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The roles of STRA6, EFNB1/B2 and ARMC5 in T cell function and autoimmune diseases

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Résumé

Les récepteurs tyrosine kinases sont un groupe de molécules clés de signalisation, qui ont 2 fonctions: la détection des stimuli de l'environnement extérieur des cellules et la transmission de ces signaux à l'intérieur des cellules. Dans les 20 dernières années, notre laboratoire a choisi d'étudier la fonction d'Ephb6 kinase, un récepteur tyrosine kinase fortement exprimé dans les lymphocytes T.Comme Efnb1 et Efnb2 sont tous des ligands pour Ephb6, nous avons ensuite procédé à étudier leur rôle dans la fonction des cellules T *in vitro* et *in vivo*. Des cellules T spécifiques mutants (KO) dans les gènes *Efnb1* ou *Efnb2* ainsi que les doubles mutants *Efnb1/b2* (double KO) ont été générés, mais il n'y avait que les souris double KO qui ont démontré de la déficience dans le développement des thymocytes, fonction de Th1 et Th17, la signalisation du récepteur d'IL-6, et les réponses antivirales.

Des preuves solides indiquent que la reconnaissance d'auto-antigène par les cellules T est un événement précoce dans la pathogenèse de la PR. Donc, nous avons postulé que les cellules T spécifique *Efnb1 / b2* double KO chez la souris peuvent protéger les souris de l'arthrite induite par collagène (CIA), un modèle de souris de la PR humaine. Nous avons trouvé que Efnb1et Efnb2 dans les cellules T étaient essentielles pour la production d'anticorps pathogéniques et de la migration des lymphocytes T vers les pattes enflammées chez les souris CIA. Notre étude clinique suggère que l'expression de EFNB1 dans les cellules T pourrait être un paramètre utile pour surveiller l'activité de la maladie de RA et la réponse de traitement.

Pour élucider les événements dans le programme d'activation des lymphocytes T, nous avons exploré par l'analyse des micropuces d'ADN pour identifier des molécules qui ont été exprimées de manière différente dans le WT par rapport aux cellules T Ephb6 KO dans le stade précoce de l'activation des cellules T. Environ 30 molécules étaient sur ou sous exprimées plus de 3 fois dans les cellules T WT par rapport aux cellules T KO pendant les 16 premières heures après stimulation par l'anti-CD3. Stra6 (stimulée par le gène de l'acide rétinoïque 6) et Armc5 (Armadillo répéter contenant 5) ont été parmi ceux qui ont été validées pour leur expression altérée.

STRA6 est un récepteur de haute affinité pour le plasma rétinol-binding protéine (RBP) et un médiateur pour absorption cellulaire de vitamine A. Cellules T KO et WT étaient similaires en

termes de prolifération et les réponses immunitaires anti-virales de virus de la chorioméningite lymphocytaire (LCMV). Ainsi, la sur-régulation de Stra6 est soit un événement parallèle qui ne soit pas essentiel pour le programme d'activation des lymphocytes T, ou il est très essentiel que la redondance existe, et sa suppression ne montre aucun effet apparent sur l'activation des cellules T.

ARMC5 est une protéine intracellulaire contenant sept répétitions en tandem d'armadillo et un domaine BTB. Les fonctions du ARMC5 dans le système immunitaire ne sont pas encore connues. Nos résultats d'hybridation *in situ* ont montré une expression élevée de Armc5 dans le thymus, et une expression modérée dans les ganglions lymphatiques et la rate. Nous avons généré des souris KO *Armc5*. Fait interessant, les cellules T Armc5 KO présentaient de la prolifération diminuée et de la différenciation compromise vers Th1 et Th17 *in vitro*. Les souris KO étaient résistantes à l'induction expérimentale d'encéphalite auto-immune, et ont été compromises dans les réponses immunitaires anti-LCMV. En utilisant de la levure 2-hybride test, nous avons identifié 8 protéines ARMC5-associantes, qui sont connues pour les rôles dans l'activation de la cellule, le cycle cellulaire et l'apoptose. Une étude mécanique est en cours. Nos résultats montrent que Armc5 est essentiel dans la programme d'activation/de prolifération/de différenciation des lymphocytes T.

Nos études ont augmenté nos connaissances sur EFNB1, EFNB2, STRA6 et ARMC5 en biologie des lymphocytes T et leur pertinence à des troubles immunitaires dans des modèles animaux ainsi que chez l'être humaine.

Mots-clés : cellules T, EFNB1, EFNB2, STRA6, ARMC5, la polyarthrite rhumatoïde, la sclérose en plaques

Abstract

Receptor tyrosine kinases are a group of key signaling molecules, which have dual functions: sensing the environmental stimuli outside the cells and transmitting them into the cells. 20 years ago, our laboratory started to study the function of Ephb6 kinase, a receptor tyrosine kinase highly expressed in T lymphocytes. As both Efnb1 and Efnb2 are the ligands for Ephb6, we then proceeded to study their roles in T cell function *in vitro* and *in vivo*. T cell-specific *Efnb1*, *Efnb2* single gene knockout (KO), as well as *Efnb1/b2* double KO mice were generated, but only the double KO mice showed compromised thymocyte development, Th1 and Th17 function, IL-6 receptor signaling, and anti-virus responses.

Strong evidence indicates that T cells play a crucial role in the pathogenesis of rheumatoid arthritis (RA). Thus, we postulated that T cell-specific *Efnb1/b2* double KO in mice may protect mice from collagen-induced arthritis (CIA), a mouse model for human RA. We found that Efnb1 and Efnb2 in T cells were essential for pathogenic antibody production and T cell migration to the inflamed paws in mice with CIA. Our clinical study suggests that the expression of *EFNB1* in T cells might be a useful parameter for monitoring RA disease activity and treatment responses.

Naïve T cells have the ability to expansion and differentiation into effector cells once they encounter foreign antigens, during which a large number of molecules are modulated. Some of these molecules play essential regulatory roles, while others exert house keeping functions and/or act as supporters to cope with increased or changed metabolic demands. To fully elucidate events in the T cell activation program, we undertook unbiased exploration with DNA microarray analysis to identify molecules that were differentially expressed in WT versus Ephb6 KO T cells in the early T-cell activation stage. About 30 molecules were up- or down-regulated more than three folds in WT T cells compared with KO T cells. *Stra6* (stimulated by retinoic acid gene 6) and *Armc5* (Armadillo repeat-containing 5) were among those that had been validated for their altered expression. We generated mice with these two genes deleted to study their roles in T cell function *in vitro* and *in vivo*.

STRA6 is a high-affinity receptor for plasma retinol-binding protein (RBP) and mediates cellular vitamin A uptake. *Stra6* KO mice manifest normal spleen and thymus in size,

cellularity and lymphocyte subpopulations. KO and WT T cells were similar regarding proliferation, differentiation and anti-viral immune responses to lymphocytic choriomeningitis virus (LCMV). Thus, the up-regulation of *Stra6* is either a parallel event which is not essential for the T cell activation program or it is so critical that heavy redundancy exists.

ARMC5 is an intracellular protein containing seven tandem armadillo repeats and one BTB domain. Functions of ARMC5 in the immune system are not known previously. Our *in situ* hybridization results showed high expression of Armc5 in the thymus and moderate expression in the spleen and lymph nodes. A transient increase of *Armc5* expression in T cells after TCR activation was found. To investigate its roles in T cell function, *Armc5* KO mice were generated. The KO mice weighed 40% less than their WT counterparts. Lymphoid organs (the thymus, spleen and lymph nodes) of the KO mice appeared to be of normal size, weight, cellularity, and lymphocyte subpopulations. Intriguingly, *Armc5* KO T cells presented decreased proliferation and compromised differentiation towards Th1 and Th17 *in vitro*. The KO mice were resistant to experimental autoimmune encephalitis induction and were compromised in anti-LCMV immune responses. Using yeast 2-hybrid assay, we have identified 8 ARMC5-assciating proteins, which have known functions in cell cycling and apoptosis. Further mechanistic study is underway. Our results reveal that Armc5 is vital in the T cell activation/proliferation /differentiation program.

Our studies have augmented our knowledge about EFNB1, EFNB2, STRA6 and ARMC5 in T cell biology and their relevance to immune disorders in animal models as well as in humans.

Keywords: T cells, STRA6, EFNB1, EFNB2, ARMC5, rheumatoid arthritis, multiple sclerosis

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Statement of authorship

Here is a statement of authorship regarding all the coauthors and myself to the papers included in this thesis:

Chapter 2

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Conceived and designed the experiments: YH XW YW XF JM GS JW HL. Performed the experiments: YH XW YW WJ BC JP SQ. Analyzed the data: YH XW MK JP SQ GS JW HL. Wrote the paper: YH XW YW WJ BC XF JM MK JP SQ GS JW HL. For each particular figure in this chapter, the authors who performed experiments are listed below: Figure 2.1: *YH XW SQ;* Figure2.2: *YH XW;* Figure2.3: *YH XW;* Figure2.4: *YH JP;* Figure2.5: *YH WJ;* Figure2.6: *YW BC.*

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Terra R, Wang Xh, **Hu Y(Co-first)**, Charpentier T, Lamarre A, Zhong M, Sun H, Mao J, Qi S, Luo H, Wu J. To investigate the necessity of Stra6 upregulation in T cells during T cell immune responses. **PLoS One.** 2013 Dec 31;8(12):e82808.

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Appendix:

Wang X, **Hu Y**, Charpentier T, Lamarre A, Qi S, Wu J, Luo H. TNF-like ligand 1A (TL1A) gene knockout leads to ameliorated collagen-induced arthritis in mice: implication of TL1A in humoral immune responses. **The Journal of Immunology.** 2013 Dec 1;191(11):5420-9. *Conceived and designed the experiments: XW HL JW. Performed the experiments: XW YH TC SQ. Analyzed the data: XW YH AL HL JW. Wrote the paper: XW JW HL.* For each particular figure in this chapter, the authors who performed experiments are listed below: Figure1: *XW;* Figure2: *XW TC;* Figure3: *XW YH SQ;* Figure4: *XW YH;* Figure5: *XW YH;* Figure6: *XW*.

