TRANSCRIPTIONAL REGULATION OF THE INDUCIBLE COSTIMULATOR (ICOS) IN T CELLS

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TABLE OF CONTENTS

Chapter 2 Materials and Methods

Chapter 3 Results

SUMMARY

The inducible costimulator (ICOS), a member of the CD28 family of costimulatory molecules, is rapidly induced upon T cell activation. Although the critical role of ICOS in T-cell-mediated immunity is well documented, little is known of the intracellular pathways that modulate ICOS expression. We first investigated ICOS induction during early activation of T cells by T cell receptor (TCR) and CD28 co-engagement. We found that the ectopic expression of the transcription factor NFATc2 or a constitutively active form of MEK2 that activates ERK amplified *icos* transcription by acting on a 288-bp region of the *icos* promoter in luciferase reporter assays. We also identified a site on the promoter that is sensitive to ERK signalling and further showed the *in vivo* binding of NFATc2 to the promoter, the intensity of which is diminished when Fyn signalling is ablated. The normal activation of ERK but reduced nuclear translocation of NFATc2 in Fyn-deficient ($Fyn^{-/-}$) CD4+ T cells imply that Fyn and NFATc2 act in a common axis, separate from ERK, to drive *icos* transcription.

Following initial activation, T cells differentiate into Th1 or Th2 cells, depending on the nature of the immune response. Because ICOS expression was found to be differentially expressed in these cells, we next examined the control of ICOS expression by Th1-specific T-bet and Th2-specific GATA-3, which drive respective lineage commitment, as well as NFATc2, which is broadly expressed across lineages. We observed that the over-expression of T-bet or GATA-3 could enhance, and NFATc2 could further synergize with either of them to increase, *icos* transcription. While T-bet acted on the *icos* promoter, GATA-3 operated via an *icos* 3′UTR element. Interestingly, NFATc2 was found to bind promiscuously the *icos* promoter in developing Th0, Th1 and

Th2 cells but became selectively associated with T-bet at the promoter and with GATA-3 at the 3′UTR in fully differentiated Th1 and Th2 cells, respectively. The binding dynamics of these transcription factors coincided with the chromatin accessibility of these regulatory regions in the different Th cells as assessed by histone trimethylation.

Finally, we also found ICOS expression to be regulated at the post-transcriptional level by a recently discovered RING-type E3 ubiquitin ligase, roquin. Enforced expression of wild-type but not a *sanroque* mutant form of roquin accelerated the decay of ICOS mRNA in a T cell line. Collectively, our findings indicate that during the initial TCR/CD28-mediated activation of T cells, Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 signalling pathways cooperate to induce ICOS expression. As Th cells differentiate along the Th1 or Th2 lineage, the non-selectively expressed NFATc2 synergises with Th-restricted T-bet or GATA-3 in a temporally evolving fashion to direct *icos* transcription via distinct regulatory elements in Th cells undergoing differentiation. In addition to the transcriptional control of ICOS expression in Th cells, there exists a post-transcriptional level of ICOS regulation, in terms of mRNA turnover, mediated in part by the *ROQ* domain of roquin.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ab	antibody
AILIM	activation-inducible immunomodulatory molecule
AP-1	activator protein-1
APC	antigen presenting cell
Bcl	B-cell lymphoma
CD	cluster of differentiation
ChIP	chromatin immunoprecipitation
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis
Elf-1	E74-like factor-1 (Elf-1)
Elk-1	E26-like protein-1
EAMG	experimental autoimmune myasthenia gravis
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal-regulated kinase
Ets-1	E26 transformation-specific-1
FoxP3	forkhead box protein 3
GATA-3	GATA binding protein 3
GFP	green fluorescent protein
GVHD	graft versus host disease
ICOS	inducible costimulator
ICS	intracellular staining

IFN-γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
iono	ionomycin
JNK	c-Jun NH_2-terminal kinase
LAT	linker for activated T cells
LICOS	ligand for ICOS
LSF	late SV40 transcription factor
luc	luciferase
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
mTOR	mammalian target of rapamycin
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor binding immunoglobulin κ light chain enhancer in B cells
PI-3K	phosphatidylinositol-3-kinase
PLC-γ1	phospholipase C-gamma1
PMA	phorbol-12-myristate-13-acetate
RAPA	rapamycin
SAP	SLAM-associated protein
SCID	severe combined immunodeficiency
SH	Src homology
SLAM	signalling lymphocytic activation molecule

SLP-76	signaling leukocyte protein of 76 kD
STAT	signal transducer and activator of transcription
SV40	simian virus 40
Syk	spleen tyrosine kinase
T-bet	T-box expressed in T cells
TCR	T cell receptor
T_{FH}	follicular B helper T
Th	T helper
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
T_{reg}	regulatory T
TSS	transcription start site
UTR	untranslated region
WM	wortmannin
WT	wild-type
XLP	X-linked lymphoproliferative syndrome
ZAP-70	zeta-associated protein of 70 kD

CHAPTER 1

INTRODUCTION

1.1 Two-signal model of T cell activation

Mounting an appropriate immune response depends on the careful regulation of lymphocyte activation. To this end, lymphocytes require two independent signals to become optimally activated. The first, an antigen-specific signal is sent via the unique antigen receptor: TCR on T cells or surface Ig on B cells. The TCR is cross-linked by an agonist peptide or antigen (Ag) presented in the context of the major histocompatibility complex (MHC) II for cluster of differentiation (CD)4+ and MHC I for CD8+ T cells on the surface of antigen presenting cells (APCs), which include macrophages, dendritic cells (DCs) and B cells. The second signal, termed costimulation, is critical for full activation, sustaining cell proliferation, inducing differentiation and preventing anergy and/or apoptosis (Frauwirth and Thompson, 2002; Chambers and Allison, 1997; Chambers, 2001), and in the context of T cells, is delivered by costimulatory ligands expressed on activated APCs (Coyle and Gutierrez-Ramos, 2001; Sharpe and Freeman, 2002; Greenwald et al., 2005) (Figure 1.1). The two most prominent superfamilies mediating costimulation are those of B7/CD28 (Carreno and Collins, 2002; Lenschow et al., 1996b; Shahinian et al., 1993) and tumour necrosis factor / receptor (TNF/TNFR) (Croft, 2003a; Croft, 2003b), the signals of which are in turn negatively regulated by inhibitory receptors expressed upon lymphocyte activation. Members of the CD28 family of receptors, part of the broader immunoglobulin (Ig) superfamily, are type I transmembrane glycoproteins and share 20 – 35% identity in their amino acid sequences (Table 1.1). Despite such low homology in primary amino acid composition, these molecules share a similar secondary structure: single Ig V and Ig C-like extracellular

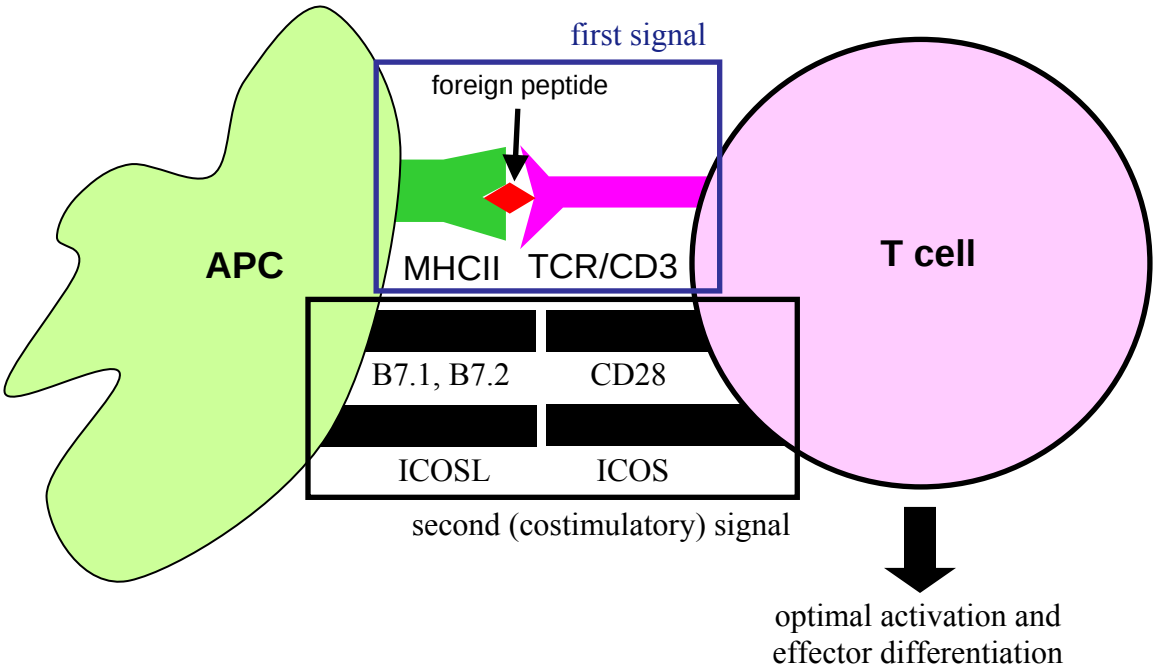


Figure 1.1 **Two-signal model of T cell activation.**

Table 1.1 **Comparison of CD28 family of receptors.**

	CD28	**CTLA-4**	**ICOS**	**PD-1**	**BTLA**
% identity	100 %	30 %	27 %	23 %	23 %
Chromosome Human Mouse	2q33 1C1	2q33 1C2	2q33 1C2	2q37 1D	3q13.2 16A1
Structure Ligand binding motif Cytoplasmic domain	MYPPPY PI3K motif, PP2A	MYPPPY PI3K motif, PP2A, ?SHP2	FDPPPF PI3K motif	? ITIM motif, SHP2, ITSM motif, SHP1	? Two ITIM motifs
Expression Cell type	T	T	T, NK	T, B, M	T, B

T = T cell; B = B cell; NK = natural killer cell; M = macrophage; ITAM = immunoreceptor tyrosine-based activation motif; ITIM = immunoreceptor tyrosine-based inhibition motif; ITSM = immunoreceptor tyrosine-switch motif.

domains. Four cysteine residues, which are involved in the formation of the disulfide bonds of the Ig V and Ig C domains, are well-conserved. The receptors for the B7 family are members of the CD28 family, and possess a single Ig V-like extracellular domain. Their cytoplasmic tails contain putative Src homology (SH)2- and SH3-motifs thought to be involved in signal transduction (Rudd and Schneider, 2003; Wang and Chen, 2004). APC-expressed B7-1 (CD80) and B7-2 (CD86) bind the costimulatory CD28 and inhibitory cytotoxic T lymphocyte-associated antigen (CTLA)-4 on T cells. Engagement of the prototypical CD28 receptor on naïve T cells by either B7-1 or B7-2 augments the TCR signal (Riley and June, 2005), which leads to enhanced interleukin (IL)-2 transcription (Civil and Verweij, 1995), expression of CD25 (IL-2Rα), and entry into cell cycle (Parry et al., 2003). Critical survival, as distinct from proliferation, signals are also conferred via the B-cell lymphoma (Bcl)-X_L pathway (Burr et al., 2001; Okkenhaug et al., 2001; Sperling et al., 1996; Boise et al., 1995; Marinari et al., 2004). In contrast, the engagement of CTLA-4 delivers negative signals, which inhibit IL-2 synthesis, cell cycle progression and terminate T cell responses.

1.2 Fyn and Lck signalling downstream of TCR

The src-family tyrosine kinases $p59^{fyn}$ (Fyn) and $p56^{lck}$ (Lck) are expressed in T cells and constitute the most proximal signalling molecules to be activated downstream of TCR. They are responsible for the initial tyrosine phosphorylation of the receptor, leading to the recruitment of the zeta-associated protein of 70 kD (ZAP-70) tyrosine kinase, as well as the subsequent phosphorylation and activation of ZAP-70, linker for activated T cells (LAT), and phospholipase C-gamma1 (PLC-γ1), leading to calcium flux, activation

of calcineurin and dephosphorylation and nuclear translocation of the nuclear factor of activated T cells (NFAT). Although closely related, these signalling molecules have distinct functions during development, maintenance and activation of peripheral T cells. For example, during thymopoiesis, albeit Fyn can substitute for a subset of signals which Lck is uniquely able to provide for pre-TCRβ selection, Fyn is largely dispensable for gross T cell development (Appleby et al., 1992). In naïve peripheral T cells, either Lck or Fyn can transmit TCR-mediated survival signals, and yet only Lck is able to trigger TCR-mediated expansion signals under lymphopenic conditions. Stimulation of naïve T cells to proliferate and produce IL-2 by antigenic stimuli is also severely compromised in the absence of Lck, but hardly impaired by the absence of Fyn. Interestingly, more profound defects in the Lck-dependent signalling pathway were observed with stimulation of Fyn-deficient T cells with anti-CD3 and anti-CD28 antibodies, which was rescued by coaggregation of CD3 and CD4 (Sugie et al., 2004). This suggests that Fyn positively regulates Lck when stimulation of CD4+ T cells is restricted to TCR with no involvement of MHCII and CD4 co-receptor.

Another study, examining the ability of Fyn to mediate TCR signal transduction in an Lck-deficient Jurkat T-cell line (JCaM1), found that the signalling leukocyte protein of 76 kD (SLP-76) adapter protein, the Ras mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 4,5-biphosphate signalling pathways, but not NFAT and IL-2 production, were activated in the absence of Fyn (Denny et al., 2000). This indicates Fyn mediates an alternative form of TCR signalling which is independent of ZAP-70 activation and imply which Src family kinase is used to initiate signalling may dictate the outcome of TCR signal transduction, at least in human T cells.

1.3 CD28 costimulatory receptor

CD28, being constitutively expressed, is critical for the activation of naive T cells and plays an important role in the initial phase of an immune response, while not being as effective in regulating effector and memory T cell responses. Despite its status as a key costimulatory molecule, CD28 does not account for all costimulatory functions in T cells. Immune responses are not totally nullified in the absence of CD28 because mice lacking CD28 exhibit normal Th cell activation and are able to produce IgG in response to infections by some viruses (Whitmire and Ahmed, 2000). In addition, priming of T helper (Th) cells, Ig class switching and IgM responses are reportedly normal in CD28-deficient mice (Wu et al., 1998), while memory Th2 effector cells can develop in the absence of B7-1/2 and CD28 interactions, after sensitisation with an intestinal nematode parasite *Heligmosomoides polygyrus* (Ekkens et al., 2002). Reactivation of antigen-experienced memory and effector T cells are far less dependent than naive T cells on CD28/B7 costimulation (London et al., 2000), consistent with the idea that CD28 signalling is less important for the function of the former population of T cells. CD28 deficiency paradoxically promotes the development of diabetes in the nonobese diabetic (NOD) mouse strain (Lenschow et al., 1996a), although this observation was later attributed to the profound decrease of immunoregulatory CD4+CD25+ T cells in CD28-deficient NOD mice. Another study found that the production of effector cytokines such as interferon (IFN)-γ and IL-4 can be stimulated to normal levels by APCs lacking both B7-1 and B7-2 (Schweitzer et al., 1997). Furthermore, naive TCR-transgenic T cells lacking CD28 are able to initiate a primary Ag-specific response, but fail to sustain proliferation as the cells acquire effector status (Lucas et al., 1995). This is possibly due to

compensation for costimulatory requirement by prolonged TCR signalling (Acuto and Michel, 2003), made obvious in the case of T cells genetically engineered to express a TCR transgene, and/or functional substitution for CD28 by other costimulatory molecules. These results suggest that other molecules can compensate for the absence of CD28 signalling.

1.4 Inducible costimulator (ICOS) receptor

1.4.1 ICOS structure and signalling

One such recently discovered candidate belonging to the CD28 family is the inducible costimulator or ICOS. Alternatively termed activation-inducible lymphocyte immunomodulatory molecule (AILIM), the rat ICOS homologue (Tamatani et al., 2000; Tezuka et al., 2000), or H4 (Buonfiglio et al., 2000), ICOS is a T-cell specific molecule structurally and functionally related to CD28 and shares 30% to 40% sequence similarity to CD28 and CTLA-4 but does not carry the conserved MYPPPY motif necessary for binding B7-1 and B7-2 (Beier et al., 2000; Hutloff et al., 1999) (Table 1.1). Murine, rat and canine ICOS share respectively 71.7% (Mages et al., 2000), 67% (Tamatani et al., 2000) and 79% (Lee et al., 2004b) sequence identities with their human counterpart. It is rapidly induced on T cells upon activation and binds its cognate ligand B7-H2 (also known as GL50 (Ling et al., 2000), B7RP-1, B7h, LICOS or ICOSL) which is expressed constitutively on APCs, including B cells and macrophages, and can be induced in cultured fibroblasts and non-lymphoid tissues by treatment with inflammatory agents such as LPS and TNF-α (Liang and Sha, 2002; Swallow et al., 1999). LICOS was first identified as the homologue of B7-1 expressed by chicken macrophages and of

mammalian B7-H2 and may be the contemporary form of a primordial vertebrate costimulatory ligand (Brodie et al., 2000).

Similar to CD28 signalling, ICOS engagement costimulates T cell proliferation, promotes Th differentiation and production of IFN-γ, TNF-α, IL-4, IL-5, IL-10 and IL-13 (Beier et al., 2000; Coyle et al., 2000; Nurieva et al., 2003b; Vieira et al., 2004; Yoshinaga et al., 1999) but unlike CD28, it does not enhance IL-2 production, as ICOS blockade did not attenuate IL-2 secretion by mice administered superantigen (SAg) (Gonzalo et al., 2001a). However, ICOS strikingly potentiated secretion of IL-2, IFN-γ and TNF-α, but not IL-4 in human naive CD4+ T cells stimulated through CD3 and CD28 (Mesturini et al., 2006), alluding to differences between mouse and human ICOS signalling. It has been suggested that ICOS signalling may be more important for regulating activated, effector and memory T cells (Coyle et al., 2000), whereas CD28 functions to prime naive T cells, given its absence or very low levels of expression on naive T cells and its up-regulation upon stimulation in both CD4+ and CD8+ effector T cells (Beier et al., 2000; Tafuri et al., 2001; Yoshinaga et al., 1999). Consistent with this view, delayed ICOS blockade during chronic allograft rejection, the progression of which is mediated by effector/memory T cells, was associated with down-regulation of local intragraft expression of several cytokines and chemokines (Kashizuka et al., 2005). Blocking ICOS on effector T cells during the efferent phase of the immunopathogenesis of murine experimental autoimmune encephalomyelitis (EAE) abrogated disease (Rottman et al., 2001). Similar blockade during the effector phase of murine experimental autoimmune uveoretinitis ameliorated disease, while doing so during the induction phase had no significant effect (Usui et al., 2006b). Moreover, ICOS/B7-H2 interactions were

shown to play an important role in controlling the entry of effector/memory T cells across the endothelium into inflamed tissues in the periphery via activating the PI3-kinase/Akt and Rho family cascades (Nukada et al., 2006; Okamoto et al., 2004a).

Initial characterisation of either ICOS or B7-H2 deficient mice revealed a primary phenotype of severe defects in germinal centre (GC) formation and B cell immunoglobulin class switching. (Dong et al., 2001a; McAdam et al., 2001; Tafuri et al., 2001; Wong et al., 2003; Dong et al., 2001b). ICOS deficient mice were highly resistant to clinical experimental autoimmune myasthenia gravis development, exhibited defective T cell-mediated humoral immunity, and had a diminutive GC reaction in secondary lymphoid tissues (Scott et al., 2004). Consistent with the pivotal role of ICOS in GC development, ICOS has been implicated in the maintenance of CXCR5+ follicular B helper T cells (T_{FH}) *in vivo* (Akiba et al., 2005). Murine ICOS deficiency is associated with a severe reduction of CXCR5+CD4+ GC Th cells (Bossaller et al., 2006). Human ICOS deficiency, on the other hand, results in a clinical phenotype indistinguishable from the monogenic disease known as adult-onset common variable immunodeficiency (Grimbacher et al., 2003; Warnatz et al., 2005). In the context of viral and parasitic infections, ICOS regulates CD28-dependent and CD28-independent CD4+ T cell subset polarisation but not cytotoxic T lymphocyte responses (Bertram et al., 2002; Kopf et al., 2000; Loke et al., 2005). In addition, a recent study demonstrated a critical role for ICOS costimulation in immune containment of pulmonary influenza virus infection (Humphreys et al., 2006). Substantial similarity between the immune phenotype and responses of ICOS and B7-H2 deficient mice (Dong et al., 2001a; Mak et al., 2003; McAdam et al., 2001; Tafuri et al., 2001; Wong et al., 2003), combined with interaction

studies using recombinant protein (Nurieva et al., 2003b; Swallow et al., 1999), brought about the notion of a monogamous receptor-ligand relationship between ICOS and B7-H2. So far, there has been a paucity of evidence to suggest otherwise.

ICOS expression is optimally induced when both TCR and CD28 are activated, whereas its up-regulation is significantly reduced in the absence of CD28/B7 interaction (Coyle et al., 2000; McAdam et al., 2000), although physiologically relevant levels of ICOS are expressed on T cells subjected to submitogenic stimulation with anti-CD3 antibody (Ab) (McAdam et al., 2000; Yoshinaga et al., 1999). It is therefore anticipated that one of the possible reasons behind attenuated ICOS/B7-H2 signalling in CD28 KO mice is defective ICOS up-regulation. To address the issue of the costimulatory capacity of ICOS in the absence of CD28, mice doubly deficient in CD28 and ICOS were generated and their Ab responses against environmental Ags, T-dependent protein Ags, and vesicular stomatitis virus were compared with those of CD28 deficient and wild-type (WT) counterparts. Doubly deficient mice manifested profound defects in their immune responses that far exceeded those observed in CD28 deficient mice, suggesting that in the absence of CD28, ICOS assumes the major T cell costimulatory role for humoral immune responses (Suh et al., 2004). Besides its dominant role in humoral immunity, ICOS signalling is also involved in the proliferation of effector T cells, driving the initial clonal expansion of primary and primed Th1 and Th2 cells in response to immunisation (Smith et al., 2003). Furthermore, its signalling regulates DC-mediated clonal expansion of human Th2, but not Th1 cells (Vieira et al., 2004), and dictates the expansion of polarised Th subsets developed in response to infection with *Trichuris muris* or *Toxoplasma gondii* (Wilson et al., 2006).

1.4.2 ICOS in Th1 and Th2-associated immunity

After engagement of the T-cell receptor (TCR) by the appropriate peptide-MHC complex, naïve CD4+ T cells residing in peripheral lymphoid organs undergo differentiation into the canonical Th1 or Th2, or the more recently characterised IL-17-producing T helper (Th17) or regulatory T (T_{reg}) cell subsets (Reinhardt et al., 2006; Murphy and Reiner, 2002). It is increasingly appreciated that CXCR5+ T_{FH} cells constitute a lineage distinct from Th1 or Th2 cells, although the presence of IFN-γ- and IL-4-producing Th cells in the follicles obscures the unique identity of these cells (Chtanova et al., 2004). Which lineage program is adopted by the naïve Th cell depends on the nature of the pathogen activating effector immune or immunomodulatory responses. In host defence, effector Th1 cells are responsible for cell-mediated immunity, providing protection against intracellular pathogens such as some bacteria, viruses and protozoa, whereas Th2 cells are responsible for extracellular immunity associated with protection against parasitic helminths (Liu et al., 2004). Both subsets have also been implicated in pathological responses. The very recently identified Th17 rather than Th1 cells have been shown to mediate several organ-specific autoimmune diseases. Several lines of evidence indicate that Th1 cells (and IFN-γ) are actually anti-inflammatory in some mouse models of autoimmunity. For example, IFN-γ-deficient mice were more susceptible to EAE due to exacerbated expansion of encephalitogenic CD4+ T cells (Chu et al., 2000) and deficiency in IL-12 signalling worsened collagen-induced arthritis (Murphy et al., 2003). Th2 cells, on the other hand, are involved in asthmatic and allergic responses. The final composition of the Th cell response to antigen can therefore potentially determine the outcomes of inflammatory, infectious or autoimmune

responses, resulting in beneficial resolution of the disease or its harmful protraction and consequent bystander damage to the host.

Numerous studies define an apparent role for ICOS in determining the polarisation of Th function, with early reports linking ICOS preferentially to Th2-type responses. Indeed, ICOS-deficient T cells exhibited defects in Th2 cytokine secretion (Dong et al., 2001a; Tafuri et al., 2001), being selectively impaired in IL-4 production after *in vitro* differentiation or *in vivo* priming by protein antigen (Dong et al., 2001a). In agreement with this, normal IL-5-mediated lung eosinophilic infiltration but defective IL-4-dependent IgE production were observed in ICOS-deficient mice that developed asthma, a Th2-type condition (Dong et al., 2001a). Using TCR transgenic mice subjected to the same asthma model, another study reported that monoclonal antibody (mAb)-mediated neutralisation of ICOS signalling diminished both eosinophilic inflammation and IgE production (Gonzalo et al., 2001b). Consistent with this, ICOS blockade was also reported to inhibit Th2 effector function, although not Th2 differentiation, in a model of allergic airway disease (Tesciuba et al., 2001). Moreover, treatment with a blocking anti-ICOS Ab strongly suppressed Th2-type chronic graft versus host disease (GVHD) (Ogawa et al., 2001), and resulted in decreases in TNF-α, IL-4 and IL-5 and total serum IgE levels induced by the gastrointestinal helminth *Trichinella spiralis*, although the expulsion of adult parasites was unaffected (Scales et al., 2004). As opposed to driving Th2-associated immunopathology, the ICOS/B7-H2 pathway surprisingly facilitated the development and inhibitory function of IL-10-producing T_{reg}s important in respiratory tolerance and in down-regulating pulmonary inflammation in airway hypersensitivity (Akbari et al., 2002). More recently, *in vitro* Th polarisation experiments demonstrated

that ICOS transcriptionally costimulates Th2 differentiation by an IL-4-driven mechanism involving the transcription factors NFATc1 and c-Maf (Nurieva et al., 2003a).

Despite structure-function analyses that associate ICOS signalling with Th2 differentiation (Andres et al., 2004; Rudd and Schneider, 2003), various *in vivo* infection, autoimmune, hypersensitivity and transplantation models suggest that ICOS could also be important in controlling Th1-mediated responses. Anti-ICOS therapy profoundly reduced both chronic and Th1-dependent acute allograft rejection (Ozkaynak et al., 2001). In a mouse model of cardiac allograft transplantation, delayed ICOS blockade prolonged graft survival while early blockade produced a reciprocal effect (Harada et al., 2003). B7RP-1-Fc chimaeric protein stimulated Th1-dominated contact hypersensitivity when given near either the time of sensitisation or challenge with oxazolone (Guo et al., 2001). Furthermore, neutralisation of the ICOS/B7-H2 pathway respectively exacerbated or ameliorated disease during the sensitisation or established phases of EAE (Rottman et al., 2001), and effectively inhibited anti-nucleolar autoantibodies and total serum IgE production in a mouse strain susceptible to mercury-induced autoimmunity (Zheng et al., 2005). Blockade of the ICOS pathway increased susceptibility of CD28-deficient mice to Th1-type *Toxoplasma gondii* infection. Administration of anti-B7-H2 mAb before the onset of renal disease significantly delayed the onset of proteinuria, prolonged survival, effectively inhibited all subclasses of IgG autoantibody production and accumulation of both Th1 and Th2 cells during the pathogenesis of murine lupus nephritis (Iwai et al., 2003). Blocking ICOS on the surface of CD28-deficient Th1 cells abrogated development of murine colitis, whereas blocking CD28 or ICOS alone had almost no effect on disease

induction, suggesting that CD28 and ICOS collaborate to promote colitic development by aggressor Th1 cells (de Jong et al., 2004). In conclusion, blockade of the ICOS/B7-H2 costimulatory pathway affects both Th1 and Th2 responses, although the effect is more pronounced in highly polarised Th2 responses.

As further confirmation of the context-dependent association of ICOS with Th polarisation, a study found that ICOS+CD4+ Th cells expressed strikingly different cytokines depending on the type of infection encountered, the chronicity of the immune response, and the cells' anatomical localisation. In Th2-dominated immunity against *Schistosoma mansoni*, ICOS expression of hepatic CD4+ cells was strongly associated with IL-5, IL-10 and IL-13 expression, but not with the chemokine receptor CXCR5, a pattern reflective of Th2 effector cells. However, in the secondary lymphoid organs of schistosome-infected mice, ICOS expression was randomly correlated with Th2 cytokines, but positively correlated with CXCR5 expression, the hallmark of T_{FH} cells. During infection with *Toxoplasma gondii* and in the severe combined immunodeficiency (SCID)-transfer colitis model, ICOS expression was positively correlated with IFN-γ production (Bonhagen et al., 2003). A separate study observed that $ICOS^{low}$ T cells in unchallenged mice were loosely associated with IL-2, IL-3, IL-6 and IFN-γ; $ICOS^{medium}$ cells with the Th2 cytokines IL-4, IL-5, and IL-13, and $ICOS^{high}$ cells with the anti-inflammatory cytokine IL-10 (Lohning et al., 2003). Consistent with the specific link between T cells highly expressing ICOS and IL-10 synthesis, interaction of ICOS on human effector T cells with B7-H2 on mature DCs strongly and selectively promoted IL-10 secretion (Witsch et al., 2002).

