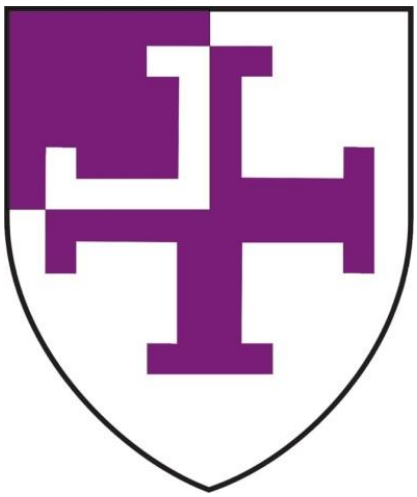
Regulatory T cells in Rheumatoid Arthritis: the Role of TNF-α Receptor Signaling and DNA Methylation



Wen-Yi Tseng

St Cross College

& Nuffield Department of Orthopaedics,

Rheumatology and Musculoskeletal Sciences,

University of Oxford

Supervisors: Dr. Richard Williams & Prof. Sir Marc Feldmann

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Abstract

Anti-TNF-α therapy has revolutionised the management of inflammatory arthritis, leading to improvement of symptoms and signs of rheumatoid arthritis, ankylosing spondylitis and psoriatic arthritis, as well as inhibiting radiographic progression in rheumatoid arthritis, and psoriatic arthritis. However, despite its success in the majority of patients with inflammatory arthritis, about 20-40% of patients are resistant to anti-TNF-α treatment and few patients (10% or less) achieve long-term remission. Several studies suggest that TNF-α receptor 2 (TNFR2) plays an immunoregulatory role in arthritis and that the interruption of TNF/TNFR2 interaction contributes to resistance to anti-TNF-α therapy and to some of the adverse effects associated with anti-TNF-α therapy. The objective of the work presented in this thesis was to gain a deeper understanding of TNFR signalling in rheumatoid arthritis with particular emphasis on a sub-population of lymphocytes known as regulatory T cells (Tregs).

Firstly, an association was shown between TNFR2 signaling and TGF-β in regulating the expression of the Treg associated transcription factor, FoxP3. I further assessed the effect of TNFR signaling on CpG methylation at the *FoxP3* locus by bisulfite sequencing. The result showed that TNFR2 deficiency leads to increased level of DNA methylation in the proximal promoter of *FoxP3*.

Secondly, I investigated the role of TNFR signaling in arthritis. As expected, $TNFR1^{-/-}$ mice were found to be relatively resistant to arthritis. In contrast to $TNFR1^{-/-}$ mice, higher incidence and increased disease activity were observed in $TNFR2^{-/-}$ mice compared to wild-type mice. In addition, the results showed that TNFR2 deficiency leads to decreased numbers of Tregs, reduced FoxP3 expression of Tregs, reduced expression of immune inhibitory markers and increased production of pro-inflammatory cytokines in arthritis.

Thirdly, I investigated the therapeutic potential of DNA demethylating agents in the treatment of RA. It showed that the DNA demethylating drug, decitabine, ameliorated collagen-induced arthritis (an animal model of rheumatoid arthritis) and promoted Treg suppressive function whilst inhibiting pathogenic Th1/Th17 cells.

These findings highlight the therapeutic potential of strategies to boost Tregs by TNFR2 agonists or DNA demethylating agents and suggest that it may be possible to achieve long term inhibition of pathogenic inflammation using this approach.

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Declaration:
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Abbreviations

Abbreviation	Full
2-ME	2-Mercaptoethanol
ACPA	Anti-citrullinated protein/peptide antibodies
ANOVA	Analysis of Variance
ADAM17	A Disintegrin and Metallopeptidase Domain 17
AhR	Aryl hydrocarbon receptor
AS	Ankylosing Spondylitis
APC	Antigen Presenting Cell
CD	Cluster of Differentiation
CAIA	Collagen Antibody Induced Arthritis
cDNA	Complementary Desoxyribonucleic Acid
CFA	Complete Freund's Adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
CIA	Collagen Induced Arthritis
CIAP	Cellular Inhibitor of Apoptosis Protein
CTLA-4	Cytotoxic T-lymphocyte-associated Protein 4
CXCL-1	Chemokine (C-X-C motif) ligand 1
DMARDs	Disease Modifying Antirheumatic Drugs
DNA	Desoxyribonucleic Acid
dNTP	Deoxyneucleotide Triphosphates
DNMT	DNA MemethylTransferase

DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EAE	Experimental autoimmune encephalomyelitis
FoxP3	Forkhead Box P3
FADD	Fas-associated Protein with Death Domain
FACS	Fluorescence-activated Cell Sorting
FSC	Forward-Scattered Light
$FLIP_L$	FLICE-like Inhibitory Protein
GITR	Glucocorticoid-induced TNFR-related Protein
GM-CSF	Granulocyte-macrophage Colony-stimulating Factor
GVHD	Graft-Versus-Host Disease
HDAC	Histone Deacetylase Activity
HLA	Human leukocyte antigen
HPRT	Hypoxanthine-guanine Phosphoribosyltransferase
HOIL1	Haeme-Oxidized IRP2 ubiquitin Ligase 1
HOIP	HOIL1-ineracting protein
IBD	Inflammatory Bowel Disease
IFN-γ	Interferon-γ
IL-1	Interleukin 1
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-8	Interleukin 8

IL-10	Interleukin 10
IL-17	Interleukin 17
IL-21	Interleukin 21
IL-23	Interleukin 23
IL-23R	Interleukin 23 Receptor
ILCs	Innate Lymphoid Cells
iTregs	Induced Regulatory T Cells
JIA	Juvenile Idiopathic Arthritis
JNK	c-JUN N-termianl kinase
LAG3	Lymphocyte-activation gene 3
LUBAC	Linear Ubiquitin Chain Assembly Complex
LNs	Lymph Nodes
mAb	Monoclonal Antibody
MAPKs	Mitogen-activated Protein Kinase
MFI	Median Fluorescent Intensity
MHC	Major Histocompatibility Complex
NEMO	Nuclear factor κB Essential Modulator
mRNA	Messenger Ribonucleic Acid
MTX	Methotrexate
MLKL	Mixed Lineage Kinase domain-like protein
MSD	MesoScale Discovery MultiSpot assay
NFκB	Nuclear Factor Kappa-light-chain-enhancer of Activated B cells
MS	Multiple Sclerosis

NKT cells	Natural Killer T cells
nTregs	Natural Regulatory T cells
PAD	Pepetidyl arginine deiminase
PD1	Programmed Death 1
PD-L1	programmed death Ligand 1
PsA	Psoriatic Arthritis
PCR	Polymerase Chain Reaction
PMA	Phorbol myristate acetate
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RORγt	Retinoid-Acid Receptor-related Orphan Receptor Gamma t
RPMI medium	Roswell Park Memorial Institute Medium
RIPKI	Receptor-Interacting serine/threonine-Protein Kinase I
RT	Reverse Transcription
RT-qPCR	Reverse Transcription Polymerase Chain Reaction
SEM	Standard Error of the Mean
SLE	Systemic Lupus Erythematosus
SSC	Side-scattered Light
STAT3	Signal Transducer and Activator of Transcription 3
SHARPIN	SHANK-Associated RH domain-interacting protein
TACE	TNF-converting enzyme
TAKI	Transforming Growth Factor-Activated Kinase I

TAB	TAKI-binding protein
TCR	T Cell Receptor
Teff	Effector T cells
TGF-β	Transforming Growth Factor Beta
Th1	Type 1 T Helper Cells
Th17	Type 17 T Helper Cells
TLR	Toll Like Receptor
TNF-α	Tumour Necrosis Factor-α
sTNF-α	Soluble form of Tumour Necrosis Factor-α
mTNF-α	Membrane form of Tumour Necrosis Factor-α
TNFR1	Tumour Necrosis Factor Receptor 1
TNFR2	Tumour Necrosis Factor Receptor 2
TNFRSF1A	Tumor Necrosis Factor Receptor Superfamily Member 1A
TNFRSF1B	Tumor Necrosis Factor Receptor Superfamily Member 1B
TRADD	Tumor Necrosis Factor Receptor Type 1-associated DEATH Domain Protein
TRAF	Tumor necrosis factor receptor-associated factor
Tregs	Regulatory T cells
TSDR	Treg-Specific Demethylated Region
UE	Upstream Enhancer
WT	Wild Type

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Chapter 1: Introduction

1.1 Overview of Rheumatoid Arthritis

1.1.1 Epidemiology, Etiology and Genetics

Rheumatoid arthritis (RA) is the most common type of arthritis following osteoarthritis and gouty arthritis and affects around 0.5-1% of the world population[1]. The prevalence of RA can vary from 6% in native American-Indian populations to around 0.1-0.3% in populations from rural Africa and South-East Asia, including China, Japan and Taiwan[2-8]. There has been a trend towards decreased incidence of RA over the past decade, suggesting the role of environmental factors in the etiology of the disease[9, 10]. RA can occur at any age but the peak age of onset is between 40-60 years of age. Like a number of rheumatic diseases, RA is more frequent in females than in males (ratio 3:1). The disease is characterized by symmetrical joint swelling and tenderness in multiple joints, synovial inflammation and hyperplasia, with cartilage and bone destruction. The progression of RA usually results in disability due to severe joint deformity without proper treatment. In addition to joint symptoms, extra-articular

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manifestations are also common in patients with RA, such as rheumatoid nodules, keratoconjunctivitis sicca, Sjögren's syndrome, interstitial lung disease and pleuritis[11]. The mortality rates are increased 47% among RA patients compared to the general population[12]. The development of RA is frequently accompanied by a significant reduction in employment, productivity and significant economic loss to patients and society.

Although the cause of RA is still unknown, many findings suggest both environmental and genetic factors contribute to the cytokine dysregulation that is characteristic of the disease. Microbial infections, such as Epstein–Barr virus, cytomegalovirus, proteus species, and *Escherichia coli*, are believed to play an important role in the initiation and/or perpetuation of RA[13]. Compared with the healthy population, patients with established, active, RA have a markedly increased incidence of periodontitis and patients with periodontitis have a higher risk of developing RA than people without periodontitis[14]. Pepetidyl arginine deiminase (PAD), expressed by *PorphyromonasGingivalis,* can promote citrullination of human fibrinogen and α-enolase, and has been proposed to drive the autoimmune response in RA[15]. Several studies reveal the potential role of

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the gastrointestinal microbiome in animal models of RA, although human data is still lacking[16]. Furthermore, the efficacy of antibiotics against anaerobic bacteria in the treatment of RA points to the involvement of bacteria in the pathogenesis of the disease[17-19].

Smoking has also been reported to be strongly associated with the risk and severity of RA, particularly anti-citrullinated protein/peptide antibodies (ACPA)-positive RA[20, 21]. A large cohort study showed a positive correlation between the risk of RA and the amount and duration of cigarette use[22]. Many studies suggest that the interaction between bacterial infection, environmental factors and RA is associated with citrullinated proteins, a process that is catalyzed by peptidyl arginine deiminase[23].

An early population survey carried out in Lancashire showed the familial clustering of RA[24]. The result implicated the role of hereditary factors in the pathogenesis of RA. The hypothesis was further explored by studying twins[25, 26]. The concordance rates for seropositive RA were 6 times as high in monozygotic twins than in dizygotic twins. In 1987, a strong association between

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human leukocyte antigen (HLA)-DRB1 and RA was identified[27]. In addition to MHC associated single-nucleotide polymorphisms, many additional risk alleles in seropositive rheumatoid arthritis have been identified, which are associated with nuclear factor κB (NF-κB)-dependent signaling (e.g., TRAF1–C5 and c-REL) and T cell function (e.g., PTPN22 and CTLA4)[28-31]. The fact that many of these polymorphisms are associated with immune-related genes reinforces the notion that RA results from a dysregulated immune system.

1.1.2 Cytokine dysregulation in RA

Although the pathophysiology of RA is still not fully understood, it is generally believed that the interaction between T cell, B cell, and a complex series of cytokines and chemokines play an indispensable role in the pathogenesis of RA. The analysis of cytokine expression in RA patients detected the augmented expression of IL-1 and IL-6 in synovial fluid[32, 33]. Further studies, including mRNA expression in synovium and immunostaining of biopsies, showed the expression of pro-inflammatory cytokines such as IL-1 and TNF-α in the synovium of RA patients[34, 35]. Similarly, immunoassays and bioassays of the

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culture supernatant of mixed cells isolated from the synovium of RA patients showed the production of IL-1 and TNF-α[34, 36]. IL-1 and IL-6, together with TGF-β, IL-21 and IL-23, promote Th17 differentiation and inhibit Treg differentiation, thus skewing T-cell homeostasis toward inflammation[37]. The complex of pro-inflammatory cytokines leads to the expression of endothelial cell adhesion molecules, trafficking of immune cells, promotion of angiogenesis and production of autoantibodies, thus exacerbating the inflammatory process.

In an attempt to elucidate the role of cytokines in the pathogenesis of RA, Brennan et al showed the inhibitory effect of TNF-α-specific antibodies on synovial cell IL-1 production in RA patients[38]. Further studies showed the effect of anti-TNF antibody on suppression of other pro-inflammatory cytokines, such as IL-6, GM-CSF and IL-8 synthesized by synovial cells from RA patients[39-41]. These studies illustrate that TNF plays a dominant role in the inflammatory process by driving the production of additional pro-inflammatory cytokines.

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1.2 Overview of TNF signaling

1.2.1 Tumour Necrosis Factor

Tumour necrosis factor (TNF) is a potent inflammatory mediator exerting effects on many different pathological and physiological functions. The discovery of TNF can be traced back over a century ago, with the observation by P. Brunes that some cancer patients experienced tumour regression following acute bacterial infection. This finding led to the practice by William B. Coley[42]of injecting cancer patients with bacteria or bacterial products, referred to as Coley's toxin. In 1962, O'Malley et al. reported tumour necrotizing activity in the serum obtained from mice after injection of *Serratia marcescens* polysaccharide[43]. The name "TNF" was first used by E.A. Carswell et al. to name the substance in the serum of mice treated with endotoxin, which resulted in haemorrhagic necrosis of tumours[44]. In 1984 and 1985, two proteins that caused lysis of tumour cells were purified, structurally identified and cDNA cloned[45-47]. They were named TNF-α and TNF-β. These proteins showed around 50% sequence homology and interacted with the same membrane receptors but were produced from different cells and exhibited different physiological functions[45-47]. Beutler et al. reported

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on the high degree of homology between mouse cachectin and human TNF-α [48]. The advancement in our understanding of TNF-α, coinciding with improvements in molecular biology capabilities, has resulted in the generation of recombinant TNF-α (rTNF-α), which led to investigations into its clinical utility.

TNF-α was first identified as a nonspecific anti-tumour therapy. Early studies demonstrated the efficacy of utilizing purified TNF-α against various kinds of murine and human tumours heterotransplanted into nude mice[49]. However, all phase I clinical trials of intravenous, subcutaneous or intramuscular-administered rTNF-α showed symptoms of toxicity, including fever, chills, headache, fatigue, dyspnea, tachycardia and hypotension, without significant clinical efficacy in advanced cancer patients[50-53]. Although TNF-α was not shown to be an effective treatment for cancer, several studies indicated that TNF-α was a potent mediator of inflammation via induction of IL-6 [54, 55].. The importance of TNF-α in the pathogenesis of RA was confirmed in clinical trials in which intravenous administration of chimeric anti-TNF-α mAb (infliximab, Remicade®) caused clear reductions in the level of disease activity and radiographic progression[56-58]. Similar findings were subsequently reported for soluble

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TNF-α receptor-Fc fusion protein (etanercept, Enbrel®)[59-61]. Anti-TNF-α therapy was approved for treatment, in combination with methotrexate, of RA patients in 1998. In addition to RA, anti-TNF-α therapy has now been approved for the treatment of ankylosing spondylitis (AS), psoriasis, psoriatic arthritis (PsA), juvenile idiopathic arthritis (JIA), inflammatory bowel disease (IBD), and most recently, hidradenitis suppurativa and noninfectious intermediate and posterior uveitis and panuveitis [62, 63]. However, clinical trials with anti-TNF-α therapy in multiple sclerosis (MS) patients resulted in disease exacerbation[19, 64]. Moreover, it has been reported that the use of anti-TNF-α therapy in RA resulted in the development of autoimmune disease characteristic of systemic lupus erythematosus (SLE) and MS[65, 66]. The paradoxical effects of anti-TNF-α therapy suggest that TNF-α plays both proinflammatory and regulatory roles in the immune system.

1.2.2 TNFR1 and TNFR2 signalling

TNF-α is a pleiotropic pro-inflammatory cytokine, mainly produced by activated monocytes/macrophages, but also by various cell types such as NK cells, T

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lymphocytes and eosinophils in response to various stimuli[67]. TNF-α is initially synthesized as a type II transmembrane protein of 233 amino acids, then expressed on the cell surface as a trimer. Subsequent cleavage by TNF-converting enzyme (TACE, also named ADAM17) liberates the trimer into the circulation. Both the membrane form of TNF-α (mTNF-α) and the soluble form of TNF-α (sTNF-α) exert physiologic functions by binding to two structurally and functionally distinct receptors on target cells: TNF receptor 1 (also named as TNFR1, CD120a, p55 and TNFRSF1A) and TNF receptor 2 (also named as TNFR2, CD120b, p75 and TNFR12A) on target cells.

TNFR1 is ubiquitously expressed, bears a cytoplasmic death domain, and is activated by both mTNF-α and sTNF-α [68]. The death domain enables TNFR1 to recruit TNFR1 associated death domain protein (TRADD), which mainly leads to apoptosis and inflammation[69]. By contrast, TNFR2 is expressed on a restricted range of cell types including activated $CD4^+$ and $CD8^+$ lymphocytes, endothelial cells, astrocytes, oligodendrocytes, thymocytes, myocytes, and human mesenchymal stem cells[70-76], and is preferentially activated by mTNF-α [68]. Due to the lack of a death domain, TNFR2 recruits TNFR associated factor 1

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(TRAF1) and TRAF2 rather than TRADD[69, 77, 78]. In contrast to TNFR1-TRADD signalling, TNFR2 signalling through TRAF1 and TRAF2 mediates a homeostatic effect, including cell survival and tissue regeneration[79]. However, further studies have revealed a degree of receptor crosstalk and overlapping function between TNFR1 and TNFR2, which seems to depend on multiple factors, such as cell type, intracellular or extracellular environment, stimuli and age [80, 81].

Upon engagement by TNF-α, TNFR1 translocates to lipid rafts in the plasma membrane and forms a homotrimer[82, 83]. The binding of TNF-α to TNFR1 induces a conformational change in the cytoplasmic death domain of TNFR1, which results in the recruitment of TRADD and receptor-interacting serine/threonine-protein kinase I (RIPKI)[77, 84, 85]. The co-localisation of TFNR1, TRADD and RIPKI initiates the assembly of distinct complexes, named complexes I, IIa, IIb and IIc, which leads to activation of distinct downstream signalling pathways[86, 87]. The formation of these three complexes is mainly determined by the ubiquitination status of RIPK1. Complex I is composed of TRADD, RIPK1, TRAF2 or TRAF5, cellular inhibitor of apoptosis protein 1 (cIAP1) or cIAP2 and the linear ubiquitin chain assembly complex (LUBAC)[77,

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84, 86, 88-90]. Initially, both TRAF2/5 and cIAP1/2 are E3 ubiquitin ligases that mediate K63-linked ubiquitination of RIPKI[91-93]. Subsequently, the LUBAC complex, which consists of haeme-oxidized IRP2 ubiquitin ligase 1 (HOIL1), HOIL1-ineracting protein (HOIP) and SHANK-associated RH domain-interacting protein (SHARPIN), attaches an M1-linked polyubiquitin chain to RIPK[89, 94-96]. Both K63- and M1-linked polyubiquitination events stabilize complex I and facilitate further signalling. Ubiquitin chains attached to RIPK1 enable the recruitment and activation of two signalling complexes, transforming growth factor (TGF)-activated kinase I (TAKI) complex and the inhibitor of κB (IκB) (IKK) complex[89, 94]. The TAKI complex, which is composed of TAKI, TAKI-binding protein 2 (TAB2) and TAB3, phosphorylate mitogen-activated kinase (MAPK) and consequently lead to the activation of the c-JUN N-terminal kinase (JNK), p38 and AP1 transcription factor[97, 98]. The IKK complex, comprising nuclear factor κB (NFκB) essential modulator (NEMO), IKK subunit-α (IKKα) and IKKβ, activate NFκB pathway signalling[99, 100]. Induction of NFκB and AP1 target genes plays an indispensable role in inflammation, host defense, cell proliferation and survival[86].

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In contrast, the formation of complex IIa and IIb depends on non-ubiquitinated RIPK1. For the formation of complex IIa, the attached K63- and M1- linked polyubiquitin chain are removed from RIPK1 in complex I by cylindromatosis (CYLD) and result in dissociation of the deubiquitinated RIPK1 from membrane bound complex I[101-103]. The released deubiquitinated RIKP1 interacts with cytosolic TRADD, FAS-associated death domain protein (FADD), pro-caspase 8 and the long isoform of FLICE-like inhibitory protein ($FLIP_L$) to form complex IIa[104, 105]. Alternatively, complex IIb is formed when the RIPK1 is not ubiquitinated due to the depletion or degradation of cIAPs[91]. The non-ubiquitinated RIPK1 dissociates from membrane bound complex I and interacts with RIPK3, pro-caspase 8 and $FLIP_L$ to from complex IIb. Both complexes IIa and IIb generate active caspase 8 from pro-caspase 8 and lead to activation of the downstream caspase cascade and thus induce cell death via apoptosis. Concurrently, the deubiquitinated RIPK1 and RIPK3 must be cleaved by the pro-caspase 8- $FLIP_L$ heterodimer, or active caspase 8, to prevent cells from undergoing necroptosis[104, 106-109]. The formation of complex IIc is similar to complex IIa and complex IIb formation in that the released deubiquitinated or non-ubiquitinated RIPK1 is caused by deubiquitination mediated by CYLD or

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depletion of cIAPs. However, in some circumstances, RIPK1 and RIPK3 cannot be cleaved due to inactivation of caspase and therefore aggregate to form complex IIc (also named as necrosome). Complex IIc activates mixed lineage kinase domain-like protein (MLKL) and leads to the induction of necroptosis[77, 85, 110]. In contrast to apoptosis which is a form of highly controlled programmed cell death, necroptosis results in plasma membrane rupture, which leads to leakage of intracellular contents, including DAMPs, and local inflammation[77, 78] (See Figure 1.1).

TNFR2 lacks TRADD, but instead binds to TRAF1 or TRAF2 to recruit cIAP1 or cIAP2. The binding of TRAF2 to TNFR2 is much weaker in comparison with that of TRAF2 to TRADD[111]. The aggregation of TRAF1/2 and cIAP1/2 triggers the formation of complex I and downstream MAPK and NFκB signaling. TNFR2-TRAF signaling mediates a homeostatic effect including tissue generation, cell proliferation and cell survival[79]. The crosstalk between TNFR1 and TNFR2 has not been fully elucidated. The difference of binding affinity between TRAF2-TNFR2 and TRAF2-TRADD suggests that TNFR2 may have a regulatory effect on TNFR1 signaling in the same cell[110-112] (See Figure 1.1).

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Previously, it has been proposed that TNFR2 is required for TRAF2 degradation, leading to activation of the alternative NFκB pathway and contributing to MAPK and classical NFκB signalling. Ruspi et al studied the signalling functions of TNFR2 in primary macrophages[113]. TNF-α induced TRAF2 degradation, which was blocked in TNFR2$^{-/-}$ macrophages. However, TRAF2 degradation was not required for TNF-α -induced tolerance of p38 MAPK activation. In addition, the alternative NFκB pathway was not activated in primary macrophages by TNF-α or lipopolysaccharide. As expected, TNFR2 was required for activation of p38 MAPK and NFκB pathways by TNF-α. Of interest was the finding that, although TNFR2 was not required for p38 MAPK activation and IκBα degradation at high concentrations of TNF-α, it did play a role in activation of these pathways at lower concentrations of TNF-α. These findings suggest that TNFR2 plays an additional, or sensitising, role to TNFR1 in activation of p38 MAPK and NFκB pathways[113]. Indeed, a similar concept was proposed earlier by Tartaglia et al[114].

The genes encoding TNFR1 and TNFR2 (TNFRSF1A and TNFRSF1B) have been mapped to 12p13 and 1p36, respectively[115-117]. Numerous polymorphisms

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within these genes have been described[118-120] and several studies have investigated their possible association with RA[121-123]. TNFR-associated periodic syndrome is attributed to mutations in TNFRSF1A [124]. In contrast, the A36G polymorphism in exon 1 of TNFRSF1A plays a protective role in patients with familiar RA[125]. An association have been reported between a specific single nucleotide polymorphism (SNP) in TNFRSF1B and susceptibility to familial RA[121, 126]. This +196 SNP (T/G) in the fourth extracellular domain of TNFR2 (exon 6) results in a non-conservative amino acid substitution (methionine to arginine). Functional analysis of this +196 SNP in TNFRSF1B transfected cells showed increased IL-6 production and enhanced cytotoxic activity[127]. Another study showed an association with this same SNP and impaired ability to induce NF-κB signalling resulting in reduced TNF-α induced apoptosis[128]. In addition, it has been suggested that the +196 SNP in TNFRSF1B may affect receptor assembly, conformation, and shedding and is associated with levels of soluble TNFRs in patients with RA [127, 129]. As the function of soluble TNFR is to inhibit excessive TNFR signaling, these findings point to a potential molecular mechanism for the involvement of polymorphisms in TNFRS1B in the pathogenesis of RA and other chronic inflammatory disorders.

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While the studies described have demonstrated the association between TNFR SNPs and RA susceptibility, the association of TNFR SNPs with RA severity is less clear. Two studies showed that neither TNFRSF1A nor TNFRSF1B is associated with levels of disease activity[130, 131]. One study investigated the association between the +196 SNP in TNFR2 and disease severity of RA in Southern Europe[132]. Although no statistically significant associations between the +196 SNP with RA severity were found, a trend towards higher prevalence of the GG genotype of the +196 SNP was observed in treatment refractory RA patients with more aggressive disease. One study demonstrated an association between a polymorphism in TNFRSF1B (676T>G) and a lower response to anti-TNFα therapy[133], suggesting that polymorphisms in TNFR may have utility in predicting response to anti-TNFα therapy. However, it is worth pointing out that a relatively small number of the reported associations between SNPs in TNFRSF1A or TNFRSF1B have been reproduced across different RA patient populations and there is currently no consensus regarding their clinical importance.

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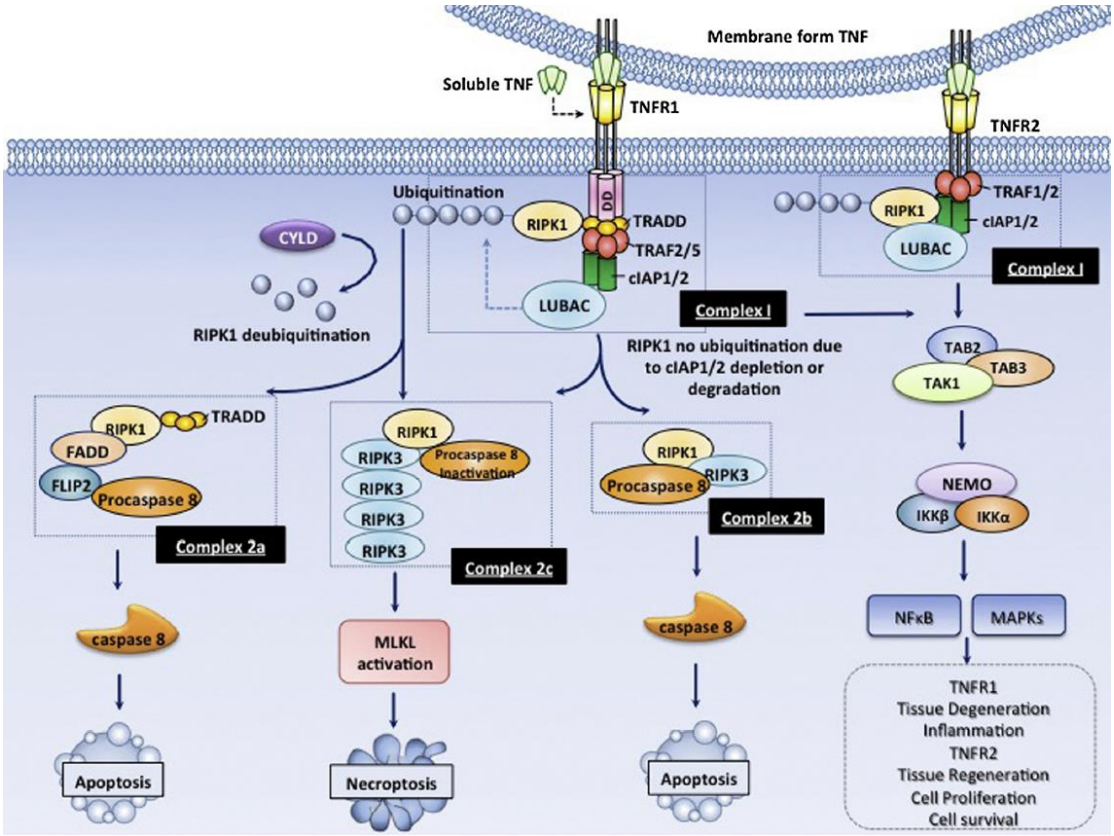


Figure 1.1 TNF-α and downstream signalling. TNF-TNFR1 is activated by both sTNF-α and mTNF-α, and TNFR2 is preferentially activated by m TNF-α. Ligation TNFR1 by TNF-a results in the recruitment of TRADD and RAPKI. According to the ubquitination status of RIPKI, the complexes I, IIa, IIb, and IIc are generated. TNFR1-complex I signaling induces tissue degeneration, inflammation, host defense, cell proliferation and survival via activation of NF-jB and MAPKs. Alternatively, the formation of complexes IIa and IIb cause apoptosis and complex IIc results in necroptosis and inflammation. TNFR2 lacks the TRADD and binds to TRAF1/2 directly, leading the formation of complex I and the downstream activation of NF-κB and MAPKs. TNFR2 signalling mainly mediates a homeostatic effect, including cell proliferation and survival and tissue regeneration. This figure is reproduced from a review article I wrote and published (Wen-Yi et al. 2016, Cytokine, 101, January 2018, Pages 19-25) with the permission from all the co-authors and the publisher.

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1.2.3 Role of TNF-α in health and disease

TNF-α is required for the normal development of the immune system, defense against pathogens and cancer, development of germinal centers and granuloma formation[67, 134, 135]. However, uncontrolled production or function of TNF-α has been shown to be associated with the pathogenesis of many inflammatory diseases, including RA, AS, PsA, JIA and IBD. TNF-α plays a role in mediation of pain pathways, through triggering of hyperalgesia by inducing peripheral and central sensitization in experimental arthritis, which is partly independent of inflammation. Anti-TNF-α therapy reverses the effect and results in analgesia[136-140]. TNF-α is also implicated in the pathogenesis of atherosclerosis and Dupuytren's disease[141, 142].

