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Effects of two classes of immunosuppressive agents, mTOR inhibitors vs. calcineurin inhibitors, on the generation and function of human alloreactive T helper cells (Th1, Th17 and Treg)

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Ho pensato molto ai ringraziamenti di questa tesi e sono giunta alla conclusione che probabilmente mi servirebbe piu' di una pagina o addirittura un'ulteriore tesi per poter ringraziare una per una tutte le persone che mi sono state vicine e mi hanno supportata durante il mio corso di dottorato. Quindi ho deciso di racchiudere i miei ringraziamenti in due piccole parole: Italia e America.

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Some things will always be stronger than time and distance, deeper than languages and ways, like: "Following your dreams and learning to be yourself, sharing with others the magic you have found."

Sergio Bambaren:"The Dolphin: Story of a dreamer"

SUMMARY

Successful transplantation requires the prevention of allograft rejection and complete reduction of the effects on the immune system by any immunosuppressive agent. The commonly immunosuppressive drugs used in clinical such as calcineurin inhibitor (tacrolimus, TAC) and mTOR inhibitor (sirolimus, SRL) affect naïve T cell differentiation and memory T cell expansion; however, the biological and biochemical changes induced by these drugs on the generation and expansion of different subpopulations of T helper cells are not fully elucidated yet. CD4 positive ($CD4^+$) T cells are known to orchestrate and regulate adaptive immune responses and play an important role in allograft rejection or tolerance. $CD4^+$ T cells, upon activation and expansion, develop into different effector T helper cell subsets and produce distinct cytokine profiles and mediate separate effector functions. From a functional perspective, $CD4^+$ T cells can be classified into effector T helper cells (Th1, Th2, Th17, Tfh) and regulatory T cells (Tregs).

In the transplant setting, prevailing evidence shows that both effector Th1, Th17 cells and cytokines IFN-γ, IL-17 are involved in the process of allograft rejection whereas Treg and Th2 cells favor long-term graft survival. The effects of Tacrolimus and Sirolimus on these subsets were studied because a balance between graft-destructive effector T cells and graft-protective regulatory T cells toward dominance of Tregs may promote clinical transplant tolerance. Therapies targeting inhibition of pathogenic effector T cells, promotion of Treg cells and directing against the mediators of intragraft inflammation may have profound effects on the rejection process and induce long-term graft acceptance.

In this study, alloreactive $CD4^+$ T cells in a MLR culture (Mixed-Lymphocyte reaction) with responder naïve T cells and allogeneic APCs (antigen presenting cells), were generated. Alloreactive CD4+ T cells were enriched and restimulated with autologous APCs plus anti-CD3 stimulation in the absence or the presence of TAC or SRL or their combination. Although both TAC and SRL inhibit alloreactive T helper cell proliferation and various cytokine productions, the intensity and kinetics for TCR-induced T helper subpopulations are markedly differently affected between the two drugs. TAC at 2-5ng/ml significantly inhibited over 90% of the productions of IFN-γ and IL-17 from the supernatants of bulk cell cultures, and the percentages of IFN-γ (Th1) and IL-17-secreting cells (Th17), whereas SRL at high concentration (20ng/ml) had moderate inhibition on IFN-γ and IL-17 productions (30% and 60% respectively). When IL-2 was added to the culture, TAC still exerted similar inhibition while SRL completely lost its inhibition on IFN-γ expression. In contrast, in the presence of IL-2, FOXP3 expressing cells (Tregs) were markedly increased in SRL treatment compared to the controls (Averagely 2-fold increase), whereas TAC treatment was not changed and did not show a decreased trend. When the two drugs were used in combination, we found that TAC at 2ng/ml with SRL at 2-5ng/ml achieved the maximal effect in inhibiting the productions of IFN-γ and IL-17 while maintaining a high level of FOXP3 expression. SRL treatment did not affect the plasticity or reprogramming of Tregs, but significantly decreased $FOXP3^+$IFN-γ and $FOXP3^+IL\text{-}17^+$ populations when used in combination with very low dose of TAC. When an inflammatory setting was mimicking by adding proinflammatory cytokines (IL-1, IL-6, TNF-α) to the cell culture, there was a significant

decrease of the generation of SRL-derived FOXP3^{+}Treg cells. SRL-derived Tregs expressed normal Treg surface markers, were anergic to allostimulations, and functionally suppressed the proliferation of allogeneic effector T cells, and Th1 and Th17 alloimmune responses. Without affecting FOXO3/FOXP3 interaction, SRL markedly decreased DNMT1 expression. DNMT1 is a FOXP3 promoter demethylation and it may account for its long-term induction. Furthermore, it is a prerequisite for stable FOXP3 expression and suppressive phenotype of Tregs. These findings can help to guide the clinical use of immunosuppressive drugs to promote Treg expansion and to control Th1 and Th17 alloimmunity.

RIASSUNTO

Il successo di un trapianto richiede la prevenzione del rigetto d'organo e la completa riduzione degli effetti sul sistema immunitario da parte di qualsiasi agente immunosoppressore.

I piu' comuni farmaci immunosoppressori usati in clinica al giorno d'oggi, sono gli inibitori della calcineurina (tacrolimus, TAC) e gli inibitori mTOR (sirolimus, SRL) che agiscono a livello immunitario sulla differenziazione e l'espansione delle cellule linfocitarie T *naïve*; però i cambiamenti biologici e biochimici indotti da questi farmaci sulla generazione e l' espansione di diverse sottopopolazioni di cellule T helper non sono ancora state chiarite completamente.

Le cellule linfocitarie T CD4 positive ($CD4^+$), sono conosciute come cellule che regolano la risposta immune adattativa e giocano un ruolo importante nel rigetto dei trapianti e nella tolleranza immunologica. Queste cellule, mediante attivazione, possono indurre la produzione di differenti sottopopolazioni di cellule T effettrici e delle loro differenti citochine.

Le cellule T $CD4^+$, dunque, possono differenziarsi nelle seguenti sottopopolazioni: cellule effettrici T helper (Th1, Th2, Th17, Tfh) e cellule T regolatrici (Treg). Nello studio dei trapianti e dei rigetti vi sono forti evidenze che mostrano che cellule linfocitarie Th1 Th17 e le loro citochine IFN- γ, IL-17 sono coinvolte nel processi di rigetto, mentre cellule linfocitarie Treg (T regolatrici) e Th2 e le loro citochine favoriscono la sopravvivenza a lungo termine dell'organo trapiantato.

Considerando che un bilancio, tra le cellule effettrici Th1 Th17 (con il ruolo di distruggere e attaccare immunologicamente l'organo trapiantato) e le cellule T effettrici Treg e Th2 (con il ruolo di proteggere l'organo

trapiantato), potrebbe promuovere lo sviluppo della tolleranza clinica al trapianto; studiare gli effetti di Tacrolimus e Sirolimus su queste quattro differenti popolazioni cellulari risulta fondamentale per permettere il controllo della risposta imunologica in soggetti trapiantati.

In questo studio, sono state prima generate cellule alloreattive $CD4^+$ T in una coltura MLR (Reazione di Mix Linfocitario) con cellule T *naïve* autologhe e APC allogeniche. Le cellule alloreattive $CD4^+$ vengono ristimolate con cellule autologhe APC autologhe e stimolate con anti-CD3 in assenza o presenza di TAC, SRL o entrambi i farmaci. Sebbene sia TAC che SRL inibiscano la proliferazione delle cellule alloreattive T *helper* e la produzione di diverse citochine, l'intensità e la cinetica con le quali le sottopopolazioni di cellule T sono influenzate e' differente. TAC ad una concentrazione di 2-5ng/ml inibisce significativamente oltre il 90% della produzione di IFN-γ e IL-17, prodotti dalle cellule Th1 e Th17, mentre SRL utilizzato ad alta concentrazione (20ng/ml) ha un'inibizione moderata sulla produzione di IFN-γ e IL-17 (rispettivamente del 30% e 60%). Quando IL-2 veniva aggiunto alla cultura cellulare, TAC continuava ad esercitare ancora un' inibizione, mentre SRL perdeva completamente la sua inibizione sull'espressione di IFN-γ. Al contrario, in presenza di IL-2, le cellule esprimenti FOXP3 (Tregs) erano piu' numerose (di almeno il doppio) nelle colture trattate con SRL, mentre nelle colture cellulari trattate con TAC non si notava alcuna modifica o alcun decremento di cellule Treg. Quando i due farmaci sono stati usati in combinazione, utilizzando TAC a 2ng/ml e SRL a 2-5ng/ml, veniva raggiunto il massimo effetto nell'inibire la produzione di IFN-γ e IL-17, pur mantenendo un alto livello di espressione di FOXP3. Il trattamento con SRL non influenzava la plasticità o riprogrammazione delle cellule T

regolatrici (Treg), ma significativamente diminuiva $FOXP3^+$ IFN-γ e $FOXP3^+$ $IL\text{-}17^+$ popolazioni quando usato in combinazione con dosi molto basse di TAC. Inoltre, quando un ambiente infiammatorio viene ricreato con l'aggiunta di citochine proinfiammatorie (IL-1, IL-6, TNF-α) nella coltura cellulare, si può notare una diminuzione significativa della generazione di cellule Treg $FOXP3^+$. Cellule Treg derivanti dalla cultura contenenti SRL esprimevano normale marcatori di superficie per le cellule Treg, e funzionalmente sopprimevano la proliferazione di cellule T effettrici allogeniche, e le risposte Th1 e Th17 alloimmuni. Senza influenzare l'interazione FOXO3/FOXP3, SRL marcatamente riduceva l'espressione di DNMT1, che può spiegare la stabilità di espressione di FOXP3, in quanto promotore di demetilazione di FOXP3, sottolineando che questo è un prerequisito per la stabile espressione di FOXP3 e per il fenotipo soppressivo delle cellule Treg. In conclusione, questi risultati possono aiutare a guidare l'uso clinico di farmaci immunosoppressori per promuovere l'espansione di cellule Treg e per controllare la risposta allo-immune da parte delle cellule Th1 e Th17, considerando inoltre che le cellule Tregs sono ritenute fondamentali per la tolleranza immunologica.

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INTRODUCTION

1.1 *Transplantation and the rejection mechanisms*

Transplantation is the act of surgically removing an organ, cells or tissue, called a graft, from one individual and placing into a different individual. Transplantation occurs because the recipient's organ has failed or has been damaged through illness or injury. The individual who provides the graft is called the donor, and the individual who receives the graft is called either the recipient or the host. In clinical practice, transplantation is used to overcome a functional or anatomic deficit in the recipient and this approach to treatment of human diseases has increased steadily during the past 40 years, and transplant of kidneys, hearts, lungs, livers, pancreas and bone marrow is widely used today.

There are several different kinds of graft:

- Autologous graft: tissue transferred from one site on the body to another in the same individual
- Syngeneic graft: tissue transferred between genetically identical individuals
- Allogeneic graft: tissue transferred between genetically individuals of the same species
- Xenogeneic graft: tissue transferred between different species.

The molecules that are recognized as foreign or allografts are called allo-antigens, and those on xenograft are called xenoantigens.

Some of the problems in transplantation are the surgical difficulties (largely overcame, now), the graft rejection (still a major problem) and the lack of available organs.

Alloantigens elicit both cell-mediated and humoral immune responses and the major histocompatibility complex (MHC) molecules are responsible for almost all strong (rapid) reactions. In fact, MHC molecules play a critical role in normal immune responses to foreign antigens, namely, the presentation of peptides derived from protein antigens in a form that can be recognized by T cells. In fact foreign MHC molecules (especially the class I molecules) present a strong barrier to transplant survival, and it has been estimated that 5% to 10% of an individual's CD8 positive (CD8+) T cells can recognize and bind fragments of foreign MHC class I.

So, the recipient immune system recognizes peptide fragments presented by MHC class I or II molecules, whether those fragments are derived from infectious organisms or from the degradation of self-molecules encoded by host genes. In the case of transplanted tissues, the genes of the engrafted cells may encode no-self molecules that also can be detected by the recipient immune system and function as histocompatibility antigens. T cells can be detected and be activated against histocompatibility antigens through two different pathways of recognition: direct and indirect.

Direct presentation of alloantigens.

The direct recognition involves antigen presentation by donor antigen-presenting cells (APCs) to recipient T cells. Direct recognition can occur when some of the MHC class I or II molecules on the donor cells are identical to those on recipient cells. Like other cytosolic proteins, MHC class I and II molecules can be degraded by proteosomes and the resulting fragments presented on the cell surface by intact MHC class I molecules. If the donor and recipient have MHC class I molecules in common, APCs of donor origin may be able to present those peptide fragments directly to

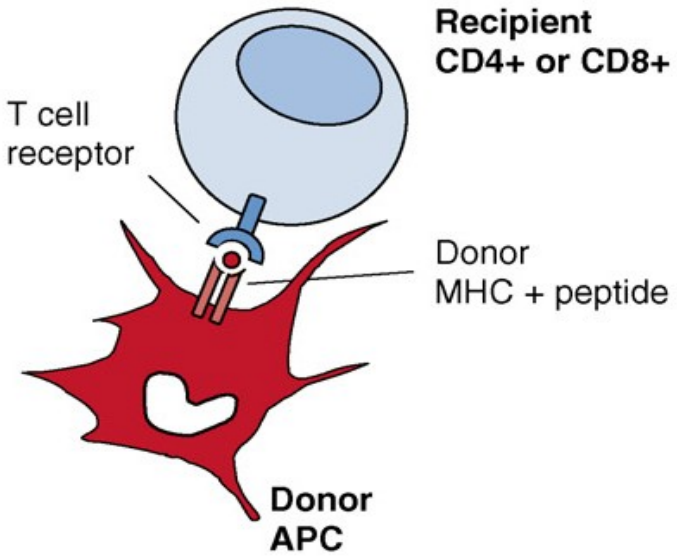
the TCRs of recipient CD8+ T cells. Because the MHC class I molecules on the donor cells are the same as those present in the host thymus during thymic education, the recipient TCRs are able to recognize and bind the pMHC I molecules on the donor cells. Direct recognition may also occur if donor APCs ingest cellular debris of donor origin and process/present it via MHC class II molecules to recipient to recipient $CD4^+$ T cells (Fig.1) (Gould and Auchincloss 1999)

Indirect presentation of alloantigens:

The indirect recognition involves antigen presentation by recipient APCs to recipient T cells. Indirect alloantigeneic recognition occurs when allogeneic MHC molecules from graft cells are taken up and processed by recipient APCs, and peptide fragments of the allogenic MHC molecules containing polymorphic amino acid residues are bound and presented by recipient (self) MHC molecules.

Thus the recognition of foreign histocompatibility antigens and activation of the T cells against them involve processes very similar to those involved in the initiation of responses against antigens derived from infectious organisms. Indeed, the recipient immune system may view the transplanted cells as just another batch of infected cells-infected by no-self genes (Fig. 1) (Gould and Auchincloss 1999)

A.



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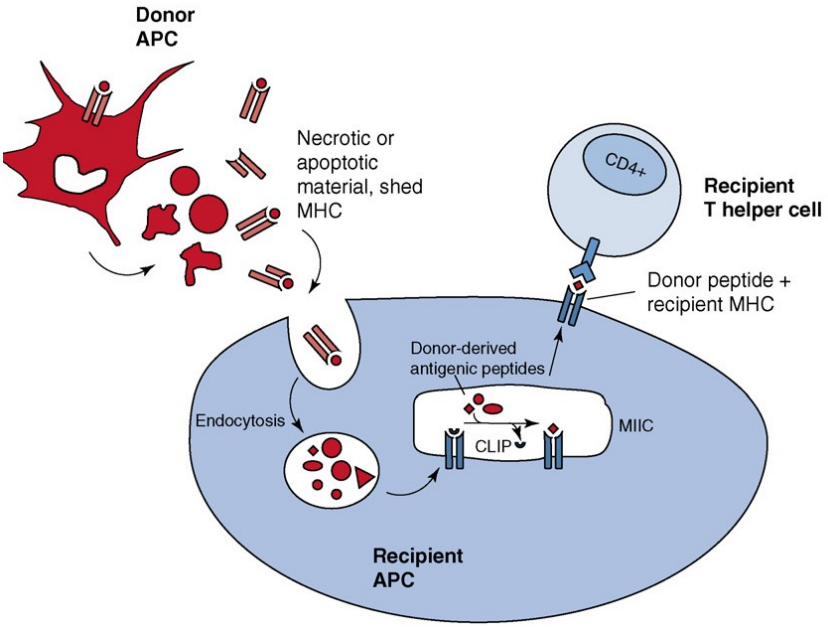


Figure 1: Direct and Indirect pathway

It is possible to say that a major limitation in the success of transplantation is the immune response of the recipient to the donor tissue, in fact the failure of graft is caused by an inflammatory reaction, called rejection.

Graft rejection occurs when the recipient's immune recognizes the graft as foreign, and destroys it.

Rejection responses fall into three general categories: hyper-acute, acute, and chronic, depending upon timing and intensity. Each type involves particular sets of immune responses and is determined in part by the genetic mismatch between donor and recipient.