Liste des sigles

aa: Amino acid

ACPAs: Anti-citrullinated protein antibodies

ACR: American College of Rheumatology

ADH: Alcohol dehydrogenase

AF6: ALL1-fused gene from chromosome 6

AITD: Autoimmune thyroid disease

APCs: Antigen-presenting cells

APC: Adenomatous polyposis coli

ARMC5: Armadillo repeat containing 5

AS: Ankylosing spondylitis

BCR: B cell receptor

Breg: Regulatory B cell

CAIA: Collagen-antibody-induced arthritis

CBP: CREB-binding protein

CD: Crohn's disease

CIA: Collagen-induced athritis

CIS: Clinically isolated syndrome

CK1α: Casein kinase 1α

CNV: Copy number variations

CRBP1: Cellular retinol binding protein-1

CRD: Cysteine-rich region

CRP: C-reactive protein

CSF: Cerebrospinal fluid

CTD: Carboxyl-terminal domain

CTLA-4: Cytotoxic T lymphocyte associated antigen-4

DH-PH: Dbl-homology-pleckstrin-homology

DMARDs: Disease-modifying anti-rheumatic drugs

EBV: Epstein-Barr virus

EFN: ephrin

EGF: Epidermal growth factor

Eph: Erythropoietin-producing hepatocyte kinase

ESR: Erythrocytes sedimentation rate

EULAR: European league against rheumatism

FAK: Focal adhesion kinase

FGF: Fibroblast growth factor

FNIII: Fibronectin type III

FoxP3: Forkhead box P3

GC: Germinal center

G-CSF: Granulocyte colony stimulating factor

GD: Graves' disease

GEF: Guaninenucleotide exchange factor

GITR: Glucocorticoidinduced tumor necrosis factor receptor

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GRIP: Glutamate receptor interacting protein

GSK3β: Glycogen synthase kinase 3β

GWAS: Genome-wide association scans

HGF/SF: Hepatocyte growth factor/scatter factor

HHV-6: Human herpesvirus 6

HLA: Human leukocytes antigen

HSC: Hepatic stellate cell

HSV-1: Human herpesvirus 1

ICOS: Inducible T cell co-stimulator

JAK/STAT: Janus kinase/Signal transducer and activator of transcription

KO: Knockout

LAP: Latency-associated peptide

LBD: Ligand binding globular domain

LEF: Lymphoid enhancer-binding factor

LRAT: Lechithin retinol acyl transferase

MAPK: Mitogen-activated protein kinase

MHC: Major histocompatibility complex

MMP: Matrix metalloproteinase

MRI: Magnetic resonance imaging

MS: Multiple sclerosis

MTX: Methotrexate

MWS: Mathew Wood syndrome

NTD: Amino-terminal domain

OCB: Oligoclonal IgG band

PD1: Programmed cell death 1

PI3K: Phosphatidylinositol 3'-kinase

PICK: Protein interacting with C kinase

PMAH: Primary macronodular adrenal hyperplasia

PPMS: Primary-progressive MS

PSA: Psoriatic arthritis

PTB: Phosphotyrosine binding

PTP-BL: Rrotein tyrosine phosphatase BAS-like

RA: Rheumatoid arthritis

RALDH: Retinal dehydrogenase

RANK: Receptor activator of nuclear factor Kb

RANKL: RANK ligand

RAR: Retinol acid receptor

RBD: Receptor-binding domain

RF: Rheumatoid factor

RRMS: Relapsing-remitting MS

RTK: Receptor tyrosine kinases

RXR: Retinoid X receptors

SCID: Severe combined immunodeficiency

SF: Synovial fluid

SH2: Src homology 2

SLE: Systemic lupus erythematosus

SM: Synovial membrane

STRA6: Stimulated by retinoic acid 6

T1D: Type 1 diabetes

T-bet: T-box transcription factor

TCF: T cell-specific factor

TEC: Thymic epithelial cell

Tfh: T follicular helper

TM: Transmembrane

TMEV-IDD: Theiler's murine encephalomyelitis virus-induced demyelinating disease

TPC: Tumor-propagating cell

Treg: Regulatory T cell

VEGF: Vascular endothelial growth factor

WT: Wild type

βTrCP: β-transducin repeat containing protein

Liste des abréviations

ARM: Armadillo

PSO: Psoriasis

Chapter 1 Introduction

Autoimmune diseases are conditions with an inappropriate activation of the immune responses, which can cause diseases such as rheumatoid arthritis (RA) and multiple sclerosis (MS) (Davidson & Diamond, 2001). There are about 80 types of autoimmune diseases. According to Cooper *et al.*, more than 3% of the US population suffers from one or more of these diseases. The incidence of diseases are higher in women than in men (Cooper & Stroehla, 2003). It is a chronic disease which makes it a contributor to decreased life quality for individuals and also increase the costs of the whole health care system.

1.1 Risk factors for autoimmune diseases

Earlier twin studies and familial studies proved that genetic factors are essential for the development of autoimmune diseases. More recent studies show that non-genetic factors, such as hormones, infections, and environmental factors, interact with the existing genetic factors to trigger a particular kind of autoimmune disease in individuals bearing certain genetic variations.

1.1.1 A variety of genetic factors related to distinct signaling pathways are involved in the initiation of autoimmune diseases

In the past decade, the extraordinary technical advances in this area have lead to the discovery of new factors that may contribute to human autoimmune diseases. For example, genome-wide association scans (GWAS) is a major approach to detect the relatively common (>1% or more) genetic variants, which is the dominant form of genetic variation for autoimmune diseases. These genetic changes also present in healthy populations. What's more, the genetic associations found by GWAS turned out to be modest. The predisposition of autoimmune diseases represents the total effects of the genetic variations in different genes which influence the position and the strength of genetic signals and functions (Encinas & Kuchroo, 2000). Moreover, autoimmune diseases share a common set of susceptibility genes. For example, a variation within the human leukocytes antigen (HLA) is associated with almost every autoimmune disease. Copy number variations (CNV) and rare genetic variants are also present in the population. CNVs in genes like *CCL3L1*, *FCGR3B*, *DEFB4, etc.* have been linked to

many types of autoimmune diseases. A rare variant in *TREX1*, which is a DNA exonuclease, is found to be associated with sporadic lupus. Current published data suggest a variety of genetic heterogeneities related to distinct signaling pathways are involved in the initiation of autoimmuny

1.1.2 Infectious agents, sex hormones, smoking, and ultraviolet radiation play a role to initiate autoimmune diseases

Environmental factors are also critical in the initiation of autoimmune diseases (Fagnani et al., 2015). Components of pathogens bear the potential to evoke autoreactivity through molecular mimicry (Cusick, Libbey, & Fujinami, 2012), which is due to the antigens from some microbes that share epitopes with endogenous antigens. For instance, peptides from Epstein-Barr virus can be presented to $CD4^+$ T cells, which can also react with myelin basic protein (Wucherpfennig & Strominger, 1995). Moreover, infectious agents will lead to inflammation and lymphocytes activation, both of which lower the threshold of autoimmunity.

Women, especially women in their fertile period are most often affected, indicating an essential part of sex hormones in disease onset and perpetuation (Cutolo et al., 2006). Estrogen exerts dose- and time-dependent effects on cell growth and apoptosis for B cells and synovial fibroblasts in RA (Kawasaki, Ushiyama, Inoue, & Hukuda, 2000). It also has influences on Th1/Th2 cytokines in RA (Janele et al., 2006).

Smoking is related to SLE, RA, and myositis (Wahren-Herlenius & Dorner, 2013). It triggers inflammatory responses and sustains autoimmunity by the toll-like receptor pathways, autophagy, and apoptosis in the lung through reactive oxygen species.

Ultraviolet radiation can trigger systemic autoimmunity via immune pathways or by induction of apoptosis in diseases like SLE and dermatomyositis. Self-antigen-exposure during apoptosis prolongs the contact with autoantigen-specific B cells, which will lead to autoimmunity in individuals with genetic defects (Wahren-Herlenius & Dorner, 2013).

1.2 Mechanism of autoimmune diseases

Our immune system is designed to have a low level of autoreactivity. Exaggerated immune responses result in tissue damages and autoimmune diseases. For decades, studies from animal

models and humans have led to a hint that immune cells and the molecules they produce vary in different stages of disease pathogenesis.

1.2.1 Rheumatoid arthritis

RA is a chronic autoimmune disease that involves the activation of immune response both systemically and locally. Joints are the dominant organ that are targeted in the inflammatory responses.

1.2.1.1 ‘‘RA is the most common inflammatory joint disease’’

‘‘RA is the most common inflammatory joint disease’’ (Cooper & Stroehla, 2003). The annual incidence of RA has been estimated to be around 40 per 100, 000 (Cooper & Stroehla, 2003). The disease prevalence is about 0.9 percent of the Canadian population (Canada, 2011). However, the prevalence varies between 0.1 percent (in rural Africans) and 5 percent (some populations in India, Australia, New Zealand and Netherlands), indicating the initiation of RA can be influenced by ethnic and/or environmental elements (Helmick et al., 2008; Peschken & Esdaile, 1999). While affects all age groups, more than one-half of new RA cases occur between ages of 40 and 75 years (Cooper & Stroehla, 2003). Females have two to three times’ higher prevalence rate than that of males. A decreasing trend has been observed in the US (Doran, Pond, Crowson, O'Fallon, & Gabriel, 2002), as well as in high-risk populations in Indians (Jacobsson et al., 1994). RA causes economic burden due to disability in the workforce and the cost of treatment, especially the cost of biologic agents in recent years (Michaud, Messer, Choi, & Wolfe, 2003). RA increases the mortality rate in patients, and this death rate has not changed since 1990. Whether this is due to an inflammatory pathology, or due to the exposure to anti-rheumatic drugs, or due to both, is unclear (Wolfe et al., 1994).

1.2.1.2 Clinical manifestation of RA

1.2.1.2.1 ‘‘The 2010 ACR/EULR classification criteria for RA’’

RA is characterized by symmetrical synovitis involving multiple diarthrodial joints with extra-articular manifestations. In 2010, the criteria for the classification of ‘‘RA were revised by the American College of Rheumatology (ACR)/European League Against Rheumatism (EULAR) ’’ (Aletaha et al., 2010). The 2010 criteria help to identify patients earlier and also helps to

identify those who may respond to ‘‘disease-modifying anti-rheumatic drugs (DMARDs)’’ (Aletaha et al., 2010).

1.2.1.2.2 Chronic pain and swelling leading to bone damage in RA joints

The disease onset of RA is usually insidious, with the observation of tenderness and subtle swelling of one or more joints in hands and wrists. Some patients with RA have a slowly progressive course with exacerbations, which usually takes over weeks to months. However, some patients have a waxing and waning course known as “palindromic rheumatism”, which may take several years before the chronic, persistent features of RA become evident. Typically, RA involves the ‘‘metacarpophalangeal joints and the proximal interphalangeal joints of the fingers, the interphalangeal joints of the thumbs, the metatarsophalangeal joints of the toes, the wrists, elbows, shoulders, hips, knees, and ankles’’ (Aletaha et al., 2010). RA patients commonly have stiffness, which usually presents in the morning (morning stiffness) or after long periods of inactivity.

Seventy percent of patients with RA display radiographic evidence featured by ‘‘joint space narrowing and bone erosions’’ in small joints of hands and feet within few years after diagnosis. However, about 25% of patients with early RA may not develop erosive damage even after eight years of follow-up. The development of magnetic resonance imaging (MRI) is particularly useful for the measurement of the inflammatory process in the synovial membrane (McQueen, 2000). The use of ultrasonography enables the visualization of bone erosions, joints fluids, and synovial hypertrophy (Terslev et al., 2003).

1.2.1.2.3 Systemic symptoms, elevated autoantibodies and acute phase proteins in RA patients

Systemic symptoms, including fatigue, emaciation, low-grade fever, weakness, and depression often accompany the early signs of joint inflammation. Anemia of chronic inflammation, thrombocytosis, and sometimes mild leukocytosis are common hematologic abnormalities in active RA. The majority of RA patients have the positive test for rheumatoid factor (RF), anti-citrullinated protein antibodies (ACPA), or both. Some of them are antinuclear antibodies positive. The acute phase proteins, including the erythrocytes sedimentation rate (ESR) and C-reactive protein (CRP), are often elevated in patients with active RA. Significant correlations

are frequently observed between the degrees of elevation of these proteins and RA activity (Cylwik, Chrostek, Gindzienska-Sieskiewicz, Sierakowski, & Szmitkowski, 2010).

