneficial effects not only on acute but also on chronic rejection as a result of suppression of IL-10 secretion. However, the exact phenotype and mechanisms of action of regulator T cells remain controversial (Womer et al., 2001). The Th1 and Th2 paradigm which holds that rejection is dependent on the expression of Th1 cytokines (IL-2, IFN- γ) and that tolerance is dependent on Th2 cytokines (IL-4, IL-5, IL-10 and IL-13) has been recently challenged. The existence of specific cytokine gene knock out mice indicates that Th1 cytokines may function not only to initiate cell-mediated immune responses but also to regulate T-cell responses (Womer et al., 2001).

Summary: In this study, we demonstrated the inhibition effect of MPA on lymphocyte proliferation and on the expression of cell surface antigens. The immunosuppressive effect of MPA in clinical transplantation therefore may be related not only to inhibition of lymphocyte proliferation but also to the inhibition of cell surface antigen expression, which may be an important additional factor in limiting the alloresponse. MPA has no effect on expression of NFAT-regulated genes and their proteins.

4.2 Inhibition of lymphocyte function by immunosuppressant combination

4.2.1 Synergistic effects of MPA with CsA and Tac *in vitro*

Our *in vitro* studies revealed a greater overall inhibition of lymphocyte function for the combination MPA plus CsA or Tac. *In vitro*, addition of MPA to low concentrations of CsA (25ng/mL) or Tac (2ng/mL) led to a synergistic effect on inhibition of lymphocyte function. Analysis of CFSE staining showed complete inhibition of lymphocyte division when MPA combined with CsA or Tac (Table 3.3, figure 3.6). This effect was equal to MPA treatment alone. In combination with MPA, CsA or Tac acted synergistically at low concentrations to inhibit lymphocyte proliferation. More cells were blocked in G0/G1 phase than MPA or CNI monotherapy groups. Increasing concentrations of MPA enhanced inhibitory effect and 86-91% lymphocytes were blocked in G0/G1 phase (Table 3.4, Figure 3.8). This seems to be related to the capacity of PBMC to divide in response to residual IL-2 produced in the presence of these levels of CNI, whereas the cell cycle blocking effect of MPA under these *in vitro* conditions is more complete (Ogawa et al., 2001).

An increased maximal inhibition of T-cell surface antigens expression was noticed after combination drug therapy compared with MPA or CNI monotherapy. MPA showed an effective dose dependent inhibition of the expression of T-cell surface antigens (Table 3.5 and 3.6, Figure 3.9). MPA plus CNI, especially with Tac, almost completely inhibited expression of CD154 on activated T cells.

Our data indicated that Tac and CsA specifically suppress inflammatory cytokine production triggered by T cell activation. However, addition of MPA did not show additive inhibitory effect on the expression of NFAT regulated cytokine as well as on the expressions of their mRNA.

Barten et al (Barten et al., 2003) recently showed that the total inhibitory effect on both lymphocyte proliferation and activation was enhanced when MPA was combined with either CsA or Tac, compared to CsA, Tac mono-treatment. For the combination of Tac plus MPA, the extent of inhibition of lymphocyte function was greater

compared to the effect of the other drug combinations. In combination with CsA or Tac, MPA acted synergistically at low concentrations to inhibit lymphocyte proliferation. Increasing concentrations of MPA antagonized the CsA or Tac effect on lymphocyte proliferation. MPA concentrations potentiated synergistically the effect of Tac inhibiting the expression of T cell surface antigens. In contrast, in combination with CsA the effect of MPA on T cell surface antigens was antagonistic regardless of the concentrations used. However, contrary to Barten's results, we found that either low concentration of CsA or Tac was synergistic in combination with MPA in the inhibition of lymphocyte proliferation and expression of activation surface antigens. We assume that different drug combinations and distinct stimulation of lymphocyte are the main cause of differences between two experiments.

