EFFECTS OF DNA METHYLTRANSFERASE-INHIBITION ON FOXP3 EXPRESSION IN $CD4^+$ T CELLS

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STATEMENT OF CONTRIBUTIONS

All experimental data contained in this work was entirely performed, analyzed, and compiled by myself, Edmund Yao, under the guidance of Dr. Ciriaco Piccirillo.

STATEMENT OF INTEGRITY

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ABSTRACT

$CD4^+Foxp3^+$ regulatory T cells (T_{REG}) are pivotal in the maintenance of immune tolerance to self-antigens and act as regulators of autoimmune reactions. There are two subsets of T_{REG}: naturally occurring (nT_{REG}) which develop in the thymus and inducible (iT_{REG}) which are generated from conventional $Foxp3^-$ effector T cells (T_{CONV}) in the periphery or *in vitro*. While both subsets require Foxp3 expression to function, iT_{REG} cells gradually downregulate Foxp3 *in vitro* and lose their suppressive capabilities. In contrast, nT_{REG} cells show constitutive Foxp3 expression and demonstrate stable suppressive function *in vitro*. Recent studies reveal a region of the *Foxp3* locus that is differentially methylated between nT_{REG} and iT_{REG}. The methylation-sensitive region, known as the T_{REG}-specific demethylated region (TSDR), is completely unmethylated in nT_{REG}, partially methylated in iT_{REG}, and heavily methylated in T_{CONV}, suggesting that differential methylation patterns of the TSDR may account for unstable Foxp3 expression in iT_{REG}. Methylation is mediated by DNA methyltransferase (DNMTs) enzymes, and several inhibitors are known to block their activity. In this study, we use 5'-Aza-2'-deoxycytidine (Aza), a nucleoside analog inhibitor, and RG108, a non-nucleoside inhibitor to determine whether inhibition of DNMTs and thus methylation, impacts Foxp3 expression and iT_{REG} development in culture. Our results show that DNMT inhibition in the absence of TGF-β was unable to induce Foxp3 expression in T_{CONV} cells. However, in T_{CONV} cells that were pre-exposed to TGF-β, Aza and RG108 induced Foxp3 expression in a significant number of cells. Moreover, a higher proportion of Foxp3-expressing cells with stronger Foxp3 MFI were induced when Aza was used in conjunction with TGF-β suggesting an additive and non-redundant effect of DNMT-inhibition. Although iT_{REG} cells typically downregulate Foxp3 in the absence of TGF-β, treatment with Aza alone prolongs the persistence of Foxp3 expression. Neither Aza nor

RG108 was as effective as TGF-β in maintaining Foxp3 expression. Collectively, our data shows that DNMT inhibition without TGF-β is insufficient to induce Foxp3 expression but in conjunction, can increase induction and strengthen Foxp3 expression. DNMT inhibition can also prolong Foxp3 expression in the absence of TGF-β possibly by disrupting epigenetic silencing mechanisms.

ABRÉGÉ

Les lymphocytes T régulateurs $CD4^+Foxp3^+$ (T_{REG}) sont des pivots dans le maintien de la tolérance immunitaire aux antigènes autologues et agissent en tant que régulateurs des réactions auto-immunes. Il y a deux sous-ensembles de T_{REG} : les naturelles (nT_{REG}) qui se développent dans le thymus et les inductibles (iT_{REG}) qui sont générés à partir des lymphocytes T effecteurs $Foxp3^-$ conventionnels (T_{CONV}) en périphérie ou *in vitro*. Bien que les deux sous-ensembles requièrent l'expression de Foxp3 pour fonctionner, les lymphocytes iT_{REG} régulent graduellement de manière négative Foxp3 *in vitro* et perdent leur capacité suppressive. En revanche, les lymphocytes nT_{REG} présentent une expression constitutive de Foxp3 et démontrent une fonction suppressive stable *in vitro*. Des études récentes révèlent une région du locus *Foxp3* qui est différentiellement méthylée entre les nT_{REG} et les iT_{REG}. La région sensible à la méthylation, connue sous le nom de la région déméthylée spécifique à T_{REG} (T_{REG}-specific demethylated region, TSDR), est complètement non méthylée dans les nT_{REG}, partiellement méthylée dans les iT_{REG}, et fortement méthylée dans les T_{CONV}. Cela suggère que les modèles de méthylation différentielle du TSDR pourraient expliquer l'expression instable de Foxp3 dans les iT_{REG}. Les enzymes ADN méthyltransférases (DNA methyltransferases, DNMTs) servent de médiatrices dans la méthylation, et quelques inhibiteurs sont connus pour bloquer leur activité. Dans cette étude, nous utilisons le 5'-Aza-2'-déoxycytidine (Aza), un inhibiteur analogue nucléosidique, et RG108, un inhibiteur non-nucléosidique, pour déterminer si l'inhibition des DNMTs et ainsi la méthylation, aurait un impact sur l'expression de Foxp3 et le développement de iT_{REG} en culture. Nos résultats montrent que l'inhibition de DNMT en l'absence de TGF-β n'a pas pu induire l'expression de Foxp3 dans les lymphocytes T_{CONV}. Toutefois, dans les lymphocytes T_{CONV} qui étaient pré-exposés au TGF-β, Aza et RG108 ont induit l'expression de Foxp3 dans un nombre important de

lymphocytes. De plus, une proportion plus grande de lymphocytes exprimant le Foxp3 avec un Foxp3 MFI plus élevé étaient induits lorsque Aza était utilisé conjointement avec TGF-β, suggérant un effet additif et non redondant de l'inhibition de DNMT. Ma1gré que les lymphocytes iT_{REG} régulent généralement de façon négative Foxp3 en l'absence de TGF-β, le traitement par Aza seul prolonge la persistance de l'expression de Foxp3. Ni Aza, ni RG108 n'est aussi efficace que TGF-β dans le maintien de l'expression de Foxp3. En somme, nos données montrent que l'inhibition de DNMT sans TGF-β est insuffisant pour induire l'expression de Foxp3, mais que conjointement, peut en augmenter l'induction et la renforcer. L'inhibition de DNMT peut également prolonger l'expression de Foxp3 en l'absence de TGF-β possiblement en perturbant les mécanismes d'inhibition épigénétique.

LIST OF ABBREVIATIONS

3dTx: day-3 thymectomy

APC: antigen-presenting cell

ATF: activating transcription factor

Aza: 5'-Aza-2'-deoxycytidine

CNS: conserved non-coding sequence

CREB: cyclic-AMP-responsive-element-binding protein

CTLA-4: cytotoxic T lymphocyte antigen-4

DNMT: DNA methyltransferase

Foxp3: forkhead box P3

GATA: trans-acting T-cell-specific transcription factor

HAT: histone acetyltransferase

HDAC: histone deacetylase

IFN: interferon

LAG: lymphocyte activation gene

MHC: major histocompatibility complex

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

IBD: inflammatory bowel disease

IL: interleukin

IPEX: immunodysregulation polyendocrinopathy enteropathy X-linked syndrome

iT_{REG}: inducible $CD4^+Foxp3^+$ regulatory T cell

JAK: Janus kinase

MFI: median fluorescence intensity

MS: multiple sclerosis

nT_{REG}: naturally-derived $CD4^+CD25^+Foxp3^+$ regulatory T cell

Smad: mothers against decapentaplegic homolog

STAT: signal transducer and activator of transcription

T1D: type 1 diabetes

T_{CONV}: $CD4^+CD25^-$ conventional T cell

TCR: T cell receptor

TGF-β: Tumour growth factor-beta

Th1: T-helper type 1

Th2: T-helper type 2

TNF-α: Tumour necrosis factor alpha

T_{REG}: regulatory T cell

TSDR: T_{REG}-specific demethylated region

RG108: 2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl)propionic acid

INTRODUCTION

1. Autoimmune disease and peripheral tolerance mechanisms

In North America, it is estimated that approximately 7% of the total population suffers from autoimmune disease[1]. Autoimmune disease is characterized by an aberrant inflammatory response which is mediated by the host's own immune system against self-molecules. Over 80 autoimmune diseases have been described thus far, of which inflammatory bowel disease (IBD), type 1 diabetes (T1D), and multiple sclerosis (MS) are some of the most extensively studied. Collectively, autoimmune disease is considered as one of the most important public health concerns of the developed world[1, 2]. Canada for example, has one of the highest incidence and prevalence rates of IBD in the world[3, 4]. Epidemiological studies have reported that the prevalence rates of autoimmune disease have increased dramatically in the last 40 years, particularly in Westernized, developed countries[4, 5]. While the precise etiology behind the development and progression of autoimmune diseases is unclear, it is generally accepted that auto-reactive T cells are mediators of the disease[2, 6].

A functioning adaptive immune system must be able to protect its host against a lifetime of unanticipated pathogens. Through a process of gene recombination, lymphocytes generate a repertoire of receptors of incredible diversity capable of recognizing innumerable antigens[7]. Sometimes, randomly generated receptors that can recognize self-molecules arise, and the immune

system employs several self-tolerance mechanisms to prevent these cells from becoming pathogenic. For the most part, T-cells with receptors that recognize self-antigens with high avidity, are eliminated in the thymus through a process known as clonal deletion[8, 9]. Despite only 2-4% of developing thymocytes surviving the selection process and reaching maturity[7], self-reactive T-cells nevertheless manage to escape these deletion mechanisms; even in healthy individuals, there is evidence of potentially hazardous self-reactive lymphocytes in the periphery[10]. In these cases, the cells may be rendered functionally inactive (anergy) or further eliminated through apoptosis[8].

2. Regulatory T cells (T_{REG})

These passive or recessive mechanisms of self-tolerance undoubtedly play an important role in limiting the potential of auto-reactive lymphocytes to cause disease. However it is now established that there is a subset of $CD4^+$ T cells that plays a dominant role in the maintenance of immune homeostasis and prevention of autoimmunity by actively inhibiting the activation and expansion of auto-reactive lymphocytes[11]. The existence of a regulatory subset of T cells was first demonstrated in classic experiments using neonatal day-3 thymectomized mice (3dTx), which would result in the development of widespread organ-specific autoimmunity characterized by autoantibody formation and cell-mediated immune destruction[12, 13]. Subsequent studies showed that 3dTx-induced autoimmunity resulted from lack of a specific subset of $CD4^+$ T cells that constitutively expressed the IL-2 receptor α chain (CD25)[14]. In normal mice,

$CD4^+CD25^+$ T cells appeared in the periphery on day 3 and were preceded by $CD4^+CD25^-$ T cells, which were detectable immediately after birth[14]. Therefore, it was shown that removal of the thymus at day 3 resulted in depletion of a $CD4^+CD25^+$ T cell subset with immunosuppressive characteristics and that these cells were required to prevent potentially auto-reactive $CD4^+CD25^-$ T cells from causing severe autoimmune disease[15]. These cells became known as naturally-occurring regulatory T cells (nT_{REG}) because of their ability to suppress immune responses immediately after export from the thymus[11]. Initially, nT_{REG} cells were identified by CD25 which is constitutively expressed on the surface of nT_{REG} cells, but this was inadequate as conventional T cells (T_{CONV}) also expressed CD25 upon activation in both mice and humans[16]. The discovery of the gene responsible for the function and specific-lineage commitment of nT_{REG} cells would not be discovered until later, using a natural mutant mouse strain called *scurfy*. *Scurfy* is an X-linked recessive disease in mice characterized by excessive and uncontrolled proliferation of lymphocytes[17], multi-organ infiltration[18], and increased production of proinflammatory cytokines[19] resulting in death 16-25 days after birth[20]. In humans, the disease equivalent to *scurfy* is known as IPEX (immunodysregulation, polyendocrinopathy, enteropathy X-linked syndrome) and is characterized by similar symptoms[21]. In 2001, the etiology behind *scurfy* and IPEX was discovered to be a mutation in a gene called *Foxp3*[20, 22]. A direct association linking Foxp3 and the nT_{REG} cell subset implicated in 3dTx experiments, was established in 2003 with the observations that $Foxp3^{-/-}$ mice completely lacked $CD4^+CD25^+$ T cells with immunosuppressive function,

developed aggressive lymphoproliferative autoimmunity almost identical in nature to *scurfy* mice, and that retroviral transduction of *Foxp3* into naive non-regulatory T cells was sufficient to convert them toward a regulatory T cell phenotype with suppressive function[23-25]. Today, it is well established that T_{REG} cells are an essential component of peripheral tolerance representing a unique lineage of $CD4^+$ T-cells that depend on expression of Foxp3 for their development and function[16].

2.1 Mechanisms of T_{REG} Suppression

Considerable efforts have been made since the discovery of $Foxp3^+$ T_{REG} cells to elucidate the mechanisms behind their suppressive function. T_{REG} cells appear to use a variety of regulatory mechanisms, which can be divided into four basic modes of action (*Fig. A*): secretion of inhibitory cytokines, cytolysis, metabolic disruption, and modulation of the function of antigen presenting cells (APCs)[26, 27]. The inhibitory cytokines IL-10, IL-35, and TGF-β are secreted by T_{REG} cells and are believed to be important mediators of suppression. IL-10 is a potent immunoregulatory cytokine which can down-modulate the production of Th1-polarizing cytokines such as TNF-α, IL-12, and IFN-γ by APCs[28]. Moreover, IL-10 production by T_{REG} cells appears to be essential in the prevention of colitis in mouse models for IBD[29] and can reduce airway hypersensitivity in allergic inflammation models[30]. IL-35 was shown to be constitutively expressed by murine nT_{REG} cells and knockout of IL-35 resulted in disruption of the regulatory capacity of nT_{REG}[31]. TGF-β has many important functions in the context of

immune suppression and Foxp3 function (see details below). In spite of the evidence, the overall importance of cytokine-mediated suppression is contested because of evidence suggesting that T_{REG} suppression is dependent on cell-cell contact[32, 33]. Another mechanism of suppression employed by T_{REG} cells is cytolysis, mediated through the secretion of granzymes. It has been shown that T_{REG} cells, through a granzyme B-dependent and perforin-dependent mechanism, could target killing of B-cells, cytotoxic lymphocytes, and NK cells, thereby inhibiting B cell function and tumour clearance respectively[34, 35]. Suppression of effector T cells can also be achieved by T_{REG}-mediated metabolic disruption. It has been established that T_{REG} cells are unable to produce IL-2, due to transcriptional repression of the *Il2* gene by Foxp3; this, coupled with the fact that CD25 (IL-2Rα) is constitutively expressed by T_{REG} cells, led to the theory that local consumption of IL-2 by T_{REG} cells causes IL-2-deprivation-mediated apoptosis in effector T cells[32, 33]. This theory however, is still a matter of controversy as some groups report that IL-2-depletion is insufficient for T_{REG} suppression[36]. T_{REG} cells may modulate APC function through cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), which is constitutively expressed on the surface of T_{REG}, and lymphocyte activation gene 3 (LAG-3), which upon interacting with CD80/CD86 and MHC class II respectively, results in the reduced ability of APCs to activate T_{CONV} cells[31, 32]. Furthermore, T_{REG} was shown to induce the expression of indoleamine 2,3-dioxygenase (IDO) in dendritic cells, which triggers production of pro-apoptotic metabolites, resulting

in effector T cell suppression[37]. Collectively, these studies demonstrate that T_{REG} cells mediate suppression through a variety of non-exclusive mechanisms.