1.2.1.2.4 Subcutaneous nodule formation, pleuropulmonary disease, and cardiovascular disorders are also found in RA patients

Other systemic manifestations, including subcutaneous nodule formation, pleuropulmonary disease, and cardiovascular disorders are also found in RA patients. Rheumatoid nodules are the most frequently observed manifestation. Typically, subcutaneous nodules are hard and non-tender. These nodules vary in sizes, being associated with the present of serum rheumatoid factors, erosive joint destruction, necrotizing vasculitis and other ‘‘extra-articular manifestations of RA’’ (Vlak, 2003). The pleuropulmonary disease is a primary reason for the morbidity and mortality of RA. Interstitial lung disease, pleural thickening, and effusions are the most common pleuropulmonary manifestation of rheumatoid arthritis lung disease (Shaw, Collins, Ho, & Raghu, 2015). RA patients are more inclined to have myocardial infarction and stroke (Dhawan & Quyyumi, 2008). Rare manifestations, such as Felty’s syndrome which features with neutropenia and splenomegaly, were also reported. RA patients have a similar incidence of cancer than their healthy controls in general. However, RA patients have a higher incidence of lymphomas and lung cancer. Until now, how rheumatoid arthritis causes systemic illness remains obscure.

1.2.1.3 Animal models of RA

There are two major reasons for the extensive use of animal models in RA. Firstly, the mechanisms of RA, which can trigger articular disease onset and define chronicity of the local and systemic inflammation, remain obscure. Secondly, the rarely obtainable human tissues, such as synovial tissues, lymph node, bone marrow and spleen, hinds the research of finding out contributors to immune responses in human. Currently, numerous arthritis models including the induced- and spontaneous- models are developed (Asquith, Miller, McInnes, & Liew, 2009). Even though none of them can entirely mimic human RA, studies based on animal models have been proved to be helpful in understanding the potential mechanism and treatment of RA.

1.2.1.3.1 CIA is ‘‘the gold standard animal model of human RA’’, while CAIA proves a role of humoral immunity in RA

CIA is considered as ‘‘the gold standard animal model of human RA” (Brand, Latham, & Rosloniec, 2007). DBA/1 mice are the most popular strain for CIA model in research. These mice are susceptible to CIA because of their MHC class II haplotype (I-A^q). CIA bears similarity to human RA regarding the breach of tolerance, the role of lymphocytes in the initiation and persistence of the disease, and the histological findings in the arthritis joints. Studies reveal that mouse MHC A^q peptide binding part are conformationally similar to that of human HLA DR4 (DRB1*0401/DRA), which is the human MHC highly associated with RA (Holmdahl, Bockermann, Backlund, & Yamada, 2002). Also, the majority of collagen II-specific T cells in both human and mouse recognizes a peptide from aa256-270 of the collagen II peptide. Moreover, CIA mouse shares local features with human RA regarding the infiltration of immune cells in the rheumatoid synovium, the synovial lining cell hyperplasia, and the secretion of inflammation mediators, which resulting in local joint damage. The detailed mechanism of lymphocytes in the development of CIA will be discussed in detail in the “Pathogenesis of RA” part.

The biggest problem of the CIA model in DBA/1 background is its limited usefulness in genetically modified mice. This is due since most genetically modified mice are on the C57BL/6 background, which is not a CIA-susceptible strain. Studies showed that the use chicken collagen II and a boost with IFA (Incomplete Freund’s Adjuvant) overcome the resistant issue of C57BL/6 mice (Inglis, Simelyte, McCann, Criado, & Williams, 2008). After the refinement of the protocol, C57BL/6 mice share the basic pathological features with DBA/1 mice. However, they develop less severe CIA with later onset, lower incidence, and lower clinical scores. Also, intergroup inconsistency is observed in C57BL/6 mice, underscoring that precise, independent internal controls are necessary.

The injection of antibodies against type II collagen leads to collagen-antibody-induced arthritis (CAIA) (Khachigian, 2006). The antibodies trigger similar clinic development of arthritis as that of CIA. CAIA model itself proves the importance of auto-antibodies in the development of joint damage. Other antigens are also reported to induce arthritis (Asquith et al., 2009; Maffia et al., 2004). Arthritis can also be caused by other factors related to the T

cell-dependent signalling pathway as that of CIA; others may trigger cell signaling pathways in other immune cells. For example, zymosan, a component of *Saccharomyces cerevisiae* can trigger TLR2 signaling in macrophages (Frasnelli, Tarussio, Chobaz-Peclat, Busso, & So, 2005).

1.2.1.3.2 Spontaneous arthritis models, including human "TNF-α transgenic mice, K/BxN mice, and SKG mice", have similarities with human RA

The spontaneous arthritis models are characterized by the development of chronic and progressive polyarthritis, which is similar to the human disease. These include "human TNF-α transgenic mice" (Li & Schwarz, 2003), "K/BxN mice" (Monach, Mathis, & Benoist, 2008) and "SKG mice" (Hata et al., 2004). In the human TNF-α transgenic arthritis model, treatment with anti-human TNF-α monoclonal antibody completely rescued mice from the disease. This model is particularly useful in exploring novel treatment targeting downstream signaling molecules of the TNF-α pathway. The K/BxN mice also develop spontaneous arthritis. The K/BxN mice express the TCR transgene "KRN, the MHC class II molecule A^{g7}", as well as autoantibodies against "glucose-6-phosphate isomerase" in sera (Monach, Mathis, & Benoist, 2008). This model provides a useful tool for investigating the innate immune systems in the initiation of arthritis. SKG model is caused by a point mutation of ZAP-70, which depends on environmental factors to induce inflammatory arthritis. Severe combined immunodeficiency (SCID) mice have also been used to exploit the underlying mechanism of RA by implanting them with human synovial tissues (Pierer et al., 2003). This model reveals the contribution of a local factor in cartilage destruction and bone erosion. It is also useful in identifying novel targets for the prevention of local damage in RA.

1.2.1.4 Pathogenesis of RA

1.2.1.4.1 Macrophages orchestrate in RA inflammatory responses

Macrophages are a subpopulation of white blood cells, which function in infectious microbe clearance, normal tissue remodeling, wound repair and angiogenesis. The classically activated M1 and the alternatively activated M2 are activated macrophages with distinct functions (Sica & Mantovani, 2012). In normal synovial membrane, macrophages (type A synovial cells) are

not the predominant cell type. However, in rheumatoid condition, the macrophage number significantly increases (Maruotti, Cantatore, Crivellato, Vacca, & Ribatti, 2007).

Macrophages play important roles in RA by phagocytosis. They differentiate into M1 macrophages in response to LPS and secrete proinflammatory cytokines like IFN-γ and TNF-α. These proinflammatory cytokines act as autocrine stimulator, as well as potent paracrine inducer of other inflammatory cytokines. For example, TNF-α can induce ‘‘IL-1, 6, 8 and granulocyte-macrophage-colony-stimulating factor (GM-CSF) in RA joints’’ (Butler, Maini, Feldmann, & Brennan, 1995; Haworth et al., 1991). It also induces matrix metalloproteinases (MMPs) in the synovium. MMPs are responsible for cartilage matrix destruction. Moreover, in the synovium, TNF-α induces NO production, which could promote synovial cells to produce TNF-α (Chae et al., 1997; McInnes et al., 1996).

Conversely, the activation of macrophages with IL-4 or IL-13 promotes the M2 phenotype, which suppressing destructive immunity, while promoting angiogenesis and matrix remodeling. Macrophages in rheumatoid arthritis synovium contribute to pathological angiogenesis by producing vascular endothelial growth factor (VEGF). Deletion of certain domain of VEGF receptor-1 in mice protects mice from a murine model of RA. This protection is due to the decrease of the phagocytosis and the secretion of IL-6 and VEGF-A (Murakami et al., 2006). Macrophages can also promote pathological angiogenesis through the release angiogenic chemokines and cytokines. The former includes CXCL5 (Koch et al., 1994), while the latter includes ‘‘IL-8, IL-15, IL-17, *et al.*’’ (Koch et al., 1991; Pickens et al., 2010). In contrast, antiangiogenic factors such as thrombospondin 2, are also produced (Park et al., 2004).

1.2.1.4.2 ‘‘B cells play a role in RA pathogenesis through autoantibody production’’, antigen presentation and costimulation, neogenesis of lymphoid microstructures and cytokine secretion

The discovery of autoantibodies in the 1950s’ (Holman & Deicher, 1959; Rose, Ragan, & et al., 1948) had brought the humoral autoimmunity into the center stage, which was followed by the identification of numerous autoantibodies as the diagnostic markers of autoimmunity. However, as plasmapheresis showed disappointing results, treatment strategies no longer

focused on this area. It was the unexpected success of anti-CD20 antibody treatment to deplete B cells that brought them back to the central of the stage in autoimmunity.

Autoreactive B cells

Numerous evidence showed that subtle alterations in B cell receptor (BCR) signaling pathways triggered by autoantigens can be the predisposing causes for autoantibody production in both mice and human beings (LeBien & Tedder, 2008). Also, any aberrant in B-cell selection centrally in the bone marrow or peripherally in the lymphoid tissues during B cell development and/or disruption of the normal BCR somatic hypermutation during affinity maturation within the germinal center can lead to autoimmunity. An early break in B cell tolerance is proposed as different studies proved the presence of autoantibodies in the patients' serum many years before the presence of any clinical symptoms. However, the exact role of autoantibodies in RA remains elusive.

Diverse functions of B cells in autoimmune diseases

1. Autoantibody production

Autoantibody production is a characteristic feature in most autoimmune diseases. Some autobodies have remarkable specificity and can be used as biomarkers for disease diagnosis and indicators of therapeutic interventions. Autoantibodies are usually high-affinity, somatically mutated IgG, which might lead to pathogenic consequences. Autoantibodies exert their function through direct binding or forming immune complexes. The former usually happens in organ-specific autoimmune diseases, like myasthenia gravis, where they directly damage the target organ. The latter often occurs in systemic autoimmune diseases, where tissue injury is caused by immune complex composed of autoantibodies conjugated with free molecules, cell surface antigens, and nucleoprotein antigens.

Autoantibodies may also cause damage through the engagement of complement and/or Fc receptor (FcR) effector pathways. In the K/BxN mouse model, the administration of autoantibodies activates the alternative complement pathway. This activation attracts neutrophils, leading to the accumulation of proteolytic enzymes and cytokines in the inflammatory joints (Monach, Benoist, & Mathis, 2004; Wipke, Wang, Nagengast, Reichert, & Allen, 2004).

It is still elusive to define the role of autoantibodies in the initiation of autoimmune diseases like RA. Autoantibodies alone are unable to initiate autoimmune diseases in multiple mice

models. Some of them are even proven to have protective functions (Shoenfeld & Toubi, 2005). What's more, autoantibodies themselves are often not pathological. The co-existing secondary triggers of autoantibodies, such as infectious agents, will lead to a vicious chronic state of inflammation (McClain et al., 2005).

2. Antigen presentation and costimulation

Besides antibody production, B cells can also promote disease development by presenting antigens to T cells. B cells are required to start autoimmune diseases in mice model for arthritis, lupus, diabetes, *etc.* (Chan, Hannum, Haberman, Madaio, & Shlomchik, 1999; O'Neill et al., 2005; Wong et al., 2004). However, their antibody-secreting capabilities are not required that early. B cells capture antigen through their B-cell receptors, which is over 1,000-fold more efficient than other APCs. Therefore, B cells can present antigens at extraordinarily low concentrations and also focus the immune response to a specific antigen in certain autoimmune diseases (Rivera, Chen, Ron, Dougherty, & Ron, 2001). What's more, B cell may have specific roles in providing costimulatory signals to antigen-specific T cells. In NZB/NZW mice, a spontaneous lupus model, elevated expression of costimulatory molecules are found in B cells from spleen (Wither, Roy, & Brennan, 2000).