Paradoxically, TNF-α suppresses excessive immune responses and protects tissue from damage[79]. Faustamn et al demonstrated that administration of low dose TNF-α selectively killed autoreactive T cells, leading to reversal of type I diabetes[143]. A subsequent study showed that a TNFR2 agonist killed autoreactive $CD8^+$ T cells and exhibited less systemic toxicity compared with administration of TNF-α. Apart from TNFR2 signalling induced apoptosis of

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autoreactive T cells, TNF-α exerts immune suppressive function via regulatory T cells (Tregs). TNFR2 is expressed on most Treg and plays an indispensable role in maintaining the stabilization of the Treg phenotype in the inflammatory environment[144, 145]. Furthermore, $TNFR2^{+}$ Tregs exhibit higher suppressive function than $TNFR2^{-}$ Tregs[146-148]. In synergy with IL-2, TNF-α was shown to increase the expression of CD25 and FoxP3 on Tregs. However, the stimulatory effect was not seen in mice deficient in TNFR2[149]. Various defects in TNFR2, including polymorphisms in the TNFR2 gene or TNFR2 shedding have been implicated in the pathogenesis of several autoimmune diseases[126, 150, 151]. These findings suggest that TNFR2 plays an important immunoregulatory role in the immune system. In addition to its immunoregulatory function, TNF-α is critical in tissue regeneration, including neuronal remyelination, cardiac remodeling and cartilage regeneration, effects thought to be mediated principally via TNFR2[152-154]. These results suggest TNF-α plays an indispensable role in maintaining homeostatic status, which is partly attributed to TNFR2 signalling[79].

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1.2.4 Anti-TNF-α therapy has revolutionized the treatment of RA

Aspirin, a non-steroidal anti-inflammatory drug, which was developed by Bayer Company in 1900, was the first drug used in RA patients for symptomatic relief. However, aspirin did not affect the disease course and resulted in many severe side effects, such as epigastralgia, gastro-intestinal hemorrhage and coagulopathy. In the 1950's, cortisol, the first steroid medication, dramatically improved the symptoms of RA such as joint tenderness, stiffness and swelling. However, steroids did not halt the process of bone erosion and joint destruction. Moreover, sustained use of steroid resulted in many systemic and irreversible side effects, such as Cushing's syndrome, osteoporosis, immunodeficiency, high blood pressure, dyslipidemia, diabetes, glaucoma and cataract.

In the following years, many medications ranging from gold injection, antibiotics, to anti-malarial drugs were prescribed for RA patients. Methotrexate (MTX), a folate analogue synthesized in 1948, was shown to result in significant improvement in two randomized, placebo-controlled studies[155, 156]. Based on the results from these studies, MTX was approved as a therapy for RA by the US Food and Drug Administration in 1988. These drugs, including anti-malarial

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drugs, MTX, azothioprine, sulfasalazine, and leflulomide, that can be used to modify the course of RA, are referred to as disease modifying anti-rheumatic drugs (DMARDs). However, for most RA patients the improvement from conventional DMARDs is unsatisfactory, and only a small number of patients achieve remission even with combined treatment of DMARDs.

Several studies indicated that TNF-α was a potent mediator of inflammation via induction of IL-6[54, 55]. Subsequently, in a series of key experiments it was shown that blockade of TNF-a in cultures of synovial cells from rheumatoid arthritis (RA) patients abrogated the expression of IL-1 and other pro-inflammatory cytokines, suggesting that TNF-a was pivotal in driving the production of multiple mediators of inflammation[38]. Based on the aforementioned studies of the pathogenic role of TNF-α in RA, a small, open label, Phase I/II clinical trial of TNF blockade was carried out in 1992[56]. There was a dramatic improvement in all 20 patients and the efficacy sustained for 8 to 26 weeks. This inspiring result contributed to the setting up of further phase II/III, randomized, double blind, and placebo-control trials[58, 157, 158]. These trials

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confirmed the efficacy of TNF-α blockade and also demonstrated the synergistic effect with MTX treatment in RA patients. Moreover, the trials showed the bone protective effect of TNF-α blockade in more than 50% of patients[158]. Based on the Phase III clinical trial, infliximab (Remicade) was approved for use in RA patients, in combination with MTX.

The development of TNF-α inhibitors has revolutionized the management of a number of autoimmune diseases. To date, there are five anti-TNF-α agents, infliximab (Remicade), etanercept (Enbrel), adalimumab (Humira), certolizumab (Cimzia) and golimumab (Simponi) that have been approved for use in RA patients. The use of anti-TNF-α therapy has now extended to a variety of rheumatic diseases, including ankylosing spondylitis (AS), psoriatic arthritis (PsA), psoriasis, inflammatory bowel disease (IBD) and juvenile RA[63]. Despite the great success of anti-TNF-α therapy, there are still drawbacks. Thus, in clinical trials or post marketing registry studies, around 10-30% patients are resistant to anti- TNF-α therapy (primary failure) and more patients exhibited a decrease clinical response to anti-TNF-α therapy after sustained treatment (secondary failure)[60, 158-160]. Moreover, some patients undergoing anti-TNF-α therapy

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developed additional autoimmune diseases, including type I diabetes, SLE, MS and psoriasis[161-163]. A clinical trial of using lenercept (a soluble TNFR-Fc fusion protein) for the treatment of relapsing remitting MS was discontinued owing to unexpected aggravation of the disease in the lenercept treatment group[19]. The reasons for the contradictory results are still elusive. One hypothesis put forward to explain the paradoxical effect of anti-TNF-α therapy is an ascendant effect of dysregulated Tregs and increased Th17 responses due to interruption of TNFR2 signalling[164].

1.3 Overview of Tregs

1.3.1 Development of Tregs

Regulatory Tregs are a sub-population of T cells characterized by immuno-suppressive activity, which play an indispensable role in maintaining hemeostasis and inhibiting excessive immune responses. Tregs were first investigated following the finding of destruction of ovaries and thyroiditis in mice after neonatal thymectomy[165, 166]. Intriguingly, transferring normal $CD4^+$ T cells from normal mice prevented disease development[167, 168]. These results

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showed that the normal thymus continuously produces a group of $CD4^+$ T cells with immune-suppressive function. The paucity of specific markers for defining this group of $CD4^+$ T cells with immune-suppressive function initially led to skepticism as to their true relevance to immune tolerance. This group of $CD4^+$ T cells was first identified by the expression of a cell surface molecule, CD25, in 1995[169] and a transcription factor, FoxP3, in 2003[170, 171]. In mice, FoxP3 is expressed specifically on T cells with regulatory function[171]. Deficiencies or mutations in FoxP3 were shown to result in lymphoproliferation and multi-organ autoimmunity in scurfy mutant mice and human IPEX patients (Immune-Dysregulation Polyendocrinopathy Enteropathy X-linked)[172]. Additionally, it has been reported that over-expression of FoxP3 induced Treg-like cells with suppressive function[170]. FoxP3 has therefore been considered as a lineage specific transcription factor for Tregs and as a master regulator of their function.

Tregs can be generated in the thymus (thymus-derived, tTregs) and in the periphery, where they are differentiated from conventional $CD4^+$ T cells (induced Treg, iTregs). Currently, there is no reliable marker to discriminate between these

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two subsets. However, some studies reported there is a difference of the methylation level at cis-regulatory elements of *FoxP3* locus between tTregs and iTregs[173-176]. It has also been reported not only FoxP3 expression, but also Treg-cell-specific DNA hypomethylation, is required for defining Tregs or stably maintaining Treg cell suppressive function[177].

1.3.2 Mechanism of Treg-Mediated Immune Suppression

The mechanisms which Tregs utilize to suppress immune response are various. Tregs can secrete inhibitory soluble factors, such as TGF-β, IL-10, IL35 and adenosine, to generate a potent suppressive effect within the surrounding microenvironment[178]. In addition, Tregs can deliver intracellular inhibitory signals to dendritic cells via the binding between CTLA-4 and CD80/CD86. This leads to increased expression of immunoregulatory molecules, such as indolamine 2,3-dioxygenase (IDO), and decreased CD80/86 expression and impaired maturation of dendritic cells[179]. In contrast, CTLA-4 on Tregs also competes with CD28 for interaction with CD80/86, thus blocking the co-stimulatory pathway for Teff cells. Tregs are also capable of direct killing of target immune

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cells by secreting granzyme A, granzyme B and perforin, or expressing FAS ligand[180-182]. Tregs can also deprive IL-2 or the amino acid tryptophan through high CD25 expression or induction of IDO expression in dendritic cells[183, 184]. These different suppressive mechanisms employed by Tregs may be necessary to modulate diverse immune effector cell types in inflammatory settings and it is not clear whether they represent functions of different sub-types of specialized Tregs.

1.3.3 Regulatory T cells in Rheumatoid Arthritis

Dysfunction of Tregs results in a breakdown of immunological tolerance and has been implicated in the pathogenesis of autoimmune diseases, including SLE, RA and type I diabetes[185-188]. Many studies have investigated whether RA are associated with defective numbers of Tregs. However, the results are controversial. Some studies showed that the frequency of Tregs among CD4+ T cells was higher in healthy controls[189-191]. Other studies showed that the frequency of Tregs was higher in RA[192-194]. Interestingly, many studies showed similar frequency of Tregs cells in peripheral blood between RA patients and healthy

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controls[195-198]. The inconsistency might be accounted for by different definitions of Tregs. A range of markers were used to identify Tregs in these studies, including $CD25^+$ alone, $CD25^{high}$, $FOXP3^+$, $CD25^+$ $FOXP3^+$, $CD25^{high}$ $CD25^+$ $CD127^-$. Stricter definitions of Tregs, including function related markers (CD45RA) and demethylation of FoxP3 CNS2 might become important in the further studies. In addition, further study is required to investigate whether numbers of antigen-specific Tregs differs in RA and healthy controls.

The correlation between Treg function and the pathogenesis of RA is still elusive. It has been reported that Tregs isolated from patients with RA and from healthy controls showed similar suppressive function on Teff proliferation[195, 197]. However, one study reported a defect in the capacity of Tregs from RA patients to suppress Teff proliferation[199]. Some studies suggest that Tregs from RA patients are defective in their ability to inhibit the production of IFN-γ and TNF by Teff[195]. In addition, some evidence showed Teff can become resistant to suppression by Tregs in patients with autoimmune disease such as type I diabetes and RA[200, 201], which may interfere the suppressive function of Tregs on Teffs.

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These studies investigating Tregs defects in number and function have some limitations. First, disease status in RA patients including disease activity and disease duration was not consistent across studies. In addition, RA treatments were not identical across studies, either. These treatments may influence on the proportion and function of Tregs in RA patients. It has been reported MTX can restore the suppressive function of Tregs in RA patients[202]. Other studies suggested biologic therapies, such as anti-TNF and anti-IL6R can expand functionally suppressive Tregs in RA patients but not healthy control[203, 204]. Further investigation on early stage and treatment-naïve RA patient is warranted to determine the correlation between Tregs and RA.

The expression of Foxp3 and its stability have indispensable roles in the maintenance of Treg cell function[205]. Conditional deletion of a Foxp3 allele in mature Treg cells results in not only the loss of their suppressive function but also the effector T cells that are capable of causing inflammation[206]. Lack of IL-2 signaling impairs FOXP3 expression in Tregs which contributes to the induction of autoimmune diseases[207, 208]. It has also been reported maintenance of

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FOXP3 expression is impaired in RA, which is accounted for the enhanced stimulation of TNF [199]. Moreover, some studies indicate that there is a plasticity between both Tregs and T effector cells[209]. In RA patients, increased IL-17–expressing exFoxp3 T cells accumulates in inflamed joints, which is mediated by synovial fibroblast-derived IL-6. Similarly, increased IL-17 secreting $FOXP3^{lo}$ T cells is observed in type I diabetes children compared to healthy controls[210]. Overall, these defects in stability and plasticity of Tregs[211], which is mediated at least partially by epigenetic modifications, may lead to inadequate numbers or function of Treg in sites of inflammation[212].

1.3.4 Epigenetic regulation of regulatory T cells

Epigenetic modification is a process that affects gene expression without altering the sequence of bases in the DNA which including DNA methylation, histone modifications, and RNA interference[213]. It can target histones or the DNA directly, and affect gene transcription by altering the accessibility of distinct DNA regions to transcription factors and other DNA-binding molecules[214]. DNA methylation is a fundamental process by which methyl groups are added to the

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DNA molecule at the 5th carbon of the cytosine ring in the context of CpG dinucleotide[215]. This process is catalyzed by DNA methyltransferases (DNMTs). In mammalian cells, maintenance of DNA methylation is mediated by DNMT1, while de novo methylation is managed by DNMT3a and DNMT3b [216]. CpG methylation at the promoter directly represses gene expression by impeding the binding of transcription proteins to the gene and, in contrast, promoting recruitment of repressor molecules, such as methyl-CpG-binding domain proteins (MBDs) [217]. In general, DNA methylation is associated with the silencing of gene expression, whereas the actively transcribed genes are typically hypomethylated. DNA methylation is indispensable for normal development and has profound effects on a number of key processes including genomic imprinting, X-chromosome inactivation, repression of transposable elements, aging and carcinogenesis [218].

Some studies in mice suggested that the transcription of FoxP3 is regulated by methylation within *foxp3* locus [173, 174]. It has been reported that the Treg-specific demethylated region (TSDR), a conserved, CpG -rich region, is heavily demethylated in human nTregs, whereas it is completely methylated in

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iTregs and other non-suppressive T cells that also express FoxP3[173, 176]. Moreover, FoxP3 induction by TGF-β is associated with partial demethylation of these CpG sites and transient expression of FoxP3. The phenotype of iTregs is unstable in vitro, since the FOXP3 expression is reversed upon restimulation in the absence of exogenous TGF-β [173]. Similar results were also observed in human Treg cells. Several studies indicated that human nTreg cells can be distinguished from FoxP3(+) conventional CD4(+) T cells by analysis of methylation status in the foxp3 locus[176, 219, 220]. Despite of FoxP3 expression in activated CD4+T cells, these cells display FoxP3 DNA hypermethylation as compared with nTregs.

The studies mentioned above suggest that hypomethylation at TSDR play an indispensable role in maintaining stable FOXP3 expression. In addition, several key factors for Treg cell development, such as CD25[221] and galectin-1 [222], are also regulated by the methylation of CpG islands in their promoter regions. A recent study shows that DNA demethylation enzymes, the Tet family members including Tet 1, Tet 2 and Tet 3, have essential role in maintaining DNA demethylation of FoxP3 enhancer [223]. These findings suggest the importance of

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DNA methylation in FOXP3 expression and indicate that induction of iTregs *in vitro* may be achieved by modifying DNA methylation at *foxp3* locus.

In the case of RA, it has been shown that loss of Treg cell function is accompanied by reduced expression of FoxP3 and CTLA-4 due to aberrant CpG methylation in the cis-regulatory regions of the two genes[202, 224]. Indeed, the importance of DNA demethylation at the *foxp3* locus in determining Treg cell function has been confirmed in a number of independent studies[173, 174, 225-228]. Some work suggested that the loss of CTLA-4 expression in Treg cells resulted in inhibition of indoleamine 2,3-dioxygenase mediated generation of kynurenine, a positive regulator of Treg cell differentiation[224].

These findings raise the question of whether transient reversal of DNA methylation could epigenetically re-wire Treg cells leading to sustained upregulation of their suppressor activity. Two DNA demethylating agents, decitabine and azacytidine, have been approved for use in myeloid malignancies due to their ability to promote re-expression of silenced tumour suppressor genes. Decitabine and azacytidine act on proliferating cells by incorporating into DNA and inhibiting the activity of DNA methyltransferases. Serendipitously, it has

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been shown that numbers of circulating Treg cells are increased in the peripheral blood of patients following treatment with azacytidine[229, 230]. In addition, DNA demethylating agents are reported to generate induced Treg (iTreg) cells and to promote their suppressive function in vitro[231] [232] and to protect against experimental autoimmune encephalomyelitis and allogeneic cardiac transplant rejection[233]. Overall, these studies indicate the therapeutic potential of DNA demethylating agents in the treatment of RA via modifying methylation status at the *foxp3* locus.

1.3.5 Application of Tregs as cellular therapy

Dysfunction of Tregs results in a breakdown of immunological tolerance and has been implicated in the pathogenesis of autoimmune diseases, including SLE, RA and type I diabetes[185-188]. Several approved drugs are reported to restore function or increase numbers of Tregs, and this effect may contribute to the therapeutic potential of these drugs in RA[188, 234-238].

These findings suggest the therapeutic potential of exploiting Tregs to treat autoimmune diseases. There are several possible Treg therapeutic strategies. First, Tregs can be expanded in vivo by administration of low dose of IL-2. Several

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clinical trials have shown efficacy of low dose IL-2 in the treatment of graft-versus-host disease (GVHD), hepatitis C virus-induced cryoglobulinaemic vasculitis and type I diabetes[239-241]. Apart from IL-2 treatment, anti-CD45RB has been reported as a potent therapeutic agent to promote expansion of antigen-specific Tregs by augmenting intergrin-dependent Treg-DC interactions[242].

A second strategy is to transfer in vitro expanded autologous Treg to patients with autoimmune diseases. The efficacy of Treg therapy in pre-clinical animal studies of organ transplantation has been clearly shown. However, minimal therapeutic effect was achieved in several Phase I/II clinical trials in GVHD and type I diabetes[243-246].

The unsatisfactory result might be explained by the poor survival or the plasticity of the transferred Tregs in these patients. The hypothesis is supported by the finding that the infused Tregs rapidly decreased with loss of FoxP3 expression[246, 247]. The expansion of Tregs in vitro can be achieved by antigenic stimulation in the presence of IL-2[248]. The other strategy of Treg therapy is to convert Teff into Treg by antigenic stimulation combined with IL-2,

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TGF-β, all-trans retinoic acid, interferon-γ or cilostimide[249-252].

1.4 Aims of the thesis

Anti-TNF-α therapy has revolutionised the management of inflammatory arthritis, leading to improvement in symptoms and signs of RA, AS and PsA, as well as abrogating radiographic progression in RA and PsA[253]. However, despite its success in the majority of patients with inflammatory arthritis, about 20-40% of patients are resistant to anti-TNF-α treatment and few patients (10% or even less) achieve long-term remission[254]. In addition, some patients experience significant side effects, such as infections or paradoxical autoimmunity during treatment with anti-TNF-α therapy[255]. A further investigation of the effect of TNF-α on the immune system is needed to improve the standard care of RA.

Regulatory T cells (Tregs) are a subpopulation of T cells, which are essential for immunological homeostasis. Dysfunction of Tregs results in a breakdown of immunological tolerance and has been implicated in the pathogenesis of autoimmune diseases, including SLE, RA and type I diabetes[185-188]. It has

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been reported that TNF-α promotes proliferation, survival, stability and suppressive function of Tregs[147, 148, 256]. In addition, TNFR1 blockade, but not TNFR1/2 blockade, promoted generation and suppressive function of Tregs in CIA[257]. Based on these findings, we hypothesized that TNFR2 plays an immunoregulatory role in arthritis and interruption of TNF/TNFR2 interaction contributes to the resistance to anti-TNF-α therapy and anti-TNF-induced autoimmunity observed in some patients. The objective of the work presented in this thesis was to gain a deeper understanding of TNFR signalling in rheumatoid arthritis with particular emphasis on Tregs.

Specifically, the first aim of this project is to investigate the effect of TNFR signalling on the phenotype of Tregs and to determine whether there is an association between changes in phenotype and the methylation status of the *FoxP3* locus. The next aim was to determine the role of TNFR signalling in the pathogenesis of collagen-induced arthritis (CIA), a widely studied model of RA. Thirdly, I set out to explore the therapeutic potential of DNA demethylating agents in the treatment of RA via promotion of Treg generation and suppressive function.

Chapter 2 Materials and Methods

2.1 Animal and cell Preparation

2.1.1 Animals

The generation of TNFR1-/- and TNFR2-/- (C57BL/6 background) which were used in FACS analysis for Treg gene expression analysis has been described previously[258]. Homozygous colonies were maintained at the Kennedy Institute of Rheumatology. For the CIA studies in Chapter 4, male, wild type H-2^q mice were bred by backcrossing C57BL/6 mice (expressing A^b) to congenic C57BL/6N.Q mice (expressing A^q). For the studies in Chapter 5, male DBA/1 mice were purchased from purchased from Envigo (Huntingdon, UK). Mice were housed in groups of 5 in ventilated cages, maintained at a 21°C ±2°C and a 12-hour light/12- hour dark cycle, with food and water available *ad libitum*. All experimental procedures were approved by the UK Home Office.

2.1.2 DNA isolation for genotyping

DNA from mouse ear tissue was extracted using Extraction solution (Cat. No.

E7526, Sigma-Aldrich, St. Louis, USA) and Tissue Preparation Solution (Cat. No. T3073, Sigma -Aldrich) at a ratio of 4:1 at room temperature for 30 min. Then the samples were incubated on a heat block for 15 min. Neutralization Solution B (Cat. No. N3910, Sigma-Aldrich) was added into heated extract and mixed by vortexing or spinning. The extract solution was stored at 4 °C until used in PCR.

2.1.3 WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$, H2q and H2b genotyping

The sample DNA was mixed with MangoMix (Cat. No. Bio-25034, Bioline), primers (p60-B, P60-E, p60-spe, pgk-5' for TNFR1$^{-/-}$ genotyping, p80-Kas, p80i-1, pgk5' for TNFR2$^{-/-}$ genotyping, H2q_f_1, H2q_r_2 for H2q genotyping, and H2Bee_F_2, H2Bee_R_1 for H2b genotyping) (Table 1) and ddH_2O on ice. The amount of PCR reaction buffer was listed as follows:

Reagents	Volume
MangoMix	12.5λ
Template	2 λ
Primers	8 λ
ddH_2O	2.5 λ
Total volume	25 λ

The sample DNA was amplified under the cycling condition:

Process	Temperature and Times	Cycles
Pre-denaturation	94 °C for 2 mins	1
Denaturation	94 °C for 60 sec	
Annealing	63 °C for 1 min	40
Extension	72 °C for 30 sec	
Extension	72 °C for 2 mins	1
End and store	4 °C	∞



The PCR product was run on a 3% agarose gel alongside Hyperladder 1kb plus (Cat. No. Bio-33053, Bioline, London, UK).

Table 1. Primers used for genotyping of mice

Gene	Primer Sequence
p60-B	GGA TTG TCA CGG TGC CGT TGA AG
p60-E	TGA CAA GGA CAC GGT GTG TGG C
p60-spe	TGC TGA TGG GGA TAC ATC CAT C
pgk5'-66	CCG GTG GAT GTG GAA TGT GTG
p80-Kas	AGA GCT CCA GGC ACA AGG GC
p80i-1	AAC GGG CCA GAC CTC GGG T
H2q_f_1	CCA CAT GGG ATT TTA GCT GTT ATA AC
H2Bee_F_2	AGG AAA TCA TAT CTC TCA CCT CAT AG
H2Bee_R_1	CCA CCA TGG CTC TGT GGT G
H2q_r_2	GCT TCT GGA AAA GAG ACA GTG AG

2.1.4 Validation of H2q and H2b genotyping by FACS

To validate the genotyping result of PCR, 500 µl blood was collected from mice using a heparin coated syringe and was transferred into centrifuge tubes. The blood was incubated with 5 ml red blood cell lysis buffer (Cat. No. R7757, Sigma-Aldrich) for 10 min. The blood was centrifuged at 1500 rpm for 5 min. The pellet was washed and then resuspended in FACS buffer (1XPBS contains 0.01% NaN3 and 1% Bovine Serum albumin) and plated into a 96 'V' bottom plate. The cells were then centrifuged at 1500 rpm for 5 min. The pellet was resuspended in FACS buffer with fluorochrome conjugated antibodies against I-Aq, I-Ab and CD11b (Table 2). The labelled cells were placed at 4ºC for 30 min, protected from light. The labelled cells were washed with FACS buffer 3 times and resuspended in FACS buffer before analysis on the flow cytometer.

2.1.5 Cell Isolation

Mice were culled and spleens, lymph nodes and thymuses were harvested into incomplete RPMI medium (Cat. No. 61870-010, Life technologies), (10% heat inactivated fetal bovine serum (Cat. No. 10500-064, Life technologies, Carlsbad,

USA), 1% Penicillin/Steptomycin (Cat. No.17-602E, Lonza, Bazel, Switzerland) and 0.1µl β-Mercaptoethanol (Cat. No. 31350-010, Life technologies)). The spleen, thymus and lymph nodes were dispursed by pushing through a Falcon 70 µm strainer (Cat. No. 352350, BD) atop a 50 ml Falcon tube (Cat. No. 352070, BD). The spleen cells were incubated in 1 ml of red blood cell lysing buffer (Cat. No. 10492-01, Life technologies) for 1 min, then diluted in 20 ml incomplete RPMI medium and centrifuged at 1500 rpm for 5 min. The cell pellets of spleen, lymph nodes or thymus were resuspended by complete RPMI medium and counted with a hemocytometer. The cell suspension was diluted to 2 or 4×10^6 cells/ml for further analysis.

2.2 Flow Cytometric Analysis

2.2.1 Cell Staining for FACS analysis

100 µl of cell suspension (2 or 4×10^6 cells/ml) were plated into a 96 well U-bottom tissue culture plate (Cat. No. 353077, Fisher Scientific). The cells were washed in FACS buffer (1X PBS contains 0.01% EDTA and 1% Fetal Bovine Serum), then centrifuged at 1500 rpm for 5 min and resuspended in FACS

buffer with flurochrome conjugated antibody against cell surface markers (Table 2) and Zombie NIR™ Fixable Viability Kit (Cat. No. 423106, Bioledgend). The labelled cell sample was placed at 4ºC for 20 min, protected from light. After washing, the cells were fixed and permeabilized using a mixture of Fixation/Permeabilization concentrate (Cat. No. 00-5123-43, eBioscience) and Fixation/Permeabilization Diluent (Cat. No. 00-5223-56, eBioscience). After fixation, the cells were washed with 1X permeabilization buffer (Cat. No. 00-8333-56, eBioscience) then stained for intracellular markers (Table 2) (4ºC for 20 min, protected from light). The labelled cells were washed in permeabilization buffer and resuspended in FACS buffer before running on the flow cytometer (BD Canto II or Fortessa).

To determine cytokine production, 100 µl cell suspension (2×10^6 cells/ml) isolated from spleen and lymph node of mice with CIA (CC57Bl/6 WT $H2^q$) was placed in each well of a U-bottomed 96 well falcon plate and stimulated with 20 ng/ml of phorbol myristate acetate (Cat. No. 524400, Calbiochem) and 2 µM ionomycin (Cat. No. 407950, Calbiochem) in the presence of 12.5 µg/ml Brefeldin A (Cat. No. B7651, Sigma-Aldrich) and monesin (Cat. No. 554724, BD Bioscience). The stimulated cells were incubated at 37ºC for 3 hr. The cells were then washed with FACS buffer and stained with surface marker (Table 2) first and stained with intracellular cytokine marker (Table 2) after fixation.

Table 2. Fluorochrome conjugated antibodies for FACS analysis

Flurochrome conjugated antibodies (mouse)	Company	Cat. No.	Diluation Ratio
anti-CD4 -PECy7	eBioscience	25-0042-82	1/200
anti-CD8-FITC	BD Biosciences	553031	1/200
anti-CD11b-APC-Cy7	BD Biosciences	14-0112-81	1/200
anti-I-Aq-AF647	BioLegend	553614	1/200
anti- I-A^b-PE	BioLegend	116407	1/200
anti-IL17-PE	BioLegend	506904	1/200
anti-INFγ-Alexa Fluor® 647	BioLegend	505814	1/200
anti-CD4-BV510	BioLegend	100559	1/200
anti-CD25-PerCP-Cy5.5	BioLegend	102030	1/200
anti-Foxp3-PeCy7	eBioscience	25-5773-82	1/200
anti-GATA3-eFluor® 660	eBioscience	50-9966-42	1/100
anti-RoRγt-PE	BD Biosciences	562607	1/200
anti-Helios-PB	BioLegend	137220	1/100
anti-Tbet-BV711	BioLegend	644819	1/100
anti-CD4-PerCP-Cy5.5	BioLegend	100434	1/200
anti-CD25-BV605	BioLegend	102035	1/200
anti-CTLA4-PE	BD Biosciences	553720	1/200
anti-PD-1-BV421	BioLegend	135218	1/200
anti-LAG3-APC	BioLegend	125210	1/100

2.3 Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

2.3.1 RNA isolation

The cell sample was lysed by using buffer RLT (Cat. No. 79216, Qiagen) with 2M dithiothreitol (DTT) (Cat. No. 74106, Qiagen, Limburg, Netherlands) or 1% 2-mercaptoenthanol (2-ME) (Cat. No. M3148, Sigma-Aldrich). The resulting cell lysate was used for RNA column extraction using the manufacturer instructions of RNeasy Mini Kit (Cat. No. 74106, Qiagen). RNA was eluted using RNase and DNase free water and stored at -80°C until converted to cDNA. The concentration of RNA was measured using a Nanodrop.

2.3.2 cDNA synthesis

For each cDNA reaction, RNA (25 ng/ul, total 500ng) was mixed with equal volume of 2X Master Mix that contains 10X RT buffer, 25X dNTP mix, 10X random primers, reverse transcriptase (High capacity cDNA reverse transcription kit, Cat. No. 4368814, Life Technologies), and Nuclease-free H_2O. The RNA was converted to cDNA under the cycling condition: 25°C 10 min, 37°C 120 min, 85°C 5 min and 4°C ∞. cDNA was stored at -20ºC until used in qPCR reaction.

2.3.3 Real-Time Quantitative Reverse Transcription PCR

For each 6 µl RT-qPCR reaction, 2.4 µl the template cDNA (Table 3) was placed into wells of MicroAmp® Optical 384-Well Reaction Plate with Barcode (Cat. No. 4309849, Life Technologies) with of 3 µl Takyon MasterMix (UF-LPMT-B0701, Eurogentee), 0.3 µl TaqMan primer (table 3) and 0.3 µl Nuclease-free H_2O. Non-template controls were included on each plate. The plate was sealed with MicroAmp ® Optical Adhesive Film (Cat. No. 10299204, Life Technologies) and loaded into Applied Biosystems® ViiATM 7 Real-Time PCR System. The target DNA was amplified under the cycling condition: 95°C 10 min (95°C 15 sec, 60°C

1min) × 45 cycles. Data were normalized to HPRT expression and calibrated relative to a WT mice sample. There are at least two calculation methods used for relative quantification; the standard curve method and the "Delta Delta CT" (ΔΔCT) method. When using the standard curve method, the amount of each experimental sample is first determined using a standard curve and is then expressed relative to a calibrator sample. Therefore, a standard curve must be generated separately for each target gene and housekeeping gene. The ΔΔCT method is based on two assumptions: that the amplification efficiency between primers are equal and the target and reference gene amplification efficiency are equal to 2. Apart from the standard curve method, the generation of a standard curve is unnecessary in ΔΔCT method and can increase throughput. For limited amount of sample, the relative expression ratio was calculated using the ΔΔCT method in my study.