4.2.2 Monitoring of surface markers of PBMC in liver transplant recipients

Because the clinical relevance of combination therapy is certainly more important, we compared the CNI monotherapy with combination therapy of MPA with low dose of CNI (MMF/CNI reduction group). Interestingly, we found that the percentage of CD8 cells (CTL), CD56⁺CD3⁺NK T cells and memory T cells (CD4⁺CD45RO⁺) significantly decreased in MMF/CNI reduction group. The expression of CD71 on CD4 cells was significantly reduced in MMF/CNI reduction group. We also found a trend of decreased CD154 expression on CD4 cells. This result is in accordance with previous studies which also demonstrated that MPA in a dose-related fashion inhibits the generation of CTLs and the rejection of allogeneic cells (Allison and Eugui, 2000).

Generation of antigen-specific CTL is a major immunological effector mechanism in allograft rejection. Today, it seems clear that CD8⁺ CTL play an important role in allograft rejection (Rocha et al., 2003). CD8⁺ CTL are primed and activated by recognition of donor MHC class I antigens. These antigens also serve as targets for the mature cytolytic effector. In the prevailing view, recipient CD8⁺ T cells are primed by direct presentation of donor antigens by 'passenger' APCs from the graft (Arakelov and Lakkis, 2000). Although donor APCs are the major pathways for CTL

induction, Kreisel et al (Kreisel et al., 2002) reported a novel mechanism of direct antigen presentation by activated donor vascular endothelial cells. This pathway can induce responder CD8⁺ T cells with an effector phenotype that is sufficient to cause acute rejection. Although the role of class I MHC antigens is unequivocal, the requirement for co-stimulatory molecules in the allogeneic priming of CTL is controversial. After cardiac transplantation, blockade of CD40–CD154 (CD40L) interactions has been variously reported to impair (Zhai et al., 2003) or to have no effect (Jones et al., 2000) on activation of allospecific CD8⁺ T cells.

CD40 and its ligand CD154 represent conventional members of a second family of co-stimulatory molecules that belong to the TNF receptor and TNF superfamilies, respectively (Larsen and Pearson, 1997; Yamada and Sayegh, 2002). CD40 is constitutively expressed on APCs, such as B cells, macrophages and dendritic cells, and can also be induced on endothelial cells and fibroblasts (Larsen and Pearson, 1997). CD154 is expressed after activation on CD4⁺ T cells, as well as on some CD8⁺ T cells, NK cells, and eosinophils (Rothstein and Sayegh, 2003). While there is limited evidence to suggest that CD154 directly provides signals to the T cell, CD40 plays a major role in the activation and maturation of B cells and dendritic cells (Blair et al., 2000; van Essen et al., 1995). Thus, the CD40-CD154 interaction initially provides costimulatory signals to APCs rather than T cells. However, because such costimulation significantly augments the ability of APCs to present antigen, it strongly enhances T-cell activation (Rothstein and Sayegh, 2003). Thus, inhibition of expression of CD154 by MPA may have benefit for organ transplant recipients by interfering with the “second signal” of T cell activation.

In MMF/CNI reduction group, the percentage of $\gamma\delta$ TCR⁺ T cells was significantly reduced. $\gamma\delta$ TCR⁺ T cells are relatively frequent in mucosal epithelia and have a specific repertoire of T-cell receptors biased towards certain bacterial/viral antigens. Current opinion is that these cells may have an important role in protecting the mucosal surfaces of the body. We hypothesize that over suppression of $\gamma\delta$ TCR⁺ T cells by immunosuppressants may make patients more susceptible to some

opportunistic infections.

Summary: All our *in vivo* and *in vitro* data indicated that combination of MPA and sub-therapeutic levels of CNI increased the overall inhibition of lymphocyte function compared with CNI monotherapy. The synergistic effect of MPA with CNI does not only inhibit directly lymphocyte proliferation and activation marker expression, but blocks cytokine production as well. This effect of MPA is dose-dependent (Barten et al., 2002a).