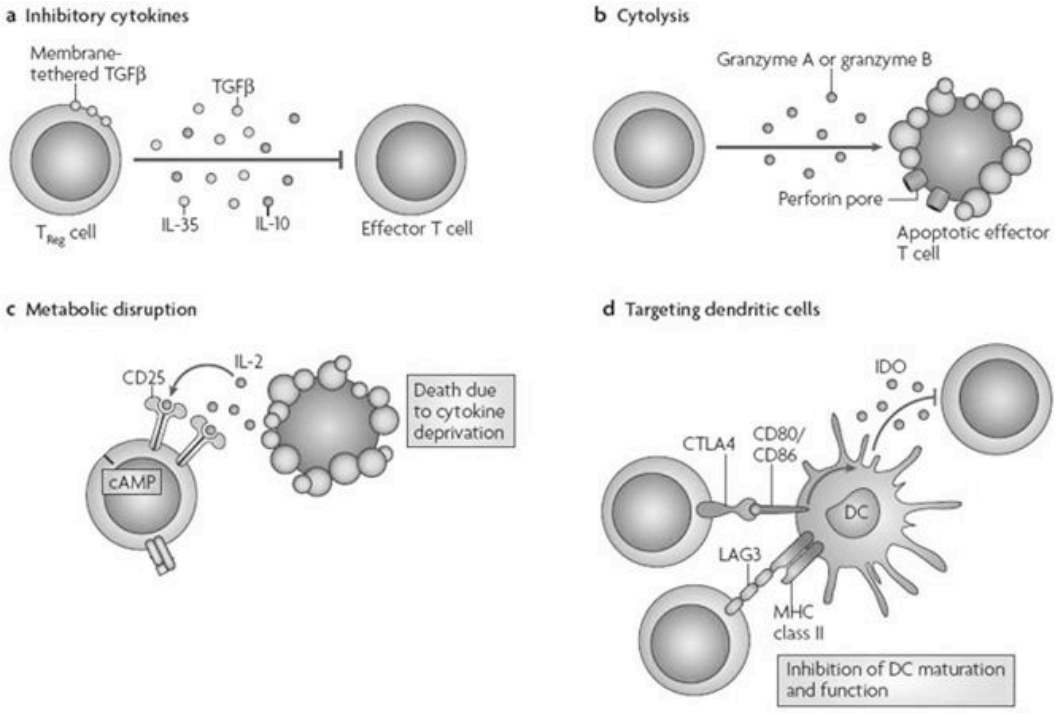

Figure A: **T_{REG} cells exert their suppressive functions via four basic modes of action**. a) secretion of inhibitor cytokines, IL-10, IL-35, and TGF-β, b) granyzme-mediated cytolysis, c) metabolic disruption through IL-2 deprivation, and d) modulation of dendritic cell function. ***~Adapted from Vignali et al., 2008[32].***

2.2 The transcription factor forkhead box protein 3

Foxp3 belongs to the forkhead box (Fox) family of transcription factors which are defined by their characteristic forkhead (FKH) domain. The FKH domain (amino acids 338-421) is responsible for nuclear localization and DNA-binding and is therefore critical for the regulatory functions of Foxp3[38]. Indeed,

the mutation in *scurfy* mice was a 2-bp frameshift mutation resulting in a product lacking the FKH domain[20]. In addition to the FKH domain, Foxp3 also contains a proline-rich N-terminal domain (amino acids 70-105) associated with inhibition of cytokine gene expression[39] and a leucine zipper (amino acids 240-261) which is required for homodimerization of Foxp3[40]. These functional domains were also shown to be essential for T_{REG} function as mutations affecting these regions were associated with IPEX disease[41] (*Fig. B*). The role of Foxp3 as a transcriptional repressor has been well documented. Foxp3 physically associates with positive regulatory transcription factors such as nuclear factor of activated T cells (NFAT) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), effectively blocking the expression of certain activation-induced cytokines such as IL-2, IL-4, and IFN-γ[42, 43], thus inhibiting effector T cell function and Th1/Th2 lineage differentiation. In addition to its repressor functions, Foxp3 acts as a transcriptional activator, targeting genes that are constitutively expressed in T_{REG} cells such as CD25 and CTLA-4[16, 44]. Foxp3 also maintains a positive auto-feedback loop in the regulation of its own expression[45]. Recent chromatin immunoprecipitation assays in conjunction with microarray analyses have identified up to 700 potential gene targets that are either activated or repressed by Foxp3[42], indicating that Foxp3 probably coordinates an extensive network of transcription factors that ultimately governs the overall functional programme of T_{REG} cells[42].

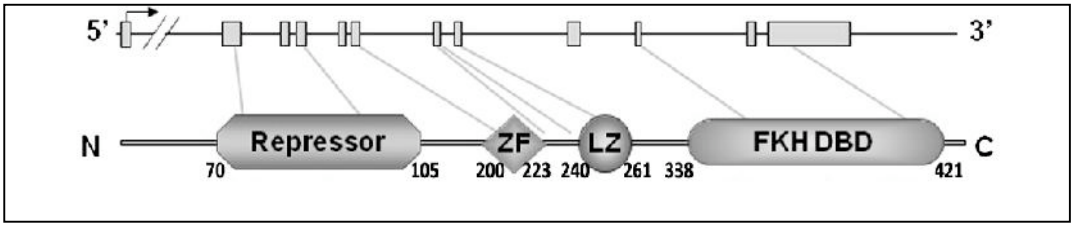

Figure B: Schematic of the *Foxp3* gene (above) and Foxp3 protein (below) showing its essential functional domains: Proline-rich N-terminal domain (Repressor), leucine zipper (LZ), and the Forkhead domain (FKH DBD). **~***Adapted from d'Hennezel et al., 2012*[41]

3. Adaptive/Inducible regulatory T cells (iT_{REG})

In addition to thymically-derived nT_{REG} cells, it is now clear that $Foxp3^+$ cells with regulatory function can also be induced in the periphery from $CD4^+CD25^-$ T_{CONV} cells both *in vivo* and *in vitro*[33, 46-48]. In an experimental system whereby naive T_{CONV} cells were transferred into a lymphocyte-deficient recipient, massive T-cell activation and symptoms resembling acute graft-versus-host disease occurred[49]. This was immediately followed by a recovery phase which was associated with the *de novo* generation of $Foxp3^+$ cells with regulatory function[49]. The experiment demonstrated that these 'inducible' $Foxp3^+$ T cells (iT_{REG}) represented a distinct lineage apart from nT_{REG}. Accumulating evidence now supports the view that TGF-β-induced iT_{REG} cells generated from T_{CONV} cells resemble nT_{REG} in that they are anergic and suppressive[44, 45]. Transfer of *in vitro*-generated iT_{REG} cells have demonstrated anti-inflammatory potential in a number of animal models of organ-specific autoimmune disease, including encephalomyelitis[50], autoimmune diabetes[51], colitis[48], graft versus host disease[52], allergic asthma[46], and *scurfy*[53]. Given their effectiveness at potentiating

suppression in autoimmune disease models, iT_{REG} cells have garnered considerable interest for use as a therapeutic treatment. Thus, efforts have been made to elucidate the conditions in which their conversion is favoured. Like nT_{REG} cells, the iT_{REG} subset also depends on the induction of Foxp3-expression for their differentiation and suppressive function[33, 46-48]. To date, the precise mechanisms by which the expression of Foxp3 is regulated are still uncertain. However it is well established that T cell receptor (TCR) stimulation in the presence of TGF-β and IL-2 can lead to efficient conversion of naive $CD4^+$ T_{CONV} to $Foxp3^+$ iT_{REG} *in vitro*[46]. Therefore, we will discuss each of these pathways and their contributions to Foxp3 expression and T_{REG} function.

3.1 T-cell receptor signaling

Both nT_{REG} and iT_{REG} require TCR signaling pathways for Foxp3 expression. In human studies, TCR activation leads to the binding of transcription factors, nuclear factor of activated T cells (NFAT), and activator protein 1 (AP1) to the *Foxp3* promoter[54]. Studies in mice revealed that TCR activation results in the binding of cyclic-AMP-responsive-element-binding protein (CREB) and activating transcription factor (ATF) to an intronic enhancer element in the *Foxp3* gene[55]. Although contributions of the TCR signaling pathway are required for induction of Foxp3, some evidence suggests that suboptimal TCR stimulation can result in upregulation of Foxp3-expression[56]. Furthermore, premature termination of TCR signalling was shown to promote expression of Foxp3 in mice[57].

Collectively, the data suggests that the duration and strength of TCR signaling is important for Foxp3 regulation.

3.2 Tumour growth factor-beta

Tumour growth factor-beta (TGF-β) family cytokines are comprised of three isoforms in mammals: TGF-β1, -β2, and -β3 with TGF-β1 being the predominantly expressed isoform within the immune system[14, 15]. Signal transduction is induced upon binding of TGF-β to its heterodimeric receptor complex, which activates kinase activity of the TGF-βRII subunit which in turn phosphorylates TGF-βR1 ultimately leading to the phosphorylation and activation of effector Smad proteins[58, 59]. Activated Smad2 and Smad3 then forms a heterodimer with Smad4, resulting in translocation into the nucleus where it can regulate TGF-β1-dependent gene expression[60]. Some T-cell specific target genes of TGF-β include trans-acting T-cell-specific transcription factor 3 (GATA3), T-bet, signal transducer and activator of transcription 4 (STAT4), interferon-gamma (IFN-γ), and granzyme-B, all of which are suppressed by TGF-β[61-64]. The critical importance of the TGF-β signaling pathway to the regulation of immune suppression was demonstrated in studies using TGF-$\beta^{-/-}$ mice, which developed fatal autoimmune disease characterized by increased inflammatory cytokine production and multiple organ failures[65, 66]. Specific T-cell deletion of receptor subunit TGF-βRII also resulted in onset of fatal multifocal autoimmunity and was associated with a significantly reduced pool of peripheral T_{REG} cells[67]. Interestingly, intrathymic development of nT_{REG} cells was mostly unaffected[58, 68],

suggesting that although TGF-β is indispensible for the maintenance and homeostasis of peripheral T_{REG} cells, it is not required for the development of thymic-derived nT_{REG}[69]. In addition to TGF-β/Smad-dependent transcriptional repression, TGF-β is perhaps the most prominent factor for Foxp3 induction and generation of iT_{REG} cells from naive T_{CONV} cells[46, 47]. TGF-β-mediated induction of Foxp3 involves the cooperation of transcription factors Smad3 and NFAT, which are downstream of TGF-β-receptor and TCR signaling respectively[70]. Recently, it was discovered that the *Foxp3* gene contains a highly conserved Smad3-NFAT responsive enhancer element known as the TGF-β sensor[70].

3.3 Interleukin-2 (IL-2) and common receptor γ-chain (γ_c)

Since the discovery of nT_{REG} cells and their constitutive CD25 expression, IL-2 has been known to be critical for T_{REG} function and homeostasis[49, 71-74]. Because Foxp3 represses the transcription of *Il2*, T_{REG} cells are unable to produce their own IL-2 and are completely dependent on paracrine sources of IL-2 for survival and growth[43, 75]. Upon binding to its receptor, IL-2 initiates a signaling cascade mediated by the common receptor γ-chain (γ_c), which in turn activates Janus kinase 1 and 3 (JAK,1 and JAK3), and signal transducer and activator of transcription 5 (STAT5). IL-2-induced STAT5 binds directly to highly conserved regions in the *Foxp3* locus to induce transcription[76]. Early studies showed that mice deficient for IL-2, IL-2Rα (CD25) or IL-2Rβ (CD122) developed widespread autoimmunity resembling that of *scurfy* mice[77-80]. Interestingly, studies showed that IL-2 or IL-2R deficiency led to a reduction, but not a

complete loss of T_{REG} frequencies, suggesting that IL-2 was dispensable for Foxp3 expression and nT_{REG} development in the thymus[74, 81]. Mice that are deficient for the common receptor γ-chain (γ_c) however, showed a complete lack of T_{REG} cells[81]. Therefore, it is thought that other γ_c-containing receptors, such as IL-7 and IL-15 can compensate for IL-2 deficiency[71].

3.4 Differences between stability of Foxp3-expression in iT_{REG} and nT_{REG}

Although iT_{REG} cells share many of the same Foxp3 regulatory pathways as nT_{REG} cells, one critical difference remains - whereas Foxp3 expression is stable and permanent in the nT_{REG} lineage, the expression of Foxp3 in induced iT_{REG} cells appears to be transient. Thus, most iT_{REG} cells lose Foxp3 expression and suppressive function following restimulation in the absence of TGF-β[82-84]. Indeed, the plasticity of Foxp3-expression in iT_{REG} cells is among the chief caveats associated with the therapeutic transfer of iT_{REG} cells, as diminished Foxp3 expression in iT_{REG} led to the acquisition of effector T cell functions, including production of IL-2, IL-4, IL-17, and IFN-γ[85], potentially exacerbating disease. Therefore, understanding the mechanisms behind iT_{REG} plasticity, and instability of Foxp3-expression, has become a top priority. Evidence has now emerged suggesting that stable Foxp3 expression may depend on epigenetic modifications of the *Foxp3* locus[84, 86-89].

4. Epigenetic Modification of Foxp3

In addition to signal transduction pathways, gene expression can be further regulated by epigenetic modifications, which include methylation of DNA as well as the acetylation or methylation of histone[90] (*Fig. 3*). These modifications affect gene transcription by altering the accessibility of DNA to transcription factors and other DNA-binding molecules[87, 90]. Methylation of DNA occurs at the carbon-5 position of cytosine residues within the CpG dinucleotide, and is catalyzed by a family of enzymes called DNA methyltransferases (DNMTs). There are three distinct phylogenic mammalian DNMTs: DNMT1, DNMT3A, and DNMT3B, each of which has different roles[91, 92]. DNMT1 is mainly responsible for maintenance by copying existing methylation patterns following DNA replication[93]. DNMT3A and DNMT3B exhibit *de novo* methyltransferase activity and are required for establishing new methylation patterns during embryonic development[94]. The methylation reaction occurs immediately after DNA replication and involves a transfer of a methyl moiety from the donor, S-adenosyl-L-methionine to the 5' carbon position of the cytosine ring[90]. DNA methylation ultimately results in gene silencing by directly interfering with the binding of transcription factors[95] or by recruiting methyl-binding domain proteins (MBDs) which function as adaptors between methylated DNA and chromatin-remodelling enzymes[96, 97]. These enzymes, which include histone deacetylases and histone methyltransferases, can covalently modify amino-terminal residues on histone. For instance, methylation of lysine at position 9 in histone H3 and deacetylation

of lysine at position 16 in histone H4, is associated with heterochromatin and gene silencing[98]. Whereas methylation results in silencing, the opposite occurs following demethylation of CpG residues, which results in chromatin decondensation and increased accessibility of DNA to transcription factors[90]. The lineage differentiation of helper T cells is largely dependent on epigenetic modifications to orchestrate the generation of a restricted set of progeny from a multi-potent progenitor cell. For instance, the genes encoding for effector cytokines *Ifn-γ* and *Il4*, are tightly regulated by DNA methylation and chromatin remodelling, as mediated by the transcription factors T-bet and Gata-3, respectively[99, 100].