Table 3 TaqMan primers (ABI assay ID, Life Technologies) used in RT-qPCR experiments

Gene	Murine
HPRT	Mm00446968_m1
CTLA-4	Mm00486849_m1
FoxP3	Mm00475165_m1
GITR	Mm00437136_m1
TGF-β	Mm00441724_m1
IL-10	Mm00439616_m1
IL-4	Mm00445259_m1
IL-17	Mm00439619_m1
IL-23R	Mm00519943_m1

2.3.4 384 well microfluidic card Taqman gene expression assays

The microfluidic card coated with target Taqman primers (table 4) was placed at room temperate with sun light protection before the experiment. The target genes selected for measurement were based on (1) T cell subset specific genes, particularly Treg related genes (2) T cell cytokines (3) immune check points (4) TNFR1/2 (5) genes associated with monocyte lineage. 110 ul mixed reaction buffer for per reaction which contains 26.4 ul cDNA sample (500 ng), 28.6 ul nuclease free water and 55 ul Takyon MasterMix (UF-LPMT-B0701, Eurogentee) was prepared. 100 μL of the PCR reaction mixture was loaded into the fill port of the microfluidic card. The filled microfluidic card was inserted into a card holder, placed in a bucket, and then was centrifuged at 1200 rpm for 1 min twice. After the PCR reaction mixture was distributed into each well of the Taqman array card, the filled wells were isolated using The TaqMan® Array Micro-Fluidic Card

Sealer. Following that, the fill strip of Taqman array card was removed by scissors. Then, the Taqman array card was loaded into Applied Biosystems® ViiATM 7 Real-Time PCR System for further RT-qPCR reaction according to the protocol in 2.3.3. The result was analysed using ExpressionSuite Software v1.1.

Table 4 The layout of the 384 well microfluidiccard Taqman gene expression assays

Gene	Assay ID	Gene	Assay ID
Hprt	Mm03024075_m1	Tnfaip3	Mm00437121_m1
Gapdh	Mm99999915_g1	Cd4	Mm00442754_m1
Tnfrsf1a	Mm00441883_g1	Cd8a	Mm01182107_g1
Tnfrsf1b	Mm00441889_m1	Cd86	Mm00444543_m1
Tnf	Mm00443258_m1	Cd80	Mm00711660_m1
Il6	Mm00446190_m1	Cd68	Mm03047343_m1
Il10	Mm01288386_m1	Kmo	Mm01321343_m1
Il12a	Mm00434169_m1	Lag3	Mm00493071_m1
Il12b	Mm01288989_m1	Tigit	Mm03807522_m1
Il23r	Mm00519943_m1	Havcr2	Mm00454540_m1
18s rRNA	Hs99999901_s1	Il23a	Mm00518984_m1
Tbx21	Mm00450960_m1	Cd40lg	Mm00441911_m1
Il4	Mm00445259_m1	Icos	Mm00497600_m1
Gata3	Mm00484683_m1	Tgfb1	Mm01178820_m1
Il17a	Mm00439618_m1	Ifng	Mm01168134_m1
Il2ra	Mm01340213_m1	Rorc	Mm01261022_m1
Foxp3	Mm00475162_m1	Tnfrsf9	Mm00441899_m1
Ctla4	Mm00486849_m1	Ikzf2	Mm00496108_m1
Tnfrsf18	Mm00437136_m1	Irak3	Mm00518541_m1
Tnfrsf4	Mm00442039_m1	Cd274	Mm03048248_m1
Pdcd1	Mm01285676_m1	Ahr	Mm00478932_m1
Pdcd1lg2	Mm00451734_m1	Gzmb	Mm00442837_m1
Ido1	Mm00492590_m1	Cd27	Mm01185212_g1
Ido2	Mm00524210_m1	Cd28	Mm01253994_m1

2.4 Methylation analysis for regulatory T cells

2.4.1 Isolation of mice Tregs by FACS sorting

Single cell suspensions from spleens and lymph nodes were collected by using the cell isolation method described in 2.1.5, and then pooled for performing enrichment of $CD4^+$ T cells using a commercial kit (Cat. No. 130-104-454, Miltenyi Biotec). After collecting the enriched $CD4^+$ T cells, cells were stained with flurochrome conjugated anti CD4 and CD25 antibodies (Table 2) for 20 mins at 4°C, and then washed with FACS buffer at least twice. Finally, the mice Tregs (purification >95%) were collected from labelled $CD4^+$ T cells by FACS sorting (BD FACSAria™ III), and then lysed by using buffer RLT plus (Cat. No. 1053393, Qiagen) with 2M DTT or 1% 2-ME for further genomic DNA and RNA extraction.

2.4.2 Isolation and bisulfite conversion of genomic DNA

The resulting cell lysate from buffer RLT plus was used for genomic DNA and RNA extraction according to the manufacturer instructions of ALL prep® DNA/RNA Mini Kit (Cat. No. 80204, Qiagen). Genomic DNA and RNA were

eluted by using DNase and RNase free water, respectively. And then, RNA samples were stored at -80°C until converted to cDNA. Genomic DNA samples were stored at -20°C until performing bisulfite-conversion. The concentration of genomic DNA and RNA were measured using a Nanodrop.

The nucleotide cytidine (C) of genomic DNA was converted to uracil (U) by using EpiTect Fast Bisulfite Conversion Kit (Cat. No. 59824, Qiagen) according to the manufacturer instructions.

2.4.3 PCR amplification of *FoxP3* bisulfite-converted fragments

The bisulfite DNA was used as template, with the mixture of My Taq HS Red mix (Cat. No. Bio-25407) and the following primers listed in the (table 5) to amplify the upstream enhancer (UE), proximal promoter and Treg-specific demethylated region (TSDR). The procedure we used to amplify proximal promoter and TSDR is nested PCR protocol, which uses two separate rounds of PCR. Nested PCR uses two sequential sets of primers, the first primer (OF and OR) followed by the second primer (IF and IR). PCR was performed with the following parameters:

Process	Temperature and Times	Cycles
Pre-denaturation	95°C for 15 mins	1
Denaturation	95°C for 45 sec	
Annealing	55°C for 45 sec	40
Extension	72°C for 60 sec	
Extension	72°C for 10 mins	1
End and store	4°C	∞



Table 5. List of primers in the methylation of FoxP3 study

Primer	Sequence(5'-3')
UpEnhnc_F	AATGTGGGTATTAGGTAAAATTTTT
UpEnhnc_R	AAACCCTAAAACTACCTCTAAC
Promoter OF	TTTTGTGATTTGATTTATTTTTTTT
Promoter OR	ATACTAATAAACTCCTAACACCCACC
Promoter IF	TATATTTTTAGATGATTTGTAAAGGGTAAA
Promoter IR	ATCAACCTAACTTATAAAAAACTACCACAT
TSDR OF	TATTTTTTTGGGTTTTGGGATATTA
TSDR OR	AACCAACCAACTTCCTACACTATCTAT
TSDR IF	TTTTGGGTTTTTTTGGTATTTAAGA
TSDR IR	TTAACCAAATTTTTCTACCATTAAC

2.4.4 Purification of PCR products

The purified *FoxP3* bisulfite-converted DNA fragments were collected by using QIA quick PCR Purification kit (Cat. No. 28104, Qiagen) according to manufacturer instructions.

2.4.5 TOPO cloning ligation

The purified *FoxP3* bisulfite-converted DNA fragments were ligated into pCR4-TOPO vectors by the TOPO cloning kit (Cat. No. K4595-01, Invitrogen). The reaction buffers were listed in the following:

Reagents	Volume
pCR4-TOPO vector	1 λ
Salt solution	1 λ
Purified DNA fragments	4 λ
Total volume	6 λ

The mixture was incubated at RT for 10 mins.

2.4.6 Transformation

The 50 µl competent cells from TOPO cloning kit were thawed on ice and then 6 µl of TOPO ligation solution was added to the tube on ice for 30 min. The

transformation mixture was heat-shocked on the water bath for 30 seconds at 42°C and then placed on the ice for 5 mins. 300 µl S.O.C medium from TOPO cloning kit was added into the each tube and incubated at 37°C with gentle shaking for 30 minutes. Taking 150~200 µl transformed bacteria solution to place on the LB plates containing 50 µg/ml kanamycin for selecting the transformed bacteria, and incubated overnight at 37°C.

2.4.7 Colony PCR

Before sequencing, colony PCR was an easy and fast method to identify the positive clones containing inserts. Sterile tips were used to pick the single bacterial colonies from LB plates, followed by touching the tips on to a new LB plate for growth and then the tips were dipped into 20 λ of colony PCR reaction buffer. The amount of colony PCR reaction buffer was listed as follows:

Reagents	Volume
MangoMix	10λ
M13 Forward primer (5 µm)	2 λ
M13 Reverse primer (5 µm)	2 λ
d_2H_2O	6 λ
Total volume	20 λ

PCR tubes of the mixture were placed into T3 Thermocycler (Biometra). The cycling parameters for the colony PCR were presented as follows:

Process	Temperature and Times	Cycles
Pre-denaturation	95°C for 5 mins	1
Denaturation	95°C for 30 sec	
Annealing	55°C for 30 sec	40
Extension	72°C for 30 sec	
Extension	72°C for 10 mins	1
End and store	4°C	∞



2.4.8 Bisulfite genomic sequencing analysis

After overnight growth of selected clones, tips were used to pick correct clones confirmed by colony PCR, and then dipped into FrameStar® PCR 96 wells plate (Eurofins) for growth over night. Genomic sequencing was performed by Eurofins. The methylation rate was determined by the BiQ Analyzer software.

2.5 Collagen-induced arthritis

2.5.1 Collagen purification

Powdered bovine cartilage was weighed and stirred with 5 ml of Tris-buffered guanidine per gram of cartilage overnight in a 500 ml flask at 4°C. The cartilage containing solution was centrifuged at 14000 g, 4ºC for 1 hr using Thermo Scientific™ Sorvall™ Evolution™ RC Superspeed Centrifuge. The pellet was resuspended with 5 ml of Tris-buffered guanidine per gram of cartilage and transferred to a 500 ml flask. The cartilage containing solution was stirred overnight and centrifuged at 14000 g, 4ºC for 1 hr.

The cartilage pellet was resuspended in 20 ml of acetic acid (0.5M, pH: 2.8) per gram of cartilage and 1g pepsin for every 20 g of cartilage was added. The cartilage containing solution was stirred at 4ºC overnight and then centrifuged at 14000 g, 4ºC for 48 hr. The supernatant was collected into a 500 ml flask and NaCl was added gradually into the supernatant to a final concentration of 0.89 M. The NaCl containing solution was stirred at 4ºC overnight and centrifuged at 14000 g, 4ºC for 1 hr.

The supernatant was discarded, and the pellet was dissolved in 100 ml 0.1M

acetic acid. In order to inactivate the residual pepsin, the cartilage containing solution was placed in a dialysis tube and dialysed against 5 L 0.02 M, pH: 9.4 Na_2HPO_4 at 4ºC for 3 days. The dialysate was changed every day. The solution was taken from the dialysis tube and centrifuge at 14000 g, 4ºC for 1 hr.

The collagen containing solution was divided into 50 ml falcon tubes and placed at -80ºC overnight. The falcon tubes were transferred to the freeze dryer for lyophilisation. The lyophilised collagen stored at 4ºC.

2.5.2 Immunization

Immunizing emulsion was produced by mixing a solution of bovine type II collagen with an equal volume of complete Freund's adjuvant (CFA) (Cat. No.263910, Difco Laboratories) on ice. Male mice (C57BL/6 WT $H2^q$ or DBA/1) were anaesthetized using isoflurane and immunized by subcutaneous injection of 100 µl emulsion at two sites (one at the base of the tail and one on the flank).

2.5.3 DNMT inhibitors treatment for CIA

The mice were injected intraperitoneally from the day of arthritis onset with

DNMT inhibitors for 4 days (Decitabine (Cat. No. A3656, Sigma-Aldrich) 1 mg/kg once a day, Psammaplin A (Cat. No. sc258049, Santa Cruz) 10 mg/kg twice a day and Procainamide (Cat. No. P9391, Sigma-Aldrich) 100 mg/kg twice a day) from the day of arthritis onset by intraperitoneal injection.

2.5.4 Disease Assessment

The mice were monitored daily for arthritis starting from 2 weeks after immunization and scored from day of onset. The severity of arthritis was scored as following: 0=normal, 1=slight swelling and/or erythema, 2=marked swelling, 3=ankylosis for each paw. The hind paw thickness was also measured daily using calipers. The mice with arthritis were culled at day 10 after arthritis onset.

2.5.5 Histology Scoring

Paws were harvested and fixed in 10% neutral buffered formalin for at least 48 hours, followed by decalcification with 5.5% EDTA in buffered formalin for 4 weeks. Fixed paws were embedded in paraffin, and sections were cut and stained with hematoxylin and eosin. Histopathologic changes in the joints were scored on

a scale of 0–3, where 0 = normal, 1 = minimal synovitis without cartilage/bone erosion, 2 = synovitis with some marginal erosion but joint architecture maintained, and 3 = severe synovitis and erosion with loss of normal joint architecture.

2.6 In vitro cell culture assay

2.6.1 T cell proliferation assay

Single cell suspensions of spleens and lymph nodes were collected using the cell isolation method described in 2.1.5, and then counted. 1 x 10^7 cells were stained with CFSE dye first by using CellTrace™ CFSE Cell Proliferation Kit (Cat. No. C34570, Invitrogen) according to the manufacturer's instructions. And then, 4 x 10^5 of cells were plated into a 96 well U-bottom tissue culture plate and stimulated with bovine type II collagen (50 μg/ml) or anti-CD3 antibody (0.1 μg/ml) at 37°C for 3 days. The cells were stained with flurochrome conjugated anti CD4 antibody (Table 2) and Zombie NIR™ Fixable Viability Kit for 20 mins at 4°C, and then washed with FACS buffer at least twice and run on the flow cytometer (BD Canto II).

2.6.2 *In vitro* differentiation of $CD4^+CD25^+FoxP3^+$ induced Tregs

Single cell suspensions of spleens and lymph nodes were collected using the cell isolation method described in 2.1.5, and then the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Cat. No. 130-091-041, Miltenyi Biotec) was used to isolate the non-$CD4^+$ T cells (APCs), $CD4^+CD25^-$ T cells (Tconvs) and $CD4^+CD25^+$ T cells (Tregs) according to the manufacturer instructions. 1 x 10^5 APCs (50 uL) treated with or without mitomycin C were plated into 96 wells culture plate first, then 1 x 10^5 Tconvs (50 ul) were plated into the same wells. The ratio of APCs to Tconvs was 1:1. 50 uL of cytokine and antibody mixture were added to the same wells (Final concentration: anti-CD3: 1 μg/ml, TGFβ: 5 ng/ml and IL-2: 10 ng/ml). 50 ul of Decitabine was added to the wells (final concentration is 0.05, 0.1, 0.2, 0.5, 0.7, 1 and 2 μM), Procainamide (Final concentration is 12.5, 25, 50,100 and 200 uM) or Psammaplin A (Final concentration is 0.05, 0.1, 0.2, 0.5, 0.7, 1 and 2 μM). The plates were incubated for indicated duration at 37°C and then the cells were stained with fluorochrome conjugated anti CD4, CD25 and FoxP3 antibodies (Table 2) according to the protocol in 2.2.1 and run on the flow cytometer (BD Canto II).

2.6.3 Treg suppression assay

Single cell suspensions of spleens and lymph nodes were collected using the cell isolation method described in 2.1.5, and then the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Cat. No. 130-091-041, Miltenyi Biotec) was used to isolate the Non-$CD4^+$ T cells as APCs, $CD4^+CD25^-$ T cells as responder cells and $CD4^+CD25^+$ T cells (Tregs) as suppressor cells according to the manufacturer instructions. The responder cells were labelled with CSFE by using CellTrace™ CFSE Cell Proliferation Kit according to the manufacturer instructions. 1 x 10^5 Tregs (100 ul) were plated first and serial dilutions were performed on the plate (50 ul per well). 5 x 10^4 responder cells (50 ul) and 5 x 10^4 of APCs (50 ul) treated with mitomycin C were then added to the same wells to acquire the different ratio of Treg: Responder: APC from 2:1:1 to 0.0675:1:1. 50 uL of 4 μg/ml anti-CD3 monoclonal antibody was added to the wells to make a total of 200 μl. The culture plate was incubated at 37^0C for 3 days. The cells were stained with fluorochrome conjugated anti CD4 antibody (Table 2) and Zombie NIR™ Fixable Viability Kit for 20 mins at 4°C and then washed with FACS buffer for least twice and run on the flow cytometer (BD Canto II) to analyze CFSE dilution.

2.6.4 Statistical analyses

All the data were analyzed by one-way ANOVA followed by Dunnet's or Tukey's multiple comparison test, as appropriate. All calculations were made using GraphPad Prism 6 software. A p-value less than 0.05 was considered significant.

Chapter 3 TNFR2 deficiency is associated with decreased FoxP3 gene expression and increased CpG methylation at the proximal promoter

3.1 Introduction

TNFR2 is expressed on Tregs[145] and a previous study revealed that sustained exposure to TNF-α increased the proliferation of Tregs and restored their suppressive function on proliferation and cytokine production of effector T cells. In addition, in synergy with IL-2, TNF-α up-regulated the expression of CD25 and FoxP3[259]. However, the stimulatory effect of TNF-α on Tregs was not seen in $TNFR2^{-/-}$ mice following sublethal cecal ligation and puncture[149]. Further studies showed $TNFR2^{+}$ Tregs exhibit higher suppressive function compared with $TNFR2^{-}$ Tregs[146, 148] and TNFR2 plays an indispensable role in maintaining the stabilization of Treg phenotype in the inflammatory environment[144]. In CIA, it was shown that the selective TNFR1 blockade not only reduced disease severity to the same extent as TNFR1/2 blockade, but also expanded and activated Tregs, an effect that was not seen following TNFR1/2 blockade[257]. These studies

indicate an immunoregulatory role for TNFR2 in promoting Tregs and suggest that TNFR1 specific blockade (TNFR2 sparing treatment strategy) may be superior than TNFR1/2 blockade in treating RA.

Based on the aforementioned findings, I hypothesized that deficiency of TNFR2 signaling may lead to decreased Treg number by inhibiting FoxP3 expression, a master regulator of Tregs. Thus, the objective of the work in this chapter was to assess the differences in phenotype of Tregs between WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice using flow cytometry analysis and RT-qPCR.

Epigenetic modification is defined by mitotically and meiotically heritable changes without changing the sequence of DNA. DNA methylation and histone modification are two major mechanisms that alter the DNA accessibility and chromatic structure, thereby regulating gene expression. It has been reported three cis-regulatory elements within *FoxP3* locus are susceptible to methylation and are involved in regulating generation and maintenance of Tregs. Another hypothesis tested in this chapter was that TNFR2 deficiency would alter the methylation degree of cis-regulatory elements of FoxP3 locus, thus leading to

changes of the phenotype of Tregs. To address the effect of TNFR signaling on methylation of regulatory elements of FoxP3, I measured the methylation level of upstream enhancer, proximal promoter and TSDR of FoxP3 locus in Tregs isolated from WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice by bisulfite sequencing.

3.2 Results

3.2.1 TNFR1 and TNFR2 Genotyping

To clarify the role of TNFR1 and TNFR2 in the development of Tregs, I bred mice deficient for either TNFR1 or TNFR2. I used different primer pairs specific for each gene on genomic DNA from the ear. Wild type (WT), TNFR1-/- and TNFR2 genomic DNA produced a single band of expected size (WT 120 bp in Figure 3.1A and 275 in Figure 3.1B, TNFR1-/- 155 bp, TNFR2 -/- 160 bp). Heterozygous genomic DNA produced two bands of expected size. (Figure 1A and 1B)

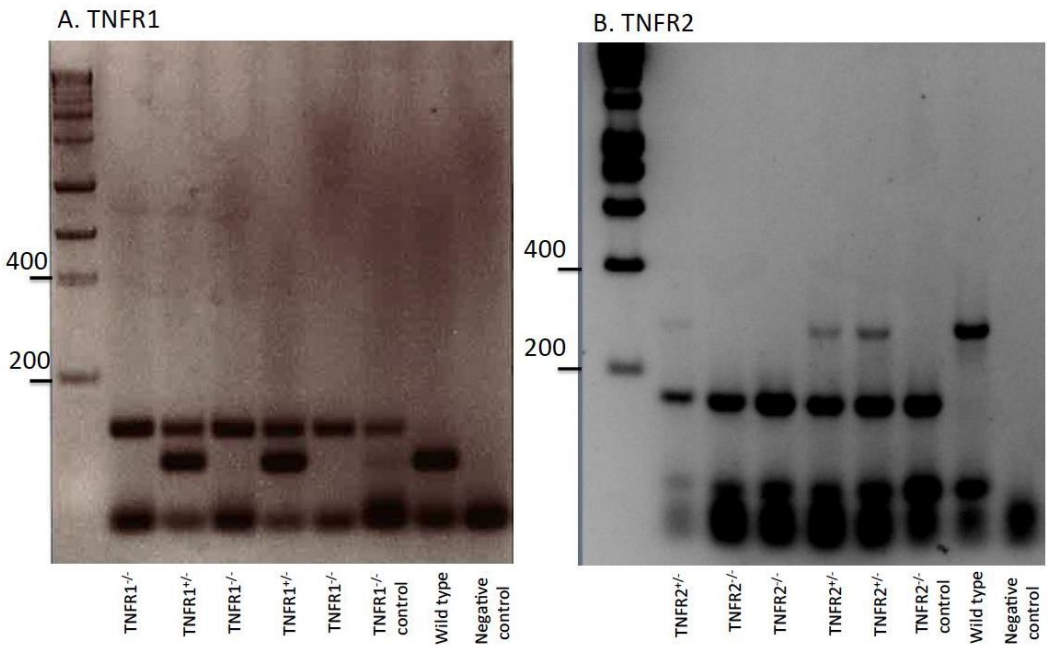


Figure 3.1 (A) TNFR1$^{-/-}$ and (B) TNFR2$^{-/-}$ genotyping. DNA was extracted from ear biopsies and genotyped by PCR. Each reaction contained four primers: P60B, P60E, P60-spe and PGK5-66, for TNFR1$^{-/-}$ amplification and three primers: p80-Kas, p80i-1, and PG-66 for TNFR2$^{-/-}$ amplification (see Materials and Methods for primer details). Amplified DNA fragments were separated using 3% agarose gels alongside molecular weight markers. TNFR1$^{-/-}$ and 2$^{-/-}$ control are the samples had been genotyped previously as TNFR1 and TNFR2 knock mice.

3.2.2 Decreased expression of FoxP3 in TNFR2$^{-/-}$ mice.

Tumour necrosis factor (TNF), a pleiotropic cytokine, mediates inflammatory and immunity response via two functionally and structurally distinct receptors: TNFR1 and TNFR2. TNFR1 is expressed on most cell types and is responsible for most inflammatory, cytotoxic and apoptotic effects of TNF. In contrast, the expression of TNFR2 is limited to certain cell types such as human mesenchymal stem cells, some neuronal subtypes and some subpopulations of immune cells, including regulatory T cells[260]. Some studies showed TNFR2 plays an important role in regulatory T cells such us stabilizing the expression of FoxP3 and maintaining the suppressive function of Tregs[144].

To assess the influence of TNFR1 and TNFR2 on numbers of Treg cells, I first assessed the proportion of CD4^{+}CD25^{+}FoxP3^{+} Treg cells in the spleen, LNs and thymus of WT, TNFR1$^{-/-}$, TNFR2$^{-/-}$ mice. In the spleen, the percentage of CD4^{+}CD25^{+}FoxP3^{+} Treg cells in TNFR2$^{-/-}$ mice was slightly lower than WT and TNFR1$^{-/-}$ mice, although not reaching statistical significance (Fig 3.2A). The same trend was also shown in LNs (Fig 3.2B). In contrast, there was no difference in

the percentage of $CD4^+CD25^+FoxP3^+$ Treg cells in total $CD4^+CD8^-$ T cells of thymus between WT, $TNFR1^{-/-}$, $TNFR2^{-/-}$ mice (Figure 3.2C). Our findings are different from the previous study that showed reduced numbers of Tregs in the thymus, spleen and LNs in TNFR2 deficient mice[144]. Intriguingly, medium fluorescence intensity (MFI) of FoxP3 in $CD4^+CD25^+$ cells decreased in the spleen and thymus from $TNFR2^{-/-}$ mice. This finding is compatible with a previous study that showed TNF, in synergy with IL-2, up-regulated the expression of FoxP3 in Tregs[149]. My result indicates that TNF plays an indispensable role in expression of FoxP3 in Tregs through TNFR2.

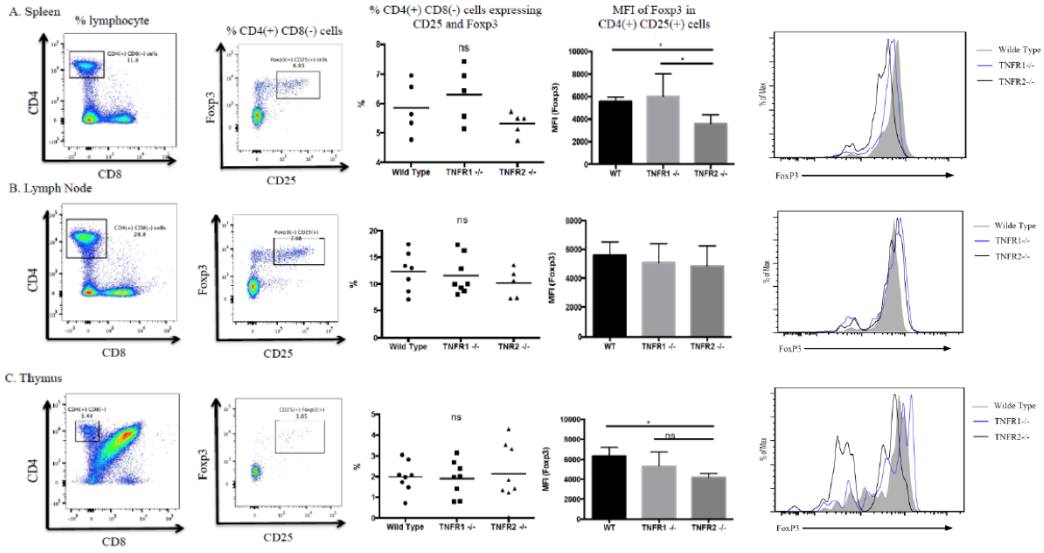


Figure 3.2 The absence of TNFR1 or TNFR2 does not affect numbers of Tregs. Cells from (A) spleen, (B) lymph nodes and (C) thymus in wild type (WT) mice, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (n=5 - 8) were stained with Ab to CD4, CD8, CD25 and FoxP3. The expression of CD25 and FoxP3 were analyzed by FACS, gating on CD4^{+}CD8^{-} cells. In (A), (B) and (C), left three panels show the gating strategy and grouped data of Tregs, and right two panels show the representative histograms and grouped data on FoxP3 MFI for each group of mice. The horizontal lines represent the mean of each group. The comparison of the mean of proportion of Tregs and the MFI of FoxP3 on Tregs was determined by one-way analysis of variance with Tukey's multiple comparison post-test (*=$p<0.05$, ns= not significant).

3.2.3 The numbers of iTreg and tTreg cells are not affected by TNFR1 and TNFR2 signaling

Tregs are composed of two main populations: thymus derived natural Tregs (tTregs) and peripherally derived Treg cells (iTregs). Helios protein, encoded by the *IKZF2* gene, was proposed to be a valid marker expressed by tTregs but not iTregs. However, other studies have demonstrated expression of Helios in both $CD4^+$ and $CD8^+$ T cells upon stimulation, and also in iTregs generated *in vitro*[261-263]. In addition, it was also suggested that $Helios^+$ Tregs represent a functional subset with superior suppressive function[264]. Despite these contradictory findings, the consensus view is that Helios is preferentially but not exclusively expressed by tTreg cells. A previous report showed that TNF-α signaling is required for in vivo function of tTregs but not iTregs[265]. To investigate further the role of TNFR1 and TNFR2 on tTregs versus iTregs, I assessed the proportion of $CD4^+FoxP3^+Helios^+$ Treg cells and $CD4^+FoxP3^+Helios^-$ Treg cells in the spleen, LNs, and thymus of WT, $TNFR1^{-/-}$, $TNFR2^{-/-}$ mice. The percentage of CD4+FoxP3+Helios+ Treg cells was around 10% in spleen and LNs and 3% in thymus. The percentage of

CD4+FoxP3+Helios- cells is lower than CD4+FoxP3+Helios+ Treg cells and around 5% in spleen and LNs and 1% in thymus. No significant difference of the proportion of CD4+FoxP3+Helios+ Treg cells and CD4+FoxP3+Helios- cells in spleen, LNs and thymus was noted between the 3 groups of mice (Figure 3.3). Therefore, genetic ablation of TNFR1/TNFR2 didn't result in a significant change of the proportion of CD4+FoxP3+Helios+ Treg cells and CD4+FoxP3+Helios- cells in spleen, LNs or thymus.