3. Neogenesis of lymphoid microstructures

Neogenesis of lymphoid microstructures is found ectopically in multiple chronic inflammations. The size and organization of these neogenesis lymphoid structures vary depending on the context. These structures have been described in SLE, T1D, Sjögren's syndrome, MS and RA (Aloisi & Pujol-Borrell, 2006; Drayton, Liao, Mounzer, & Ruddle, 2006). The exact function of these structures remains unknown. However, the locally generated plasma cells are found to secrete autoantibodies (Salomonsson et al., 2003), suggesting their potential role in the maintenance of autoimmunity.

4. Cytokine secretion

Activated B cells ''produce multiple cytokines, including IL-6, IFN-γ, lymphotoxin-α, TGF-β, IL-4, and IL-10'' (Duddy, Alter, & Bar-Or, 2004; Harris et al., 2000; Lund, Garvy, Randall, & Harris, 2005). B cell-derived cytokines play pathologic roles in regulating functions of multiple cells, such as macrophages, dendritic cells and T cells. In addition, signals from these cells can activate B cells in turn, creating a feedback loop They also play protective roles in immune responses to autoantigens, as in the case of IL-10, which will be described below.

5. Regulatory B cells

Regulatory B cells (Bregs) (Mizoguchi & Bhan, 2006) are B cells that can produce anti-inflammatory cytokines like IL-10, TGF-β. Bregs can be found in a variety of B-cell subsets and participate in the pathogenesis of autoimmune diseases, including SLE (Lenert, Brummel, Field, & Ashman, 2005), RA (Mauri, Gray, Mushtaq, & Londei, 2003), and MS (Matsushita, Yanaba, Bouaziz, Fujimoto, & Tedder, 2008). The regulatory properties of B cells in autoimmune diseases were originally reported in B10.PL mice. These mice are lack of B cells and are unable to recover from EAE as compared to their healthy counterparts (Wolf, Dittel, Hardardottir, & Janeway, 1996).

Like it is in Tregs, the IL-10 secreted by B cells also (Fillatreau, Sweenie, McGeachy, Gray, & Anderton, 2002) suppresses Th1 polarization and inhibits macrophage functions. B cells can regulate the T cell co-stimulation pathways by direct cell-cell contacts with CD40 and B7.

Autoantibodies in RA

The autoantibodies found in RA patients target quite diverse molecules, including ‘‘cartilage components, chaperons, enzymes, nuclear proteins and citrullinated proteins’’ (Song & Kang, 2010). In recent years, new technologies, like protein arrays, have been used to detect RA-associated autoantibodies with better specificity and sensitivity (Hansson et al., 2012; W. H. Robinson, Steinman, & Utz, 2003). However, only two of them are used in clinical practice currently.

1. Rheumatoid factor

RFs are autoantibodies that are against the Fc region of IgG. The IgM isotype is the primary type that exists in the sera of RA patients (Nell et al., 2005). RFs can be detected physically in ordinary old people, but also in other diseases like Sjögren’s syndrome, SLE. When the titer goes higher than 50IU/ml (RF50), it is quite specific for RA (Song & Kang, 2010). High titer IgM RF status is associated with poor disease prognosis. Notably, the co-presence of high titer IgM RF and IgA isotype usually indicate erosive RA (Jonsson et al., 1998; Scott, 2000). Furthermore, higher RF does not occur in experimental models of RA. Up to now, the role of RF in RA is still not entirely clear.

2. Anti-citrullinated protein antibodies (ACPAs)

The identification of ACPAs is one of the most meaningful event in RA diagnostic marker studies. Comparing with RF, it has a better diagnostic value regarding specificity and

sensitivity. ACPAs are referred to antibodies against ‘‘perinuclear factor, keratin, filaggrin and cyclic citrullinated peptide (CCP)’’ (Song & Kang, 2010). Citrulline is the common epitope of these antibodies, which can be generated post-translationally from arginine (Schellekens, de Jong, van den Hoogen, van de Putte, & van Venrooij, 2015). The ACPAs can be found in 70-90% of RA patients, with a specificity of 96% (Suzuki et al., 2003). They are highly specific for RA. As IgM RF, they are indicators of worse outcome in RA (Im et al., 2009; Kroot et al., 2000). Of all the ACPAs, anti-CCP is the most wildly used one in clinical diagnosis.

1.2.1.4.3 T cells are critical for disease onset and to sustain of RA

Role of T cell activation and tolerance in RA

T cell activation is essential for the onset and maintenance of RA. To fully activate T cells, the signaling transduce through both TCR complex and the co-stimulators are needed. The former is identified as signal 1. It is stimulated when the major histocompatibility complex (MHC) with antigen peptide (pMHC) binds to TCR. The latter is called signal 2. It is stimulated when B7-1 and B7-2 on antigen presenting cells conjugate to CD28 on T cell surface (Figure 1.1). The engagement of signal 1 leads to TCR aggregation, which enables closer pMHC/TCR contact. TCR aggregation and activation of signal 2 will then initiate the phosphorylation and the activation of a variety of molecules, resulting in the signaling transduction through PI3K/AKT/mTOR pathway, MAPK/Erk pathway, PKCθ pathway, and Ca^{2+}-mediated signaling pathways (Smith-Garvin, Koretzky, & Jordan, 2009). These signaling pathways are regulated by a variety of molecules at multiple levels in response to particular environment stimulation. Abnormal T cell activation involves both breakdowns of T cell tolerance and aberrant T cell response, leading to the autoimmune disease state.

$CD4^+$ T helper cells control both the cell-mediated and humoral immune responses, which makes it the most important subpopulation of T cells in immune tolerance. T cell tolerance may occur during their maturation in the thymus (central tolerance) or occur in peripheral when they meet antigens (peripheral tolerance) (Xing & Hogquist, 2012). In central tolerance, the majority of immature T cells that recognize self-antigens with high avidity are deleted, while some of them differentiate into regulatory T cells (Tregs). In the past decades, the underlying mechanism of central tolerance has been well studied in animal models. However, it is still not known about how much it contributes to a human autoimmune disease. The

peripheral tolerance mechanism is another important aspect for T cell tolerance. Prolonged TCR activation may result in anergy. Defects and/or lack of the co-stimulation will contribute to peripheral tolerance. Moreover, the activation of death signal and the dysfunction of Tregs may also lead to the breakdown of peripheral tolerance.

Role of co-stimulation (signal 2) in T cell activation

As shown in Fig 1.1, signal 2 (co-stimulatory) is an essential part of T cell activation. The B7.1/B7.2 expressed on APCs conjugate both CD28 and (or) CTLA4 on T cells, depending on the cell type and context. The interaction of B7.1/B7.2 and CD28 promotes T cell activation, as well as T cell differentiation. In contrast, their interaction with CTLA-4 will lead to the suppression of T cell activation. The binding capacity of CTLA-4 with B7.1/B7.2 is much higher than that of CD28, making it efficient in regulating T cell activation signaling (Greene et al., 1996; Peach et al., 1994). CTLA-4-Ig (Abatacept) is approved by FDA for the treatment of RA (Kremer et al., 2005). It is a protein fuse with ‘‘the extracellular binding domain of CTLA-4 and the Fc region of IgG’’ (Kremer et al., 2005). Thus, abatacept is highly efficacious in treating RA, leading to well-improved physical function. It significantly reduces the progression of structural damage in patients with established RA (Provan et al., 2015; N. Takahashi et al., 2015). A combination of methotrexate (MTX) and abatacept can deliver significant clinical benefits and radiographic improvement (T. Hirota et al., 2015; Mochizuki et al., 2015). Abatacept is currently prescribed to patients with RA unresponsive to regular doses of existing DMARDs.

Later studies proved that many co-stimulation and co-inhibition molecules are involved in T cell regulation, such as ‘‘ICOS, PD1, OX40 (also known as CD134, TNFRSF4) *et al.*’’ (L. Chen & Flies, 2013).

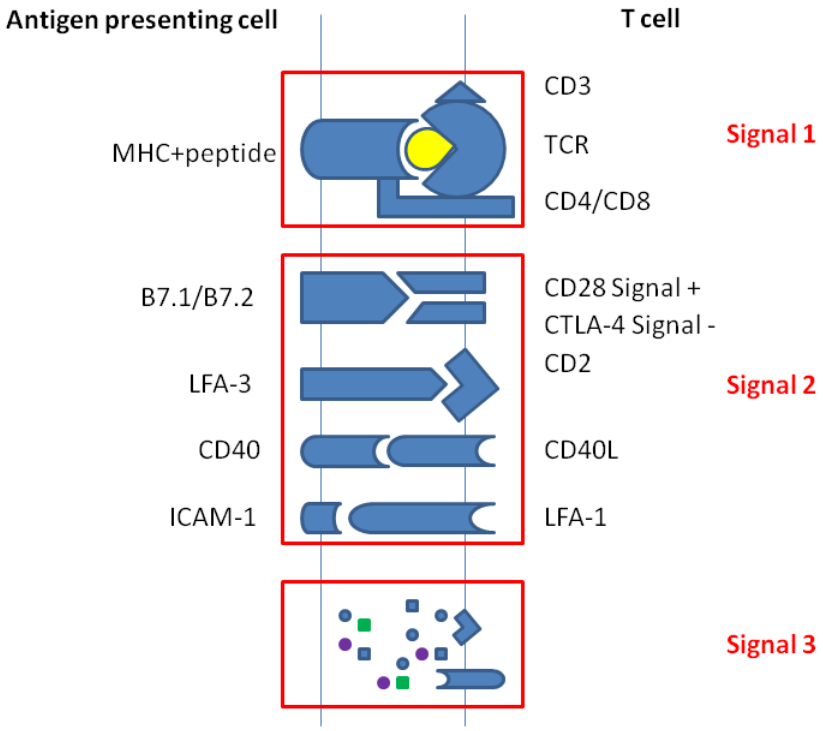

Figure 1.1 T cell activation

Role of $CD4^+$ *T cell differentiation in RA*

The etiology of autoimmune diseases has not been fully investigated up till now, however, accumulating evidence proves that $CD4^+$ T cells is essential for the development and maintain of both systemic and local inflammation. Studies show that about one-third of cells infiltrated into the inflammatory joint are T cells. Naive $CD4^+$ T cells will be driven into different subsets, named Th1, Th17, and Tregs, depending on the cytokine environments (signal 3) (Figure 1.1). Activated T cells can infiltrate into the inflammatory sites. These effector T cells will secrete molecules, such as chemokines and cytokines, which will further recruit other inflammatory cells and enhance local inflammation.

1. Th1 & Th17 cells are the principal players in the pathogenesis of RA

TCR activation and cytokine IL-12 will drive naive $CD4^+$ T cells into IFN- γ-secreting Th1 subpopulation. T-box transcription factor (T-bet) is the master regulator that promotes Th1 differentiation. T-bet can also suppress the development of opposing cell lineages like Th2 and Th17. Th1 subpopulation mainly functions as the pro-inflammatory factor, while Th2

basically works as the anti-inflammatory factor. Studies showed that early administration of a monoclonal antibody against IFN-γ could protect mice from CIA (Boissier et al., 1995). The frequency of Th1 cells with citrulline-specific epitopes are increased in rheumatoid arthritis, and this is influenced by therapy and disease activity (James et al., 2014). In the past, the balance of Th1/Th2 was once thought to be critical in the pathogenesis of autoimmune disease including RA. In 2005, the recognition of Th17 subpopulation refreshed this notion (described in detail in the MS part) (Cua et al., 2003).