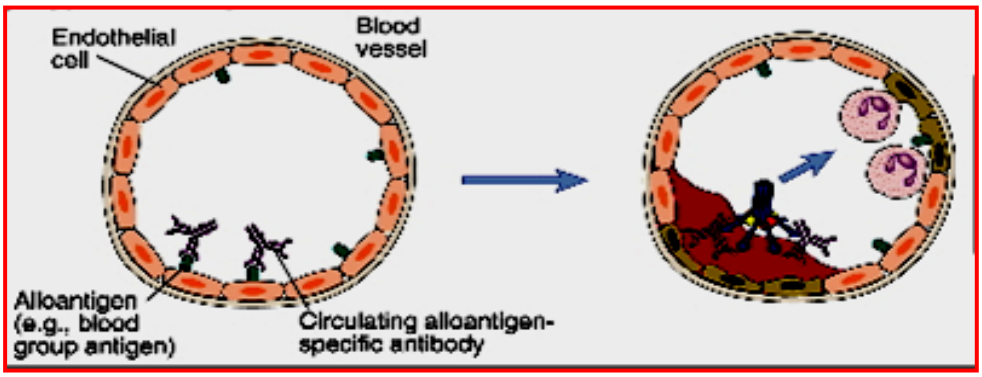
Hyperacute rejections are the most rapid type of rejection. They are initiated and completed within a few days of graft placement, usually before the grafted tissue or organs can establish connection with the recipient vasculature. In fact, hyperacute rejection is characterized by thrombotic occlusion of the graft vasculature that begins within minutes to hours after host blood vessels are anastomosed to graft vessels and is mediated by pre-existing antibodies in the host circulation that bind to donor endothelial antigens (Abbas, Lichtman et al 2011). The immune attack is typically mediated (in various situations) by complement, natural killer (NK) cells, and/or pre-existing antibodies (Fig.2A).

Acute rejections occur much sooner after graft emplacement than do chronic rejections. The graft establishes vascular connection and function normally for a relatively short period of time (e.g., two to four weeks) before the first signs of rejection appear. Unlike chronic rejections, acute rejections proceed rapidly once underway. Acute rejection is a process of vascular and parenchymal injury mediated by T cells and antibodies that usually begins after the first week of transplantation. Effector T cells and

antibodies that mediate acute rejection develop during a few days or weeks in response to the graft, accounting for the time on onset of acute rejection. T lymphocytes play a central role in acute rejection by responding to alloantigens, including MHC molecules, present on vascular endothelial and parenchymal cells. The activated T cells cause direct lysis of graft cells or produce cytokines that recruit and activate inflammatory cells, which injure the graft (Abbas, Lichtman et al 2011) (Fig.2B).

Chronic rejections are the slowest and the least vigorous type of rejection. The transplanted tissue or organs establish a vascular connection and proceed to function for weeks, months, and even years before signs of deterioration due to immune attack become evident. Chronic rejection is characterized by fibrosis and vascular abnormalities with loss of graft function occurring during a prolonged period. As therapy for controlling acute rejection has improved, chronic rejection has emerged as the major cause of allograft loss. The pathogenesis of chronic rejection is less well understood that of acute rejection. The fibrosis of chronic rejection may result from immune reactions and the production of cytokines that stimulate fibroblasts, or it may represent wound healing after the parenchymal cellular necrosis of acute rejection. Perhaps the major cause of chronic rejection of vascularized organ graft is arterial occlusion as a result of proliferation of intimal smooth muscle cells. This process is called accelerated (or graft) arteriosclerosis and it is frequently seen in failed cardiac and renal allografts (Abbas, Lichtman et al 2011) (Fig.2C).

A.



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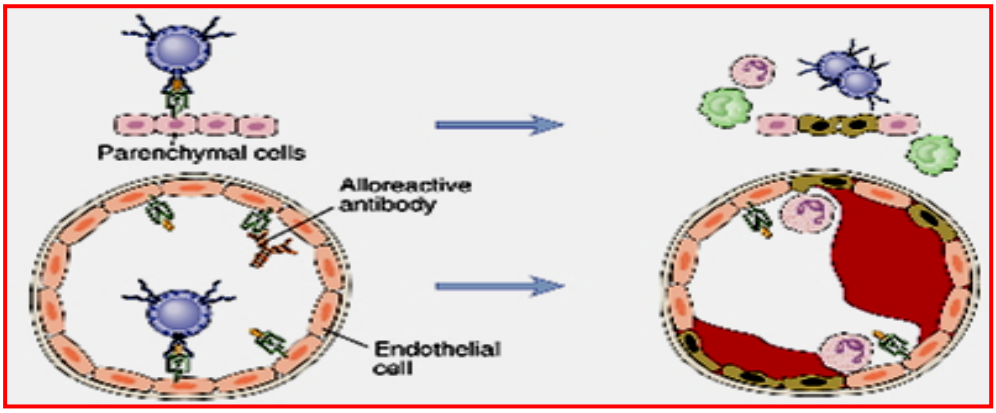

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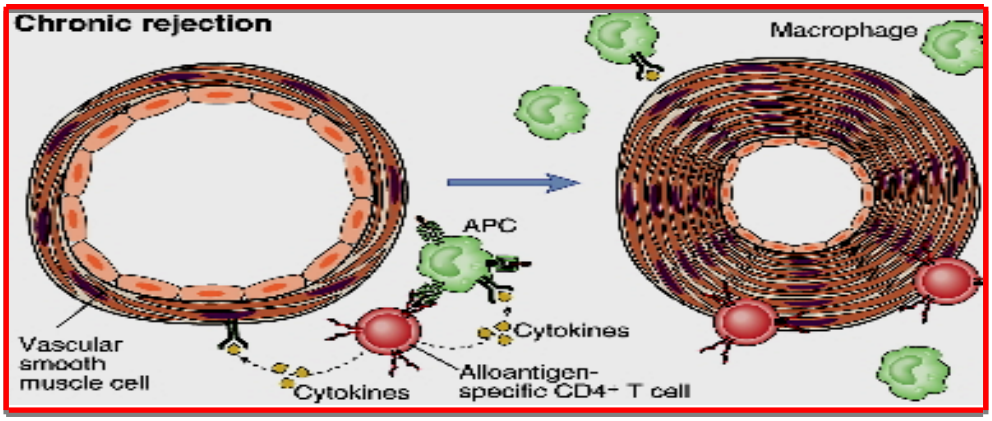


Figure 2: Type of graft rejection. A. Hyperacute rejection, B. Acute and C. Chronic.

1.2 Immunosuppressive agents in solid organ transplantation

Effective immunosuppression is an essential pre-requisite for successful organ transplantation and improvements in outcome after transplantation have to a large extent been dependent of developments in immunosuppressive therapy. Immunosuppressive drugs that inhibit or kill T lymphocytes are the principal treatment regimen for graft rejection. Organ transplantation is now the optimal treatment for many patients with end-stage organ failure and national and international registries report 1-year graft survival rates of around 85% after kidney, liver, and heart transplantation (Denton, Magee et al. 1999).

The current success of organ transplantation is in very large part attributable to advances in immunosuppressive therapy and very few allograft are now lost as a result of acute rejection.

A historical perspective on the development of immunosuppression for organ transplantation will be analyzed, with a focus of individual mechanism of action and efficacy.

The first successful kidney transplant was in 1954, performed by Murray and co-workers, only possible because the donor and recipient were monozygotic twins. The immune system was slowly being characterized, but there were no effective immunosuppressive agents. The breakthrough in chemical immunosuppression for transplantation come with the observation that 6-mercaptopurine (6-MP) could induce immunological unresponsiveness to a foreign protein (human serum albumin) (Hanidziar and Koulmanda 2010). Around the same time, a number of nucleotide analogous was created in the hope of finding novel chemotherapy agents.

One of the compounds, BW57-322 (Azathioprine), stood out in terms of efficacy and tolerability. Azathioprine was much less toxic than 6-MP and

afforded better prolongation of allograft survival. It rapidly moved into clinical use and, while better than total or subtotal body irradiation, it was not potent enough to permit most recipients to keep their graft. Also the corticosteroids were used to try to prolong graft survival, and it was only when corticosteroids were combined with azathioprine in the early 1960s that effective chemical immunosuppression became a reality.

Azathioprine and steroids remained the mainstay of immunosuppression for the next 25 years, as efforts were made to develop compounds that affected lymphocyte function. It took the discovery of cyclosporine in 1976 for thoracic organ and liver transplantation to be truly successful. Cyclosporine was initially studied for its potential as an anti-fungal compound, but when it was discovered to have potent anti-lymphocytes properties its development was temporarily halted. Although initially used alone, Cyclosporine proved more successful when combined with steroid and azathioprine as triple therapy.

The 1970s were also notable for the development of monoclonal antibodies (mAbs), for example, OKT3 (muromonab-CD3), a mouse anti-human CD3 mAb was used initially to treat acute rejection, and is still used occasionally for steroid resistant acute rejection or as a induction agent (Schreiber and Crabtree 1992).

Other two agents with interesting results were identified in the late 1980s, namely Tacrolimus (FK506) and Sirolimus (rapamycin).

And now we will explain their mechanisms of action:

Calcineurin inhibitors (CNIs) Cyclosporine and Tacrolimus are licensed for use in organ transplantation. The molecular mechanisms whereby CNIs inhibit T cell activation are well understood. T cell receptor engagement with donor MHC/peptide normally triggers calcium-

dependent intracellular signaling resulting in activation of calcium/calmodulin-dependent phosphatase calcineurin (Fig.3). This leads to the dephosphorylation of NF-AT allowing translocation into the nucleus where it enhances binding of transcription factors to genes encoding for pro-inflammatory cytokines such as IL-2, IL-3, IL-4, IFN-γ and TNF-α. After entering the cytoplasm, CNIs form complexes with their immunophilins. Cyclosporine binds to cyclophilin and tacrolimus bind to the 12 kDa FK506-binding protein (FKBP-12). The CNI-immunophilins complex, inhibits the calcineurin activity, and hence prevents nuclear translocation NF-AT and cytokine gene transcription. The net result is that CNIs block the production of cytokines such as IL-2 and inhibit T cell activation and proliferation, which so it leads to a reduced function of both effector T cells and Tregs (Schreiber and Crabtree 1992; Demirkiran, Hendrikx et al. 2008).

The introduction of cyclosporine into clinical practice ushered in the modern era of transplantation.

Before the use of cyclosporine, the majority of transplanted hearts and livers were rejected, but now the majority of these allografts survive for more than 5 years.

Sirolimus and Everolimus (The mTOR inhibitors) belong to the group of immunosuppressive agents called mammalian target of rapamycin (mTOR) inhibitors. Both drugs are macrocyclic lactones, with Sirolimus being a naturally occurring fermentation product of the actinomycete Streptomyces hygroscopicus, while Everolimus represents a chemical modification of sirolimus to improve absorption. Sirolimus (SRL) and Everolimus (EVL) bind to the 12 kDA intracellular immunophilin FK506 binding protein (FKBP12) but, unlike Tacrolimus, do not inhibit

calcineurin activity. Instead the SRL/FKBP12 and EVL/FKBP12 complexes are highly specific inhibitors of mammalian target of rapamycin (mTOR). MTOR is a serine/threonine kinase involved in the phosphadyt-inositol 3-kinase (P12K)/AKT (protein kinase B) signaling pathway (Fig.3). Inhibition of mTOR has a profound effect on the cell signaling pathway required for cell-cycle progression and cellular proliferation. mTOR inhibitors directly bind the mTOR Complex 1 (TORC1) and inhibit the PI3K/AKT/mTOR signaling, which is part of CD28 costimulatory and IL-2 receptor signaling pathways known for fully T cell activation, thus results in decreased phosphorylation mTOR/p70S6 kinase and 4E-BP BP (Abraham and Wiederrecht 1996). Long-term treatment with mTOR inhibitors also affects TORC2, which is required for fully activation of AKT kinase (Sarbassov, Ali et al. 2006; Janes, Limon et al. 2010). Activation of AKT via PI3K/AKT/FOXO signaling may directly phosphorylate FOXO family transcription factors, subsequently excludes them from the nucleus, and thus diminishes their coactivating function for de novo FOXP3 induction whereas the PI3K/AKT/mTOR signal phosphorylation of p70S6K and 4E-BP1 may account for its anti-proliferation effect on effector T cells (Ouyang, Beckett et al. 2010; Merkenschlager and von Boehmer 2010) with an increasingly important role for mTOR in directing T cell activation and differentiation has become apparent. Further dissecting the underline mechanisms for the induction/expansion Tregs by mTOR inhibitors and the environmental effects on the differentiation, activation and proliferation of CD4 Th cells may develop novel strategies to prevent graft rejection and promote the induction of tolerance to the transplant (Mitchell, Afzali et al. 2009; Li and Turka 2010; McMurchy, Bushell et

al. 2011). So, combinations of cyclosporine (which blocks IL-2 synthesis) and rapamycin (which blocks IL-2 driven proliferation) are potent inhibitors of T cell responses.

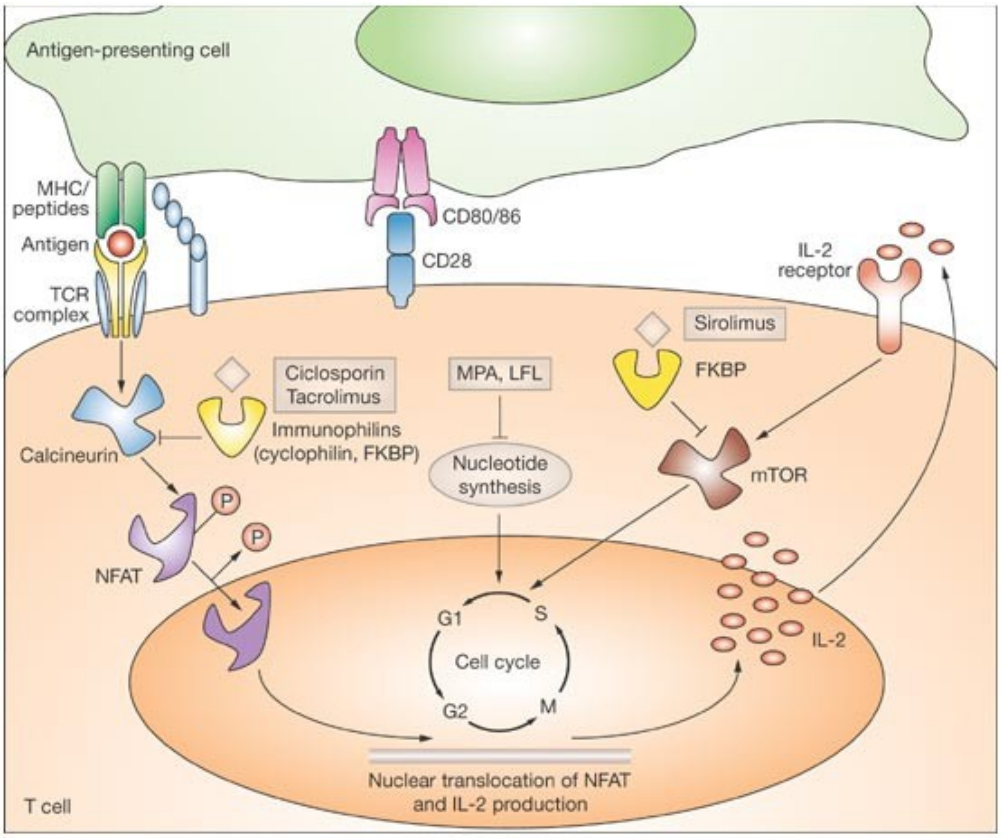


Figure 3: CNI vs mTOR inhibitors
Mechanisms of action of maintenance immunosuppressive agents. CNIs (ciclosporin and tacrolimus) bind to their respective immunophilins, and inhibit calcineurin. Calcineurin is then unable to dephosphorylate NFAT, which will prevent translocation of NFAT to the nucleus and thereby production of IL-2. Sirolimus is an mTOR inhibitor. It binds to FKBP and inhibits mTOR, which in turn inhibits transition of the cell cycle from G1 to S phase. MPA and LFL are also cell-cycle inhibitors, and act via inhibition of nucleotide synthesis. Abbreviations: CNI, calcineurin inhibitor; FKBP, FK506-binding protein; IL-2, interleukin-2; LFL, leflunomide; MHC, major histocompatibility complex; MPA, mycophenolic acid; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; TCR, T cell receptor.

1.3 Functional diversity of helper T lymphocytes (Th1 Th2 Th17)

Despite the dramatic advances of modern immunosuppression in reducing acute graft rejection, long-term allograft survival has remained disappointing. The current pharmacological agents that so effectively prevent acute graft rejection are inadequate for averting late graft loss caused by chronic rejection. The mechanism of action of most of these agents is based on preventing T cell activation and/or proliferation, but no drug directly targets the differentiation of $CD4^+$ effector T cells.

Until recently, it was believed that there were two distinct types of effector $CD4^+$ T helper (Th) cells based on the types of cytokines they produced when stimulated to differentiate (Abbas, Murphy et al. 1996) Th1 cells, which produce large quantities of Interferon-γ (IFN-γ), considered to be the major mediators of allograft rejection, and, Th2 cells characterized by the production of Interleukin-4 (IL-4), IL-5, and IL-13 have been proposed to favor long-term graft survival in some models. However, the validity of the Th1/Th2 paradigm in transplantation has been questioned. More recently, a new Th cell subtype was identified that produced IL-17 and was also found to mediate allograft rejection in T-bet knockout recipients of bm12 cardiac grafts (O'Shea and Paul 2010). Moreover, a regulatory $CD4^+$ T cell phenotype (Treg) was also discovered with dominant production of IL-10 and TGF-β, associated with marked capability of suppressing pathogenic effector T cells in the transplant setting. From a functional perspective, CD4 T cells can be classified into effector T helper cells (Th1, Th2, Th17, Tfh) and regulatory T cells (Tregs) (Fig.4).

In sum, immune homeostasis in transplantation seems to be the result of the balance among different T helper subtypes.