1.4.3 ICOS in immune tolerance

Although the ICOS/B7-H2 pathway is extremely important for costimulating effector T cell responses and T cell-dependent B cell responses, it also plays a critical role in regulating T cell tolerance primarily through promoting T_{reg} (especially the IL-10-producing subpopulation) cell development, homeostasis and/or function (Greenwald et al., 2005), corroborated by evidence derived from several studies summarised below. Firstly, ICOS deficient mice were found to have decreased numbers of FoxP3+ T_{reg}s and impaired *in vitro* T_{reg} suppressive function compared with WT mice, leading to enhanced atherosclerosis (Gotsman et al., 2006). Secondly, in a mouse model of myelin oligodendrocyte glycoprotein (MOG) peptide-induced oral tolerance, CD4+ T cells from MOG-fed WT mice could not transfer suppression to ICOS deficient recipients, suggesting again that ICOS may have a direct role in controlling the effector functions of T_{reg}s (Miyamoto et al., 2005). Thirdly, the ICOS/B7-H2 pathway was essential for the induction of inhalation tolerance after sensitisation with a mucosal allergen, aerosolised OVA, albeit redundant for the generation of Th2 responses (Gajewska et al., 2005). Fourthly, the development and ability to inhibit allergen-induced airway hyperreactivity of IL-10-producing T_{reg} cells required T cell costimulation by mature pulmonary DCs via the ICOS/B7-H2 pathway. T_{reg} cells, through the ICOS/B7-H2 signalling axis, downregulate pulmonary inflammation and maintain respiratory tolerance in asthma (Akbari et al., 2002). A fifth study exploring the relationship between expression levels of self-antigen and the function of self-reactive T cells in the periphery concluded that IL-10-producing T_{reg} cells developed when self-antigen expression is sufficiently high and exert self-tolerance via ICOS signalling (Kohyama et al., 2004). Moreover, T_{reg} cells

operating within prediabetic lesions to keep them from destructive progression are correlated with significantly higher levels of IL-10 and ICOS expression than their lymph node counterparts (Herman et al., 2004). Findings with an EAE-induced model of mucosal tolerance were similar (Miyamoto et al., 2005). Interestingly, two independent studies provided evidence that only maturing human plasmacytoid DCs (pDCs) as opposed to myeloid DCs (mDCs) up-regulated ICOSL expression to high levels, endowing them with the ability to promote the differentiation of naive CD4+ T cells to IL-10-producing T_{reg} cells (Ito et al., 2007; Janke et al., 2006).

1.5 Th1 or Th2 cell lineage decision primed by TCR and CD28 signalling

The first echelon of signalling that activates alternate cytokine fates to prime naïve Th cells to differentiate into a particular lineage involves the strength and quality of the TCR and costimulatory signals. Accumulating evidence implicates nuclear factor binding the immunoglobulin κ light chain enhancer in B cells (NF-κB) and extracellular signal-regulated kinase (ERK) cascades in modulating TCR signal strength. The level of ERK activity at the early phase of naïve Th cell stimulation appears to be critical for deciding in part the Th differentiation outcome. Transient ERK activation due to weak engagement of TCR by low concentrations of cognate peptide induced IL-2-dependent signal transducer and activator of transcription (STAT)-5 phosphorylation, IL-4-independent early GATA-3 expression, and IL-4 production, which were abolished by high-affinity TCR signalling that induced sustained ERK activation and subsequent Th1 differentiation (Yamane et al., 2005). In developing Th2 cells, pharmacological inhibition of ERK decreased GATA-3 protein levels through promoting GATA-3 degradation by

the ubiquitin-proteasome pathway (Yamashita et al., 2005). Furthermore, ERK signalling facilitates GATA-3-mediated chromatin remodelling at Th2 cytokine gene loci. Another important family of transcription factors triggered by TCR signalling is the NFAT family, which are key regulators of inducible gene expression in the immune system. Upon TCR stimulation, NFAT members undergo calcineurin-mediated dephosphorylation and translocate to the nucleus where they cooperate with the activator protein (AP)-1 complex to activate target genes such as IL-2. Without their transcriptional partners, NFAT alone binding to gene promoters results in T cell anergy (Rao et al., 1997). Interpreting the role of NFAT members in Th differentiation is more confounding. Naive Th cells doubly deficient in NFATc2 and NFATc3 intrinsically differentiate into Th2 cells, even in the absence of IL-4 production (Rengarajan et al., 2002). Such an observation is perhaps not surprising, considering the fact that NFAT regulates the expression of a plethora of cytokines that influence Th development, including partnering with T-bet and GATA-3 to drive IFN-γ and IL-4 transcription respectively.

1.6 Molecular circuitry of Th1 and Th2 cell differentiation programs

Extensive work in the last decade has uncovered a complex and highly plastic picture of the interplay between the polarising cytokines and the transcriptional apparatus responsible for instructing the Th differentiation program (Rao and Avni, 2000; Glimcher and Murphy, 2000; Dong and Flavell, 2000) (Figure 1.2). The signature cytokines IL-12 and IL-4 are known to promote respectively the development of Th1 and Th2 cells by causally inducing transcription factors such as STAT4 and STAT6 respectively, leading to lineage-selective gene expression (Ho and Glimcher, 2002). In the same regard, the

T-box transcription factor expressed in T cells (T-bet) has been shown to direct Th1 lineage commitment (Szabo et al., 2000; Szabo et al., 2002; Szabo et al., 2003), inducing both transcriptional proficiency of the *Ifnγ* locus and responsiveness to IL-12-transduced growth signal (Mullen et al., 2001). These events establish an IFN-γ/STAT1 autocatalytic and autocrine loop to further enhance T-bet expression in the developing Th1 cell. T-bet in turn induces the expression of IL-12Rβ2, leading to acute IFN-γ transcription by potentiating the IL-12/STAT4 pathway (Afkarian et al., 2002; Mullen et al., 2001). The crucial importance for T-bet in the development Th1-mediated responses *in vivo* is underscored by the susceptibility of T-bet-deficient mice to *Leishmania major* infection (Szabo et al., 2002) and their predisposition to asthma (Finotto et al., 2002). On the other hand, the zinc finger transcription factor GATA-3 is a master regulator of Th2 differentiation, being both necessary and sufficient to drive development of this subset (Zheng and Flavell, 1997), although the b-ZIP (basic-region leucine-zipper) transcription factor c-Maf initially skews naïve Th cells toward a Th2 phenotype via early induction of IL-4 (Ho et al., 1996). This precipitates an IL-4/STAT6 pathway which rapidly stimulates GATA-3 expression to a high level in committed Th2 cells. Whereas embryonic lethality caused by germline disruption of the GATA-3 gene precludes direct assessment of the role of GATA-3 in T cell development and Th2 differentiation (Kuo and Leiden, 1999), conditional deletion of GATA-3 in CD4+ T cells diminished Th2 differentiation, eliminated Th2 responses and allowed the generation of IFN-γ-producing cells in mutant compared to WT mice challenged with *Nippostrongylus brasiliensis* (Zhu et al., 2004). Moreover, sound biochemical evidence exists for GATA-3 specifying transcriptional competence of the Th2 cytokine cluster, which includes the genes encoding IL-4, IL-5

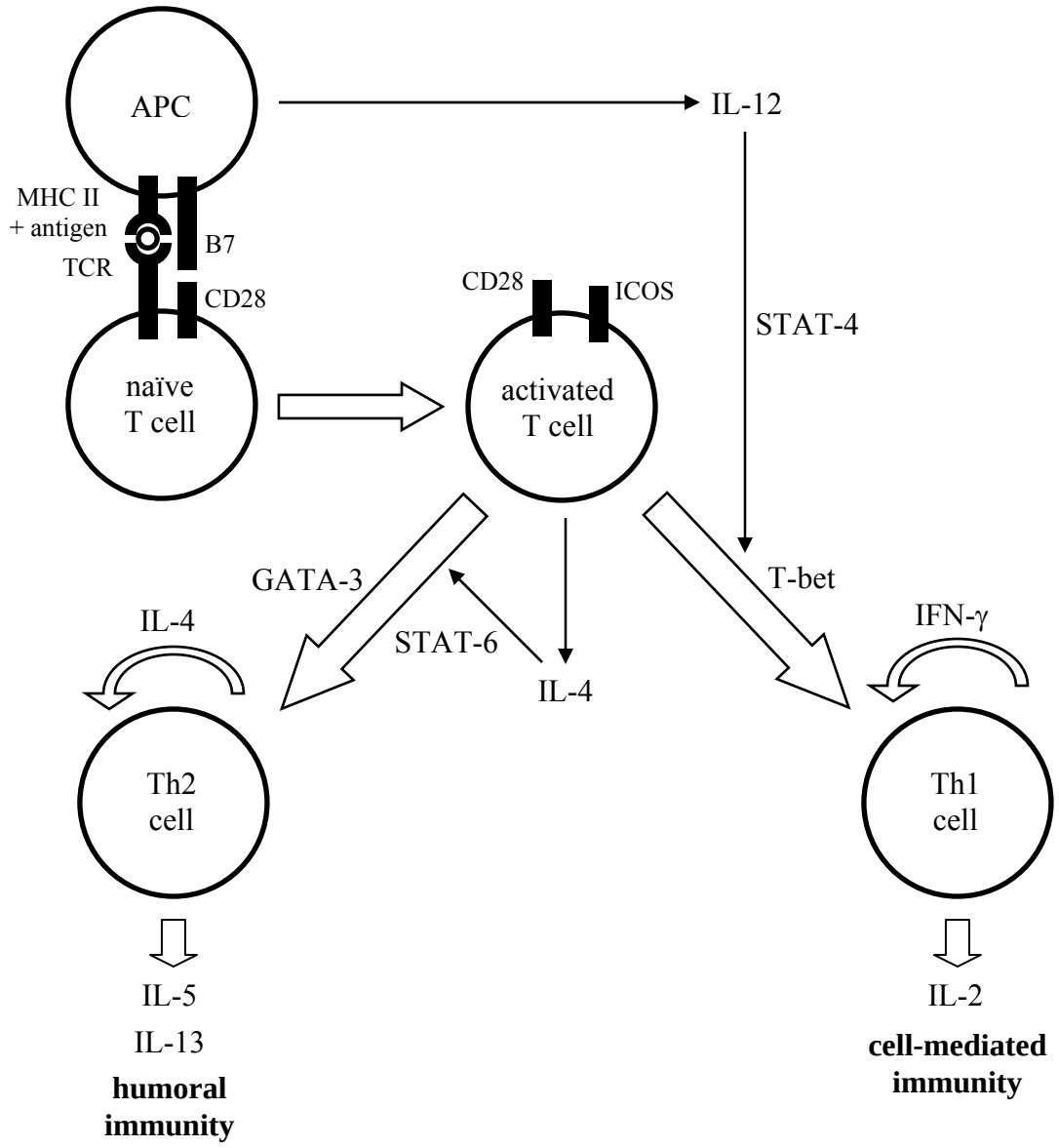


Figure 1.2 Cytokines and transcriptional apparatus governing Th1 and Th2 differentiation.

and IL-13 (Lee et al., 2001; Lee et al., 2000). It is now generally accepted that a dualistic view of Th development is overly simplistic, partly because many complexities underlie the crosstalk between master regulators of this process. For example, Itk-mediated phosphorylation of T-bet facilitates its physical interaction with GATA-3, sequestering the latter from the Th2 cytokine locus (Hwang et al., 2005). Consistent with this, a recent study concluded that the principal function of T-bet in developing Th1 cells is to negatively regulate GATA-3 rather than positively regulate the *ifn-γ* gene (Usui et al., 2006a). GATA-3, in contrast, appears to suppress Th1 development by down-regulating STAT4 and not through effects on IL-12Rβ2 or T-bet (Usui et al., 2003).

1.7 Rationale and aims of study

Despite extensive studies pointing to a crucial role for ICOS in cellular and humoral immunity, little is known about the intracellular mechanisms underlying the regulation of ICOS expression in T cells, especially CD4+ T (Th) cells which play a crucial role in humoral immune responses. It is envisaged that the ability to enhance ICOS expression at the appropriate time of an ongoing host response could increase its effectiveness against pathogens. Conversely, the ability to down-regulate ICOS expression could modulate the rejection of organ and tissue transplantation and alleviate the severity of autoimmune diseases. Hence, in the first part of the ensuing results, we elucidate the signalling pathways originating from TCR and CD28 co-engagement that regulate ICOS induction as well as delineate the *cis*-acting regulatory region and *trans*-acting transcription factors governing ICOS transcription during the initial 48 h of Th cell activation. We found that the Fyn-calcineurin-NFATc2 and MAPK/ERK kinase (MEK)2-ERK1/2 signalling pathways operate independently but converge on the *icos* promoter to induce ICOS transcription. Moreover, we identified an ERK-sensitive site on the *icos* proximal promoter which is critical for TCR and CD28-mediated regulation of the mouse *icos* gene.

Following ICOS induction by TCR and CD28 co-signalling which dominate during the early phase of Th cell activation, how ICOS expression is controlled during the subsequent phase of Th cell differentiation forms the subject of the second part of the findings. Although the role of ICOS in the overall differentiation program of Th cells *in vivo* remains controversial, its specific contribution to the production of Th2 cytokines and preferential expression in Th2 cells as described before led us to hypothesise that the

cytokine networks and downstream signalling molecules driving Th lineage commitment may influence ICOS expression in developing Th1 and Th2 cell subsets, in a manner commensurate with the differential levels of ICOS found in these subsets. We present evidence for the lineage-selective transcription factors T-bet and GATA-3, in cooperation with the more broadly expressed NFATc2 and ERK, to regulate ICOS transcription in a temporally dynamic and Th-specific fashion. These data, together with earlier work demonstrating an important role for ICOS in the transcriptional regulation of Th2 differentiation (Nurieva et al., 2003a), argue for a bidirectional cross-talk between the transcriptional machinery mediating Th differentiation and the expression of a single costimulatory gene, *Icos*.

Last but not least, we asked if the *icos* transcript is regulated at the post-transcriptional level. Our investigations show that a novel RING-type E3 ubiquitin ligase, roquin, plays an important role in mediating the decay of ICOS mRNA via mechanisms which are presently unclear.

CHAPTER 2

MATERIALS AND METHODS

2.1 Mouse strains

WT C57BL/6 mice were obtained from the Singapore Biological Resource Centre. The generation of *Tbx21*$^{-/-}$ (T-bet-deficient) and B6.129S7-*Fyn*^{tm1Sor}/J (*Fyn*$^{-/-}$) mice were previously described (Finotto et al., 2002; Stein et al., 1992) and they were purchased from the Jackson Laboratories (Bar Harbor, ME). All mice were bred and maintained in accordance with Institutional Animal Care and Use Committee (IACUC) regulations and used between 8 – 12 weeks of age.

2.2 T cell lines

2.2.1 Murine EL4 T cell line

This cell line was maintained in complete RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (HyClone, Logan, UT), *L*-glutamine (2 mM), penicillin (50 mg/ml), streptomycin (50 mg/ml), HEPES (100 mM) and β-mercaptoethanol (55 μM).

2.2.2 AE.7 Th1 and CDC35 Th2 cell clones

The AE7 Th1 cell and CDC35 Th2 cell clones were kind gifts from Dr. I-Cheng Ho (Harvard Medical School, Brigham and Women's Hospital, Boston, MA) and cultured according to conditions described previously (Hecht et al., 1983; Tony et al., 1985) with some modification. Briefly, antigen presenting cells (APCs which were splenocytes) were first isolated from Balb/c and AKR/J mice, treated with 50 μg/ml of mitomycin C (Sigma, MO) at 37°C for 45 min to mitotically arrest them and then washed with medium at least 3 times to eliminate traces of mitomycin C. $1 - 2 \times 10^6$ resting AE7 Th1 cells

were incubated with 1×10^7 growth-arrested syngeneic AKR/J splenocytes (presenting I-E^k MHC) and 5 μM of pigeon cytochrome C in 8 ml of complete RPMI 1640 medium, supplemented with 10% FCS (HyClone, Logan, UT), *L*-glutamine (2 mM), penicillin (50 mg/ml), streptomycin (50 mg/ml), HEPES (100 mM), Na pyruvate (1 mM), non-essential amino acids (100 μM) and β-mercaptoethanol (55 μM) for 48 h in a 6-well plate. The cell culture was then expanded in a ratio of 1:5 into 10% rat concanavalin A (con A) conditional medium and thereafter further expanded every 3 to 4 days. The AE7 cells were restimulated with antigen every 2 weeks when necessary. The conditional medium is α-methylmannoside (Sigma, MO)-containing supernatant from Lewis rat spleen cells that had been stimulated with 2.5 μg/ml of concanavalin A for 48 h and added at 10% to the complete medium described above as a source of lymphokines. CDC35 Th2 cells were cultured in similar fashion except with Balb/c splenocytes (presenting I-A^d MHC) in the presence of 100 μg/ml of rabbit γ-globulin.

2.3 Chemical inhibitors

Pharmacological inhibitors FK506 (10 μM), CsA (low dose: 5 ng/ml; high dose: 50 ng/ml), both of which inhibit calcineurin, wortmannin (WM) (100 nM), which inhibits phosphatidylinositol-3-kinase (PI-3K), and rapamycin (RAPA) (10 nM), which inhibits mammalian target of rapamycin (mTOR), were obtained from Sigma-Aldrich (St Louis, MO). PP2 (5 μM), a broad Src family kinase inhibitor, damnacanthal (100 nM), which specifically inhibits Lck, piceatannol (50 μM), a Syk family kinase inhibitor, SB203580 (10 μM), which inhibits p38 MAPK, JNK inhibitor I (5 μM) and NF-κB competitor

peptide SN50 (10 μM) were purchased from Calbiochem (San Diego, CA). U0126 (50 μM) was obtained from Cell Signalling Technology (Beverly, MA).

2.4 Primary murine CD4+ T cells

2.4.1 CD4+ T cell purification

Single cell suspensions were prepared from the peripheral lymph nodes and spleens of WT, Tbx21$^{-/-}$ and Fyn$^{-/-}$ mice and treated with red blood cell lysis solution (0.15 M NH_4Cl and 0.1 mM Na_2EDTA in PBS) for 5 min at 4°C to eliminate erythrocytes. CD4+ T cells were positively isolated using anti-CD4 antibody (mAb)–coupled magnetic cell-sorting (MACS) microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) to more than 95% purity (as analysed by flow cytometric staining with anti-CD4 and anti-CD8 Abs, data not shown).

2.4.2 CD4+ T cell activation

Purified CD4+ T cells (5×10^5) were either untreated or seeded into 24-well tissue culture plates pre-coated for 2 h at 37°C with 1 μg/ml anti-CD3 and 4 μg/ml soluble anti-CD28 mAbs. In some experiments, CD4+ T cells were incubated with chemical inhibitors 1 h prior to activation.

2.4.3 CD4+ T cell differentiation *in vitro*

Purified CD4+ T cells (2×10^6/ml) were stimulated with 1 μg/ml plate-bound anti-CD3 and 4 μg/ml soluble anti-CD28 mAbs in 48-well tissue culture plates under Th0 (3 μg/ml anti–IL-12 plus 5 μg/ml anti–IFN-γ plus 10 μg/ml anti–IL-4), Th1-skewing (5 ng/ml

IL-12 plus 10 μg/ml anti–IL-4) or Th2-skewing (10 ng/ml IL-4 plus 3 μg/ml anti–IL-12 plus 5 μg/ml anti–IFN-γ) conditions. 100 U/ml recombinant mouse IL-2 was added after 24 h, and the cells were expanded in complete medium to culture Th cell lines as described above but containing IL-2 for 2 or 8 d. Recombinant mouse IL-2, IL-4 and IL-12 were purchased from PeproTech Inc. (Rocky Hill, NJ). NA/LE-grade anti-CD3 (145-2C11), anti-CD28 (37.51), anti–IL-12 (C17.8), anti–IFN-γ (XMG1.2) and anti–IL-4 (11B11) were obtained from BD Biosciences.

2.4.4 Retroviral Constructs and Retroviral Transduction of CD4+ T cells activated by anti-CD3 and anti-CD28

The bicistronic vectors green fluorescent protein(GFP)-RV and T-bet-RV, kind gifts of Dr. Laurie H. Glimcher (Harvard School of Public Health, Boston, MA), were described previously (Szabo et al., 2000). The Phoenix-Eco packaging cell line was obtained from Dr. Gary P. Nolan (Hofmann et al., 1996). The GFP-RV vector was constructed by inserting the encephalomyocarditis virus internal ribosomal entry sequence (IRES) and the GFP allele into the MSCV2.2 retroviral vector (Ouyang et al., 1998). Full-length murine GATA-3 cDNA was generated by PCR using pcDNAHis-GATA-3 as template. The primers used were 5′-GAA GAT CTT CGG AAT TCG ACA TGG AGG TGA CTG CG-3′ and 5′-CCG CTC GAG CGG GCT CTA GAC TAA CCC ATG GCG GTG A-3′. The PCR product, digested with both BglII and XhoI, was then ligated to similarly digested GFP-RV vector to generate GATA-3-RV. The vectors express two cDNAs, one encoding T-bet or GATA-3 and the other GFP, and simultaneously use an IRES to initiate translation of each mRNA separately. Transfection of the packaging cell line and

retroviral transduction of primary CD4+ T cells were described elsewhere (Ouyang et al., 1998) and performed with little modification. CD4+ T cells activated with plate-bound anti-CD3 and soluble anti-CD28 were infected after ~24 h using a 1:2 volume of viral supernatant and polybrene (Sigma, MO) at 8 μg/ml, centrifuged at 1800 rpm for 45 min at room temperature, and incubated at 37°C for between 12 and 24 h before being replaced with media containing IL-2 and expanded for an additional 48 h after primary activation. Flow cytometric analysis of surface CD4 and ICOS on GFP-positive cells was then performed (see below for details on how flow cytometry was performed). To isolate RNA or extract protein from cells, they were first sorted (to greater than 98% being GFP-positive, data not shown) on a FACS-Vantage Flow Cytometer/Cell Sorter (Becton Dickinson, Mountain View, CA).

2.5 Intracellular cytokine staining (ICS) and flow cytometric analyses

Naïve, activated, retrovirally transduced or polarised Th cells were stained with optimal amounts of anti-CD4 FITC and anti-ICOS PE Abs. CD4+ T cells stimulated with anti-CD3 and anti-CD28 and retrovirally infected were stained with anti-CD4 biotin and anti-ICOS PE Abs. PE-Cy5-conjugated streptavidin was used in a secondary step to reveal biotinylated CD4 staining. ICS of Th cells was performed using the BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Solution Kit with BD GolgiStop™ according to manufacturer's instructions. Th cells (~ 2×10^6) were first treated with 4 μl of BD GolgiStop™ Protein Transport Inhibitor (containing monensin) for every 6 ml of culture media and PMA (50 ng/ml) and ionomycin (0.5 μg/ml) for 6 h at 37°C. Cells were then harvested and stained with anti-CD4 biotin followed by PE-Cy5-conjugated

streptavidin. After 2 washes with staining buffer (3% FCS, 0.1% NaN_3 in PBS), cells were fixed and permeabilised for 20 min at 4°C. Cells were then washed twice with 1 × BD Perm/Wash™ buffer and thoroughly resuspended in 50 μl of Perm/Wash™ buffer containing pre-determined optimal concentrations of anti-IFN-γ FITC and anti-IL-4 PE or their corresponding isotype controls. After incubation for 30 min at 4°C in the dark, cells were again washed with Perm/Wash™ buffer and resuspended in about 300 – 400 μl of staining buffer. Cells were stained with fluorochrome-conjugated anti-CD4 and anti-ICOS Abs and analysed on a FACScan flow cytometer using CellQuest software (Becton Dickinson, Mountain View, CA). The following Abs were purchased from BD Pharmingen (San Diego, CA): NA/LE-grade anti-CD3 (145-2C11) and anti-CD28 (37.51) Abs for cell stimulation, FITC and biotin-conjugated anti-CD4 (GK1.5), anti-CD8 (53-6.7), PE- and biotin-conjugated anti-ICOS (7E.17G9), PE-Cy5-conjugated streptavidin, anti-IFN-γ FITC (XMG1.2) and anti-IL-4 PE (11B11).

2.6 RNA isolation and real-time RT-PCR analyses

Total RNA was isolated using TRIzol and treated with DNase I (Invitrogen Life Technologies, Carlsbad, CA) to avoid DNA contamination; cDNA was prepared using SuperScript III Reverse Transcriptase (Invitrogen) with oligo$(dT)_{12\text{-}18}$ as primer. Primers used for real-time PCR are: ICOS, 5′-TGA CCC ACC TCC TTT TCA AG-3′ and 5′-TTA GGG TCA TGC ACA CTG GA-3′; β-actin, 5′-GAT CTG GCA CCA CAC CTT CT-3′ and 5′-ACC AGA GGC ATA CAG GGA CA-3′. Each reaction (95°C, 2 min; 95°C, 10 s; 60°C, 30 s; 72°C, 30 s, 40 cycles) was performed in a Mx3000P QPCR System (Stratagene) using QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA) or the

SYBR Green PCR Kit (Invitrogen). All experiments were done at least twice, and mRNA levels of ICOS were normalised to those of the housekeeping gene, β-actin. The levels of mRNA in activated, retrovirally transduced or differentiated cells were expressed as a fold change relative to levels in naïve cells. Statistical analysis (unpaired two-tailed Student's *t* test) was performed with PRISM software (GraphPad Software, San Diego, CA).

2.7 Western blotting

Cells ($2 - 5 \times 10^6$) were lysed on ice for 15 min in a buffer containing 1% (v/v) Nonidet P-40, 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.2 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin, and the debris was removed by centrifugation at 13,000 rpm for 12 min at 4°C. Following determination of protein concentrations of lysates using Bradford assay (Bio-Rad Laboratories, Hercules, CA), they were then loaded in equal amounts and electrophoresced in a 7–10% SDS-PAGE, electroblotted onto polyvinylidene difluoride (PVDF) membrane, and probed with Abs that recognise specific proteins. Protein bands were visualised using horseradish peroxidase-coupled Abs and the enhanced chemiluminescence detection system (SuperSignal West Pico Chemiluminescent Substrate System from Pierce Biotechnology, Rockford, IL). Abs against T-bet (4B10), GATA-3 (HG3-31), phospho-ERK (E-4), ERK2 (C-14), NFATc2 (G1-D10), HDAC1 (H-51), TFIID (58C9) and β-actin (C-11) were all obtained from Santa Cruz Biotechnology.

2.8 Plasmid constructs

The *icos* promoter, spanning −1478 and −1 relative to the first nucleotide of the mouse cDNA (NCBI GenBank accession number NM_017480) was amplified from C57BL/6 mouse genomic DNA by PCR using primers carrying restriction sites for *Kpn*I and *Hind*III, 5′-GAG CAG TCA TTG AGA GGC CAG AAG AG-3′ and 5′-CCC AAG CTT AGT GCT CAA AAG TGT CAG-3′, and cloned into the promoterless luciferase (luc) plasmid vector, pGL3-Basic (pGL3B; Promega, Madison, WI) to create mICOSp(−1478)-Luc. 5′ deletion mutants of this promoter fragment were either generated after suitable restriction enzyme digestion and religated into pGL3B or subcloned by nested PCR to create mICOSp(−900)-Luc, mICOSp(−599)-Luc, mICOSp(−471)-Luc, mICOSp(−288)-Luc, mICOSp(−147)-Luc, mICOSp(−109)-Luc and mICOSp(−52)-Luc. A 259-bp fragment of the *icos* 3′UTR (NCBI GenBank™ accession no. AK030827) containing putative NFATc2 and GATA-3 binding sites was generated by PCR from C57BL/6 mouse genomic DNA using the primers 5′-GAT GTT CCC ATA TTC TCC-3′ and 5′-CCA GGA GAA TGT TTG CCC-3′ and cloned into the BamHI / SalI sites of mICOSp(-52)−Luc to generate a luc reporter vector driven by a minimal 52-bp *icos* promoter containing a 259-bp *icos* 3′UTR fragment downstream of the *luc* gene. A 3-bp region CTT between 142 and 144 bp within mICOSp(−288)-Luc was replaced by AGA using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) to create mICOSp(−288)-ERK-mut-Luc. The same protocol was used to replace a 2-bp section CC between 231 and 232 bp with AA in mICOS(−288)-Luc to generate mICOSp(−288)-NFAT-mut-Luc. The plasmids encoding roquin WT and roquin M199R,

kind gifts of Dr. Carola G. Vinuesa (John Curtin School of Medical Research, The Australian National University, Canberra) were described previously (Vinuesa et al., 2005a). Briefly, the coding sequence of roquin mRNA was amplified using PCR from C57BL/6 spleen cDNA, A-tailed with Taq DNA polymerase and cloned in-frame with GFP in the pCDNA3.1 CT-GFP TOPO TA fusion vector, to generate roquin WT vector. The Met (amino acid position 199) → Arg substitution was introduced by site-directed mutagenesis to yield roquin M199R, which carries a single base (T → G) mutation in a novel ROQ domain. The pUSEamp vector expressing a constitutively active form of MEK2 (S222D/S226D double mutant) was purchased from Upstate Biotechnology. The pBJ5 and pBJ5-NFATc1 vectors were generously provided by Dr Neil A. Clipstone (Northwestern University, Chicago, IL). The pBJ5-NFATc2 (pSH210) and pBJ5-NFATc3 (pSH250A) vectors were kind gifts of Dr Gerald R. Crabtree (Stanford University, Stanford, CA). Sequences of all DNA constructs were confirmed by automated sequence analyses.