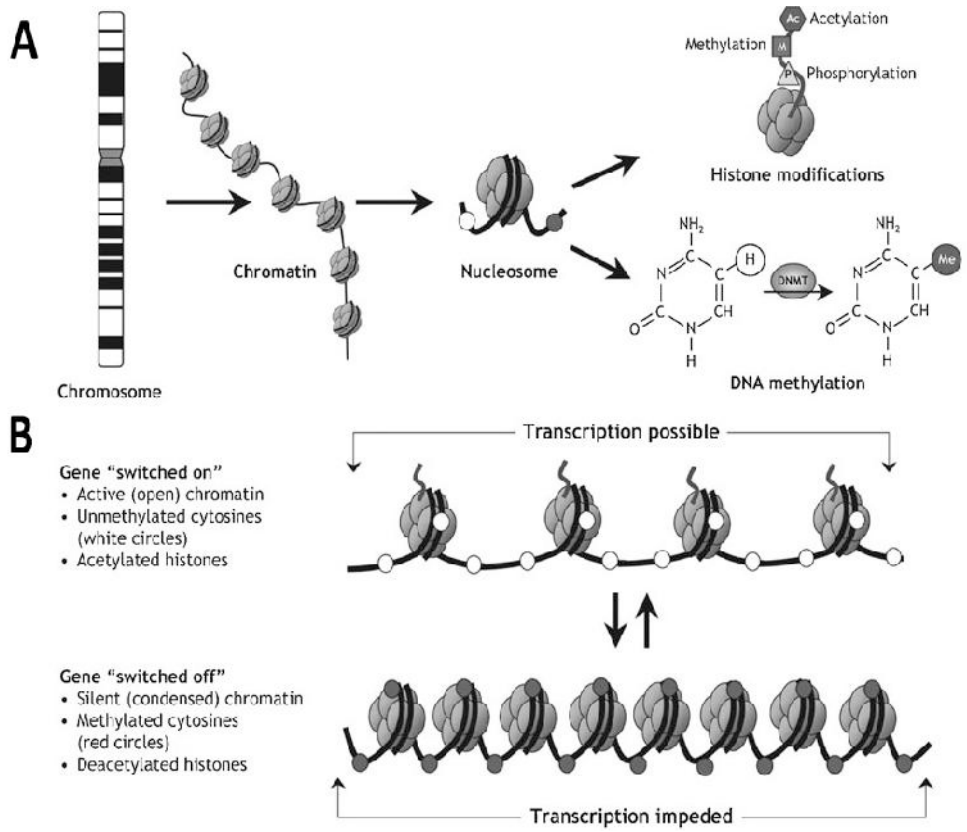

Figure 3 – Schematic of Epigenetic Modifications A) Chromosomes are made up of chromatin - tightly packed DNA that is bound to proteins called histones. The basic unit of DNA packaging is the nucleosome, which consists of a DNA strand wrapped around eight histone protein cores. Epigenetic modifications can either target histones, through acetylation, methylation and phosphorylation of amino-terminal residues, or can target DNA through methylation of cytosine residues in a reaction catalyzed by DNMT. B) Epigenetic modifications alter gene expression through changes in the structure of chromatin organization: genes are expressed (switched on) when the chromatin is an open (active) configuration, and they are inactivated (switched off) when the chromatin is in a condensed (silent) configuration. White circles = unmethylated cytosines; gray circles = methylated cytosines.

~ Adapted from Luong, P. 2009, (http://cnx.org/content/m26565/1.1/)

Hence, it is likely that differentiation of iT_{REG} and induction of Foxp3-expression in naive T_{CONV} cells is also regulated by epigenetic modifications. Three distinct regions of the *Foxp3* locus have been discovered recently which show differential patterns of both DNA methylation and histone modifications between T_{REG} subsets and T_{CONV} cells. These highly conserved non-coding regions have been identified through sequence analyses and all have been connected with epigenetic modifications during regulation events of Foxp3.

4.1 *Foxp3* promoter

The *Foxp3* promoter is located approximately 6.5kb upstream of the first coding exon and contains classic TATA- and CAAT-box sequences[54]. The *Foxp3* promoter contains several binding sites for the transcription factors NFAT, and AP-1 and specific deletion of these sequences resulted in lower transcriptional activity[54]. Analysis of the chromatin structure surrounding the promoter revealed that the *Foxp3* promoter is accessible in both resting and activated $CD4^+CD25^-$ T_{CONV} cells and this open chromatin configuration likely confers the ability for conversion of T_{CONV} cells to iT_{REG}[54]. Analysis on the methylation patterns revealed that CpG motifs within the *Foxp3* promoter are nearly completely demethylated in T_{REG} and partially methylated in T_{CONV} cells[55]. Furthermore, it was shown that *in vitro* activation of T_{CONV} cells led to an increase of methylation in the *Foxp3* promoter, thereby restricting access to transcription factors and silencing expression[55].

4.2 TGF-β sensor

As described previously, TCR stimulation of T_{CONV} cells with TGF-β induces Foxp3 expression through cooperative actions of NFAT and Smad3 via binding to a highly conserved non-coding enhancer of the *Foxp3* gene. This region, which is located approximately 2kb upstream from the first coding exon of *Foxp3*, was termed the TGF-β sensor[70]. Analysis of histone H4 acetylation levels revealed that $Foxp3^+$ nT_{REG} and iT_{REG} cells exhibited an open chromatin configuration whereas $Foxp3^-$ T_{CONV} cells did not. Although the TGF-β sensor does not contain any CpG motifs, there is evidence that recruitment of NFAT and Smad3 to the TGF-β sensor may mediate demethylation of the *Foxp3* promoter, resulting in expression[55].

4.3 T_{REG}-specific demethylated region

The most compelling evidence linking DNA methylation and stability of Foxp3 expression came with the discovery of a third, highly conserved CpG-rich region situated within the 5' UTR of the *Foxp3* gene. Studies reported that nearly 100% of the CpG motifs in this area were methylated in $CD25^-CD4^+$ T_{CONV} cells[84]. However, these same CpG motifs were almost completely demethylated in *ex vivo* nT_{REG} cells, giving rise to the naming of this region as the T_{REG}-specific demethylated region (TSDR). Interestingly, TGF-β-induced $Foxp3^+$ cells also exhibited demethylation but to a far lesser degree than that of nT_{REG} cells[84]. Upon restimulation, TGF-β-induced $Foxp3^+$ cells rapidly downregulated Foxp3 in the absence of TGF-β and this was associated with nearly complete loss

of demethylation in the TSDR. In contrast, e*x vivo* nT_{REG} cells maintained both the expression of Foxp3, and demethylation of TSDR upon reactivation[84]. High levels of acetylated histones H3 and H4 were associated in the TSDR of nT_{REG} cells but not T_{CONV} cells[84], indicating that demethylation of the TSDR promoted an open configuration of chromatin. Further studies on this region revealed binding sites for CREB/ATF[55] and STAT5[73], signifying that the activity of the TSDR is under the control of TCR and IL-2 signaling. Methylation of the TSDR was inversely correlated with CREB binding, indicating that binding sites overlapped with CpG dinucleotides[55]. Together, the evidence suggests that the demethylation of the TSDR confers stable Foxp3 expression as seen in nT_{REG} cells. Along these lines, it may be possible to recapitulate stable Foxp3 expression in TGF-β-induced iT_{REG} by promoting demethylation of the TSDR.

5. Inhibitors of DNA Methyltransferase

Inhibition of DNMT should effectively prevent *de novo* methylation. Of the known methyltransferase inhibitors, 5'-aza-2'-deoxycytidine (Aza) is one of the best studied and has been approved by the Food and Drug Administration as an anti-tumour agent for the treatment of myelodysplastic syndrome[90, 92]. Aza is a nucleoside analog of cytidine, and is incorporated randomly into DNA during synthesis. DNMT enzymes are unable to distinguish between cytosine residues and Aza, resulting in the formation of a covalent bond with the 5-aza-cytosine ring in DNA[92, 101]. Consequently, the transfer of the methyl moiety does not occur and DNMT is trapped on the DNA forming a covalent protein-DNA adduct. As

synthesis of DNA progresses with the incorporation of Aza, levels of soluble DNMT are effectively depleted and passive loss of methylation results[101]. A major caveat associated with the use of Aza is that protein-DNA adduct formation triggers DNA-damage response mechanisms resulting in cell death[92, 101]. In addition to DNMT inhibitors that are derived from nucleosides, some molecules directly bind to the catalytic domain of DNMTs and do not demonstrate the inherent cytotoxicity associated with nucleoside inhibitors that cause covalent enzyme trapping. After the advent of a three-dimensional homology model for the catalytic domain of human DNMT1, *in-silico* screening identified RG108 (2-(1,3-Dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl)propionic acid) to be a candidate inhibitor[102]. Studies showed that RG108 effectively blocked the active site of purified recombinant DNMTs, and that its inhibition was specific[102]. Experiments on human cancer cell lines confirmed that administration of RG108 effectively demethylated and reactivated tumour suppressor genes, did not deplete cellular levels of DNMT, and led to considerably less cell death than Aza administration[96, 102]. Hence, these results indicate that administration of DNMT-inhibitors, Aza and RG108, should effectively block DNA methylation reactions by inactivating DNMT enzymes in the cell.

SUMMARY AND EXPERIMENTAL OBJECTIVES

Given the rise in prevalence of autoimmune disease in recent decades, there is considerable demand for the innovation of novel therapeutic approaches. Since the discovery of nT_{REG} cells and their essential role in the maintenance of peripheral tolerance and prevention of autoimmunity, nT_{REG} cells have been extensively studied as a potential candidate for therapy. Unfortunately, nT_{REG} cells only constitute a small fraction of $CD4^+$ T cells in the periphery (~5-10%) and are difficult to expand their numbers *in vitro* due to their state of anergy[11, 16]. Consequently, harvesting sufficient numbers of nT_{REG} cells for the treatment of autoimmunity is often not viable[16]. In contrast, the iT_{REG} subset of regulatory cells can be generated in large numbers from T_{CONV} cells following TCR stimulation in the presence of IL-2 and TGF-β. TGF-β-induced iT_{REG} cells have demonstrated the ability to suppress inflammation and inhibit autoimmunity in a number of animal disease models. However, it has been shown that Foxp3-expression within *in vitro*-generated iT_{REG} cells is unstable and transient, resulting in concomitant loss of suppressive function. Hence, understanding the different regulatory mechanisms between nT_{REG} and iT_{REG} in terms of the stability of Foxp3-expression remains a top priority. Recent evidence has emerged which establishes a link between stable Foxp3-expression, as seen in the nT_{REG} cell subset, and epigenetic modifications of key regulatory elements of the *Foxp3* gene. Therefore, we hypothesize that in addition to signal transduction pathways, DNA methylation, which is carried out by DNMT enzymes, also contributes to the

regulation of Foxp3 in T_{CONV} and iT_{REG} cells. To investigate this notion, we propose that blocking methylation via DNMT-inhibitors should enhance stability of Foxp3 expression in iT_{REG}, and promote the capacity of signaling transduction pathways to induce Foxp3-expression in T_{CONV} cells. We also aim to further our understanding of the relative contributions of signaling transduction pathways, and epigenetic modifications, to the regulation of Foxp3.

MATERIALS AND METHODS

Antibodies and Reagents

5'-Aza-2'-deoxycytidine and RG108 were kindly contributed by our collaborators in the Dr. Moshe Szyf lab (Department of Pharmacology, McGill University). Recombinant human IL-2 (rhIL-2) was a gift from the Surgery Branch at the *National Cancer Institute*. Recombinant human TGF-β1 was purchased from *R&D Systems*. Functional grade purified anti-mouse CD3ε (145-2C11), functional grade purified anti-mouse CD28 (37.51), PE-CD4 (RM5), Pacific Blue-Ly5.1 (A20), PE-Cy7-CD25 (PC61), APC-TNF-α (MP6-XT22), eFluor-670 proliferation dye, and eFluor-780 viability dye were purchased from *eBioscience* (San Diego, CA). Cells were cultured in 'complete' RPMI-1640 (*Invitrogen*) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin mixture, 0.1mM non-essential amino acids, 10mM Hepes, 1mM sodium pyruvate, 1% gentamycin, and 50μM 2-ME.

Mice

B6.Foxp3^{GFPKi} reporter mice were obtained from Alexander Rudensky (Memorial Sloan-Kettering Cancer Center). Congenic Ly5.1 (CD45.1) C57BL/6 mice were purchased from The Jackson Laboratory. Both strains are maintained in a specific pathogen-free animal facility at the McGill University Health Center in accordance with approved animal protocols. All of the mice used in experiments were 4-8 weeks old.

Organ Harvest and Cell Preparation

B6.Foxp3^{GFPKi} mice were sacrificed and harvested for the axial, inguinal, brachial, and mesenteric lymph nodes along with the spleen. The organs were dissociated into single cell suspensions and pooled together. Red blood cells were removed using hypotonic ammonium chloride potassium lysis buffer.

Purification of CD4$^+$CD25$^-$ Conventional T Cells

Single-cell suspensions of lymph nodes from B6.Foxp3^{GFPKi} mice are resuspended in PBS at a concentration of 100x10^6 cells/ml for all staining steps and incubated in the dark for 15 minutes at 4^{o}C with washing in between. To remove contaminating regulatory T cells, CD25$^+$ cells are depleted by first staining with PE-anti-CD25 (35µg/100x10^6 cells) followed by anti-PE magnetic beads (75µl/100x10^6 cells). Cells are then resuspended at 50x10^6 cells/ml in PBS/2% FBS and a 'sensitive positive selection' program is run by magnetic-associated cell sorter (Miltenyi Biotech). The resulting CD25$^-$ sample is then stained with PE-anti-CD4 (35µg/100x10^6 cells) followed by anti-PE magnetic beads (75µl/100x10^6 cells). A 'positive selection' program is run and the CD4$^+$ sample is retained. Flow cytometry confirms that these cells are >98% pure for CD4$^+$CD25$^-$ cells with <1% contaminating Foxp3^{GFP+} cells.

Conditions for the Generation of iT_{REG} in vitro

$CD4^+CD25^-$ T cells ($5x10^5$) were cultured in 48-well, flat-bottom microtiter plates that were previously coated with anti-CD3 (2μg/ml) and anti-CD28 antibody (2μg/ml) for 1 hour at 37°C. TGF-β1 (5ng/ml), and rhIL-2 (5ng/ml) were provided to each well and cultured in 1ml of 'complete' RPMI-1640. Control cells are cultured in identical conditions except that they are not given any TGF-β1.

Fluorescence-Activated Cell Sorting (FACS)

Cell samples were stained with specific antibodies in order to distinguish desired cell subsets and resuspended in PBS/2% FBS at a concentration of $20x10^6$ cells/ml. Cell sorting was performed by the FACSAria cell sorter (*BD Biosciences*) at the McGill University Health Centre (Montreal, Quebec) immunophenotyping platform and was executed by the technician, Marie-Hélène Lacombe.

Foxp3 Maintenance Assay

TGF-β-induced $Foxp3^+$ cells were generated from MACS purified $CD4^+CD25^-$ T_{CONV} cells. At day 3 post-activation, cultures are harvested and purified for $Foxp3^{GFP+}$ cells by FACS. These $Foxp3^{GFP+}$ cells are plated ($5x10^4$ cells/well) in a 96-well microtiter plate and restimulated under plate-bound conditions: αCD3/αCD28 (1μg/ml), with Aza (5μM) or RG108 (500μM). All cultures were supplemented with rhIL-2 (5ng/ml).

iT_{REG} Suppression Assay

$CD4^+CD25^-$ T_{CONV} responder cells were isolated from congenic Ly5.1 (CD45.1) mice as previously described and labeled with eFluor-670 proliferation dye at a final concentration of 5μM for 5 minutes at 37°C in PBS. Cells were washed with cold 'complete' RPMI-1640 three times before being plated. Antigen presenting cells (APCs) were prepared from the MACS sorted $CD4^-$ $CD25^-$ cell population of B6.$Foxp3^{GFPKi}$ reporter mice and irradiated for 15 minutes. iT_{REG} cells were generated from B6.$Foxp3^{GFPKi}$ reporter mice as previously described and sorted with FACS to obtain a pure population of Foxp3-expressing cells. The cells were then co-cultured into a 96-well flat-bottom microtitre plate in 'complete' RPMI medium and rhIL-2 (5ng/ml) and activated using soluble αCD3 (1μg/ml). Each well contained $5x10^4$ T_{CONV} cells, $2x10^5$ APCs, and various numbers of iT_{REG} in different ratios with T_{CONV} cells. By gating on the Ly5.1 congenic marker during analysis, proliferation of only the responder cells can be accurately measured.