In the transplant setting, prevailing evidence shows that both effector Th1, Th17 cells and cytokines IFN-γ, IL-17 involve in the process of allograft rejection whereas Treg and Th2 cells favor long-term graft survival although it is not currently possible to draw any conclusions regarding a specific role for these cells (Strom and Koulmanda 2009; Mitchell, Afzali et al. 2009; Li and Turka 2010; Wood, Bushell et al. 2011) therefore, a balance between graft-destructive effector T cells and graft-protective regulatory T cells toward dominance of Tregs may promote clinical transplant tolerance (McMurchy, Bushell et al. 2011). On the other hand, the environmental factors, especially pro-inflammatory cytokines, prevent the commitment of donor-activated T cells into Treg lineage, instead they foster generation of the Th1/Th17 phenotypes. In addition, the inflammatory milieu may disarm existing Treg cells, converting them into inflammatory effector T cells, or may render them resistant to suppression. Therefore, therapies targeting inhibition of pathogenic effector T cells, promotion of Treg cells and directing against the mediators of intragraft inflammation may have profound effects on the rejection process and induce long-term graft acceptance (Hanidziar and Koulmanda 2010; Li and Turka 2010).

Recent data has shown that Cyclosporin A (CsA) blocks IL-15-mediated production of IL-17 in the joints of rheumatoid arthritis patient. CsA inhibits activity of human IL-17 promoter via NFAT as a crucial sensor of TCR signaling in the IL-17 promoter (Mitchell, Afzali et al. 2009). Further, CsA decreased CD3 induced IL-17 production in a dose dependent manner (Li and Turka 2010). Sirolimus (SRL) has been reported to selectively expand both murine and human functional natural Tregs in vitro while depleting $CD4^{+}CD25^{-}$ effector T cells. On the other

hand, SRL but not CsA permits thymic generation and peripheral preservation of murine Tregs (Wood, Bushell et al. 2011). Further, SRL-conditioned dendritic cells are poor stimulators of allogeneic CD4+ T cells, but enrich for antigen-specific $Foxp3^+$ T regulatory cells and promote murine cardiac tolerance. Using reporter mice for Treg marker FOXP3, demonstrated that SRL promotes de novo conversion of alloantigen-specific Treg cells, whereas CsA completely inhibits this process. Upon transfer in vivo, converted Treg cells potently suppressed the rejection of donor but not third party skin grafts. Thus, the differential effects of SRL and CsA on Teff and Treg cells favor the use of SRL in shifting the balance of aggressive to protective type allo-immunity.

SRL, by inhibiting mTOR activity, blocks almost completely, mitogen and cytokine-induced proliferation of T effector cells. However, its effect, as stated above, on the inhibition of proliferation is less profound in CD25hi Tregs, and in following mitogenic activation they survive and by selection expand (Hippen, Merkel et al. 2011). The reason for these differential effects of SRL on each T cell subset is uncertain, but potential mechanisms have been suggested. One proposed explanation is that the PI3K/Akt/mTOR pathway is activated to a lesser extent in Treg cells after activation with IL-2, in comparison to conventional T effector cells, and therefore that Treg cells use alternative survival pathways independent of mTOR (Hippen, Merkel et al. 2011; Tresoldi, Dell'Albani et al. 2011). Interestingly, it has also been suggested that activation of the PI3K/Akt/mTOR pathway may even be detrimental to the function of Tregs. Indeed, when Akt is overexpressed in naïve T cells, the expression of FoxP3 is inhibited after stimulation with TGF-β and IL-2. It is

therefore possible that the PI3K/Akt/mTOR pathway may inhibit the differentiation of T cells into regulatory $CD4^+$ T cells.

We are now starting to recognize how different classes of immunosuppressive agents can differentially alter the alloimmune responses. It is then imperative to study how different immunosuppressive agents can modulate the immune system so that appropriated combinations and/or modifications of immunosuppressive drugs can be used to prevent graft loss due to rejection or to chronic calcineurin inhibitors (CNIs) nephrotoxicity.

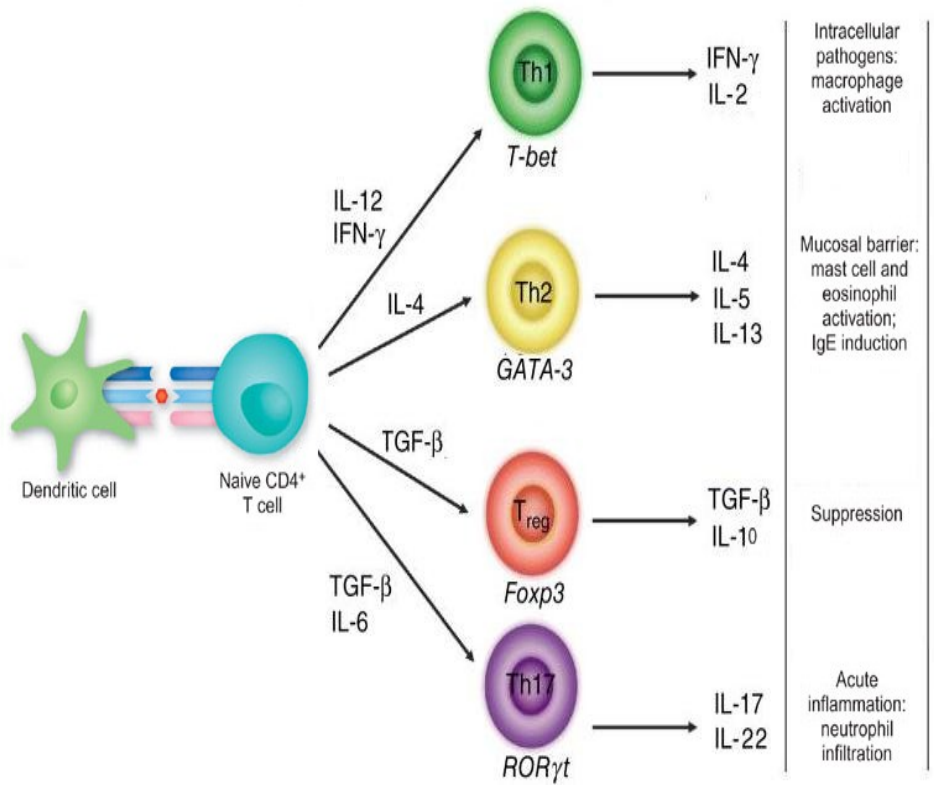


Figure 4: New paradigm for T helper cell differentiation.

1.4 Scientific hypothesis and aim of the work

Despite the improvements in immunosuppression leading to a reduction in acute rejection rates, long-term allograft survival remains disappointing. CD4+ T helper cells are known to orchestrate and regulate adaptive immune responses and play a key role in allograft rejection/tolerance. From a functional perspective, they can be classified into CD4+ effector T cells (Th1, Th2, Th17) and $CD4^+$ regulatory T cells (Treg). While IFN-γ producing effector T cells are major mediators of graft rejection (Th1), Th2 cytokine producing T cells and Treg have been considered protective of the allograft. More recently, IL-17-producing cells were also demonstrated to be involved in allograft loss.

Based on that, we may conclude that the outcome of the allograft is dependent on the balance among these different T cell subtypes and manipulation of T helper cell differentiation might permit increased graft acceptance.

Little is known about the impact of immunosuppressive drugs on human allospecific T cell subpopulations. In this work we will generate different subpopulation (Th1, Th17 and Treg) of human alloreactive $CD4^+$ T cells and test the effects of mTOR inhibitors vs CNIs alone and in different combination on the generation of Th1, Th17 and Treg cells. We will also study, in the system, the role of inflammatory cytokines in altering the balance of Tregs towards Th1/Th17.

In this study, we were interested in how alloactivated T cells responded to immunosuppressive agents and their differentiation/expansion to different T cell subsets and exerted their functions in alloimmunity.

By assessing in vitro cytokine production by alloreactive $CD4^+$ T helper subsets from healthy donors, it will be possible to show that although both

TAC and SRL inhibited alloreacitve T helper cell proliferation and various cytokine productions, the intensity and kinetics for TCR-induced T helper subpopulations are differently affected between the two drugs.

Using an in vitro system, we will study the effects of mTOR inhibitors vs CNIs alone and in different combination on the generation of Th1, Th17 and Treg cells. The hypothesis is: mTOR inhibitors cause a differential effects on human Th1, Th17 and Treg compared to CNIs and the combination of these two immunosuppressive agents tested at different concentrations will allow us to achieve a maximal effect in controlling Th1 and Th17 responses while maintaining and sparing Treg function subsets (Hippen, Merkel et al. 2011; Tresoldi, Dell'Albani et al. 2011). Furthermore, if SRL decreased the expression of DNMT1, which can epigenetically modify DNA methylation in the *FOXP3* locus, this drug might account for a gradual accumulation of the $FOXP3^+$ population and a suppressive phenotype of Tregs in this system (Huehn, Polansky et al. 2009; Josefowicz, Wilson et al. 2009; Daniel, Wennhold et al. 2010).

MATERIALS AND METHODS

2.1 Reagents

Sirolimus (SRL) and Tacrolimus (TAC) were obtained from Axxora (San Diego, CA). Recombinant human IL-1β, IL-6 and TNFα and IL-10 neutralizing antibody were purchased from R&D system (Minneapolis, MN). Anti-CD3 monoclonal antibody (UCHT1) and anti-CD28 monoclonal antibody (L293) were obtained from BD Biosciences (San Diego, CA). Anti-CD3/CD28 coated microbeads were obtained from Invitrogen (Carlsbad, CA). PMA (phorbol 12-myristate 13-acetate), and Ionomycin (Ionomycin calcium salt from Streptomyces conglobatus), EDTA (Ethylenediaminoetracetic acid disodium salt) and LPS were obtained from Sigma-Aldrich (St. Louis, MO). Culture medium RPMI 1640 1x with L-glutamine, PBS 1x (Phosphate-Buffered Saline), Lymphocyte Separation Medium, Sodium-Pyruvate, MEM (non-essential aminoacids) were provided by Mediatech, Inc. 1M Hepes Buffer in normal saline, 2-Mercaptoethanol were purchased from VWR. MACS Separation columns (LS columns), CD14 Microbeads human, CD4 Microbeads human, CD45 Microbeads human were provided by MACS Miltenyi Biotech. Human Regulatory T cell staining Kit (Ache some antibody) were provided by eBioscience. FBS (fetal bovine serum) was provided by ATLAS biological

2.2 Cell culture

Medium RPMI 1640 with L-glutamine was supplemented with 10% heat-inactivated FCS (HyClone Laboratories), 100 U/ml penicillin/100 µg/ml streptomycin, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 1x nonessential amino acids, 0.05mM β-

mercaptoethanol (all from MediaTech). The cell cultures were incubated at 37°C in an atmosphere of 5% CO2.

2.3 Magnetic isolation of monocytes and T cell subsets

PBMC were isolated by Ficoll-Hypaque gradient centrifugation of heparinized venous blood obtained from a group of 30 healthy volunteers. The Informed consent was obtained from each subject, and research protocols were approved by the Institutional Review Board of Northwestern Memorial Hospital in accordance with regulations mandated by the Department of Health and Human Services.

Cells recovered from the gradient interface were washed one time at 2000 rpm and twice at 1200 rpm, with RPMI 1630.

All isolations of monocytes and T cell subsets were performed using magnetic beads and reagents from Miltenyi Biotec. The buffer for monocytes and T cell subsets selection washing was 1xPBS pH 7.2, 0.5 % FBS, 2mM EDTA.

$CD14^+$ monocytes were first isolated from PBMC by immunomagnetic positive selection of $CD14^+$ and the flow through was used for $CD45RA^+$ naïve T cell selection ($CD14^+$ and $CD45RA^+$ Cell Isolation kits) according to the manufacturer's instructions. Memory-like $CD45RO^+$ T cells were purified from the primary culture by depletion of $CD45RA^+$cells and positive selection of $CD4^+$ T cells (CD4+ cell isolation kit), $CD4^+CD25^{high}CD127^-$ Tregs were purified from SRL-treated monocyte/T cell culture (see below). The purity of each separated population was assessed by immunofluorescence flow cytometry, and the purity was >95% in all experiments.

2.4 MLR culture

As we have discussed, graft rejection is often a mediated process that is initiated by recognition of allogeneic MHC molecules. The *mixed leucocytes reaction* (MLR) is a useful in vitro model of direct T cell recognition of allogeneic MHC gene products and is used as a predictive test of cell/mediated rejection. MLR is induced by culturing mononuclear leukocytes cells (which include T cells, B cells, natural killer cells, mononuclear phagocytes, and dendritic cells) from one individual with mononuclear leukocytes derived from another individual, and in humans, these cells are typically isolated from peripheral blood. (Abbas, Lichtman et al. 2011)

Purified $CD4^{+}CD45RA^{+}$ naïve T cells (1.5 x 10^{6}/well) and allogeneic CD14+ cells (7.5 x 10^{5}/well) were co-cultured for 7 to 11 days in 24-well culture plates in complete RPMI 1640. Effector/Memory $CD45RA^{-}$ T cells were then purified and rested for overnight.

2.5 Secondary Monoyte/T cell culture

Alloresponsing memory-like $CD4^{+}CD45RA^{-}$ T cells (1 x 10^{5}/well) co-cultured with 100ng/ml of soluble anti-CD3 antibody and autologous CD14 cells (0.5 x 10^{5}/well) in 96-well U-bottom plates in the presence or absence of 100U/ml IL2 and for 5-6 days. In some experiments, TAC, SRL, or the combinations of the two agents were added at the beginning of the cultures. Different inflammatory cytokines IL-1 (10ng/ml), IL-6 (20ng/ml), or TNF- (50ng/ml) were added alone or in combination in the presence of TAC or SRL or the combinations of the two agents.

2.6 Cell surface and intracellular staining

In these experiments, in the monoyte/T cell culture, cells were restimulated with 20ng/ml PMA and 500 ng/ml ionomycin for 6 h. During the last 4 h, GolgiStop (BD biosciences) was added to cultures to prevent cytokine secretion.

Cells were first stained for surface markers with fluorochrome-conjugated CD4, CD25, CD127, CD45RO, CTLA-4, GITA Abs (all from BD Biosciences). For intracellular IFN-γ and IL-17 staining, cells were followed fixed and permeabilized with a BD Cytofix/Cytoperm Fixation/Permeabilization Solution Kit and incubated with PE-conjugated anti-IL-17 and FITC-conjugated anti-IFN (all from BioLegned). For intracellular FOXP3 staining, after appropriate surface staining, cells were fixed and permeabilized with eBioscience Human Regulatory T cell Staining Kit and incubated with FITC-anti-IFN- γ, -IL-10 and PE-anti-IL-17, TGF-β (R & D system) and PE-Cy5-conjugated anti-FOXP3 (PCH101) or primary rabbit anti-DNMT1 (sc-20701, Santa Cruz, CA) for 30 min at 4°C followed with PE-anti-rabbit Ig together with PE-Cy5-FOXP3. For phosflow staining of phospho-FOXO3a and phospho-AKT, at the end of monoyte/T cell culture, cells were harvested, washed and fixed for 10 min at 37°C using Cytofix buffer (BD Biosciences), pelleted, and permeabilized in PERM III buffer (BD Biosciences) for 30 min on ice. The cells were washed twice in staining buffer (BD Biosciences) and rehydrated for 30 min on ice in the staining buffer. Cells were stained with anti-pFOXO3a (Ser253) antibody for 30 min at room temperature. Washed twice and were restained with FOXP3–PE-Cy5, anti-pAKT (Ser473)–PE or anti-rabbit-Ig–PE antibodies for 30 min at room temperature. Data were acquired on a FACSCalibur flow cytometer and analyzed by FlowJo software (Tree Star, Ashland, OR).

The flow cytometer is an instrument that can be used to analyze specific cell populations. One or two laser, as well as light detectors, are used to gather information about the cells as they are acquired. To gather information about each cell individually, the flow cytometer uses hydrodynamic focusing to prevent multiple cells from passing through the laser at the same time. In short, the cell sample is in a fluid that is injected into the center of a cylinder of sheath fluid from the flow cytometer. As they move forward, their path narrows, causing the cells to line up in a row to pass in front of the laser. Two properties of the cells that can be investigated are size and granularity (complexity). The size of the cells is measured by the *Forward Scatter* (FSC) of the light as it passes through the cell. The granularity (complexity) is measured by the *Side Scatter* (SSC) of the light as it passes through the cell. Figure 4 illustrates the laser light passing from left to right and being deflected to the forward or side light detectors (Fig.5).

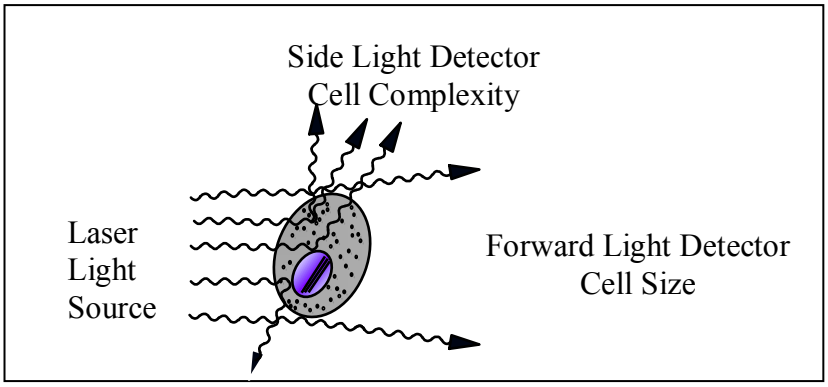


Figure 5. Forward and Side Scatter

In addition to cell size and complexity, additional light detectors can measure the light emission from stains used to label the cells. The

fluorochromes used in these stains are *excited* by the laser, and *emit* a different wavelength of light. When using several stains, their fluorochromes are all excited by the same laser, but they emit different specific wavelengths of light from each other, allowing the light detectors to detect each stain individually. Using the data collected, plots and histograms can be used to identify and analyze cell populations of interest.