2.9 Transient transfections in EL4 cells

For luc assays, 0.98 μg of the luc reporter plasmid and 0.02 μg of pRL-TK (*Renilla*) plasmid (50:1 ratio) (total amount of DNA = 1 μg) were premixed in OPTI-MEM I reduced serum medium (Invitrogen) and transiently transfected with Lipofectamine 2000 reagent (Invitrogen) in triplicate into EL4 cells seeded in 24-well flat-bottom plates at 1×10^6 cells/ml in 0.5 ml of RPMI 1640 medium without antibiotics following manufacturer's recommendations. For example, to transfect 1 μg of DNA, 3 μl of Lipofectamine 2000 was first mixed with 47 μl of OPTI-MEM I for 5 min at room

temperature. Concurrently, 1 μg of DNA was also mixed with the same volume of OPTI-MEM I and the two mixtures were incubated together after 5 min for a further 20 min to allow liposome-DNA complex formation. This total mixture was subsequently added to each well of plated cells, which were incubated at 37°C in 5% CO_2 for 4 h before stimulation with 10 ng/ml PMA and 0.5 μg/ml ionomycin. Cells were cultured for another 14 to 16 h before being harvested by centrifugation.

2.10 Luc reporter assays

Following overnight culture as described in the previous section, cell pellets were solubilised in 1 × passive lysis buffer (Promega) and incubated on a Spiramix roller mixer for 15 min at room temperature. 20 μl of cell lysate was assayed for both fire-fly and *Renilla* luc activities using the Dual-Luciferase Reporter Assay System (Promega), and relative light units (RLU) were measured in a TD-20/20 single-tube luminometer (Turner BioSystems, Sunnyvale, CA). RLU from the fire-fly luciferase (luc) was normalised for transfection efficiency to the *Renilla* luc RLU in each lysate (normalised RLU = $RLU_{\text{fire-fly luc}}$ / $RLU_{\textit{Renilla}\ \text{luc}}$). For *icos* promoter or 3′UTR analysis, the activity of a reporter construct following stimulation was calculated as: Reporter Activity = normalised $RLU_{\text{stimulated}}$ / normalised $RLU_{\text{unstimulated}}$. For effect of over-expressed proteins on promoter or 3′UTR activities, the fold induction was calculated as: Fold Induction = normalised $RLU_{\text{protein expressing vector, unstimulated or stimulated}}$ / normalised $RLU_{\text{null vector, unstimulated}}$.

2.11 siRNA knockdown of T-bet and GATA-3 respectively in AE7 and CDC35 cells

AE7 cells were transfected using Lipofectamine 2000 with 100 nM of Tbx21 siRNA ID#181595 (which targets exon 3 and designated T-bet siRNA #1), 100 nM of siRNA ID #181596 (which targets exon 6 and designated T-bet siRNA #2) or 100 nM of *Silencer*® GFP (eGFP) siRNA (cat #AM4626) as negative control. CDC35 cells, on the other hand, were transfected with 1 μM of GATA-3 siRNA ID #61725 (which targets exon 2 and designated GATA-3 siRNA #1), 1 μM of ID #61780 (which targets exon 5 and designated GATA-3 siRNA #2) or 100 nM of GFP siRNA as negative control. Both T-bet siRNAs were pre-designed using Tbx21 gene sequence information derived from GenBank acc. no. NM019507 while those for GATA-3 were designed using GenBank acc. no. NM008091 as reference. All siRNA duplex oligonucleotides with catalog IDs as indicated above were purchased from Ambion, Inc., Applied Biosystems, Austin, TX. Following transfection, cells were rested for 24 h before additional transfection with appropriate luc reporter vectors and stimulation for luc assays or stimulation alone for ChIP assays.

2.12 Chromatin immunoprecipitation (ChIP)

ChIP was performed according to the manufacturer's protocol (Upstate Biotechnology, Lake Placid, NY) with some modifications. Naïve, anti-CD3/CD28-activated CD4+ T and differentiating Th cells ($2 – 5 \times 10^6$) stimulated or not by PMA and ionomycin for 6 h were cross-linked with formaldehyde at a final concentration of 1% for 10 min at 37°C, washed with ice-cold PBS containing 1 mM PMSF, harvested and lysed in 10% SDS

buffer. DNA was sheared by ultrasonication using a High Intensity Ultrasonic Processor (Heat Systems) for 8 × 12 s pulses at 30% amplitude. Lysates were cleared by centrifugation, diluted in ChIP dilution buffer and pre-cleared using salmon sperm DNA/protein A-agarose and a sample of input DNA was collected at this point. Protein-DNA complexes were immunoprecipitated with 6 μg of an Ab against the protein of interest or isotype-matched control overnight at 4°C. Ab-protein-DNA complexes were then captured using salmon sperm DNA/protein A-agarose for 1 h at 4°C. After washing beads with low and high salt, LiCl and TE buffers, the protein/DNA complexes were eluted using 1% SDS, 0.1 M $NaHCO_3$ buffer and disrupted by heating at 65°C for 4 h. DNA was then phenol/chloroform- extracted, ethanol-precipitated and subjected to PCR using various primers (15 s at 94°C; 30 s at 60°C and 1 min at 72°C, completed by 10 min at 72°C, 28 – 30 cycles). The following primers were used: a pair spanning –288 to TSS of *icos* promoter (ICOS P): sense 5′-CCG CTC GAG CAT GCA TGC ATC CAT C-3′; antisense 5′-CCC AAG CTT AGT GCT CAA AAG TGT CAG-3′ (288-bp product); a pair spanning a 259-bp stretch of *icos* 3′UTR (ICOS E), 5′-GAT GTT CCC ATA TTC TCC-3′ and 5′-CCA GGA GAA TGT TTG CCC-3′ (259-bp product). The antibodies used were anti-NFATc2 (G1-D10) and mouse IgG_{2a} isotype control, anti-T-bet (4B10) and mouse IgG_1 isotype control, and anti-GATA-3 (HG3-31) and mouse IgG_1 isotype control from Santa Cruz. Anti-H3K4me3 Ab was from Upstate Biotechnology, Lake Placid, NY and its control is rabbit anti-mouse serum. PCR products were finally separated on 2% agarose gels.

2.13 Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL) with minor adjustments. Cells (10×10^6) were untreated or stimulated for 16 h and washed in PBS at 4°C. Nuclear extracts were prepared by hypotonic lysis and high salt extraction, and subsequently cleared of insoluble material by centrifugation at 13,000 rpm for 5 min at 4 °C before being concentrated with a microcon-3 column (Millipore Corp., Billerica, MA). The extracts were stored at −80°C prior to use, and protein content measured using Bradford assay (Bio-Rad Laboratories). For gel shift assays, double-stranded oligonucleotide probes were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Labeled probes were purified using ProbeQuant G-50 micro columns (Amersham Pharmacia Biotech, Piscataway, NJ). Binding reactions were conducted at room temperature for 20 min in a 20 μl total volume containing 0.5 ng of labeled probe, 5 μg of nuclear extract, 1 μg of poly(dI·dC) (Amersham Pharmacia Biotech) and gel shift buffer (12 mM HEPES, pH 7.9, 4 mM Tris-HCl, pH 7.9, 60 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT) and 12.5% glycerol). For competition assays, competing oligonucleotides were preincubated with the reaction mix at room temperature for 15 min before the addition of radiolabeled probe. Binding reactions were size-fractionated using a 5% non-denaturing PAGE in 0.5 × TBE and electrophoresed at 120 V for 4 h at room temperature. The gel was subsequently transferred onto 3MM chromatography paper, dried and exposed to autoradiographic film for 1 to 3 days at −80°C. The probe sequences were: p154_105, 5′-TCA CCG GGT ACT TGC CAT GCA GGA GGC GCT GTG ATA GCT CTC CAA GTA GA-3′ and p154_130, 5′-TCA CCG

GGT ACT TGC CAT GCA GGA G-3′. Competitor sequences were: p154_105, as above; p154_130, as above; p129_105, 5′-GCG CTG TGA TAG CTC TCC AAG TAG A-3′; p154_130_mut, 5′-TCA CCG GGT AAG A[1]GC CAT GCA GGA G-3′; NF-κB consensus, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′; NFATc consensus, 5′-CGC CCA AAG AGG AAA ATT TGT TTC ATA-3′; Ets-1 consensus, 5′-GAT CTC GAG CAG GAA GTT CGA-3′; Elk-1 consensus, 5′-GGG CTG CTT GAG GAA GTA TAA GAA T-3′; Elf-1, 5′-CTT GGG GGC AGG ACT TCC TGT TTC T-3′ (Sarafova and Siu, 1999); CP2/LSF consensus, 5′-GTC TGA TTT CAC AGG AA-3′ (Casolaro et al., 2000); Sp1 consensus, 5′-ATT CGA TCG GGG CGG GGC GAG C-3′; AP1 consensus, 5′-CGC TTG ATG ACT CAG CCG GAA-3′.

[1] The underlined sequences denote the mutant binding site for Erk-sensitive transcription factors.

CHAPTER 3

RESULTS

3.1 Induction of ICOS expression by TCR and CD28 co-engagment

3.1.1 Induction of ICOS by TCR and CD28 is subject to transcriptional control

ICOS expression is induced upon T cell activation and, in particular, in response to TCR and CD28 co-engagement (McAdam et al., 2000). To examine in detail the kinetics of ICOS induction, we first determined the level of ICOS cell surface expression and correlated that with the induction of ICOS mRNA in murine primary CD4+ T cells stimulated with anti-CD3 and anti-CD28 Abs, which engaged the TCR and CD28 receptor respectively (henceforth designated as TCR/CD28). As shown in Figure 3.1A, an increase in cell surface expression of ICOS could be detected as early as 6 h of T cell activation using a fluorochrome-conjugated anti-ICOS antibody in flow cytometry analysis, and the expression level seemed to peak at 40 h of T cell stimulation. At the transcriptional level, an increase in ICOS mRNA could be detected via real-time RT-PCR as early as 3 h after TCR/CD28 stimulation and the mRNA level appeared to peak and plateau from 6 to 18 h before declining (Figure 3.1B). Thus, it was evident from the temporal increase in mRNA and cell surface protein expression that ICOS was subjected to some level of transcriptional control.

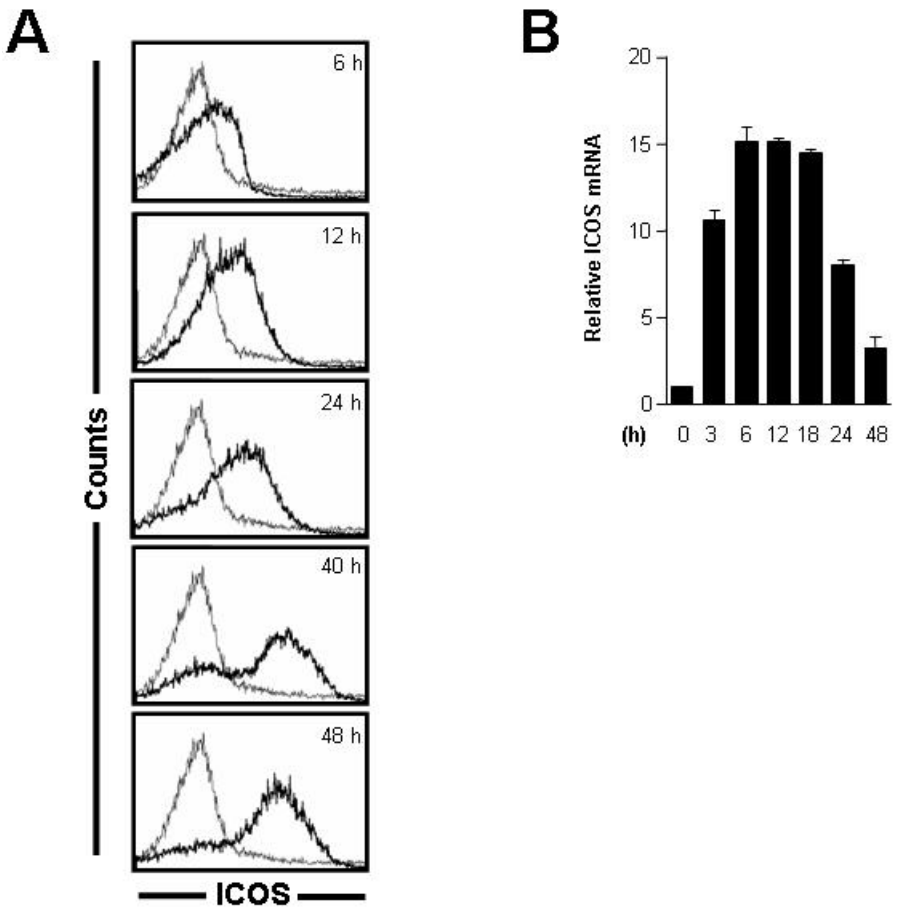


Figure 3.1 ICOS expression is induced at the transcriptional level upon T cell activation.

A, Purified murine primary CD4+ T cells were stimulated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (4 μg/ml) antibodies for various times as indicated and analysed for cell surface expression of ICOS by flow cytometry. ICOS expression on stimulated cells (*thick line*) is compared with that of unstimulated cells at 0 hr (*thin line*). Data shown are representative of 2 independent experiments. **B**, ICOS mRNA levels in T cells activated for various times were analysed by quantitative real-time RT-PCR. Data shown had been normalised to those from unstimulated cells at 0 hr and were expressed as mean values ± SEM of at least 2 independent experiments.

3.1.2 ICOS expression is regulated by distinct pathways downstream of TCR and CD28 signalling

Since TCR/CD28 stimulation could lead to an increase in ICOS mRNA and cell surface protein expression, we were interested to characterise the signalling pathways that regulate ICOS induction. To accomplish this, we first utilised a range of chemical inhibitors to block intracellular signalling pathways known to be activated by TCR/CD28 engagement in primary CD4+ T cells and assessed their effects on the induction of cell surface ICOS expression (Figure 3.2A). Our data shows that FK506 (tacrolimus), which is known to inhibit calcineurin, substantially diminished cell surface expression of ICOS on activated T cells, thus suggesting a possible role for this pathway in ICOS induction (Figure 3.2A, left panel). This was consistent with a previous study that showed an inhibitory effect of CsA, which also deactivates calcineurin, on ICOS induction in human CD4+ T cells (Beier et al., 2000). Furthermore, as calcineurin is Ca^{2+}-sensitive, blocking Ca^{2+} elevation with EGTA, also mitigated the induction of ICOS (data not shown). To determine whether the reduction in cell surface expression of ICOS on activated T cells by FK506 treatment was in part a consequence of reduced transcription, we assayed the amount of ICOS mRNA in FK506- and CsA-treated TCR/CD28-stimulated CD4+ T cells. Indeed, as seen in Figure 3.2C, pre-treatment of these activated cells with FK506 and CsA both reduced the amount of ICOS mRNA up-regulation compared to untreated cells, indicating that this signalling pathway probably acts at the level of ICOS transcription. In addition, we showed that PP2 but not piceatannol also attenuated TCR/CD28-induced ICOS induction, hence implicating Src rather than spleen tyrosine kinase (Syk) family of protein tyrosine kinases in regulating ICOS expression (Figure

3.2A, left panel). Again, the regulation of ICOS by the Src family kinases appeared to be at the transcriptional level as PP2-treated TCR/CD28-activated CD4+ T cells had reduced levels of ICOS mRNA compared to control cells (Figure 3.2B). To ascertain which of the Src kinases were involved (Fyn and Lck being the most highly expressed in T cells), we treated cells with damnacanthal, a highly potent and selective inhibitor of Lck activity (Faltynek et al., 1995). ICOS induction at both the cell surface protein and mRNA expression levels were unaltered by damnacanthal treatment, thus implicating Fyn but not Lck as the primary tyrosine kinase involved (Figure 3.2A, left panel and B). Although we could not rule out a possible role for Src kinase, our analysis of $Fyn^{-/-}$ CD4+ T cells indicated that Fyn likely plays a major role in inducing ICOS expression as these cells expressed reduced levels of cell surface ICOS protein or ICOS mRNA compared to Fyn-sufficient cells upon TCR/CD28 stimulation (respectively Figure 3.2C and D).

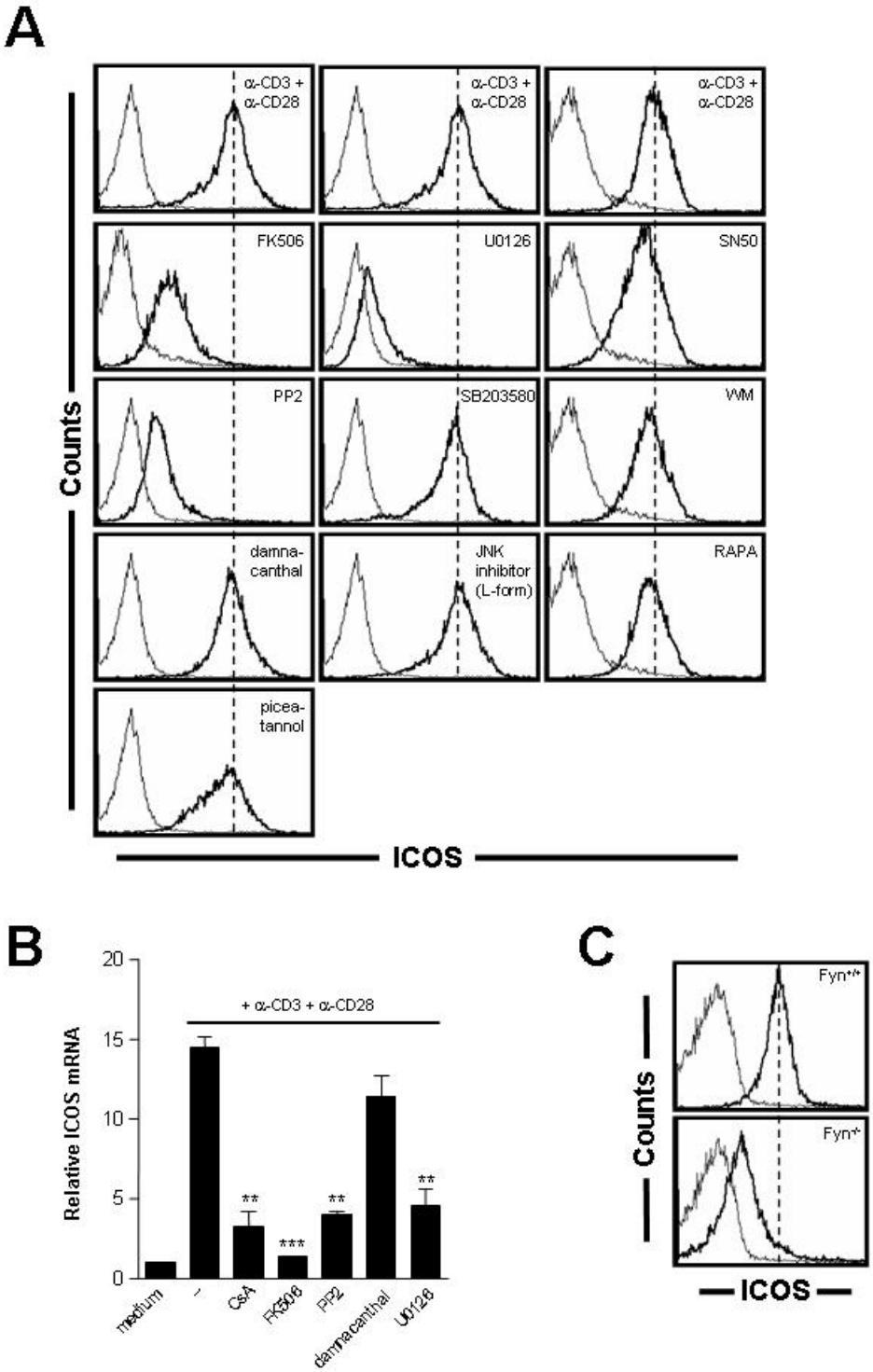
TCR/CD28 engagement is also known to activate the various MAPKs (Rudd and Schneider, 2003). We found that extracellular signal-regulated protein kinase (ERK), but not JNK (c-Jun NH_2-terminal kinase) or p38 MAPK, played a critical role in ICOS induction. ICOS induction was virtually abrogated when TCR/CD28-activated CD4+ T cells were pre-treated with the ERK inhibitor, U0126, but not with the JNK inhibitor (*L*-form) or p38 inhibitor, SB203580 (Figure 3.2A, middle panel). This inhibition appeared to act also at the transcriptional level, similar to that mediated by CsA and PP2 (Figure 3.2B).

Despite a number of studies emphasizing the importance of PI-3K in CD28 signalling leading to cell activation, proliferation and survival (Pages et al., 1994; Prasad et al., 1994; Stein et al., 1994), ICOS induction did not seem to be dependent on PI-3K or

mTOR pathways because treatment of cells with wortmannin (WM) or rapamycin (RAPA) respectively, did not affect TCR/CD28-mediated ICOS up-regulation (Figure 3.2A, right panel). We also did not observe any effect of SN-50 inhibitor peptide on ICOS induction, thus indicating a negligible or redundant role for NF-κB in TCR/CD28-mediated ICOS induction.

As a control to ensure that the dampening of ICOS induction by some of the chemical inhibitors examined above was not due to their effect on the surface expression of CD3 and/or CD28 molecules leading to diminution of ICOS induction, we treated CD4+ T cells with these inhibitors for 16 h and found that the cell surface expression of CD3 (data not shown) and that of CD28 were unaffected by these inhibitors (Figure 3.2F).

Finally, we also addressed the feasibility of using the mouse EL4 T cell line as a model for subsequent study of ICOS regulation. Although there was already a high level of basal ICOS transcript in EL4 cells, the level of ICOS mRNA in these cells could be further elevated upon TCR/CD28 or PMA and ionomycin (henceforth designated as P/I) treatment (Figure 3.2E and data not shown). Importantly, like in primary CD4+ T cells, TCR/CD28-induced ICOS mRNA induction in EL4 cells could also be blocked by PP2, U0126 and CsA. Thus, taken together, our results using both murine primary CD4+ T cells and EL4 T cells indicate that Fyn, calcineurin and ERK are the primary signalling molecules involved in the induction of ICOS transcription upon TCR/CD28 stimulation.


A
α-CD3 + α-CD28
α-CD3 + α-CD28
α-CD3 + α-CD28
FK506
U0126
SN50
PP2
SB203580
WM
damna-canthal
JNK inhibitor (L-form)
RAPA
picea-tannol
Counts
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20
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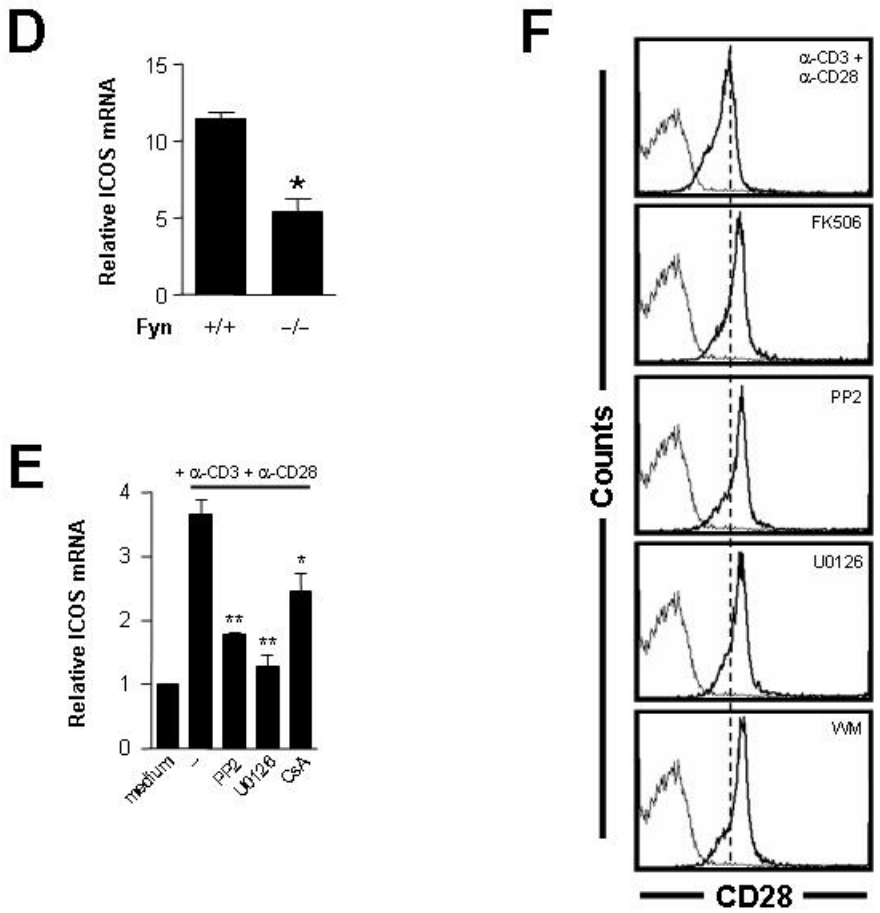


Figure 3.2 ICOS induction by TCR and CD28 engagement is regulated by distinct downstream signalling pathways.

A, ICOS induction on activated CD4+ T cells pre-treated with various chemical inhibitors. Purified CD4+ T cells were pre-treated with various inhibitors for 1 hr and stimulated as in Figure 3.1 for 40 – 48 h prior to examination of surface ICOS expression via flow cytometry (*thick* and *thin lines* are as depicted in Figure 3.1). Data shown are representative of 2 independent experiments. **B**, Measurement of ICOS mRNA levels in activated CD4+ T cells pre-treated with inhibitors. Cells were stimulated as above and harvested 6 h later to assess ICOS mRNA levels by real-time RT-PCR. Data were normalised to those from unstimulated cells and shown as mean ± SEM of at least 2 independent experiments. **C** and **D**, Purified CD4+ T cells from $Fyn^{+/+}$ and $Fyn^{-/-}$ mice were stimulated and harvested as in Figure 3.1. Cell surface ICOS expression and ICOS mRNA levels were assessed by flow cytometry and real-time RT-PCR respectively. Data shown are mean values ± SEM of 2 independent experiments. **E**, Murine EL4 thymoma cells were treated as in **A** and harvested after 14 – 16 h, and ICOS mRNA levels were quantified by real-time RT-PCR. **F**, CD28 expression on naive CD4+ T cells pre-treated with various chemical inhibitors. T cells were treated as in **A** and harvested after 14 – 16 h prior to assessment of surface CD28 expression via flow cytometry (*thick* and *thin lines* are as depicted in Figure 3.1). ***** indicates $p < 0.05$; ****** $p < 0.01$; ******* $p < 0.001$.

3.1.3 Fyn induces ICOS transcription in part through NFATc2 independently of ERK

Since the above study with U0126 implicated ERK in TCR/CD28-mediated ICOS induction, we expressed a constitutively active form of MEK2 (caMEK2), which is the upstream kinase of ERK, in EL4 cells to determine if hyperactivation of ERK would result in enhanced ICOS induction. As shown in Fig. 3.3A, the expression of increasing amounts of caMEK2 resulted in a dosage-dependent induction of ICOS mRNA levels. This result, together with our previous data using U0126, unambiguously established a role for ERK in ICOS induction.

As we demonstrated earlier that the signalling pathways leading to TCR/CD28-mediated ICOS induction were sensitive to CsA and FK506 (Figure 3.2A, B and E), we hypothesised that NFAT proteins could be involved in TCR/CD28-mediated ICOS induction. Three NFAT family members are known to be expressed in T cells, namely, NFATc1, NFATc2 and NFATc3 (Macian, 2005). As shown in Figure 3.3B, over-expression of NFATc2 in P/I-treated EL4 cells led to a significant increase in ICOS mRNA levels whereas the levels of ICOS mRNA induction were modest or negligible in P/I-treated EL4 cells respectively over-expressing NFATc1 or NFATc3. Thus, NFATc2 is involved in TCR/CD28-induced ICOS mRNA up-regulation.