Th17 is a subpopulation of T helper cells, which secrete IL-17A, IL-17F, and IL-22. RORγt is the master transcription factor of Treg. Cytokines like TGF-β and IL-6 drive naive $CD4^+$ T cells differentiation into Th17 subpopulation, while IL-23 promotes Th17 cells expansion. Fossiez and colleagues reported the first evidence of the involvement of Th17 in RA. They showed that IL-17 is capable of stimulating synovial fibroblast *in vitro* from RA patients. (Fossiez et al., 1996). Emerging reports coming from animal models show that less severe arthritis is found in genetically modified mice of Th17-related cytokines and transcription factors, including ‘‘IL-17 knockout mice, IL-23 p19 subunit knockout mice, and STAT4 knockout mice’’ (Langrish et al., 2005; Hildner et al., 2007).

Th17 cells are the primary T cell subpopulation in the pathogenesis of RA. The receptor of IL-17 is found to be expressed in cells from the immune system (monocytes and macrophages), as well as cells in the RA joint (fibroblasts and osteoblasts), which are essential for RA inflammation. First of all, Th17 cells exert their function through IL-17, which is able to generate other pro-inflammatory cytokines once the IL-17 receptors are activated. Secondly, these pro-inflammatory cytokines will further enhance the Th17 function in turn, which might trigger autoimmunity in genetic sensitive individuals. Thirdly, IL-17 can synergize with other proinflammation mediators, like TNF-α and IL-1β in RA. Particularly, IL-17 promotes germinal center (GC) formation in the K/BxN model (H. J. Wu et al., 2010). Recently, it was reported that Toll-like receptors expressed by synovial fibroblasts could induce IL-17 production (F. Hu et al., 2014).

Besides cytokines, Th17 also triggers chemokines production, which is critical for the recruitment of other inflammatory cells to the inflamed joint. This recruitment will lead to the local inflammatory cascade. Particularly, Th17 subset with the expression of $CCR6^+$ will be recruited to the inflammatory site in response to the CCL-20 produced by synovial fibroblasts.

What makes it more interesting is the treatment against CCR6 in Th17 has proved to be effective. More over, IL-17 can also upregulate enzymes, small molecules, and cell surface receptors in cells such as synovial fibroblasts, causing bone and cartilage damage (Nakashima et al., 2011).

2. Tregs play a regulatory role in RA

Tregs, either natural or inducible, are subsets of T cells which play regulatory roles in immune inflammatory processes. Nature Tregs (nTregs) express CD25 (IL-2R**α**) on the cell surface and FoxP3 in the cell nucleus. nTregs are developed in the thymus during the positive selection stage with a higher than the normal affinity for self-antigens. Inducible Tregs (iTregs) are generated out of thymus. IL-10 and TGF-β are two major cytokines which can drive naive $CD4^+$ T cells to iTregs. Self-antigen-specific nTregs are the major subset of T cells that is critical for the maintenance of self-tolerance and the prevention of autoimmune diseases. iTregs, on the other hand, are a key subset of T cells in fighting against foreign pathogens.

CD25 and FoxP3 are cell surface markers for Tregs. However, recent studies showed that both CD25 and FoxP3 could also be found in newly activated human effector $CD4^+$ T cells. The IL-7 receptor α chain (CD127) is found to be inversely correlated with nTreg status (Liu et al., 2006). Thus, the combination marker $CD4^+CD25^+FoxP3^+CD127^{low}$ is widely used to identify human nTregs. Fletcher and colleagues reported that $CD39^+FoxP3^+$ T cells also have the suppressive effect (Schuler, Harasymczuk, Schilling, Lang, & Whiteside, 2011). Further potential surface markers for the assessment of nTregs are ‘‘CTLA-4, GITR, CD73, and LAP’’ (T. Takahashi et al., 2000; McHugh et al., 2002; Deaglio et al., 2007; M. L. Chen, Yan, Bando, Kuchroo, & Weiner, 2008).

In autoimmune diseases, regulatory T cells not only offer a new perspective of self-tolerance establishment and maintenance, but also actually engaging in systemic and local inflammation, memory and resolution. Autoimmunity with immune dysregulation, polyendocrinopathy, enteropathy X-linked syndrome (IPEX) in children links to several mutations of Foxp3 gene (Bennett et al., 2001), while in mice mutation or depletion of the Foxp3 gene resulted in fatal autoimmune diseases (Brunkow et al., 2001). Data about the frequency of Tregs in RA are contradictory, especially in the peripheral blood (PB) (Kawashiri et al., 2011; van Amelsfort, Jacobs, Bijlsma, Lafeber, & Taams, 2004). Accumulating

evidence indicates the enrichment of Treg population in the synovial fluid (SF) and synovial membrane (SM) (Jiao et al., 2007; Moradi et al., 2014). The transfer of $CD4^+Foxp3^+$ Treg cells reduces the severity of CIA when it is done prior to collagen immunization (Wright et al., 2009). The administration of monoclonal antibody specific for CD25 targeting $CD4^+CD25^+$ Tregs on collagen-induced arthritis (CIA) worsens the disease (Morgan et al., 2003). Some studies showed that Tregs from RA patients, especially Tregs from PB, have impaired suppressive function (Ehrenstein et al., 2004).

Tregs are capable of inhibiting other proinflammatory cell function in both contact-independent and contact-dependent manner. IL-10 impairs the ability of dendritic cells to promote Th1 differentiation and suppress the production of IL-12 by Th1 cells (Rubtsov et al., 2008). Blockade of TGF-β expression on Tregs with neutralizing antibodies resulted in the abolishment of Treg suppression (Nakamura, Kitani, & Strober, 2001). The contact-dependent inhibition involves the cell surface receptors on Tregs. Receptors like CTLA-4, GITR, and OX40 could trigger the suppressive effects of Tregs. CTLA-4 gene polymorphisms have been reported to be correlated with RA (Daha et al., 2009; Lei et al., 2005). Tregs from RA patients showed a less CTLA-4 expression, as well as a reduced function compared to healthy Tregs (Walker et al., 2009). Furthermore, signaling pathways triggered by CTLA-4 can regulate the balance between the anti- and pro-inflammatory cytokines, resulting in the inhibition effect (Kormendy et al., 2013). The third mechanism is that Tregs can deplete IL-2 through CD25, which is also known as the IL-2 receptor. With the high expression of CD25, Tregs will deprive IL-2, which is necessary for other effector $CD4^+$ T cells. Effector $CD4^+$ T cells will undergo apoptosis because of this deprivation (Pandiyan, Zheng, Ishihara, Reed, & Lenardo, 2007). Lastly, Tregs can execute target cells by making its own granzyme A and perforin (Cao et al., 2007) or releasing metabolites like adenosine to exert their immunosuppressive functions (Ohta & Sitkovsky, 2014).

3. Tfh cells are crucial for T-cell helped function in RA

T follicular helper (Tfh) cells are characterized by the expression of CXCR5 on $CD4^+$ T cells. Bcl-6 is the signature transcription factor, and IL-21 is the signature cytokine. ‘‘PD-1, ICOS, CD40 ligand (CD40L)’’ (Ueno, Banchereau, & Vinuesa, 2015) *et al.* are their surface markers, which usually apply in combination with CXCR5. Tfh cells are essential for T-cell helped function, including B cell maturation and antibody production in germinal centers.

Tfh cells play important roles in the inflammatory responses of RA. Elevated circulating Tfh and IL-21 are found in patients with RA, and they are correlated with disease activity (Arroyo-Villa et al., 2014; Y. Zhang, Li, Lv, Yin, & Wang, 2015). Tfh cells could also be found in rheumatoid synovium tissues from RA patients, but are absent in OA synovium (Chu, Wang, Zhou, Chen, & Lu, 2014). In RA synovium and synovial fluid, enrichment of PD-1^+ T cells is found, reflecting the existence of negative feedback at the inflammatory sites (Raptopoulou et al., 2010). Several strategies targeting Tfh-related molecules and cytokines showed impressive therapeutic effects, opening new venues for possible immune regulatory therapy in RA (Jang et al., 2009; Odegard et al., 2008).

4. $CD8^+$ cells are predominantly proinflammatory T cells in RA

Earlier studies of $CD8^+$ T cell functions in RA yield conflicting results, causing the research in this cell type became almost complete oblivion (Carvalheiro, da Silva, & Souto-Carneiro, 2013). However, recently, more and more evidence support that $CD8^+$ T cells present in the inflammatory joint mainly contribute to the chronic inflammation locally (Fekete et al., 2007; Maldonado et al., 2003; Masuko-Hongo et al., 1997; Wagner et al., 1998). $CD8^+$ T cells promote the local inflammation not only through their secretion of proinflammatory cytokines, but also kill the target cells by their lytic enzymes. It is also approved that $CD8^+$ T cells can exert their suppressive role in an IL-10 dependent manner in RA (Carvalheiro et al., 2013).

1.2.2 Multiple sclerosis

1.2.2.1 MS is the second cause of disability in young adults

About 350,000 people suffer from MS in US, and this number is 2.5 million worldwide. MS affects adults of 20-40 years old. It is the second cause of disability in young people. The incidence of MS in women is about twice higher than that of men. Epidemic studies showed that MS has a familial distribution pattern (Weinshenker, 1996). It is also reported that monozygotic twins got higher chance to have MS than the dizygotic twins (Hansen et al., 2005). The prevalence rates of MS have been reported to be high in northern parts of Europe and North America (5-30 per 100,000), but low in Asia and South America (<5 per 100,000) (Pugliatti et al., 2006). Some reports show that the epidemic pattern of MS can be changed due to migration. In the majority of the case, MS does not influence longevity. However, due to

the long duration of disease, the loss of productivity, the need for assistance in activities of daily life and the use of immunomodulatory treatments, MS is a heavy socioeconomic burden (Sospedra & Martin, 2005).

1.2.2.2 Clinical manifestation of MS

1.2.2.2.1 Symptoms of MS

The clinical features of MS are highly variable, but an attack (relapse or exacerbation) of neurologic dysfunction heralds the onset of the disease in approximately 90% of patients. Common symptoms of an MS attack include optic neuritis, long tract symptoms/signs (e.g., numbness, paresthesia, or weakness), a brainstem syndrome (e.g., internuclear ophthalmoplegia), or a spinal cord syndrome (e.g., transverse myelitis). Symptoms due to cortical syndromes such as aphasia or visual field disturbances are less common. Symptoms of MS range from mild to severe, depending on lesion locations and the extent of tissue destruction.

1.2.2.2.2 Relapsing-remitting with chronic, persistent progression is the most common clinical pattern of MS

Relapsing-remitting with chronic, persistent progression is the most common clinical pattern of MS. Eighty-five to ninety percent of patients initially present with relapsing-remitting MS (RRMS) (Nylander & Hafler, 2012). The first attack typically occurs acutely. The duration for each attack lasts from days to weeks. Recovery from each attack in the early course of the disease is often virtually complete. Most RRMS will become secondary-progressive MS (SPMS), which is featured by the progressive accumulation of disability. RRMS may relate to myelin-reactive T cells, which will migrate into the CNS resulted in acute inflammation, while SPMS may be caused by progressive chronic CNS inflammation.

Approximately 10% of patients have primary-progressive MS (PPMS). In this form of disease, patients usually present spinal syndrome, a spastic paraparesis usually with no apparent effect on sensory level. The time course of the disease is commonly found to be associated with the progressive deterioration of neurologic function. Plateaus and slight fluctuations may occur occasionally, but no relapses (Rice, Cottrell, Wilkins, & Scolding, 2013).