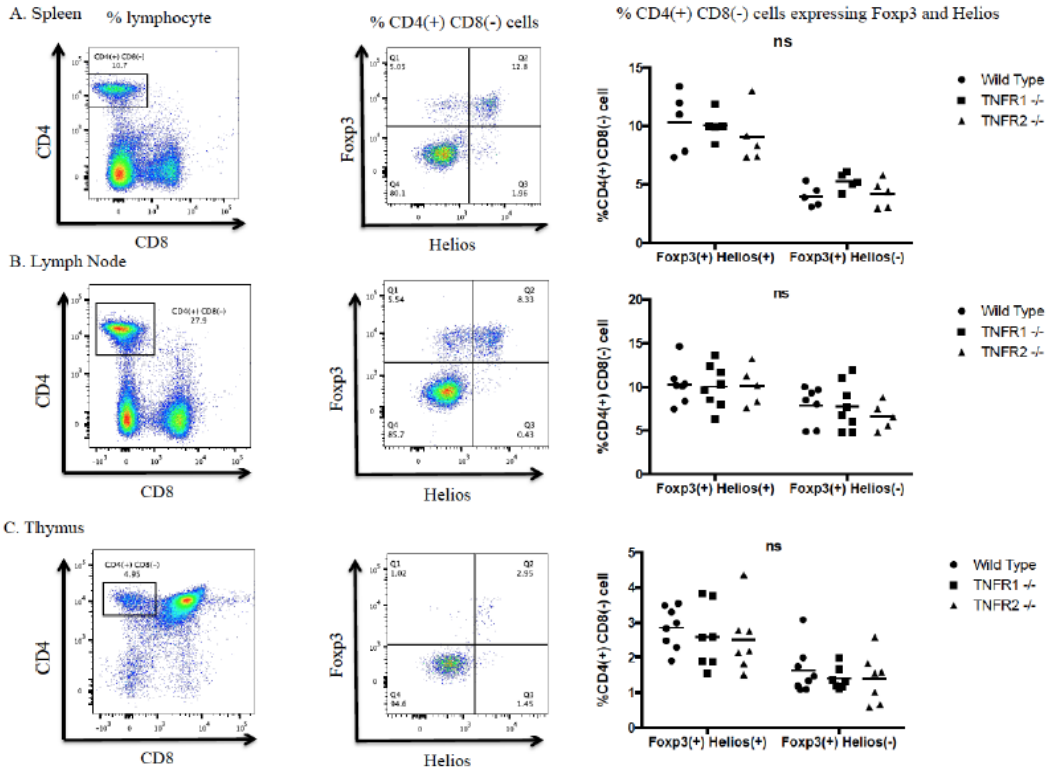


Figure 3.3 TNFR1 and TNFR2 are not required for development of CD4+FoxP3+Helios+ Treg cells and CD4+FoxP3+Helios- Treg cells development. Cells from (A) spleen, (B) lymph nodes and (C) thymus in WT mice (C57BL/6), TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (n=5-8) were stained with Ab to CD4, CD8, FoxP3 and Helios. The expression of FoxP3 and Helios were analyzed by FACS, gating on CD4^{+}CD8^{-} cells. In (A), (B) and (C), left and middle panels show the gating strategy, and right panels show the grouped data. The horizontal lines represent the mean of each group. The comparison of the mean proportion of nTreg cells and iTreg cells between three groups of mice was determined by one-way analysis of variance with Turkey's multiple comparison post-test (ns=not significant).

3.2.4 Reduced gene expression of FoxP3 and TGF-β in the spleen and thymus of TNFR2$^{-/-}$ mice.

There is some evidence that TNFR2 defines the most suppressive subgroups of Tregs[148] and TNFR1 specific blockade, which spares TNFR2 function, increased the suppression function of Treg cells[257]. To further probe the association between TNFR1, TNFR2 and phenotype and function of Treg cells at the gene expression level, the level of messenger RNA for *foxp3, ctla4, gitr, tgfβ, il10, il4, il17and il23r* were measured by RT-qPCR.

In spleen, there was a reduction of gene expression of *foxp3* in TNFR2$^{-/-}$ mice compared with WT mice. The gene expression of *tgfβ* was decreased in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. Compared to WT mice, *il23r* gene expression showed a marked increase in TNFR1$^{-/-}$ mice (Figure 3.4).

In LNs, there was no significant difference of the gene expression level of all the target genes, except *il10*, which was increased significantly in TNFR2$^{-/-}$ mice (Figure 3.5).

In the thymus, there was a similar trend in the gene expression level of *foxp3* and *tgfβ* to the expression in spleen. The gene expression level of *foxp3* and *tgfβ* decreased in TNFR2$^{-/-}$ mice. Regarding IL-23R, the gene expression level in TNFR1$^{-/-}$ mice was higher than WT and TNFR2$^{-/-}$ mice, although not reaching statistical significance (Figure 3.6).

FoxP3 is critical for the development and function of Tregs and the expression of FoxP3 is regulated by many factors, including IL-2, TGF- β, TCR, CTLA-4 and TLR. The finding of decreased gene expression of *foxp3* and *tgf- β* in the spleen and thymus of TNFR2$^{-/-}$ mice indicates that TNFR2 may play a role in maintaining the expression of FoxP3 in a TGF-β dependent manner.

IL-23 signalling plays an important role in maintaning proliferation of Th17 and stimulating the production of IL-17. The result of increased gene expression of IL-23R in the spleen of TNFR1$^{-/-}$ mice suggests a role of TNF/TNFR1 signaling in regulation of IL23R expression but this finding will need to be confirmed in further studies.

Ct (threshold cycle) is the value where the amplification curve crosses the threshold line. It is a relative measure of the concentration of target genes in the qPCR reaction. In general, when a Ct value is obtained, the sample tested is considered to be positive Higher the Ct, the less the cDNA detected is present. However, many factors other than the concentration of the target template may affect the value of Ct, including amplification efficiency of the PCR reaction, concentration of reference dye, and master mix components. Therefore, Ct values above an arbitrarily defined cutoff value (>35 in my study) is considered as negative (or not trustworthy), which may be generated by degradation of the fluorophore or by nonspecific amplification of background nucleic acids. According to the fluorophore chemistry used, some procedures are used to distinguish fluorescence signal artifacts. For instance, in SYBR Greena dye-based assays, validating methods include melting curve analysis, DNA electrophoresis gel, and sequencing. In contrast, probe-based assays, such as TaqMan probe (the method used in my study), which are based on the detection of a specific sequence within the template, limit the signals from the nonspecific amplification. In addition, a no-template control is always included to detect cross contamination from positive samples with negative samples

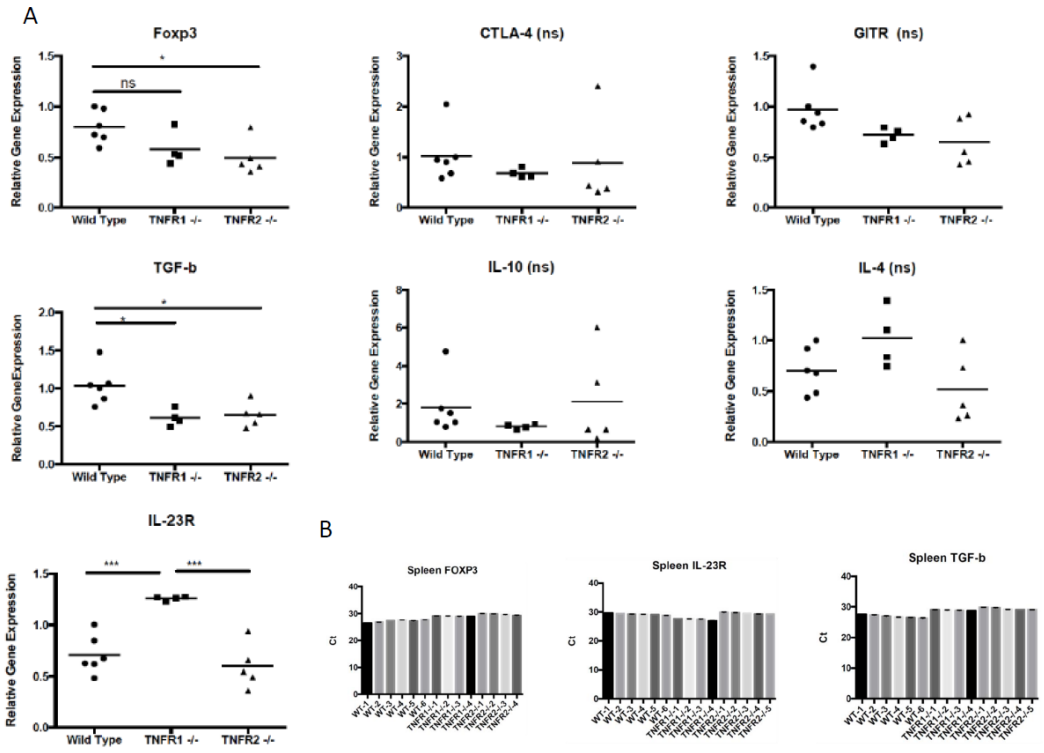


Figure 3.4 Reduced expression of the genes encoding FoxP3 (*foxp3)* and TGFβ (*tgfβ1)* in TNFR2$^{-/-}$ mice and increased expression of IL-23R (*il23r)* in TNFR1$^{-/-}$ mice. RNA was isolated from spleens of WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (n=4-6), and reverse transcribed to cDNA for RT-qPCR analysis using Taqman primer probes. (A) Data were normalized *to hprt* expression and were calibrated relative to WT. Horizontal lines show the means. (B) Ct value of the genes with statistical difference between groups are provided. The comparison of the mean of target gene expression was determined by one-way analysis of variance with Tukey's multiple comparison post-test (*=P <0.05, **=P<0.01, ***=P <0.001, ns=not significant).

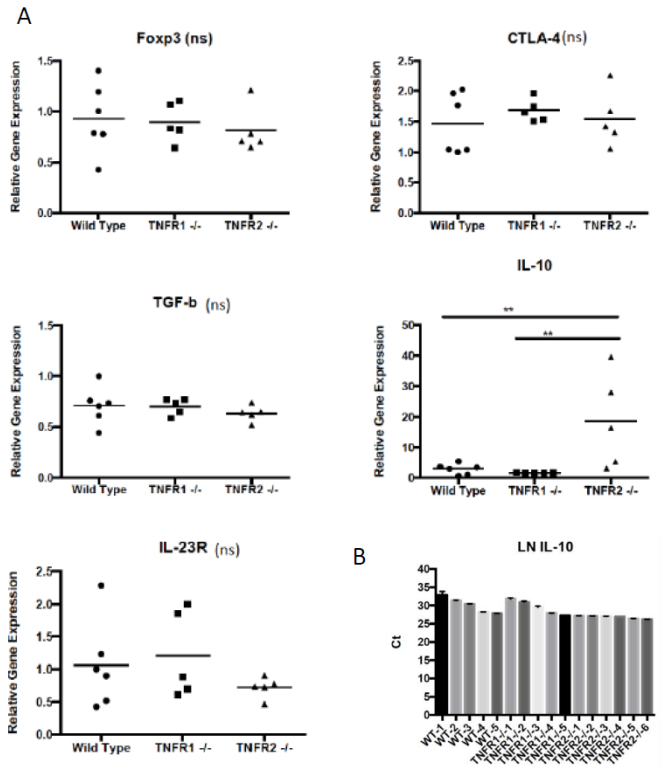


Figure 3.5 Increased expression of *il10* in the lymph nodes of TNFR2$^{-/-}$ mice. The RNA was isolated from inguinal lymph nodes of WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (n=5-6), and reverse transcribed to cDNA for RT-qPCR analysis using Taqman primer probes. (A) Data were normalized to HPRT expression and were calibrated relative to WT. Horizontal lines show the mean. (B) Ct value of the gene with statistical difference between groups are provided. The comparison of the mean of target gene expression was determined by one-way analysis of variance with Tukey's multiple comparison post-test (**=P <0.01, ns=not significant).

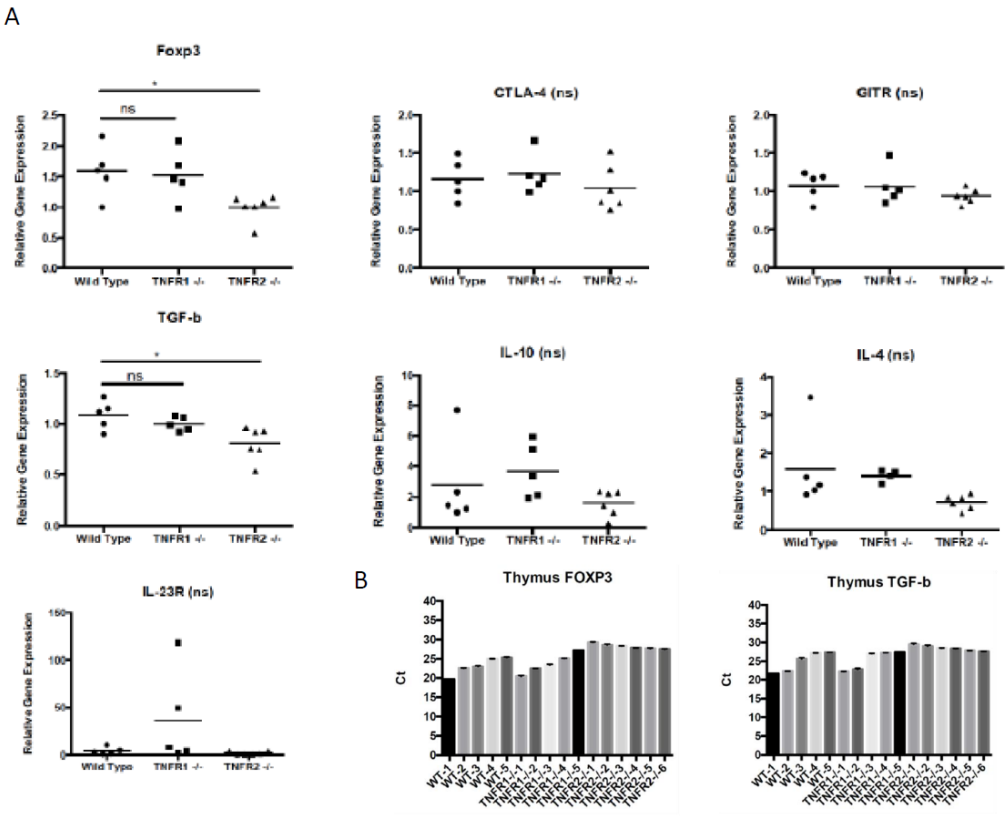


Figure 3.6 Reduced expression of *foxp3* and *tgfβ1* **in the thymus of TNFR2$^{-/-}$ mice.** The RNA was isolated from thymus of WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (n=5-6), and reverse transcribed to cDNA for RT-qPCR analysis using Taqman primer probes. (A) Data were normalized to HPRT expression and were calibrated relative to a WT mouse. Horizontal lines show the mean. (B) Ct value of the gene with statistical difference between groups are provided. The comparison of the mean of target gene expression was determined by one-way analysis of variance with Tukey's multiple comparison post-test (*P=<0.05, ns=not significant).

3.2.5 Increased degree of methylation at FoxP3 locus in TNFR2$^{-/-}$ mice

My study showed that TNFR2 deletion is associated with the decreased expression of FoxP3. It is generally accepted that eukaryotic transcription is regulated by various mechanisms, which takes place in three stages: transcriptional initiation (includes epigenetic modulation and transcription factors), elongation of RNA and termination of the polymerase. There are several *cis*-regulatory elements that have been identified in the *foxp3* locus, which present upstream of the transcriptional start site (upstream enhancer and proximal promoter) and between non-coding exons (intronic enhancer, also referred as Treg specific demethylated region (TSDR). The methylation status of these elements negatively correlates with *foxp3* gene expression and plays an indispensable role in controlling the differentiation and stabilization of Tregs[213, 266]. I further investigated the association between TNFR and epigenetic modulation of FoxP3.

To understand whether the decreased expression of FoxP3 in TNFR2$^{-/-}$ mice is associated with the epigenetic modulation at *foxp3 cis*-regulatory elements, I assessed the methylation status of the upstream enhancer, proximal promoter and

TSDR of Treg from WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice (Figure 3.7A). Since the *foxp3* gene is located on X-chromosome, male mice were used to avoid mosaicism in female mice. Splenic cells and LN cells were harvested from WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ male mice (3 to 4 mice in each group) and pooled. Tregs were isolated from pooled spleen and LN cells by FACS-sorting (Figure 3.7B). Genomic DNA was extracted from Tregs, followed by bisulfite conversion, purification, PCR amplification, and cloning (Figure 3.7C). The degree of methylation of each regulatory element was determined by bisulfite sequencing. While the methylation degree of upstream enhancer is comparable between 3 groups (Figure 3.8), significantly increased levels of methylation were seen in proximal promoter of TNFR2$^{-/-}$ mice compared with that seen in WT mice (Figure 3.9). Notably, there was also a trend of increased level of methylation in TSDR of TNFR2$^{/-}$ mice compared with that observed in WT mice, although not reaching statistical significance (Figure 3.10). The results indicated that TNFR2 signaling regulates the expression of FoxP3 in Tregs by affecting the methylation degree in proximal promoter, and possible TSDR, in FoxP3 locus.

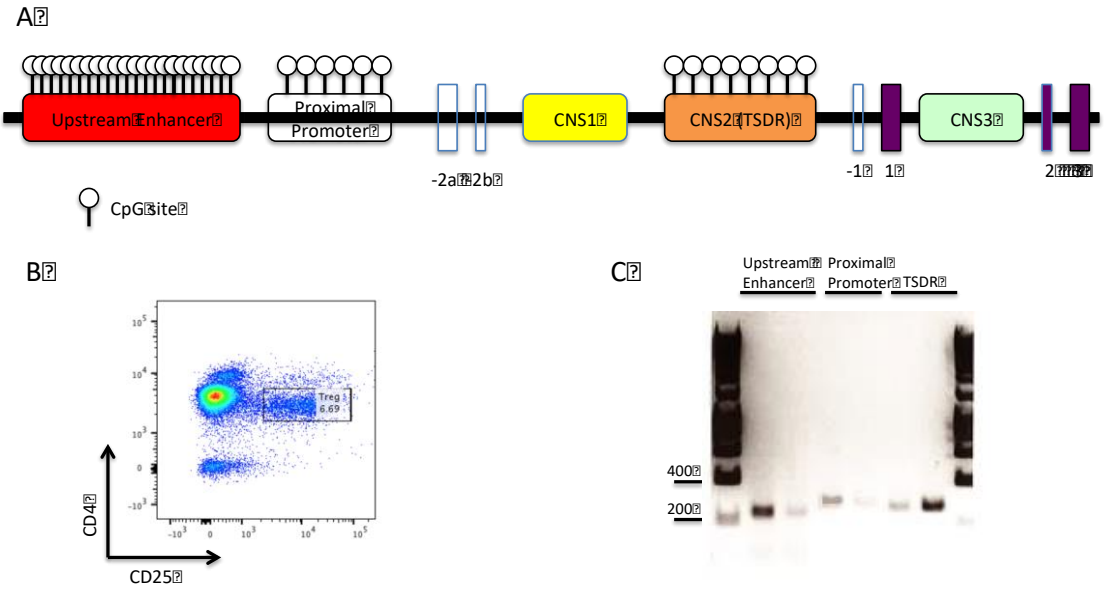


Figure 3.7 Different regulatory *cis*-elements in the FoxP3 locus. (A) Schematic of the CpG islands in the mice FOXP3 locus. Splenic cells and LN cells were harvested from WT, TNFR1$^{-/-}$and TNFR2$^{-/-}$ male mice (3 to 4 mice in each group) and pooled. Genomic DNA was isolated from FACS-sorted Treg cells (B) of WT, TNFR1$^{-/-}$ and TNFR2-/- male mice, bisulfite converted, and then the upstream enhancer region, proximal promoter region and TSDR were PCR amplified using regional specific primers. Amplified DNA fragments were separated using 3% agarose gels alongside molecular weight markers (C).

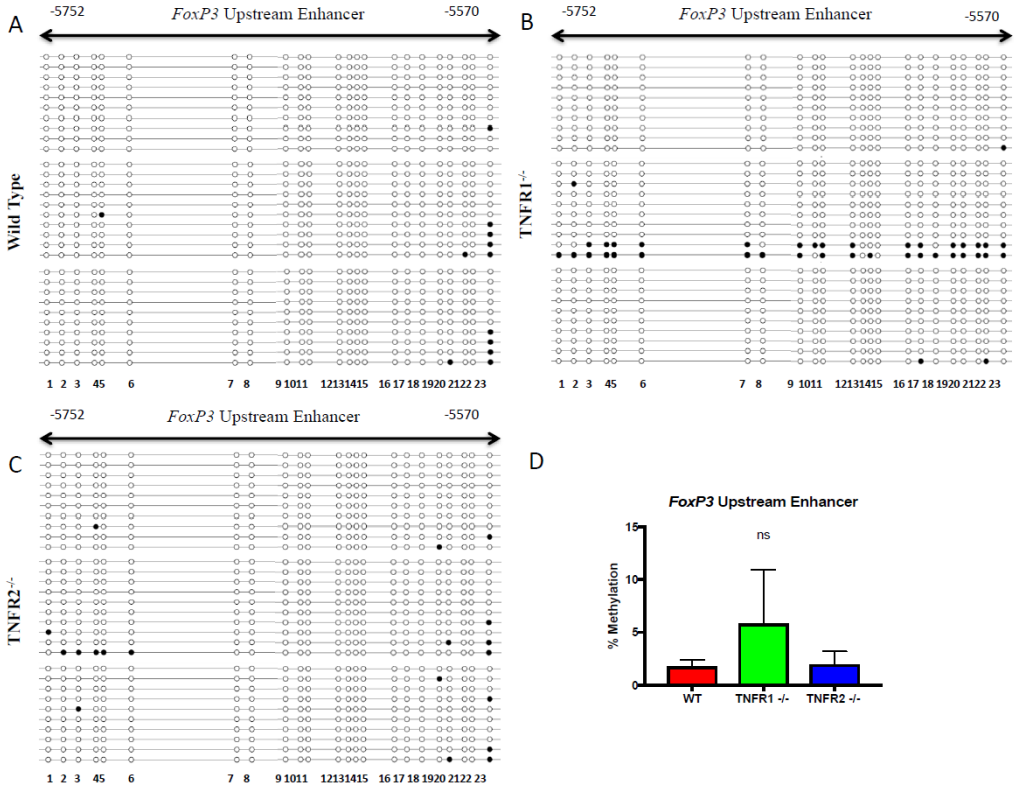


Figure 3.8 The absence of TNFR2 does not affect FoxP3 upstream enhancer methylation status. Genomic DNA was isolated from the FACS-sorted Tregs, modified with sodium bisulfite, PCR amplified, cloned into the pCR4-TOPO vector, and individual clones were sequenced. Each line represents a different clone. The methylation pattern of each clone obtained is shown. ●, Methylated CpG;○, nonmethylated CpG. Data are representative of 3 independent experiments for each strain (A to C). (D) Methylation degree of each experiment was calculated. Horizontal lines with error bars in D show the mean ± SEM, ns=not significant versus WT mice; by one-way analysis of variance with Dunnett's multiple comparison post-test.

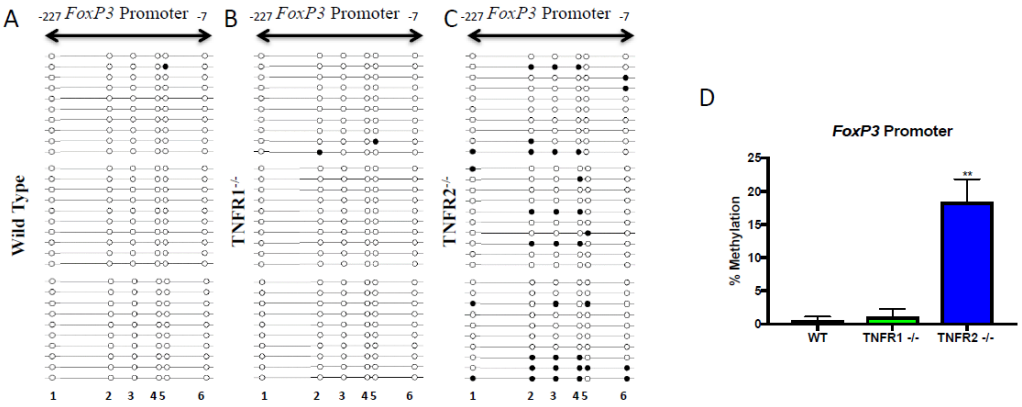


Figure 3.9 FoxP3 proximal promoter methylation status is altered in TNFR2$^{-/-}$ mice compared to WT controls. Genomic DNA was isolated from the FACS-sorted Tregs, modified with sodium bisulfite, PCR amplified, cloned into the pCR4-TOPO vector, and individual clones were sequenced. Each line represents a different clone. The methylation pattern of each clone obtained is shown. ●, Methylated CpG;○, nonmethylated CpG. Data are representative of 3 independent experiments for each strain (A to C). (D) Methylation degree of each experiment was calculated. Horizontal lines with error bars in D show the mean ± SEM, **=$p<0.01$ versus WT mice; by one-way analysis of variance with Dunnett's multiple comparison post-test.

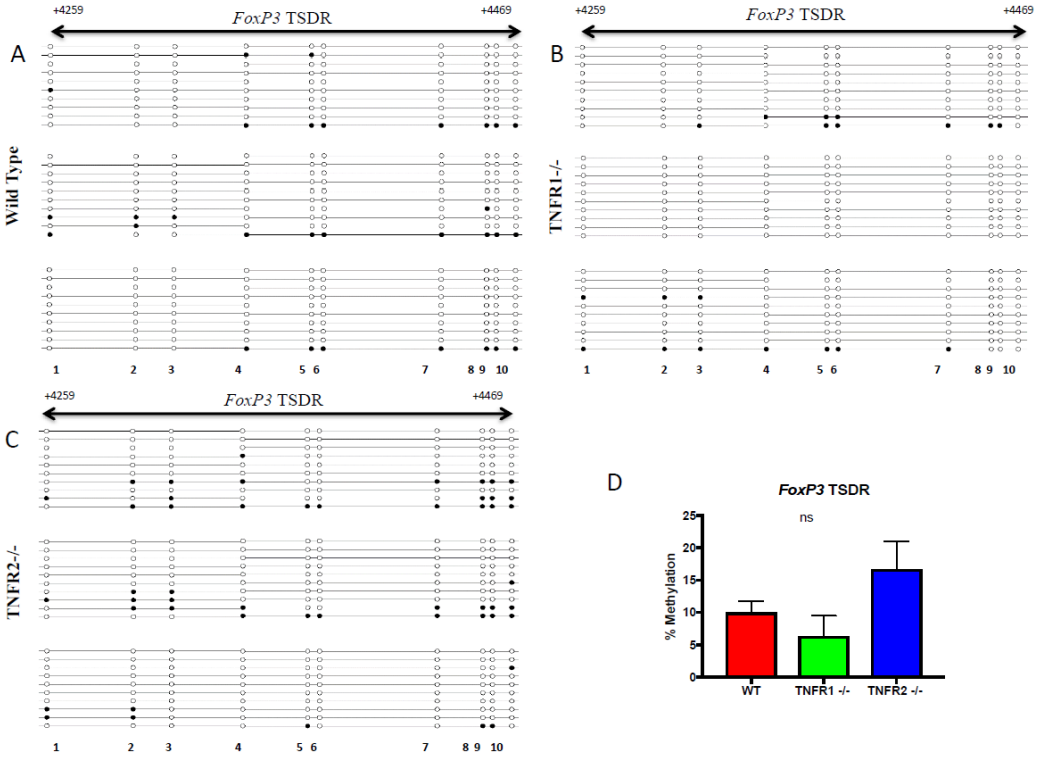


Figure 3.10 The absence of TNFR2 does not affect FoxP3 TSDR methylation status. Genomic DNA was isolated from the FACS-sorted Tregs, modified with sodium bisulfite, PCR amplified, cloned into the pCR4-TOPO vector, and individual clones were sequenced. Each line represents a different clone. The methylation pattern of each clone obtained is shown. ●, Methylated CpG; ○, nonmethylated CpG. Data are representative of 3 independent experiments for each strain (A to C). (D) Methylation degree of each experiment was calculated. Horizontal lines with error bars in D show the mean ± SEM, ns=not significant versus WT mice; by one-way analysis of variance with Dunnett's multiple comparison post-test.

3.3 Discussion

In this chapter I had addressed the role of TNFR1 and TNFR2 in the development and function in Tregs. I first established whether there were any differences in numbers of Tregs in WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. The results showed that there was no effect of deletion of TNFR1 or TNFR2 on the number of CD4+FoxP3+ Tregs. This result is different from a previous study, which reported that there was a significant reduction in the proportion of Tregs in CD4 single positive thymocytes and CD4+ splenic cells in TNFR2$^{-/-}$ mice[144]. Although the difference of Treg number between three groups in our study did not reach statistical significance, it showed a trend towards decreased number of Tregs in spleen and thymus of TNFR2$^{-/-}$ mice. Moreover, there was a marked decrease in the MFI of FoxP3 in Tregs in the spleen and thymus from TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. This finding suggests that TNFR2 plays a role in inducing or stabilizing the expression of FoxP3.

Tregs are not a single population but can be divided into subsets, including tTregs which originate in the thymus, and iTregs which are induced in the periphery. I

further assessed the numbers of iTregs and tTregs in WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice by labeling the Tregs with the antibody to Helios, which is suggested as one of the markers of tTregs[267]. The expression of Helios in peripherally induced tTregs precludes its use as a marker of tTregs. However, high Helios expression has been reported to be associated with a set of Treg-known markers including Foxp3, CD25, CD39, CTLA-4 and low levels of CD127[262]. In addition, Helios enriched, CD103+ GITR+ Treg might represent a population with enhanced suppressive capacity in comparison with Helios-Treg. The FACS analysis showed that there was no difference in Helios expression between the 3 groups of mice, indicating comparable numbers of Heilos+Tregs and Helios-Tregs in TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice versus WT mice.

I further investigated the difference of FoxP3 staining intensity between WT, TNFR1$^{-/-}$, and TNFR2$^{-/-}$ mice by checking the gene expression level. This result confirmed that gene expression of FoxP3 was decreased in spleen and thymus of TNFR2$^{-/-}$ mice. The result is compatible with a previous study, which indicated that TNFR2 is critical for the stabilization of Treg phenotype and FoxP3 expression *in vivo*[144]. In addition, decreased expression of TGF-β was also

noted in spleen and thymus of TNFR2$^{-/-}$ mice. Some studies reported that TGF-β is able to induce FoxP3 expression[268, 269]. Moreover, other studies showed that TNF-α increased the production of TGF-β in different cell types[270, 271]. Taken together, these results show an association between TNFR2 signaling, TGF-β and the expression FoxP3 in Tregs. Moreover, the results may lead to a hypothesis that transmembrane TNF, the main ligand for TNFR2, promotes homeostasis by increasing production of TGF-β and subsequent FoxP3. When inflammation occurs, enhanced activity of TACE might lead to the cleavage and release of soluble TNF, which results in a shift towards pro-inflammatory TNFR1 signaling activity and a reduction in homeostatic TNFR2 signaling. Thus, an imbalance of TNFR1/TNFR2 signalling may contribute to a failure in resolution of inflammation. For example, reduced production of soluble TNFR (which neutralizes free TNFα) in some individuals may lead to increased TNFR1 signalling and reduced TNFR2 signalling, leading to increased inflammatory responses. Further studies are needed to support the hypothesis. To establish the importance of TNFR2 and resolution of arthritis, I propose to assess the effect of TNFR2 deficiency in the antigen-induced arthritis (AIA) model. AIA is an acute model which resolves rapidly after the initial flare of disease. I hypothesize that

AIA would be more severe and more chronic in TNFR2-/- mice than in WT mice. There is accumulating evidence to show that TNFR2 expression identifies the most suppressive subset of mouse and human Tregs[144, 147]. One previous study also showed increased Treg cell suppression of T cell proliferation and cytokine production in TNFR1$^{-/-}$ mice compared to TNFR2$^{-/-}$ mice[257]. In my study, there was no difference of gene expression of *il4* (a suppressor of Tregs) or *gitr* (an activator of Tregs) between WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. Similarly, no difference in gene expression of *ctla4*, a key molecule involved in Treg suppressive function[272], was noted between the three groups of mice, either. Intriguingly, there was a marked increase of *il-10* gene expression noted in the LNs of TNFR2$^{-/-}$ mice, but this was not seen in spleen or thymus. However, the amplification curve for *il10* in RT-PCR was later than anticipated therefore the expression level of IL-10 was difficult to quantify reliably. IL-10 is an important anti-inflammatory cytokine which is produced by T cells (including Tregs), mast cells, neutrophils, natural killer cells, macrophages and dendritic cells[273]. The validity and origin of the increased IL-10 gene expression, particularly in Tregs, will need to be further investigated.