Extracellular/Intracellular Staining and Flow Cytometric Analysis

Staining was performed with the specific antibody (0.5μg/10^6 cells) at 4°C for 15 minutes in the dark. For the detection of cytokine secretion, cells were stimulated for 4 hours at 37°C with PMA (20 ng/ml), ionomycin (1 nM) *Sigma-Aldrich* (Oakville, ON, Canada), and GolgiStop (1:1000 dilution) *BD Biosciences*, before cells were fixed, permeabilized, and stained with specific antibody (0.75μg/10^6 cells) at 4°C for 60 minutes in the dark. All cell samples are stained with eFluor-780 viability dye to facilitate exclusion of non-viable cells during analysis.

Approporiate 'fluorescence minus one' (FMO) and isotype controls were used to identify gating boundaries. Cells were acquired using the FACSCanto flow cytometer (*BD Biosciences*) and data was analyzed using FlowJo (*Tree Star*).

Statistical Analysis

All MFI values reported are actually given as *median* fluorescence intensity to compensate for logarithmic data. Bar graphs are plotted with the mean ± standard error of the mean (SEM). All statistical analysis was conducted with GraphPad Prism v.5 (GraphPad Software). Analyses comparing multiple treatment conditions were performed using *1-way ANOVA* with *Bonferroni's* or *Dunnett's Multiple Comparison* post-test. Values of $p<0.05$ are considered significant. All data is representative of at least three individual experiments unless otherwise stated.

RESULTS

Activation of CD25$^-$ CD4$^+$ T_{CONV} coupled with exogenous TGF-β results in generation of Foxp3-expressing cells.

To study the effects of DNMT-inhibition on Foxp3 expression, we first needed to establish a model whereby *bona fide* Foxp3$^+$ regulatory T cells could be generated *in vitro* from naive CD4$^+$CD25$^-$ conventional T cells (T_{CONV}). Previous studies have established that induction of Foxp3-expression in naive T_{CONV} cells occurs upon TCR-stimulation in the presence of TGF-β and IL-2[46, 103]. Since we aim to study the effects of DNMT-inhibition in the context of *de novo* induced iT_{REG} cells, it is imperative that our starting populations of T_{CONV} cells be devoid of any circulating nT_{REG} cells. Therefore, cell suspensions isolated from Foxp3^{GFP} reporter mice were purified by MACS to obtain a population of CD4$^+$CD25$^-$ T_{CONV} cells that were >98% pure (*Fig. 4-A*). These purified T_{CONV} cells were then activated as previously described under plate-bound conditions with or without TGF-β.

Expression of Foxp3 was detectable as early as 24 hours in cultures treated with TGF-β, with about 10-20% of cells expressing Foxp3 (*Fig. 4-B*). The proportions of Foxp3-expressing cells in the culture grew progressively until reaching a maximum by day 4, at which point proportions of Foxp3-expressing cells stabilized at around 75-85% (*Fig. 4-B*). Cultures were maintained for up to 9 days with fresh rhIL-2 and 'complete' RPMI added on days 4 and 7 accompanied with splitting of cultures to prevent overcrowding. It is interesting to note that the

induction of Foxp3-expression in T_{CONV} cells never reached 100%, despite being cultured under optimal conditions for differentiation. Thus, there was always a proportion (~15-20%) of T_{CONV} cells which remain Foxp3-negative. By day 7, the proportion of Foxp3-expressing cells appeared to decrease (~65% at day 7, 62% at day 9). However, this was not a statistically significant decrease from the proportion of Foxp3-expressing cells at day 4 (*Fig. 4-B*), and the decrease was associated with increased cell death in cultures beyond day 4 (*data not shown*). These observations indicate that Foxp3-expression is maintained until at least day 9 post-activation, when cultures were terminated. This result is consistent with previous studies indicating that downregulation of Foxp3-expression in iT_{REG} was only observed upon re-stimulation through TCR-signaling[84, 86]. Control cultures that were not treated with TGF-β failed to express Foxp3 at any of the measured time points (*Fig. 4-C, only data at day 3 is shown*), indicating that Foxp3-expression in T_{CONV} cells requires contributions from TCR-stimulation, IL-2, and TGF-β signaling.

In vitro generated Foxp3$^+$ cells demonstrate suppressive function.

It has been previously shown that Foxp3-expressing cells induced by TGF-β *in vitro* could suppress T cell proliferation[32, 104]. Therefore, we attempted to verify that our TGF-β-induced Foxp3-expressing cells had indeed acquired suppressive function. To do this, we assessed their capacity to inhibit the proliferation of responder T cells using a suppression assay. Responder T_{CONV} cells (Ly5.1$^+$), antigen presenting cells, and TGF-β-induced Foxp3^{GFP}-expressing

cells were prepared according to the methods previously described, and co-cultured at the indicated ratios (*Fig. 5*). After 3 days, cultures were harvested and acquired by flow cytometry. By gating on $CD4^+Ly5.1^+$ cells, which were labeled with eFluor-670 proliferation dye, accurate proliferation of only the responder cells could be tracked as each division of cells decreases the intensity of proliferation dye by half. By day 3 post-activation, nearly all T_{CONV} cells (~97%) underwent division when cultured alone, without any iT_{REG} cells (*Fig. 5-A, 'T_{CONV} only'*). Suppression was measured by determining the proportion of undivided T_{CONV} cells in the culture. As ratios of iT_{REG}:T_{CONV} increased, so did the proportions of undivided T_{CONV} cells, with significant suppression being observed at a 1:2 ratio (19% ± 4%, $p<0.05$) and 1:1 ratio (47% ± 7%, $p<0.001$) (*Fig. 5-B*). Since the number of T_{CONV} cells remained constant in all wells, reduction in proliferation must be attributed to the presence of co-cultured iT_{REG} cells, with suppression being dosage-dependent on the number of iT_{REG}. Therefore, our *in vitro* TGF-β-induction model efficiently converts naïve T_{CONV} cells into $Foxp3^+$ cells with potent suppressive function resembling that of iT_{REG} cells[32, 104].

DNMT-inhibitors, Aza and RG108 exert differential effects on cell viability and proliferation.

Before investigating the effects of DNMT-inhibitors on Foxp3-expression, we wanted to evaluate whether DNMT-inhibitors exerted any general effects on murine primary lymphocytes. Numerous studies on human cancer cell lines have shown that 5'-aza-2'-deoxycytidine (Aza) exerts cytotoxic and anti-proliferative

effects resulting from DNA damage caused by the formation of covalent protein-DNA adducts[92, 101, 102, 105] and that the non-nucleoside inhibitor RG108 is comparatively less toxic due to its specificity for the catalytic domain of DNMTs[96, 102]. To determine if DNMT inhibitors exhibit cytotoxicity on *ex vivo* primary T cells, we purified $CD4^+CD25^-$ T_{CONV} cells by MACS as previously described and activated them under plate-bound conditions with various doses of Aza and RG108 as indicated (*Fig. 6-A*). To assess cytotoxicity, cells were stained with eFluor-780 viability dye following the harvest of cells at day 3 post-activation. Only cells that are dead are permeable to eFluor-780 viability dye and stain positive. In untreated controls, 72% ± 2% of cells remained viable, whereas cultures treated with Aza at concentrations of 1μM and 5μM resulted in significantly fewer viable cells (59% ± 2, $p<0.05$, and 43% ± 2, $p<0.001$, respectively) than untreated controls, indicating that Aza exerts dosage-dependent cytotoxicity in agreement with previous studies[90, 99, 100, 103]. Cultures treated with 0.1μM of Aza showed no discernible difference from untreated cells (*Fig. 6-A*). In contrast to Aza, cultures treated with RG108 showed no significant change in viability from untreated cells at any of the concentrations tested (*Fig. 6-A*), indicating that RG108 exerts negligible effects on cytotoxicity. Although non-significant, TGF-β-treated cultures had the highest number of viable cells (81% ± 3%), which is consistent with the known ability of TGF-β to promote T cell survival during expansion[60].

In addition to cytotoxicity, studies on cancer cell lines have reported that DNMT-inhibitors are anti-proliferative[92, 102]. To determine whether DNMT-

inhibitors exert anti-proliferative effects on *ex vivo* primary cells, we labeled T_{CONV} cells with eFluor-670 proliferation dye prior to activation in order to track subsequent cell divisions. Nearly 100% of cells in non-treated activated cultures underwent cell division by day 3 post-activation (*Fig. 6-B 'untreated'*). We assessed the degree of suppression by determining the proportion of undivided cells. In cultures treated with RG108, significant levels of suppression were not observed (*Fig. 6-C*); however, there appeared to be a trend as increasing doses of RG108 resulted in slight reductions in proliferation (4% ± 2% undivided cells at 100μM, and 8% ± 1% undivided cells at 500μM). Moreover, there appeared to be fewer cells that underwent multiple divisions in cultures treated with 500μM, as indicated by the larger areas under each defined division peak relative to untreated controls (*Fig. 6-B*). Overall, it was shown that RG108 exerts mild, but non-significant effects on cell proliferation. In Aza-treated cultures (*Fig. 6-B, C*), significant suppression was observed at doses of 1μM (24% ± 5% undivided cells, $p<0.01$) and at 5μM (72% ± 3%, $p<0.001$). Even at doses of 0.1μM of Aza, cell division was inhibited to comparable levels as that of the highest dosage of RG108 (10% versus 8% undivided cells, respectively; *non-significant*). Collectively, our results are in agreement with previous studies regarding the cytotoxic and anti-proliferative effects mediated by DNMT-inhibitors[90, 100]. Aza was shown to be considerably more potent than RG108 in terms of both cytotoxicity and anti-proliferation in primary lymphocytes.

Treatment with Aza, but not RG108, delayed the loss of Foxp3 expression in iT_{REG} in the absence of TGF-β.

Instability of Foxp3 expression in iT_{REG} cells has been well documented and shown to be associated with increased methylation of the *Foxp3* gene and TSDR[82, 84]. Therefore, we wanted to investigate whether blocking methylation by DNMT-inhibitors could abrogate or delay the loss of Foxp3 expression. TGF-β-induced $Foxp3^+$ cells were generated from methods previously described. $Foxp3^+$ cells were then re-stimulated in the presence of TGF-β (5ng/ml), Aza (5μM), and RG108 (500μM). Loss of Foxp3-expression is measured by determining the proportion of Foxp3-expressing cells remaining in culture at the various time points. Since all cells are initially $Foxp3^+$, loss of Foxp3-expression would correspond to a reduction in the proportions of $Foxp3^+$ cells. In agreement with previous studies[84], significant downregulation of Foxp3 was not observed in our cells without re-stimulation, even in the absence of TGF-β (*Fig. 4-B*). However, upon re-stimulation through TCR, downregulation of Foxp3 was rapid, and observed as early as 24 hours in untreated cultures, which contained 70% ± 2% $Foxp3^+$ cells. By day 3, untreated cultures contained only 19% ± 4% $Foxp3^+$ cells (*Fig. 7–A, B*). The degree of Foxp3 downregulation in untreated cultures was used as a benchmark to compare the efficiency of other treatments to maintain Foxp3-expression. Cultures treated with TGF-β contained 94% ± 1%, $p<0.05$ $Foxp3^+$ cells at 24 hours and 82% ± 1%, $p<0.05$ by day 3, indicating that TGF-β is a highly effective mediator of sustained Foxp3-expression. Aza-treated cultures

contained 80 ± 1%, ($p<0.05$) and 39% ± 4%, (*non-significant*) $Foxp3^+$ cells by 24 hours and day 3, respectively. The data shows that Aza administration delays the loss of Foxp3, although not as efficiently as TGF-β. Although the day 3 time point is statistically insignificant, there is a trend demonstrating its maintenance effect on Foxp3-expression, and it is expected that increasing the number of trials should increase the statistical significance of this result. RG108-treated cultures contained 65% ± 2% (*non-significant*) and 18% ± 1% (*non-significant*) $Foxp3^+$ cells by 24 hours and day 3, respectively. These results indicate that RG108 has no significant effect on the maintenance of Foxp3-expression and shows indiscernible differences from untreated cultures (*Fig. 7-A, B*). Collectively, the data supports the notion that DNMT-inhibition can augment the stability of Foxp3 expression in iT_{REG} cells. We showed that Aza-treatment approximately doubled the proportions of cells that remain $Foxp3^+$ as compared to untreated controls. Lastly, the proportion of viable cells in Aza-treated cultures at day 3 post-restimulation was comparable to that of TGF-β-treated cultures (*Fig. 7-C*), precluding the possibility that Aza might exert selective cytotoxicity to $Foxp3^-$ cells. It should be emphasized however, that Aza was not as effective at sustaining Foxp3-expression as TGF-β, suggesting that other dominant factors in addition to demethylation may be important for Foxp3 maintenance.

Effects of DNMT-inhibitor treatment on the induction of Foxp3-expression in naive T_{CONV} cells.

The previous experiments were intended to determine whether DNMT-inhibitors could *delay* the downregulation of Foxp3 expression in established iT_{REG} cells. In the next series of experiments, we investigated whether DNMT-inhibitors could augment the *induction* of Foxp3 expression in naive T_{CONV} cells. We activated e*x vivo* $CD25^-$ $CD4^+$ T_{CONV} cells in plate-bound conditions and treated with Aza and RG108, either alone or in combination with TGF-β. By day 3 post-activation, cultures were harvested and analyzed with flow cytometry to determine the proportion of Foxp3-expressing cells. In these assays, proportions of Foxp3-expressing cells generated by TGF-β treatment were used as a benchmark to compare the efficiency of treatments to induce Foxp3-expression. Cultures treated with only Aza or RG108, without TGF-β, failed to induce Foxp3-expression (*Fig. 8–A*), suggesting that DNMT-inhibition by itself is insufficient to trigger expression of Foxp3. Treatment with Aza plus TGF-β, and RG108 plus TGF-β resulted in lower proportions of Foxp3-expressing cells than cultures treated with TGF-β alone (23% ± 1%, *non-significant*, 21% ± 1%, $p<0.05$, and 28% ± 1%, respectively). Despite generating a lower frequency of $Foxp3^+$ cells, treatment with both RG108 plus TGF-β, and Aza plus TGF-β resulted in higher median fluorescence intensity (MFI) of $Foxp3^{GFP}$ and appeared to be dependent on the dosage of DNMT-inhibitor (*Fig. 8–B*). Collectively, these results indicate that DNMT-inhibition and demethylation, may not be essential for the conversion

of iT_{REG} and induction of Foxp3-expression, but may augment the expression of Foxp3 once the gene has already been induced by signal transduction pathways. It is important to emphasize that DNMT-inhibition without TGF-β, results in no Foxp3-induction, suggesting that methylation-mediated regulation may be secondary to TGF-β-mediated signal transduction.

Delayed DNMT-inhibition and induction of Foxp3

Previously, we demonstrated that Aza exerts potent cytotoxic and anti-proliferative effects (*Fig. 6*). The anti-proliferative effect of DNMT-inhibitors poses a problem when assessing its effect on gene expression. Because the methylation reaction catalyzed by DNMT requires *de novo* synthesis of DNA[90, 92], compromised cell division would effectively limit the demethylation potential of DNMT-inhibitors. The caveats (cytotoxicity and anti-proliferative effects) associated with DNMT-inhibitor usage must be taken into consideration when interpreting the results. Therefore, we wondered if the decrease in proportions of Foxp3-expressing cells seen previously, upon DNMT-inhibitor treatment, was due to impaired cellular proliferation. To test this, purified T_{CONV} cells were activated under plate-bound conditions, and to offset the negative effects on proliferation, we delayed the administration of DNMT inhibitors and TGF-β by 36 hours after activation to allow sufficient time for cells to undergo cell division. At 3 days post-activation, cultures were harvested and analysed by flow cytometry. Delayed administration of DNMT-inhibitors by 36 hours effectively restored proliferative capacity of T_{CONV} cells, with all culture conditions showing similar levels of

proliferation as untreated controls (*Fig. 9–A*). Delaying administration of TGF-β abrogated its ability to induce Foxp3-expression (*Fig. 9–A*), indicating that there is a specific window of time for TGF-β signaling, relative to TCR-stimulation, to be effective. Previous studies have reported that TGF-β-mediated induction of Foxp3 is inhibited in pre-activated T cells[86, 106], and cells which have initiated lineage commitment[107]. Combined with reports showing that activated T cells express high levels of STAT3 which in turn upregulates DNMT1 expression[108], our data suggests that 36 hours may be sufficient time for DNMT-mediated silencing, presumably through restricting access of the *Foxp3* gene to transcription factors. Interestingly, Aza seemed to restore the capacity of TGF-β to induce Foxp3-expression (*Fig. 9–B*), as a significant proportion of Foxp3-expressing cells were generated in cultures treated with Aza plus TGF-β (13% ± 2%, $p<0.001$). Other culture-conditions (RG108, RG108 plus TGF-β, and Aza alone) failed to generate significant numbers of $Foxp3^+$ cells (*Fig. 9–B*). Together, these results demonstrate that the timing, relative to TCR-stimulation, at which TGF-β and DNMT-inhibition is administered, is critical to the regulation of the *Foxp3* gene.