1.2.2.2.3 McDonald criteria 2010 for the diagnosis of MS

From its first historical depictions, MS has always been described and diagnosed by its clinical semiology, as no specific laboratory test is available for the disease. Currently, the use of McDonald criteria 2010 has allowed for earlier diagnosis of MS with higher specificity and sensitivity, making it possible for the earlier treatment and better counseling of patients (Polman et al., 2011). MRI provides a non-invasive support for diagnosis and monitors the prognosis of MS (Traboulsee & Li, 2006). Oligoclonal IgG in CSF helps in diagnosis of MS in the appropriate clinical context (Rojas, Patrucco, Tizio, & Cristiano, 2012).

1.2.2.3 Animal models of MS

Animal models have been critical for addressing MS pathogenesis due to the rarity of diseased human tissues. Even though each animal model has its drawbacks, they did contribute to our understanding of MS, as well as to the exploration of the new treatment for MS in the past decades. The representative models are described below (Ransohoff, 2012).

1.2.2.3.1 Both EAE and TMEV-IDD are immune-models for human MS, with the former mainly induces $CD4^+$ T cell responses

EAE model shares many similarities with human MS, including breach of tolerance, and many of the pathological changes observed in the CNS lesions (Croxford, Kurschus, & Waisman, 2011; A. P. Robinson, Harp, Noronha, & Miller, 2014). The generation of autoreactive T cells against self-antigen specific for myelin proteins is the initial step in the pathogenesis of both EAE and MS. Then, these cells will migrate to the central nerves system to trigger the immune-inflammatory responses locally. This will lead to the accumulation of other immune cells, the release of inflammatory mediators, and finally demyelination or even axonal loss in both EAE and MS lesions. EAE also creates the opportunity to investigate the role of Th1 and Th17 effector $CD4^+$ T cells in the development of MS (see in the "Adaptor immune system in MS" part).

Mice EAE model and human MS share common pathologic features, including infiltrating T cells and other inflammatory cells in the CNS and the demyelination. C57BL/6 background mice are the most popular strain for this model (Tse et al., 2012). Ascending paralysis typically starts from the hind limps, which is related to the pathologic changes in the axon.

Moreover, adoptive transfer of Th1 and Th17 cells from immunized donors can directly induce effector phase of EAE. However, relapse rarely happens in EAE model (Ransohoff, 2012). Thus, it can't be used in studying remyelination, which often happens in MS patients. Moreover, due to the strong inflammatory responses after EAE induction, the predictive value for treatment efficacy of this model is limited (Ransohoff, Howe, & Rodriguez, 2002). Lastly, EAE induction mainly induces $CD4^+$ T cell responses, which is not ideal for $CD8^+$ T cell responses and B cell functions in MS pathogenesis.

The TMEV-IDD model, which is short for Theiler's murine encephalomyelitis virus-induced demyelinating disease, also features with immune-mediated demyelination. Mononuclear cell infiltration and $PLP_{131-151}$-specific $CD4^+$ Th1 cells are found early in TMEV-IDD CNS lesions (Katz-Levy et al., 2000). Autoantibodies against other myelin antigens appear in serum with the progress of the disease. Inflammation and demyelination similar to that in MS are found. However, unlike MS, virus infections are continually found in the CNS during the persistence of TMEV-IDD (Miller et al., 1997).

1.2.2.3.2 Administration of toxins enables the study of remyelination, and some spontaneous EAE models are explored

Administration of toxins, such as cuprizone, enables the study of remyelination. The copper chelator, cuprizone, is fed to mice in chow for 4-6 weeks to induce demyelination (Matsushima & Morell, 2001). Cuprizone lead to the apoptosis of oligodendrocytes, which results in the demyelination of axons. In this model, remyelination occurs once cuprizone is discontinued, making it an ideal model for study the underlying mechanism of oligodendrocyte death and remyelination.

Myelin-specific peptide TCR transgenic mice are generated as a spontaneous demyelination model of EAE (Bettelli et al., 2003; Waldner, Whitters, Sobel, Collins, & Kuchroo, 2000). Approaches to induce selective expression of suicide gene expression in oligodendrocyte (Pohl et al., 2011; Traka et al., 2010) are also explored. These approaches are relative novel, the application of which might bring unique insights into the pathogenesis of MS.

1.2.2.4 Innate immune system in MS

1.2.2.4.1 Viral infections can be the trigger of MS

Viral infections, like human herpesvirus 6 (HHV-6), Epstein-Barr virus (EBV), human herpesvirus 1 (HSV-1), *et al.*, have been considered as environmental triggers of MS. This fact indicates the involvement of innate immune mechanisms in MS.

1.2.2.4.1 γδ T cells undergo massive expansion, produce cytokines, chemokines, and cytokine receptors in the CNS

γδ T cells are important in the autoimmune inflammation of the CNS. γδ T cells are found in the CSF and CNS inflammatory lesions of both MS patients and EAE model (Battistini et al., 1995; Wohler, Smith, Zinn, Bullard, & Barnum, 2009). In EAE model, γδ T cells are found to have the ability to migrate into the local lesions in the CNS. These cells undergo massive expansion there, and then quickly leave the CNS (Wohler et al., 2009). γδ T cells secrete cytokines, chemokines (Murzenok, Matusevicius, & Freedman, 2002; Rajan, Klein, & Brosnan, 1998). Recently, γδ T cells are found to be the primary source of IL-17 in demyelinating diseases (Blink & Miller, 2009). γδ T cells can also directly mediate cytotoxic responses to kill primary human oligodendrocytes (Saikali et al., 2007). Also, γδ T-cell knockout mice have attenuated EAE (Spahn, Issazadah, Salvin, & Weiner, 1999). Even though studies using anti-γδ T cell antibodies show controversial results, most of these studies show attenuated EAE severity (Dandekar & Perlman, 2002; Rajan, Gao, Raine, & Brosnan, 1996). What's more, features like restricted TCR usage and tissue-specific location make γδ T cells a therapeutic advantage in targeting T cell subpopulation for MS.

1.2.2.5 Adaptor immune system in MS

1.2.2.5.1 Role of B cells in MS

For decades, T cells had been regarded as the major contributor in MS pathogenesis. However, recent studies suggested that this point of view is overly simplistic. Numerous researchers reported the presence of B cells and autoantibodies in the CNS of MS patients, indicating the potential role of B cells in the propagation of MS (Cepok et al., 2005; von Budingen, Harrer, Kuenzle, Meier, & Goebels, 2008; M. J. Walsh & Tourtellotte, 1986).

Generally, the effector mechanisms of B cells and autoantibodies are similar to those in RA, which were described in detail previously. The most important point about B cells and autoantibodies is the oligoclonal IgG bands (OCBs) in CNS. Interestingly, it does not exist in

serum, indicating the production of IgG might occur in the CNS inflammatory lesion (Kabat, Freedman, & et al., 1950). Moreover, the exhibition of OCBs in CSF correlates with the clinical and MRI findings, making it helpful in the MS diagnosis (Disanto, Morahan, Barnett, Giovannoni, & Ramagopalan, 2012).

Autoantibodies against the components of myelin, including proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG), are important in the pathogenesis of MS. Antibodies against these proteins are found in both human MS and in animal models. In CSF and (or) serum of MS, these antibodies can be found. However, none of them are sensitive or specific enough as diagnostic markers of disease activity (Egg, Reindl, Deisenhammer, Linington, & Berger, 2001; Reindl et al., 1999; Sellebjerg, Christiansen, & Garred, 1998).

1.2.2.5.2 T cells are the major population in the pathogenesis of MS

Like they are in RA, T cells are the driving force in the pathogenesis of MS. Therefore, the mechanisms involving T cell activation, co-stimulation and tolerance also apply to the pathogenesis of MS. For this part, I will highlight on different T helper subtypes in the pathogenesis of MS.

Th1 and Th17 are the main effector T cells responsible for the pathogenesis of MS

Before the discovery of Th17 cells, Th1 cells were considered as the major driving force for the pathogenesis of MS. Earlier studies show that IL-12 is increased in MS lesions (Windhagen et al., 1995). Further *ex vivo* studies show that the T cells from MS patients have the ability to produce more Th1 cytokines, such as IL-12 and IFN-γ (Balashov, Smith, Khoury, Hafler, & Weiner, 1997). The knockout of Th1 master transcription factor, T-bet, leads to less severe EAE in mice (Bettelli et al., 2004). Also, glatiramer acetate directly kills Th1 cells in the treatment of MS (Racke & Lovett-Racke, 2011; Ziemssen & Schrempf, 2007). What's more, treatment of MS patients with the recombinant IFN-γ results in exacerbation of disease (Panitch, Hirsch, Haley, & Johnson, 1987). However, knockout of IFN-γ in mice is found to develop more severe EAE (Ferber et al., 1996).

The Th17 subset was initially recognized through the studies of EAE mice. In this study, Cua *et al.* proved that IL-23, but not IL-12, is the critical cytokine for EAE (Cua et al., 2003). After that, extensive study on IL-23 and Th17 in MS and other autoimmune diseases started.

Like Th1 cells, IL-17 expressing Th17 cells can be detected in MS brain lesions. Moreover, in MS patients, the association of IL-17$^+$ population in the peripheral blood with disease activity are also confirmed (Durelli et al., 2009; Lock et al., 2002). What's more, *in vitro* induced PLP-specific Th17 cells are capable of inducing EAE after passive transplantation (Langrish et al., 2005). Furthermore, neutralization of IL-17 by anti-IL-17 antibody and IL-17-receptor-Fc-protein protects mice from EAE (Hofstetter et al., 2005; Langrish et al., 2005). Also, IL-17 can mediate BBB breakdown by activating neutrophils to secrete MMP-3 and proteases, which directly participate in BBB disruption (Yong, Power, Forsyth, & Edwards, 2001). "IL-17 can stimulate the secretion of ROS" (Huppert et al., 2010), which activates the brain endothelial cell contractile machinery. Nevertheless, mice lacking IL-17 are not protected from disease (Haak et al., 2009). Currently, numerous findings support the consensus view that IL-17 and other Th17-derived cytokines are important for both mice EAE and human MS.

Since the identification of Th17, Th1 cells have been out of the attentions of researchers for a long time. Recently, it is reported that Th1 is another arm for the pathogenesis of EAE. Harrington *et al.* reported that adoptively transferred with Th1 induces EAE. Th17 cells, however, are not able to do that (Harrington et al., 2005). This indicates that Th17 cells do not have the ability to migrate into the CNS to initiate the disease. Moreover, the Th1 cytokine, IL-12 can recruit macrophages into the inflammatory site. The Th17 cytokine, IL-23, on the other hand, can recruit neutrophils (Kroenke, Carlson, Andjelkovic, & Segal, 2008). More recently, it is reported that a new population of T cells, which are IL-17$^+$IFN-γ^+, are found in MS and EAE. This IL-17$^+$IFN-γ^+ population is preferentially recruited into CNS (Kebir et al., 2009). In addition, T-bet$^+$ Th1 cells could be sub-divided into T-bet$^+$/RORγt$^+$ IL-17-producing cells to induce EAE, suggesting the existence of plasticity within these populations (Abromson-Leeman, Bronson, & Dorf, 2009; Damsker, Hansen, & Caspi, 2010).