When reading the data output from the flow cytometer, the first plot you will want to look at is Forward Scatter (FSC) vs. Side Scatter (SSC). As showed in Figure 6, there are several populations of cells (clusters of dots) that are present. It will be important to know which population the cells you are interested in looking at are located in order to proceed.

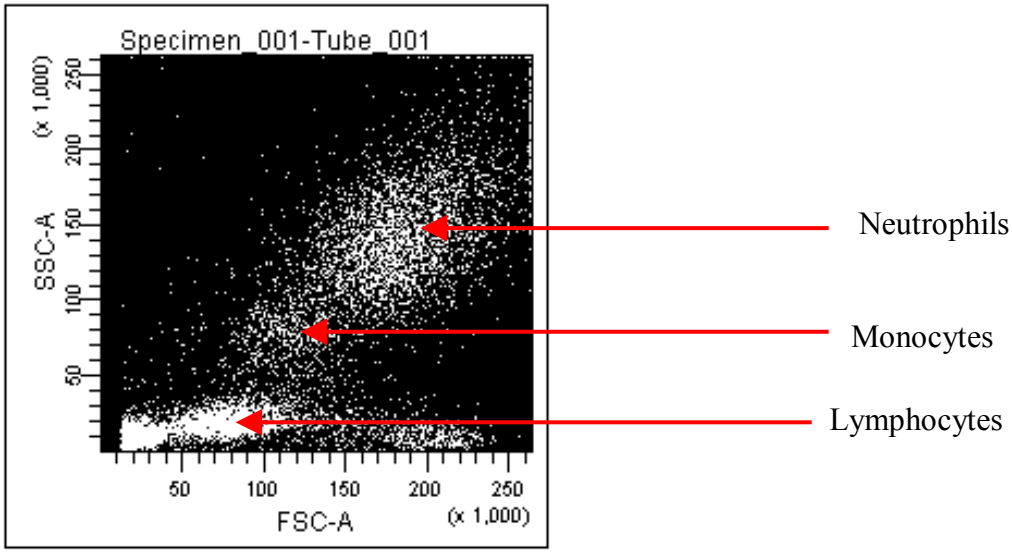


Figure 6: Several populations of cells reading the data output from the flow cytometer

From the FSC vs. SSC plot, you will want to 'Gate' on the population that your cells of interest are in. Gating on a population simply means that you are selecting the cells that you want to look at in future plots.

2.7 Cytokine assays

The cytokine assays is commonly used to detect and quantify cytokine from different samples is Flow cytometry combining intracellular cytokine staining, to investigate either the spontaneous production of cytokines or the stimulated (i.e.) induced production of citokines, and multiparameter flow cytometry allows for simultaneous detection of two or more cytokines in a single cells of the lineage defined by expression of one or more surface markers. (Prussin 1997)

Citokine flow cytometry is an antibody-based technique amenable to signal amplification by biotinylation of the reagents. Its specificity and sensitivity are strictly dependent on anticytokine antibodies selected for use.

Supernatants were collected on day 6 of monocyte/T cell cultures and stored at -80°C. Cytokine secretions from the supernatants were quantified with flowcytomix cytokine assay kit (eBioscience) as per the manufacturer's instructions. Approximately 2,000-gated events were collected on a Beckman Coulter CMP500 flow cytometer, and FlowCytomix Pro 2.4 software (eBioscience) was used for data analysis.

2.8 Quantitative real-time PCR analysis

In molecular biology, real-time polymerase chain reaction, also called quantitative real time polymerase chain reaction (Q-PCR/qPCR/qrt-PCR) or kinetic polymerase chain reaction (KPCR), is a laboratory technique based on the PCR, which is used to amplify and simultaneously quantify a targeted DNA molecule. For one or more

specific sequences in a DNA sample, Real Time-PCR enables both detection and quantification. The quantity can be either an absolute number of copies or a relative amount when normalized to DNA input or additional normalizing genes.

The procedure follows the general principle of polymerase chain reaction; its key feature is that the amplified DNA is detected as the reaction progresses in real time. This is a new approach compared to standard PCR, where the product of the reaction is detected at its end. Two common methods for detection of products in real-time PCR are: (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

Frequently, real-time PCR is combined with reverse transcription to quantify messenger RNA and Non-coding RNA in cells or tissues (Logan J, Edwards K et al. 2009).

In this experiment, for the quantitative real time PCR, total RNA samples (2 μg) was extracted from cells using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The first strand of cDNA was obtained using High Capacity RNA-to-cDNA kit and transcripts were quantified by real-time quantitative PCR using TaqMan Fast Universal PCR kit on an ABI 7500 Fast Real-Time PCR System (all RT-PCR reagents from Applied Biosystems, Foster City, CA). Human transcription factors T-bet (Hs00203436_m1), RORgt (Hs01076112_m1), GATA-3 (Hs00231122_m1), and FOXP3 (Hs00203958_m1) predesigned Gene Expression Assays also from Applied Biosystems were used according to the manufacturer's

instructions. Reactions were carried out using TaqMan Universal PCR Fast Master Mix and the following amplification conditions: 95°C for 10 min, 40 cycles of 95°C for 3 sec, and 60°C for 30 sec. Specific gene expression was normalized to the housekeeping genes GAPDH. Expression of specific mRNA levels was calculated by first determining the average threshold cycle (Ct) for each culture, which corresponded to the following: (average specific gene threshold cycle – average GAPDH threshold cycle). Triplicate samples were used to calculate the average threshold cycle. The replicate threshold cycle (Ct) was then calculated with the following formula: ($Ct_{with\ drug} - Ct_{without\ drug}$). Transcription factors T-bet, RORgt, GATA-3, and FOXP3 mRNA were expressed as expression fold value (2^{-Ct}).

2.9 Suppression assays

$CD4^+CD25^+CD127^-$ Tregs from the monocyte/T cell culture treated with 10 μg/ml SRL were purified with magnetic bead separation. The capacity of the Tregs to suppress T cell responses were assessed by their addition (at increasing cell doses) to a newly set-up co-cultures of $CD4^+CD25^-$ responder (5×10^4) stimulated with original donor or third party irradiated PBMC stimulators (1×10^5; 3000 rad, ^{137}Cs source) in complete RPMI1640 medium. Original donor group vs. the third party are set for testing allospecific suppression. Two methods were applied for the evaluation of suppressive capacity. In the first series of experiments CFSE dilution technique was used. CFSE (Carboxyfluorescein succinimidyl ester) dilution technique is a technique where CFSE labeling infuses cells with a dye that is diluted during successive rounds of cell division. Facs analysis of CFSE labeled cells provides an assay to identify the number of cell division that cell populations under go following

stimulation up to 8 generations CFSE is used for staining cells prior to flow cytometric analysis of cell proliferation or cell division. This dye can be used to monitor lymphocyte proliferation, both in vitro and in vivo, due to the progressive halving of CFSE fluorescence within daughter cells following each cell division(Lyons and Parish 1994; Xu, Zhang et al. 2008). Techniques currently available for determining cell division are able to show one or, at best, a limited number of cell divisions. This technique, in which an intracellular fluorescent label, is divided equally between daughter cells upon cell division. The technique is applicable to in vitro cell division, as well as in vivo division of adoptively transferred cells, and can resolve multiple successive generations using flow cytometry. The label is fluorescein derived, allowing monoclonal antibodies conjugated to phycoerythrin or other compatible fluorochromes to be used to immunophenotype the dividing cells.

In this experiment, $CD4^+CD25^-$ responder cells were labeled with CFSE and then activated for 7 days by stimulators with addition of various numbers of PKH26 labeled Treg cells. Tregs were labeled with PKH26 (a *red fluorescent dye* that can, in principle, be used for the study of asymmetric cell divisions) aiming to separate their potential proliferation, which may affect the responders proliferation readout. Alternatively, suppressive capacity was assessed with thymidine incorporated proliferation assay for 7 days by adding 3H-Thymidine for the last 12-18 hours. Background proliferation was determined from cultures with $CD4^+CD25^-$ T effectors alone and $CD4^+CD25^+CD127^-$ Treg cells alone.

2.10 Western blotting analysis

At the end of culture, cells were washed and lysed, 50 μg of proteins were loaded and separated on SDS-PAGE, and western blot was

done as described from Xu L, Immunol Lett 2008; Xu, Zhang et al. 2008. The following antibodies were used in this study: rabbit polyclonal anti-DNMT1 (H-300, Santa Cruz, CA), anti-phospho AKT (Ser473), and anti-phospho FOXO3a (Ser253) (both from Cell Signaling Technologies, MA).

2.11 Statistical analysis

Data were analyzed by Student's two-tailed t test. A value of $p < 0.05$ was considered statistically significant. Data are presented as mean ± SD.

RESULTS

3.1 Alloreactive naïve $CD4^+CD45RA^+$ T cells can differentiate into effector Th1, Th17 and Treg cells

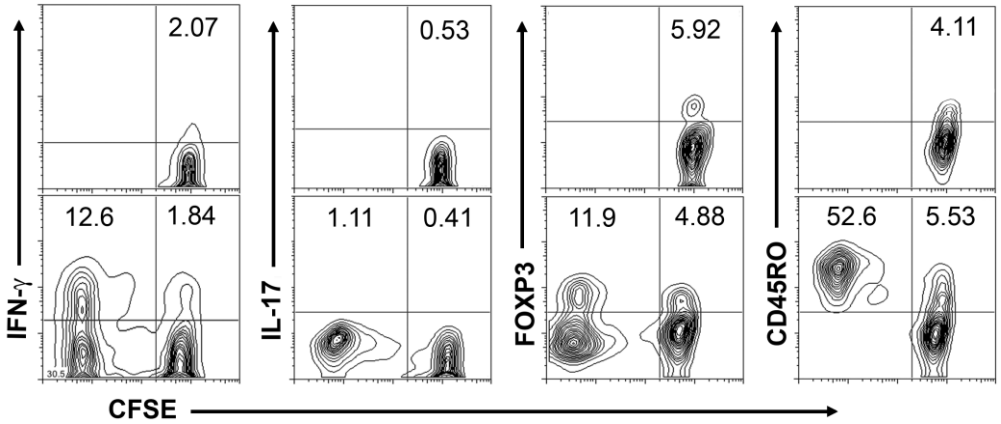
Subpopulation of Th1, Th17 and Treg cells and their signature cytokines IFN-γ, IL-17, and the forkhead box transcription factor (FOXP3) are best documented for their relation to acceptance and rejection of transplanted organs (Mitchell, Afzali et al. 2009; Strom and Koulmanda 2009). Most studies on the differentiation/expansion of Th cell subsets have used strong anti-CD3/CD28 stimulation and inflammatory settings by modulating levels of polarizing cytokines in vitro or in vivo, but this approach might not be ideal to evaluate the physiologic role of T cell subsets during immune responses (Li, Kim et al. 2010). We studied the differentiation and functions of alloreactive effector Th1, Th17 or Treg cells in the absence of exogenous polarizing cytokines or insults, and tested the role of immunosuppressive agents affecting the differentiation/expansion processes and their possible clinical relevance to the transplantation.

Human naïve $CD4^+$ $CD45RA^+$ T cells purified from ex vivo PBMCs were CFSE-labeled and co-cultured with allogeneic APCs or $CD14^+$ monocytes, which include myeloid $CD14^+$ monocytes and $CD11^+$ dendritic cells for 9-11 days. At the end of culture, we tested the IFN-γ, IL-17 and FOXP3 expressing cells with intracellular staining method. We found that $CD4^+CD45RA^+$ naïve T cells can differentiate into alloreactive effector IFN- γ^+/Th1, IL-17^+Th17 and FOXP3+/Treg subsets at single cell level (Fig. 7A), and the proliferated cells were found to gain the most expression of IFN- γ, IL-17 or FOXP3 than non-dividing cells. As human

Th17 differentiation from naïve T cells needs more strict conditions, in addition to TGF-β and IL-6, they need additional cytokines like IL-23 and IL-21, which are not secreted by monocytes, but activated T cells (Zhou, Ivanov et al. 2007; Volpe, Servant et al. 2008; Yang, Anderson et al. 2008), so we did not expect to see much IL-17 secretion from these cells and, as predicted, we saw very few IL-17-secreting alloresponsive T cells in the culture (Fig. 7A, 1% of $CD4^+$ T cells expressing IL-17 cultured with monocytes). As control, when autologous APCs were used as stimulators in the primary cultures, we saw few CD4+T cells upregulated CD45RO expression in conjunction with IFN-γ, IL-17 or FOXP3 expression to differentiate to alloresponsive T cells (data not shown).

Alloresponsive T cells are defined as T cells proliferating in response to allogeneic APCs, and the cell population changes and their phenotypes are defined by expression effector/memory-like marker $CD45RO^+$. Further analysis by cell surface staining of CD45RO (human memory T cell marker) vs. CD45RA (human naïve T cell marker) showed that these cells highly expressed $CD45RO^+$ and this depends on the cell stimulation conditions. Compared to more strong stimulation by a classic MLR, $CD4^+CD45RA^+$ naïve T cells co-cultured with allogeneic APCs have a delay expression of $CD45RO^+$, but can be induced to a maximum expression similar to the classic MLR at day 9 (Fig. 7B). From these experiments, it can be concluded that human $CD4^+CD45RA^+$ naïve T cells can differentiate into effector Th1, Th17 and Treg cells in a similar way as classic MLR by the stimulation with allogeneic APCs.

A.



B.

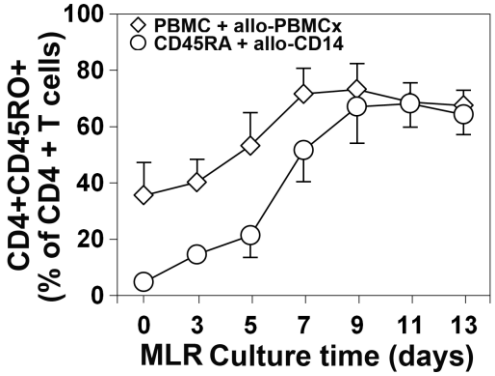


Figure 7. Alloreactive naïve CD4$^+$CD45RA$^+$ T cells can differentiate into effector Th1, Th17 and Treg cells. Naïve CD4$^+$CD45RA$^+$ T cells labeled with CFSE co-cultured with allogeneic CD14$^+$ monocytes.

A. The proliferated CD4 T cells (CFSE low) were capable to produce cytokine IFN-γ of Th1, IL-17 of Th17 and to express transcription factor FOXP3 of Tregs and to differentiate into effector CD4 T cell subsets. Upper panel: at day1; Lower panel: at day7.

B. After co-cultured for 9 days, 74±12% of naïve CD45RA$^+$T cells (open circles) were activated and expressed CD45RO$^+$ marker, which is similar to a classic MLR (open squares).

3.2 APC, TGF-β and IL-2 are essential for the differentiation/expansion of alloreactive $CD4^+CD45RA^-$ T cells into effector Th17 and Treg cells

To study how alloreactive effector/memory CD4 T cells were affected by different immunosuppressive drugs as these types of cells are enriched in the blood circulation and intragraft in post-transplant recipients, we isolated viable alloresponsive effector/memory-like $CD4^+CD45RA^-$ T cells from the above culture by depleting unstimulated $CD45RA^+$ and positively recovering $CD4^+$ cells. Cells were cultured in complete RPMI 1640 medium and rested overnight for subsequent experiments.

With regard to mechanisms, it is believed that TCR (T cell receptor) together with co-stimulation signal plus various cytokines are required for the induction of Th cell subsets. Fresh monocytes were most efficient in inducing Th17 from both memory and naïve T cells (Evans, Suddason et al. 2007; Crome, Clive et al. 2010; Kryczek, Wu et al. 2011) while in vivo induction of functional suppressive Treg was best achieved by subantigenic activation of T cells under conditions that avoid functional activation of APCs. In addition, it was shown that in human alloreactive $CD4^+$ T cell are biased to a Th17 response, which is inversely related to the number of HLA class II mismatches (Litjens, van de Wetering et al. 2009). In these regards, to mostly mimic physiologic environment, we stimulated alloreactive CD4 T cells with submitogenic dose of anti-CD3 (e.g. 100ng/ml) in the absence or the presence of costimulatory signal from monoclonal anti-CD28 or autologous APCs for 5-6 days. The results showed that strong activation of alloreactive $CD45RA^-$ T cells by anti-CD3 plus CD28 beads induced higher expression of both IFN-γ^+ and

$FOXP3^+$ cells, with conversely a lower percentage of IL-17-secreating cells. In contrast, anti-CD3 plus autologous APCs induced significant IL-17 production than those of anti-CD3 alone or anti-CD3 plus anti-CD28 in the absence of APCs (Fig. 8A, and data not shown). The data suggested that under non polarizing conditions, efficient differentiation/expansion of Th17 cells relies on the presence of APCs (Evans, Suddason et al. 2007; Allan, Crome et al. 2007), and transient high FOXP3 expression by anti-CD3 plus anti-CD28 in conjunction with IFN- γ secretion, which accounts for high percentage of IFN- γ^+FOXP3^+ double positive T cells (1.7% vs. 10.6% for anti-CD3/APC vs. anti-CD3 plus anti-CD28), are contrast to the induction of functional Treg cells. Finally, we tested TLR ligands, which mimic pathogenic stimulation, and we found that strong activation of monocytes by both TLR4 ligand (LPS) and TLR7/8 (R848) induced prefunded activation of IFN-γ-producing Th1 cells with accordingly lower levels of IL-17-producing Th17 cells (data not shown).