In comparison to WT mice, the gene expression of *il23r* in splenic cells of TNFR1$^{-/-}$ mice showed a significant increase. There was also a trend toward increased gene expression of *il23r* in thymus, although the difference did not reach statistical significance. IL-23 is a heterodimer cytokine composed of one p19 subunit and one p40 subunit, which is shared with IL-12[274]. IL-23 is produced by antigen presenting cells and plays an indispensable role in the maintenance of Th17 cell proliferation and IL-17 production through IL-23R signaling[275, 276]. In addition to Th17 cells, IL-23 acts on cells of the innate immune system, including subsets of γδ Tcells[277], natural killer T (NKT)[278] cells and innate lymphoid cells (ILCs)[279]. These IL-17 producing cells are characterized by expression of IL-23R and the transcription factor retinoic acid receptor-related orphan receptor-γt (RORγt)[280].

There is accumulating evidence to show that IL-23 signaling plays an indispensable role in host defense against bacterial infection[281] and pathogenesis of autoimmune disease, such as multiple sclerosis[282], psoriasis, inflammatory bowel disease[283] and RA[284]. Some studies have shown that the expression of IL-23R is induced by TGF-β[285]. Further published studies

revealed that IL-6 and IL-21 could induce the expression of IL-23R via a STAT3 dependent pathway[286]. In addition, the induction of IL-23R might be RORγt dependent, as reduced expression of IL-23R was noted in RORγt deficient mice[287]. All the studies imply that IL6/IL-21, together with TGF-β, induce the expression of IL-23R through RORγt and STAT3 signaling. The unexpected result of increased expression of IL-23R in TNFR1$^{-/-}$ mice may account for the previous finding that anti-TNF monoclonal antibody expanded the population of Th17 cells and the expression of IL-12/IL-23 p40 was up regulated in LN from TNFR1$^{-/-}$ mice but not TNFR2$^{-/-}$ immunized mice[288]. This paradoxical increase of Th17 cells has been confirmed by a study of anti-TNF therapy in RA patients, which showed increased IL-17 production after anti-TNF therapy[289, 290]. These results suggest that TNF signaling is an important regulatory factor, which is independent of TGF-β, in the development and function of Th17 cells. However, the mechanism is still elusive, and I was going to investigate the correlation between TNFR1 and 2, IL-23, IL-6, IL-17 and TGF-β in CIA model in order to understand the relationship between TNFR signaling and Th17 cells in the next chapter.

Epigenetic regulation by CpG methylation at specific sites in T cells plays an indispensable role in regulating differentiation of T helper cells[173, 213]. Several *cis*-regulatory elements, which are susceptible to methylation, are expressed in the *foxp3* locus, including upstream enhancer, proximal promoter and TSDR. It has been reported epigenetic modifications at different *cis*-regulatory elements in the *FoxP3* locus have different effect on regulating the generation and maintenance of Treg cells in response to various kinds of intrinsic or extrinsic stimulation[291].

The methylation status of the CpG residues in the proximal promoter region plays an indispensable role in FoxP3 expression. Demethylation at proximal promoter region induced by a demethylating agent, decitabine, in human NK cells can lead to FoxP3 expression[292]. It has been reported that methylation level in *FoxP3* proximal promoter ranges from 10-45% in $CD4^+CD25^-$ cells, whereas the counterpart in tTregs in completely demethylated. Interestingly, treatment TGF-β induces partial demethylation at this site in $CD4^+CD25^-$ cells, thus leading to FoxP3 expression[174].

It has been reported the CpG residues in TSDR are fully demethylated in tTreg, and completely methylated in $CD4^+CD25^-$ T cells in mice and in human[173, 174, 176]. In TGF-β induced Tregs, the TSDR showed partially demethylated despite high FoxP3 expression. Furthermore, TGF-β induced Tregs lost both FoxP3 expression and suppressive function upon restimulation in the absence of TGF-β[173, 174, 176]. The result indicates methylation modification of TSDR plays an indispensable role in maintaining the stability and function of Tregs.

The upstream enhancer is heavily methylated in naive $CD4^+CD25^-$ T cells, activated $CD4^+$ T cells, and TGF-β induced $FoxP3^+$Tregs, but is demethylated in tTregs[175]. Incubating $CD4^+CD25^-$ T cells with azacytidine demethylates the upstream enhancer, leading to stable increased FoxP3 mRNA and protein expression, and enhanced suppressive function. Different from proximal promoter and TSDR, TGF-β was unable to modify the methylation status and function of upstream enhancer.

My data showed there was an increased methylation level in proximal promoter and TSDR in $TNFR2^{-/-}$ mice compared to WT mice, but not in upstream enhancer.

This study was limited by a small number of reads. However, if confirmed in a larger study this indicates that TNFR2 signaling regulates the expression and stability of FoxP3 expression by modifying methylation degree in the two regions. Additionally, the results of RT-qPCR showed decreased gene expression of TGF-β in TNFR2$^{-/-}$ mice compared with WT mice. Taken together, these results hints TNFR2 signaling regulates methylation modification in *FoxP3* locus in an TGF-β dependent manner, that could explain the comparable methylation level in upstream enhancer in TNFR2$^{-/-}$ mice. However, the association between TNFR2 and TGF-β remains unclear will need to be further investigated. For example, it would be informative to assess the effect of TNFR2 blockade on TGF-β production using a specific inhibitor and to determine the effect of increasing TGF-β using an agonist of TNFR2.

In conclusion, the work in this chapter has shown that TNFR2 deficiency does not alter the number of Tregs. However, the level of expression of FoxP3 is decreased in TNFR2$^{-/-}$ mice, which is compatible with the reduced gene expression of FoxP3 in spleen and thymus of TNFR2 $^{-/-}$ mice. Moreover, elevated gene expression of IL-23R was unexpectedly noted in the spleens of TNFR1$^{-/-}$ mice which need to be

further explored in the future. This increase may contribute to expansion of Th17 and production of IL-17 during inflammation. In addition, increased methylation level was noted in proximal promoter and TSDR in *FoxP3* locus in $TNF2^{-/-}$ mice. This increase may lead to less stability and suppressive function of Tregs in $TNFR2^{-/-}$ mice. Finally, the notion that global deletion of TNFR2 leads to artifacts due to the absence of the receptors on non-hematopoietic cells is important to consider. To overcome this, it would be possible to create bone marrow chimeras in which irradiated WT mice are reconstructed with bone marrow cells from $TNFR2^{-/-}$ mice.

Chapter 4 TNFR2 plays a disease-limiting role in collagen-induced arthritis

4.1 Introduction

Despite the success of TNF-α blockade, there are still unmet needs. As discussed above, in clinical trials or post marketing registry studies, a proportion of patients are resistant to anti-TNF-α therapy (20-40%). In addition, some patients exhibit a decrease in clinical response to anti-TNF-α therapy after sustained treatment with anti-TNF-α antibodies[254]. The reasons for lack of response are still elusive. One hypothesis put forward to explain resistance to anti-TNF-α therapy is an ascendant effect of dysregulated Tregs and increased Th17 responses due to TNF-α blockade. Serial studies demonstrated that TNFR2 is critical for stabilization and suppressive function of Tregs[144, 257] and impaired TNF-TNFR2 signaling contributes to Th17 polarization[164, 293]. This raises the possibility that TNFR2 signaling could be a potential therapeutic target in the treatment of autoimmune disease. Several studies had shown that TNFR2 agonism can selectively kill autoreactive $CD8^+$ T cells and expand active Tregs ($CD4^+CD25^+FoxP3^+RO$) in type I diabetes[294, 295]. In addition to this regulatory effect on immune cells, a selective TNFR2 specific agonist was reported to rescue human neurons from oxidative stress induced cell death[296]. However, to date, the role of TNFR2 signaling in the pathogenesis of RA remains unclear.

My data (in Chapter 3) confirmed that TNFR2 plays an important role in the expression of FoxP3 in Tregs via epigenetic control of the FoxP3 promoter. Based on

the findings, I hypothesized that blocking TNFR2 signaling may lead to higher incidence and disease activity of CIA by inhibiting both Treg generation and suppression function. Thus, the objective of the work in this chapter was to assess the differences of clinical manifestations of CIA between WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice. Another hypothesis tested in this chapter was that in mice with inflammatory arthritis, TNFR2 deficiency would lead to an increase in the number of Th1 and Th17 cells, as a secondary effect of the decrease in the number/activity of Tregs. This could explain the finding of elevated circulating Th1/Th17 cells in RA patients after anti-TNF therapy, which could potentially lead to resistance to anti-TNF therapy. To address the effect of TNFR signaling on Th1, Th17 and Treg cells, spleen and LN cells, harvested from arthritic mice, were first analyzed by flow cytometry. Next. in order to be able to simultaneously measure a range of target cytokines in the serum samples, MesoScale Discovery MultiSpot assay (MSD) were used. Additionally, 384 well microfluidic card Taqman gene expression assays were used to measure the expression level of target genes in spleen and LNs of WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice with CIA.

4.2 Results

4.2.1 H-2^{q} and H-2^{b} genotyping for the back-crossed C57BL/6 mice (expressing A^{b}) to congenic C57Bl/6N.Q mice.

CIA is an animal model of RA that is widely used to address questions of disease pathogenesis and to validate therapeutic targets. Susceptibility to CIA is strongly associated with major histocompatibility complex class II genes, with H-2^{q} and H-2^{r}

being the most arthritis-susceptible[297]. The DBA/1 strain (H-2^q) is the most commonly used strain for the use of CIA. However, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ strain are available on a C57BL/6 (H-2^b) background, a strain that is resistant to CIA induced by immunization with bovine type II collagen[298]. To overcome this problem, I bred WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ strains onto a congenic C57BL/6N.Q background to generate mice expressing the arthritis-susceptible H-2^q allele. To differentiate the mice expressing A^b or A^q, we amplified the genomic DNA by PCR with the primer pairs specific for the allele. H-2^q and H-2^b genomic DNA produced a single band of expected size. Heterozygous genomic DNA produced two bands of expected size and a fainter non-specific band (Figure 4.1A).

To confirm the result of $H2^q$ and $H2^b$ genotyping, we collected the blood from the mice and labeled the leukocytes with antibodies: I-A^q-AF647, I-A^b-PE and CD11b-APC-Cy7. The phenotype (MHC class II) of the leukocytes was assessed by FACS analysis and shown as unambiguously A^q, heterozygous $A^{q/a}$ or A^b (Fig 4.1B). This confirms the validity of the PCR data.

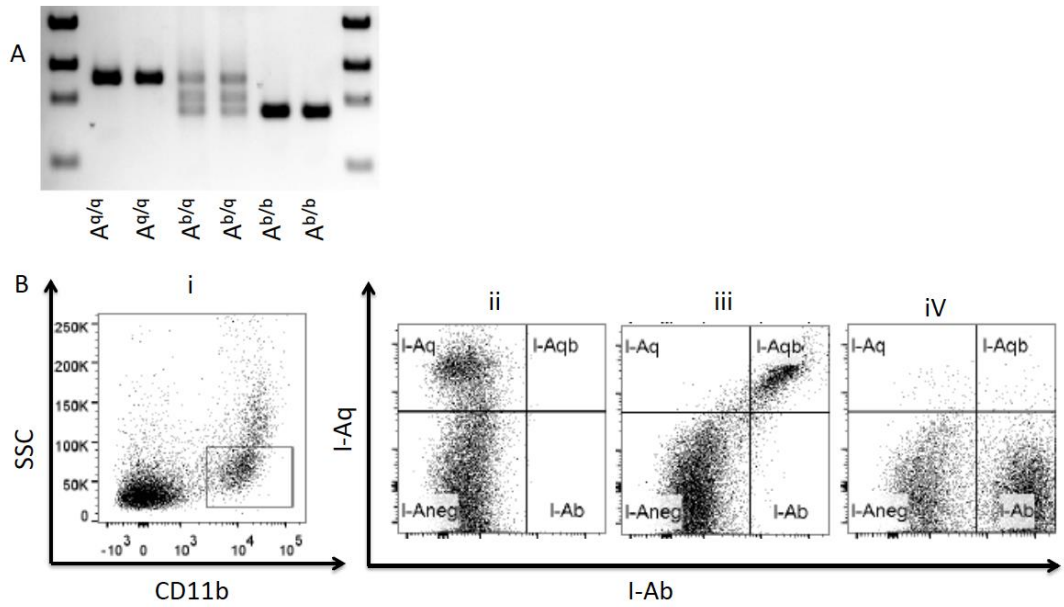


Figure 4.1 $H2^q$ and $H2^b$ genotyping for C57BL/6 mice (expressing A^b) back-crossed to congenic C57Bl/6N.Q mice (expressing A^q) . (A) The DNA was extracted from ear biopsies and genotyped by PCR. Each reaction contained four primers H2q_f_1, H2Bee_F_2, H2Bee_R_1 and H2q_r_2. Amplified DNA fragments were separated using 1.5% agarose gels alongside molecular weight markers. (B) To validate the genotyping result, whole blood was collected in a heparin-coated tube by cardiac puncture. The cells were then stained with Ab to I-A^q, I-A^b and CD11b. After gating on peripheral blood monocytes (i), the haplotype was determined unambiguously as A^q (ii), heterozygous (iii) or A^b (iv).

4.2.2 TNFR2 signalling exerts a protective function against inflammation in arthritis

To test the effect of TNFR signaling in arthritis, WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ C57/BL6N.Q (H-2^{q}) mice were immunized with bovine type II collagen emulsified in complete Freund's adjuvant. The incidence of arthritis in WT mice was 46.7% (14/30). TNFR1$^{-/-}$ mice had a significantly lower incidence of arthritis (13.8%, 4/29), as compared with WT. In the contrast, there was an increased incidence of arthritis in TNFR2$^{-/-}$ mice (69.6%, 16/23) (Figure 4.2A).

There was a similar trend of disease severity and number of paws affected. Thus, TNFR1$^{-/-}$ mice showed minimal disease activity and self-limiting pattern throughout the 80-day observation period. WT and TNFR2$^{-/-}$ mice showed similar clinical scores in early arthritis (Figure 4.2B). However, from day 75 to 80 after immunization, TNFR2$^{-/-}$ mice had a higher clinical score in comparison with WT mice. It was also observed that most of the paws of TNFR1$^{-/-}$ mice were disease-free and TNFR2$^{-/-}$ mice had more arthritic paws compared to WT mice, reaching statistical significance from day 70 to day 80 (Figure 4.2C).

Histologically, while the paws of TNFR1$^{-/-}$ mice were largely protected from arthritis, with the majority of joints remaining unaffected, the joints of mice from TNFR2$^{-/-}$ mice showed a significant degree of inflammatory infiltration and bone erosion (Figure 4.2D). Representative images of a proximal interphalangeal joint from mice WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice are shown in Figures 4.1E-G. WT mice showed increased cell infiltration and a thickening layer of synovial membrane covering the

joint capsule, which are characteristic features of arthritis in diarthrodial joints (Figure 4.2E). TNFR2$^{-/-}$ mice showed extensive inflammation, with large areas of infiltrating cells. Articular cartilage showed an uneven surface, which indicates loss of chondrocytes and proteoglycan (Figure 4.2G). Distinct regions of bone erosion were also evident, with pannus invading the subchondral bone, resulting in joint destruction. Joints from TNFR1$^{-/-}$ mice (Figures 4.2F), in contrast, were largely free of inflammatory infiltration and demonstrated clear joint spaces and intact architecture. To summarize the findings of clinical and histological severity, deficiency of TNFRI provides significant joint protection from the inflammatory process that leads to bone destruction. In contrast, more severe clinical and histologic changes observed in TNFR2$^{-/-}$ mice shows that TNFR2 signaling plays a protective role in preventing mice from arthritis and maintaining remission of disease.

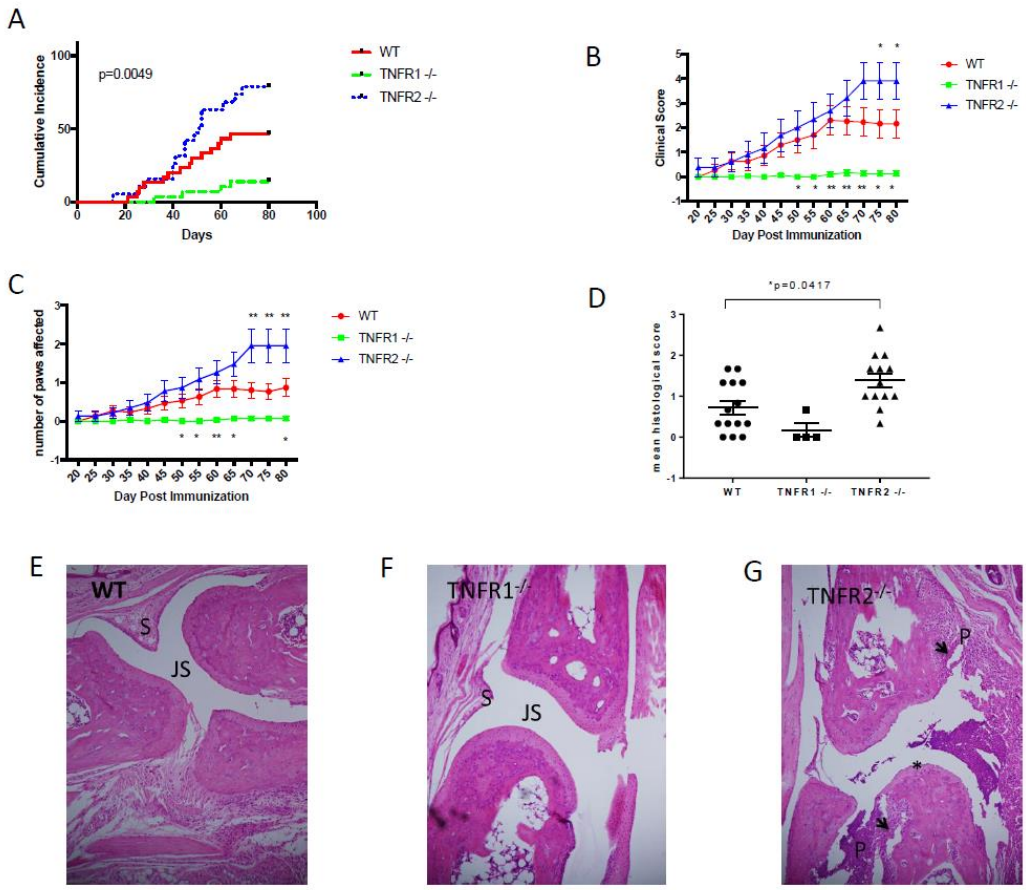


Figure 4.2 Development of CIA in TNFR1 and TNFR2-deficient mice. WT (n=30), TNFR1$^{-/-}$ (n=29) and TNFR2$^{-/-}$ (n=23) C57/BL6 (H2^{q}) were immunized with bovine type II collagen emulsified in complete Freund's adjuvant. The immunized mice were evaluated daily. The incidence of arthritis (A), clinical score (B) and number of affected paws (C) in each treatment group summarized from three experiments are shown. Values in B and C are the mean ± SEM. *=P<0.05; **=P<0.01 for TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice versus WT mice, by one-way analysis of variance with the Dunnett's multiple comparison post-test. (D) On day 80 after immunization, paws were harvested and prepared for histologic analysis. Paraffin-embedded sections were stained with hematoxylin and eosin and histopathologic changes in the joints were scored. Horizontal lines with error bars in D show the mean ± SEM, *=P<0.05 versus WT mice, by one-way ANOVA with Dunnett's multiple comparison test. Also shown are representative images of proximal interphalangeal joint sections from WT (E), TNFR1$^{-/-}$ (F), and TNFR2$^{-/-}$ (G) mice. Arrows indicate bone loss. Asterisks indicate damage of articular cartilage. P = pannus; S = synovial membrane; JS = joint space.

4.2.3 TNFR2 deficiency impairs the generation of thymus derived Tregs in inflammation.

Several studies have shown that TNFR2 signaling plays an indispensable role in the generation, suppressive function and maintenance of stability of Tregs[147, 299]. It has also been reported TNFR1 specific blockade, which spares TNFR2 signaling, ameliorated severity of CIA without interfering the function and phenotype of Tregs[257]. It is thus possible that in TNFR2 deficient mice with arthritis, Treg cell function is impaired, resulting in increased pathogenic T cell activity. I therefore assessed the effect of TNFR1/2 deficiency on the numbers of effector and regulatory T cells in inguinal LN and spleen cells from arthritic mice.

Harvested spleen and LN Cells were surface stained for CD4, CD8 (Figure 4.3 A) and CD25, and subsets were identified based on co-expression of intracellular IFN-γ (Th1 cells), IL-17A (Th17 cells) (Figure 4.3B), or FoxP3 (Treg cells) (Figure 4.4 A). Strikingly, while there was a significant increase of percentage of $CD4^+$ T cells expressing IFN-γ in spleen and inguinal LN cells from $TNFR1^{-/-}$ mice (Figure 4.3C & D), the percentage of Th17 cells in spleen and inguinal LN cells was comparable in all groups (Figure 4.3E & F). Additionally, the percentage of $CD4^+$ T cells expressing the Treg cell signature transcription factor, FoxP3, was significantly increased in spleen and LN cells from $TNFR1^{-/-}$ mice but was decreased in inguinal LN cells from $TNFR2^{-/-}$ mice as compared to WT mice (Figure 4.4 B).

I next measured the expression of functional markers, FoxP3 and CD25, on Tregs. A significant decrease in FoxP3 MFI in the $CD25^+FoxP3^+$ Tregs was observed in

TNFR2$^{-/-}$ mice with arthritis compared with WT mice (Figure 4.4C). No differences were found in CD25 MFI in classical CD25^{+}FoxP3^{+} Tregs (Figure 4.4D). Helios is thought to be preferentially expressed by thymus-derived Tregs (tTregs) and is also a marker of activated Tregs, which have more suppressive characteristics, compared with FoxP3^{+}Helios^{-} Tregs[300, 301]. There was a significant decrease of percentage of FoxP3^{+} Helios^{+}Tregs in spleen and inguinal LN cells from TNFR2$^{-/-}$ mice, compared with WT mice (Figure 4.5A & B). Taken together, these data demonstrate that TNFR2 deficiency impairs the generation of tTregs and inhibits the expression of FoxP3 in Tregs in inflammation. There was also a significant increase in Th1 responses in TNFR1$^{-/-}$ mice in inflammation.

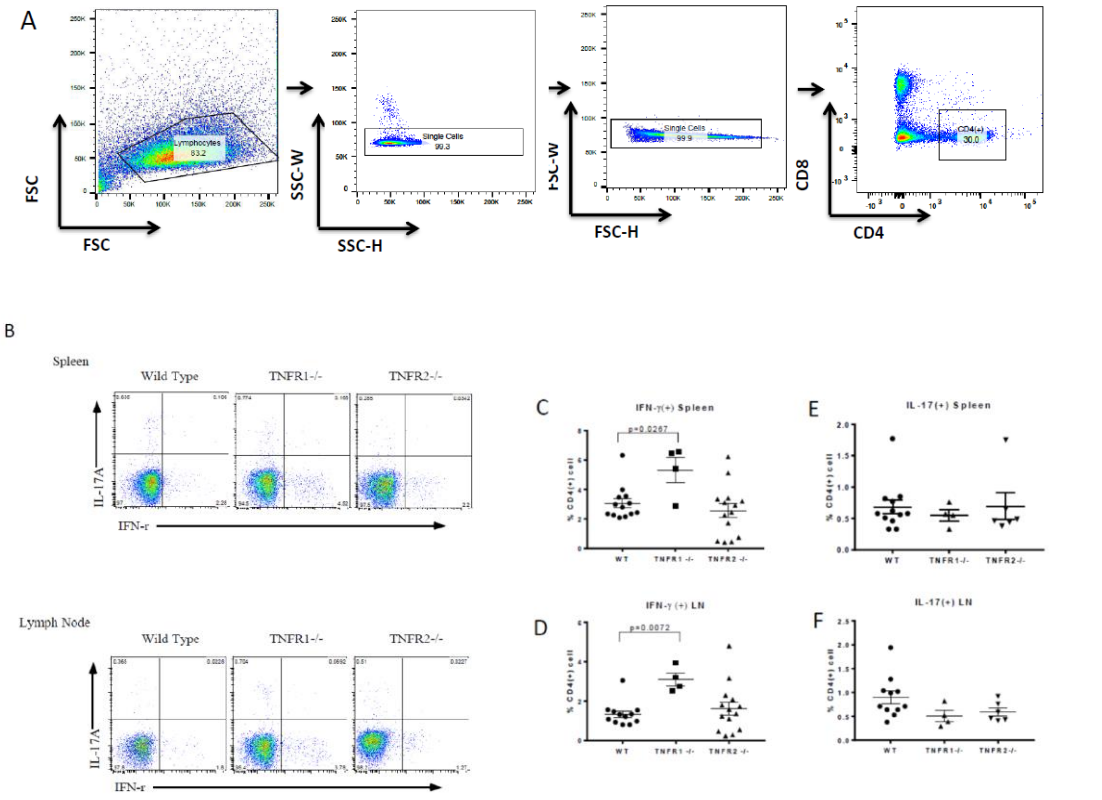
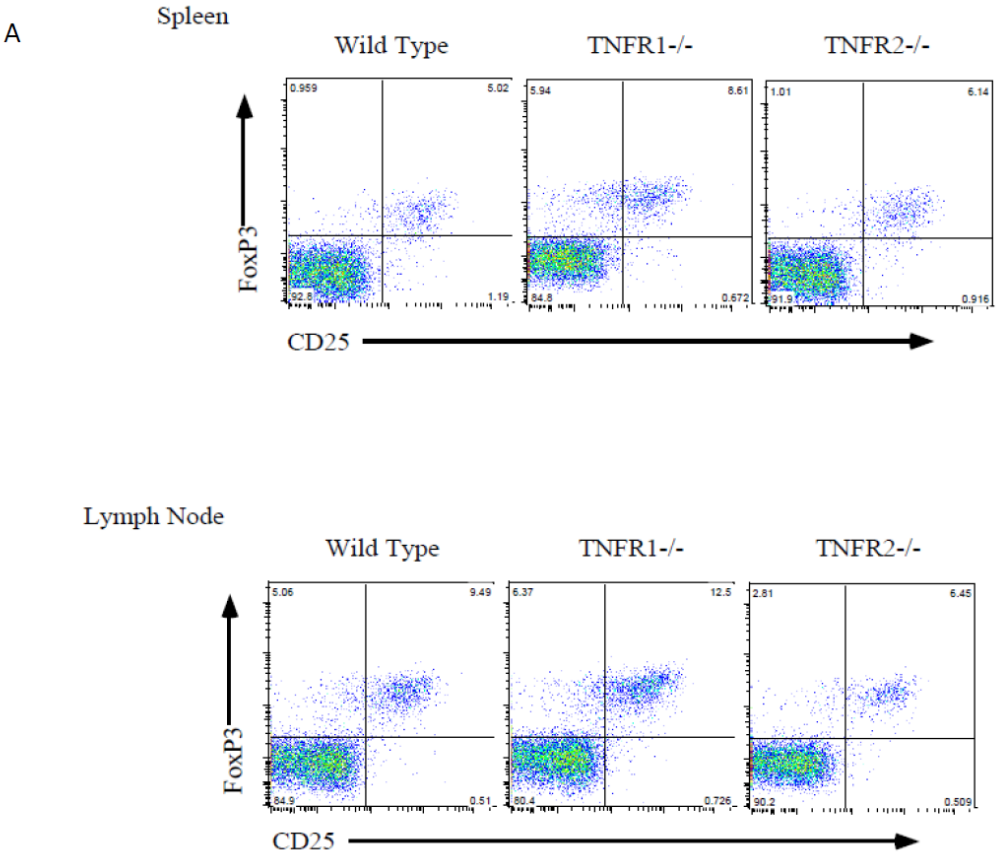


Figure 4.3 Increased proportion of Th1 cells in the spleen and lymph nodes (LNs) of TNFR1$^{-/-}$ mice with CIA. On day 10 following CIA onset, cells were isolated from the draining LNs and spleens of WT (n=12), TNFR1$^{-/-}$ (n=4) and TNFR2$^{-/-}$ (n=11) arthritic mice, and the percentages of CD4^{+} cells **(A)** expressing interferon-γ (IFN-γ; Th1 cells) (B-D) and interleukin-17A (IL-17A; Th17 cells) (B, E & F) were calculated. Horizontal lines with error bars in C to F show the mean ± SEM. Groups were compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

A


Spleen
Wild Type
TNFR1-/-
TNFR2-/-
0.959
5.02
92.8
1.19
5.94
8.61
84.8
0.672
1.01
6.14
91.9
0.916
FoxP3
CD25
Lymph Node
Wild Type
TNFR1-/-
TNFR2-/-
5.06
9.49
84.9
0.51
6.37
12.5
80.4
0.726
2.81
6.45
90.2
0.509
FoxP3
CD25