4.3 Further study

Current therapeutic challenges associated with immunosuppressive drugs are directed toward minimizing toxicity through the use of effective combination therapy with new drugs such as MMF. On account of the narrow therapeutic ranges of CsA and Tac, the wide intra- and interindividual variations in their pharmacokinetics, as well as the potential for significant drug-drug interactions, monitoring of blood levels and monitoring of the immunosuppressive drug effects over time are recommended to optimize therapy (Dambrin et al., 2000). Several studies revealed that trough MPA levels differ significantly according to the type of CNI (Cantin et al., 2002; Pou et al., 2001). MMF also induces alterations of CsA metabolism (Pape et al., 2003). Infectious complications with the use of MMF have been questioned due to the perceived implication of increased immunosuppression (Smallwood et al., 2002). Therefore, monitoring of individual drug levels does not, however, provide enough information on the combined immunosuppressive effects of the different immunosuppressive drug protocols.

Barten et al (Barten et al., 2002b) found that dose-dependent inhibition of lymphocyte function was higher for the combination of Tac and MMF than after a single drug treatment *in vivo*. High correlations were found between the pharmacodynamic (PD) parameters AUC_{0-24} and I_{max} of lymphocyte function and drugs, thus suggesting a “diagnostic range” that may be a useful means to monitor drug combination therapy.

MPA levels were related to efficacy and major adverse events in liver graft

recipients. Plasma MPA concentrations within patient groups were not closely correlated with the dose of mycophenolate mofetil but were dependent upon the indication for prescribing MMF, age and serum albumin and creatinine concentrations (Tredger et al., 2004). Tredger et al (Tredger et al., 2004) reported that a therapeutic range of predose MPA plasma levels of 1 to 3.5 mg/L (by immunoassay) is applicable in liver allograft recipients. The potential for MPA toxicity at higher predose plasma levels is in agreement with the findings in renal transplant recipients of Mourad and colleagues (Mourad et al., 2001) who identified at 3 mg/L threshold using ROC curve analysis. MMF with the benefit of MPA monitoring, only 6.3% were considered to need 1.5 g twice a day clinically, and 44.7% were prescribed less than 1 g twice a day (Tredger et al., 2004). The drug levels were also influenced by the type of immunosuppressive comedication (Shaw et al., 2002). In a study of 61 children, MPA clearance was greatest during CsA comedication, least during Tac comedication and intermediate when MMF was given without either CNI agent. The underlying mechanism is suggested to be an inhibition of the excretion and enterohepatic recirculation of MPA glucuronide by CsA compared with inhibition of mycophenolate glucoronidation by Tac (Brown et al., 2002; Zucker et al., 1999). In a study of 33 renal transplant patients whose immunosuppression was based on triple therapy regimen (MMF, CsA and steroids), 21% patients required one or more dose adjustments (Shaw et al., 1998). MPA PD exhibits large inter- and inpatient variability and may be altered in specific patient populations due to changes in protein binding, concomitant disease states, or interactions with concurrent immunosuppressants. Therefore, clinical PD monitoring can provide more information regarding efficacy of MMF than clinical judgment alone (Cox and Ensom, 2003). Lymphocyte function may be used to measure PD of immunosuppressants *in vivo* and could be helpful to optimize immunosuppressive therapy in transplanted patients. Additional monitoring of PD data of MPA such as AUC_{0-24} and C_{max} may help to avoid side effects associated with over-immunosuppression (Shaw et al., 2002).

4.4 Conclusion

Synergistic effects on inhibition of lymphocyte function were observed for the combination of MPA plus CNI by blocking cells in the G0/G1 phase of the cell cycle, decreasing activation marker expression and NFAT-regulated gene expression. Adjustment of the MMF dosage by detection of the AUC₀₋₂₄ may be required to avoid over-immunosuppression. FACS analysis and assessment of cytokine expression of PBMC represent important tools for monitoring and optimizing the immunosuppressive regimen within the transplant setting.