It is known that TGF-β is indispensible for in vitro induction of iTreg cells (Induced T regulatory), and IL-2 is required for the expansion and survival of Treg, both in vitro and in vivo (Davidson, Di Paolo et al. 2007; Chen, Kim et al. 2011). As previous data argued that medium with fetal bovine serum contains TGF-β, our culture medium contains 3ng/ml of TGF-β, too. We therefore continued to use this culture medium without adding extra TGF-β. Then, we checked the effect of IL-2 in our system. In the presence of normal levels of IL-2 (e.g., 20U/ml), $CD4^+IFN\text{-}\gamma^+$ and $CD4^+IL\text{-}17^+$ cells were increased from average of 15.5% to 22.1% and 6% to 11.8% respectively while higher levels of IL-2 (100U/ml) slightly decreased the percentages of $CD4^+IFN\text{-}\ \gamma^+$ and CD4+$IL\text{-}17^+$ cells (Fig. 8B). The reason may be that high amount of IL-2 induced more apoptosis

to effector T cells (Refaeli, Van Parijs et al. 1998). Of note, previous studies showed contrary results for IL-2 in the differentiation of Th17 cells. IL-2 may inhibit Th17 differentiation, or only attenuates IL-17 production without inhibiting its differentiation, or instead is required for Th17 differentiation (Deknuydt, Bioley et al. 2009). Considering that IL-2 is necessary for the initial development and survival of memory effector T cells, thus, the overall influence of IL-2 on the Th17 differentiation program may be more complex than anticipated. In the presence of high levels of IL-2 (100U/ml), $FOXP3^+$ cells were significantly induced up to 12.9% in culture (Fig. 8B). This confirmed that high concentration of IL-2 is required for the induction and stability of FOXP3 expression and the survival and expansion of $FOXP3^+$Treg cells (Chen, Kim et al. 2011). It is to be noted that a small fraction of IFN- γ^+FOXP3^+ and IL-17^+Foxp3^+ cells was existed in our system, and again, in contrast to the induction of IFN-γ^+FOXP3^+ cells, autologous APCs are needed for inducing appreciable levels of IL-17^+Foxp3^+ T cells without exogenous cytokines (Figure 10D and data not shown) (Crome, Clive et al. 2010; Kryczek, Wu et al. 2011). Thus, from the above results, optimal induction of Th1, Th17 and Treg cells in our system required weak TCR stimulation plus APCs and the concomitant presence of IL-2 and TGF-β.

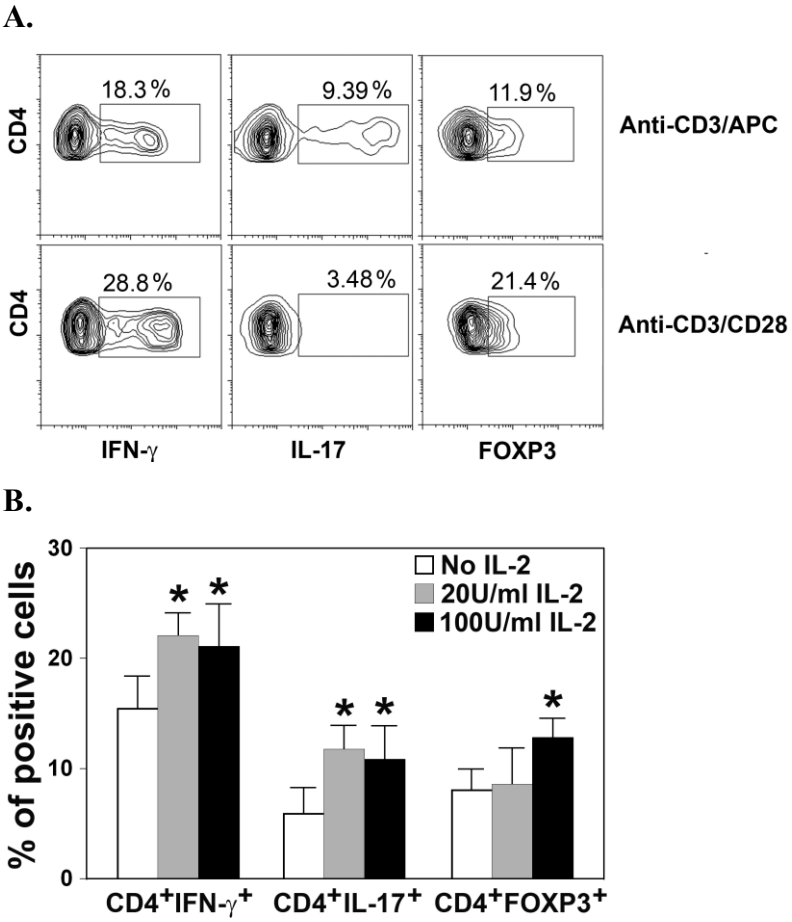


Figure 8. APC, TGF-β and IL-2 are essential for the differentiation/expansion of alloreactive Th1, Th17 and Treg subsets in monocyte/T cell culture. Purified alloreactive memory-like $CD4^+CD45RA^-$T cells generated from allogeneic activated naïve $CD4^+CD45RA^+$ T cell as in Figure 1 (without CFSE labeling) were rested overnight and re-stimulated with soluble anti-CD3 and autologous APCs (thereafter as monocyte/T cell culture) or anti-CD3/CD28 beads in the presence of IL-2 for 5 to 6 days. The induction of IFN-γ^+/Th1, IL-17^+/Th17 and FOXP3+/Treg cells were tested by intracellular staining of IFN-γ, IL-17 or FOXP3 proteins.

A. APCs were essential for the induction of IL-17-secreating Th17 cells or $FOXP3^+$Treg cells in monocyte/T cell culture compared to anti-CD3/CD28 stimulation which induced higher transient expression of FOXP3 with barely affected IL-17-secreating Th17 cells.

B. IL-2 was differently needed for the induction of IFN-γ^+Th1, IL-17^+Th17 and $FOXP3^+$Treg cells. High concentrations of IL-2 (> 100U/ml) were specifically required for the induction of FOXP3+Tregs. * $p < 0.05$ compared to No IL-2 treated group. Values represented mean ± SD and were obtained from 4 healthy donors.

3.3 Calcineurin inhibitor and mTOR inhibitor differently inhibit Th1 and Th17 cells in alloreactive CD4 T cells

Calcineurin inhibitors (CsA, TAC) and mTOR inhibitor (SRL, Rapalogs) have been used for a long time for the treatment of graft rejection and have proved to inhibit the activation of conventional effector T cells in both naïve and memory compartments. We started to check how alloreactive $CD4^+$ T cells, defined here as effector/memory-like $CD4^+CD45RA^-$ cells, are affected by these two immunosuppressive drugs in the generation and expansion of Th1 and Th17 cells. Again, in contrast to the previous work with strong TCR/CD28 stimulation of both naïve and memory CD4 T cells under polarizing conditions, we stimulated alloreactive $CD4^+CD45RA^-$ cells with subantigenic anti-CD3 (100ng/ml) in the presence of autologous APCs with or without TAC or SRL for 5 days and systemically we determined IFN-γ and IL-17 production at both single cell level by intracellular staining and bulk culture supernatants with multiplex cytokine assay. We found that TAC at low dose (2ng/ml), blocked over 90% of the productions of IFN- γ and IL-17, even in the presence of high concentration of IL-2 (100U/ml). In the contrary, SRL at high concentration (10ng/ml) had moderate inhibition on IFN- γ and IL-17 productions (30% and 60% respectively, Fig 9A). Interestingly, this inhibition of IFN- γ production by SRL but not TAC was reversed in the presence of high concentration of IL-2 (Figure 9B). The combination of the two drugs showed significantly inhibition on both IFN- γ and IL-17 productions (N= 6-8 donors, * $p < 0.05$; ** $p < 0.01$).

In parallel experiments, we examined the cytokine production of supernatants collected from bulk culture with different treatments.

Compared with the supernatants collected from TAC or SRL or the combination of the two treated cells, we found that in agreement with intracellular staining, TAC strongly blocked IFN-γ production, while SRL showed more profound inhibition of IFN-γ compared to intracellular staining (Fig. 9C). This is obvious that other immune cells such as IFN- γ -secreting monocytes in our system could be inhibited by SRL. As we could not detect any IL-17 production in the supernatants collected from monocyte/T cell culture in the absence of IL-2, we determined IL-17 production in our system in the presence of IL-2. Both TAC and SRL inhibited IL-17 production although in this situation. TAC was less efficient in blocking IL-17 production as compared to intracellular staining (Fig. 9C). The reason for this discrepancy maybe is because TAC has minor effect on monocytes than on T cells, as such monocyte produced Th17 polarizing cytokines IL-1β and IL-6 play a role for the high percentage of IL-17+Th17 cells (Zhou, Ivanov et al. 2007); and also because in the presence of high amount of IL-2, calcineurin inhibitor may reverse activated T cells from activation-induced cell death and IL-21, IL-23 produced by these cells are important for the induction of Th17 cells. These results, together with the intracellular staining at single cell levels, suggest that TAC is more efficient than SRL to inhibit differentiation and expansion of preformed alloreactive CD4 T cells. At molecular levels, TAC blocks T cell activation signaling and subsequently production of T cell survival cytokine IL-2, and SRL most likely affects IL-2-induced later G1-S cell cycle transition in T cells, thus prevents their proliferation and favor the establishment of T cell anergy, or prevents T cell anergic reversal by the presence of IL-2 (Powell, Lerner et al. 1999).

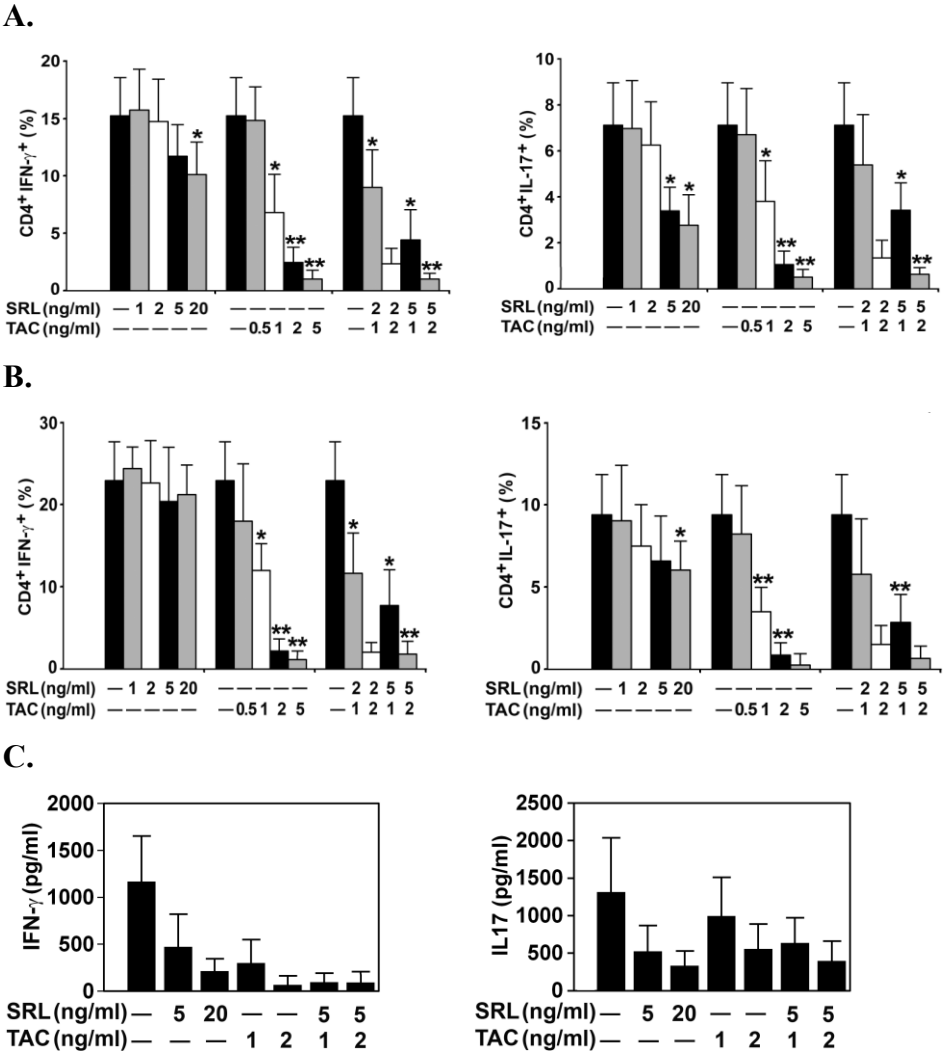


Figure 9. TAC and SRL potentially inhibit IFN-γ and IL-17 producing cells in monocyte/T cell cultures. Purified alloreactive $CD4^+$ $CD45RA^-$ T cells (1 $x10^5$/well) generated from allogeneic activated naïve $CD4^+$ $CD45RA^+$ T cell as in Figure 8 were rested overnight and re-stimulated with low dose of anti-CD3 (100ng/ml) and autologous APCs (0.5 $x10^5$/well, thereafter as monocyte/T cell culture) in the absence or in the presence of 100U/ml of IL-2 for 5 to 6 days. TAC, SRL or the combination of the two drugs at indicated concentrations were added at the start of cultures. At the end of culture, the cells were harvested, and intracellular cytokines were stained at single cell levels.

At the end of culture, the cells were harvested, and intracellular cytokines were stained at single cell levels.

A. No IL-2 was added to the monocyte/T cell culture.

B. In the presence of 100 U/ml of IL-2. Values represented mean ± SD and were

obtained from 6–8 healthy donors. * $p < 0.05$; * * $p < 0.01$ compared to no treatment
C. Cytokine productions from bulk culture supernatants collected from the same monocyte/T cell cultures were determined with flow-based multiplex cytokine assay.

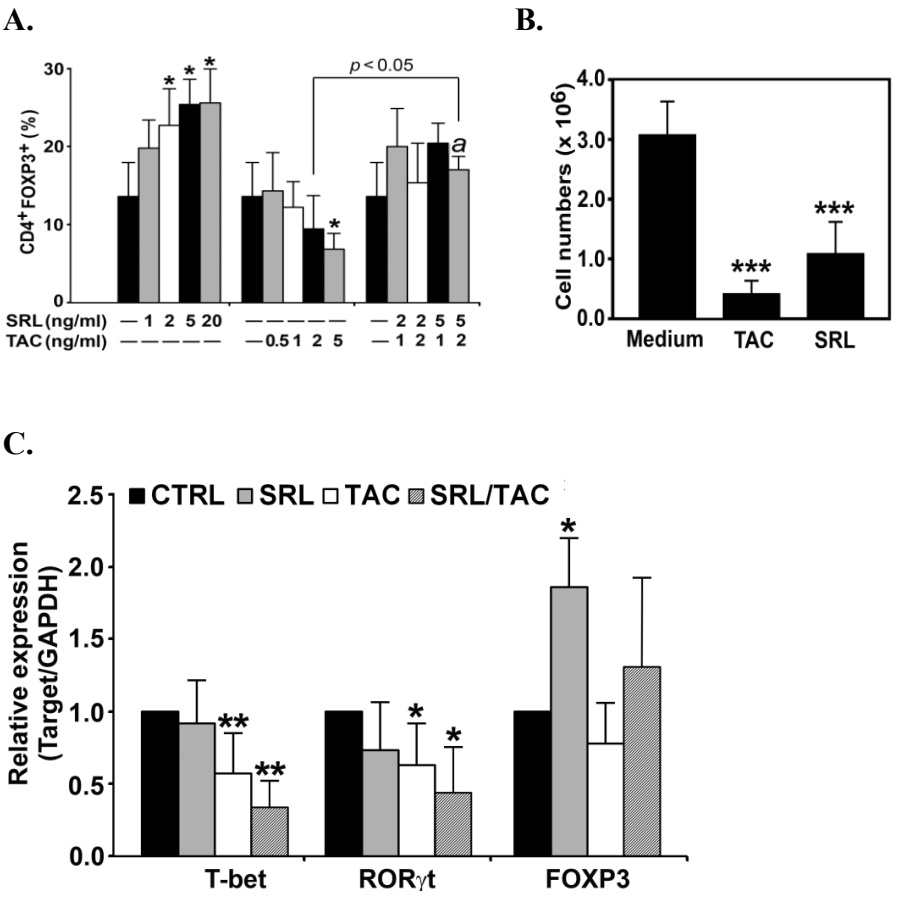
3.4 Restimulation of alloreactive CD4 T cells in the presence of SRL leads to increased FOXP3 expression

TAC and SRL inhibit proliferation and differentiation of conventional T cell in both naïve and memory T cell compartments (Demirkiran, Hendrikx et al. 2008), but the results in this study showed that only SRL favors Treg cells generation and expansion (Coenen, Koenen et al. 2006; Strauss, Whiteside et al. 2007). In this system, the SRL and TAC were tested directly on the Treg generation from alloreactive $CD4^+$ T cells. Given that the expression levels of both FOXP3 and CD25 are proportional in human $FOXP3^+$ T cells, and FOXP3 is a more specific marker than CD25 for Treg cells, we first tested different combinations of Treg markers for detecting this population in our system. In pilot experiments, we found that various amounts of SRL (0.5 to 20ng/ml) parallel increased similar percentages of $CD4^+FOXP3^+$ cells compared to $CD4^+CD25^+FOXP3^+$ cells in the presence of IL-2 in our monocyte/T cell culture. So we used % of $CD4^+FOXP3^+$ cells instead of % of $CD4^+CD25^+FOXP3^+$ T cells as Treg readouts for the remaining experiments (Kryczek, Wu et al. 2011); and data not shown). We next started out to check how alloreactive T cells defined here as $CD4^+CD45RA^-$ cells were affected by these two immunosuppressive drugs. We restimulated rested alloreactive $CD4^+CD45RO^+$ T cells with soluble anti-CD3 mAb (100ng/ml) in the presence of autologous APCs with rhIL-2 (100U/ml), and SRL or TAC or the combination of the two at indicated concentration for 5 days. At the

end of culture, the cells were harvested and analyzed by flow cytometer for Foxp3 expression. In the absence or normal culture condition of IL-2 (no more than 20U/ml), both SRL and TAC have no significant effects on the expression of FOXP3 in our culture system (data not shown). This also proved that IL-2 is needed for the steady expression of FOXP3 and the survival of FOXP3+ Tregs (Figure 8B. In the presence of IL-2 (100U/ml), the percentage of FOXP3 expression in SRL-treated Tregs was increased significantly in comparison with those in the medium only (26.6%5.7 vs. 14.1%5.3; n=6; $p<0.05$), whereas TAC-treated iTregs were not changed or showed a decreased trend (Figure 10A). When used in combination at low doses, TAC at 2ng/ml with SRL at 2-5ng/ml achieved the maximal effects in inhibiting the productions of IFN-γ and IL-17 while maintaining a high level of FOXP3 expression (Fig. 10A and Fig. 10B). From the same culture as above, the absolute cell number of T cells recovered by counting trypan-blue negative cells was markedly reduced in T cells exposed to TAC and SRL compared with control cultures (average fold expansion: 0.3 ± 0.2 in TAC cultures vs. 1.1 ± 0.4 in SRL cultures vs. 3 ± 1.2 in medium cultures; n = 6) (Figure 10B). Taken together, proliferation of alloreactive T cells is reduced in the presence of SRL, yet, the percentage of $FOXP3^{+}CD4$ T cells is increased in our system, supporting the previous data that SRL favors Treg expansion by binding to mTOR complex and selectively blocking proliferation of AKT/mTOR-sensitive effector T cells while sparing Tregs (Bensinger, Walsh et al. 2004; Crellin, Garcia et al. 2007).