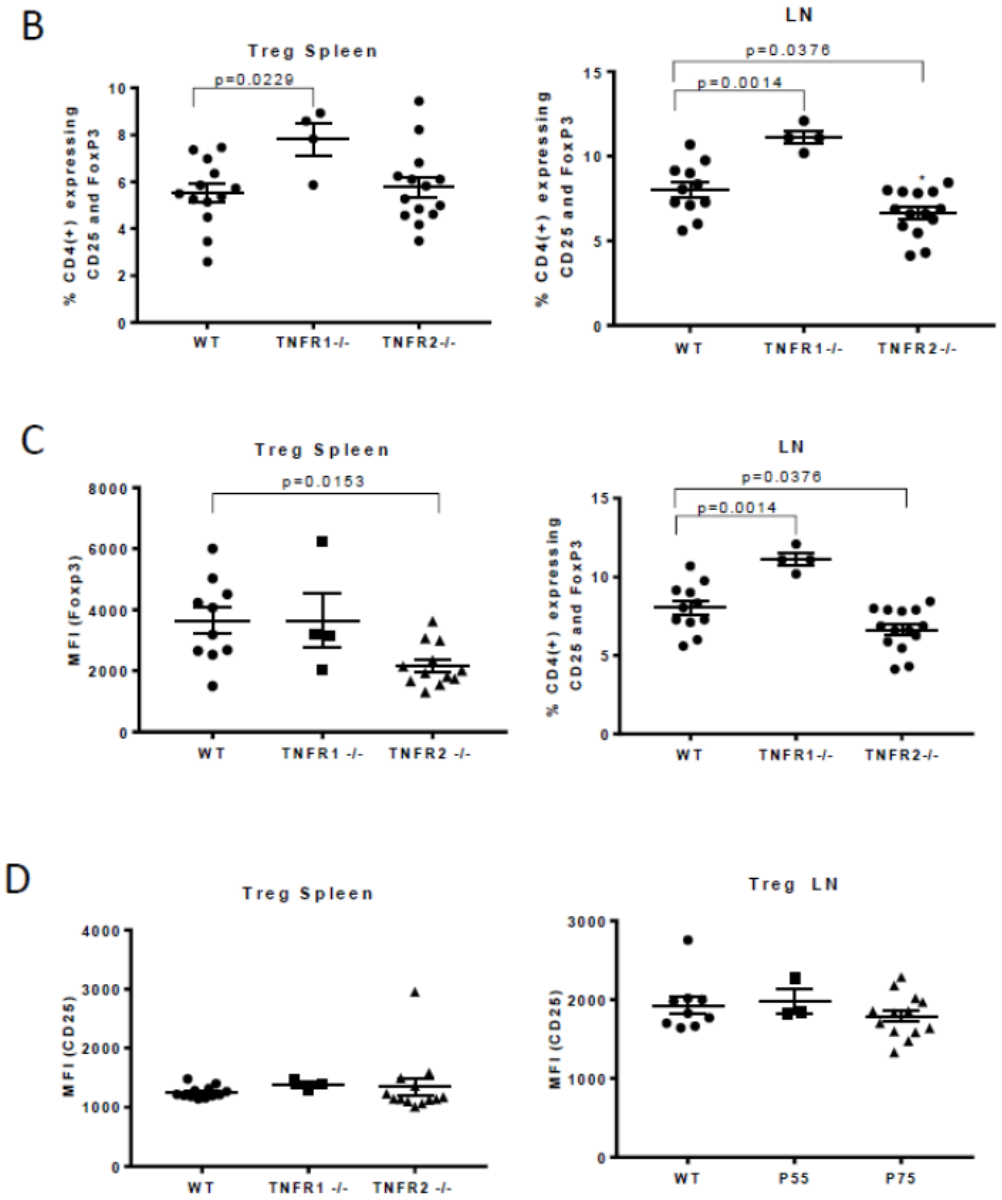


Figure 4.4 Decreased expression of FoxP3 in Tregs cells of TNFR2$^{-/-}$ mice with CIA. On day 10 following CIA onset, cells were isolated from the inguinal LNs and spleens of WT (n=12), TNFR1$^{-/-}$ (n=4) and TNFR2$^{-/-}$ (n=11) arthritic mice, and the percentages of CD4^{+} cells expressing FoxP3 and CD25 (A & B) were calculated. Mean fluorescence intensity (MFI) showing expression levels of FoxP3 (C) and CD25 (D) protein in Tregs (CD4^{+}CD25^{+}FoxP3^{+}) was also determined. Horizontal lines with error bars in B to D show the mean ± SEM. Groups were compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

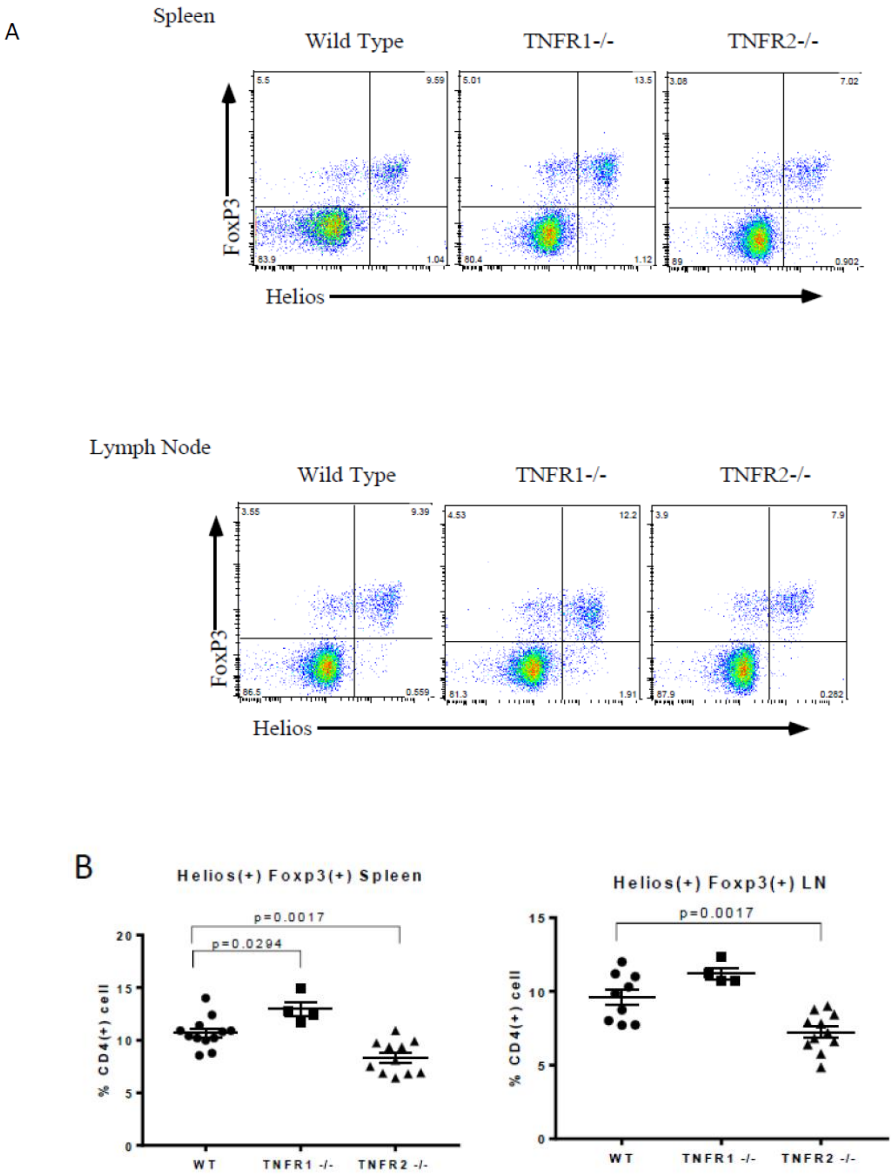


Figure 4.5 Decreased proportion of Helios^{+}FoxP3^{+} Tregs in TNFR2$^{-/-}$ mice with CIA. On day 10 following CIA onset, cells were isolated from the inguinal LNs and spleens of WT (n=12), TNFR1$^{-/-}$ (n=4) and TNFR2$^{-/-}$ (n=11) arthritic mice, and the percentages of CD4^{+} cells expressing FoxP3 and Helios (A & B) were calculated. Horizontal lines with error bars in B show the mean ± SEM. Groups were compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

4.2.4 TNFR2 signalling regulates the expression of PD-1 in CIA

The inhibitory receptor, programmed death 1 (PD-1; also known as PDCD1), is known as a negative regulator of activated T cells, by delivering a negative signal that leads to the recruitment of SHP-2 to the phosphorylated tyrosine residue in the cytoplasmic region, results in a state known as T cell exhaustion[302, 303]. It has also been reported that PD-1^+ T cells are enriched in RA synovium and may act as a negative regulator in the pathogenesis and progression of the disease[304, 305]. Several studies showed that TNF-α can upregulate the expression of PD-1 in cancer and chronic virally-infected patients[306]. To investigate the correlation between TNFR signalling and T cell exhaustion in arthritis, I measured the expression of PD-1 in $CD4^+$ T cells by flow cytometry. There was a significant decrease in the percentage of $CD4^+$ T cells expressing PD-1 in spleen and inguinal LN cells from $TNFR2^{-/-}$ mice, as compared with WT mice (Figure 4.6). However, the MFI of PD-1 on CD4+PD-1+ cells was comparable between groups, indicating that TNFR2 affects the overall size of the PD-1+ T cell pool but does not affect to level of PD-1 expression in CD4+PD-1+ cells.

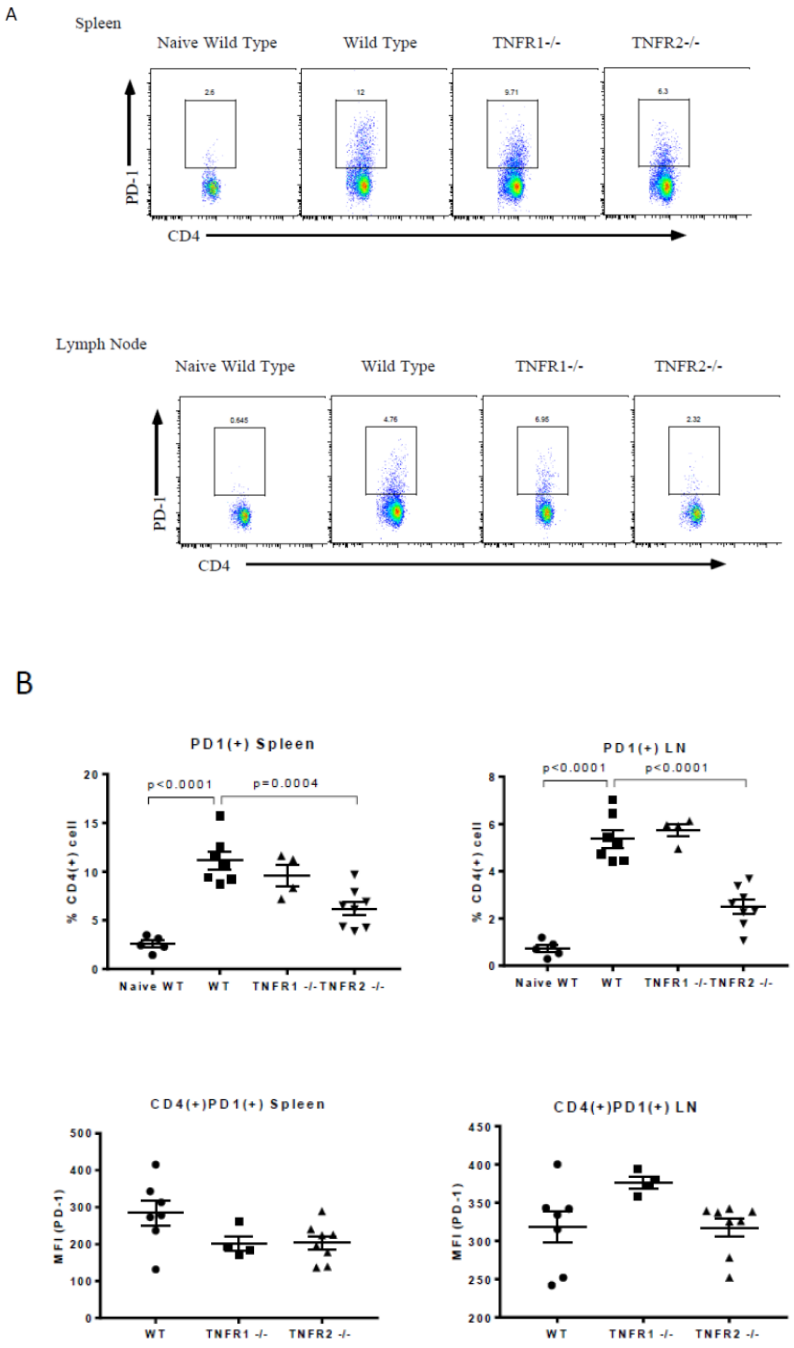


Figure 4.6 Decreased proportion of PD-1^{+}CD4^{+} in TNFR2$^{-/-}$ mice with CIA. On day 10 following CIA onset, cells were isolated from the inguinal LNs and spleens of WT (n=7), TNFR1$^{-/-}$ (n=4) and TNFR2$^{-/-}$ (n=8) arthritic mice, and the percentages of CD4^{+} cells expressing PD-1 and MFI of PD-1 on CD4^{+}PD-1^{+} cells were calculated (A & B). Spleen and LN cells of healthy WT mice were also collected for comparison. Horizontal lines with error bars in B and C show the mean ± SEM, Groups are compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

4.2.5 Increased IFN-γ production in arthritic TNFR1$^{-/-}$ mice and increased TNF-α production in arthritic TNFR2$^{-/-}$ mice

Ten days following arthritis onset, mice were bled, and serum levels of IFN-γ, TNF-α, IL-12p70, IL-5, IL-1, IL-10, CXCL1 and IL-4 were assayed (Figures 4.7 A–H). The circulating levels of IFN-γwere significantly higher in TNFR1$^{-/-}$ mice than in WT mice. The result is compatible with my previous finding that there was a significant increase of Th1 cells in spleen and LN cells from arthritic TNFR1$^{-/-}$ mice compared with arthritic WT mice. Interestingly, TNFR2$^{-/-}$ mice had higher circulating levels of TNF-α, but lower levels of IL-4 in comparison with WT mice. TNF-α is a pro-inflammatory cytokine, which plays an indispensable role in the pathogenesis of RA. IL-4 is a cytokine that induces differentiation and proliferation of Th2 cells. The result suggests that TNFR1 signaling inhibit development of Th1 cells and TNFR2 signaling suppresses the production of TNF-α, but promotes Th2 differentiation.

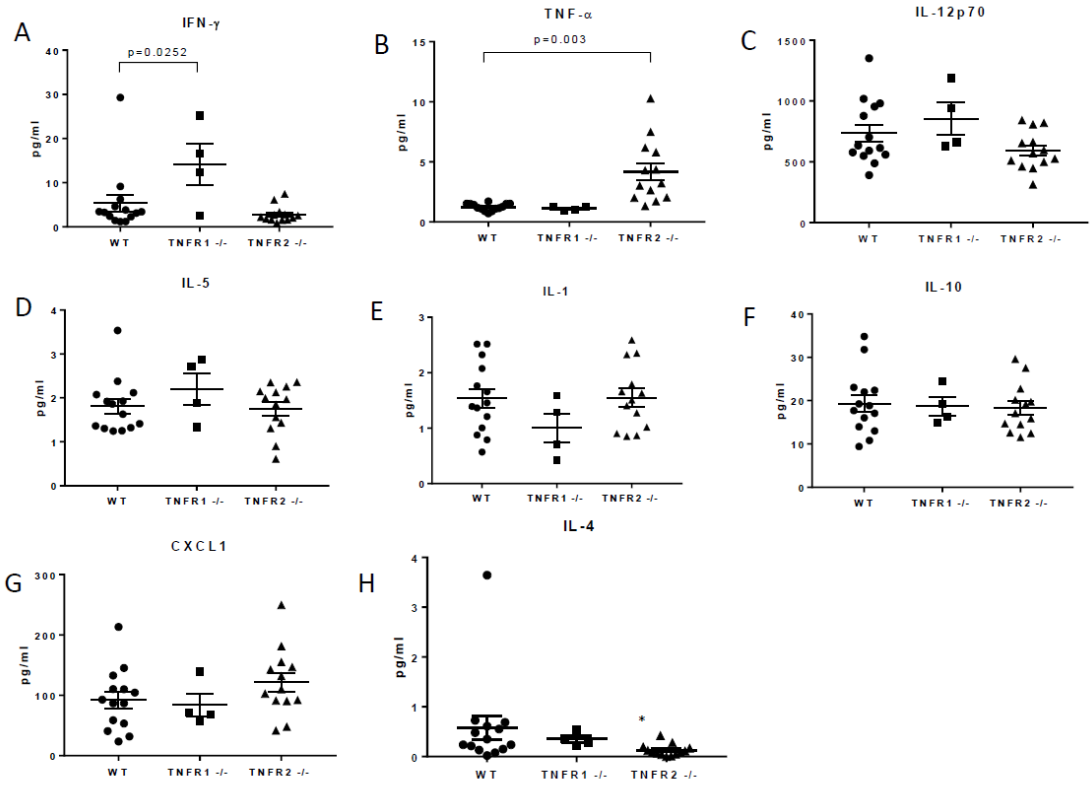


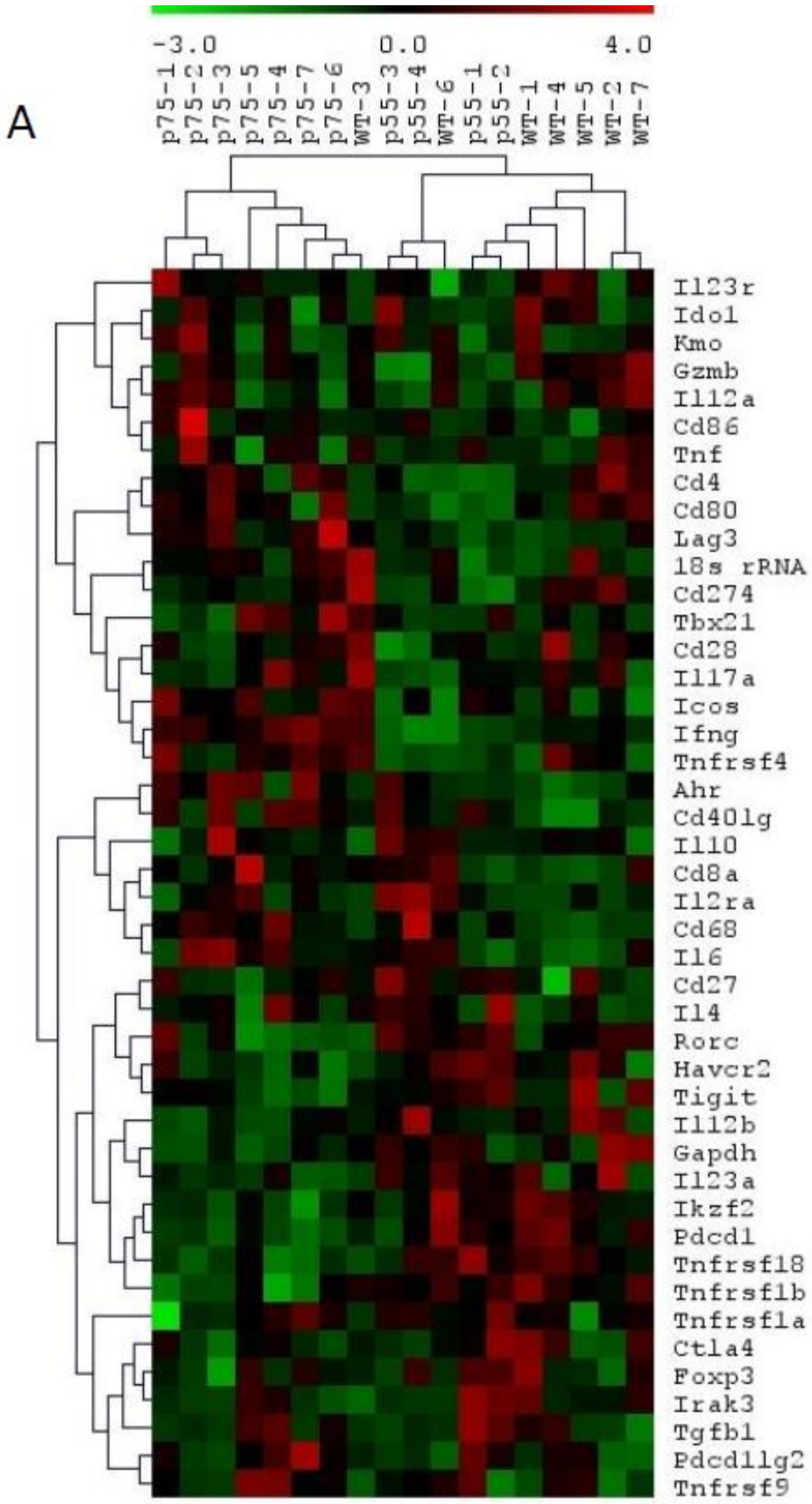
Figure 4.7 Serum cytokine levels in WT, TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice with CIA. On day 10 following arthritis onset, arthritic mice were bled, and serum was separated. Serum levels of the cytokines IFN-γ(A), TNF-α (B), interleukin-12 p70 (IL-12p70) (C), , IL-5 (D), IL-1 (E), IL-10 (F), CXCL1 (G), and IL-4 (H) were measured using a Meso Scale Discovery cytokine multiplex array (WT n=13; TNFR1$^{-/-}$ n=4; TNFR2$^{-/-}$ n=12). Horizontal lines with error bars in A to H show the mean ± SEM. Groups are compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

4.2.6 Decreased expression of immune checkpoints in arthritic TNFR2$^{-/-}$ mice

Pathogenesis of CIA is characterized by deregulated interaction between Th1, Th17, Treg, B cell and macrophages. To analyze the effect of TNFR signaling on the immune-profile during the course of CIA, mRNA level of multiple immune-regulatory markers in draining LNs were determined (Figure 4.8). We used whole LNs, rather than CD4 T cells, because we wanted to measure monocyte-associated genes, which may be important for antigen presentation, in addition to T cell associated genes. The result showed there was significant increase in the gene expression of *il6*, *cd40* and *cd68* in TNFR2$^{-/-}$ mice compared with WT mice. The result indicates activation of macrophages that leads to increased level of pro-inflammatory cytokines in TNFR2$^{-/-}$ mice. Additionally, there was significant decrease in the gene expression of *pd-1* and *lag3*, which negatively regulates cellular proliferation, activation, and homeostasis of T cells[307-309]. Aryl hydrocarbon receptor (Ahr), the receptor of tryptophan derivatives, such as kynurenine, has been reported to play an important role in inhibiting Th17 cells and promoting Tregs[310, 311]. The gene expression of *ahr* in TNFR2$^{-/-}$ is higher than in the WT. In TNFR1$^{-/-}$ mice, there was a significant decrease in the gene expression of PD-L1 and LAG3, which negatively regulates the function of T cells. The result is compatible with finding of low disease activity in TNFR1$^{-/-}$ mice and also indicates TNFR1 signaling is strongly associated with the expression of PD-L1 and LAG3 in inflammation. The expression of *foxp3* was comparable between the three groups, which is inconsistent with the results in Fig. 4.4 which showed decreased *foxp3* expression in TNFR2 deficient Treg cells. The discrepancy might be accounted for by the transient expression of FoxP3 on activated effector T cells in LNs during inflammation, which may interfere the

interpretation of the result. Further measurement of expression of *foxp3* in purified Tregs from FoxP3^{GFP} mice would be needed to definitively address this question.

In summary, my data indicate that TNFR2 deficiency impairs FoxP3 expression and numbers of PD-1^{+} cells in arthritis and promotes the production of TNF-α, which is likely to contribute to the higher disease activity and joint destruction observed in TNFR2 deficient mice with CIA.


A
-3.0
0.0
4.0
p75-1
p75-2
p75-3
p75-5
p75-4
p75-7
p75-6
WT-3
p55-3
p55-4
WT-6
p55-1
p55-2
WT-1
WT-4
WT-5
WT-2
WT-7
Il23r
Ido1
Kmo
Gzmb
Il12a
Cd86
Tnf
Cd4
Cd80
Lag3
18s rRNA
Cd274
Tbx21
Cd28
Il17a
Icos
Ifng
Tnfrsf4
Ahr
Cd40lg
Il10
Cd8a
Il2ra
Cd68
Il6
Cd27
Il4
Rorc
Havcr2
Tigit
Il12b
Gapdh
Il23a
Ikzf2
Pdcd1
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Pdcd1lg2
Tnfrsf9

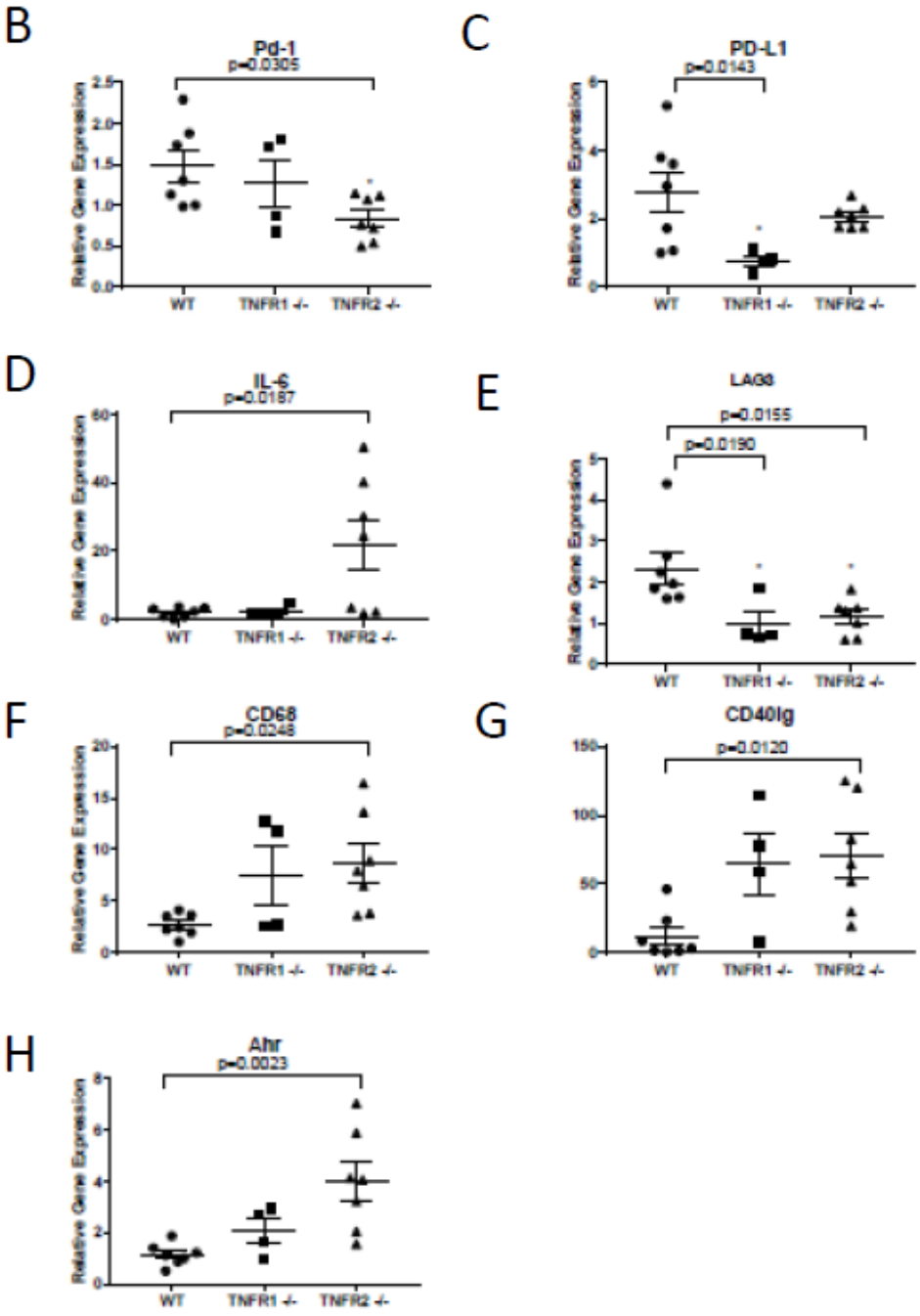

B
Pd-1
p=0.0305
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-
C
PD-L1
p=0.0143
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-
D
IL-6
p=0.0187
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-
E
LAG3
p=0.0155
p=0.0190
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-
F
CD68
p=0.0248
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-
G
CD40lg
p=0.0120
WT
TNFR1 -/-
TNFR2 -/-
H
Ahr
p=0.0023
Relative Gene Expression
WT
TNFR1 -/-
TNFR2 -/-

Figure 4.8 Profile of gene expression in LNs from WT, TNFR1 and TNFR2 mice with CIA. The RNA was isolated from LNs harvested from WT (n=7), TNFR1$^{-/-}$ (n=4) and TNFR2$^{-/-}$ mice (n=7) with CIA on day 10 following arthritis onset, and reverse transcribed to cDNA for RT-qPCR analysis using 384 well microfluidic card Taqman gene expression assays. Data were normalized to HPRT expression and were calibrated relative to WT. Data are presented in heat map format combined with hierarchical clustering using ΔCt values of gene expression (A). Each row represents a gene, and each column represents a mouse. Gene expression is relative to the median of ΔCt values. Genes in red and green indicate expression above and below the median, respectively. (A) Hierarchical cluster analysis using all selected genes. (B to H) Genes with significant difference of expression level between groups are shown. Horizontal lines with error bars in B to H show the mean ± SEM, Groups are compared by one-way analysis of variance with Dunnett's multiple comparison post-test.

4.3 Discussion

I attempted to explore the roles of TNFR1 and TNFR2 in arthritis by studying the effects of knocking out TNFR1 or TNFR2 signalling in CIA as a model of RA. I first established that TNFR1$^{-/-}$ mice are relatively resistant to arthritis, both clinically and histologically, indicating that TNFR1 is the major pro-inflammatory receptor in CIA. This finding is consistent with previous reports that contributed to the development of anti-TNFα therapy in RA[312, 313], but contradicts those of Yoshifumi Tada *et al[314]*. In the study, Yoshifumi Tada *et al* reported similar severity and incidence of CIA in TNFR1$^{-/-}$ mice and TNFR1$^{+/-}$ mice. The discrepancy in findings may be accounted for by differences in the methods of inducing CIA. Yoshifumi Tada *et al.* immunized DBA/1 mice twice on day 0 and 21, whereas I only immunized C57BL/6N.Q mice once on day 0. The double boosting may induce arthritis in a TNFR1 independent manner. A TNFR1 specific antagonist was shown to have comparable efficacy with conventional TNFR1/2 inhibitors in ameliorating disease progression of CIA[257]. Similarly, it has also been shown that expression of TNFR1 on mesenchymal cells is necessary and sufficient for induction of arthritis[315]. These findings suggest TNFR1 signalling plays an indispensable in the induction of arthritis.

While TNFR1 deficiency limits the progression of CIA, I found there was an expansion of Th1 cells in the spleen and LN cells of TNFR1$^{-/-}$ mice. Further analysis by MSD confirmed that the serum level of IFN-γ in TNFR1$^{-/-}$ mice was significantly greater than in WT or TNFR2$^{-/-}$ mice. The finding is supported by a previous study of Notley *et al[288].* However, there was not found to be an expansion of Th17 cells, or increased production of IL-17 in TNFR1$^{-/-}$ mice, which was observed in the study of Notley *et al.* The discrepancy in findings may be accounted for differences in the time

course of disease. In this study, Notley *et al* checked the activity of Th1 and Th17 cells on day 14 following immunization, whereas I assessed it on day 10 following arthritis onset. My results show conclusively that inhibition of Th1 responses occurs via the TNFR1 and not the TNFR2 in arthritis. This increase of Th1 activity may explain some of the adverse effect of anti-TNF therapy, such as new onset or exacerbation of vasculitis or lupus[316].