5 Summary

Complications of Calcineurin inhibitor (CNI), in particular nephrotoxicity, have a major effect on morbidity and mortality within the transplant setting. We randomized liver transplant patients with renal dysfunction under CNI treatment to either a) receive mycophenolate mofetil (MMF) up to a dose of 2 g/day followed by consecutive reduction of CNI (CNI reduction group) or b) to continue their current CNI dose (control group). Peripheral blood mononuclear cell (PBMC) were isolated from patient blood at baseline and after conversion to MMF/low dose CNI regimen. The expression of CD3, CD4, CD8 on PBMC and the expression of activation marker was determined by FACS analysis. In *in vitro* experiments freshly isolated PBMC from healthy volunteers were treated with mycophenolic acid (MPA) (1 up to 10 μ M), cyclosporine A (CsA) (25-100 ng/mL), tacrolimus (Tac) (2-10 ng/mL) and combined CsA/MPA or Tac/MPA. T cell proliferation was measured by carboxyfluorescein diacetate succinimidyl ester and FACS analysis. T cell cycle analysis was performed by bromodexoxyuridine staining and FACS. Furthermore activation marker and nuclear factor of activated T cell (NFAT)-regulated cytokine expression was analyzed by FACS. The expression of cytokine mRNA was measured by real-time RT PCR. Combined MMF and low dose CNI therapy leads to reduction of percentages of CD8⁺ T cells, CD4⁺CD45RO⁺ T cells, CD3⁺CD56⁺ T cells and inhibition of activation marker expression. *In vitro* experiments have demonstrated that 1 μ M MPA completely blocks the proliferation of CD4 and CD8 T cells. MPA stops the cell cycle of activated T cells at G0/G1 phase and this effect is dose-dependent. Both MPA and CNI inhibit the expression of activation T cell markers and this effect is potentiated by combined exposure to MPA and CNI. MPA has no detectable effect on NFAT-regulated genes such as IL-2, IL-4, IFN- γ and their protein expression. Our results suggest that FACS analysis and assessment of cytokine expression of PBMC represent important tools for monitoring and optimizing the immunosuppressive regimen within the liver transplant setting. Adjustment of the MMF dosage by detection of the AUC may be required to avoid over-immunosuppression. Combined MMF and low dose CNI exhibit anti-proliferative effects in T cells by a) blocking the S phase of the cell cycle, b) decreasing NFAT-regulated gene and activation marker expression.

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7. Abbreviation

7-AAD	7-amino-actinomycin D
APCs	antigen-presenting cells
APC	allophycocyanin
AUC	area-under-the concentration-time curve
AZA	azathioprine
BCR	B-cell receptor
BrdU	bromodexoxyuridine
BSA	bovine serum albumin
CFSE	carboxfluorescein diacetate succinimidyl ester
C _{max}	maximal concentration
CNI	calcineurin inhibitor
CsA	cyclosporine A
CTL	cytotoxic T lymphocyte
DEPC	diethylpyrocarbonate
DMSO	dimethylsulfoxide
ELISA	enzyme-linked immunosorbent assay
ET	endothelin
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
HLA	human leucocyte antigen
IFN	interferon
IL	interleukine
I _{max}	maximal inhibition
IMPDH	inosine monophosphate dehydrogenase
MHC	major histocopatibility complex
MMF	mycophenolate mofetil
MPA	mycophenolic acid
NFAT	nuclear factor of activated T cell
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline
PD	pharmacodynamic
PE	phycoerythrin
PerCP-Cy5.5	peridinin chlorophyll protein-cyanin5.5
ROC	receiver operator characteristic
mRNA	messenger riboneucleic acid
RT PCR	reverse transcriptase polymerase chain reaction
Tac	tacrolimus
TCR	T-cell receptor
Th cell	helper T cell
TNF	tumor necrosis factor

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Publications:

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