We also tested whether there were also any changes at transcriptional RNA levels. Expression of specific T cell subset transcription factor T-bet (Th1), GATA3 (Th2), RORγt (Th17) or FOXP3 (Tregs) at mRNA levels

was determined by real-time PCR. In agreement with the flow cytometry data, in the presence of IL-2 (100U/ml), SRL at 5ng/ml had little effects on T-bet and GATA3 while moderate inhibition on RORγt mRNA expressions. TAC at low dose (2ng/ml) effectively blocked both T-bet and RORγt mRNA expressions (Fig. 10C). As for FOXP3 mRNA expression, SRL augmented while TAC moderately inhibited its expression, and the combination of the two showed an increase of its expression (Fig. 10C).



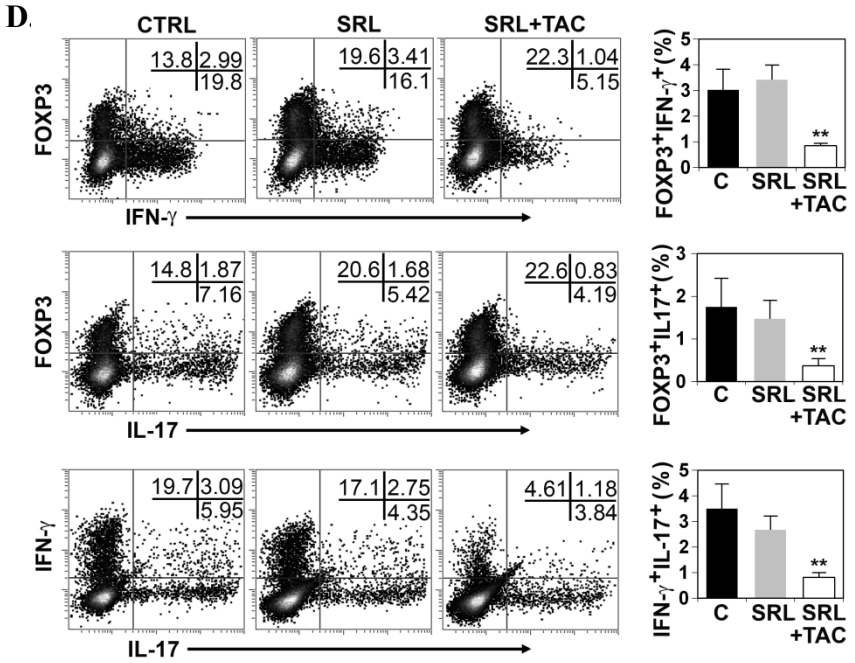


Figure 10. Restimulation of alloreactive CD4 T cells in the presence of SRL leads to increased FOXP3 expression. Purified alloreactive $CD4^+CD45RA$- T cells (1 $x10^5$/well) generated from allogeneic activated naïve T cell as in Figure 8 were rested overnight and re-stimulated with low dose of anti-CD3 (100ng/ml) and autologous APCs (0.5 $x10^5$/well) in the presence of 100U/ml of IL-2.

A. At the end of culture, the cells were harvested and intracellular FOXP3 expression was stained at single cell levels. (* $p < 0.05$ and [a] $p > 0.05$ compared to no treatment). Values represented mean ± SD and were obtained from 6–8 healthy donors.

B. TAC and SRL inhibited the proliferation of alloreactive $CD4^+CD45RA^-$ T cells. Cell growth was assessed by manual counting at the end of cultures. There was a significantly higher amount of cells recovered in medium cultures than those in TAC or SRL cultures (** $p< 0.01$, *** $p< 0.001$ compared to medium). Values represented mean ± SD and were obtained from 4 healthy donors.

C. Effects of TAC and SRL on the lineage-defining transcription factor expressions for T cell subtypes. Real-time PCR were performed to determine gene specific transcription factors for T cell differentiation, e.g. transcription factor T-bet for Th1, RORγt for Th17, and FOXP3 for Treg differentiation. The results were normalized to the housekeeping genes GAPDH and shown here as fold changes. Values represented mean ± SD and were obtained from 3 separate donors with quadruplicates.

D. Low concentration of SRL (e.g. 5ng/ml) alone did not increase percentage of IFN-γ+$FOXP3^+$, IL-17+$FOXP3^+$ or IFN-$γ^+$IL-17^+ T cell subsets. However, SRL at this concentration plus very low concentration of TAC (e.g. 1ng/ml) significantly inhibited the production of these three T cell subsets (*$p < 0.05$, ** $p < 0.01$ compared to control). Values represented mean ± SD and were obtained from 6–8 healthy donors.

3.5 SRL together with very low dose of TAC inhibits the induction of IFN-γ$^+$FOXp3$^+$ and IL-17$^+$FOXP3$^+$ Treg subsets

In addition to nTreg (Naturally occurring regulatory T) and iTreg (Induced T regulatory) subsets, there is evidence of phenotypic and functional heterogeneity and plasticity within the FOXP3$^+$ Treg population, which potentially include IFN-γ$^+$FOXP3$^+$ and IL-17$^+$FOXP3$^+$ cells (O'shea jj science 2010), and has been implicated as the pathologic effector T cells in autoimmune inflammatory diseases and in cancers, but incomplete data in transplantation settings (Kryczek, Wu et al. 2011; Hippen, Merkel et al. 2011). As a consequence, we wondered whether SRL-derived alloreactive FOXP3$^+$Tregs in our system also contained significant IFN-γ$^+$FOXP3$^+$ and IL-17$^+$FOXP3$^+$ cells. Thus, we tested the IFN-γ and IL-17-producing cell content in SRL treated cells vs. control group. Although we only detected low amount of IL-17$^+$FOXP3$^+$ or IFN-γ$^+$FOXP3$^+$ cells present in the alloreactive CD45RA- T cells irrespective of the presence of SRL compared to the controls (Fig. 10D), more importantly, SRL-treated group did not show any increase of IL-17$^+$FOXP3$^+$ or IFN-γ$^+$FOXP3$^+$ cells. Furthermore, SRL together with very low dose of TAC diminished significantly the percentage of these Treg subpopulations. Considering other recent data that showed these small populations of induced or expanded Treg subsets still possessed suppressive function both in vitro and in vivo in a GVDH model, and the most important aspect of SRL-treated Tregs that display the same % of FOXP3$^+$ cells and are plasticity resistant to further exposure to Th17 polarizing cytokines and possesses a regulatory activity as good as those prior to Th17-cell condition exposure TGFβ$^+$IL-21$^+$IL-23 (Hippen, Merkel et al. 2011; Tresoldi, Dell'Albani et al. 2011). These results

showed that SRL inhibits the differentiation and growth of IL-17^{+}Treg or IFN- γ^{+}Treg cells in our system, and we may conclude that a clinically practical application of SRL-derived Tregs for future adoptive Treg-based therapies in transplantation.

3.6 Production of TGF-β and IL-10 in Tregs and monocyte/T cell culture

Although Treg cell-mediated suppression is not fully understood, anti-inflammatory cytokines TGF-β and IL-10 represent one of multiple means for them to accomplish suppression of different inflammatory responses (Vignali, Collison et al. 2008). To examine whether SRL affects cytokine production from Treg cells, intracellular staining of TGF-β and IL-10 in FOXP3+Tregs and TGF-β and IL-10 productions in the supernatants of monocyte/T cell culture treated with SRL, were measured after 5 days. There was no significant difference in TGF-β production from both FOXP3^{+}Treg cells and supernatants of cell culture among the control and SRL-treated groups (data not shown), whereas IL-10 production slightly decreased with SRL-derived Treg cells by intracellular staining compared to untreated controls. In consistent with our Treg-MLR suppressive assay, it also showed that although IFN- γ, IL-17 productions from the culture supernatants were significantly inhibited, TGF-β was not affected (Figure 12.1A). In sum, soluble TGF-β and IL-10 are not important factors for their suppressive function by SRL-derived Tregs.

3.7 IL-1β, TNF-a, and to a lesser extent IL-6 down-regulate FOXP3 expression in alloreactive CD4 T cells

Cytokines have seemed increasingly important for the induction, regulation and function of distinct Th subsets in addition to antigen

strength and type of costimulation. TGF-β plus IL-2 are essential for the induction of iTreg (Davidson, DiPaolo et al. 2007; Horwitz, Zheng et al. 2008; Chen, Kim et al. 2011), whereas TGF-β plus IL-6 or TGF-β plus IL-21 or IL-1β or TNF-α are recently shown involved in differentiation/ expansion of human Th17 cells from both naïve, and memory $CD4^+$ T cells (Volpe, Servant et al. 2008; Yang, Anderson et al. 2008). During early inflammatory process, the acute phase cytokines IL-1β, IL-6 and TNF-α are produced and they are important mediators of early inflammatory events in allografts that undermine graft survival. In this regard, we determined the effects of these important proinflammatory cytokines on the generation of Th1/Th17, Treg cells were certain because our culture media contain about 3ng/ml of TGF-β, which is similar amount for the most in vitro study for both Treg and Th17 cell inductions. Because the primary purpose for the current study is to induce appropriate amount of Th1, Th17 and Treg cells aiming to test any effect by IS (immunosuppresor) drugs, we did not add extra exogenous TGF-β to our culture system. Alloreactive $CD4^+CD45RA^-$ T cells were stimulated for 5 days with subantigenic dose of anti-CD3 in the presence of autologous APCs and varied of concentrations of TAC and SRL. Three cytokines (10ng/ml of IL-1, 50ng/ml of TNF-α, and 20 to 100ng/ml of IL-6) were added individually or in the combinations at the beginning of culture. IFN-γ, IL-17 and FOXP3 expressions were determined by intracellular staining and flow cytometry. In the first set of experiments for individual cytokines, IL-1, but not IL-6 (up to 100ng/ml) could augment the productions of IFN-γ and IL-17, while TNF-α could only show a minimal effect on IFN-γ production (Fig. 11A and 11B). A combination of these three components, however, increased about 41% of IFN- γ^+ or 23% of

IL-17$^+$ cells compared to the control (Fig. 11A and 11B). Under the above inflammatory conditions, low doses of TAC or the combination of low doses of TAC and SRL still effectively blocked over 90% of IFN- γ or IL-17 production, while SRL alone moderately inhibited IFN- γ production or to a less extent for IL-17 production (Fig. 11A and 11B). The data showed here that IL-1β is a stronger stimulus while IL-6 alone has little or no effect on human naïve or memory T cells, consistent with previous human work (Yang, Anderson et al. 2008). The data in this study may also prove previous conclusion that IL-6 may need IL-23 to promote the differentiation/expansion of Th17 cells (Liu, Lin et al. 2004). TNF-α may exert its role through enhancement of IL-6 to induce Th17 cells, but we cannot rule out the possibility that TNF-α stimulates APCs to produce IL-6 and IL-23 for Th17 cell induction (Iwamoto, Iwai et al. 2007).

In the presence of above each single inflammatory cytokine (IL-1β, IL-6 or TNF-α), FOXP3 expression in CD4$^+$ T cells was marginally affected, but it was significantly down-regulated in the presence of all three cytokines compared to the absence of these cytokines in monocyte/T cell culture (Figure 11C, 11.93.2% vs. 6.083.01%, $p< 0.05$).

At molecular levels, these results are consistent with previous data that IL-6 may through IL-6/Stat3 downregulated FOXP3 expression or IL-6 induced re-methylation of CpG residues and decreased AcH3 in the upstream enhancer of the FOXP3 gene, thereby inhibiting FOXP3 expression. TNF-α may inhibit the expansion and function of Tregs via TNFRII which shown higher expression on Treg than Teff cells (Valencia, Stephens et al. 2006). Given the fact that TNF-α blockade constitutes one of the major therapeutic options in the treatment of some chronic inflammatory diseases in humans, such as rheumatoid arthritis

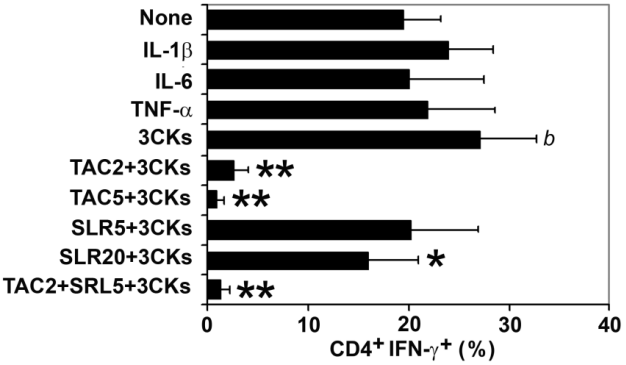
and inflammatory bowel disease, the above results make the role of TNF-α in Teff/Treg crosstalk even more important to understand. Finally, simultaneous activation of naive T cells and Treg cells in the presence of APCs under neutral condition, or directly stimulation of naïve Tregs in Th17-polarizing condition induced the differentiation of Tregs into IL-17 producing cells, and IL-1β was mandatory for this function as IL-1R highly expressed on Tregs than on naïve T cells (Deknuydt, Bioley et al. 2009; Li, Kim et al. 2010). We concluded that proinflammatory environment reverent to the downregulation of FOXP3 expression are necessary to be reconsidered for the future adoptive Treg-based therapies (Li and Turka 2010; O'Shea and Paul 2010).

In this condition, SRL at 5ng/ml still can significantly increase $CD4^+FOXP3^+$ Treg though the percentage was lower than that induced by the same concentration of SRL without the presence of any above cytokines (Fig. 11C). When used in combination, low doses SRL at 5ng/ml and TAC at 2ng/ml still showed an increased expression of FOXP3 compared to a decreasing expression of FOXP3 with the treatment of TAC alone though the difference did not reach a statistical level (Fig. 11C).

Of note, conventional effector T cells activated in the presence of strong co-stimulatory signals or in pro-inflammatory microenvironments are refractory to Treg cell-mediated suppression (Beriou, Costantino et al. 2009; Crome, Clive et al. 2010). According to the results, FOXP3 expression is downregulated by the presence of proinflammatory stimulation as well as the presence of pro-inflammatory cytokines IL-1β and IL-6 can induce human Treg cells to secrete pro-inflammatory cytokines and concomitantly affect the ability to suppress (Beriou,

Costantino et al. 2009), it is likely as important to control inflammation as to consider inducing transplantation tolerance in alloimmunity. Considering that SRL-derived Tregs did not increase IFN- $\gamma^{+}FOXP3^{+}$ and IL-$17^{+}FOXP3^{+}$ cells (Figure 10D) and its anti-inflammatory properties, SRL prove to be an ideal choice for clinical transplantation.

A.