In TNFR2$^{-/-}$ mice, there was significantly enhanced clinical and histological disease activity compared with WT mice. This result indicates TNFR2 plays a protective role in CIA. This is consistent with many previous reports, which showed TNFR2 protects neurons from oxidative stress and neurodegenerative disease[296, 317]. I next sought to identify the mechanism by which TNFR2 signaling protects the mice from CIA. While TNFR2 deficiency aggravated the severity of CIA, I found that the number of Tregs, more specifically tTregs, was decreased in the TNFR2$^{-/-}$ mice compared with WT mice. Additionally, decreased expression of FoxP3 was observed in arthritic TNFR2$^{-/-}$ mice. These findings show that TNFR2 signaling promotes Treg generation and suppressive function in arthritis. This is supported by the fact that TNFR2 plays an indispensable role in stabilizing the phenotype of Tregs in inflammation[144].

In addition, increased gene expression of pro-inflammatory cytokines, TNF-α and IL-6, were detected in TNFR2$^{-/-}$ mice compared with WT mice. The elevated level of TNF-α and IL-6 could potentially be explained by the increased activity of macrophages in TNFR2$^{-/-}$ mice, which is supported by the genes encoding CD40 and CD68 in the draining LNs of TNFR2$^{-/-}$ mice. To conform this finding at the protein level, it would be important to determine the level of expression of CD40 and CD68

expression by FACS. Macrophages express both TNFR1 and TNFR2 and it has been reported TNFR2 signalling mediates TNFα-induced cell death in macrophages[318]. Thus, TNFR2 deficiency may result in increased number of macrophages in inflammation. Moreover, $TNFR2^{+}$ Tregs represent a maximally suppressive subset of Treg[148]. Tregs can exert direct suppressive effect on monocyte/macrophages, thereby affecting subsequent immune response[319]. These findings suggest $TNFR2^{-/-}$ Tregs may not be sufficient to inhibit activation of macrophages in inflammation.

Continuing exposure to antigen and inflammatory signalling during chronic viral infection and cancer generally reduces T cell function in a process known as 'T cell exhaustion'. T cell exhaustion is characterized by the expression of inhibitory receptors, such as LAG3 and PD-1 which are expressed on $CD4^{+}$ T cells, $CD8^{+}$ T cells, NKT cells, B cells and activated monocytes. The ligation of PD-1 by PD-L1 antagonizes TCR signalling and prevents viral and tumour clearance. In RA, high levels of expression of both PD-L1 and PD-1 are found in synovial T cells and macrophages of patients[320]. Elevated sPD-1 levels are detected in the synovial fluid and serum of patients with RA and correlate with disease activity and inflammatory cytokine concentration. Intraperitoneal injection with sPD1-Fc fusion protein aggravates CIA and joint destruction, promoting autoantibody production and Th1/Th17 response[321]. Conversely, administration of PD-L1-Fc fusion protein decreases severity of CIA and inhibits production of IL17 and IL23[322]. Taken together, these findings suggest PD-1-PD-L ligation plays a pivotal role in the pathogenesis of RA.

Persistent elevated concentrations of TNF-α in chronic viral infection result in $CD4^{+}$ T cell exhaustion via promoting NF-κB expression[323]. However, my data showed

TNFR2$^{-/-}$ mice had significant lower levels of PD-1 and LAG3 expression in arthritis when its mean clinical score and level of TNFα was higher. The lower expression of PD-1 and LAG3 may also contribute to higher disease of CIA. The finding suggests TNFα may promote expression of immune checkpoint markers via TNFR2 signalling, not TNFR1 signalling, although the mechanism needs to be further explored.

In conclusion, the results of this chapter showed that TNFR2 signaling plays several immunoregulatory roles in CIA. First, it contributes to promoting Treg generation and FoxP3 expression, and second, it plays an inhibitory role by limiting production of TNF-α. TNFR2 also regulates the expression of PD-1 during inflammation. The results support the rationale to develop TNFR1 specific antagonists and TNFR2 agonists for the treatment of autoimmune diseases.

Chapter 5 DNA demethylating agents ameliorate collagen-induced arthritis and alter the balance between Th1, Th17 and Treg cells

5.1 Introduction

Despite the success of chemical and biologic DMARDs, around 30% of patients with RA are resistant to conventional therapy and few patients (10% or even less) achieve long-term remission[254]. In addition, the use of biologic agents increases the risk of infection in patients[324]. Regulatory T cells (Tregs) are a subpopulation of T cell, which are essential for immunological homeostasis. However, chronic inflammation is associated with loss of Treg cell function, a phenomenon that has been observed in a number of autoimmune diseases, including RA, systemic lupus erythematosus and type 1 diabetes[185-188]. Several approved drugs including MTX and biologic agents are reported to restore function or increase numbers of Tregs, and this effect is likely to contribute to the partial efficacy of these drugs in RA[188, 234-238].

These findings suggest that Tregs could be an ideal target for therapies to induce long term remission of autoimmune diseases. Tregs are ideal for this purpose because they suppress inflammation in an antigen-specific manner without leading to systemic immune suppression, which is a problem encountered with conventional immunosuppressive therapies. In addition, short-term therapy with Tregs can lead to durable inhibition of autoimmune disease[325].

The phenotypic stability of Treg is a major challenge for using Tregs as an immunotherapy in the clinic. For example, $FoxP3^+$ Tregs can convert into Th1 or Th17-like cells within the proinflammatory microenvironment[326-328]. Several studies have shown that DNA demethylation at FoxP3 regulatory elements helps to maintain Treg phenotype and is also a reliable marker of stable Tregs in mice[173, 174, 227, 228]. DNA demethylating agents can promote suppressive function of human Tregs and have been shown to expand numbers of Tregs in patients treated for malignancy[232, 329]. I hypothesized that boosting Tregs by treating with demthylating agents could achieve long term and specific inhibition of inflammation by promoting Treg responses.

To provide the rationale for treatment with DNA demethylating agents in RA, I first established the effect of DNA demethylating agents on Treg differentiation from Teff cells. I then tested the efficacy of demethylating agents in the treatment of CIA. I further investigated the effect of DNA demethylating agents on T helper cell populations at inflammatory sites. The results confirmed the therapeutic potential of DNA demethylating agents in the treatment of RA.

5.2 Results

5.2.1 Treatment with Decitabine or Psammaplin A , but not procainamide induces Treg differentiation from Teff cells.

To determine whether different DNA methylation inhibitors can promote Treg differentiation from Teff cells with the same efficiency, we first used 5-azacytidine (decitabine), which is a cytosine nucleoside analogue that inhibits DNA methyltransferase (DNMT) and which was shown to induce FoxP3 expression in human NK cells[292]. Naïve T cells ($CD4^+$ $CD25^-$ $CD44^{low}$ $CD62L^{high}$) were stimulated with anti-CD3, IL2, TGF-β1, mitomycin treated antigen-presenting cells (APCs), in the presence or absence of decitabine for 4 days. Decitabine induced FoxP3 protein (Figure 5.1A) and mRNA expression (Figure 5.1B) in naive $CD4^+$ $CD25^-$ T cells in a dose-dependent manner.

I also tested the effect of Psammaplin A, which is a DNMT inhibitor and also a histone deacetylase, on Treg differentiation. The data showed that Psammaplin A has similar efficacy as decitabine in promoting FoxP3 expression (Figure 5.2).

DNMT1, DNMT3a, and DNMT3b are the three active methyltransferases responsible for the establishment and maintenance of DNA methylation, which have been identified in mammals. DNMT1 plays a central role in regulating the expression of FoxP3[174]. To avoid the unwanted effect of DNMT inhibitors on DNMT3a and DNMT3b, I tested the ability of procainamide, a DNMT1 specific inhibitor, to promote FoxP3 expression. In contrast to the finding of the previous study[175], our results showed that procainamide failed to induce the expression of FoxP3 on day 4 following incubation (data not shown), or with a longer incubation time (Figure 5.3).

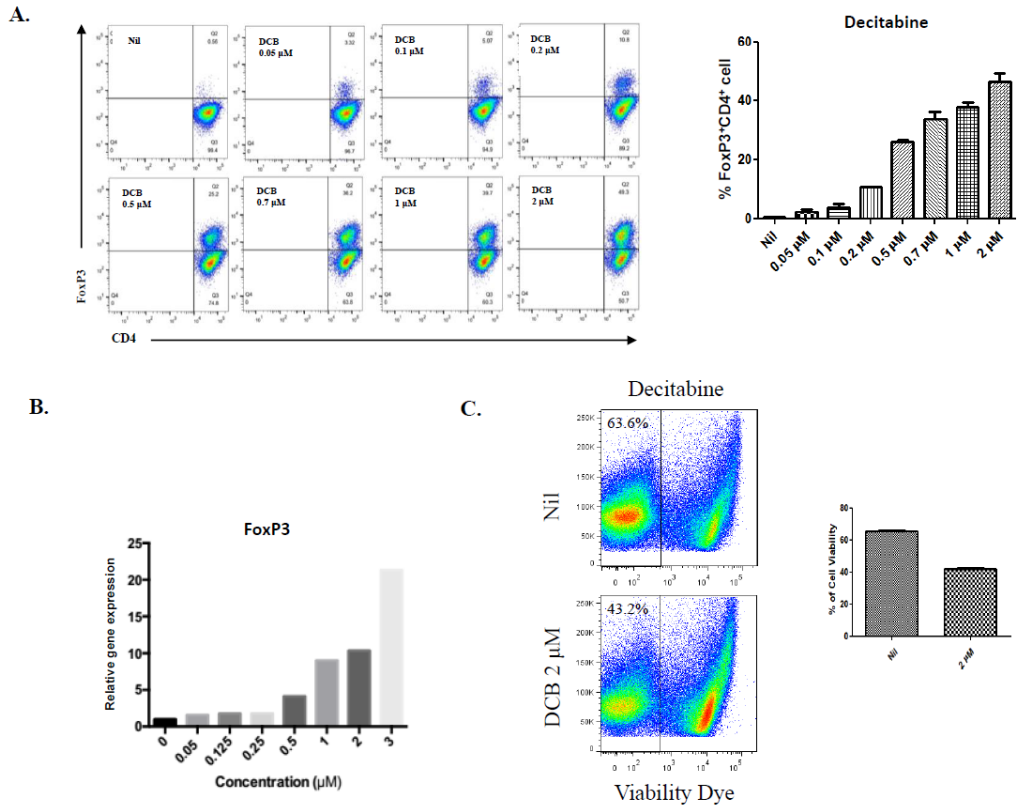


Figure 5.1 Decitabine promotes Treg differentiation from Teff cells. (A) Naïve T cells ($CD4^+$ $CD25^-$ $CD44^{low}$ $CD62L^{high}$) were isolated from cell suspensions prepared from spleen and LNs of 6-8 weeks old DBA/1 mice using the MACS isolation kit. Isolated naïve cells ($4x10^6$/ml) were suspended in complete RPMI1640 with anti-CD3, IL2, TGF-β1, mitomycin treated antigen-presenting cells and decitabine, as indicated. These cells were incubated for 96h in 5 % $1CO_2$ incubator at 37°C. After 4 days, the number of the induced Tregs was determined by FACS (n=3). (B) RNA was isolated from cell lysate, and reverse transcribed to cDNA for RT-qPCR analysis (n=1). Data were normalized to HPRT expression and were calibrated relative to the group in absence of decitabine. (C) Cell viability at 2 μ M decitabine was determined by flow cytometry (n=3). Values in A and C are the mean ± SEM in each group treated with indicated concentration of decitabine.

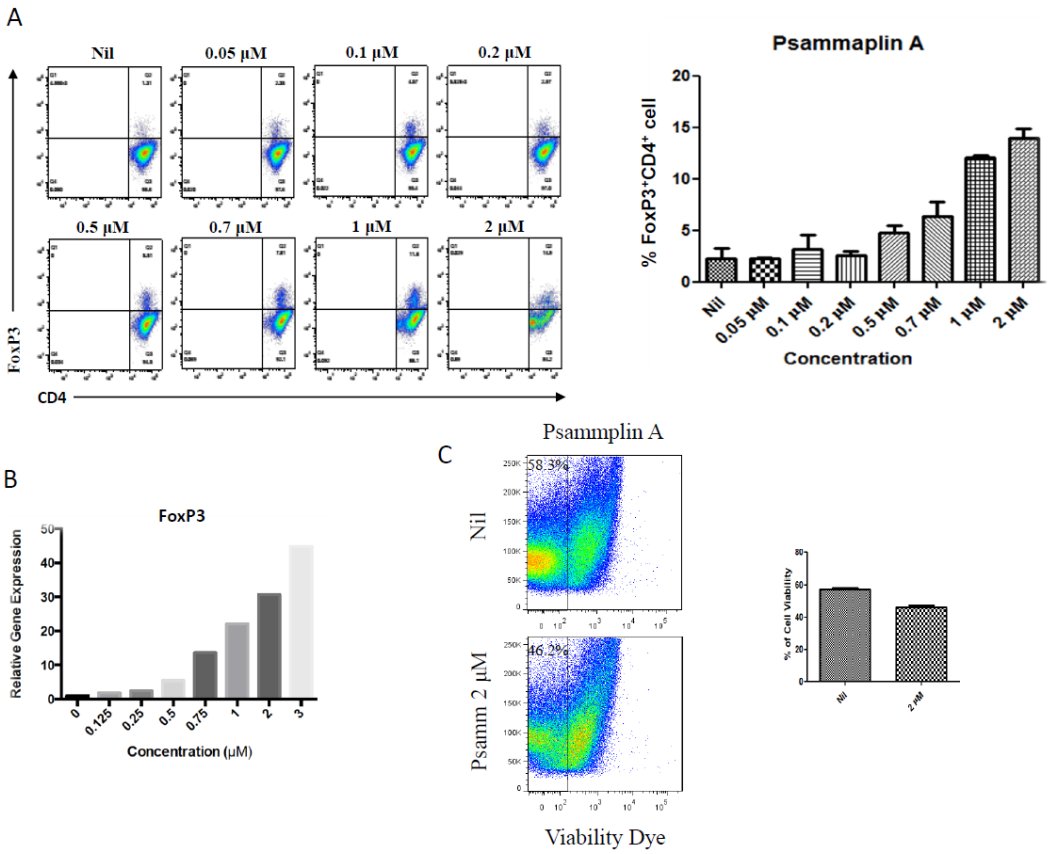


Figure 5.2 Psammaplin A can also promote Treg differentiation from Teff cells. (A) Naïve T cells ($CD4^+$ $CD25^-$ $CD44^{low}$ $CD62L^{high}$) were isolated from cell suspensions prepared from spleen and LNs of 6-8 weeks old DBA/1 mice using MACS isolation kit. Isolated naïve cells ($4x10^6$/ml) were suspended in complete RPMI1640 with anti-CD3, IL2, TGF-β1, mitomycin treated antigen-presenting cells and psammaplin A, as indicated. The cells were incubated for 96h in 5 % CO_2 incubator at 37°C. After 4 days, the number of the induced Tregs was determined by FACS (n=3). (B) The RNA was isolated from cell lysate, and reverse transcribed to cDNA for RT-qPCR analysis (n=1). Data were normalized to HPRT expression and were calibrated relative to the group in absence of pasmmaplin A. (C) Cell viability at 2 μ M of psammaplin A was determined by flow cytometry (n=3). Values in A and C are the mean ± SEM in each group treated with indicated concentration of psammaplin A.

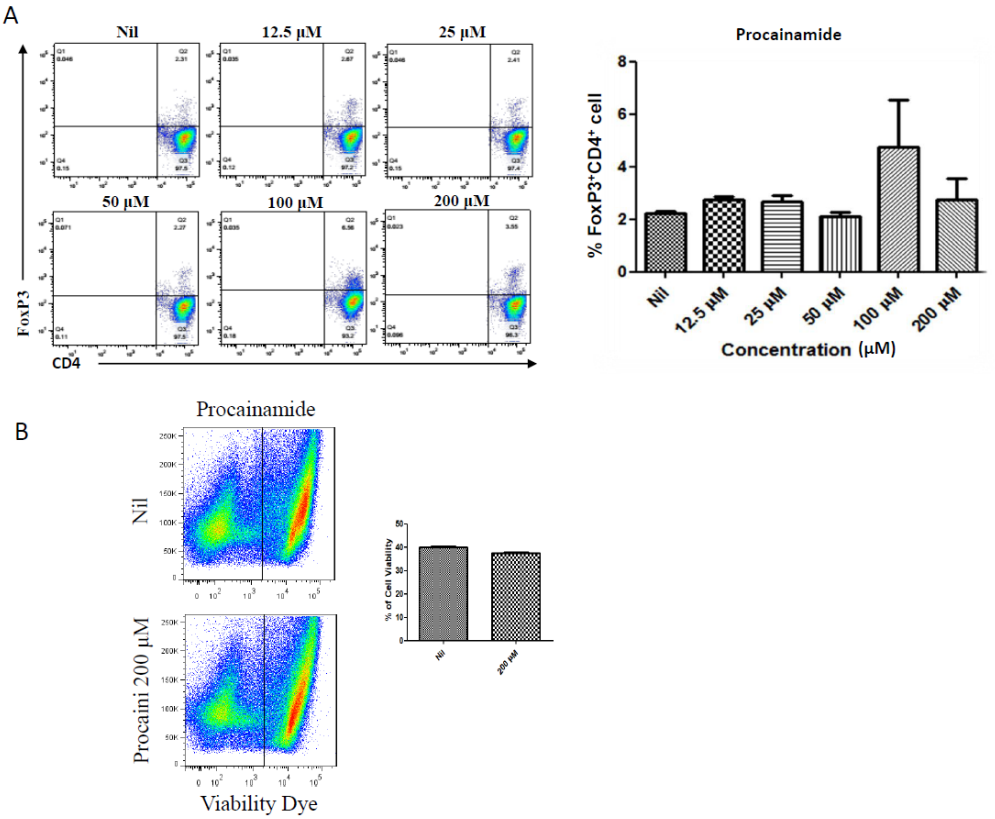


Figure 5.3 Procainamide is a poor promoter of Treg differentiation from Teff cells. (A) Naïve T cells ($CD4^+$ $CD25^-$ CD44low CD62Lhigh) were isolated from cell suspensions prepared from spleen and LNs of 6-8 weeks old DBA/1 mice using MACS isolation kit. Isolated naïve cells ($4x10^6$/ml) were suspended in complete RPMI1640 with anti-CD3, IL2, TGF-β1, mitomycin treated antigen-presenting cells and procainamide, as indicated. The cells were incubated for 168h in 5% CO_2 incubator at 37°C. After 7 days, the number of the induced Tregs was determined by FACS (n=3). (B) Cell viability at 200 μM procainamide was determined by flow cytometry (n=3). Values in A and B are the mean ± SEM in each group treated with indicated concentration of procainamide.

5.2.2 Treatment with decitabine ameliorates CIA

To explore the long-term therapeutic potential of promoting Tregs in arthritis by treating with DNMT inhibitors, mice with established CIA were treated briefly with decitabine (1 mg/kg), Psammaplin A (1 mg/kg), or vehicle control (PBS) on days 1, 2, 3, and 4 of arthritis. Both decitabine and Psammaplin A significantly reduced clinical scores from day 5 after arthritis onset, as compared to the vehicle control. However, while the effect was sustained throughout the 10-day treatment period in the decitabine-treated group, there was a significant loss of therapeutic effect in the Psammaplin A-treated group from day 8 to day 10 (Figure 5.4A).

Histologically, compared with the severe inflammatory infiltration and joint destruction in the vehicle control group, the joints of mice from both the decitabine-treated and Psammaplin A–treated groups were largely protected from disease, with the majority of joints remaining healthy or showing only mild inflammatory changes (Figure 5.4B). Representative images of a proximal interphalangeal joint sections from mice treated with PBS, decitabine, and psammaplin A are shown in Figures (5.4 C-E). Mice treated with vehicle alone showed extensive inflammation, with large areas of infiltrating cells. In addition, the articular cartilage showed significant damage with joint space narrowing (Figure 5.4C). Multiple sites of bone erosion were also evident, with pannus formation, resulting in destruction of joint architecture. Joints from mice treated with decitabine or psammaplin (Figures 5.4 D and E), in contrast, were largely free of inflammatory infiltration and demonstrated clear joint spaces and intact architecture. To summarize the findings of clinical and histological severity, decitabine provides significant joint

protection from the inflammatory process that leads to bone destruction. Psammaplin A has similar efficacy to decitabine in the early stages of disease but gradually loses the treatment effect in the late stages.

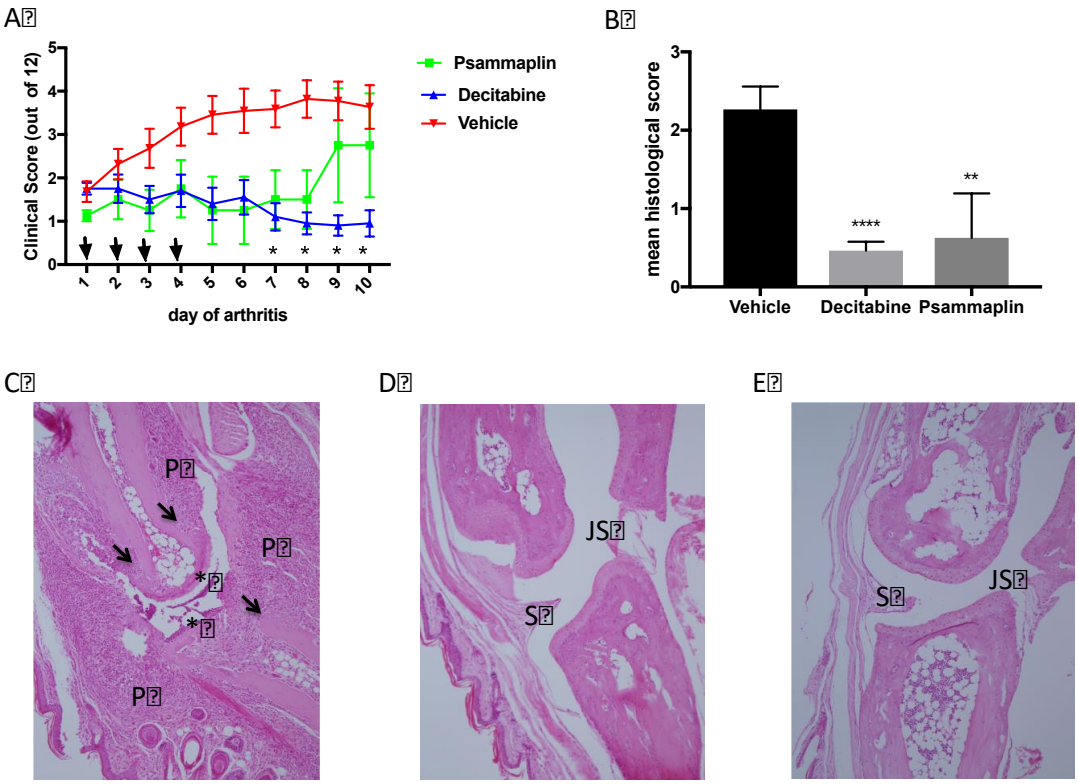


Figure 5.4. Amelioration of of CIA by treatment with DNA demethylating agents. DBA/1 mice with CIA were treated on days 1, 2, 3, and 4 after arthritis onset with vehicle (PBS), 1 mg/kg of decitabine, or 1 mg/kg of psammaplin A. **(A)** Disease severity was evaluated daily, and the mean clinical score in each treatment group was determined. Values in **A** are the mean ± SEM of 11 mice in each of the decitabine and psammaplin groups and 10 mice in the vehicle group. *P<0.05 for decitabine versus vehicle, by two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. **(B)** On day 10 following CIA onset, paws were harvested and prepared for histologic analysis. Paraffin-embedded sections were stained with hematoxylin and eosin and scored for the degree of joint destruction. Histopathologic changes in the joints were scored on a scale of 0–3. ** P<0.01; ****P<0.0001 versus vehicle, by one-way ANOVA with Dunnett's multiple comparison test. Also shown are representative images of proximal interphalangeal joint sections from mice treated with vehicle (**C**) decitabine (**D**), and psammaplin (**E**). **Arrows** indicate bone loss. **Asterisks** indicate damage of articular cartilage. **P =** pannus; **S =** synovial membrane; **JS =** joint space.

5.2.3 Decitabine can provide long-term protection from CIA independently of Treg function

It has been shown the use of decitabine results in globally decreased levels of methylation of genomic DNA, especially in promoter regions[330]. The effectiveness of decitabine in CIA raises the questions of whether the efficacy of decitabine solely relies on promoting Treg function and what is the effect of decitabine on other immune cells. To assess the role of Tregs in decitabine-mediated amelioration of CIA, I first tested the effect of Treg depletion using anti-CD25 Ab (PC61 mAb) in arthritic mice treated with decitabine. DBA/1 mice with established CIA were treated concurrently with decitabine (10 mg/kg) and PC61 mAb, or vehicle control (PBS) on days 1, 2, 3, and 4 of arthritis (Figure 5.5A). On day 10 following CIA onset, there was a significant reduction in the number of Tregs in the spleen and LN cells of mice treated with PC61, confirming the efficacy of the antibody (Figure 5.5B). Surprisingly however, decitabine combined with PC61 mAb showed comparable therapeutic efficacy to decitabine plus vehicle alone (Figure 5.5C). This result indicates that decitabine ameliorates disease in a Treg independent manner

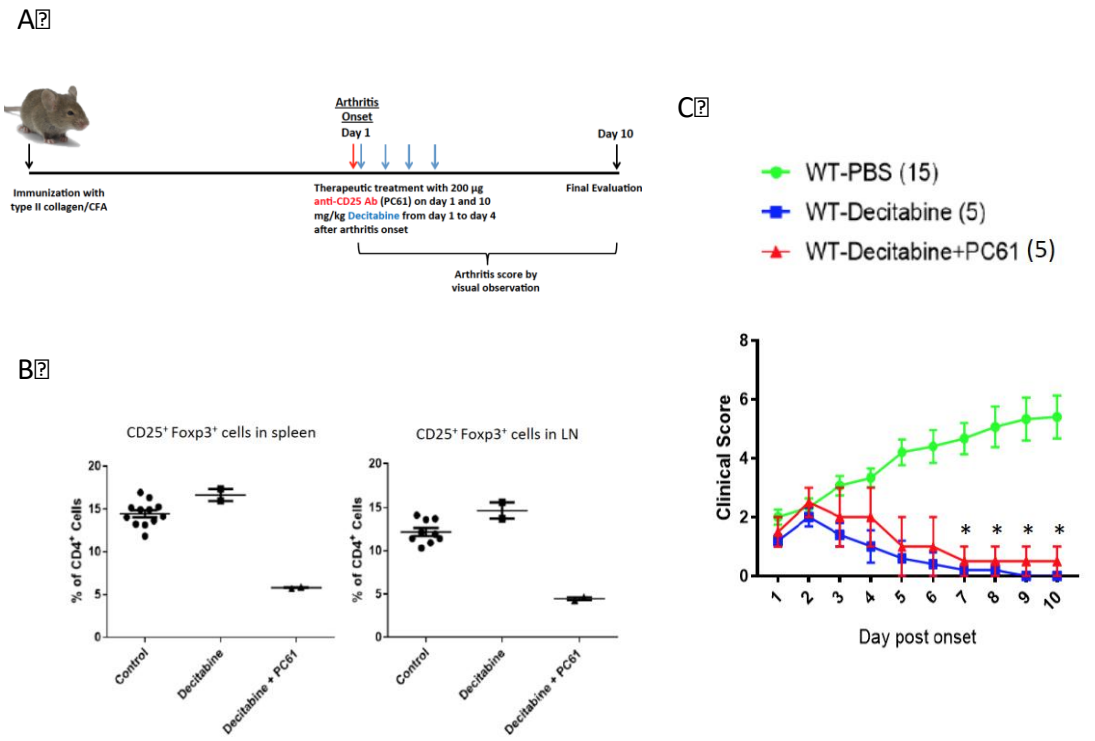


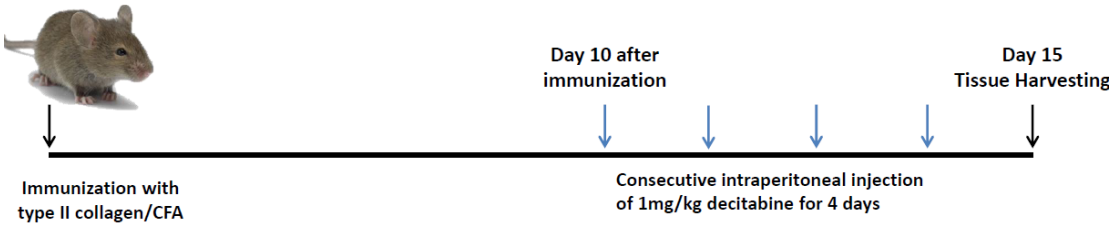
Fig 5.5 Efficacy of DNA demethylating agent in the treatment of CIA is not fully Treg dependent. DBA/1 mice with CIA were treated on days 1, 2, 3, and 4 after arthritis onset with PBS (vehicle), or 1 mg/kg of decitabine, in combination with anti-CD25 Ab (PC61) on day 1 after arthritis onset (A). (B) On day 10 following CIA onset, LN and spleen were harvested and number of Tregs was determined by FACS. (C) Disease severity was evaluated daily, and the mean clinical score in each treatment group was determined. Values in B and C are the mean ± SEM of 5 mice in each of the decitabine with or without anti-CD25 Ab groups and 15 mice in the control group. *P<0.05 for decitabine with or without anti-CD25Ab versus vehicle, by two-way analysis of variance (ANOVA) with the Dunnett's multiple comparison test.