Aza and RG108 stimulate Foxp3-expression in TGF-β pre-exposed Foxp3-negative cells.

Consistent throughout the numerous TGF-β-induction trials performed in this study, there appeared to be heterogeneity in the capacity of cells to express Foxp3 within TGF-β-induced cultures. As we showed in previous experiments (*Fig. 4-B*), the proportion of induced Foxp3-expressing cells reaches a maximum at day four post-activation and does not exceed 75-85% of total cells, which is consistent with findings from other groups[106, 109]. Despite being under optimal conditions for induction of Foxp3-expression, there remains a percentage of T_{CONV} cells that stays Foxp3-negative. By day 3, these cells have been activated, undergone several rounds of cell division, and received a TGF-β signal at time of TCR stimulation. Therefore, these cells provide an opportunity to study the effects of DNMT-inhibition on Foxp3-expression, without the confounding variables of impaired proliferation and delayed TGF-β signaling. We generated TGF-β-induced Foxp3-expressing cells as previously described and isolated the $Foxp3^{GFP(-)}$ population (hereinafter referred to as *TGF-β pre-exposed cells*) by FACS. These cells were then re-cultured with IL-2 and treated with DNMT-inhibitors or TGF-β (as indicated in *Fig. 10-A*) for an additional 3 days before harvest and acquisition. Control cells that were not exposed to TGF-β (hereinafter referred to as *non-exposed cells)* were cultured in parallel under equal conditions and are essentially the same as activated T_{CONV} cells *(Fig. 10-A*).

Starting with the results of TGF-β pre-exposed cultures (*Fig. 10-B, black bars*), it is important to note that cultures that do not receive an additional TGF-β dose, do not express Foxp3 by day 3, indicating that these cells would not spontaneously begin expressing Foxp3 simply when given additional time (*Fig. 10-B 'no TGF-β'*). When additional TGF-β is added however, induction of Foxp3 occurs and cultures contain 32% ± 1% $Foxp3^+$ cells. Interestingly, administration with both Aza and RG108 were able to trigger Foxp3 expression in TGF-β pre-exposed cells, even without additional TGF-β (*Fig. 10-B*). By day 3, Aza-treated cultures contained 17% ± 1% ($p<0.001$) $Foxp3^+$ cells, and RG108-treated cultures contained 9% ± 1% ($p<0.01$) $Foxp3^+$ cells, which represented significant induction when compared to untreated controls. Induction of Foxp3 was further enhanced in cultures co-treated with Aza plus TGF-β. These cultures contained 56% ± 2% ($p<0.001$) $Foxp3^+$ cells, which represented a significant increase over proportions of $Foxp3^+$ cells generated in cultures treated with only TGF-β, indicating an additive and non-redundant effect of Aza in the context of Foxp3-induction. Cultures treated with RG108 plus TGF-β contained 30% ± 1% $Foxp3^+$ cells, which was not significantly different from proportions of $Foxp3^+$ cells generated by TGF-β alone (*Fig. 10-B*).

In stark contrast, TGF-β non-exposed control cells were much less responsive to Foxp3-expression than pre-exposed cells under all conditions tested (*Fig. 10-B, white bars*). However, cultures treated with Aza and Aza plus TGF-β did result in very low proportions of $Foxp3^+$ cells (3.2% ± 0.2%, $p<0.05$, and 5.3% ± 0.3%, $p<0.05$, respectively). Although these proportions were very low,

they were considered significant as compared to untreated non-exposed cells (*Fig. 10-B*). This data is consistent with the results from the previous experiment, where 36-hour delayed Aza plus TGF-β could induce Foxp3 expression in 13% ± 2% cells (*Fig. 10-B*). Since the non-exposed cells used in this experiment are essentially pre-activated T_{CONV} cells, the data can be alternatively interpreted as a representation of 72-hour delayed TGF-β plus Aza, with acquisition on day 6 post-activation. Once again, we see that co-treatment with Aza and TGF-β appears to stimulate Foxp3 expression, although very slightly, in pre-activated T_{CONV} cells. Collectively, these results suggest that TGF-β pre-exposed cells are somehow repressed in terms of Foxp3-expression by a methylation-dependent mechanism whereby administration of Aza and RG108 results in demethylation and spontaneous expression of the *Foxp3* gene, even in the absence of additional TGF-β. Because of previous exposure to TGF-β, these cells may be in a state of 'partial conversion' where expression of Foxp3 is repressed by methylation. The lack of responsiveness to Foxp3 expression in TGF-β non-exposed cells might be due to differentiation progression, where extensive epigenetic modifications have restricted access to the *Foxp3* gene. Indeed, other groups have reported failure to induce Foxp3 expression in preactivated and differentiated T cells as well[86, 103, 106]. Although further experimentation will have to be performed to confirm that Foxp3 induction in pre-exposed cells is demethylation-dependent, the data nevertheless demonstrates a definitive effect of DNMT-inhibition on Foxp3-expression as well as emphasizing the critical requirement of the TGF-β pathway

on expression of Foxp3. Our data has shown repeatedly that without TGF-β signaling, efficient expression of Foxp3 in T_{CONV} cells does not occur.

TGF-β pre-exposed cultures showed marked reduction of TNF-α production than non-exposed control cells

Now that we established a model whereby DNMT-inhibitors could induce Foxp3-expression, we wanted to verify that these inhibitor-induced $Foxp3^+$ cells phenotypically resembled 'normal' iT_{REG}. Because DNMT catalyzes all methylation reactions within the cell, it is possible that DNMT-inhibition would result in global hypomethylation, causing aberrant gene expression. The cytokine profiles between iT_{REG} and activated T_{CONV} cells differ, namely that iT_{REG} cells exhibit considerably reduced production of proinflammatory cytokines such as IFN-γ and TNF-α[60, 109]. Moreover, T_{REG} cells have also been shown to inhibit TNF-α production in activated T_{CONV} cells and monocytes[60, 110, 111]. Cells were harvested at day 3 post-treatment (day 6 post-activation) and stimulated with PMA/ionomycin to stimulate cytokine production. Proportions of TNF-α-producing cells were determined by flow cytometry. TNF-α was undetectable in all conditions when gated on $Foxp3^{GFP+}$ cells (data not shown), indicating that DNMT-inhibition does not lead to aberrant expression of TNF-α in Foxp3-expressing cells. When gated on $Foxp3^{GFP-}$ cells, TGF-β pre-exposed cultures showed markedly fewer TNF-α-producing cells across all conditions than non-exposed controls (*Fig. 10-C*). Of particular interest, treatment with Aza in both populations (pre-exposed and non-exposed cells) induced the highest number of

TNF-α-producing cells (*Fig. 10-C*), suggesting that Aza may promote non-specific expression of many genes that are under the repression of methylation-dependent mechanisms. Although it is tempting to presume that the induced Foxp3-expressing cells in TGF-β pre-exposed cultures are functional and actively suppressing the production of TNF-α, it is much more likely that TGF-β itself is responsible for reduced cytokine production, which is consistent with the ability of TGF-β to repress gene expression[61-64]. At the moment, it cannot be concluded from this data whether the $Foxp3^+$ cells treated with DNMT-inhibitors functionally resemble that of 'normal' iT_{REG}.

DISCUSSION

T_{REG} cells are an indispensible component of peripheral tolerance, maintaining immune homeostasis and preventing the onset of autoimmune disease[11]. There are two distinct subsets of T_{REG} cells – naturally occurring nT_{REG} cells, which arise from the thymus during T cell development[11], and inducible iT_{REG} cells, which can be generated from T_{CONV} cells in the periphery or *in vitro*[16, 33]. Although both subsets share many of the same functions and characteristics, only nT_{REG} cells exhibit stable regulatory function and constitutive expression of Foxp3, the master regulator of T_{REG} function[16, 33]. In contrast, the iT_{REG} subset only transiently expresses Foxp3, and loss of Foxp3 is correlated with the acquisition of effector functions, such as production of inflammatory cytokines[16, 33]. Nevertheless, there is considerable interest in the iT_{REG} subset because they can be generated in great numbers *in vitro* from populations of host T_{CONV} cells. Recent studies have emerged which indicate that stable Foxp3-expression may be under the control of epigenetic modifications of the *Foxp3* locus[55, 84, 86]. Therefore, we set out to investigate whether or not stable Foxp3-expression, as seen in nT_{REG} cells, could be recapitulated in iT_{REG} cells following the inhibition of DNA methylation, which is mediated by DNMT enzymes. The goals of this project were to determine whether DNMT activity and methylation of the *Foxp3* gene plays a role in the regulation of Foxp3-expression. Via administration of DNMT-inhibitors, we aimed to block *de novo* methylation and determine the subsequent effects on the induction and maintenance of Foxp3 expression. Our

preliminary experiments established a model whereby suppressive Foxp3$^+$ cells could be generated from naïve T_{CONV} cells *in vitro* following TCR-stimulation in the presence of IL-2 and TGF-β. These TGF-β-mediated Foxp3-expressing cells demonstrated potent suppressive function as indicated by their ability to inhibit proliferation of activated T_{CONV} cells. These preliminary studies of TGF-β-induced iT_{REG} cells served as a point of reference so that we would know what is 'normal' with regards to phenomena associated with Foxp3-regulation. Importantly, we showed that the induction of Foxp3-expression in T_{CONV} cells is critically dependent on contributions from TCR-stimulation, IL-2, and most importantly, TGF-β. Moreover, we found that TGF-β must be administered within a certain time period after TCR-stimulation, as delayed-treatment resulted in marked reductions in the proportions of Foxp3-expressing cells generated. Once induced, Foxp3-expression is sustained for at least 9 days, unless re-activated through stimulation of the TCR, which triggers rapid downregulation and loss of Foxp3-expression. Furthermore, we observed that cells within TGF-β-treated cultures exhibited heterogeneity in terms of capacity to express Foxp3. Complete conversion of T_{CONV} into iT_{REG} did not occur, with proportions of Foxp3-expressing cells never exceeding 75-85%. Therefore, we investigated the effects of DNMT-inhibitor treatment on induction, and maintenance of Foxp3. Our results showed that in the absence of TGF-β, DNMT-inhibition is unable to induce expression of Foxp3 in T_{CONV} cells, indicating that demethylation alone is insufficient to stimulate transcriptional activity of the *Foxp3* gene. However, when cultures were treated with DNMT-inhibitor and TGF-β together, some

interesting effects were observed. First, the MFI of $Foxp3^{GFP}$ in cells treated with Aza and TGF-β was higher than cells treated with TGF-β alone, indicating that demethylation may augment already-active expression of Foxp3, but has little effect on the induction of Foxp3. In the context of delayed-treatment experiments, where T_{CONV} cells were pre-activated for 36 hours, TGF-β alone was unable to induce Foxp3-expression. TGF-β together with Aza however, rescued the capacity for pre-activated T_{CONV} cells to express Foxp3, suggesting that TCR-stimulation, without TGF-β, results in silencing of the *Foxp3* gene by a methylation-dependent mechanism. Lastly, in TGF-β pre-exposed cells, we showed that DNMT-inhibition, with either Aza or RG108, resulted in spontaneous Foxp3-expression, even in the absence of additional TGF-β. Foxp3-expression was further augmented in pre-exposed cultures treated with TGF-β and Aza, suggesting that DNMT-inhibition had an additive and non-redundant effect on induction of Foxp3. Together, these results indicated that TGF-β pre-exposed cells were initially repressed for Foxp3-expression by a methylation-dependent pathway. Upon DNMT-inhibition, demethylation occurs and repression of the *Foxp3* gene is lifted. It is possible that these cells are in an intermediate state of conversion between T_{CONV} and iT_{REG} cells. We also investigated the effect of DNMT-inhibition on the maintenance of Foxp3-expression. Our results showed that Aza treatment augmented the persistence of Foxp3-expression in iT_{REG} cells when compared to untreated controls, although the maintenance effect on Foxp3-expression by Aza was not as potent as TGF-β.

Taken together, our results indicate that the importance of DNMT and DNA methylation is secondary to signal transduction pathways for Foxp3-induction. In all conditions tested, TCR-stimulation, IL-2, and TGF-β had to be administered to observe any significant expression of Foxp3.

Other groups however, have reported Foxp3-expression following only Aza treatment along with TCR stimulation. Moon *et al.* were able to detect Foxp3-expression by Western blot in T_{CONV} cells following treatment with Aza 72 hours post-stimulation[89]. However, in the Moon *et al.* study, non-treated controls also showed Foxp3-expression[89], suggesting that their method of Foxp3-induction may differ from ours, as TCR stimulation in our experiments did not result in detectable levels of Foxp3. In another study, Lal *et al.* demonstrated that Aza administered at the time of TCR stimulation was able to induce Foxp3 in 35% of cells, and Aza plus TGF-β induced Foxp3 in 72% of cells[86]. Furthermore, Lal *et al.* found that Aza- and Aza plus TGF-β-treated cells had complete demethylation of the *Foxp3* CpG site[86]. In their study, Aza was removed from the culture at 24 hours post-stimulation and cells were recultured for an additional 3 days before analysis. Kim *et al.* also demonstrated that DNMT-inhibition alone could induce Foxp3-expression in activated T_{CONV} cells. They administered Aza to cultures between days 4-6 post-stimulation and observed more Foxp3-expression than TGF-β-treated controls[55]. These groups have demonstrated that Aza-treatment by itself, in the absence of TGF-β, was sufficient to induce Foxp3-expression in activated T_{CONV} cells. At the moment, further experiments will have to be conducted to be able to explain the discrepancies between our data and these

studies. However, given that our experiments have repeatedly shown that Foxp3-expression without TGF-β does not occur, it remains unclear how demethylation alone could lead to Foxp3-expression, as reported by other groups[55, 86, 89]. Assuming that Aza-treatment does result in demethylation of the *Foxp3* gene, it is presumed that specific transcription factors (such as TGF-β-dependent Smad3) are still required for expression. If DNMT-inhibition alone is indeed sufficient to induce Foxp3-expression, as reported by others, then what is regulating the expression of other genes that would also be demethylated as a result of Aza-treatment?

Although considerably more work is required to reach definitive conclusions about the role of DNMT in the regulation of *Foxp3*, our data provides some hints as to the potential mechanisms that govern the expression of Foxp3. Together with findings from other groups, we attempt to reconcile our results with known contributions of epigenetic modifications and signal transduction pathways on Foxp3-expression. Therefore, we propose the following model involving three key regulatory elements (*Foxp3* promoter, TGF-β sensor, and TSDR) of the *Foxp3* gene which we believe to be critically involved in the regulation of Foxp3-expression.