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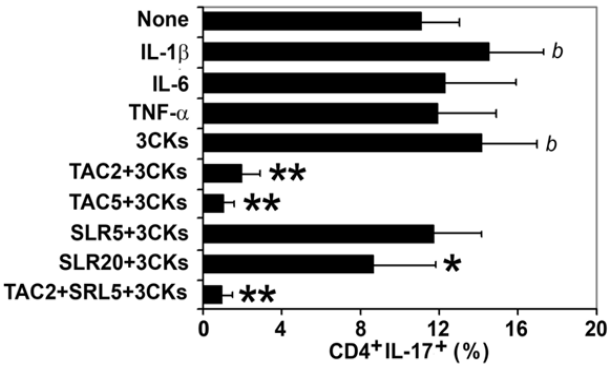

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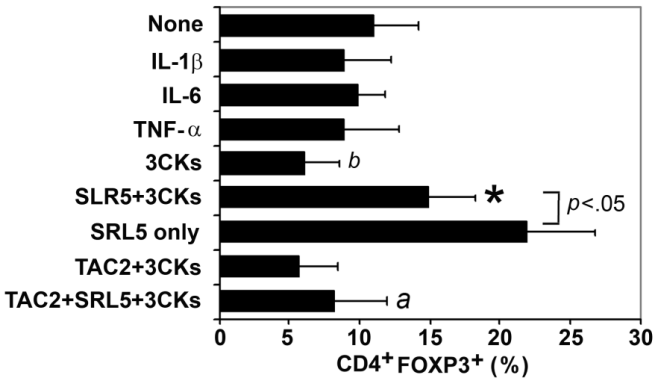


Figure 11. TAC and SRL differently affect the generation of T cell subsets in the presence of inflammatory cytokines in monocyte/T cell cultures.

A and **B** In the presence of proinflammatory cytokines (10ng/ml IL-1β, 20ng/ml IL-6 or 50ng/ml TNF-α; 3CKs were the combination of the above three individual cytokines), TAC still effectively blocked IFN-γ-producing cells (**A**) and IL-17-producing cells (**B**) while SRL was less effective to do so although inflammatory cytokines increased the % of both Th1/Th17 cells (**P*<0.05; ** *P*<0.01 vs. 3CKs; b *P*<0.05 vs. none). Values represented mean ± SD and were obtained from 4–6 healthy donors.

C. In the presence of proinflammatory cytokines as in **A** and **B**, the percentage of $FOXP3^+$Tregs was significantly decreased (*b* *P*<0.05 vs. none). Addition of SRL recovered most of Tregs (**P*<0.05 vs. 3CKs) although the percentage was lower than that induced by the same concentration of SRL without the presence of any above cytokines (Figure 5C, *P*<0.05). The combination of the two drugs had a higher % of Tregs than TAC alone though the difference did not reach a statistical level (a *P*>0.05 vs. 3 CKs). Values represented mean ± SD and were obtained from 4–6 healthy donors.

3.8 $CD4^+CD25^+CD127^-$ Tregs derived from SRL treated group suppress T cell proliferation and the differentiation of Th1 and Th17 subsets

The phenotypic changes between the control and SRL-treated culture were analyzed. SRL-derived $FOXP3^+$ Tregs from the monocyte/T cell cultures showed typically phenotypic markers of regulatory T cells, they expressed memory marker CD45RO and lacked the expression of the IL-7 receptor -chain CD127. SRL-treated Tregs also expressed higher level of CTLA-4 more than untreated controls, while expressed glucocorticoid-induced tumor necrosis factor receptor (GITR) at similar

levels compared to the control (Supplementary Figure S6). In addition, the expression levels of FOXP3 in $CD4^+$ cells from SRL-treated group were higher than that in $CD4^+$ cells compared to the control (Figures 10A and 10D).

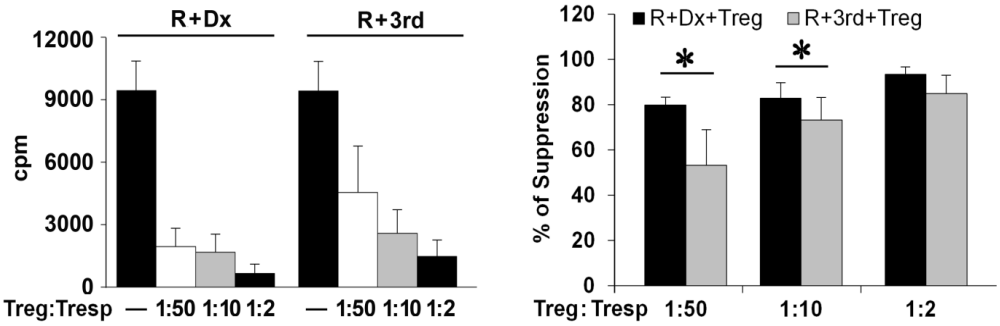
Furthermore, we tested whether the $Foxp3^+$ population of SRL-derived Tregs has a suppressive function. We isolated $CD4^+CD25^{high}CD127^{-/low}$ cells from Tregs as a $FOXP3^+$-enriched population, and found that over 90% of $CD4^+CD25^+CD127^{-/low}$ cell population isolated from SRL-treated Tregs expressed regulatory marker FOXP3.

As shown in Fig. 12A, $CD4^+CD25^+CD127^{-/low}$ cells derived from the culture treated with SRL showed significant suppression of proliferation of $CD4^+CD25^-$ T responder cells at a various range of ratio of Treg to responder T cells (Tresp). Of note, SRL-derived Tregs specifically and more efficiently inhibited allogeneic responder T cells at low ratio (Treg:Tresp < 1:5, Figure 6A, left panel). When this ratio increased, e.g Treg:Tresp = 1:2, SRL-Tregs showed a non-alloantigen specific suppression (Fig. 12A). This is reasonable as the presence of high numbers of Tregs, more anti-inflammatory cytokines (such as TGF-β, IL-10 or IL-35) produced by the Tregs and exerted non-specific suppression.

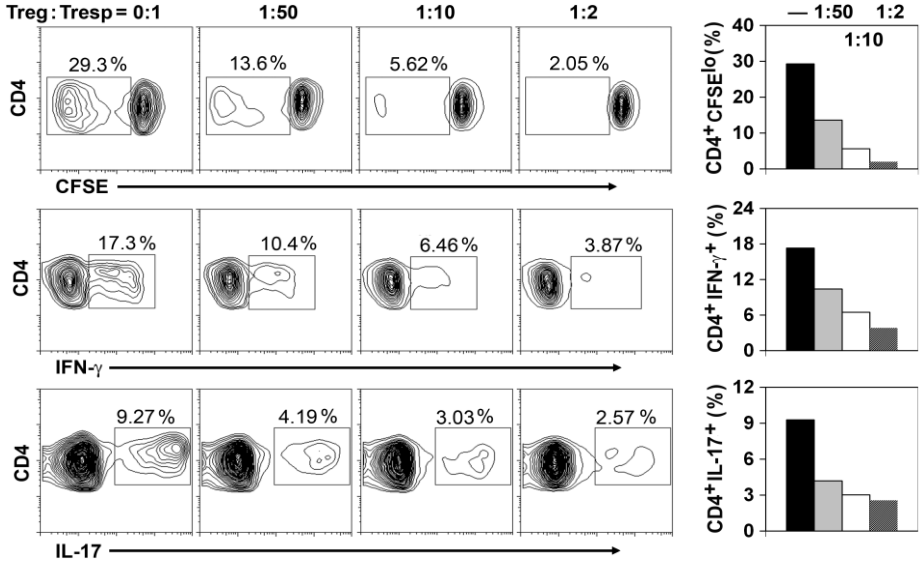
In a separate assay, responder $CD4^+CD25^-$T cells were labeled with CFSE and co-cultured with irradiated donor specific or a third party PBMCs plus a similar range of ratio of PKH26 labeled SRL-Tregs to responder T cells (Tresp) and co-cultured for 7 days. Responder T cell proliferation was determined by the dilution of CFSE in proliferated cells. The results demonstrated a dose-dependent inhibition of proliferation of CD4 T cells by SRL-Tregs (Fig. 12B, upper panel). The inhibition of alloresponse Th1 and Th17 immunity in the presence of SRL-derived

Tregs was detected by intracellular staining of IFN-γ and IL-17-secreating cells. The results showed that RL-Tregs effectively inhibited the alloresponse Th1 and Th17 activation (Fig. 12B). Finally, we checked the IFN- γ and IL-17 productions in the co-culture supernatants of above conditions, and found that in consistent to the cytokine secreting at single cell level, the production of these two cytokines were also blocked by the presence of SRL-derived Tregs (Fig. 12C).

A.



B.



C.

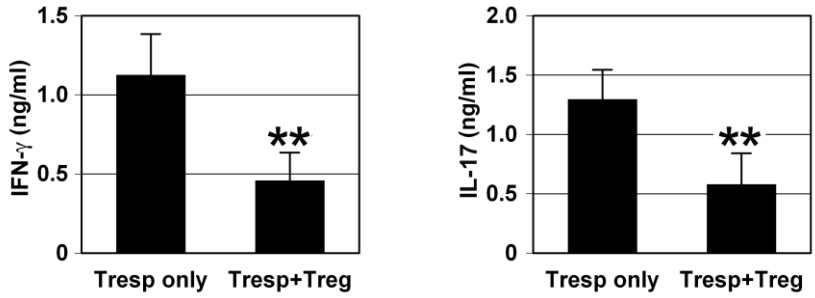


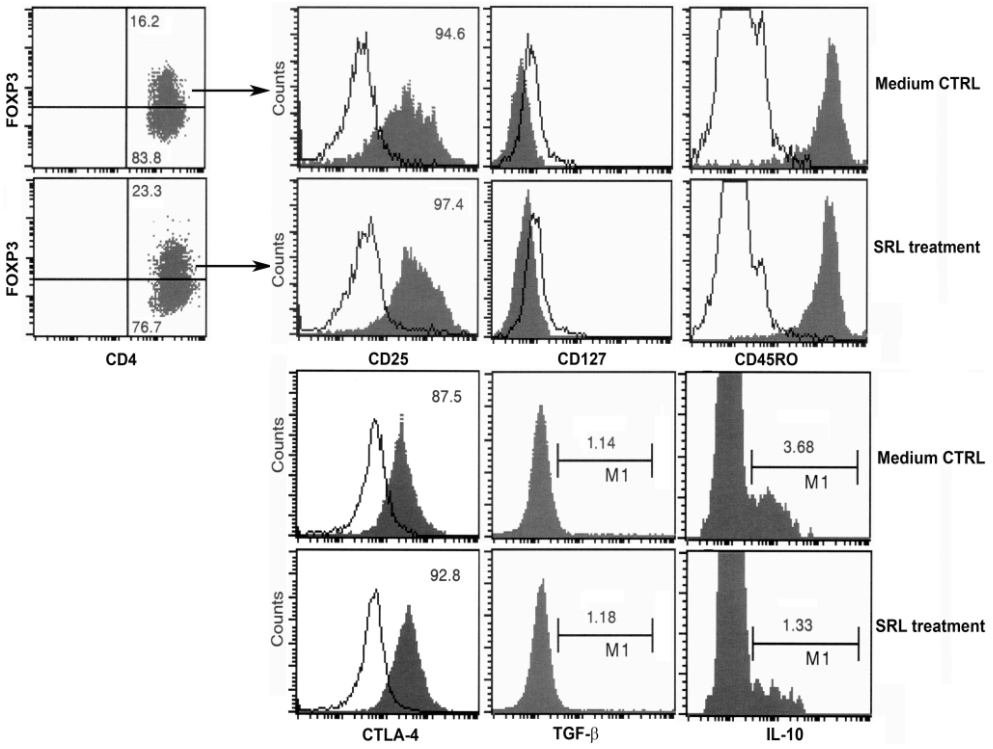
FIGURE 12. SRL-derived FOXP3+ Tregs from the monocyte/T cell cultures maintain a normal suppressive activity.

A. Normal suppressive properties of SRL-derived Tregs. Fresh $CD4^{+}CD25^{-}$responder T cells (Tresp) (5×10^4/well) and purified $CD4^{+}CD25^{+}CD127^{-/low}$ cells from SRL (10 ng/ml) treated group (1×10^3/well to 2.5×10^4/well) were co-cultured with irradiated allogeneic PBMC or a third-party at a Tregs/Tresp cell ratio of 1:2, 1:10 or 1:50, respectively. After 5 d of culture, responder T cell proliferation was determined using [^{3}H]-thymidine incorporation (left, raw data). The inhibition of responder proliferation by SRL-derived Tregs was expressed relative to that of responder T cells alone (right, percentage). The proliferation of Tregs was 424 ± 101 cpm. The control value (proliferation of responder alone) was 10,200 ± 1,403 cpm. Each value (cpm) was calculated by subtracting the proliferation (cpm) of SRL-treated Treg alone.

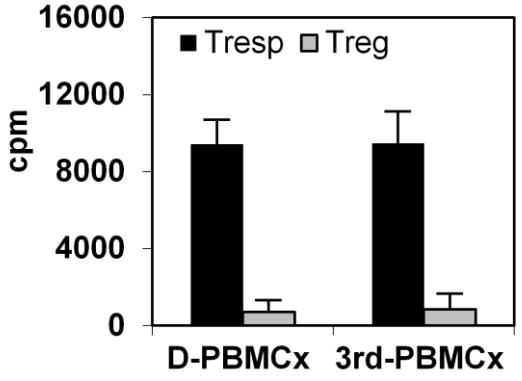
B. SRL-derived Tregs dose-dependently inhibited Th1 and Th17 alloimmune responses by detecting IFN-γ and IL-17-secreating cells. The results also demonstrated a dose-dependent inhibition of proliferation of CD4 T cells by CFSE dilution profile.

C. IFN-γ and IL-17 productions in the culture supernatants were also blocked by the presence of SRL-derived Tregs at Treg/Tresp cell ratio of 1:2. Values are the mean ±SD of at least of three experiments. Statistical analysis was performed using the Student *t* test. $^{**}p < 0.01$ vs. control.

A.



B.



Supplemetal figure 12.1.

A. Phenotypic analysis of SRL-derived Tregs. Rested alloreactive $CD4^{+}CD45RA^{--}$ T cells (5×10^{5}/well) were cultured with soluble anti-CD3 mAb (100ng/ml) in the presence of autologous APCs (2.5×10^{5}/well) plus rhIL-2 (100 U/ml), and 10ng/ml SRL for 5 d.

At the end of culture, the cells were harvested, and the surface expression of CD25, CD127, CTLA-4, CD45RO and intracellular expression of FOXP3, CTLA-4 and IL-10, TGF-β were analyzed by flow cytometry. Numbers in the corners indicate the percentage of positive cells. Representative data are shown.
B. SRL-derived Tregs were anergic to alloantigenic stimulation. $CD4^+CD25^-$ responder T cells (Tresp) and SRL-derived $CD4^+CD25^+CD127^{-/low}$ cells (Tregs) were co-cultured with irradiated allogeneic D-PBMCx or a third-party 3^{rd}-PBMCx at a ratio of 1:2, respectively. After 5 d of culture, T cell proliferation was determined using [^{3}H]-thymidine incorporation (n=3).

3.9 DNA demethylation plays a role in the stability of FOXP3 expression

Recently, compelling evidence demonstrated that epigenetic regulation played a crucial role for establishment of a stable Treg lineage (Huehn, Polansky et al. 2009). Epigenetic modifications, which can target histones or the DNA directly, affect gene transcription by altering the accessibility of distinct DNA regions to transcription factors and other DNA-binding molecules. In an in vitro TGF-β-dependent conversion of iTreg cell model, Daniel *et al* demonstrated that the stabilizing effect of everolimus, a rapamycin-derivative on FOXP3 expression is partially produced by interfering with the expression of DNMT1 (Daniel, Wennhold et al. 2010), a DNA methyltransferase 1 involving the modification of DNA methylation in the *Foxp3* locus (Baron, Floess et al. 2007; Janson, Winerdal et al. 2008; Huehn, Polansky et al. 2009). The ablating DNMT1 gene, or knocking down by siRNA, or pharmacologic inhibition of DNMT1 activity markedly increased the efficacy of induction and stability of Foxp3 expression (Huehn, Polansky et al. 2009; Josefowicz, Wilson et al. 2009). We wanted to check whether SRL vs. TAC also had a similar effect on DNMT1 changes and accounted for its role in the induction of FOXP3 expression.

As shown in Figure 7A, activated $CD4^+CD45RA^-$ T cells treated with

anti-CD3 and autologous APCs in SRL group, showed significantly lower levels of DNMT1 expression compared to untreated control or TAC treated group by western blot with bulk or total $CD4^+$ T cells. This result was further confirmed with intracellular staining of DNMT1 protein by flow analysis. SRL, compared to the untreated control or TAC group, decreased about 26% of DNMT1 expression with PI3K inhibitor LY294002, which has strong and broad inhibition at the upstream of PI3K signaling, and decreased even more by 50% that of the control (Fig. 13B). To take the advantage of flow technique, we gated on $FOXP3^+CD127^-$ Treg vs. $FOXP3^-CD127^+$ non-Treg subsets. Unexpectedly, we found that FOXP3+ cells contained higher baseline expression of DNMT1 vs. $FOXP3^-$ cells. However, the expression of DNMT1 by SRL was sharply decreased in FOXP3+ cells vs. FOXP3- cells after culture treated with SRL for 5 days (35% vs. 20%, respectively), whereas TAC had no effect on DNMT1 expression in both populations. As a positive control, the levels of DNMT1 expression decreased the most at the above conditions treated with LY294002 (potent inhibitor of phosphoinositide 3-kinases (PI3Ks) (Fig. 13C).

From these findings, it is suggested that, in humans, weak downregulation of DNMTs by treatment with TGF-β and IL-2 minimally affects the maintenance of CpG methylation during cell division (proliferation), whereas strong downregulation of DNMTs by treatment with mTOR inhibitor, together with TGF-β/IL-2, suppresses the maintenance of DNA methylation, as such resulting in a gradual accumulation of the $FOXP3^+$ population.