The studies so far had indicated that Fyn, ERK, calcineurin and NFATc2 were involved in TCR/CD28-mediated ICOS induction. However, within the TCR/CD28 signal transduction hierarchy, the participation of Fyn and ERK is proximal with respect to the downstream transcription factor NFATc2. To determine if Fyn- or ERK-mediated ICOS induction occurs in part via NFATc2, we examined the effect of NFATc2 over-

expression in TCR/CD28-stimulated EL4 cells pre-treated with PP2 or U0126 (Figure 3.3C). As expected, PP2 or U0126 treatment inhibited ICOS induction in TCR/CD28-stimulated EL4 cells. Interestingly, NFATc2 over-expression could overcome the dampening of ICOS induction in PP2-treated but not U0126-treated TCR/CD28-activated EL4 cells in a dose-dependent manner, suggesting that NFATc2 is probably downstream of Fyn but not ERK in inducing ICOS transcription.

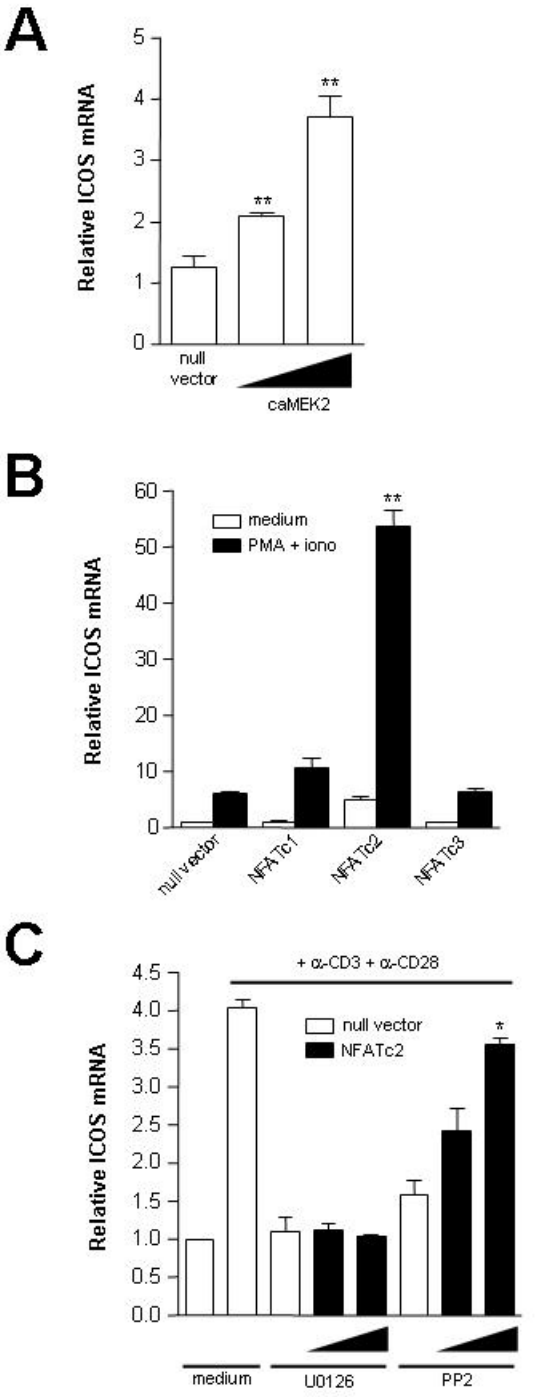


Figure 3.3 Induction of ICOS transcription by ectopic expression of MEK2 and NFATc2.

A, EL4 cells were transfected with a null vector or increasing amounts of constitutively active MEK2 (caMEK2) and the amount of ICOS mRNA was quantified by real-time RT-PCR; *******p*< 0.001. **B**, EL4 cells were transfected with vectors expressing NFATc1, NFATc2 or NFATc3, and either left unstimulated (*white bars*) or stimulated with PMA and ionomycin (*black bars*) and examined for ICOS mRNA levels via RT-PCR; ****** $p < 0.001$. **C**, EL4 cells transfected with null vector (*white bars*) or NFATc2 vector (*black bars*) were pre-treated with U0126 or PP2 and stimulated with anti-CD3 and anti-CD28 Abs and their ICOS mRNA levels were assessed by real-time RT-PCR. Data were normalised to cells transfected with null vector and shown as mean ± SEM of 2 independent experiments; ***** $p < 0.05$.

NFATc2 resides in the cytoplasm and translocates to the nucleus upon activation (Rao et al., 1997). Consistent with the above finding that NFATc2 is downstream of Fyn but not ERK signalling, we also found that PP2 but not U0126 pre-treatment of TCR/CD28-stimulated EL4 cells resulted in a reduction of NFATc2 translocation into the nucleus compared to untreated or U0126-treated cells that were subjected to the same stimulus (Figure 3.4A).

Although most studies of TCR signalling have positioned ERK downstream of Fyn in the TCR signalosome, our data seems to indicate that ERK acts in a signalling axis distinct from Fyn in regulating ICOS upon TCR/CD28 stimulation. To further clarify this, we examined ERK activation in the absence of Fyn signalling in EL4 cells. As shown in Figure 3.4B, TCR/CD28-stimulated EL4 cells, with or without PP2 pre-treatment, was comparable. This is consistent with previous work showing the same between $Fyn^{+/+}$ and $Fyn^{-/-}$ CD4+ T cells (Cannons et al., 2004). It is therefore apparent that Fyn does not act through ERK to initiate ICOS transcription and that ERK acts in an independent signalling pathway to regulate ICOS. Collectively, our data demonstrate that Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 pathways signal independently to activate ICOS transcription.

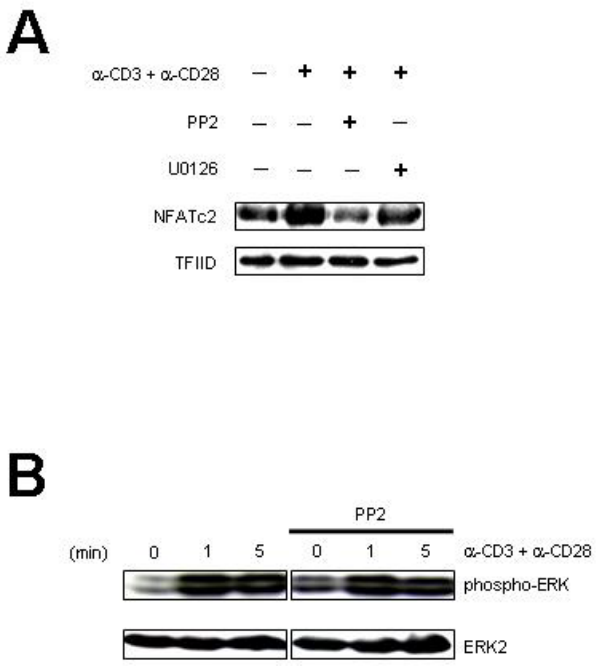


Figure 3.4 PP2 treatment affects NFATc2 nuclear translocation but not Erk activation.

A, EL4 cells were untreated or pre-treated with PP2 or U0126 and subsequently stimulated with anti-CD3 and anti-CD28 Abs for 16 h. Nuclear extracts (10 μg) were subjected to immunoblotting with anti-NFATc2 mAb. The anti-TFIID blot serves as control for equal loading of extracts. **B**, EL4 cells were either untreated or pre-treated with PP2 and then stimulated for the times indicated. ERK activation was assessed by Western blotting using anti-phospho-ERK Ab and the anti-ERK2 blot serves as a loading control.

3.1.4 A 288-bp core promoter region of *icos* confers PMA and ionomycin-induced expression of a reporter *in vitro*

To further elucidate the molecular mechanisms leading to *icos* promoter activation, we first tested a 1.48-kb *Kpn*I–*Hind*III fragment 5′ of the transcription start site (TSS) of the mouse *icos* gene for evidence of promoter activity. This fragment was linked in the sense orientation to the promoterless luc reporter vector pGL3B to create mICOSp(−1478)-Luc, which was transiently co-transfected with a normalisation vector, pRL-*Renilla* into EL4 cells. Subsequently, cells were left in medium alone or stimulated with P/I, and after a further 14 to 16 h of incubation, harvested and evaluated for luc activity. P/I were used instead of anti-CD3 and anti-CD28 Abs for stimulating cells as the former treatment gave a larger fold increase in luc activity compared to the latter (data not shown).

As seen in Figure 3.5A, the *icos* 5' 1.48-kb DNA fragment conferred significant luc activity in stimulated EL4 cells. There was an approximately 61-fold induction in luc activity in cells stimulated with P/I compared to cells in medium only. As a control, insertion of the same promoter fragment in the antisense orientation resulted in a complete loss of its ability to activate the reporter construct (data not shown). Thus, the data indicates that the 1.48-kb region upstream of *icos* TSS contains significant promoter activity.

To define more specifically the elements contributing to *icos* promoter activity, we generated a series of promoter mutants by truncating the 1.48-kb DNA fragment from the 5′ end. While the 900-bp *Bgl*II–*Hind*III (–900 to –1) and the 599-bp *Xho*I–*Hind*III (–599 to –1) fragments retained promoter activity, additional deletion of the *icos*

promoter revealed that a 288-bp fragment was the minimum region responsible for substantial transcriptional activating capability associated with the 1.48-kb parental fragment. A further 5′ truncation yielding a 147-bp fragment resulted in dramatic reduction of promoter activity (~10-fold loss in luc activity) and subsequent deletions leading to 109-bp and 52-bp fragments led to an almost complete loss of promoter activity (Figure 3.5A). Hence, based on these *in vitro* luc reporter assays, we concluded that the 288 bp region upstream of the *icos* TSS was the minimum DNA region that retained significant promoter activity capable of conferring high level of induced ICOS transcription.

3.1.5 Requirement of NFATc2 and ERK-dependent transcription factor(s) for *icos* core promoter activity

We next asked if Fyn-NFATc2 and ERK signalling cascades could influence the core promoter activity of *icos*. To this end, we assessed the effects of CsA and U0126 treatment on the promoter activity of the 288-bp, 147-bp and 109-bp DNA fragments of *icos*. We observed that CsA treatment reduced markedly the reporter activity of the 288-bp but not the 147-bp fragment following transient co-transfection of mICOSp(−288)-Luc, mICOSp(−147)-Luc or mICOSp(−109)-Luc and pRL-*Renilla* in EL4 cells (Figure 3.5B, left panel). In contrast, U0126 treatment virtually obliterated the promoter activities of both the 288-bp and 147-bp fragments, suggesting that ERK signalling has a very profound effect on the activation status and/or DNA binding activity of potential transcription factors that recognise the *cis*-regulatory elements within the 147-bp region (Figure 3.5B, right panel). In addition, our data suggests that CsA-sensitive

transcription factors such as NFAT probably act within the region between 288 and 147 bp upstream of *icos* TSS.

To further support the findings that ERK and NFAT signalling are involved in the induction of ICOS transcription through specific action within the 288-bp promoter region, we over-expressed individually caMEK2 and NFATc2 in cells harbouring mICOSp(−288)-Luc. Both caMEK2 and NFATc2 could enhance the promoter activity of mICOSp(−288)-Luc (Figure 3.5C, left panel). Strikingly, co-transfection of both led to a synergistic amplification of promoter activity, suggesting the possibility of cooperation between the ERK-responsive transcription factors and NFATc2 in accentuating *icos* promoter activity. Thus, ERK and NFATc2 could co-operatively activate the *icos* promoter. Furthermore, consistent with the data shown in Figure 5B, over-expression of caMEK2 could also enhance the promoter activity of mICOSp(−147)-Luc (but not mICOSp(−109)-Luc) comparable to that of mICOSp(−288)-Luc, indicating that the ERK-responsive transcription factors act in this region of the promoter (Figure 3.5C, right panel).

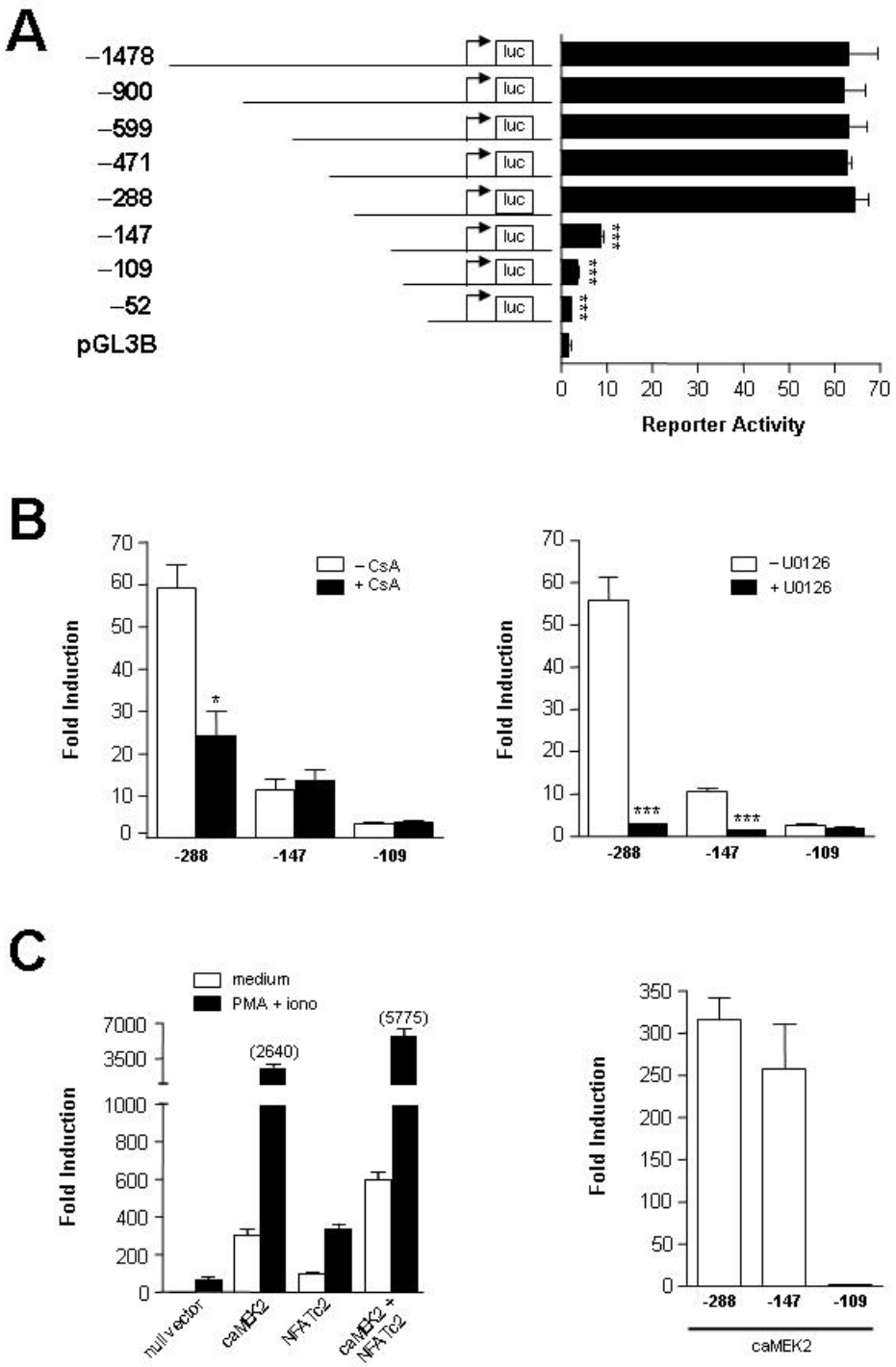


Figure 3.5 Transactivation of the putative *icos* promoter by NFATc2 and ERK signalling.

A, Delineation of the minimal *icos* promoter. A series of 5' truncations of the *icos* promoter was created as shown and linked to a luc reporter gene and transfected into EL4 cells, which were subsequently stimulated with PMA + iono. Fold induction of luc activity was calculated as the ratio of the normalised luc activity of stimulated cells to unstimulated cells. **B**, The activity of the putative *icos* promoter is affected by CsA and U0126 treatment. EL4 cells were transfected in triplicate with mICOSp(−288)-Luc, mICOSp(−147)-Luc, or mICOSp(−109)-Luc constructs and stimulated with PMA and ionomycin in the absence (*white bars*) or presence (*black bars*) of 50 ng/ml CsA (*left panel*) or 50 μM U0126 (*right panel*). **C**, Ectopic expression of a constitutively active MEK2 or NFATc2 transactivates the minimal *icos* promoter. EL4 cells were transfected in triplicate with mICOSp(−288)-Luc (*left and right panels*), mICOSp(−147)-Luc or mICOSp(−109)-Luc (*right panel*) together with the indicated expression vectors. Cells were either unstimulated (*white bars*) or stimulated with PMA + iono (*black bars*) for 16 h. Fold induction was calculated as the ratio of normalised luc activity of cells over-expressing the stated protein(s) to cells transfected with null vector. Data shown are mean ± SEM of triplicate samples and representative of 2 independent experiments. ***** $p < 0.05$; ******* $p < 0.001$.

3.1.6 NFATc2 binds *icos* 288-bp core promoter *in vivo* and is affected by Fyn signalling

In order to delineate the *cis*-regulatory elements contributing to *icos* core promoter activity, we proceeded to search for putative transcription factor-binding sites within the 288-bp region by employing the web-based software, MATINSPECTOR (www.genomatix.de) and RVISTA 2.0 (http://rvista.dcode.org) (Loots and Ovcharenko, 2005; Quandt et al., 1995), which are closely interconnected with the TRANSFAC database. These programs predicted the presence of potential AP1, C/EBPβ and Ikaros but surprisingly, not NFATc binding sites, even though we showed an important role for NFATc2 in activating the *icos* promoter. From the algorithmic predictions, we postulated that AP1 could be another candidate that regulates ICOS expression as c-*fos* was directly downstream of ERK in many signalling pathways. However, preliminary experiments exploring site-directed mutagenesis of the putative AP1 site (and other sites) within mICOSp(−288)-Luc did not lead to reproducible reduction in luc reporter activity. Conversely, over-expression of c-*fos* did not further enhance luc activity in EL4 cells co-transfected with mICOSp(−288)-Luc (data not shown).

Since NFATc2 over-expression resulted in increased mICOSp(−288)-Luc activity (Figure 3.5C, left panel), we asked if NFATc2 could bind the 288-bp fragment *in vivo*. Using chromatin immunoprecipitation (ChIP) assays, we detected a basal level of constitutive binding of NFATc2 to the *icos* promoter in resting EL4 cells (Figure 3.6A, upper panel, lane 5), possibly as a result of deregulated Ca^{2+} signalling in these cells (Fruman et al., 1995). Importantly, this level of binding was substantially increased when EL4 cells were stimulated with anti-CD3 and anti-CD28 Abs (lane 6). PP2 treatment on

similarly stimulated cells resulted in a reduction in NFATc2 binding (lane 7), suggesting that the positive impact of Fyn signalling on ICOS transcription observed earlier is via enhancing NFATc2 binding of the *icos* 288-bp promoter. As a positive control, CsA treatment was also observed to diminish, though not to the same extent, NFATc2 binding (lane 8). Binding of NFATc2 to the *icos* promoter was specific as ChIP performed using an isotype-matched antibody did not produce visible PCR products (lanes 1 – 4). We also examined the binding of NFATc2 to the *icos* promoter in primary CD4+ T cells lacking Fyn (Figure 3.6B). Consistent with the finding that Fyn and NFATc2 act in the same signalling axis to activate ICOS transcription, we show that there was reduced binding of NFATc2 to the *icos* promoter in $Fyn^{-/-}$ CD4+ T cells activated by anti-CD3 and anti-CD28 (lane 8 vs. lane 6). NFATc2 appeared to occupy a region encompassing 230 to 234 bp of the *icos* promoter, which contains an imperfectly matching consensus NFAT-binding sequence, because mutation of the bases between 232 and 231 bp led to a potent reduction in promoter activity when NFATc2 is over-expressed (compare luc activity due to mICOSp(−288)-Luc with that due to mICOSp(−288)-NFAT-mut-Luc in Figure 3.6C).

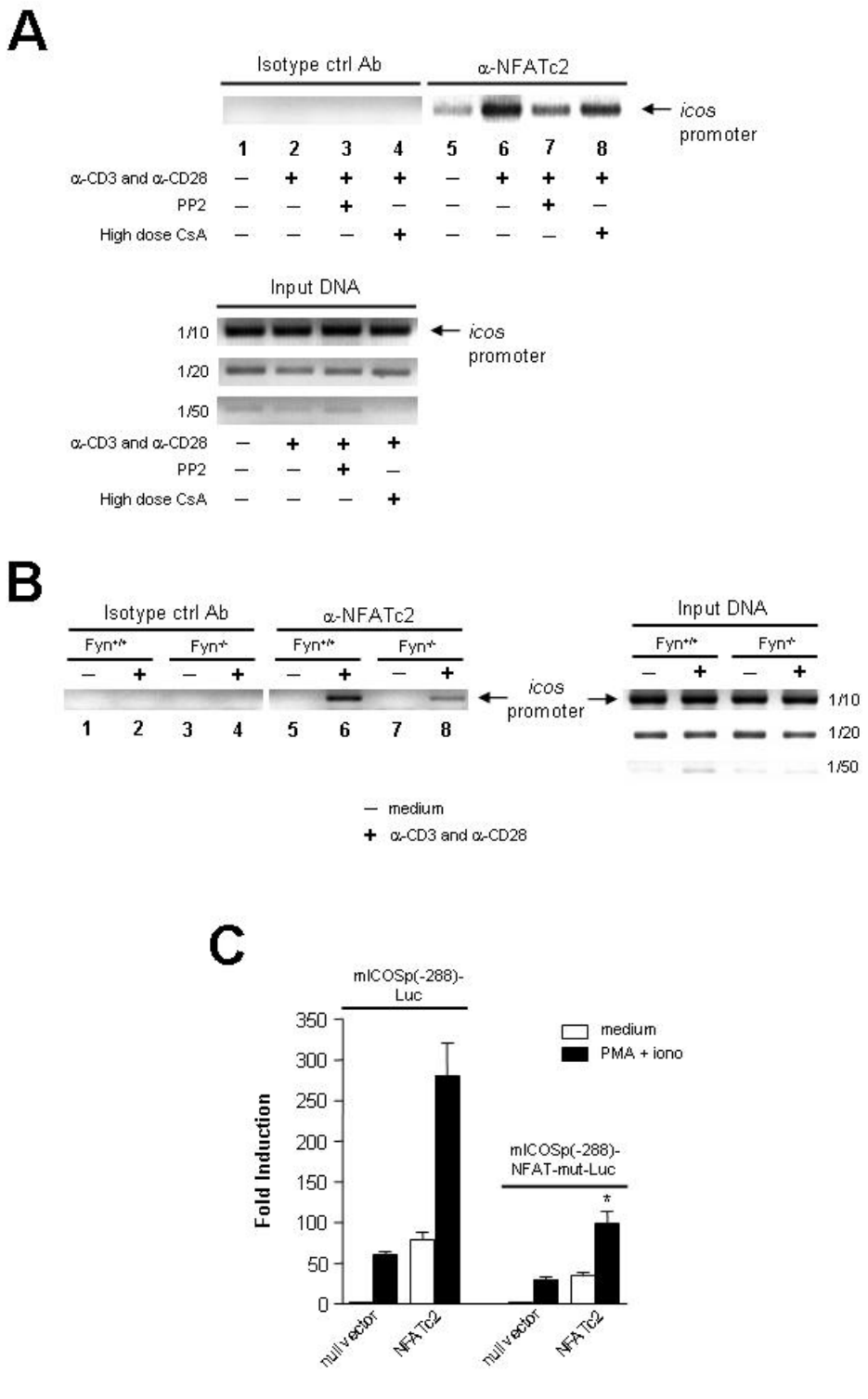


Figure 3.6 ChIP analyses of NFATc2 binding to the *icos* minimal promoter.

A, EL4 cells, either untreated or pre-treated with the indicated inhibitors for 1 h, were transfected with NFATc2 vector before stimulation with anti-CD3 and anti-CD28 Abs for 16 h. Chromatin complexes were immunoprecipitated with either isotype control (*upper panel*, *lanes 1 – 4*) or anti-NFATc2 Ab (*upper panel*, *lanes 5 – 8*) and PCR analyses of the region between –288 to –1 of the *icos* promoter were done on the precipitated DNA. To assess for equivalent loading of substrates, the same PCR was performed on serially diluted DNA before immunoprecipitation (*lower panel*). Fold-dilutions of templates are indicated on the left. **B**, Lack of Fyn signalling affects NFATc2 binding to the minimal *icos* promoter. Purified CD4+ T cells from $Fyn^{+/+}$ and $Fyn^{-/-}$ mice were either unstimulated or stimulated with anti-CD3 and anti-CD28 Abs for 16 h and ChIP was performed as in **A**. Results are representative of at least 2 independent experiments. **C**, EL4 cells co-transfected in triplicate with mICOSp(–288)-Luc or mICOSp(–288)-NFAT-mut-Luc and NFATc2 were either unstimulated (*white bars*) or stimulated with PMA and iono (*black bars*). Fold induction was quantitated as in Figure 3.5C. Data shown are mean ± SEM of triplicate samples and representative of 2 independent experiments.

3.1.7 Identification of an ERK-responsive site in the *icos* promoter

Since treatment with U0126 affected ICOS induction in TCR/CD28-stimulated T cells apparently at the promoter level, we sought to identify potential ERK-responsive site(s) within the *icos* core promoter region. To this end, we designed a set of overlapping double-stranded oligonucleotides which spanned the region 154 bp to 105 bp upstream of the *icos* TSS and used them to compete against nuclear extract binding by this region. As shown in Figure 3.7A, left panel, electrophoretic mobility shift assay (EMSA) performed with nuclear extracts obtained from P/I-stimulated EL4 cells demonstrated significant nuclear complex binding of the *icos* promoter region spanning 154 bp to 105 bp (lane 2). While molar excess of the unlabelled oligonucleotide spanning 129 bp to 105 bp (p129_105) failed to compete with the radiolabelled p154_105 (lane 5), the unlabelled p154_130 did (lane 4), suggesting that the region between 154 bp and 130 bp upstream of *icos* TSS very likely bound nuclear protein. Using a series of 3-bp scrambled mutations along the length of 154 bp to 130 bp, we refined the position of the binding site to between 148 bp to 136 bp (data not shown). Moreover, introduction of a 3-bp mutation corresponding to 144 bp to 142 bp of p154_130 (p154_p130_mut) interfered largely with the ability of unlabelled p154_130_mut to compete with the radiolabelled p154_130 (Figure 3.7A, right panel, lane 4), suggesting that the integrity of this site is important to facilitate optimal binding of as yet undetermined nuclear protein factors. EMSA assays revealed that there was considerable induction of complex binding of the stated region to nuclear extracts obtained from either P/I-stimulated EL4 cells or TCR/CD28-activated murine primary CD4+ T cells compared to those of resting cells (Figure 3.7B, lanes 2 and

5 respectively). This binding was reduced dramatically or abrogated in activated EL4 and CD4+ T cells pre-treated with U0126 (respectively lanes 3 and 6).

To confirm the functional relevance of the region spanning 154 bp to 130 bp upstream of the *icos* TSS in responding to ERK signalling, we replicated the above described mutation spanning 144 bp to 142 bp in the mICOSp(−288)-Luc construct to yield mICOSp(−288)-ERK-mut-Luc and assessed the latter's reporter activity in EL4 cells ectopically expressing a constitutively active form of MEK2. Consistent with our previous result demonstrating compromised nuclear complex binding, the introduced mutation in mICOSp(−288)-Luc led accordingly to a drastic reduction in luc activity (Figure 3.7C, left panel). Interestingly, a drastic attenuation in luc activity was also observed in EL4 cells over-expressing NFATc2 (Figure 3.7C, right panel), alluding to a possible functional hierarchy involving ERK-sensitive transcription factors and NFATc2 whereby the latter critically requires the former to operate, otherwise its own function is crippled. In other words, ERK signalling appears to be extremely important in initiating or sustaining basal ICOS transcription, whereas NFATc2 as well as other possible transcription factors played a more ancillary role in amplifying transcription. This finding is consistent with the failure of exogenous NFATc2 to rescue ICOS transcription in U0126-treated EL4 cells (Figure 3.3C) and the earlier purported cooperativity of ERK and NFATc2 in signalling ICOS expression (Figure 3.5C, left panel).

Since the region spanning 154 bp to 130 bp was responsive to ERK signalling, we attempted to identify the transcription factor(s) that bind(s) this region. For this purpose, we designed a series of unlabelled oligonucleotides bearing consensus DNA sequences that were known and had been shown to bind various ERK-sensitive transcription factors

such as E26 transformation-specific-1 (Ets-1), E74-like factor-1 (Elf-1), E26-like protein-1 (Elk-1), CP2 or late simian virus 40 (SV40) transcription factor (LSF), Specificity protein (Sp)1 and AP-1 as well as those recognising NFATc and NF-κB, and used these to compete with the binding of nuclear extracts from activated EL4 or CD4+ T cells to the *icos* promoter. None of these unlabelled oligonucleotides convincingly competed with the radiolabelled p154_130 in binding the protein complex from activated EL4 or CD4+ T cells (Figure 3.7D). Furthermore, the DNA-protein complex did not supershift with either anti-c-*fos* or anti-c-*jun* Abs (data not shown). Thus, at this juncture, we are unable to ascertain the identity of the transcription factor(s), except to report that it was or they were responsive to ERK.