It has been established that expression of *Foxp3* requires the activation and cooperation of several transcription factors downstream of TCR-stimulation, IL-2, and TGF-β, of which some notable examples include NFAT[70], CREB/ATF[55], Smad3[70], and STAT5[73]. In resting T_{CONV} cells, the *Foxp3* promoter is partially methylated[54] and this relative state of hypomethylation is associated

with an open configuration of chromatin[70, 87]. Hence, the *Foxp3* promoter is accessible to binding by transcription factors downstream of TCR and IL-2[54] and this feature likely confers the ability for naive T_{CONV} cells to be able to express Foxp3 and convert to iT_{REG}. Upon TCR-stimulation, transcription factors downstream of TCR-signaling are activated, leading to the upregulation of DNMT1 through a Stat3-dependent mechanism[55, 112]. Since lineage-specific effector cytokines such as IFN-γ and IL-4 were shown to be tightly regulated by DNA methylation[99, 100], DNMT1 is probably highly active immediately following TCR-stimulation. If TGF-β signaling is absent during these events, then DNMT1 is recruited to the *Foxp3* gene, resulting in methylation of the *Foxp3* promoter[55], and TSDR[55, 84]. Consequently, transcription factors are no longer able to bind to the DNA, and *Foxp3* is silenced (*Fig. 11-A*). In our experiments using pre-activated T_{CONV} cells, where we delayed the administration of TGF-β by 36 and 72 hours (*Fig. 9 and 10*), TGF-β treatment failed to induce Foxp3-expression. This observation may have been due to the *Foxp3* promoter being fully methylated by DNMT. Upon treatment with Aza, methylated-dependent silencing of the *Foxp3* gene would be prevented, allowing for transcription factors to bind and induce Foxp3-expression[70] (*Fig. 11-C*).

If a TGF-β signal is provided at the time of TCR stimulation, then Smad3 and NFAT are able to bind to the TGF-β sensor of *Foxp3*[70], which in turn promotes the demethylation of the *Foxp3* promoter[70], resulting in transcription of Foxp3 (*Fig. 11-B*). TGF-β-mediated expression of Foxp3 is associated with partial demethylation of the TSDR[84] as well as increased acetylation of histone

H4, suggesting that TGF-β mediates demethylation of DNA and alters chromatin remodelling of *Foxp3*. Furthermore it was shown that TGF-β somehow prevents the recruitment of DNMT1 to the *Foxp3* gene[55]. It is possible that TGF-β triggers the expression of effector molecules involved in epigenetic modifications. Such a candidate might be TGF-β-inducible early gene 1 (TIEG1), which has binding sites on the *Foxp3* promoter adjacent to NFAT[113]. Evidence suggests that TIEG1 binds to similar sequences as Sp1[114], a transcription factor that upon binding to DNA, inhibits *de novo* methylation[115, 116]. Therefore, in addition to mediating Smad3-dependent expression of *Foxp3*, TGF-β may also be responsible for disrupting the activity of DNMTs and histone modifying enzymes through an undiscovered mechanism. This might explain why in the absence of TGF-β, downregulation of Foxp3-expression occurs so rapidly, as removal of TGF-β may trigger histone-modifications that result in closing of chromatin and *Foxp3* silencing. Lastly, it was shown that the TSDR remains partially methylated in TGF-β-induced Foxp3-expressing cells[84], and that methylation of this region inversely correlated with stability of Foxp3-expression[84] and binding of CREB[55] and STAT5[73] (*Fig. 11-B*).

It still remains to be determined whether demethylation of the *Foxp3* gene occurs following treatment with DNMT-inhibitors in our experiments. Assuming that Aza and RG108 do inhibit DNMTs, we would expect uninhibited expression of Foxp3, as all CpG-islands of the *Foxp3* gene would be unmethylated, allowing for saturation of transcription factors (*Fig. 11-C*). Our results seemed to oppose this model, as the proportions of Foxp3-expressing cells generated from Aza plus

TGF-β treatment were slightly lower than cultures treated with TGF-β alone (23% versus 28% respectively, *Fig. 8-A*). To reconcile these results, we reasoned that the reduced proportions of Foxp3^{+} cells were the result of impaired proliferation by Aza. However, we and others[103, 106] showed that Foxp3-expression was detectable at 24 hours (*Fig. 4-B*), before cell division is initiated, indicating that Foxp3-expression was independent from cell division.

Under all the different experimental conditions used in this study, RG108 consistently demonstrated less potency than Aza in the context of promoting Foxp3 expression (*Fig. 7, 9, 10).* Since Aza results in covalent enzyme trapping of DNMT, its inhibition should be non-reversible. Furthermore, since Aza incorporates into DNA, the modified CpG residues should no longer be susceptible to methylation until DNA repair mechanisms can restore the original sequence. Together, these two features of Aza contribute to its high potency and effectiveness, as indicated by its strong anti-proliferative effects and cytotoxicity (*Fig. 6*). In contrast, RG108 was rationally designed to inhibit DNMTs by directly targeting the catalytic domain. Therefore, it is possible that RG108-DNMT binding is reversible and under the influence of enzyme kinetics, resulting in transient inhibition of DNMT and ineffectiveness at mediating demethylation. Nevertheless, RG108 was able to augment the MFI of Foxp3 expression when coupled with TGF-β (*Fig. 8*) as well as inducing Foxp3 in TGF-β pre-exposed cells (*Fig. 10*), indicating that RG108 is exerting some effect.

ONGOING EXPERIMENTS AND FUTURE OBJECTIVES

Although this study provides some preliminary insight on the effect of DNMT-inhibitors and Foxp3-expression, several important questions remain. First of all, whether the administration of DNMT-inhibitors actually results in demethylation of the *Foxp3* gene in T_{CONV} and iT_{REG} cells remains to be confirmed. To discount the possibility that our results are actually caused by DNMT-independent pathways, we must verify that an increase of demethylation in the *Foxp3* gene occurs following DNMT-inhibitor treatment. One approach is to compare differential methylation patterns of specific DNA regions by bisulphite sequencing. Treatment of DNA with bisulphite results in the conversion of cytosine residues to uracil while methylated cytosine residues remain unaffected[117]. Therefore, bisulphite treatment introduces site-specific changes that depend on the methylation status of a segment of DNA. Sequencing techniques are then employed to compare the ratios of C-to-U conversion between a treated and untreated sample to determine changes in methylation patterns[117]. Ongoing efforts are being made to culture sufficient numbers of Foxp3-expressing cells that have been treated with DNMT-inhibitors in order to harvest enough genomic DNA to facilitate bisulphite sequencing of the key regulatory regions of the *Foxp3* gene. It has been a challenge to harvest sufficient numbers of treated iT_{REG} cells because of the cytotoxicity of DNMT-inhibitors and also because of cell loss from multiple rounds of cell sorting in order to isolate $Foxp3^{GFP+}$ cells.

In addition to analyzing methylation patterns of the *Foxp3* gene, an analysis of histone modifications may be useful. It is possible that Foxp3-expression, in our experimental models, is simply independent of the activity of DNMT, and has more to do with the formation of protein-DNA adducts as a consequence of Aza treatment. Perhaps these adducts disrupt the recruitment of histone modifying enzymes, that would normally be recruited to methylated DNA through interactions with methyl-binding proteins (MBDs)[90]. Since the formation of adducts may prevent binding of MBDs to DNA, the configuration of chromatin could be altered, subsequently affecting Foxp3 expression. To investigate this notion, chromatin immunoprecipitation (ChIP) using antibodies specific for acetylated histone H3, and acetylated histone H4 could be performed on segments of CpG-rich DNA within the *Foxp3* promoter of DNMT-treated cells.

Lastly, for iT_{REG} cells to be considered for use as clinical therapy to treat autoimmune disorders, they must possess stable suppressive function as seen in nT_{REG} cells. We have shown that Aza can prolong the expression of Foxp3 in restimulated iT_{REG} cells (*Fig. 7*) and that Aza plus TGF-β generated higher proportions of Foxp3-expressing cells than TGF-β alone in pre-exposed cultures (*Fig. 10-B*). However, given the cytotoxicity of Aza, the function of these $Foxp3^+$ cells could be compromised and it must be concluded whether our Aza-induced Foxp3-expressing cells exert suppressive and anti-proliferative effects on effector T cells. Suppression assays akin to the procedures used in *Fig. 5* could be employed to compare the suppressive capability of Aza-induced and TGF-β-induced T_{REG} cells. Alternatively, supernatants from the two culture conditions

could be analyzed by ELISA to determine if the cytokine profile resembles that of nT_{REG} (IL-10, TGF-β)[16]. Only if Aza-induced T_{REG} demonstrated efficient and stable suppressive function and a lack of inflammatory cytokine secretion, would they qualify for consideration as a viable treatment of autoimmune disorders. Ultimately, the stability and function of Aza-treated T_{REG} would need to be tested *in vivo*. Adoptive transfer of Aza-treated T_{REG} cells into an autoimmune disease animal model, such as non-obese diabetic mice (which we breed in our animal facility), could be one approach to assess the capacity of these cells to reverse or prevent autoimmunity. As mentioned previously, attempts to culture and purify sufficient numbers of Aza-treated iT_{REG} cells were met with difficulty and ongoing efforts are being made to characterize the function of our Aza-treated Foxp3-expressing cells.

CONCLUSION

This study investigated the effects of DNMT-inhibition on the regulation of Foxp3-expression in the context of gene-induction, and gene-maintenance. We demonstrated that DNMT-inhibition generally did not lead to induction of Foxp3-expression, but instead augmented existing expression. This indicated that the effects of DNMT activity and DNA methylation on Foxp3-expression was secondary to the effect of TGF-β signaling, which was required for significant Foxp3-expression in all the conditions tested. With regards to the maintenance of Foxp3-expression, Aza appeared to delay the loss of Foxp3-expression in re-stimulated iT_{REG} cells, but due to insufficient numbers of trials, the data was not significant. Overall, the results in this study raise many questions, and further work must be done to elucidate the contributions of DNA methylation relative to signal transduction pathways to regulation of the *Foxp3* gene. Because of the potential for DNMT-inhibitors to cause global hypomethylation, modulation of expression of specific genes would be challenging. However, studies using DNMT-inhibitors are still useful for elucidation of epigenetic regulation pathways, and proof-of-principle models. With future studies, we hope to further our understanding of what confers stable regulatory function in nT_{REG} cells.

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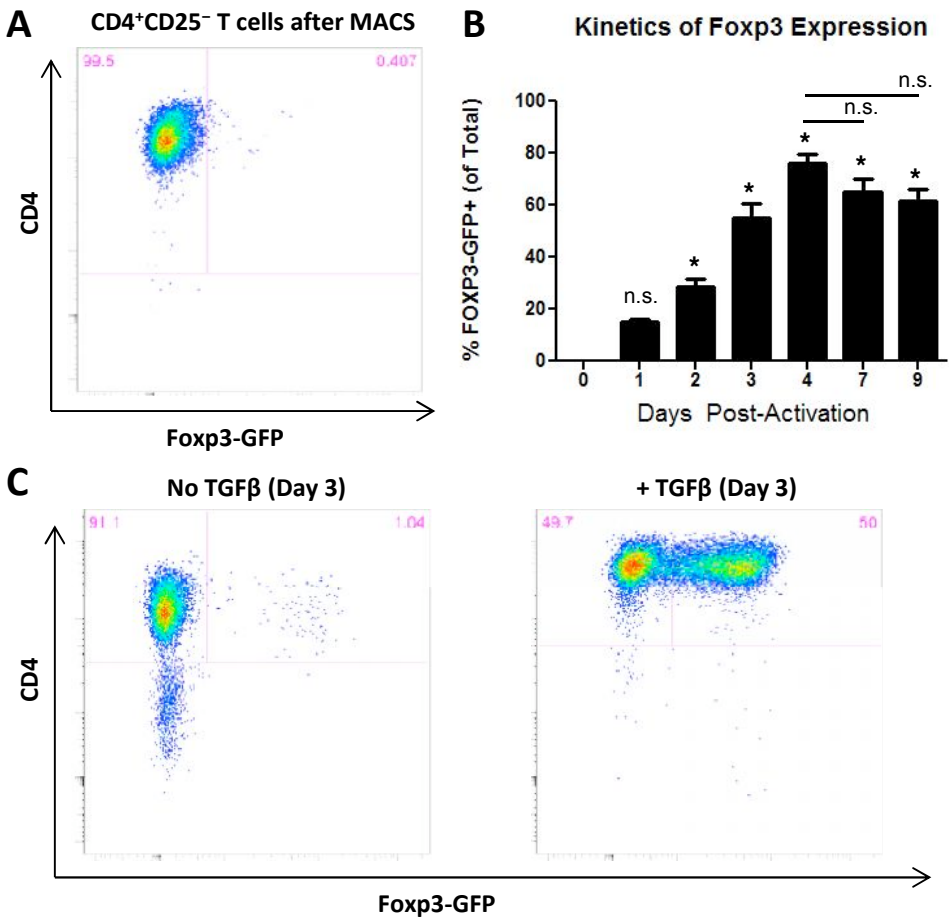

***Figure 4* - Activation of CD4$^+$CD25$^-$ T_{CONV} coupled with exogenous TGF-β results in generation of Foxp3-expressing cells.**

A) CD4$^+$CD25$^-$ cells were harvested from lymph nodes and spleens of B6.Foxp3^{GFP} reporter mice and purified with MACS. Resulting population is >98% pure with less than 0.5% Foxp3+ cells. B) CD4$^+$CD25$^-$ cells (5×10^5/well) were stimulated on day 0 in plate-bound conditions using αCD3 (2μg/ml) and αCD28 (2μg/ml) with rhIL-2 (5ng/ml) and TGF-β1 (5ng/ml). Sample wells were harvested at indicated days and analyzed by flow cytometry for Foxp3 expression by measuring GFP fluorescence. Cultures were split on days 4 and day 7 post-activation with fresh medium plus rhIL-2 (5ng/ml) to prevent overcrowding of cells. Proportions of Foxp3-expressing cells reached a maximum on day 4 and Foxp3-expression was monitored until day 9 at which point cultures were terminated. Mean ± SEM of two replicates are plotted. C) Comparison of the induction of Foxp3-expression between control cultures that receive no TGF-β1 (left) and TGF-β1-treated cultures (right) at day 3 post-activation. FACS plots are gated on CD4$^+$ cells and Foxp3-expression is detected by intensity of Foxp3-GFP. All data shown is representative of three independent experiments. * denotes $p<0.05$, n.s., not significant.