5.2.4 Decitabine can promote Treg suppressive function and inhibit Th1/Th17 cell in inflammation

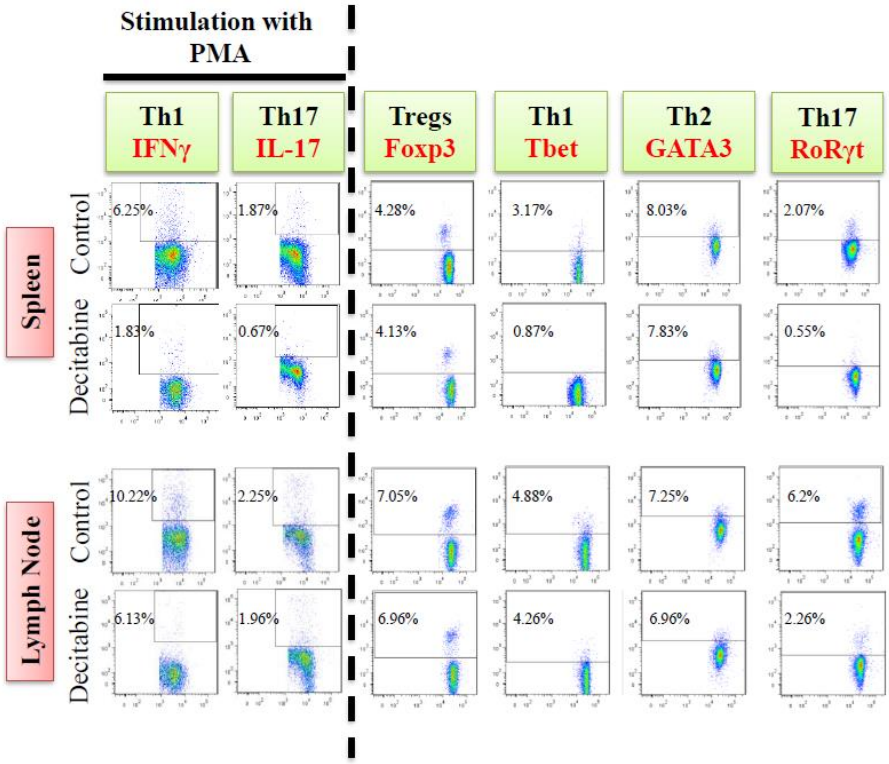
The balance between Th1, Th17 and Tregs is thought to play an indispensable role in the pathogenesis of RA. An alternative potential mechanism that enables decitabine to ameliorate disease of CIA is via inhibition of Th1 and Th17 cells. To test the effect of decitabine on generation of T helper cells, immunized DBA/1 mice were treated with decitabine (1 mg/kg) or vehicle control (PBS) on days 1, 2, 3, and 4 after immunization (Figure 5.6A). On day 15 following immunization, there was a significant reduction of number of Th1 and Th17 in the spleen and LN cell from decitabine-treated mice than the control group. The numbers of Tregs and Th2 cells are comparable in spleen and LNs of both groups (Figure 5.6B and C). This confirms the ability of decitabine to selectively deplete Th1 and Th17 cells.

I further assessed the effect of decitabine on Treg suppressive function in inflammation. The results showed that decitabine can enhance Treg suppressive function (Figure 5.7A). This was supported by the fact that there was an increased expression of CD25, which is an important functional marker of Tregs[331], on the Tregs from decitabine-treated mice, as compared to negative control groups (Figure 5.7B). In summary, my data suggests decitabine exerted its therapeutic effect for CIA by promoting suppressive function of Tregs and inhibiting Th1 and Th17 cells.

A


Day 10 after immunization
Day 15 Tissue Harvesting
Immunization with type II collagen/CFA
Consecutive intraperitoneal injection of 1mg/kg decitabine for 4 days

B


Stimulation with PMA
Th1 IFNγ
Th17 IL-17
Tregs Foxp3
Th1 Tbet
Th2 GATA3
Th17 RoRγt
Spleen
Lymph Node
Control
Decitabine
6.25%
1.87%
4.28%
3.17%
8.03%
2.07%
1.83%
0.67%
4.13%
0.87%
7.83%
0.55%
10.22%
2.25%
7.05%
4.88%
7.25%
6.2%
6.13%
1.96%
6.96%
4.26%
6.96%
2.26%

C

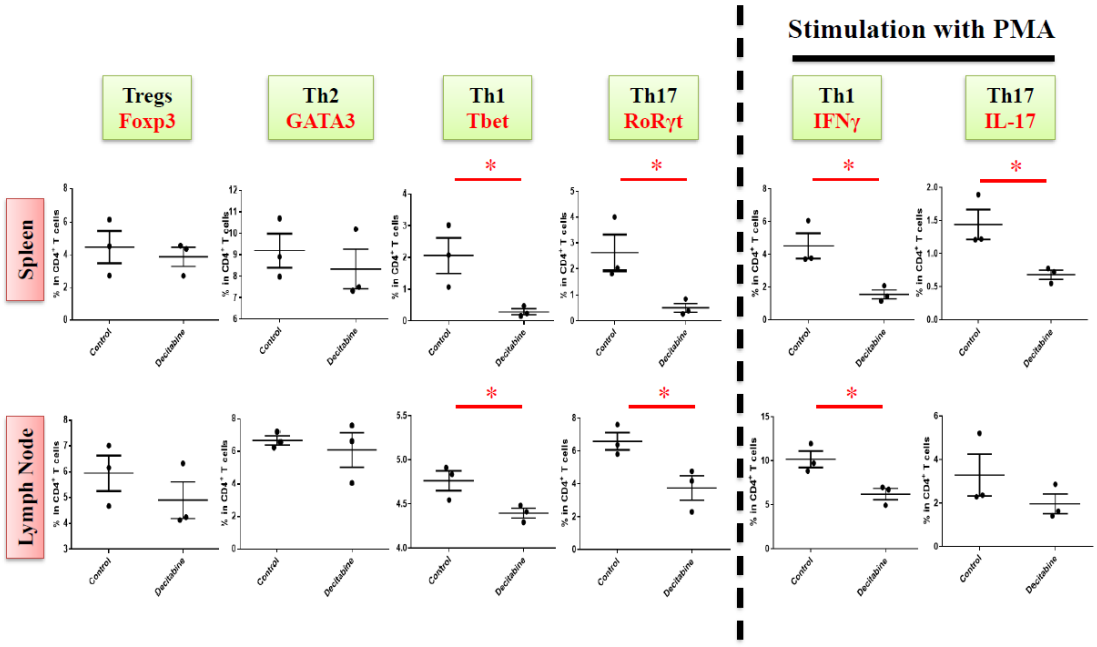


Figure 5.6. Reduced Th1 and Th17 cells following treatment with DNA demethylating agent. (A) DBA/1 mice were treated on days 10, 11, 12, and 14 after immunization with vehicle (PBS) or 1 mg/kg of decitabine. Spleen and LNs were harvested on day 15 after immunization. (B & C) The isolated splenic and LN cells were stimulated with PMA (20 ng/ml), ionomycin (1μM) and Brefeldin A (6.25 μg/ml) in complete RPMI1640 for 3h. The phenotypes of Th cells were determined by detection of cytokines and/or lineage specific transcription factors by intracellular immuno-staining (C). Values in C are the mean ± SEM of 3 mice in the decitabine groups and in the vehicle group. *P<0.05 for decitabine versus vehicle, by unpaired t-test.

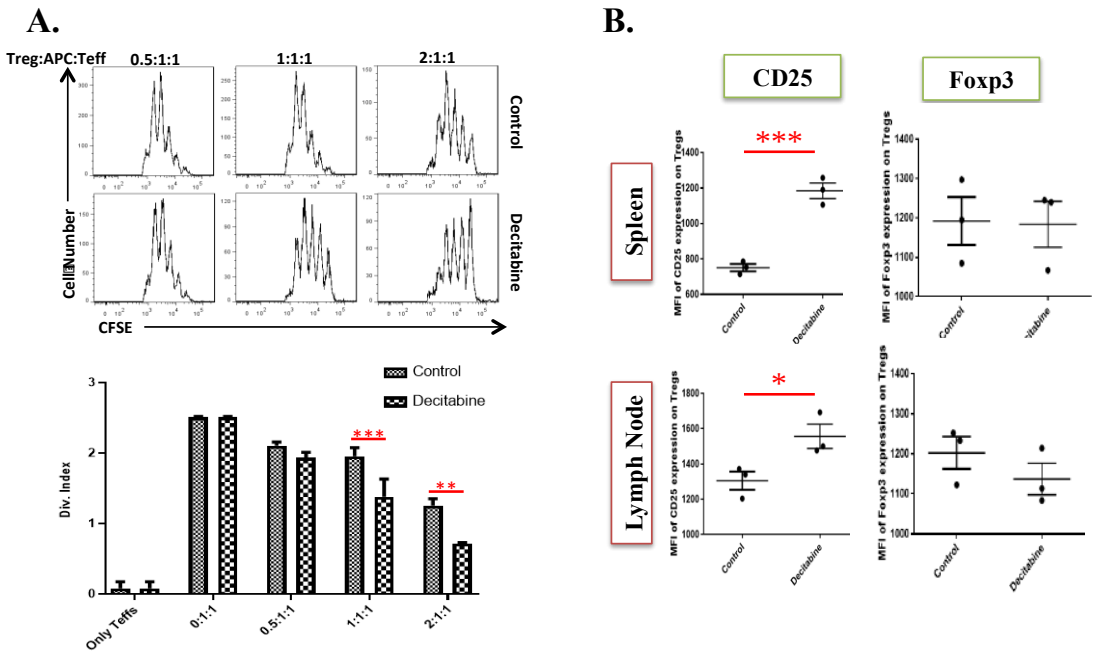


Figure 5.7 Enhanced Treg suppressive function following treatment with DNA demethylating agent. Tregs were isolated from spleen and LNs of immunized mice using MACS cell separation kit. The function of Treg was determined using Treg suppression assay. Different ratio of Treg cells and CFSE-labeled Teff cells were incubated with 5 μg/ml of anti-CD3 and mitomycin c treated antigen-presenting cells (APCs). After 72 hours, the proliferation of the CFSE-labeled Teff cells (A) and phenotype of Tregs (B) were determined by FACS. Values in **A** and **B** are the mean ± SEM of 3 mice in the decitabine groups and in the vehicle group. **P*< 0.05, ***P* < 0.01, ****P* < 0.001 for decitabine versus vehicle, by unpaired t-test.

5.3 Discussion

In this chapter, the therapeutic potential of epigenetic modification in RA is explored by studying the effect of DNA demethylating agents in CIA, as an animal model. I first established whether there were any differences between three DNA demethylating agents in promoting Treg differentiation from Teff cells *in vitro*. The results showed that both decitabine and psammaplin A could enhance Treg development in a dose dependent manner. However, procainamide failed to show a beneficial effect in promoting Treg generation, even with longer incubation times or higher doses. This result contrasts with a previous study, which reported that procainamide could induce FoxP3 expression, although to a lesser extent than decitabine[175]. A possible reason for these conflicting results is that the higher concentration of procainamide (12.5 to 100 μM) and longer incubation time used in my study.

The difference of capability in inducing Tregs in my study might be explained by different mechanisms of action of the three DNMT inhibitors. Decitabine, being a deoxyribose analog, is incorporated into DNA and replaces the carbon in the 5-position of modified pyrimidine with nitrogen. This modification induces a covalent bond with DNA and DNMT, which may induce cell death by interrupting DNA synthesis[332]. In addition, DNMT remains irreversibly bound to the incorporated DNA, preventing methylation, which leads to significant demethylation after repeated DNA replication[333]. Psammaplin A, a derivative of the marine sponge *Pseudoceratina purpurea*, have been shown to inhibit DNMT, as well as histone deacetylase activity (HDAC), an enzyme associated modulation of gene expression by histone modification[334]. Procainamide is a non-nucleoside based drug that

specifically inhibits DNMT1[335]. It has been reported that decitabine is far more effective in inhibiting DNA methylation than non-nucleoside based inhibitors, including procainamide[334]. The difference of potency in inhibiting DNA methylation may explain the difference of effectiveness in inducing Treg differentiation from Teff *in vitro*.

In my study, the results showed that DNMT inhibition led to increased expression of *foxp3* which is likely to account for the augmented Treg differentiation. However, the molecular mechanism of DNMT inhibition in promoting expression of foxp3 is hypothesied to be due to decreased DNA methylation at the *foxp3* promoter and hence increased gene expression. It has been reported that decitabine treatment led to significant inhibition of expression of DNMT1 and DNMT3B leading to enhanced expression of tumor suppressor genes in tumor cells, which would be compatible with my hypothesis[336]. In future studies, the effect of DNMT inhibitors on the expression of DNMT (DNMT1, 3a and 3b) in T cells at the mRNA and protein level will be investigated by qPCR and Western blot analysis, respectively. Due to time constraints, I did not confirm that DNMT inhibitors affected the methylation status of key genes in Tregs, such as *foxp3* and *il2ra*, and this will be an important question to address for the future. I would propose performing a digestion-based assay, using nested methylation-specific PCR, bisulfite sequencing, or ChIP-on-chip. Furthermore, whole genome methylation profiling for the T cell treated with DNMT inhibitors would elucidate how DNA methylation contributes to RA pathogenesis, and enable us to systematically analyze the DNA methylation machinery as a target for therapeutic intervention of RA. It is important to consider that all three tested drugs are non-specific DNMT inhibitors and further study is needed to address the effect of DMNT on Treg generation using specific DNMT inhibitors, SiRNA, or conditional

gene knockout, specifically targeted to DNMT1, 3a and 3b. The non-targeted nature of drugs like decitabine is likely to be an obstacle to their clinical development for chronic diseases like RA.

I further investigated the therapeutic potential of DNA demethylating agents in the treatment of RA using the CIA model. My results showed that decitabine significantly reduces disease progression and prevents inflammatory infiltration and destruction. Psammaplin A has a similarly protective effect on inhibiting disease progression and joint destruction, although its therapeutic effect was found to be less durable. The result is consistent with data obtained using decitabine in the treatment of EAE, which showed both therapeutic and prophylactic effects of decitabine in this model[233, 337, 338].

In order to determine the role of Tregs in the therapeutic effect of decitabine, I next compared the effect on CIA of decitabine treatment combined with Treg depletion. Strikingly, decitabine showed comparable efficacy in ameliorating disease progression of CIA in the absence or presence of Treg. This result indicates that the mechanism of action of decitabine in the treatment CIA is not Treg-dependent. However, apart from Tregs, activated Teffs may also transiently express CD25, and are thus also potential targets for anti-CD25 Abs. It has been reported anti-CD25 Abs may directly abrogate effector T cell responses during an inflammatory episode[339], indicating important limitations of the use of anti-CD25 Ab administration to determine Treg function during arthritis in my study. To address whether the therapeutic efficacy of DNMT inhibitors in RA relies on Tregs, more specific Treg depletion method, such as depletion of regulatory T cells (DREG) mice[340] should be considered in further studies.

I further tested the effect of decitabine on pro-inflammatory Th cell populations. While the number of Treg and Th2 cell remained unaffected, there was a significant decrease of both Th1 and Th17 cells in decitabine-treated mice compared with PBS-treated mice. Our result is not fully consistent with previous studies in EAE, which showed amelioration of disease progression with increased the percentage of Tregs[233, 337, 338]. The difference may be explained by the higher dose (1 mg/kg) of decitabine for 4 consecutive days in my experiments, compared to the low dose (0.1-0.15mg/mg) for 2-3 consecutive weeks used in the EAE study. The results suggest that long-term low-dose of DNA demethylating agents can promote FoxP3 expression, while short-term high dose DNA demethylating agents inhibit Teff proliferation. Moreover, enhanced Treg suppressive function with increased CD25 expression was shown in my result. The enhanced Treg suppressive function may also contribute to inhibition of Th1 and Th17 cells at inflammatory status. However, the fact that treatment with PC61 failed to abrogate the therapeutic effect of decitabine would argue against this. It is possible that PC61 failed to completely eliminate Tregs from the site of disease activity. As discussed above, the methylation status of key transcription factors of Th cells was not measured following decitabine treatment in my study and should be investigated by, for example, ChIP of Bisulfite-treated chromatin-Bisulfite Sequencing (CHIP-BMS) in the future.

In summary, my study reveals a potential therapeutic strategy for RA by restoring the aberrant methylation pattern during inflammation. Short-term high-dose decitabine can reduce disease activity of CIA by enhancing Treg suppressive function and inhibiting Th1 and Th17 cells. However, the challenge will be to selectively

de-repress only those genes responsible for immunoregulation and to avoid de-repression of genes which may have deleterious effects. Therefore, the safety and the effect of use of decitabine in the treatment of RA should to be further investigated and methods for targeting DNA demethylating drugs to specific promoter regions need to be explored.

Chapter 6: General Summary and Discussion

TNF-α was first described as a serum factor inducing tumor necrosis about 40 years ago and was isolated and cloned approximately 10 years later. TNF-α was identified as a therapeutic target for inflammatory diseases over 25 years ago[341] and since then anti-TNF-α therapy has changed the therapeutic landscape of inflammatory diseases, having been licensed for the treatment of RA, psoriatic arthritis, ankylosing spondylitis, Crohn's disease, ulcerative colitis, chronic psoriasis, hidradenitis suppurativa, and juvenile idiopathic arthritis[253, 342, 343]. However, despite its great success in the majority of patients with inflammatory arthritis, the use of anti-TNFα also raises some questions such as why some RA patients do not respond to the treatment (around 30%) [254]. In addition, some patients experience significant side effects, such as infections or paradoxical autoimmunity during treatment with anti-TNF-α[255]. RA is thought to be a T cell driven disease and to achieve long-term disease remission it will be necessary to modulate T cell responses. However, the effect of TNF-α on the immune system in general, and on regulatory T cells (Tregs) in particular, is poorly understood and this project seeks to address this question.

Tregs are a subpopulation of T cells, which are essential for immunological homeostasis. Dysfunction of Tregs results in a breakdown of immunological tolerance and has been implicated in the pathogenesis of autoimmune diseases, including SLE, RA and type I diabetes[185-188]. It has been reported that TNF-α promotes proliferation, survival, stability and suppressive function of Tregs[147, 148, 256]. In addition, TNFR1 blockade, but not TNFR1/2 blockade, promoted generation and suppressive function of Tregs in CIA[257]. Based on these findings, we hypothesized that TNFR2 plays an immunoregulatory role in arthritis and interruption of

TNF/TNFR2 interaction contributes to the resistance to anti-TNF-α therapy and anti-TNF-induced autoimmunity observed in some patients. The objective of the work presented in this thesis was to gain a deeper understanding of the impact of TNFR1 and TNFR2 signalling on inflammatory responsesRA with particular emphasis on Tregs.

Specifically, the main aims of this project were firstly to investigate the effect of TNFR1 and TNFR2 signaling on the phenotype of Tregs and to determine whether there is an association between changes in phenotype and the methylation status of the *FoxP3* locus. The next aim was to determine the role of TNFR1 and TNFR2 signaling in the pathogenesis of CIA, a widely-studied model of RA. Thirdly, I set out to explore the therapeutic potential of DNA demethylating agents in the treatment of RA via promotion of Treg generation and suppressive function. While this study has provided some intriguing findings, many questions remain unanswered owing to limited time and resources and which need to be further explored in the future studies. In addition, it is important to interpret the data presented in the light of thelimitations of this study. Firstly, the TNFR1$^{-/-}$ and TNFR2$^{-/-}$ mice used in my study are global knockout mice. The gene defect since birth may have a long term and unexpected impact on both the immune and non-immune systems, which may generate experimental artifacts. Therefore, conditional and cell-specific knockout mice would be a better model to address the association between TNFR signaling and Tregs. Secondly, due to limited time and funding, I mainly investigated the effect of TNFR signaling and DNMT inhibitors on Treg associated genes, without considering the effects on the whole genome. A comprehensive genome-wide RNA sequencing or DNA methylation analysis would be useful to fully understand the role TNFR

signaling and DNMT inhibitors in the immune system.

In Chapter 3, my data showed that TNFR2 deficiency, associated with decreased production of TGF-β, leads to increased CpG methylation at the proximal promoter and also a trend towards increased methylation at the TSDR of the *FoxP3* locus. The hyper-methylation status at the cis-regulatory element of the *FoxP3* locus may make the Tregs less stable and less suppressive in inflammation. To address this question in future studies, I will compare the suppressive ability of Tregs from wild-type and TNFR2$^{-/-}$ naïve mice with those from arthritic mice. In addition, it would be highly informative to compare genome-wide mRNA expression in Tregs between TNFR2$^{-/-}$ and wild-type mice with arthritis.

In Chapter 4, TNFR2 deficiency was shown to have a detrimental effect on the clinical and histological disease activity of CIA. The increased severity of arthritis was accompanied by increased production of pro-inflammatory cytokines, monocyte/macrophage activity and decreased expression of immune check points, including PD-1 and LAG3. The above findings indicate that TNFR2 plays an immunoregulatory role in inflammation in part by inhibiting DNA methylation at the *Foxp3* promoter. Based on these findings I decided to ask the question of whether reversal of DNA methylation would promote Treg responses and ameliorate CIA. The function of Treg cells is particularly sensitive to the activity of DNA methylating enzymes, such as DNA methyltransferase 1 (DNMT1). DNMT1 expression is controlled by the JAK/STAT pathway which is activated by IL-6, a cytokine expressed abundantly in inflammation. Of the three drugs were tested, decitabine was the most effective and was used for all further studies. Treatment with decitabine for

four days caused almost complete cessation of disease up to day 20. This was accompanied by greatly increased expression of Treg signature genes, FoxP3, CD25 and PD-L1, increased Treg cell function and decreased numbers of Th1 and Th17 cells. DNA demethylating drugs have previously been shown to generate iTreg cells in vitro and it was therefore surprising that numbers of Treg cells were not increased in vivo following treatment with decitabine. It is possible that additional mechanisms operate in vivo to limit the size of the Treg cell pool.

It remains to be determined whether the use of DNA demethylating drugs could be applied to human disease as there would be major safety concerns. For example, it will be important to establish the extent to which decitabine disrupts gene expression across the genome in non-immune cells and the duration of this effect. It is worth pointing out that decitabine is a cytosine analogue that inhibits DNA methylation by intercalating into DNA and "trapping" DNMT. However, like all of the nucleoside-based hypomethylating agents, decitabine causes DNA crosslinking and DNA synthesis arrest and is cytotoxic at high concentrations.

DNMT1 is the most abundant DNA methyltransferase and is the principal maintenance methyltransferase. DNMT1 acts by methylating hemimethylated CpG di-nucleotides following cell division. This is advantageous as it means that DNMT1 inhibitors target only dividing cells, such as proliferating lymphocytes. Furthermore, the expression of DNMT1 is upregulated during inflammation[344] and other studies have shown that DNMT1 expression is specifically upregulated in Tregs from RA patients with active disease[202, 225]. Hence, future work will focus on selective, non-nucleoside based DNMT1 inhibitors and on the development of novel

compounds that selectively inhibit DNMT1 activity without integration into the genome. Alternatively, a number of natural and synthetic compounds could be evaluated which have inhibitory activity on DNMT1, including RG108, MG98, SGI-1027, hydralazine, olsalazine, caffaic acid, chlorogenic acid, genistein and mithramycin.

A consistent finding to emerge from these studies has been the up-regulation of immune checkpoints following treatment with DNMT inhibitors and down-regulation of the molecules in TNFR deficient Tregs. Immune checkpoint inhibitors have revolutionized cancer therapy over the past decade. Several clinical trials have shown that modulation of the immune response can improve the overall survival of cancer patients[345, 346]. The approval of anti-CTLA-4 antibody, ipilimumab, in the treatment of metastatic melanoma highlights the therapeutic potential of strategies to block immune checkpoint in cancer therapy. Subsequently, PD-1-PD-L1 pathway blockade demonstrated significant and durable therapeutic effect in cancer patients[347-349]. Most of the aforementioned treatments are based on the mechanism of stimulating the cytotoxic arm of the immune response, and by promoting their recovery from exhaustion[350-353]. By altering the balance of the immune system, these new immunotherapies also generate immune-related adverse events (IRAEs), such as Hashimoto's disease, Myasthenia gravis, SLE and RA[354]. This finding raises the question of whether promoting immune checkpoint expression can be a potential therapeutic strategy in the treatment of autoimmune diseases. This strategy of using an immune-modulator in the treatment of autoimmune disease is supported by the use of Abatacept, which is a CTLA-4 fusion protein that blocks the

T cell co-stimulatory pathway, has shown remarkable success in the treatment of RA, PsA and JIA.

In Chapter 4, decreased expression of PD1 and LAG3 was observed in TNFR2$^{-/-}$ mice, which had increased disease activity and levels of pro-inflammatory cytokines, compared with WT mice. It has been reported that blockade of TNF during chronic infection with LCMV abrogated the expression of the inhibitory receptor PD-1, and reconstituted anti-virus immunity. The results suggest that TNFR2 plays a role in promoting the expression of immune checkpoints in inflammation. Specifically, these findings suggest that TNF can induce the expression of PD-1 on Tregs via TNFR2 rather than TNFR1 in inflammation. The could be confirmed by demonstrating increased expression of PD-1 on Tregs from immunized mice, following treatment with a TNFR2 agonist or decreased expression following treatment with a TNFR2 antagonist. Based on the results from Chapter 3 and 4, the findings raise the possibility that TNFR2 agonism has the potential in the treatment of RA by promoting Treg suppressive function and also the expression of immune check points. New approaches to promote TNFR2 signaling should be considered due to the fact that many immune checkpoint molecules, such as PD-1, LAG3 and CTLA-4, are widely expressed in cells of the immune system. Thus, attempts to target such widely expressed molecules may lead to systemic toxicity and unexpected immune response. By contrast, the effect TNFR2 agonism is likely to be more specific than immune check points inhibitors because TNFR2 expression is restricted to a subpopulation of active Tregs[147]. In addition, TNFR2 is expressed in certain tumor cells and is strongly expressed by tumor infiltrating Tregs. These findings, combined with the

limited natural expression of TNFR2, makes it as a potential next-generation anti-cancer therapeutic target. The possible anti-cancer therapeutic effect of a TNFR2 antagonistic antibody could be achieved by direct killing TNFR2 expressing tumor cells or by increasing the ability of effector T cells to eradicate cancer cells by removing the inhibitory effects of TNFR2 expressing tumor associated Tregs. Future research will explore the association of TNFR2 with immune checkpoints. The knowledge may enable us to modulate the function of T cells by targeting TNFR2 signalling and may provide a platform for future improvements in the treatment of both autoimmune diseases and malignancies.

Until now, data on the use of TNFR2 agonism in humans is quite limited. It has been reported that TNFR2 agonism is effective in selectively killing autoreactive $CD8^+$ T cells taken from patients with type I diabetes[294]. One study also showed that TNFR2 agonism is also able to expand Tregs, with superior suppressive function[355]. A further study showed TNFR2 agonism, combined with IL-2, could promote the generation of active Tregs ($CD4^+$ $CD25^+$ $FoxP3^+$ CD45RO) rather than resting Tregs ($CD4^+$ $CD25^+$ $FoxP3^+$ CD45RA) in type I diabetes [295]. The study also showed a parallel correlation between the number of active Tregs with the level of C-peptide secretion from the pancreas, which affects the control of HbA1C[295]. Moreover, TNFR2 agonism was shown to increase the purity of Tregs during expansion *in vitro* and also maintained Treg phenotype stability and suppressive function in inflammation, therefore may be of clinical utility in the generation of Tregs for immunotherapy[356]. However, the systemic effects of TNFR2 agonists need to be further investigated before this approach can be considered for human therapy.

Apart from TNFR2 signalling, methylation modification also plays an indispensable role in Treg generation and stability and could be a potential therapeutic strategy for RA. Currently, DNA demethylating agents are only approved for some blood malignancies. My study is the first one to show the therapeutic effect of DNA demethylating agents in CIA. However, there are also questions that need to be addressed before this kind of therapy can be considered for humans. The mechanism of action of DNA demethylating agents is not target specific, thus side effects are very probable. Indeed, it has been reported that procainamide and hydralazine can cause anti-nuclear antibody formation in some patients and a lupus-like disease[357]. Furthermore, murine T cells treated with these drugs showed higher auto-reactivity[358]. Moreover, in contrast to cancer therapy, the duration of treatment for autoimmune disease may be life-long. Hence, the drug toxicity of long-term use of DNA demethylating agents would be a major concern. Therefore, the safety of DNA demethylating agents and their effects on the immune system need to be further investigated. One possibility is that demethylating drugs could be used for short-term therapy to induce remission, either alone or in combination with other drugs like TNFα inhibitors. Indeed, in my experiments in CIA, short-term therapy over four days was found to give long-term protection although I was not able to address this question definitively. The use of short-term, aggressive, therapy is in fact the general approach for the treatment of cancer and is likely to be a growing trend in autoimmune disease where the goal, increasingly, is drug-free disease remission.

DNA methylation is carried out by three enzymes, DNMT1, DNMT3a and DNMT3b. Decitabine is a cytosine analogue that inhibits the activity of all three enzymes by incorporating into genomic DNA. As such, decitabine is a relatively toxic drug but

less toxic, DNMT1-specific inhibitors are being developed which could have utility in autoimmune disease.

In summary, this project has shed new light on the positive effect of TNFR2 signalling in Tregs and has, for the first time, demonstrated an association between loss of TNFR2 signalling and methylation of CpGs at the FoxP3 promoter. This knowledge strongly supports the development of selective TNFR1 antagonists for use in autoimmune diseases like RA in order to spare TNFR2 signalling. A second approach may the development of selective TNFR2 agonists to be used alone or in combination with TNFα inhibitors. Potential agonists of TNFR2 could include TNFR2 specific monoclonal antibodies or peptides that engage TNFR2 in an agonistic manner. Thirdly, it may be possible to identify the TNFR2-dependent molecular pathways that regulate suppressive Treg cell functions. This could be achieved by correlating TNFR1- and TNFR2-dependent transcriptomes with an atlas of enhancer states in order to identify novel genes and transcriptional regulatory elements involved in controlling Treg cell function via TNFR2. Ultimately, the data obtained could enable the identification of potential drug targets for diseases associated with deficient Treg cell activity (e.g. RA, type 1 diabetes) and diseases associated with excessive Treg cell activity (e.g. cancer, chronic infection). Finally, the use of DNA methylation inhibitors should be considered, provided that safety concerns can be overcome.

Publications

Publications

Review Article

Wen-Yi Tseng, Yi-Shu Huang, Hsi-Hsien Lin, Sheu-Fen Luo, Fiona McCann, Kay McNamee, Felix Clanchy , Richard Williams. TNFR signalling and its clinical implications, *Cytokine*. Oct 2016, 101, 19-25.

Richard Williams, **Wen-Yi Tseng,** Arming the regulators — new strategies to treat autoimmunity, Nature Reviews Rheumatology volume 14, pages 188–189 (2018)

Abstract

Wen-Yi Tseng, Kay McNamee, Adam Cribbs, Anviti Vyas, Richard O. Williams. TNF-Alpha Receptor II Signaling Plays an Important Role in Maintaining the Expression of Forkhead Box P3 in Murine Regulatory T Cells. American College of Rheumatology Annual Meeting 2015. San Francisco, USA.

Wen-Yi Tseng, I-Shu Huang, Felix Clanchy, Kay McNamee, D. Perocheau, P. Ericsson, H.-O. Sjogren, Z. Xue, L. Salford, A. Sundstedt, Richard O. Williams. DNA Methylation Inhibitors Produce Sustained Remission of Arthritis in Mice and Promote Regulatory T Cell Responses. Annual European Congress of Rheumatology 2017. Madrid, Spain.

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