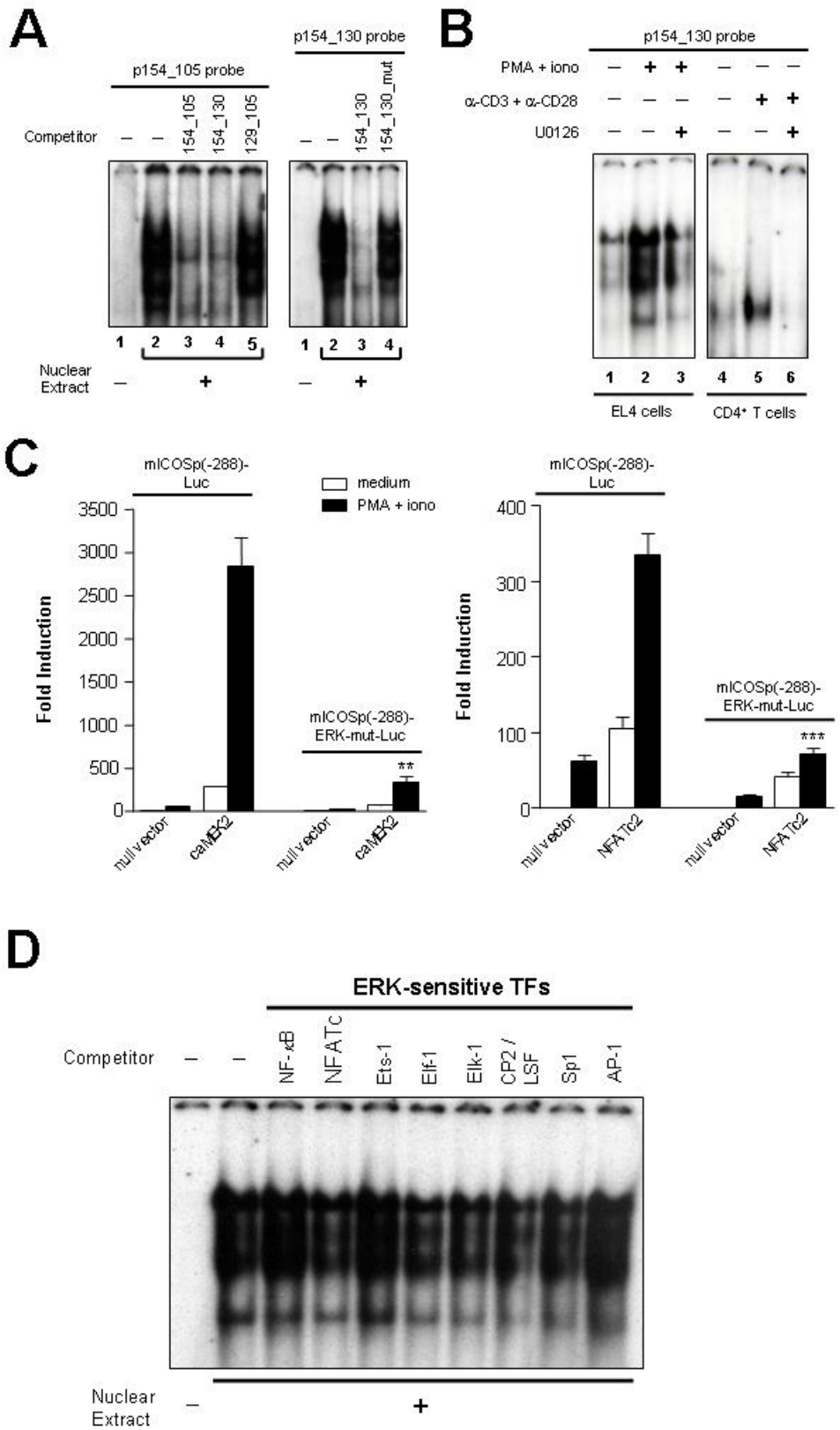


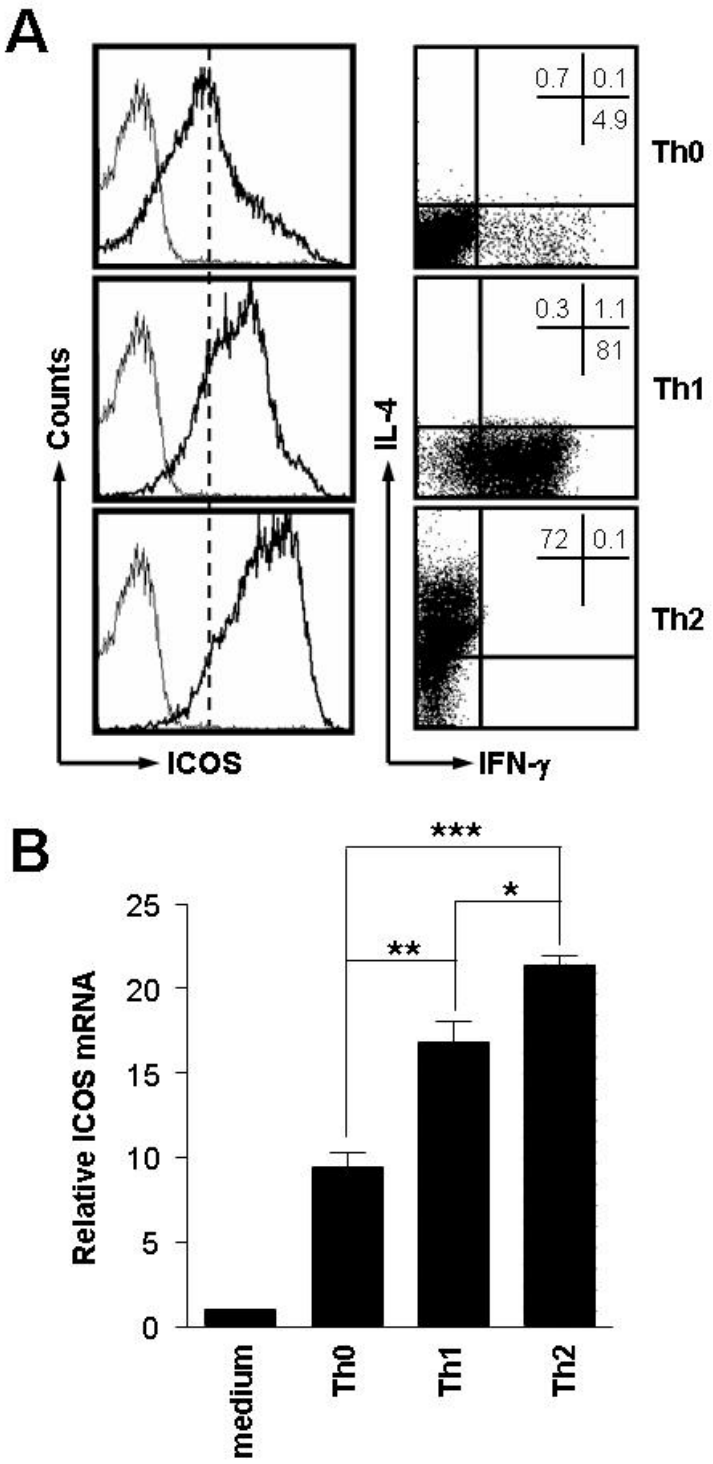
Figure 3.7 Identification of an ERK-sensitive site on the *icos* promoter.

A, Demonstration of specific transcriptional complex binding to DNA region -154 to -130 of *icos* promoter. Radiolabelled double-stranded oligonucleotides, p154_105 and p154_130, were incubated with nuclear extracts from P/I-stimulated EL4 cells either in the presence or absence of excess unlabelled competitors (*left and right panels*) and analysed via EMSA. **B**, Demonstration that the transcriptional complex binding the p154_130 probe is sensitive to the ERK inhibitor. The p154_130 probe was incubated with nuclear extracts from EL4 cells (*lanes 1 – 3*) or CD4+ T cells (*lanes 4 – 6*) that were untreated or treated as stated in the figure, and subjected to EMSA. **C**, EL4 cells were co-transfected in triplicate as in Figure 3.6C but with mICOSp(−288)-ERK-mut-Luc and caMEK2 (*left panel*) or NFATc2 (*right panel*). **D**, The p154_130 probe was incubated with nuclear extracts from PMA and iono-stimulated EL4 cells in the presence or absence of excess unlabelled competitors bearing the consensus binding sites of members of various transcription factor families; in particular, those that are known to act downstream of ERK. ****** $p < 0.01$; ******* $p < 0.001$.

3.2 Th lineage-specific regulation of ICOS expression via distinct *icos* regulatory regions by T-bet, GATA-3 and NFATc2

3.2.1 ICOS is differentially expressed in different Th cell subsets

To assess if ICOS is differentially expressed in various Th cells, we activated primary CD4+ T cells with anti-CD3 and anti-CD28 antibodies (Abs) and differentiated them for 8 days under three types of polarising conditions: Th0, Th1, or Th2 (Avni *et al.*, 2002). These treatment regimes yielded substantial fractions of IFN-γ- and IL-4-producing cells, indicating that the Th1- and Th2-polarising conditions were optimal (Figure 3.8A, right panel). Interestingly, we found the level of surface ICOS expression to vary in the different Th cell subsets: Th2 cells, consistent with earlier reports (Coyle et al., 2000; McAdam et al., 2000), expressed the highest level of ICOS, followed by Th1 and Th0 cells (Figure 3.8A, left panel). To determine if this variation in surface expression of ICOS could in part be attributed to variation in *icos* transcript levels, we quantified ICOS mRNA levels in the various Th subsets and found that these indeed correlated with levels of surface expression, with the highest amount of ICOS mRNA found in Th2, followed by Th1 and Th0 cells (Figure 3.8B). The data suggests that the expression of ICOS may be under transcriptional control in different Th cells. Incidentally, a similar pattern of ICOS protein and mRNA expression was recapitulated in AE7 Th1 (Figure 3.8C) and CDC35 Th2 cell clones (Figure 3.8D), confirming preferentially higher ICOS expression in Th2 compared to Th1 cells.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Counts
ICOS
IL-4
IFN-γ
0.7
0.1
4.9
Th0
0.3
1.1
81
Th1
72
0.1
Th2
B
Relative ICOS mRNA
25
20
15
10
5
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***
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medium
Th0
Th1
Th2

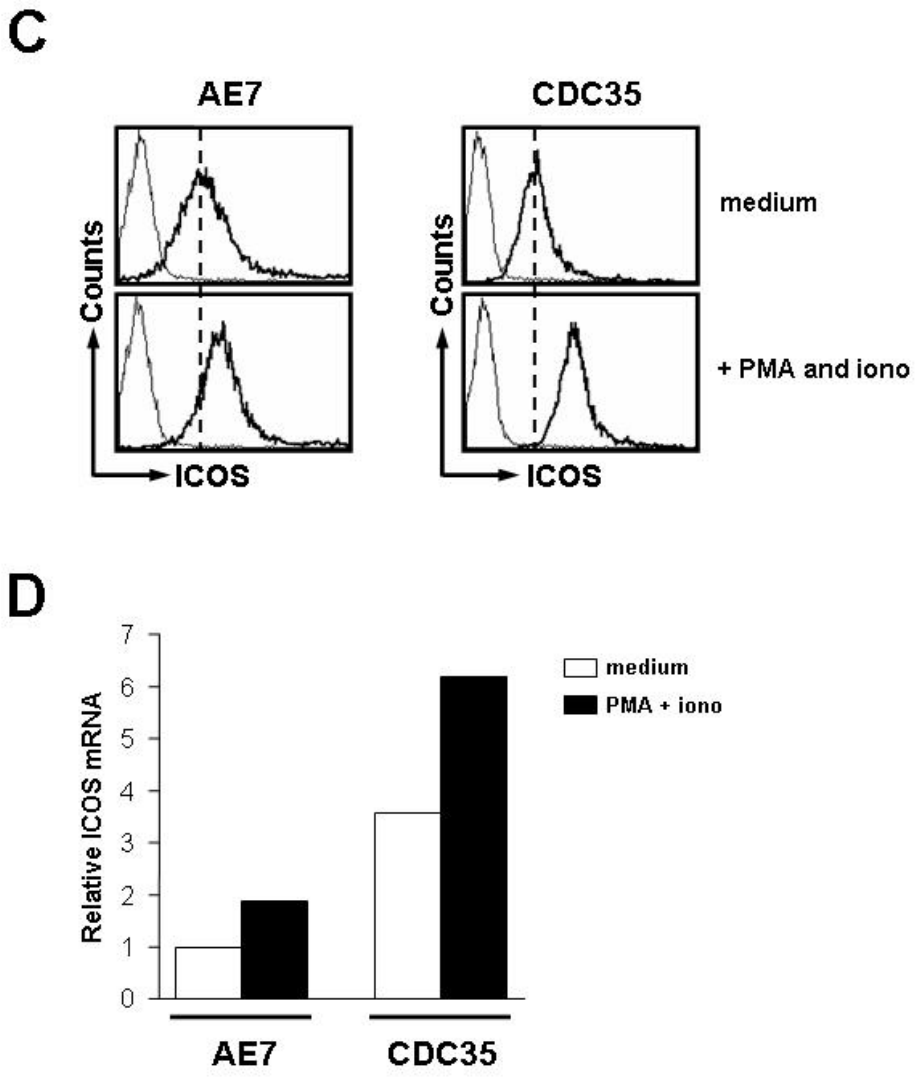


Figure 3.8 ICOS is differentially expressed in different Th cell subsets.

Purified CD4+ T cells were differentiated for 8 days under Th0, Th1 and Th2-polarising conditions and examined for **A**, cell surface expression of ICOS and, after restimulation with PMA and ionomycin for 6 h, for intracellular expression of IL-4 and IFN-γ by flow cytometry, and **B**, ICOS mRNA levels by real-time RT-PCR. Data shown in **B** had been normalised to mRNA levels in naïve T cells and expressed as the mean value ± S.E. of at least 3 independent experiments. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. AE7 Th1 and CDC35 Th2 cell clones were left in medium or stimulated with PMA and ionomycin for 6 h and assessed for **C**, surface ICOS expression and **D**, ICOS mRNA levels. Data shown in **D** had been normalised to mRNA levels in untreated AE7 cells and are representative of 2 independent experiments.

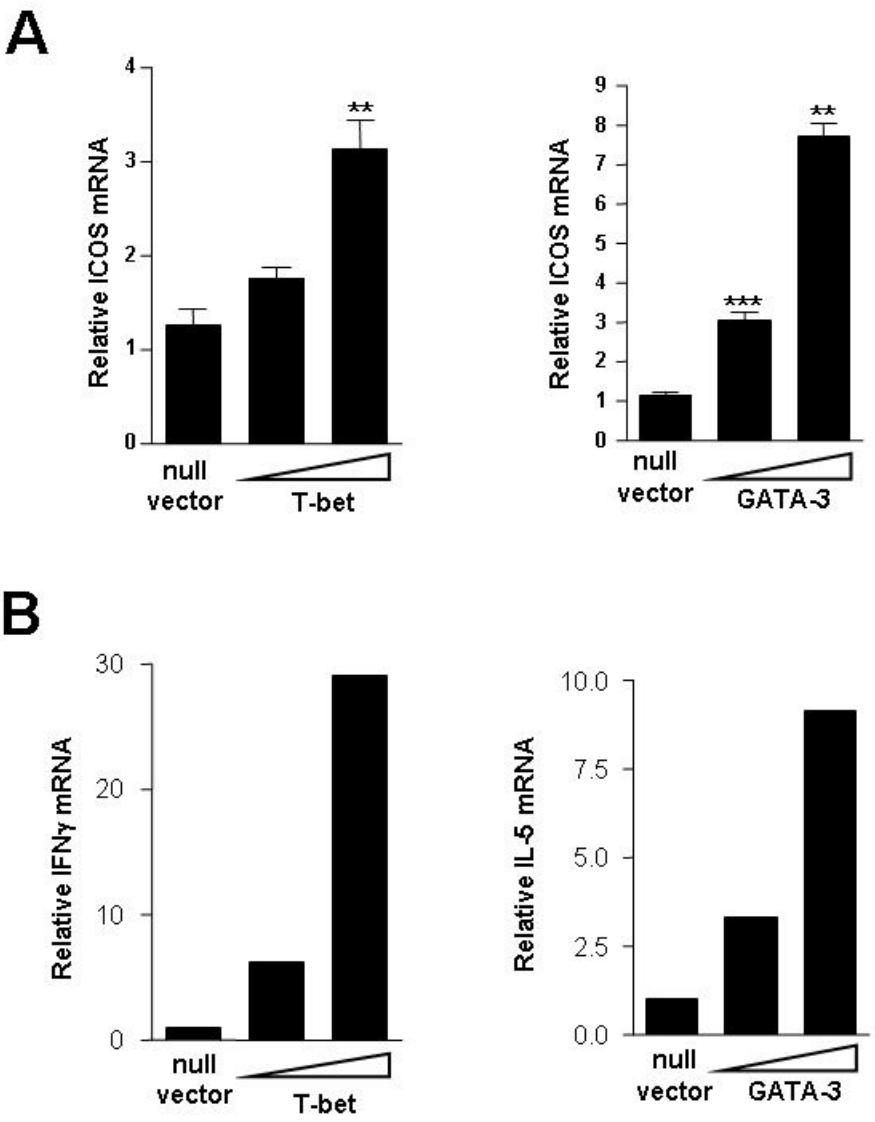
3.2.2 T-bet or GATA-3 enhances ICOS expression in T cells

Since *icos* transcripts were expressed differently in different Th cell subsets and in particular, at higher levels in Th1 and Th2 cells compared to Th0 cells, we asked if there was a role for Th1-specific T-bet and Th2-specific GATA-3, in driving ICOS expression. It was shown previously that the Th1-polarising cytokines IL-12 and/or IL-23 could enhance ICOS expression in activated human T cells independent of their pre-defined differentiation status (Wassink *et al.*, 2004). Similarly, the Th2-polarising cytokine IL-4 and growth factor IL-2 were shown to regulate ICOS expression in activated CD4+ T cells. Although retroviral transduction of GATA-3 was shown to increase surface ICOS expression in stimulated murine CD4+ T cells (Yagi *et al.*, 2003), it was not clear if this was a direct effect of GATA-3 on *icos* gene regulation. We therefore sought to ascertain if T-bet or GATA-3 *per se* may directly enhance ICOS expression in the respective Th cells.

To test the individual roles of T-bet and GATA-3 in regulating ICOS expression, we first over-expressed these transcription factors in EL4 T cells and measured the level of *icos* transcripts after overnight culture. Real-time RT-PCR analyses indicated that ectopic expression of T-bet (Figure 3.9A, left panel) or GATA-3 (right panel) could increase the level of *icos* transcripts in EL4 cells in a dose dependent manner. To control for the functionality of T-bet and GATA-3 and as shown in Figure 3.9B, we also observed respectively, concomitant increases in *ifn-*γ and *il-5* transcripts in the transfected EL4 cells (Siegel et al., 1995; Szabo et al., 2000). Next, we determined if the up-regulation of ICOS by T-bet or GATA-3 in EL4 cells was reproducible in primary CD4+ T cells. We infected CD4+ T cells activated by anti-CD3/CD28 Abs with

retroviruses that expressed GFP alone or together with T-bet or GATA-3 and examined ICOS expression in these cells 48 h after infection. Retroviral transduction of T-bet (Figure 3.9C, left panel) or GATA-3 (right panel) in activated, non-polarised CD4+ T cells led to their corresponding over-expression and also resulted in the higher expression of ICOS protein as well as mRNA in these cells when compared with control cells transduced with vector expressing GFP alone.

Finally, to establish definitively that T-bet and GATA-3 play a direct role in driving ICOS transcription, we performed converse experiments using small interference RNA (siRNA) against T-bet and GATA-3 to knock down their protein expression in AE7 Th1 and CDC35 Th2 cell lines, respectively. Knock down of *Tbx21* and *Gata3* transcripts led to the efficient abrogation of their protein expression in AE7 (Figure 3.10A) and CDC35 (Figure 3.10B) cells, whereas knock down of the irrelevant *gfp* gene hardly affected T-bet expression in AE7 or GATA-3 expression in CDC35 cells. More importantly and in agreement with data obtained so far, the knock down of T-bet significantly reduced the amount of *icos* transcripts in activated AE7 cells (Figure 3.10A), while knock down of GATA-3 profoundly diminished ICOS mRNA in CDC35 cells (Figure 3.10B). However, the reduction in ICOS mRNA was not absolute, suggesting that T-bet or GATA-3 likely cooperates with other transcription factors to regulate ICOS expression. Taken together, our over-expression and siRNA knock down data indicate that T-bet and GATA-3 play enhancing roles in driving ICOS transcription in Th cells.


A
Relative ICOS mRNA
4
3
2
1
0
**
null vector
T-bet
Relative ICOS mRNA
9
8
7
6
5
4
3
2
1
0
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null vector
GATA-3
B
Relative IFNγ mRNA
30
20
10
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null vector
T-bet
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10.0
7.5
5.0
2.5
0.0
null vector
GATA-3

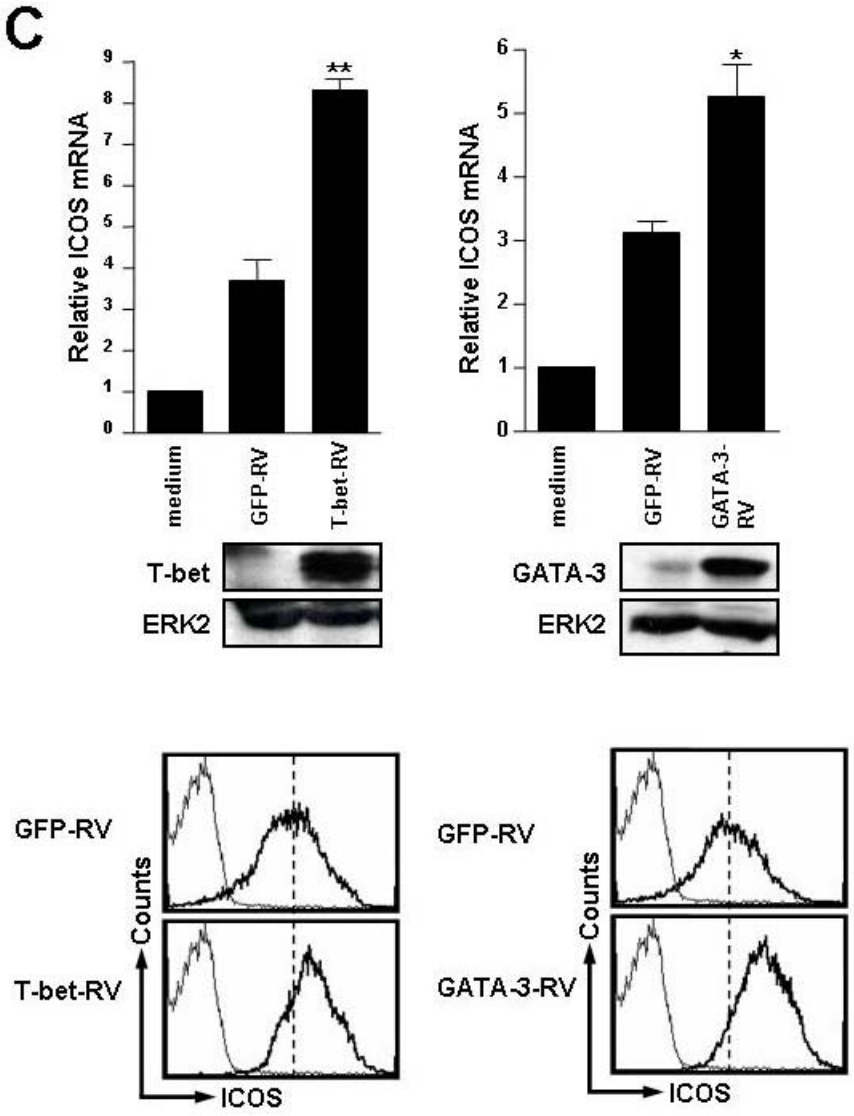


Figure 3.9 Ectopic expression of T-bet or GATA-3 enhances ICOS expression in T cells.

Real-time RT-PCR analyses of **A**, ICOS and **B**, IFN-γ and IL-5 mRNA levels in murine EL4 T cells transfected with null vector or increasing amounts of vectors encoding for either T-bet or GATA-3. * $p < 0.05$; ** $p < 0.001$; *** $p < 0.0001$. Data shown in B are representative of at least 2 independent experiments. **C**, Flow cytometry and real-time RT-PCR analyses of ICOS expression in purified CD4+ T cells transduced with retroviruses encoding GFP (GFP-RV), GFP and T-bet (T-bet-RV) or GFP and GATA-3 (GATA-3-RV). Only GFP+ cells were examined for ICOS expression by flow cytometry and FACS-sorted for the analysis of *icos* transcript levels. * $p < 0.05$; ** $p < 0.001$. Western blots were included to show over-expression of T-bet or GATA-3 in the retrovirally transduced samples. The anti-ERK-2 blot served as control for equal loading of cell lysates.

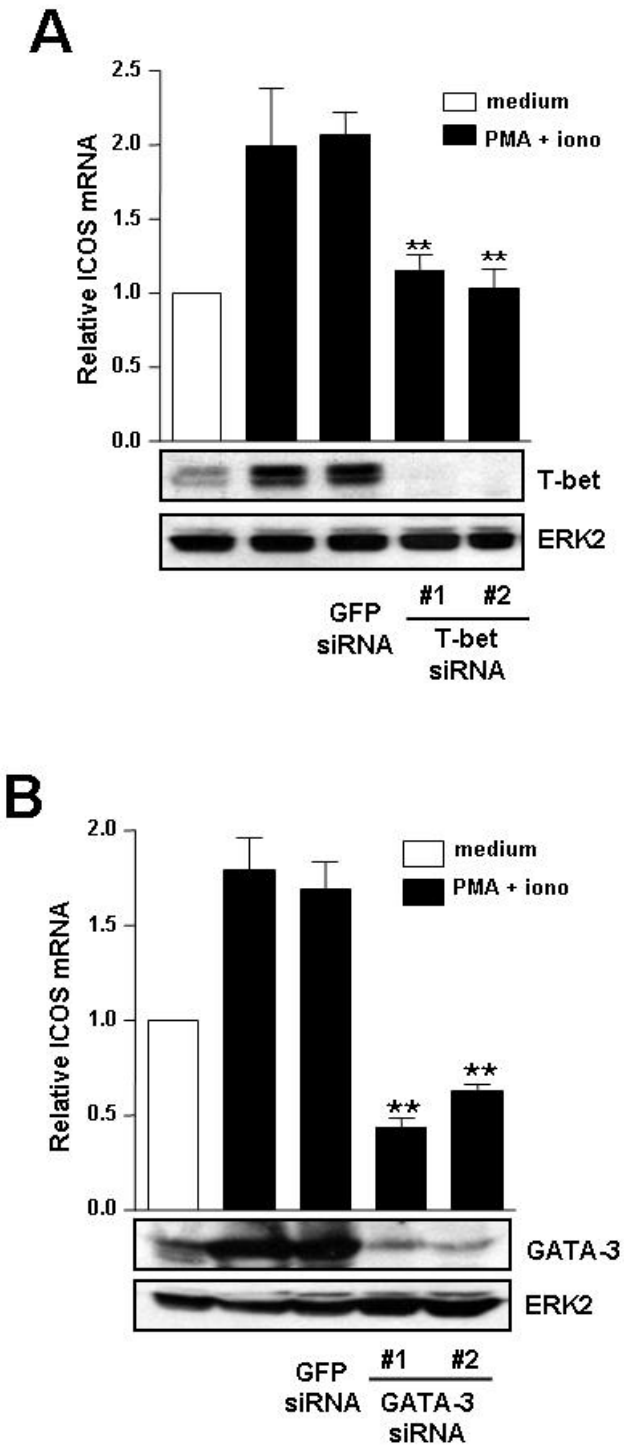


Figure 3.10 Knockdown of T-bet or GATA-3 reduces *icos* transcripts in AE7 Th1 and CDC35 Th2 cell lines.

A, AE7 Th1 or **B**, CDC35 Th2 cells were transfected with siRNA against GFP (as control), T-bet or GATA-3, rested for 24 h and stimulated with PMA and ionomycin for 16 h. The amount of *icos* transcripts present was assessed by real-time RT-PCR. The knockdown efficiencies in these cells were assessed by Western blotting as shown. The anti-ERK2 blot serves as loading control. ** $p < 0.001$.