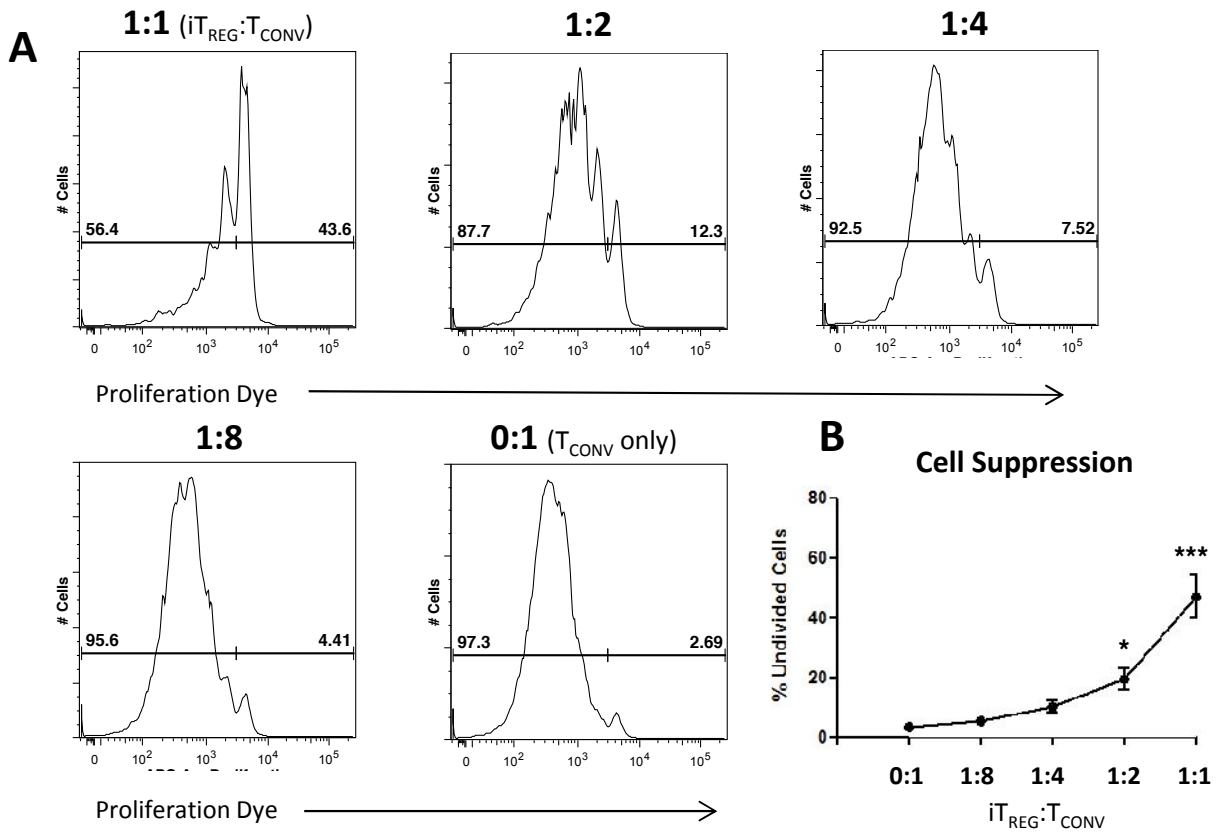

***Figure 5 - In vitro* generated Foxp3$^+$ cells (iT_{REG}) demonstrate suppressive function.**

A) $CD4^+CD25^-$ T_{CONV} responder cells were isolated from congenic Ly5.1 (CD45.1) mice as previously described and labeled with eFluor-670 proliferation dye at a final concentration of 5µM for 5 minutes at 37°C in PBS. Cells were washed with cold 'complete' RPMI-1640 three times before being plated. Antigen presenting cells (APCs) were prepared from the MACS sorted $CD25^-$ $CD4^-$ cell population of B6.Foxp3^{GFP} reporter mice and irradiated for 15 minutes. iT_{REG} cells were generated from B6.Foxp3^{GFP} reporter mice as previously described and sorted with FACS to obtain a pure population of Foxp3-expressing cells. The cells were then co-cultured into a 96-well flat-bottom microtitre plate in 'complete' RPMI medium and rhIL-2 (5ng/ml) and activated using soluble αCD3 (1µg/ml). Each well contained $5x10^4$ T_{CONV} cells, $2x10^5$ APCs, and various numbers of iT_{REG} in the indicated ratios with T_{CONV} cells. Histograms are gated on $CD4^+Ly5.1^+$ cells so that proliferation of only the responder cells are measured. The values indicate the proportions of undivided and divided cells and the data is representative of triplicates from two independent experiments. B) The proportions of undivided cells are plotted against iT_{REG}:T_{CONV} ratio. Mean ± SEM are plotted, n=2. * denotes $p<0.05$, *** denotes $p<0.001$.

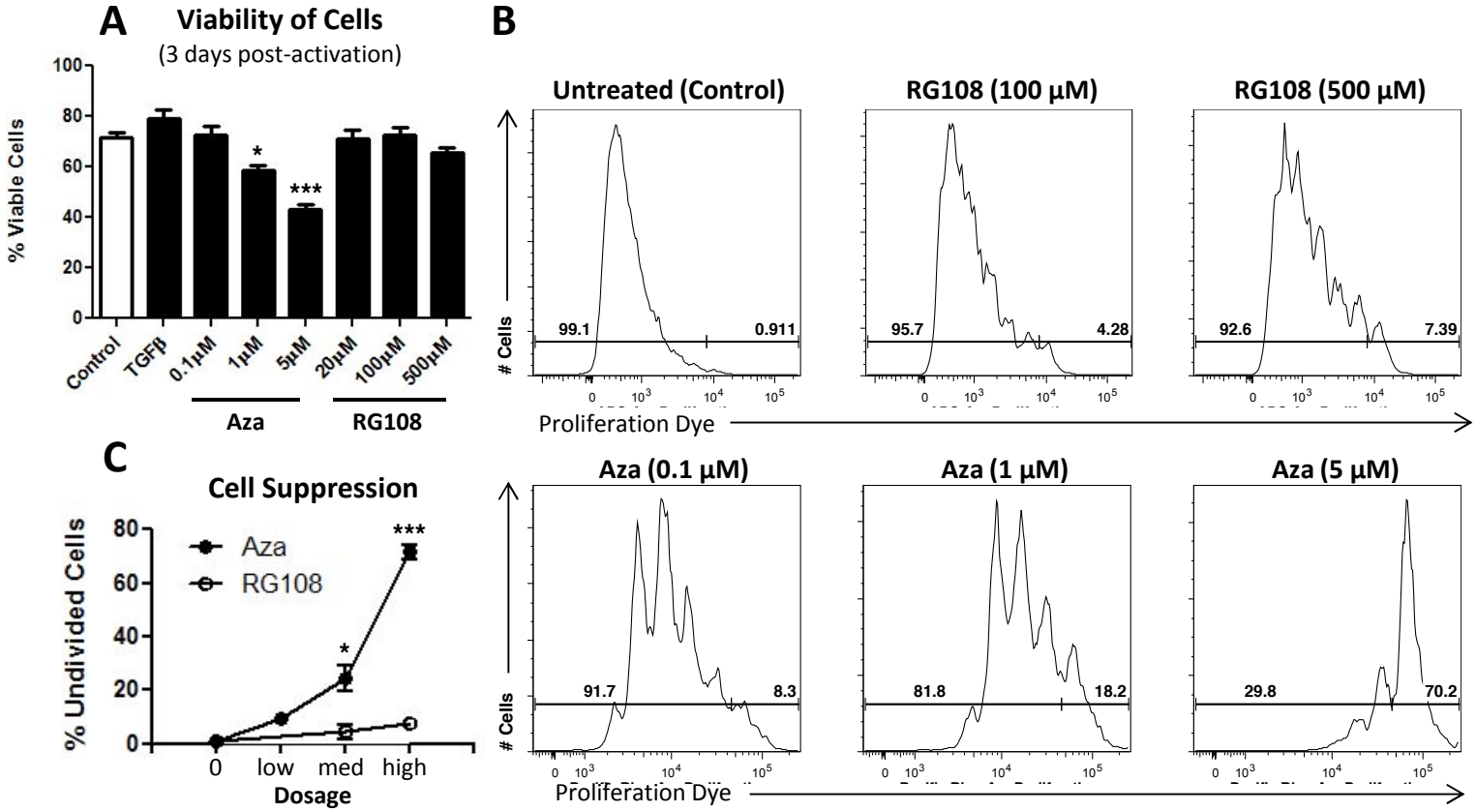

***Figure 6* – Cytotoxicity and Anti-Proliferative Effects of DNMT-Inhibitors.**

A) MACS purified $CD4^{+}CD25^{-}$ T_{CONV} cells (5×10^5/well) were activated under plate-bound conditions, αCD3/αCD28 (2μg/ml) and simultaneously administered with Aza (0.1, 1, and 5μM) and RG108 (20, 100, and 500μM). All cultures were supplemented with rhIL-2 (5ng/ml) and incubated for 3 days before harvest. Cells were stained with eFluor-780 viability dye and acquired with flow cytometry. The graph shows the proportions of viable cells in cultures as measured on day 3 post-activation. Mean ± SEM are plotted and data is representative of three independent experiments. B) Cells were labelled with eFluor-670 proliferation dye prior to activation. Histograms show cell division at day 3 post-activation of RG-treated cultures (top row) and Aza-treated cultures (bottom row) and the gate shows the proportions of undivided and divided cells. Histograms are representative from data of two independent experiments. C) Compiled cell suppression data with mean ± SEM plotted, n=2. * denotes $p<0.05$, *** denotes $p<0.001$.

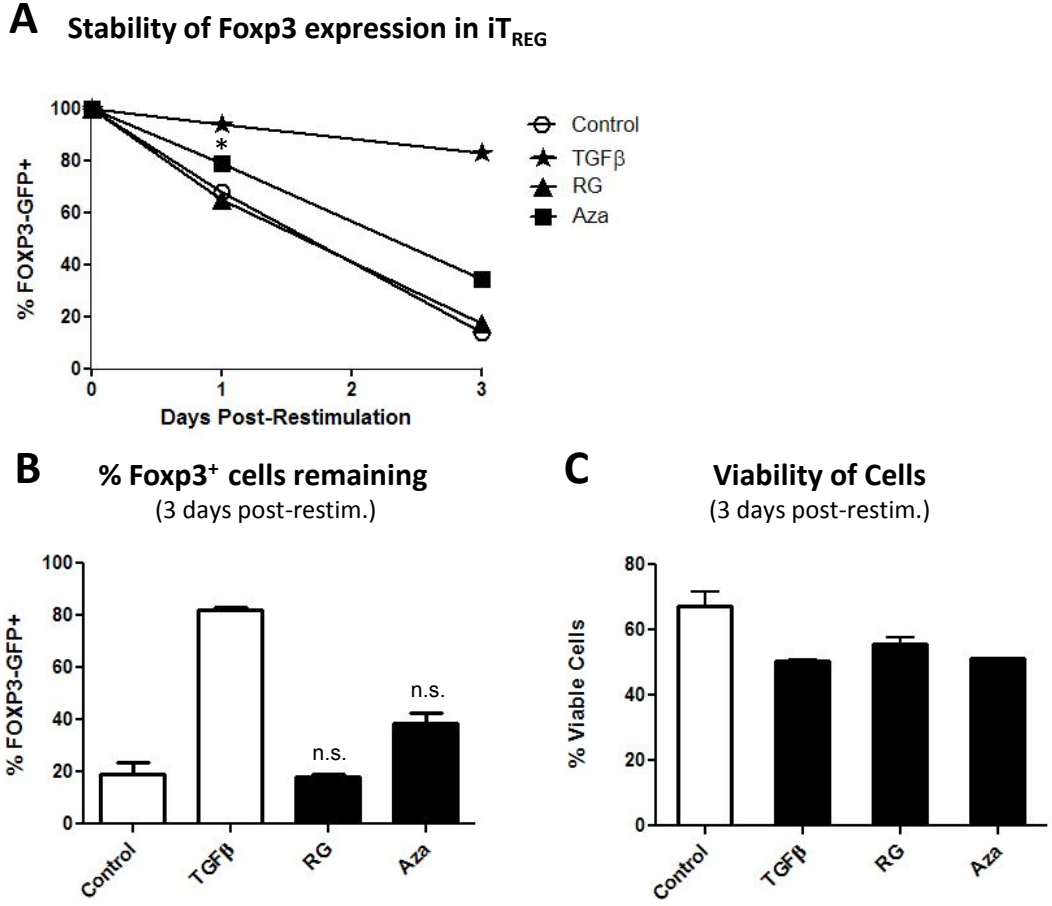

Figure 7 **– DNMT-Inhibitors and Maintenance of Foxp3 Expression.**
TGF-β-induced Foxp3^{+} cells were generated from MACS purified CD4^{+}CD25^{-} T_{CONV} cells as previously described. At day 3 post-activation, cultures are harvested and purified for Foxp3 $^{GFP+}$ cells by FACS. These Foxp3 $^{GFP+}$ cells are plated (5x10^{4} cells/well) in a 96-well microtiter plate and restimulated under plate-bound conditions: αCD3/αCD28 (1μg/ml), with Aza (5μM) or RG108 (500μM). All cultures were supplemented with rhIL-2 (5ng/ml). A) Cells were harvested at the indicated time points and proportions of Foxp3^{+} cells remaining in the culture were plotted to show loss of Foxp3-expression over the timecourse. B) Proportions of Foxp3^{+} cells remaining in the culture at day 3 post-restimulation are plotted. C) Cells are stained with eFluor-780 viability dye at day 3 post-restimulation and proportions of viable cells remaining in the culture are plotted. Mean ± SEM are plotted and data is representative of two independent experiments. ** denotes $p<0.05$.

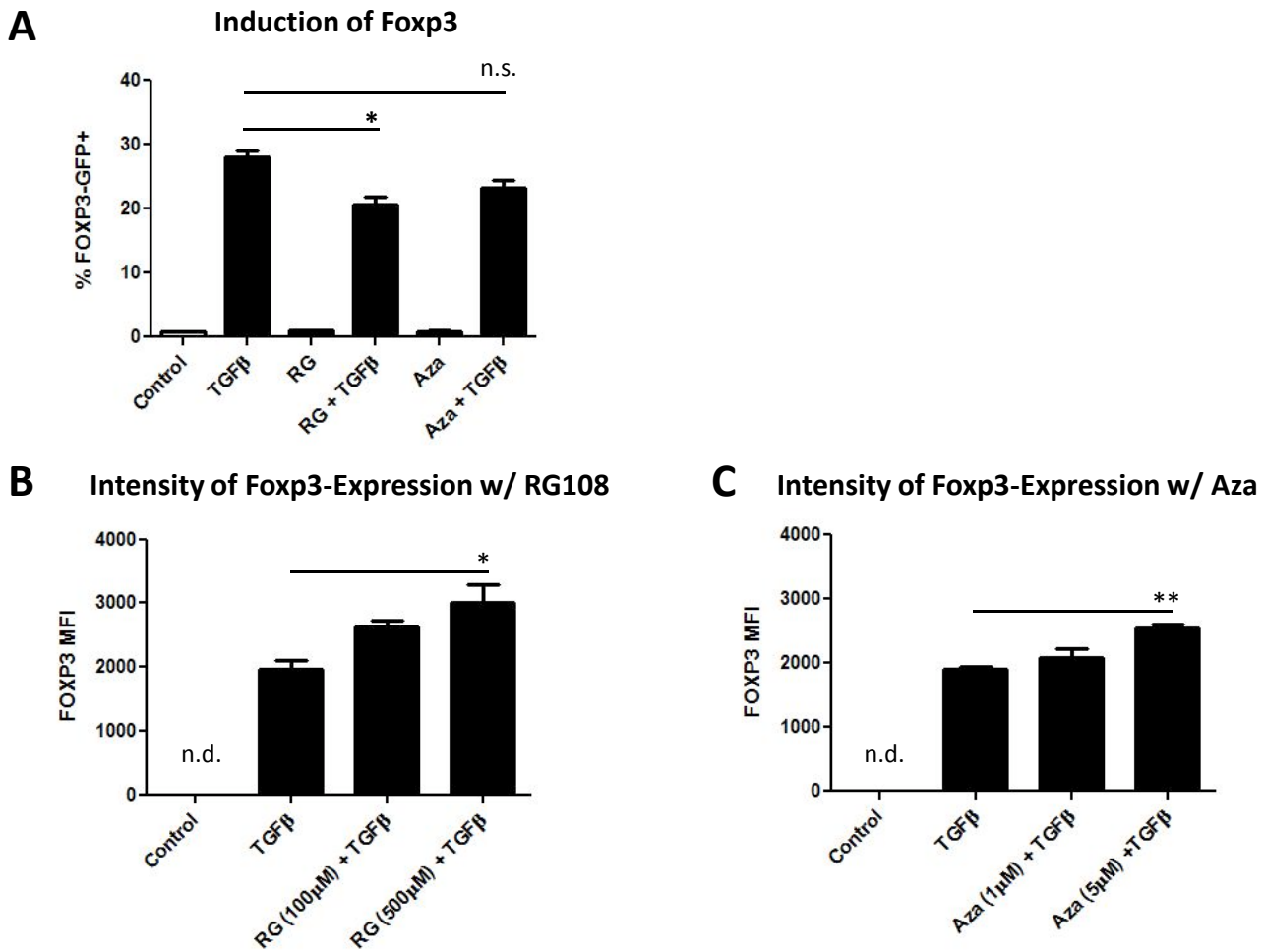

Figure 8 **– DNMT-Inhibitors and Induction of Foxp3 Expression.**

A) MACS purified $CD4^+CD25^-$ T_{CONV} cells (5×10^5/well) were activated under plate-bound conditions αCD3/αCD28 (2μg/ml) and treated with Aza (5μM), RG108 (500μM), and TGF-β1 (5ng/ml) in the indicated combinations. All cultures were supplemented with rhIL-2 (5ng/ml) and incubated for 3 days before harvest. The proportion of Foxp3-expressing cells out of total cells in culture are plotted.