Treg cells are functionally compromised in MS

Similar as they are in RA, Tregs are functionally compromised. In MS patients, the frequency of Tregs in peripheral blood is similar as their healthy controls. However, in the CSF, MS patients have more elevated Treg percentage than normal controls (Feger et al., 2007; Haas et al., 2005; Kouchaki, Salehi, Reza Sharif, Nikoueinejad, & Akbari, 2014). Interestingly, one study has found reduced Tregs in CSF of RRMS but not in SPMS, indicating a potential role of Tregs in the down-regulation of local inflammation in the former (Venken et al., 2008).

$CD39^+$ Tregs from RRMS patients has a compromised ability to suppress IL-17 (Fletcher et al., 2009), suggesting the discrepancy may partially due to a lack of representative cell surface marker for Tregs. Recently, Dhaeze *et al.* reported that MS patients have a significantly lower frequency of circulating follicular regulatory T cells accompanied by a sharply reduced suppressive function of these cells (Dhaeze et al., 2015).

In EAE models, Treg cells also controls the initiation of the disease. $CD4^+CD25^+$ Treg cells have the ability to suppress T effector cell function in an Ag-dependent manner. The transfer of $CD4^+CD25^+$ Treg cells to mice lead to the resistance of EAE (Kohm, Carpentier, Anger, & Miller, 2002). In the PLP-induced model, the percentage of Ag-specific $CD4^+CD25^+$ Treg cells inversely correlates with the susceptibility of EAE (Reddy et al., 2004). Expansion of Tregs *in vivo* by IL-2 can suppress the immune response and significantly ameliorate EAE (Rouse, Nagarkatti, & Nagarkatti, 2013).

Local factors also play critical roles in controlling Treg dysfunction. In patients with MS, their nonregulatory $CD4^+$ T cells express more granzyme B, which could inhibit Treg suppression without altering Treg viability (Bhela et al., 2015). Tregs can be skewed to Th17-like cells when stimulated by TLR2. Therefore, they will lose their suppressive function accordingly (Nyirenda et al., 2011). Similar to recent observations in mouse T cells, human T helper precursors have the plasticity to further differentiate into Th17 by TLR2 stimulation. TLR2 stimulation, with the presence of TCR activation signaling, enhances Th17 cell differentiation and expansion from Tregs.

Some *in vitro* studies prove that immunomodulators of MS, such as IFN-β, glatiramer acetate, can reverse the impairments of Treg functions from MS patients (Trinschek, Luessi, Gross, Wiendl, & Jonuleit, 2015). Part of the mechanism for the treatment effect of IFN-β is due to its ability to induce the proliferation of Treg cells (M. Chen et al., 2012). Fingolimod (FTY720) is a drug that has been proved for the treatment of MS by FDA. It reduces the autoreactive T cells egress from the secondary lymph nodes into circulation. On the other hand, it increases the frequency of circulating Treg cells, thus, increase the suppressive function (Muls, Dang, Sindic, & van Pesch, 2014; Serpero et al., 2013).

As mentioned above, Treg inhibitory effects can be mediated by cytokines, cell-cell contact, and depriving of cytokines (Takizawa et al., 2014; K. P. Walsh, Brady, Finlay, Boon, & Mills, 2009; X. Zhang et al., 2004).

The dual role of CD8$^+$ T cells in MS

Specific MHC I alleles has been shown to be associated with MS, indicating the potential role of CD8$^+$ T cells in MS (Fugger, Friese, & Bell, 2009). In MS plaques in CNS, more infiltrating CD8$^+$ T lymphocytes are found than CD4$^+$ T cells. These CD8$^+$ T cells undergo oligoclonal expansion locally (Denic, Wootla, & Rodriguez, 2013; Gregersen, Hingorani, & Monteiro, 1995; Huseby, Huseby, Shah, Smith, & Stadinski, 2012). As majority of the resident cells in the local lesion express MHC class I molecule, they could be killed by the CD8$^+$ T cells. This happens both in human MS and mice EAE lesions (Babbe et al., 2000). Also, CD8$^+$ T cells can regulate local immune-inflammatory responses through the Fas-FasL-dependent pathway. CD8$^+$ T cells can promote cell apoptosis through the upregulation of FasL and the secretion of pro-inflammatory cytokines (Chavez-Galan, Arenas-Del Angel, Zenteno, Chavez, & Lascurain, 2009; Slifka, Rodriguez, & Whitton, 1999). CD8$^+$ T cells also showed a suppressive role in a TGF-β-dependent manner (M. L. Chen, Yan, Kozoriz, & Weiner, 2009).

Receptor tyrosine kinases are a group of key signaling molecules, which are important in the immune cell functions. In the past 20 years, our laboratory has elected to study the function of Ephb6 kinase in T cells. Efnb1 and Efnb2, which are ligands for Ephb6, were also studied for their roles in T cell function in our lab and in this thesis.

1.3 Eph receptors and ephrins

1.3.1 Structure characteristics of EPH receptors & ephrins

Both erythropoietin-producing hepatocyte kinases (EPHs) and their ligands, ephrins (EFNs) are cell surface receptors. There are fourteen EPH receptors (EPHA1-8, EPHA10, EPHB1-4 and EPHB6) (Aasheim, Patzke, Hjorthaug, & Finne, 2005; Brantley-Sieders & Chen, 2004) and nine EFNs (EFNA1-6, EFNB1-3) (Pasquale, 2004). EPH receptors and ephrins are expressed in virtually all tissues of developing embryos. In adult, however, their expression is relatively less. Some tissues or regions of certain organs express high levels of some Ephs or EFNBs. Even though EPHs/EFNs have been widely studied during development, their

physiological and pathological functions in the adult are coming to light. Recent studies show that their expression is high in cells like neurons, vascular cells, immune cells, indicating their potential roles in these cells.

Eph receptors are transmembrane proteins (Figure 1.2). The protein domains that are contained in the ectodomain and the cytoplasmic region are illustrated in Figure 1.2 (Gucciardo, Sugiyama, & Lehti, 2014) (Daar, 2012). As illustrated in Figure 1.2, the EFNA subfamily members are GPI-anchored proteins, while the EFNBs are transmembrane proteins (Pitulescu & Adams, 2014). The extracellular domain of both EFNA and EFNB contains a conserved extracellular receptor-binding domain (RBD). However, unlike the membrane-bond EFNAs, EFNBs have a transmembrane helix and an intracellular domain.

1.3.2 Signal transduction of EPHs and EFNs

As both Eph receptors and ephrins are cell surface molecules, they can transduce bidirectional signals. When it goes to the receptor-expressing cells, it is called forward signalling; when it goes to the ligand-expressing cells, it is called reverse signaling (Figure 1.2). Besides bidirectional signalling, the other property of this signaling system is that the direction of signaling is highly sensitive to the microenvironment.

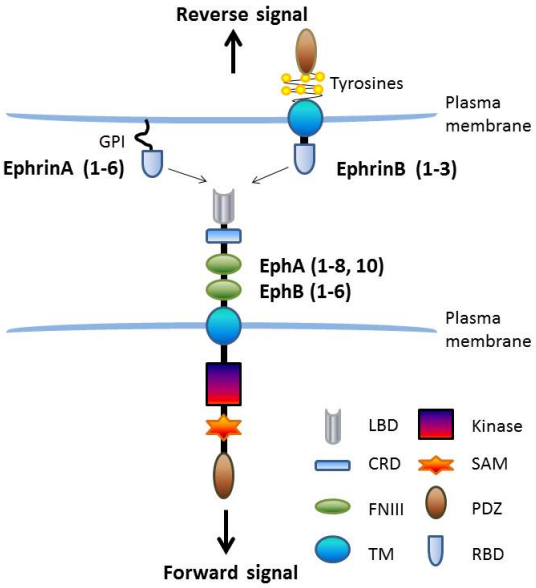

Figure 1.2 Signalling transduction of EPHs/EFNs

EPHs and EFNs interact promiscuously with each other. Usually, EPHAs selectively interact with EFNAs, while EPHBs are more likely to intact with EFNBs. Recent experiments indicate considerable crosstalk between the A and B family members. There are several exceptions, including EPHA4 can bind to EFNB2 (Hu, Li, Jiang, Li, & Zhou, 2014; Qin et al., 2010) and EFNA5 can bind to EPHB2 (Himanen et al., 2004).

1.3.2.1 Ligand-induced forward signaling transduction

The binding of ephrins to Eph receptors can activate the downstream signalling. Moreover, the clustering of ephrins after binding enhances the forward signalling (Himanen, 2012; Himanen & Nikolov, 2003). The interaction of EPH receptor and its ligand will lead to the conformation change to create more interaction interface and clustering of multiple EPH subclasses.

The signal can be transmitted in two ways, the PDZ-independent pathway and the PDZ-dependent pathway. The activation of the first pathway results in the juxtamembrane tyrosine residue phosphorylation, which will recruit adaptor proteins in the cytosol. A variety of downstream signalling pathways, like ‘‘the FAK pathway, the JAK/STAT pathway, the

RAS/MAPK pathway" (Genander et al., 2009; Lim, Manser, Leung, & Hall, 1996; Macrae et al., 2005; Miao et al., 2015), will be triggered. The second way is the PDZ-dependent signaling pathway. The PDZ-dependent pathway, will employ its PDZ-domain-binding motif at the C-terminus of Eph to bind to adaptor proteins, such as glutamate receptor interacting protein (GRIP) 1/2, Ras-binding protein ALL1-fused gene from chromosome 6 (AF6), or protein interacting with C kinase (PICK) 1 (Hock et al., 1998; Pitulescu & Adams, 2010).

1.3.2.2 Receptor-induced reverse signaling transduction

Besides serving as ligands, EFNs are also able to transduce signals into their host cells. The GPI-anchored EFNAs are reported to cluster to recruit adaptor proteins to transduce signalling. For example, EFNA5 can cluster and recruit the Fyn tyrosine kinase in regulating cell adhesion (Davy et al., 1999; Davy & Robbins, 2000). Once encounter Eph receptors, ephrin Bs are capable of triggering the reserve signaling through the activation of conserved tyrosine residues within their cytoplasmic tails. This will provide docking sites for SH2-SH3 family adaptor proteins, such as Src, Grb4, STAT3, CRK and Fyn. Their binding will lead to further signal transduction (Bong et al., 2007; Davy et al., 1999; Nagashima et al., 2002; N. J. Xu & Henkemeyer, 2009). EFNs are also able to transduce the signal by PDZ-domain-binding domain at the end of their intracellular domain. Several proteins including GRIP1, protein tyrosine phosphatase BAS-like (PTP-BL), TIAM1, Dishevelled and PDZ-RGS3 are reported to bind to EFNBs directly via their PDZ domains (Bruckner et al., 1999; Lee et al., 2006; Tanaka et al., 2004). Recently, it has been reported that EFNB2 can regulate endothelial cell morphology and motility in the presence of the C-terminal PDZ motif in the absence of EPH-receptor binding (Bochenek, Dickinson, Astin, Adams, & Nobes, 2010).

1.3.2.3 Both EPH receptors and EFNs have direct cross-talk with a variety of molecules

Besides bidirectional signal transduction, EPH receptors and EFNs do not only act in isolation, but also have direct cross-talks with other cell-surface receptors and a variety of intracellular effector proteins.