3.2.3 T-bet is more dominant in activating ICOS transcription in developing rather than fully differentiated Th1 cells

To establish a physiological role for T-bet in ICOS expression during Th1 development, we first cultured T-bet-sufficient (T-bet$^{+/+}$) and T-bet-deficient (T-bet$^{-/-}$) CD4+ T cells under Th1-polarising conditions and compared the cell surface ICOS expression in developing (Figure 3.11A) or fully differentiated cells (Figure 3.11B). After 2 days of culture, T-bet$^{+/+}$ CD4+ T cells exhibited surface ICOS expression that was comparable to that in T-bet$^{-/-}$ cells (Figure 3.11A). In contrast, ICOS mRNA levels were significantly reduced in developing T-bet$^{-/-}$ Th1 cells, even though the proportion of T-bet$^{-/-}$ cells producing IFN-γ is similar to their T-bet$^{+/+}$ counterparts. Hence, T-bet deficiency results in attenuated ICOS transcription in developing Th1 cells, at a stage when T-bet-associated defects in IFN-γ production are not yet apparent.

Surface ICOS expression of fully differentiated T-bet$^{-/-}$ T cells were observed to be marginally lower than that of T-bet$^{+/+}$ cells (Figure 3.11B). Unlike developing cells, the fraction of committed T-bet$^{-/-}$ Th1 cells that are IFN-γ+ was moderately reduced as compared to similarly cultured T-bet$^{+/+}$ cells (Figure 3.11B). There was, however, no significant difference in ICOS mRNA levels between terminally differentiated T-bet$^{+/+}$ and T-bet$^{-/-}$ Th1 cells. Thus, our data suggest a non-trivial role for T-bet in driving ICOS transcription particularly during the early Th1 ontogeny. However, this role of T-bet appears to diminish in importance as the cells approach terminal differentiation, indicating other factors may be involved in regulating ICOS transcription and could compensate for the absence of T-bet during the later phase of Th1 development. One such possible factor is ERK, the signalling of which we had earlier shown to be important

for activating basal ICOS transcription in TCR/CD28-stimulated T cells. Since the level of ERK phosphorylation (and hence activity) was perceptibly higher in the nuclei of differentiated T-bet$^{-/-}$ compared to T-bet$^{+/+}$ Th1 cells (Figure 3.12B), exacerbated nuclear ERK activity in T-bet$^{-/-}$ Th1 cells might account for comparable amounts of *icos* transcripts in T-bet$^{+/+}$ and T-bet$^{-/-}$ Th1 cells (Figure 3.11B). Nuclear occupancy of phospho-ERK in both developing T-bet$^{+/+}$ and T-bet$^{-/-}$ Th1 cells occurred with faster kinetics than Th0 or Th2 cells (Figure 3.12A), which could explain in part why ICOS transcription is not completely abolished in developing Th1 cells lacking T-bet (Figure 3.11A). Although it is presently unclear why T-bet deficiency leads to enhanced nuclear phospho-ERK in differentiated Th1 cells, our observation of sustained ERK activation in Th1 cells is consistent with a study using a high affinity peptide for stimulation of CD4+ T cells that maintained ERK activation and resulted in Th1 differentiation. If a cognate peptide of lower affinity was employed, transient ERK activity which followed dramatically increased IL-4 production and Th2 generation. (Jorritsma et al., 2003). We observed comparatively less enduring nuclear ERK activity in developing and differentiated Th2 cells (Figure 3.12A and B). Admittedly, interpretation of the role of nuclear phospho-ERK in T-bet$^{+/+}$ vs. T-bet$^{-/-}$ Th1 cells undergoing differentiation, with the currently available evidence, is at best correlative and further experiments are required to clarify how ERK signalling influences ICOS transcription during Th1 development.

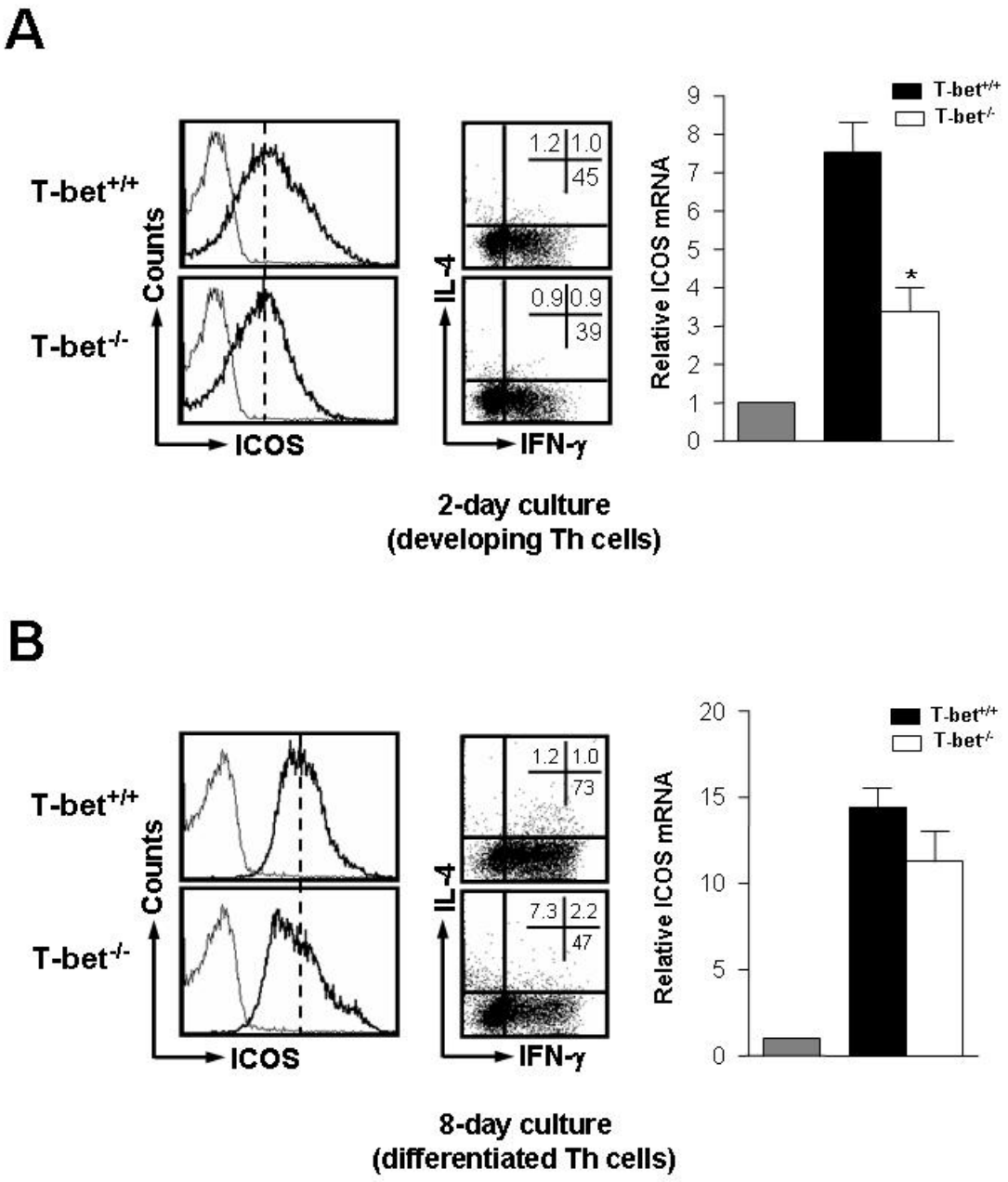


Figure 3.11 The amount of *icos* transcripts is reduced in the absence of T-bet in developing but not fully differentiated Th1 cells.

T-bet$^{+/+}$ and T-bet$^{-/-}$ CD4+ T cells were differentiated for **A**, 2 days or and **B**, 8 days under Th1-polarising condition and examined for cell surface ICOS expression or, after restimulation with PMA and ionomycin for 6 h, intracellular IFN-γ and IL-4 expression by flow cytometry. Data shown are representative of 3 independent experiments. The levels of ICOS mRNA in Th1 cells, assessed by real-time RT-PCR, had been normalised to those in naïve CD4+ T cells and expressed as the mean value ± S.E. of 3 independent experiments. * $p < 0.05$.

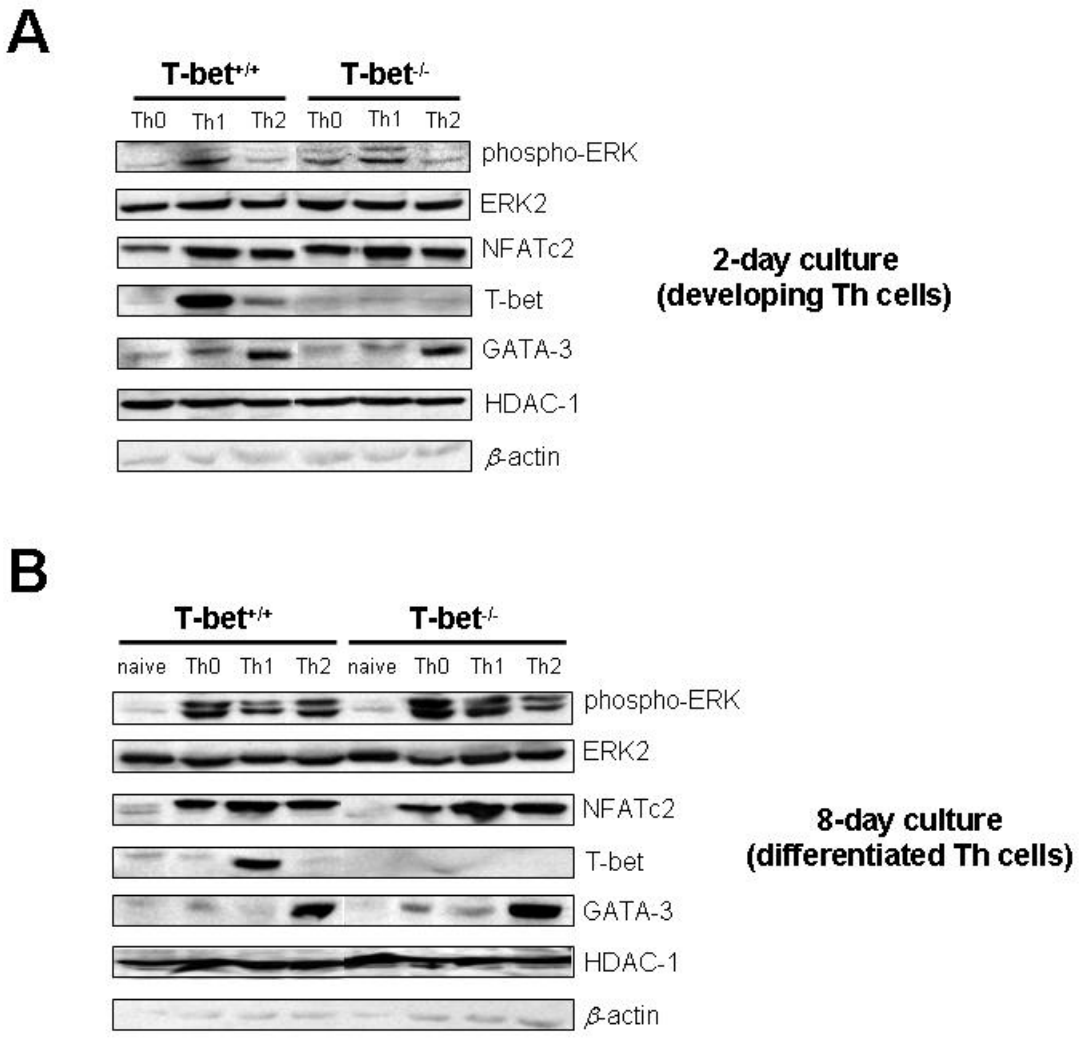


Figure 3.12 Nuclear translocation of NFATc2 precedes that of phospho-ERK in developing Th cells.

T-bet$^{+/+}$ and T-bet$^{-/-}$ CD4+ T cells were differentiated for **A**, 2 days or **B**, 8 days under Th0, Th1 or Th2-polarising conditions. Nuclear extracts were prepared from these cells and subjected to immunoblotting with anti-phospho-ERK, anti-T-bet, anti-GATA-3 and anti-NFATc2. The anti-ERK2 blot serves as control for equal loading of extracts. The anti-HDAC-1 and anti-β-actin blots indicate the effectiveness of separation between nuclear and cytoplasmic fractions.

3.2.4 T-bet cooperates with NFATc2 to transactivate the *icos* promoter

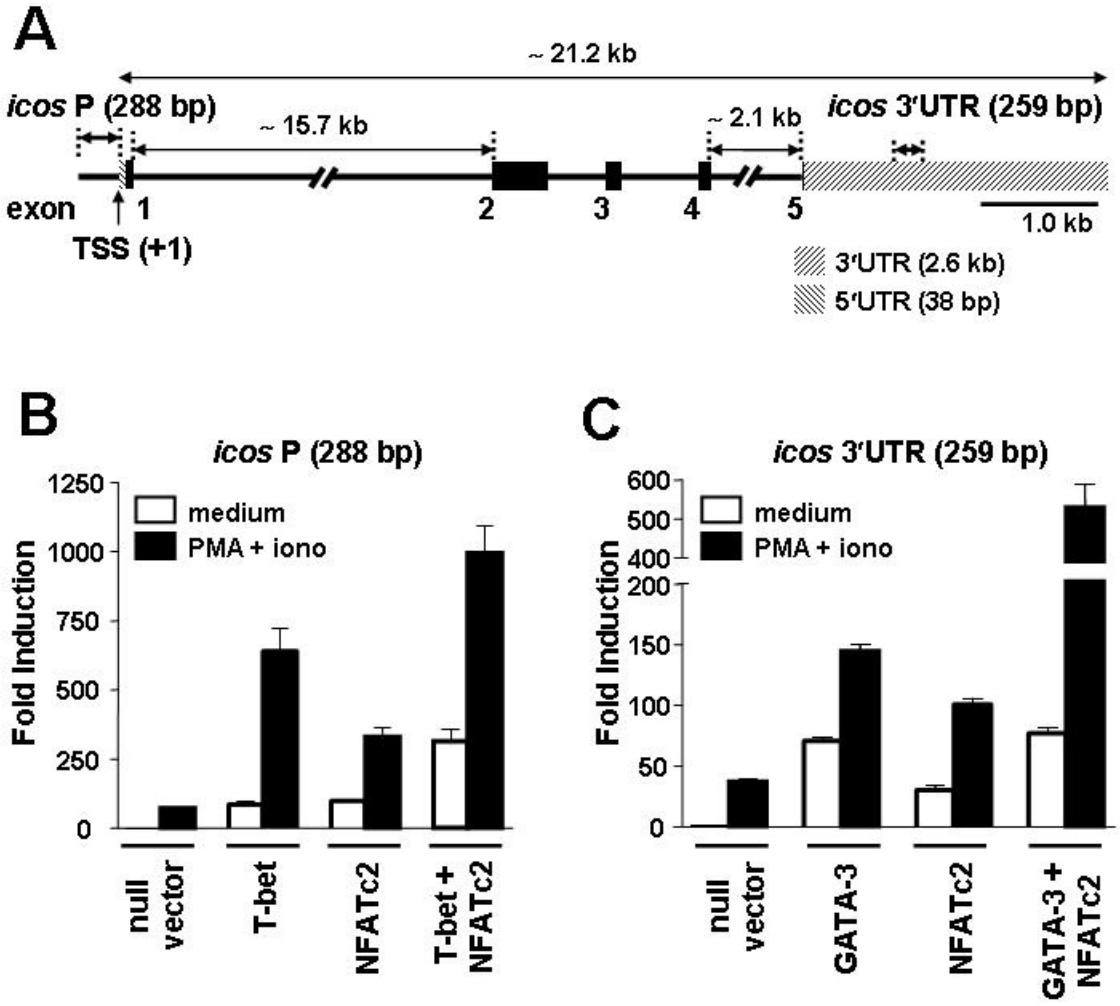
Since T-bet and GATA-3 are transcription factors shown to transactivate the promoters of *ifn-γ* (Szabo et al., 2000) and *il-5* (Zhang *et al.*, 1997) genes respectively, we were interested to determine if they would also regulate *icos* gene expression via binding to its promoter region. Initial algorithmic searches using the TRANSFAC database did not reveal putative binding sites for T-bet or GATA-3 in the *icos* promoter. We had previously shown that NFATc2 enhanced ICOS expression by acting within a 288-bp promoter region upstream of the ICOS transcription start site (TSS) (Figures 3.13A, 3.5C and Tan et al., 2006). Hence, to find out if this region could respond to T-bet and/or GATA-3, we introduced a luc reporter plasmid driven by the 288-bp *icos* promoter, with or without accompanying plasmids encoding T-bet, NFATc2 or both, into EL4 cells by transient transfection.

Ectopic expression of NFATc2 led to an increase in *icos* promoter activity as reflected by the fold induction of luc activity in PMA/ionomycin-activated EL4 cells (Figure 3.13B), concordant with our earlier work demonstrating that NFATc2 regulates ICOS expression. The introduction of T-bet also resulted in the up-regulation of luc activity in stimulated EL4 cells, suggesting that T-bet acted via the *icos* promoter to activate gene expression. Interestingly, the effect produced by co-introduction of T-bet and NFATc2 was additive, implying T-bet cooperates with NFATc2 at the promoter to drive transcription.

3.2.5 GATA-3 synergises with NFATc2 to regulate gene expression via an *icos* 3′UTR element

We repeated the above experiments to determine if GATA-3 could also act via the promoter to regulate ICOS expression. In contrast to T-bet, we observed that ectopic expression of GATA-3 could not enhance *icos* promoter activity beyond what was observed with null vector control (Figure 3.13D, left panel). This suggests the *icos* promoter does not possess GATA-3-responsive elements.

In order to delineate where GATA-3 might act in the *icos* genomic locus, we searched for potential GATA-3 binding motifs and identified a cluster of conserved GATA-3 and NFATc2 binding sites within a 259-bp stretch in the 3′-untranslated region (3′UTR) of *icos* (Figure 3.13A). To test the functional relevance of this 3′UTR site, we cloned the 259-bp fragment downstream of a luc reporter driven by a previously described 52-bp minimal *icos* promoter, and introduced this construct into EL4 cells. Stimulation of EL4 cells harbouring this construct resulted in a 40-fold induction of luc activity (Figure 3.13C, null vector), suggesting that this 259-bp segment was sufficient to confer PMA and ionomycin-mediated inducibility on ICOS transcription. To determine if this element could respond to T-bet and/or GATA-3, we co-transfected, together with the 259-bp 3′UTR reporter, vectors expressing NFATc2, GATA-3 and T-bet in EL4 cells. Over-expression of either GATA-3 or NFATc2 could augment the 3′UTR activity by approximately 150- and 100-fold, respectively. Strikingly, the co-expression of GATA-3 and NFATc2 resulted in > 500-fold increase in luc activity, suggesting GATA-3 synergises with NFATc2 to enhance ICOS transcription via this 3′UTR site. On the other hand, over-expressing T-bet failed to yield an increase in 3′UTR activity (Figure 3.13D, right panel), indicating that this region was probably non-responsive to T-bet. Hence, GATA-3 and NFATc2 appear to act on the 259-bp 3′UTR element of *icos*.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~ 15.7 kb
~ 2.1 kb
icos 3'UTR (259 bp)
exon
1
2
3
4
5
TSS (+1)
1.0 kb
3'UTR (2.6 kb)
5'UTR (38 bp)
B
icos P (288 bp)
Fold Induction
1250
1000
750
500
250
0
medium
PMA + iono
null vector
T-bet
NFATc2
T-bet + NFATc2
C
icos 3'UTR (259 bp)
Fold Induction
600
500
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medium
PMA + iono
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GATA-3
NFATc2
GATA-3 + NFATc2

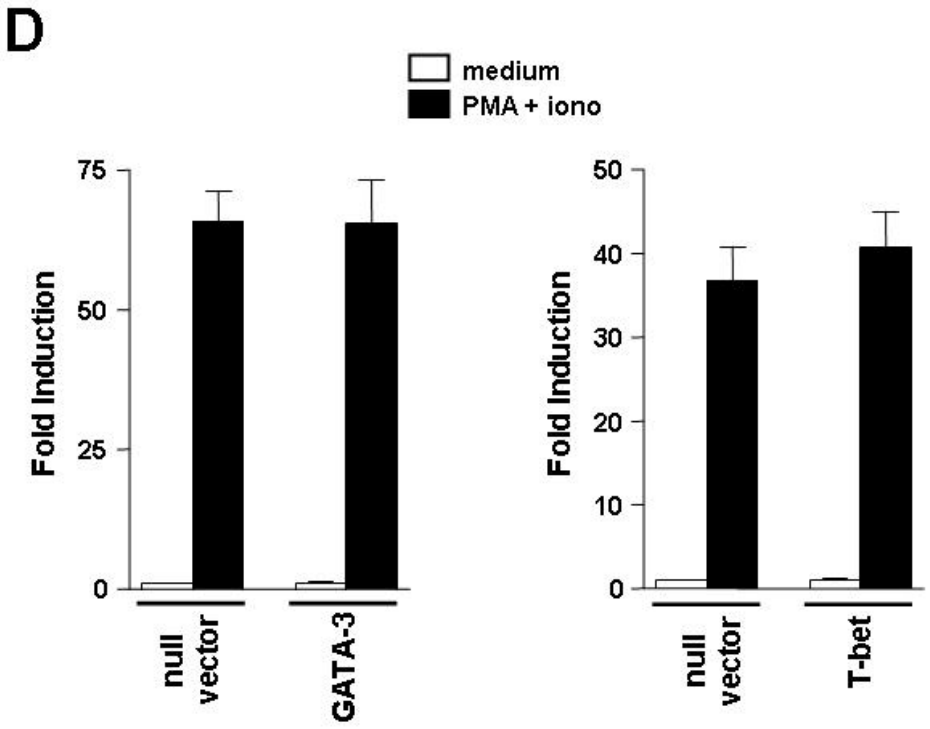


Figure 3.13 T-bet and GATA-3 act through distinct *icos* regulatory regions to regulate gene expression.

A, Schematic diagram showing putative regulatory regions within the *icos* gene locus. The promoter (P), transcription start site (TSS) and 5′ and 3′ untranslated regions (UTRs) are depicted. **B**, T-bet or NFATc2 acts alone and also cooperatively with each other to transactivate the *icos* promoter. EL4 cells were transiently transfected in triplicate with a luc reporter driven by the 288-bp *icos* promoter together with either a null vector or vector encoding T-bet, NFATc2 or both and subsequently left in medium alone (white bars) or stimulated with PMA and ionomycin (black bars) for 16 h. Fold induced luc activity was calculated as in *Materials and Methods*. **C**, GATA-3 and NFATc2 act synergistically via a 3′UTR element to transactivate *icos* expression. EL4 cells were transfected in triplicate with a 52-bp minimal *icos* promoter-driven luc reporter and a 259-bp 3′UTR segment together with either a null vector or vector encoding GATA-3, NFATc2 or both. Fold induced activity was quantified as in **B**. **D**, Over-expression of GATA-3 or T-bet does not enhance the activity of *icos* promoter or 3′UTR element, respectively. EL4 cells were transiently transfected in triplicate with luc reporter driven by the 288-bp *icos* promoter with null or GATA-3 vector, or 259-bp 3′UTR together with null or T-bet vector and subsequently left in medium alone (white bars) or stimulated with PMA and ionomycin (black bars) for 16 h. Fold induction was measured as in **B**.

3.2.6 Differential association of T-bet/NFATc2 with *icos* promoter and GATA-3/NFATc2 with *icos* 3′UTR during Th1 and Th2 differentiation, respectively

Data from the luc assays suggest that ICOS may be regulated distinctly in different Th cell subsets, likely with T-bet acting at the promoter and GATA-3 at the 3′UTR in Th1 and Th2 cells respectively and, with NFATc2 acting as the common co-activator in both Th subsets. Indeed, NFATc2 has been reported to cooperate with T-bet or GATA-3 in binding the regulatory regions of the *ifn-γ* gene in stimulated Th1 (Lee et al., 2004a) or the *il-4* gene in stimulated Th2 cells (Agarwal *et al.*, 2000), respectively. We therefore asked if T-bet, GATA-3 and NFATc2 differentially associated with the different *icos* regulatory regions during Th cell development. This was addressed by assessing the binding dynamics of T-bet, GATA-3 and NFATc2 to *icos* regulatory regions in developing and fully differentiated Th1 and Th2 cells.

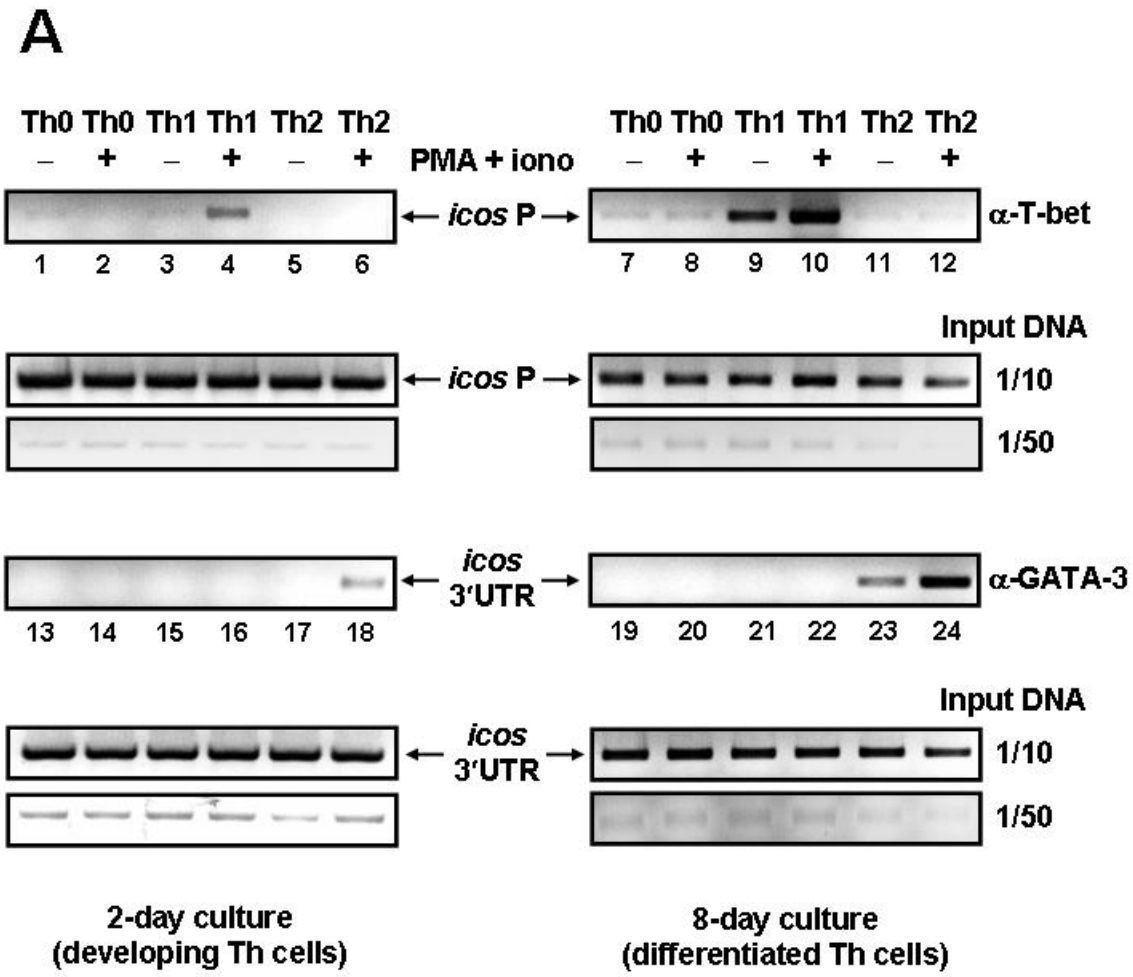
ChIP assays revealed detectable binding of T-bet to the *icos* promoter (*icos* P) in developing (2-day culture) Th1 cells after stimulation of these cells for 6 h with PMA and ionomycin (PMA + iono) (Figure 3.14A, lanes 3 and 4). This binding was significantly elevated in fully differentiated (8-day culture) Th1 cells and could be further enhanced when the cells were stimulated (lanes 9 and 10). On the other hand, T-bet was not observed to bind the promoter in developing or differentiated Th0 (lanes 1, 2, 7 and 8) and Th2 (lanes 5, 6, 11 and 12) cells, consistent with the virtual absence of T-bet expression in these cells (Figure 3.12A and B). Similarly, binding of GATA-3 to the 3′UTR element of *icos* was evident after 2 days of culture in developing Th2 cells (Figure 3.14A, lanes 17 and 18). This association was greatly augmented in Th2 cells that

approached terminal differentiation after 8 days of culture, and could be further enhanced upon activation of these cells (lanes 23 and 24). Again, GATA-3 binding to the 3′UTR of *icos* was undetectable in developing or differentiated Th0 (lanes 13, 14, 19 and 20) or Th1 cells (lanes 15, 16, 21 and 22), in agreement with the preferential expression of GATA-3 in Th2 cells (Figure 3.12A and B).