B and C) Cells are harvested 3 days post-activation and gated on $CD4^+Foxp3^+$ cells. The median fluorescence intensity (MFI) of Foxp3-GFP is plotted. For all graphs, the mean ± SEM is plotted and is representative of two independent experiments.

* denotes $p<0.05$, n.d., not detectable.

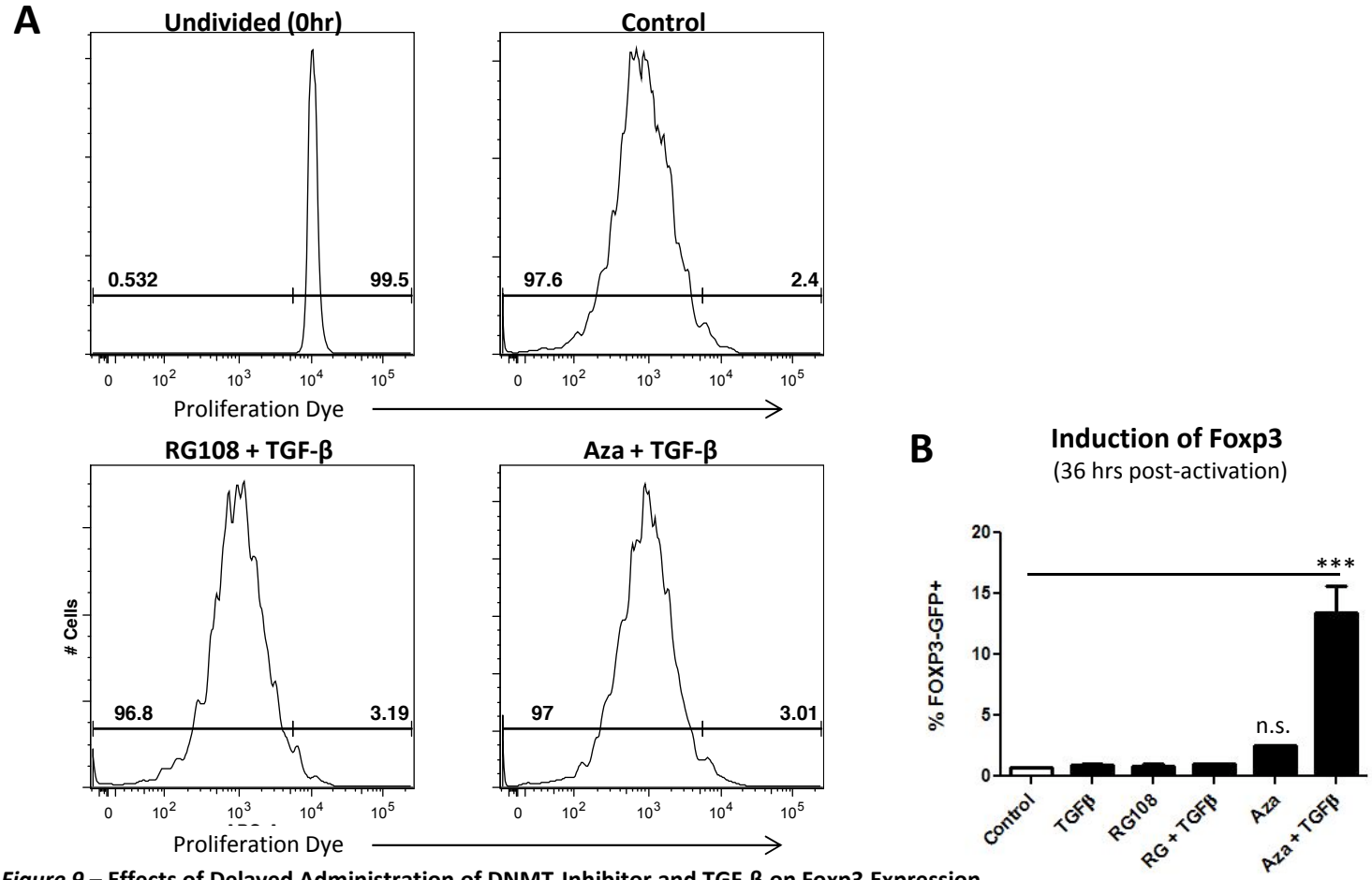

Figure 9 **– Effects of Delayed Administration of DNMT-Inhibitor and TGF-β on Foxp3 Expression.**
MACS purified $CD25^{-}$ $CD4^{+}$ T_{CONV} cells (5×10^5/well) were activated with plate-bound αCD3/αCD28 (2μg/ml). At 36 hours post-activation, cultures were administered with Aza (5μM) and RG108 (500μM) with or without TGF-β (5ng/ml). All cultures were supplemented with rhIL-2 (5ng/ml) and labeled with eFluor-670 proliferation dye.
A) At 3 days post-activation, cells are harvested. Histograms represent the proliferation of cells under the indicated culture conditions. The values indicate the proportion of undivided and divided cells. Histograms are representative of two experiments.
B) Cells are harvested 3 days post-activation and the proportion of Foxp3-expressing cells out of total cells in culture are plotted as the mean ± SEM from three independent experiments. * denotes $p<0.001$.

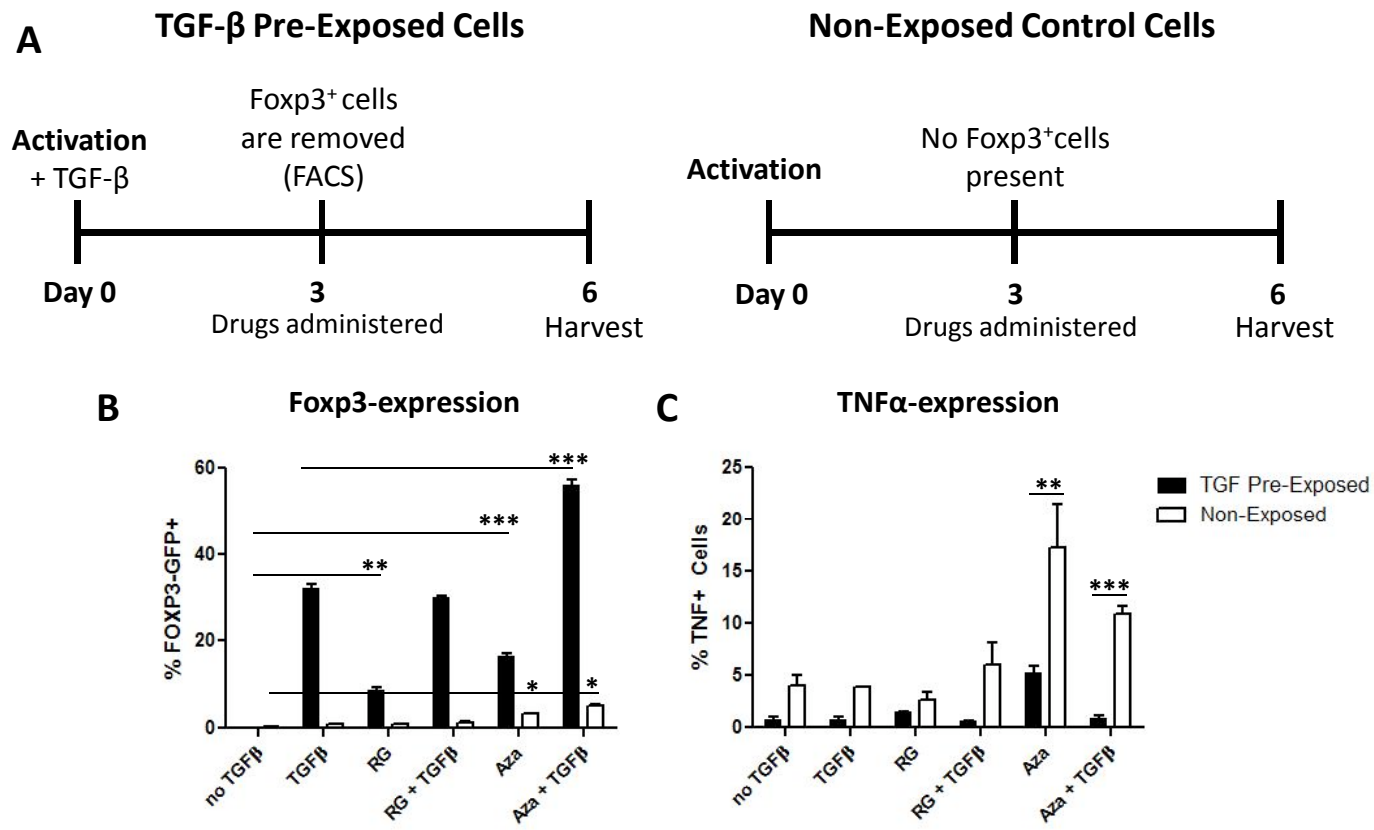

Figure 10 **- Aza and RG108 stimulate Foxp3-expression in TGF-β pre-exposed Foxp3-negative cells.**
A) TGF-β-induced Foxp3-expressing cells are generated as previously described. At day 3 post-activation the $Foxp3^{GFP-}$ population (hereinafter referred to as *TGF-β pre-exposed cells*) was purified by FACS. These cells were then re-cultured in a 96-well plate ($5x10^4$ cells/well) with IL-2 (5ng/ml) and treated with various conditions (5ng/ml TGF-β, 5µM Aza, 500µM RG) for an additional 3 days before harvest and acquisition. Control cells that were not exposed to TGF-β1 (*non-exposed control cells)* were cultured in parallel under equal conditions. B) Cultures were harvested 3 days post-treatment (6 days post-activation) and analyzed by quantitative flow cytometry for Foxp3 expression by measuring GFP fluorescence. C) Cultures are harvested 3 days post-treatment (6 days post-activation) and are fixed, permeabilized, and stained for APC-TNFα (0.75µg/10^6 cells) at 4°C for 60 minutes in the dark. Analysis by quantitative flow cytometry is done by gating on $CD4^+Foxp3^{GFP-}$ cells. TNFα was not detectable in Foxp3-expressing cells (data not shown). All bar graphs show the mean ± SEM , n=3. * denotes $p<0.05$, ** denotes $p<0.01$, *** denotes $p<0.001$

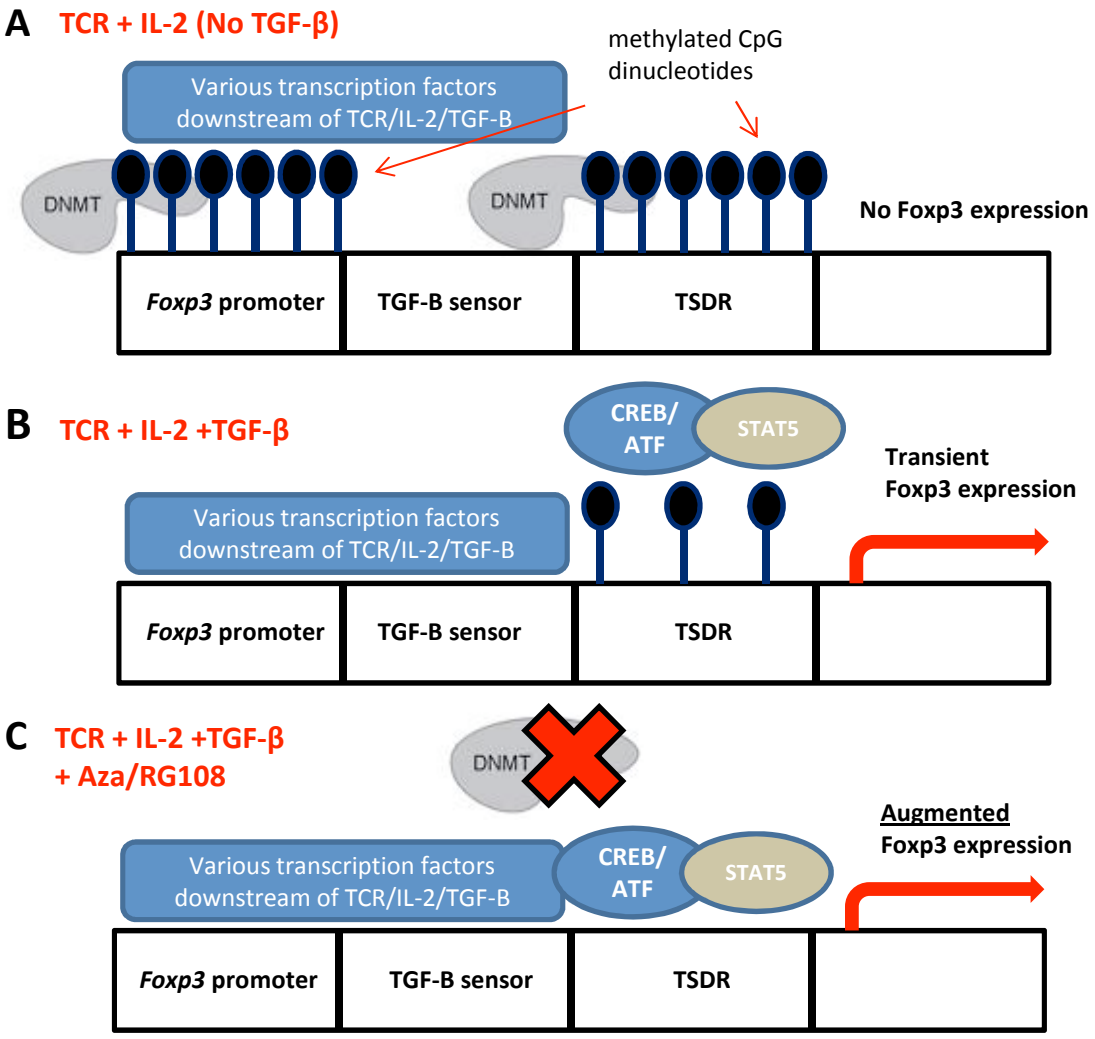

***Figure 11* – Proposed model of Foxp3 regulation with DNMT-inhibition and TGF-β**

A) Status of the *Foxp3* gene in an activated T_{CONV} cell. The *Foxp3* promoter, and TSDR are completely methylated as a result of DNMT-recruitment mediated by TCR-stimulation. Transcription factors which are necessary for Foxp3-expression are unable to bind to the DNA, and Foxp3-expression does not occur.
B) Status of the *Foxp3* gene in a TGF-β-induced Foxp3-expressing cell. Presence of TGF-β at the time of TCR-stimulation inhibits the recruitment of DNMT1 and promotes the demethylation of the *Foxp3* promoter. Consequently, transcription factors bind to the DNA and Foxp3-expression occurs. The TSDR however, remains partially methylated and binding of transcription factors CREB/ATF and STAT5 is impaired resulting in transient Foxp3-expression that diminishes in the absence of TGF-β.
C) Status of the *Foxp3* gene in DNMT-inhibitor-treated cultures.
Aza and RG108 results in inactivation of DNMTs. Consequently, the *Foxp3* gene is hypomethylated and CREB/ATF and STAT5 are able to bind to their respective binding sequences on the TSDR. Foxp3-expression is augmented as a result of saturated binding with transcription factors downstream of TCR/IL-2/TGF-β.