Whereas this result cannot prove a direct de novo induction of FOXP3+ cells from SRL treatment, we started out to check other possibility of SRL

related to de novo induction of FOXP3+ expression. We were particular interested in PI3K/AKT signaling in the induction of Tregs (Ouyang, W., O. Beckett, et al. 2010) as more recent data showed that PI3K/AKT/Foxo signaling plays a role in the development of thymic-derived natural Tregs and the *de novo* induction of TGF-β-induced iTregs (Ouyang, W., O. Beckett, et al. 2010) although PI3K/AKT/mTOR signaling has been recognized as a consistent defect in mouse and human $CD4^+CD25^+$ Treg cells compared to conventional T cells (Crellin, Garcia, et al. 2007). Although we observed a profound decrease in levels of phospho-AKT (Ser473) and phospho-FOXO3a by PI3K inhibitor LY294002 whereas SRL or TAC treatment showed no changes on either phospho-AKT or phospho-FOXO3a in alloreactive $CD4^+CD45RA^-$ T cells (data not shown). We concluded that SRL might not use PI3K/AKT/Foxo pathway as a potential *de novo* induction of $FOXP3^+$ Tregs in alloactivated CD4 T cells. However, our results suggested a possible superior usage of a combined allosteric mTOR inhibitors with a dual PI3K/mTOR kinase inhibitor for the future design of transplantation tolerance induction drugs and the importance of Tregs in alloimmune inhibition.

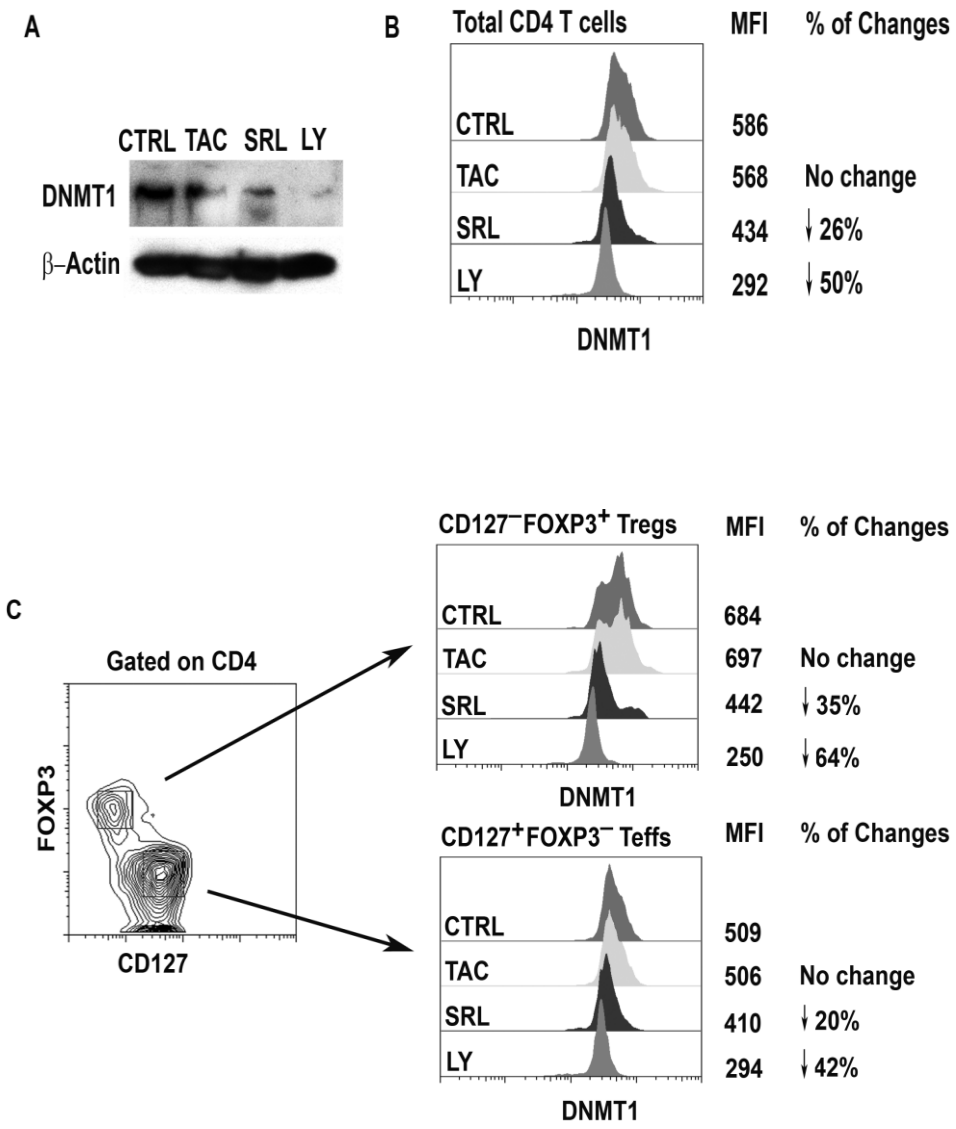


Figure 13. SRL-mediated down-regulation of DNA methylation increased FOXP3 stability. Alloreactive $CD4^{+}CD45RA^{-}$ T cells were re-stimulated with anti-CD3 and autologous APCs plus IL-2 in the presence or absence of 10 ng/ml SRL, 2ng/ml TAC or 20 μM of LY294002 for a total of 5 days.
A. DNMT1 expression was assessed at the end of the culture by Western blot analysis. Cell lysates from cultured cells as described above were separated by SDS/PAGE gel

and probed with primary polyclonal anti-DNMT1 followed by HRP-conjugated secondary antibody. SRL effectively decreased DNMT1 protein expression while TAC had no effect. Positive control of PI3K inhibitor (LY) showed profound inhibition of DNMT1 expression.

B. DNMT1 expression was alternatively assessed by flow cytometry assay. At the end of culture, cells were recovered and intracellular expression of DNMT1 was first stained with a rabbit polyclonal anti-DNMT1 antibody followed by PE-conjugated anti-rabbit Ig. Total $CD4^+$ cells were gated for analysis. Mean fluorescein intensity (MFI) changes compared to the control were calculated as: % of change = (control group - drug group)/control group. Comparable results were obtained compared to the western blot analysis.

C. Flow-based DNMT1 expression was further analyzed by gating on $CD4^+CD127^-FOXP3^+$Tregs or $CD4^+CD127^+FOXP3^-$ non-Treg effector T cell subsets, respectively. Compared to effector T cells, Tregs showed profound decrease of DNMT1 expression with SRL treatment (35% vs. 20% decrease of MFI) whereas TAC had no changes, LY as a positive control.

DISCUSSION/CONCLUSIONS

4.1 Discussion

This study confirmed that allogeneic stimulation of human naïve T cells promote the differentiation of $CD4^+$ T helper cells (Th1, Th17 and Treg cells). The results revealed that polyclonal activation of alloreactive T cells induced differentiation/expansion of effector Th1, Th17 and Treg cells, but the generation of alloreactive Th17 cell was dependent on the presence of APCs under neutral conditions or relatively physiologic stimulation. In addition, the findings indicated that alloreactive Th1 and Th17 cells were differently but effectively inhibited by calcineurin, and the component constitutions of proinflammatory Th1, Th17 cells and disease protective Treg cells had been changed. Moreover, this study confirme and extend also the previously proposed view that the Treg phenotype was not terminally differentiated (Zhou, Ivanov et al. 2007), and in the presence of SRL, $FOXP3^+$ expression in Treg cells was stable and sustained its normal functional suppression to conventional CD4 T cell proliferation and Th1/Th17 allloimmunity. Furthermore, SRL increased the percentage of $FOXP3^+$Tregs without promoting Treg reprogramming to increase $FOXP3^+$IFN-γ and $FOXP3^+$IL-17^+ Treg subsets, and that the combination of SRL and very low dose of TAC completely inhibited phenotypic $FOXP3^+$IFN-γ^+ and $FOXP3^+$IL-17^+ Treg subsets. Mechanistically, TAC blocked TCR-induced calcineurin differently prohibiting the generation of Th1 and Th17 cells, and SRL stabilized Treg cells by inhibiting DNA methyltransferase without prominent affecting FOXO3/FOXP3 interaction.

In this study, two different categories of immunosuppressive agents, calcineurin inhibitor (TAC) and mTOR inhibitor (SRL), were tested on

the generation of Th1, Th17 and Treg cells and the functional properties of in vitro generated Treg cells in activated alloreactive CD4 T cells. With regard to differential mechanisms, it becomes clear that Treg induction is best achieved by subimmunogenic TCR stimulation under conditions that avoid fully functional activation of antigen presenting cells, and the latter is also required for maximal differentiation of Th17 cells (Apostolou et al. 2005; Evans, Suddason et al. 2007; Sauer, Bruno et al. 2008; Gottschalk, Corse et al. 2010). In fact, in a steady state without inflammation or acute rejection to transplants, the in vivo protocol was more akin to an in vitro conversion method where T cells were limited for subimmunogenic stimulation and a short time activation period in the absence of exogenously TGF-β and limited activation of the PI3K/AKT/mTOR pathway (Sauer, Bruno et al. 2008). In this system, the differentiation/expansion of generated alloreactive CD4 T cells (referred as monocyte/T cell culture) was inducted with subantigenic dose of anti-CD3 stimulation (e.g. 100ng/ml) in the presence of unstimulated APCs for maximal induction of Treg and Th17 cells. High amount of IL-2, Foxp3 stabilization mediator (~ 100U/ml for human Treg induction) was included in the system (Davidson, DiPaolo et al. 2007; Horwitz, Zheng et al. 2008; Chen, Kim et al. 2011).

The two immunosuppressive agents, TAC and SRL, were compared for their ability to block the transplant destructive effector Th1/Th17 cells and maintain the stability of FOXP3 expression in graft protective Treg cells. In the past two decades, a large array of immunosuppressive agents has expanded the armamentarium used by transplant physicians and surgeons to prevent acute allograft rejection, evidenced by the greatly improved rates of short-term graft survival. The focus of transplantation

medicine is now more shifted towards tackling issues associated with side effects of long-term immunosuppression and chronic rejection. TAC and SRL are widely used to effectively prevent transplant rejection. However, the effects of SRL and TAC on the subsets of alloreactive T cells, effector Th1/Th17 versus Treg cells, were not fully studied.

The data showed that TAC and SRL in concentrations comparable or even lower than in vivo therapeutic concentrations, still strongly inhibited the proliferative capacity of human alloantigen-activated CD4 Th cells in vitro, not only at relatively to physiologic conditions, but also at stringent pro-inflammatory environments. Averagely over 90% of both IFN-γ, IL17-producing cells were blocked by TAC, and a relatively lower percentages (30 to 60% inhibition, respectively) affected by SRL. Interestingly, the presence of high concentration of IL-2 (e.g. 100U/ml) reversed the SRL effects on IFN-γ-secreting cells, but had little effect on IL-17^{+} cells. In the presence of inflammatory cytokines, such as IL-1b, IL-6 and TNF-a, TAC still effectively blocked Th1 and Th17 cell responses, while SRL only at high concentration (>10 ng/ml) or above the in vivo therapeutic concentrations could significantly inhibit IFN-γ or IL-17 production. However, the combinations of low dose of TAC irreverent of concentrations of SRL effectively inhibited IFN-r or IL-17 production than SRL used alone.

In this study, the capability of these two different categories of immunosuppressive drugs for the induction of alloantigen specific Tregs was tested. Consistent with previous work, SRL effectively induced alloantigen specific Tregs while TAC did not (Coenen, Koenen et al. 2006; Gao, Lu et al. 2007; Strauss, Whiteside et al. 2007). Considering that SRL promote while TAC decrease both percentage and *FOXP3*

mRNA levels of $FOXP3^+$ expression in alloreactive CD4 T cells (Figure 10C), we conclude that in the presence of high amount of IL-2, TAC at 2ng/ml with SRL at 2-5ng/ml achieved the maximal effect in inhibiting the production of IFN-γ and IL-17 while maintaining a high level of FOXP3 expression in alloreactive CD4 T cells.

Although Th cell subsets preferentially express particular transcription factors and produce distinct cytokines, recent studies suggest considerable levels of plasticity between different T cell lineages and in vivo reprogramming of adaptive transferred Treg cells exist, and these point toward potent peripheral regulation of effector T cell subset development in a specific microenvironment (O'Shea and Paul 2010). For instance, peripheral mature Treg cells can be converted into IFN-γ-secreting Tregs (IFN- γ^+FOXP3^+) or completely Th1 cells or IL-17-secreting Tregs (IL-17^+FOXP3^+) or completely Th17 cells in the presence of Th1 polarizing cytokine IL-12 or Th17 cytokines IL-1b or IL-6, respectively (Beriou, Costantino et al. 2009). In addition, proinflammatory conditions also promote reprogramming of Tregs (Kryczek, Wu et al. 2011). Functionally, although IL-17^+Foxp3^+ or IFN- γ^+FOXP3^+ T cells retained their suppressive capacity, they were not as strong as IL-17^-FOXP3^+ or IFN-γ^-Foxp3^+ Treg cells. They express moderate levels of effector cytokines and shared the trafficking phenotype with Treg and Th1 or Th17 cells, and may home to and play conventional T cell roles in the local microenvironment, or when encounter with several local proinflammatory stimuli, they may be able to reprogram themselves for its needs (O'Shea and Paul 2010).

In addition to the inflammatory cytokines, immunosuppressive agents and epigenetic factors may also influence the plasticity of T cell subsets. A

recent study showed that at high concentration (>100ng/ml), SRL not only completely inhibited the differentiation of IFN-γ-secreting T cells, but also inhibited Foxp3$^+$ Treg cells to produce inflammatory cytokines (IFN-γ or IL-17) when compared to untreated controls (Hippen, Merkel et al. 2011; Hippen, Merkel et al. 2011). At a mimic physiologic environment or a non-polarizing condition, clinically therapeutic dose of SRL (e.g. 5ng/ml) were effectively induced functionally alloantigen specific Tregs without increasing any IFN-γ$^+$FOXP3$^+$ or IL-17$^+$ FOXP3$^+$Treg subsets, and importantly, when combined with very low dose of TAC, SRL-derived Tregs were completely spared of any these Treg subsets. Taken together, these data suggested that although the likelihood of plasticity and in vivo reprogramming of Tregs may be context-dependent, the in vitro manipulation of Treg cells with SRL or modifications of TSDR demethylation (SRL itself affects *FOXP3* locus demethylation, (Fig. 13) of these cells may provide some degree of resistance to the reprogramming process and favor for in vivo stability of any therapeutic application of Tregs (Daniel, Wennhold et al. 2010; Hippen, Merkel et al. 2011).

Further analysis established that SRL-derived Tregs were real Tregs, which expressed typical Treg surface markers, were anergic to allostimulations, and most importantly they specifically suppressed proliferation of allogeneic effector T cells and Th1 and Th17 alloimmune responses. SRL-derived Tregs also expressed TGF-β and IL-10, two important anti-inflammatory cytokines in limiting inflammatory responses and the generation of Treg cells. TGF-β alone plays a critical role in the induction and homeostasis of Treg cells, whereas in the presence of inflammatory cytokines, it promotes Th17 differentiation, but

this TGF- β comes from activated T cells, but not from Treg cells. This suggests that Treg-derived anti-inflammatory cytokines TGF-β and IL-10 are important for promoting tolerance induction while constraining proinflammtory Th17 immunity, although SRL moderately reduced Treg-derived IL-10 production in our study.

The stability of FOXP3 expression appeared mandatory for the induction of prospective tolerance, and the in vitro induction of iTreg with unstable Foxp3 expression is correlated with lack of demethylation of the Foxp3 locus, (Baron, Floess et al. 2007; Janson, Winerdal et al. 2008; Huehn, Polansky et al. 2009). The ability of SRL and TAC to maintain the stability of FOXP3 expression was compared. SRL significantly decreased DNMT1(FOXP3 promoter demethylation) expression, which may be is a prerequisite for stable FOXP3 expression and suppressive phenotype of Tregs (Huehn, Polansky et al. 2009). The above results revealed that SRL more than TAC might interfere with the DNMT1 expression, and this underlined its appropriately future therapeutic strategies as Treg cells target.

The molecular basis of mTOR inhibitors such as SRL and everolimus increasing FOXP3 expression is presently largely unknown (Ouyang W, Beckett O, natimm2010)

Although the results in this study showed a profound decrease in levels of phospho-AKT and phosphor-FOXO3a in PI3K inhibitor LY294002 treated group, there was no evidence of either SRL or TAC has any effect on the phosphorylation of these two proteins. We concluded that SRL might not use PI3K/AKT/FOXO pathway as a potential *de novo* induction of $FOXP3^{+}$ Tregs, and any further efforts to delineate the underlie mechanism are encouraged. These results also suggested a possible

superior usage of a combined allosteric mTOR inhibitors with a dual PI3K/mTOR kinase inhibitor for the future design of transplantation tolerance induction drugs.

In sum, SRL and TAC differentially affect differentiation/expansion of alloreactive pathogenic Th1 and Th17 cells and graft-protective Treg cells. TAC used at lower than clinically therapeutic dose is more effective than SRL for inhibiting Th1 and Th17 cells, whereas SRL has advantage for de novo induction and expansion of donor-specific and functional Treg cells. Therefore, the combination of SRL and TAC should be considered for tolerance-inducing protocols, in order to recruit not only natural, but also induced $FOXP3^+$ cells into the overall Treg pool while still effective controlling the graft-destructive effector Th1 and Th17 cells. The appropriate amount of the combination of SRL and TAC used for clinical transplant patients should contribute relatively to a better tolerance induction while warrant effectively blocking acute rejection.

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