Binding of NFATc2 to the *icos* promoter was detectable in Th0 cells cultured for 2 days and can be further increased when cells were stimulated (Figure 3.14B, lanes 1 and 2), in accordance with our previous demonstration that NFATc2 was found to be associated with the promoter during anti-CD3/CD28-mediated activation of naïve CD4+ T cells. Intriguingly, the association of NFATc2 with the *icos* promoter was found initially to be promiscuous across developing Th cells (lanes 1 to 6), but became progressively Th1-restricted, Th0-diminished and Th2-extinguished as cells became terminally differentiated (lanes 7 to 12). On the other hand, the binding of NFATc2 to the 3′UTR of *icos* was marginally detectable in various developing Th cells at an early stage of differentiation (lanes 13 to 18) but became conspicuous and specifically accrued in terminally differentiated Th2 cells (lanes 23 and 24). Given the rapid nuclear localisation of NFATc2 upon TCR/CD28 stimulation and the maintenance of NFATc2 nuclear residency during the course of Th differentiation (Figure 3.12A and B), such a temporally dynamic pattern of NFATc2 association with the *icos* regulatory DNA suggests the involvement of epigenetic mechanisms that educate accessibility of the *icos* regulatory regions to transcription factors.

Taken together, the evolving patterns of T-bet, GATA-3 and NFATc2 binding to the promoter and 3′UTR of *icos* supports a model in which NFATc2 functions as the

major common transcription factor driving ICOS expression via the promoter during early Th differentiation when TCR-mediated signalling is dominant and cytokine pathways are nascent. As Th differentiation progresses and the influence of polarising cytokines increases, T-bet cooperates with NFATc2 to direct ICOS expression via its promoter in Th1 cells, while GATA-3 synergises with NFATc2 to regulate ICOS expression via the 3′UTR element in Th2 cells.


A
Th0 Th0 Th1 Th1 Th2 Th2
– + – + – +
PMA + iono
Th0 Th0 Th1 Th1 Th2 Th2
– + – + – +
icos P
α-T-bet
1 2 3 4 5 6
7 8 9 10 11 12
Input DNA
icos P
1/10
1/50
icos
3′UTR
α-GATA-3
13 14 15 16 17 18
19 20 21 22 23 24
Input DNA
icos
3′UTR
1/10
1/50
2-day culture
(developing Th cells)
8-day culture
(differentiated Th cells)

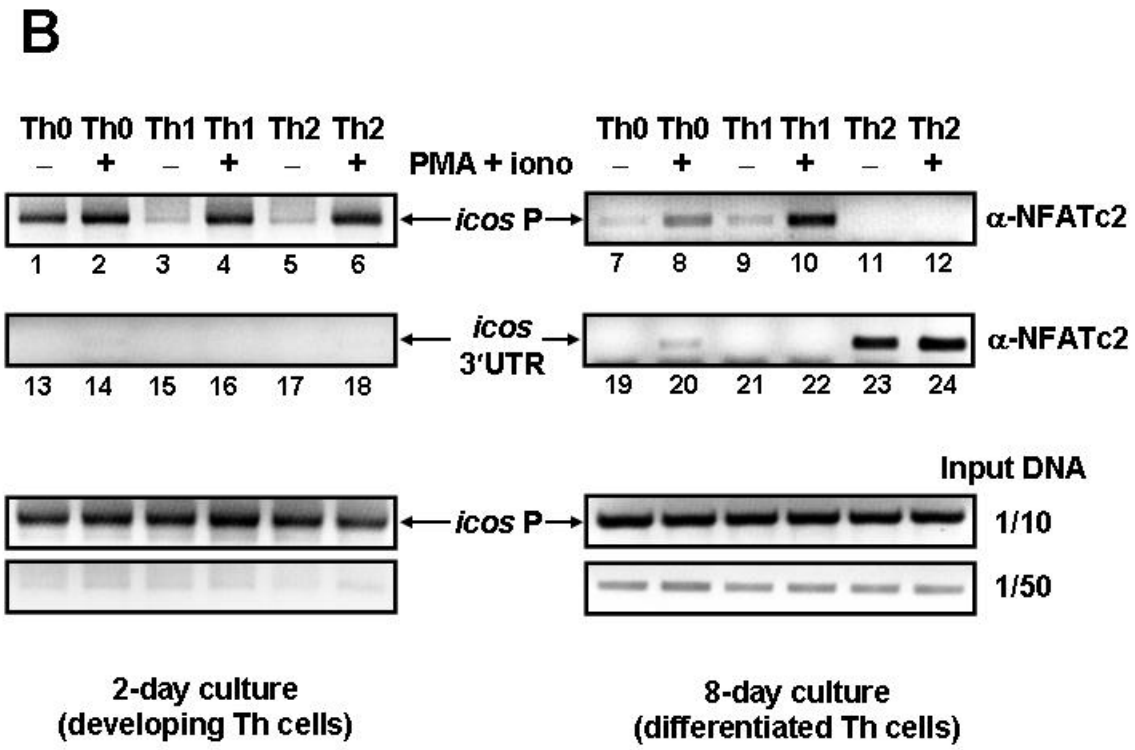


Figure 3.14 Differential association of T-bet, GATA-3 and NFATc2 with the *icos* regulatory regions during *de novo* Th1 and Th2 cell differentiation.

ChIP assays using Abs against **A**, T-bet and GATA-3 and **B**, NFATc2 were performed on primary CD4+ T cells differentiated for 2 or 8 days under Th0-, Th1- or Th2-polarising conditions and left in medium or restimulated for further 6 h with PMA and ionomycin. Primers specific for the *icos* promoter (P) or 3′UTR element (3′UTR) were used in the PCR. As input controls, DNA extracted from the samples before immunoprecipitation was serially diluted (1/10 and 1/50) and quantified by the same PCR. Immunoprecipitation with isotype-matched control Abs did not yield visible PCR bands (not shown for brevity).

3.2.7 Histone trimethylation of *icos* regulatory regions is Th-selective

Our data above revealed a temporally changing yet Th-selective pattern of binding by transcription factors to distinct *icos* regulatory regions in various Th cell subsets. It is plausible that this intricate pattern of binding by transcription factors could be influenced by epigenetic mechanisms that operate in concert to induce ICOS expression in Th cells. Hyperacetylation of histones H3 and H4 is a major mechanism through which cytokine signalling pathways preserve the transcriptional permissiveness of genes encoding the cytokines themselves in mouse and human Th cells (Ansel et al., 2006; Baguet and Bix, 2004; Morinobu et al., 2004; Mullen et al., 2001). For example, TCR stimulation of naïve T cells resulted in a transient increase in histone acetylation of the *ifn-γ* and *il-4* regulatory regions, which was stabilised by polarizing cytokines such as IL-12 and IL-4 that were required to promote Th1 and Th2 differentiation respectively (Avni *et al.*, 2002). Based on these observations, we postulated that histones associated with the *icos* regulatory regions might undergo Th-specific modification by either acetylation or trimethylation, thereby increasing the availability of these regions to binding by T-bet, GATA-3 and/or NFATc2.

To test this hypothesis, we examined histone trimethylation at the *icos* locus using an antibody that recognised trimethylated histone H3 lysine residue 4 (K4). Interestingly, a similar degree of histone trimethylation was detected across all Th subsets after 2 days of differentiation. This suggests a non-selective accessibility of the *icos* promoter (Figure 3.15, lanes 1 to 6) and 3′UTR region (lanes 13 to 18) during the early phase of Th differentiation. However, as differentiation proceeded, the pattern of histone H3 trimethylation at the *icos* locus evolved in a Th-specific manner. Hypermethylation of H3

was selectively enhanced at the *icos* promoter in differentiated Th1 (lanes 9 and 10) but not Th0 (lanes 7 and 8) or Th2 (lanes 11 and 12) cells. In contrast, an increase in H3 trimethylation was apparent only at the 3′UTR of *icos* in Th2 cells (lanes 23 and 24), but was less so in Th0 cells (lanes 19 and 20) and completely obscured in Th1 cells (lanes 21 and 22). These patterns of chromatin accessibility at the *icos* promoter and 3′UTR region strikingly resembled those of T-bet, GATA-3 and NFATc2 binding of these regions in Th1 and Th2 cells. We thus propose that such epigenetic modification of the *icos* locus could provide a mechanistic explanation for the dynamic binding of, especially, NFATc2 to *icos* regulatory DNA, as the selective association of T-bet and GATA-3 to the same regions may be ascribed largely to their lineage-restricted expression.

Taken together, our data unequivocally show that the accessibility of *icos* regulatory regions in different Th subsets is amenable to regulation by histone modification and that the binding pattern of transcription factors to these regions mirrored that of histone modifications in the various Th cells.

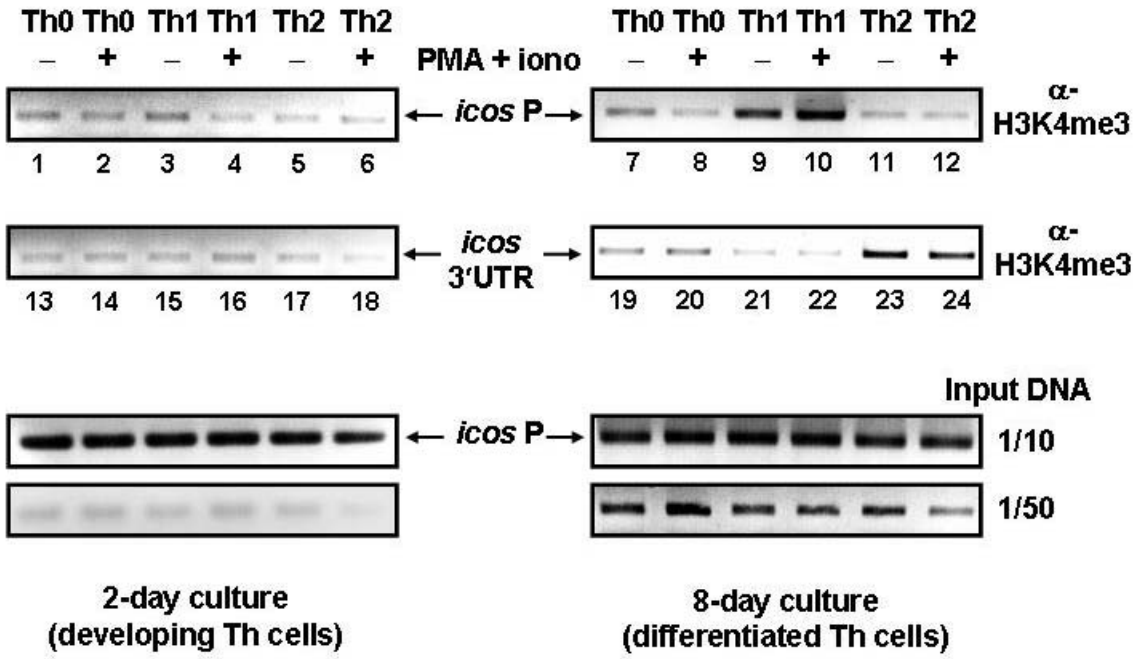


Figure 3.15 Active ICOS transcription correlates with the chromatin accessibility of the *icos* promoter or 3'UTR during Th cell differentiation.

ChIP assays using an Ab recognizing trimethylated histone H3 lysine 4 (anti-H3K4me3) were performed on CD4+ T cells that were differentiated for 2 or 8 days under Th0-, Th1- or Th2-polarizing conditions and left in medium or restimulated with PMA and ionomycin. Primers specific for the *icos* regulatory regions (as in Figure 5) were used in the PCR. Immunoprecipitation using rabbit serum as control yielded negative results (not shown). The same PCR was also performed on input DNA to control for loading of samples.

3.3 Post-transcriptional regulation of ICOS expression by RING-type E3 ubiquitin ligase, roquin

In addition to regulation of ICOS expression at the transcriptional level, there may exist mechanisms that regulate ICOS at the post-transcriptional level. Roquin was first identified, in a forward genetics approach to screen for autoimmune phenotypes generating high titers of antinuclear autoantibodies, as a novel RING-type E3 ubiquitin ligase responsible for repressing self-reactive T_{FH} cells. A methionine (M) to arginine (R) (*sanroque*) mutation in a highly conserved novel protein domain (ROQ domain) of this protein results in excessive numbers of T_{FH} cells and GCs in *sanroque* mice, which subsequently developed a lupus-like pathology (Vinuesa et al., 2005a). Other RING-type ligases important in the induction and maintenance of T cell anergy and immune self-tolerance include c-Cbl, Cbl-b, and GRAIL (Mueller, 2004). Importantly, one of the targets that roquin negatively regulates may be ICOS as *sanroque* CD4+ T cells, whether $CD44^{high}$ or $CD44^{low}$, over-express surface ICOS (Vinuesa et al., 2005a). We therefore hypothesised that since roquin contains a CCCH zinc finger domain shared by RNA-binding proteins such as TTP and Pos-1, it may, via binding its 3′UTR region, regulate ICOS mRNA stability. Alternatively, the prediction of a RING-finger in roquin suggests that it could regulate ICOS protein stability by ubiquitin-mediated proteasomal degradation. To test these hypotheses, we performed preliminary experiments to examine the role of roquin in regulating ICOS expression.

3.3.1 Roquin negatively regulates ICOS mRNA stability

To test whether roquin negatively regulates ICOS mRNA stability, we over-expressed null vector, roquin WT or roquin M199R in EL4 cells and examined ICOS mRNA levels after overnight culture. Enforced expression of roquin WT in EL4 cells dose-dependently depressed ICOS mRNA levels below those observed with null vector control. On the other hand, over-expression of roquin M199R produced a more modest dampening of ICOS mRNA (Figure 3.16A), perhaps consistent with *sanroque* mutation being recessive. We next performed similar experiments but treated the cells with actinomycin D to block *de novo* gene transcription before assessing ICOS mRNA levels over a time course. The decay of *icos* transcripts in cells over-expressing roquin WT occurred more rapidly than in cells harbouring null vector control. Suprisingly, *icos* transcripts in cells bearing roquin M199R appeared to decay marginally less slowly than null vector control, suggesting the *sanroque* mutation in this case to be dominant negative. However, over-expression of roquin M199R, resulting in copies greatly outnumbering the endogenous WT copies, may have caused such an effect, necessitating caution in interpretation of the M199R decay results.

Taken together, our results suggest that roquin may promote the destabilisation of ICOS mRNA in T cells via mechanisms that have yet to be clarified.

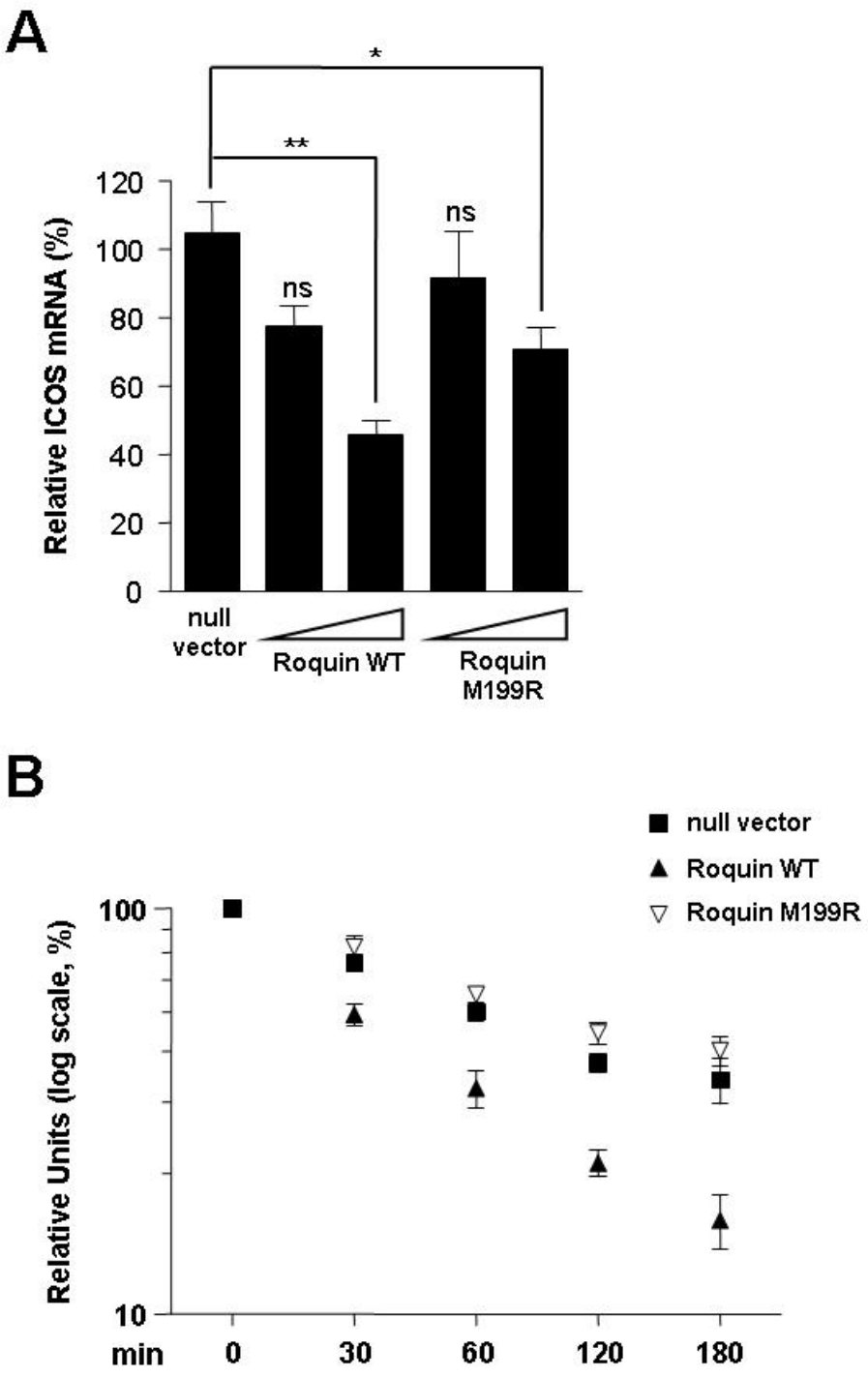


Figure 3.16 The level of *icos* transcript is more severely diminished by ectopic expression of roquin WT than M199R mutant in EL4 cells.

A, EL4 cells transfected with null vector, roquin WT or M199R mutant were stimulated with plate-bound anti-CD3 (1 μg/ml) plus anti-CD28 (4 μg/ml) and harvested after 16 h of culture. RNA was isolated from these cells and subjected to real-time RT-PCR analysis for ICOS. Levels of ICOS mRNA were normalised to those of β-actin. **B**, EL4 cells were transfected and stimulated as above for 6 h before actinomycin D (10 μg/ml) was added and total RNA isolated after 0, 30, 60, 120 and 180 min.
The normalised ICOS mRNA levels in cells **A**, transfected with null vector, or **B**, at time 0 was taken to be 100% and all other normalised mRNA levels were graphed relative to that value. Data shown are mean values ± SEM of triplicate samples and representative of at least 2 independent experiments. On a log scale, the points can be fitted to a one-phase model of exponential decay. * $p < 0.05$; ** $p < 0.001$.

CHAPTER 4

DISCUSSION AND

FUTURE DIRECTIONS

4.1 Transcriptional regulation of ICOS during early phase of T cell activation when TCR/CD28 co-stimulation is dominant

ICOS is a member of the CD28 superfamily of costimulatory receptors that is expressed at low levels on naïve T cells but rapidly up-regulated on activated T cells. Although the *in vivo* functions of ICOS in health and disease are well characterised, very little is known about the molecular mechanisms that regulate ICOS expression. In this study, we present evidence that during the initial phase of T cell activation when TCR and CD28 co-stimulatory signals are dominant, the Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 signalling pathways cooperate to induce ICOS expression.

We first demonstrated that there was a direct correlation between the up-regulation of ICOS cell surface expression and the induction of its mRNA, suggesting that TCR/CD28 signalling directly regulate ICOS expression at the transcriptional level. Both TCR and CD28 stimulation are known to activate a number of downstream signalling molecules, and it is expected that not all of them are involved in regulating ICOS. T cells possess both the Syk and Src family of tyrosine kinases, which are important in proximal TCR signalling. Our data indicate that Src family tyrosine kinases play a major role in TCR-mediated ICOS induction. Of the two major Src kinases present in T cells, namely Lck and Fyn, the latter seems to be critical for inducing ICOS. This reflects a non-redundancy of the two kinases in an important aspect of T cell physiology.

Consistent with the fact that CD28 co-stimulation is known to activate the NFAT family of transcription factors, our results clearly demonstrate an important role for NFATc2 in activating ICOS transcription (Figures 3.3, 3.5 and 3.6). TCR engagement is

known to activate the Ca^{2+}/calmodulin-dependent serine phosphatase, calcineurin, which dephosphorylates NFAT proteins and leads to their translocation into the nucleus. Nuclear NFAT has been shown to activate cytokine gene (e.g. *il-2*) promoters and enhancers, resulting in positive T cell responses (reviewed in refs. (Hogan et al., 2003; Macian et al., 2001)). Our studies expand these previous findings and implicate an additional nontrivial role for the calcineurin-NFATc2 pathway in up-regulating ICOS transcription. This was supported by our data showing the ability of the immunosuppressive drug CsA in blocking ICOS induction at both the cell surface receptor and mRNA levels (Figure 3.2A, B and E) and affecting *icos* promoter activity (Figure 3.5B, left panel). Moreover, exogenously expressed NFATc2 could enhance ICOS mRNA levels (Figure 3.3B) and promoter activity (Figure 3.5C, left panel). Thus, NFATc2 is involved in regulating ICOS expression.

The attenuation of NFATc2 nuclear translocation and *in vivo* occupation of the *icos* promoter region following PP2 treatment of activated T cells and the ability of NFATc2 over-expression to overcome PP2 inhibition of ICOS induction in activated EL4 cells places NFATc2 downstream of Fyn in the signalling axis that induces ICOS expression. Indeed, evidence that binding of the *icos* promoter by NFATc2 is compromised in $Fyn^{-/-}$ CD4+ T cells provided further support for the presence of a Fyn-calcineurin-NFATc2 signalling axis that regulates ICOS transcription.

Other major pathways activated by TCR and CD28 signalling include those involving MAPKs, of which ERK appears to play a major role in ICOS induction. This is inferred from the efficiency of the pharmacological inhibitor U0126 (but not p38 inhibitor SB203580 or JNK inhibitor I) to block surface ICOS (Figure 3.2A) and mRNA

(Figure 3.2B and E) up-regulation, as well as almost ablating *icos* promoter activity (Figure 3.5B, right panel). Co-transfection assays with a constitutively active form of MEK2 (caMEK2) demonstrated that over-expressed caMEK2 enhanced ICOS transcription (Figure 3.3A) and promoter activity (Figure 3.5C). Although we eventually identified a functional target site residing within 148 to 136 bp of the *icos* promoter region responsive to ERK signalling (because mutation of this site severely dampened reporter activity in caMEK2-over-expressed EL4 cells as shown in Figure 3.7D), it is far from clear what the identity of the transcription factors that bind this region are. Further investigation has to be carried out to determine the identity of these factors.

Our current findings also seem to suggest that ERK signals independently and critically to regulate ICOS expression as the over-expression of NFATc2 could not rescue the U0126-mediated block in ICOS induction (Figure 3.3C). Intriguingly, the ERK-responsive transcription factors appear to occupy an *icos* promoter region that is different from that recognised by NFATc2. The over-expression of NFATc2 and caMEK2 synergistically induce ICOS expression at the mRNA level. Finally, mutation of the ERK-sensitive site within the *icos* promoter not only significantly impaired the ability of ERK but also NFATc2 in driving high levels of ICOS transcription. These data collectively support a model of two signalling pathways, namely Fyn-calcineurin-NFATc2 and MEK2-ERK1/2, that independently converge on the *icos* promoter to regulate transcription, with the added complexity that ERK signalling critically initiates ICOS transcription, while NFATc2 augments the ERK-initiated signal.

The study of the transcriptional regulation of ICOS is of immense importance as the human chromosome region 2q33 that harbours the costimulatory cluster of CD28,

ICOS and CTLA-4 genes has been linked to a number of autoimmune diseases. Sequence alignment of regions flanking the first coding exon of the mouse and human *icos* genomic DNA revealed two zones of sequence homology separated by a ~250-bp mouse-specific repetitive DNA region (Ling et al., 2001). Interestingly, we found that the 288-bp region defined in this report to possess promoter activity is located within the mouse-specific repetitive region, implying possible differences in the regulation of mouse as compared to human ICOS transcription. Indeed, while ICOS plays an important role in the development of and is preferentially expressed in mouse Th2 cells (Arimura et al., 2002; Nurieva et al., 2003a), its expression appears to be enhanced in human Th1 cells (Wassink et al., 2004), although a recent study determined that polymorphisms in the human *icos* promoter are significantly associated with allergic sensitisation and Th2 cytokine production (Shilling et al., 2005). It is possible that any variants found within the mouse *icos* promoter could adversely impact NFATc2 and/or ERK-mediated transcription factor binding, leading to aberrant ICOS transcription and Th-skewing. In terms of polymorphisms in the *icos* locus associated with susceptibility to autoimmune diseases, a large family-based association study linked the short genomic interval encompassing the *cd28-ctla-4-icos* locus on human chromosome 2q33 to systemic lupus erythematosus susceptibility in two genome-wide linkage scans (Graham et al., 2006). The same gene region subjected to transmission/disequilibrium tests with coeliac disease, a common multifactorial disease, indicated that four polymorphisms, all located in the *icos* gene, showed evidence for genetic association (Haimila et al., 2004). Another study, using multiethnic DNA panels that represented a wide spectrum of human populations, demonstrated long-range linkage disequilibrium among the *cd28*, *ctla-4* and *icos* genes.

A large fraction of the variation found at the *cd28*, *ctla-4* and *icos* promoter could be attributed to the presence of extended haplotypes shaped by demographic events, environmental adaptation and/or microbial encounter (Butty et al., 2007). What these longitudinal studies emphasise is the relevance of polymorphisms in the *icos* cis-regulatory regions to interpreting its association with immune/autoimmune responsiveness. Of course, not all polymorphisms identified have functional significance. For example, no association of the microsatellite polymorphisms in the *icos* gene with susceptibility to type 1 diabetes was found when screening the IDDM12 locus (coincident with *cd28-ctla-4-icos* locus) on human chromosome 2q33 (Ihara et al., 2001). Homologous to the human *cd28-ctla-4-icos* locus, linkage analysis and congenic mapping in NOD mice have identified a syntenic region containing *ctla-4* and *icos* genes that is a susceptibility locus for type 1 diabetes, Idd5.1, on mouse chromosome 1. Higher ICOS and lower full-length CTLA-4 expression were observed on activated NOD T cells compared with C57BL/6 (B6) and C57BL/10 (B10) T cells, correlating with greater IL-10 production which may be responsible for the less severe EAE in NOD mice although they are predisposed to diabetes (Greve et al., 2004). This is an example *par excellence* illustrating that variations in the *cd28-ctla-4-icos* locus has direct effects on ICOS protein expression in T cells, underscoring the importance of delineating *icos* regulatory regions. Differences in ICOS expression, brought about by differences in its transcriptional regulation, could influence immune responses in allergic and autoimmune disease conditions.

In conclusion, this work has defined two molecular pathways downstream of TCR and CD28 signalling, namely Fyn-calcineurin-NFATc2 and MEK2-ERK1/2, in the

regulation of mouse ICOS transcription (Figure 4.1). Furthermore, we demonstrated the binding of ICOS promoter by NFATc2 and delineated an ERK-responsive site in the promoter which likely binds yet-to-be-identified transcription factors. Because ICOS plays an important role in the pathogenesis of many diseases, understanding the molecular mechanisms by which ICOS is regulated may present new opportunities for therapeutic intervention of pathologies involving this costimulatory molecule. In this regard, our current studies could shed light on human studies investigating the association of autoimmune diseases such as multiple sclerosis and type I diabetes with polymorphisms in the *cd28*/*ctla-4*/*icos* gene cluster briefly discussed above. Future work will centre on discovering other possible signalling pathways and transcription factors that regulate ICOS expression, especially those signalling pathways associated with the polarisation of T cells into different T cell subsets, which was carried out in the second part of the study.

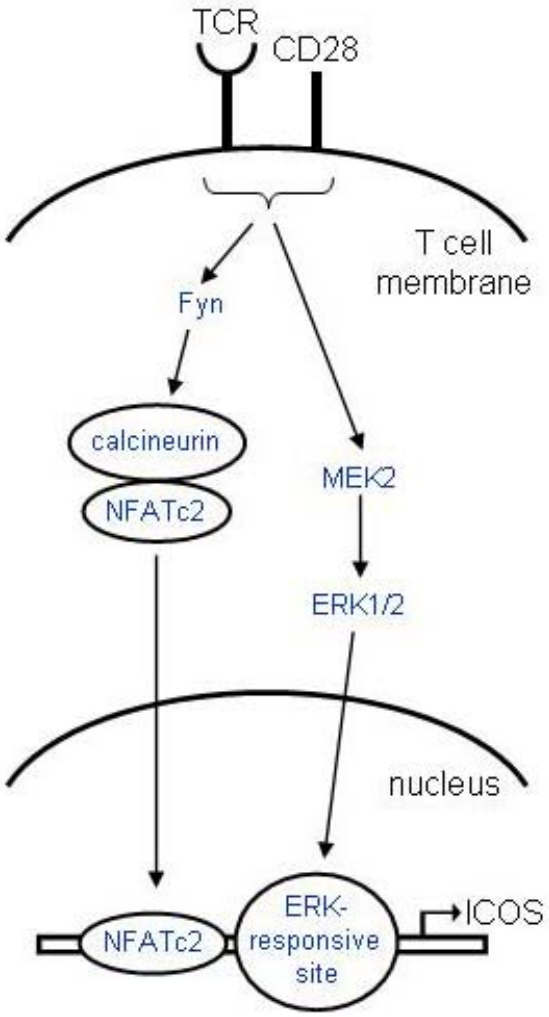


Figure 4.1 Proposed model for transcriptional regulation of ICOS expression by Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 signalling in T cells.

4.2 Transcriptional regulation of ICOS during T cell differentiation when lineage-determining cytokines and transcription factors are dominant

Early studies interrogating the roles of costimulatory molecules such as CD28, CTLA-4, ICOS and PD-1 in Th differentiation attempted to define precisely the contribution of each receptor to the specification of a certain lineage fate (Dong et al., 2003; Greenwald et al., 2005). ICOS, as mentioned earlier, was initially believed to be more important for Th2-type immune responses as ICOS deficient T cells exhibited defects in mediating B-cell antibody class switching to IgG1 and IgE during the primary response to protein antigen *in vivo* and in producing IL-4 when restimulated by the same antigen *in vitro*, although they produced normal or enhanced levels of IFN-γ (Dong et al., 2001a; Tafuri et al., 2001). A report subsequently confirmed tight correlation between ICOS expression in CD4+ T cells and their production of Th2-biased cytokines (Lohning *et al.*, 2003). Such a heightened importance for ICOS in Th2-driven immunity was evident from parasitic infection models: ICOS exerts considerable control of protective Th2 responses against *Leishmania major* and *Nippostrongylus brasiliensis*. Genetic ablation of this costimulatory pathway in or its inhibition by administration of anti-B7RP-1 mAb to mice significantly suppressed IL-4, IL-5 and IL-10 secretion from lymph node cells and serum IgE levels (Miyahira et al., 2003). More recently, it was found that ICOS does not appear to maintain a chronic cellular Th2 response during live infection by the filarial nematode, *Brugia malayi*, as the development of IL-4 producing Th2 cells and eosinophil recruitment were normal in ICOS deficient mice. Rather, in a chronic helminth infection, the primary function of ICOS might be to drive antibody switching toward type 2 isotypes, as this component of the immune response was

defective (Loke et al., 2005). Despite substantial evidence implicating ICOS in Th2 responses, numerous other studies suggest ICOS plays a nontrivial role in some Th1-mediated pathologies, most notably autoimmune disorders and graft-versus-host disease (GVHD). For instance, blockade of ICOS/B7-H2 interaction during different phases of EAE produced reciprocal disease outcomes (Rottman et al., 2001). ICOS blockade by treatment with anti-B7RP-1 mAb or genetic inactivation substantially reduced clinical progression of murine experimental autoimmune uveoretinitis (Usui et al., 2006b). Similar ICOS inhibition in mice afflicted with GVHD suppressed intragraft T cell activation and cytokine production, resulting in prolonged allograft survival (Ozkaynak et al., 2001), whereas targeting of ICOS expressed on alloreactive donor T cells inhibited GVHD and promoted bone marrow engraftment in recipient mice (Taylor *et al.*, 2005). ICOS signalling appears to function differently in humans compared to mice. Human recipients of alloreactive T cells lacking ICOS during an allogeneic hematopoietic stem cell transplantation (allo-HSCT) demonstrated significantly less GVHD morbidity and mortality as these ICOS deficient T cells were deviated toward a Th2 phenotype (Hubbard et al., 2005). In addition, unlike mouse deficiency in ICOS, human deficiency was reported to compromise naïve, switched and memory B cell generation while preserving T cell subset distribution, and is associated with common variable immunodeficiency or CVID (Grimbacher et al., 2003; Warnatz et al., 2005).

There are also studies suggesting that ICOS controls Th cell polarisation and not merely the function of polarised Th cells. For example, blocking ICOS alone had a limited but significant capacity to downregulate Th subset development upon infection with lymphocytic choriomeningitis virus (LCMV) and vesicular stomatitis virus (VSV).

ICOS inhibition in the absence of CD28 further reduced Th1/Th2 polarisation (Kopf et al., 2000). In a clinical model of experimental autoimmune myasthenia gravis (EAMG), ICOS deficiency conferred resistance to disease development, blunted lymphocyte proliferation and both Th1 and Th2 differentiation (Scott et al., 2004). A separate study found that Th1-mediated acute GVHD was inducible despite ICOS deficiency but not Th2-mediated chronic GVHD, suggesting the expression levels of ICOS are critical for determining the polarisation of Th cell function (Watanabe et al., 2006). A concept that has increasingly gained traction is one in which ICOS is not involved in dictating polarity of the Th response but regulates instead the expansion of Th subsets. ICOS-deficient mice infected with *Trichuris muris* or *Toxoplasma gondii* exhibited delay in protective immunity that was associated with a defect in infection-induced increases in the number of activated and proliferating CD4+ T cells (Wilson et al., 2006). Two separate studies examining the role of ICOS in transplantation immunity showed its importance in expanding allogeneic donor T cells. In a vascularised mouse cardiac allograft model, ICOS blockade suppressed alloreactive CD4+ T cell expansion and alloantibody production (Harada et al., 2003) while similar blockade during allogeneic bone marrow engraftment inhibited expansion of GVHD-causing effector T cells in secondary lymphoid and GVHD target organs (Taylor et al., 2005).

The complex function of ICOS in Th1- and Th2-mediated immunity revealed by the above findings prompted us to investigate whether ICOS is differentially regulated in Th1 and Th2 cells and if so, to decipher the mechanisms involved. We first showed, after differentiating naïve Th cells under Th0, Th1 and Th2-polarising conditions, that ICOS is expressed highest in terminally differentiated Th2, lowest in Th0 and intermediate

between the two in Th1 cells. In addition, ectopic expression of T-bet and GATA-3 could enhance ICOS expression both in EL4 as well as in primary CD4+ T cells and co-expression of NFATc2 led to synergy with either of them to enhance ICOS expression in the correct differentiating lineage. Hence, T-bet and GATA-3, along with NFATc2, which was previously demonstrated to drive ICOS transcription during the early phase of TCR/CD28-mediated stimulation, play important roles in driving ICOS expression in Th1 and Th2 cells.

The critical roles of T-bet and GATA-3 in the control of Th1 and Th2 lineage-associated cytokine genes have been well documented. For instance, T-bet is known to activate the transcription of *ifn-γ* through binding its promoter and enhancer (Lee et al., 2004a; Shnyreva et al., 2004) while GATA-3 is known to regulate the IL-4, -5 and -13 cytokine cluster (Murphy and Reiner, 2002; Zhou and Ouyang, 2003). On the other hand, NFATc2 has been shown to bind the *ifn-γ* 5′ conserved noncoding sequences and promoter in Th1 cells and the *il-4* enhancer in Th2 cells (Agarwal et al., 2000; Lee et al., 2004a). In this study, we showed that a single costimulatory gene, *icos*, is regulated by both T-bet and GATA-3 via distinct mechanisms in Th cells, with the former acting on the promoter while the latter via a 3′UTR element of *icos*.

What role might ERK signalling, shown in the first part to be critical for initiating ICOS transcription during TCR/CD28-mediated activation, have in the regulation of ICOS transcription during Th cell differentiation? The nuclear activity of ERK is induced more rapidly in developing Th1 compared to Th2 cells, raising the possibility that ERK signalling may play a dominant role in ICOS regulation in Th1 compared to Th2 cells, at least during the developing phase. Consistent with this, enhanced ERK activity in

terminally differentiated T-bet$^{-/-}$ Th1 cells may compensate somewhat for the loss of T-bet in driving ICOS mRNA levels in these cells (Figure 3.11B). ERK has been reported to stabilise GATA-3 protein (possibly through Ser/Thr phosphorylation) by preventing its Mdm2-mediated and ubiquitin-dependent proteasomal degradation in polarised Th2 cells (Yamashita et al., 2005). We think that such a mechanism does not sufficiently account for high ICOS transcription in Th2 cells, as pharmacological inhibition of ERK activity by U0126 in these cells led to virtually no effects on ICOS mRNA levels (data not shown). Hence, although efficient TCR-mediated activation of calcineurin-NFAT and the Ras-ERK MAPK signalling cascades are required for Th2 differentiation (Yamashita et al., 1999; Yamashita et al., 2000), the function of ERK in Th2-specific ICOS transcription remains unclear.

Separately, NF-κB has also been demonstrated to play a critical role in GATA-3 protein expression and Th2 differentiation in a mouse model of allergic airway inflammation (Das et al., 2001). Specifically, p50$^{-/-}$ mice failed to induce GATA-3 expression under Th2-differentiating conditions but showed intact T-bet expression and IFN-γ production under Th1-polarising conditions. Moreover, developing but not already committed Th2 cells pretreated with SN50 (an NF-κB competitor peptide) but not a mutant peptide prevented nuclear translocation of NF-κB and abolished GATA-3 binding to its specific motif(s) on the IL-5 promoter. This, together with the presence of putative NF-κB binding sites on the GATA-3 promoter, raises the possibility that inhibiting NF-κB activity in Th2 cells may also block ICOS up-regulation in these cells, although we observed no such effect on ICOS induction in SN50-treated Th2 cells (data not shown). Given that GATA-3 is subjected to multiple levels of transcriptional and

translational control, this likely precludes a straightforward interpretation of results yielded by simply treating developing Th2 cells with SN50 and assessing ICOS mRNA expression.

SAP (SLAM-associated protein) couples Fyn to SLAM (Signalling Lymphocyte Activation Molecule) receptors in T and NK cells (Latour and Veillette, 2004; Chan et al., 2003) and a loss-of-function mutation or deletion of *sh2d1a*, the gene encoding SAP, is responsible for the pathogenesis of X-linked lymphoproliferative syndrome (XLP) or Duncan's disease in humans upon primary Epstein-Barr virus (EBV) infection, leading to significant mortality (Engel et al., 2003). While mice lacking expression of SAP recapitulate several features of XLP following viral infection (Czar et al., 2001; Wu et al., 2001), mice expressing a Fyn-binding mutant of SAP phenocopy SAP deficient mice in terms of defective Th2 cytokine production and impaired TCR-mediated induction of GATA-3 (Cannons et al., 2004; Davidson et al., 2004). In particular, when naïve Th cells from SAP and $Fyn^{-/-}$ mice were stimulated using anti-CD3 and/or anti-CD28 Abs, GATA-3 transcript levels were markedly reduced as compared to WT cells. Furthermore, human XLP CD4+ T cells failed to up-regulate ICOS expression efficiently upon stimulation (Ma et al., 2005), resulting in insufficient IL-10 production and contributing to hypogammaglobulinemia. This, together with our earlier work which identified Fyn to be the upstream kinase activating NFATc2 and subsequent ICOS transcription, led us to hypothesise that a SAP-Fyn-GATA-3 pathway may operate to drive high levels of ICOS transcription in Th2 cells. However, retroviral reconstitution of the Fyn-binding mutant of SAP in SAP deficient CD4+ T cells rescued successfully the SAP-associated defects in ICOS expression and humoral responses, but preserved the Th2 cytokine defects

(Cannons et al., 2006). This suggests that SAP likely mediates ICOS up-regulation via yet to be discovered signalling pathway(s) independent of its Fyn-binding function. Consistent with this, inhibition of Fyn signalling by the broad Src family kinase inhibitor PP2 in developing Th2 cells had modest effects on cell surface ICOS expression in these cells (data not shown).

It is becoming increasingly appreciated that besides the classical mechanism of *trans*-acting transcription factors binding to *cis*-regulatory DNA regions to activate or repress gene transcription, epigenetic modifications at the chromatin level are essential to initiate and maintain transcriptional permissiveness or silencing. In differentiating Th cells, antigen receptor and cytokine signals act in synergy to drive reciprocal activation and silencing of the IFN-γ and IL-4 cytokine gene loci, alterations which are heritably maintained in maturing Th1 or Th2 cells and their progeny. Unpolarised transcription and chromatin remodelling of the cytokine genes in naïve Th cells is followed by consolidation of epigenetic changes and the establishment of self-reinforcing transcription factor networks in differentiated Th cells (Ansel et al., 2003). Among the epigenetic changes in chromatin structure are changes in nucleosome positioning and conformation; changes in the histone content of nucleosomes; and changes in modification status of histones and DNA. The amino tails of histones, as well as some internal sites, are amenable to a large array of covalent modifications including phosphorylation, acetylation, methylation and ubiquitination. This relates to another interesting finding in our study: the dynamic manner in which the broadly expressed NFATc2 partners with Th-restricted T-bet or GATA-3 to drive *icos* transcription. In the first 2 days of differentiation, NFATc2 activates *icos* transcription non-selectively

through its promoter, but not 3′UTR, in all Th lineages. As Th cells commit to their developmental fate, NFATc2 cooperates with T-bet via the promoter and GATA-3 via a 3′UTR element to induce ICOS expression in Th1 and Th2 cells, respectively. The binding pattern of T-bet/NFATc2 complex to the *icos* promoter and GATA-3/NFATc2 to the 3′UTR element coincides precisely with the chromatin accessibility of these regulatory regions as Th cells mature, as assessed by the trimethylation of histone 3 lysine 4. Histone trimethylation correlates with accessible or active gene loci (Lachner and Jenuwein, 2002) and is a more stable and reliable marker of active transcription than histone acetylation (Bannister et al., 2002). Such a process is likely to be carried out by histone methyltransferases, whereas the role of the converse process, histone demethylation, in reversing chromatin accessibility at *icos* regulatory regions in the appropriate Th cell subset remains to be explored. Downstream of STAT-1, T-bet is proposed to induce chromatin remodelling of the initially repressed IFN-γ locus in developing Th1 cells, resulting eventually in acute transcription when the cells become more Th1-committed (Mullen et al., 2001). Similarly, in developing Th2 cells, GATA-3, acting downstream of STAT-6, induces heritable remodelling of the *il-4* locus (Ouyang et al., 1998; Takemoto et al., 2000). GATA-3 also induced IL-4-independent changes of the chromatin structure, specifically histone acetylation, at the *il-10* locus, but did not transactivate the *il-10* promoter despite being recruited *in vivo* to two locations on the *il-10* locus (Shoemaker et al., 2006). Whether T-bet or GATA-3 participates similarly in promoting transcriptional competency of the *icos* promoter or 3′UTR, in addition to directly transactivating it, is an open question. Nevertheless, it is highly likely that chromatin remodelling of the regulatory regions of *icos*, like those of cytokine genes, and

the consequent DNA-association patterns of T-bet/NFATc2 and GATA-3/NFATc2 were conditioned by the combined action of TCR-mediated signalling and polarising cytokines responsible for moulding Th identity.

Another example of chromatin modification providing exquisite specification of transcriptional competency is the superinduction of IL-10 production by macrophages stimulated in the presence of immune complexes. In resting cells, which make essentially no cytokines, the *il-10* promoter is associated with histones containing little or no detectable modifications. Pharmacologically inhibiting activated ERK abolished rapid and dynamic phosphorylation of serine 10 on H3 (H3ser10) at specific sites on the *il-10* promoter, decreasing its accessibility to transcription factors generated in response to stimulation. A histone phosphorylation event was critical to efficiently induce *il-10* gene expression in macrophages, thus defining a unique way in which the expression of a cytokine gene is regulated in macrophages (Lucas et al., 2005; Zhang et al., 2006). It is plausible that a similar mechanism may operate at the *icos* promoter during Th1 differentiation, consistent with the more rapid dynamics of nuclear ERK accumulation in Th1 compared to Th2 cells. In agreement with ERK being recruited to chromatin and transcribed regions of genes, a recent report described the physical interaction of most MAPKs and protein kinase A subunits with the genes that they regulated, although in the yeast (*Saccharomyces cerevisiae*) genome (Pokholok et al., 2006).

A central role for ICOS in GC formation and effective development of humoral immunity has been well documented (Dong et al., 2001b; McAdam et al., 2001; Shilling et al., 2006; Tafuri et al., 2001). T_{FH} cells constitute an important part of the GC reaction and express high levels of ICOS, SAP, SLAM and CD84 mRNA (Chtanova et al., 2004;

Vinuesa et al., 2005a). These cells help GC B cells class-switch and differentiate into plasma cells. Given the unique CXCR5+ and IL-21-secreting signature of T_{FH} cells that partly distinguishes them from IFN-γ-producing Th1 and IL-4-producing Th2 cells, it will be intriguing to elucidate the signalling pathways and transcription factors that regulate ICOS expression in these cells, bearing in mind the much greater phenotypic overlap between T_{FH} and Th2 compared to Th1 cells. NFATc2 was shown to bind and directly activate the *il-21* promoter in Th2 cells, while T-bet represses IL-21 transcription by inhibiting binding of NFATc2 to the same promoter in Th1 cells (Mehta et al., 2005), suggesting NFATc2 may feature crucially in T_{FH}-specific ICOS regulation. Of course, a major caveat is the technical difficulty in distinguishing between cells of the T_{FH} and Th2 lineages, and subsequently determining which aspects of ICOS regulation are specific to each lineage.

Our data so far indicate that the cytokine networks and transcriptional apparatus initiating and reinforcing Th lineage commitment appear to instruct ICOS expression in a Th-specific manner which, together with earlier work demonstrating roles for ICOS in Th subset polarisation (Kopf et al., 2000) and the transcriptional control of Th2 differentiation (Nurieva et al., 2003a), provide the basis for feed forward amplification linking ICOS expression and Th cell differentiation (Figure 4.2A and B). Of course, Th1 and Th2 cells are by no means the only Th cell subsets found in the adaptive immune system. The concept of Th differentiation, based on the traditional Th1/Th2 dichotomy, has been under intense scrutiny and subjected to major revisions in recent years, introducing new complexities into the field of T cell biology. The list of possible Th cell subsets has grown to include T_{reg} (which can be further categorised into natural or

inducible T_{reg}, the latter comprising partly Tr1 and Th3 subpopulations), the follicular helper T (T_{FH}), IL-17-producing Th17 and the speculative IL-25-producing Th25 lineages (Tato et al., 2006). All these Th lineages likely express or can be induced to express ICOS. However, it is presently unclear how the level of ICOS expression compare between lineages and if T-bet, GATA-3 or other transcription factors regulate ICOS expression via different pathways in these subsets. Consider the example of Th17 cells, which have been causally linked to several inflammatory and autoimmune diseases including collagen-induced arthritis and experimental autoimmune myasthenia gravis, the pathogeneses of which were reportedly dependent on ICOS (Iwai *et al.*, 2002; Scott *et al.*, 2004). Both CD28 and ICOS were found to be required for the generation of effector CD4+ T cells that produce IL-17 (Park *et al.*, 2005). However, data accumulated so far suggest that the Th17 developmental pathways are independent of STAT-1/4 and T-bet as well as STAT-6 and GATA-3, which are required respectively for Th1 and Th2 differentiation. Are T-bet and GATA-3 therefore dispensable for Th17-intrinsic regulation of ICOS? A very recent study reported surface ICOS expression to be higher in Th17 compared to Th1 cells (Nakae et al., 2007), suggesting NFATc2 and ERK signalling may contribute more substantially to and/or unknown factors other than T-bet and GATA-3 direct ICOS regulation in this cell type. Combined actions of the pleiotropic TGF-β and pro-inflammatory IL-6 cytokines were shown to induce Th17 development (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006), while the retinoic acid-related orphan receptor ROR-γt was established as necessary and apparently sufficient for Th17 commitment (Ivanov *et al.*, 2006). What is the extent of contribution, if any, by these factors to ICOS expression in the Th17 lineage? Besides Th17 cells, what possible

mechanisms underlie ICOS regulation in T_{reg} cells, particularly in the CD4+CD25+Foxp3+ population expressing high levels of ICOS and are high producers of IL-10?

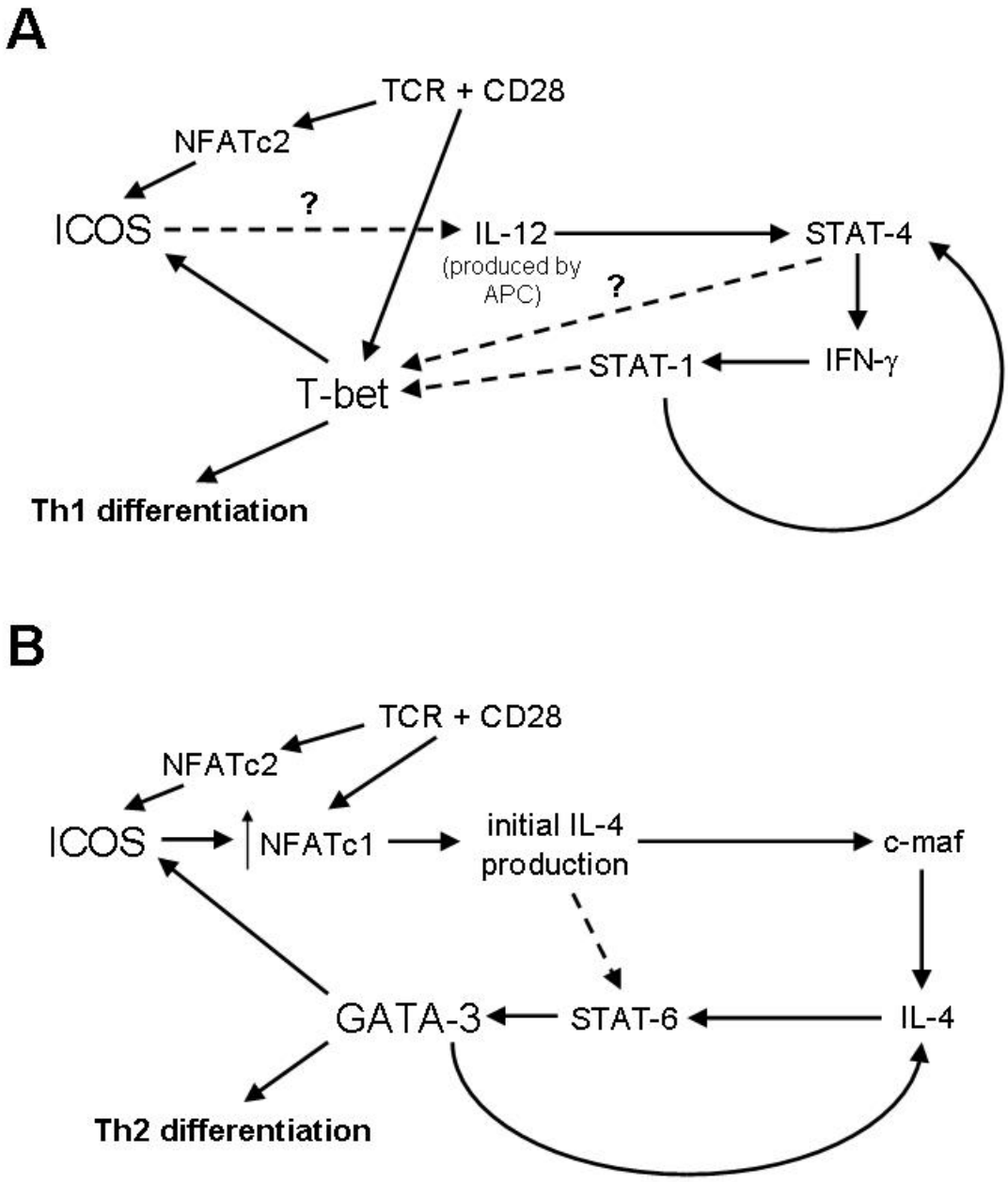


Figure 4.2 Model of feed-forward regulatory circuits linking ICOS expression, cytokine networks and transcriptional machinery directing A, Th1 and B, Th2 cell differentiation.

4.3 Post-transcriptional regulation of ICOS by E3 ubiquitin ligase, roquin

Apart from regulation at the transcriptional level, post-transcriptional mechanisms may also govern ICOS expression. As mentioned before, in *sanroque* mice carrying a mutated *Rc3h1* gene encoding for the roquin protein, T_{FH} cells hyper-proliferated and GC development was exacerbated, and these mice eventually succumbed to autoimmune disease (Vinuesa et al., 2005a), which emphasises the potential importance of T_{FH} cells in regulating autoantibody production and autoimmune pathogenesis (Vinuesa et al., 2005b). Importantly, *sanroque* CD4+ T cells expressed higher levels of ICOS compared to their wildtype counterparts, suggesting roquin may play a role in limiting ICOS expression. Roquin possesses amino-terminal RING-1 and CCCH zinc finger domains, the former being shared by E3 ubiquitin ligases and the latter by RNA-binding proteins such as Tristetraprolin (TTP). TTP is known to bind to the 3′UTR of target genes and facilitate the processing, stabilisation and translation of their mRNAs. For example, SAP mRNA contains AUUUA sequences in the 3′UTR that are targeted for ubiquitin-dependent degradation by RNA binding proteins such as AUF1 and HuR (Okamoto et al., 2004b). It is therefore conceivable that roquin may modulate ICOS expression post-transcriptionally through affecting mRNA and/or protein stability.

We show that roquin plays a nontrivial role in regulating ICOS expression at the post-transcriptional level. In particular, roquin WT, but not M199R mutant, efficiently limited ICOS mRNA levels in EL4 cells, suggesting that the ROQ domain of roquin is in part important for fulfilling this role. We also observed that *icos* transcripts in EL4 cells over-expressing roquin WT decayed with faster kinetics than in cells bearing null vector control, suggesting roquin negatively regulates ICOS mRNA stability and elucidating a

novel pathway by which ICOS expression is modulated. Consistent with roquin being a negative regulator of ICOS expression, we found roquin mRNA to be expressed highest in Th0, followed by Th1 and Th2 cells (data not shown). Via what mechanisms roquin exerts its effects on ICOS mRNA and if these mechanisms are Th-specific are unanswered questions that will be the subject of future research.

4.4 Conclusion

Our results have indicated that the initial TCR/CD28-dependent regulation of ICOS is mediated by Fyn-calcineurin-NFATc2 and MEK2-ERK1/2 signalling. As Th cells differentiate along separate lineages under the increasing influence of polarising cytokines and Th-associated transcriptional machinery, TCR-responsive NFATc2 induces ICOS transcription during the early stage of Th development, followed by Th-specific determination of the ICOS expression signature by STAT-responsive T-bet and GATA-3 as differentiation progresses to the terminal stage. What these results appear to inform is a possible model in which the early induction of ICOS in TCR/CD28-stimulated naïve Th cells is important to some extent in determining Th polarity, depending on the pathological context. When the dominant Th response is established, ICOS expression may be modulated according to requirements for its signalling in Th1- and Th2-mediated immune responses. As noted earlier, ICOS is expressed at high levels in T_{FH} and IL-10-producing T_{reg} cells, consistent with its crucial roles in facilitating T_{FH} cell help for B cell antibody class-switching in GCs and T cell-mediated tolerance, respectively. Because ICOS is implicated in the immunopathogenesis of a myriad of infectious, inflammatory and autoimmune diseases, understanding the mechanisms that govern its expression in

the various Th subsets that play differential roles in disease development may shed light on how to manipulate and fine-tune the T cell response toward desirable therapeutic outcomes, with the objective of improving host immunity whilst avoiding autoimmunity. We propose that transcriptional mechanisms are important to rapidly induce ICOS expression in naïve host T cells activated by antigen-stimulated APCs during the early phase of the immune response against pathogens, whereas post-transcriptional mechanisms are important for repressing ICOS mRNA or protein levels following immune clearance of the pathogen, the failure of which can lead to prolonged T cell activation and autoimmunity.

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LIST OF PUBLICATIONS

1. Tan,A.H., Wong,S.C., and Lam,K.P. (2006). Regulation of Mouse Inducible Costimulator (ICOS) Expression by Fyn-NFATc2 and ERK Signaling in T Cells. J. Biol. Chem. *281*, 28666-28678.

2. Yu,D., Tan,A.H., Hu,X., Athanasopoulos,V., Simpson,N., Silva,D.G., Hutloff,A., Giles,K.M., Leedman,P.J., Lam,K.P., Goodnow,C.C., and Vinuesa,C.G. (2007). Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature *450*, 299-303.

3. Tan,A.H., Goh,S.Y., Wong,S.C., and Lam,K.P. (2008). T helper cell-specific regulation of inducible costimulator expression via distinct mechanisms mediated by T-bet and GATA-3. J. Biol. Chem. *283*, 128-136.