Several growth factor receptors, such as VEGF receptors, EGF receptors, IL-7 receptor, *etc.*, are reported to interact with EPH receptors and EFNs. The common mechanism of such interaction involves different levels of cooperation between Eph receptors and many

intracellular signalling effectors. Up till now, the non-receptor tyrosine kinase Src, Rho/Rac1 axis, Akt pathway and Ras/Erk pathway can be triggered by growth factor receptor activation (Bromann, Korkaya, & Courtneidge, 2004; Hiramoto-Yamaki et al., 2010; Macrae et al., 2005; Yang, Pasquale, Owen, & Ethell, 2006). For example, growth factor receptor downstream kinase Akt phosphorylates Ser897 of ligand-unbound Epha2, thereby promoting invasion of cancer cells (Miao et al., 2015; Miao et al., 2009). Tyrosine on the cytoplasmic tail of EFNB1 can be phosphorylated by RTKs, which are associated with FGF receptor activation (Chong, Park, Latimer, Friesel, & Daar, 2000).

EPH/EFN signaling has a close connection with the cytoskeletal dynamics. Rho, Rac, and Cdc42 are the most studied Rho family proteins, which can interact with Eph receptors by guanine nucleotide exchange factors (GEFs). These interactions provid a link between Eph receptors and cytoskeleton (Noren & Pasquale, 2004). Ephexin, which is a member of Rho GEFs, can directly interact with the C-terminal of EPHA4 by its tandem Dbl-homology-pleckstrin-homology (DH-PH) domain in the modulation of growth cone collapse (Sahin et al., 2005). Other mediators like Tiam1 and Vav family GEFs are reported to interact with EPHA receptors to activate Rho GTPases, whereas Intersectin and Kalirin GEFs mediate the interaction of EPHBs and Rho GTPase (Gucciardo et al., 2014; Hunter et al., 2006; Irie & Yamaguchi, 2002).

EPHs/EFNs have also been reported to interact with chemokines (Lu, Sun, Klein, & Flanagan, 2001) and adhesion molecules (Arvanitis & Davy, 2008). For instance, binding of Eph receptor with Efnb1 can selectively inhibit CXCL12-induced CXCR4-mediated cerebellar granule cell migration via PDZ-RGS3 (Lu et al., 2001). Our group proved that Efnb1 and Efnb2 interact with IL-7Rα by stabilizing IL-7R expression at the post-translational level. Moreover, this stabilization prolongs IL-7R signaling (Luo, Wu, et al., 2011).

Also, the cellular response can be controlled by the degradation and (or) removal of EPH/EFN interaction complex mechanisms, in which the duration and intensity of the receptor/ligand interaction changed. Zimmer *et al.* reported that blockage of internalization of EPHB2 and its ligand ephrin B1 expressed on fibroblasts and neurons converts repulsion into adhesion of the cells (Zimmer, Palmer, Kohler, & Klein, 2003). A more recent report showed that the extracellular domains of Efns could be removed by ADAM10 (Kaushal & Shah, 2000;

Primakoff & Myles, 2000), MMPs (Georgakopoulos et al., 2006) and (or) γ-secretase (Tomita, Tanaka, Morohashi, & Iwatsubo, 2006). This study suggests an underlying mechanism of EPHs/EFNs in repulsion and the shaded EPHs/EFNs might have long-range paracrine functions.

1.3.3 Functional roles of EPH/EFN signaling pathways

EPH/EFN signaling contributes to a variety of processes during development, tissue homeostasis, and disease pathogenesis. In this part, I will focus on EPH/EFN signaling pathways in neural development, bone homeostasis, and cancer, which are the most extensively studied. Their roles in the immune system are also reviewed here, as it is the major focus of my thesis.

1.3.3.1 EPH/EFN signaling pathways control cell proliferation, adhesive function, and migration in neural development

EPH/EFN signaling pathways mainly participate in axon guidance and synaptogenesis during neural development, as well as neuronal plasticity in the adult. During neural development, EPH/EFN signaling pathways are known for their roles in controlling cell proliferation, adhesive function, and migration at the cellular level.

EPHs and ephrins exhibit highly complex spatial and temporal expression pattern in central nerves system, suggesting their duties in specific neurons or at a specific time point (Buchert et al., 1999; Grunwald et al., 2004; Henderson et al., 2001). Precise expression gradients and sharp boundaries are also displayed. For instance, in the recipient anterior-posterior axis in the tectum, the complementary gradient of Efna2 and Efna5 expression are found (Cheng & Flanagan, 1994; Cheng, Nakamoto, Bergemann, & Flanagan, 1995; Drescher et al., 1995).

In axon guidance and synaptogenesis, Eph receptors and ephrins co-operator with multiple other cellular pathways both in the PDZ-dependent and PDZ-independent signaling pathways. The first report about EPH/EFN signaling in axon guidance was published in 1994 (Cheng & Flanagan, 1994). With the use of Eph/Efn-specific Abs and recombinant Eph/Efns, and genetic manipulation on Eph receptors and ephrins, the function and molecular mechanism of this family of molecules have been further explored. Mice deficient for Efna2, Efna3 and

Efna5 present disrupted shape and location of eye-specific retinogeniculate layers (Pfeiffenberger et al., 2005). Truncation of the Ephb2/b3 cytoplasmic domain leads to higher frequency of axon guidance errors, indicating that reverse signaling plays an essential role in axon pathfinding (Birgbauer, Cowan, Sretavan, & Henkemeyer, 2000).

Besides axon guidance, EPHs/EFNs are critical for regulating synaptogenesis. Ephrins support presynaptic forms of long-term potentiation (LTP) by promoting presynaptic differentiation and neurotransmitter release (Klein, 2009). Bidirectional signaling is not always necessary, as in some cases Eph receptors are only needed to trigger reverse signaling. Interestingly, several groups observed a discrepancy of Ephb2 in synaptogenesis. Both overexpression and knockdown of Ephb2 *in vitro* can regulate presynaptic and postsynaptic specialization (Kayser, McClelland, Hughes, & Dalva, 2006; Kayser, Nolt, & Dalva, 2008). Surprisingly, synapse formation is normal in Ephb2 knockout mice, suggesting multiple synaptogenic pathways can compensate for the lack of Ephb2 *in vivo* (Sloniowski & Ethell, 2012). Moreover, evidence reveals that EFNA6 interacts with neurotrophin receptor TrkB and EPHB2 binds to NMDA receptor to control synaptogenesis (Dalva et al., 2000; Marler et al., 2008).

1.3.3.2 EPH/EFN signaling maintains the balance between bone formation and resorption

Bone homeostasis is strictly controlled by proper communication between osteoclasts and osteoblasts to keep the balance between bone formation and resorption (Edwards & Mundy, 2008). EPHB4 and its ligand ephrin B2 remain at the center of the focus in this regard. The forward signaling activated by EPHB4, which is expressed on osteoblasts, promote osteogenic differentiation. In contrast, reverse signaling through ephrin B2 on osteoclasts results in the suppression of osteoclast differentiation (C. Zhao et al., 2006). Furthermore, the activation of ephrin B1 and ephrin B2 expressed by mesenchymal stem/stromal cells, which are the primary source of the osteoblasts, leads to increased osteogenic differentiation (Arthur et al., 2011).

1.3.3.3 Dysregulation of EPHs and ephrins are frequently found in cancer

In human cancer, the dysregulation of EPHs and ephrins are common. EPHA2, for instance, is upregulated in many solid cancers and its expression has been found to be associated with poor clinical prognosis (Dunne et al., 2015; Shen et al., 2014). In contrast, EPHB6 is

downregulated in advanced human ovarian, prostate and colorectal cancers (Gu et al., 2015; Mohamed et al., 2015; Peng et al., 2014). Furthermore, EPH expression in cancer can be dynamic. Several EPHBs and EPHA1 are upregulated during early stages of cancer progression and can be subsequently silenced leading to tumor suppress (Batlle et al., 2005; Herath, Doecke, Spanevello, Leggett, & Boyd, 2009; Herath et al., 2012).

Functional alterations are also found in the signaling pathways of EPHs/EFNs and their crosstalk with other oncogenic signaling pathways in tumor initiation, progression, and metastasis at different levels. EPHs/EFNs, such as EPHA2 and EPHB4, are involved in the regulation of RhoA activity (Wakayama, Miura, Sabe, & Mochizuki, 2011; Yang et al., 2006). Intriguingly, increased adhesion of HEK cells and endothelial cells presents upon ligand-induced EPHB1 activation. However, EPHB3 activation in HEK cells leads to reverse effects (Miao et al., 2005).

In the past decade, tumor-propagating cells (TPCs) bearing stem cell properties were identified among cancer cells. Both EPHA2 and A3 are highly expressed in TPCs (Binda et al., 2012; Day et al., 2013). The overexpression of these two molecules on TPCs results in better self-renewal. In breast and prostate cancer, the overexpression of EPHA2 and its presence on TPCs have been reported, making EPHA2 a new therapeutic target (Sugiyama et al., 2013; X. D. Wang et al., 2007).

EPHs/EFNs also regulate the stromal compartments in cancer. The EPH/EFN system participates in the regulation of segregation at the boundary of the tumor and the normal tissues. Eph receptors and ephrins also regulate angiogenesis in tumor through the influence of vascular cell patterning. They can also influence immue cell migration to the tumor lesions (Astin et al., 2010; Ogawa et al., 2000).

1.3.3.4 EPH receptors and EFNBs can be found in different immune cells, their role in T cell biology are most extensively studied

Many studies evaluated the expression of EPHs and EFNs in immune cells. Almost all receptors and ligands of the B family are expressed in immune cells, including lymphocytes, monocytes, dendritic cells. Some of the receptors and ligands from the A family are also expressed in immune cells (Aasheim et al., 2000; de Saint-Vis et al., 2003; Ivanov &

Romanovsky, 2006; Munthe, Finne, & Aasheim, 2004). Both Eph receptor and ephrin expression are rapidly induced in T cells within 4 to 24 hours after they are cultured in medium containing heat-inactivated fetal calf serum (Luo, Yu, Wu, & Wu, 2002; Yu, Luo, Wu, & Wu, 2003a). Furthermore, a higher percentage of the Ephb6^{+} population are found in naïve (CD45RA^{+}) T cells than that of recently activated (CD45RO^{+}) T cells (Gurniak & Berg, 1996; Holen, Nustad, & Aasheim, 2010; Luo et al., 2002). EPHs and ephrins expressed on immune cells exert their role in the immune system at multiple levels, including lymphatic valve development (Makinen et al., 2005; G. Zhang et al., 2015), T cell development and function (J. Wu & Luo, 2005), as well as fighting against infection and cancer (Ivanov & Romanovsky, 2006; Tian et al., 2015).

There are two major features for the functional roles of EPHs/EFNs in the immune system. Firstly, the expression level of receptors and ligands are tightly regulated under certain conditions to modulate their functions. For instance, ‘‘the expression of EPHA1, EPHA3, EPHB3 and EPHB4 on leukocytes decreases’’ (Ivanov, Steiner, Scheck, & Romanovsky, 2005) at later stages of inflammation, thus promoting leukocytes adhesion, extravasation, and tissue transmigration. Secondly, controversial functional results are reported upon different receptor and ligand activation. One study reveals that the activation of EPHA2 with ephrin A3-Fc leads to increased adhesion of dendritic cells to fibronectin (de Saint-Vis et al., 2003), whereas another study demonstrates that stimulation of EPHA3 resulting in the loss of cell adhesion to the same matrix protein (Smith et al., 2004).

EPHs/EFNs in T cell biology are most extensively studied among all different types of immune cells. Several Eph receptors (Ephb2, b3, b4) and ephrins (Efnb1, b2) expressed on T cell progenitors and (or) thymic epithelial cells (TECs) are shown to influence T cell maturation and thymic architecture integrity (Alfaro et al., 2015; Cejalvo et al., 2015; Luo, Charpentier, et al., 2011). Previous work in our group demonstrated that T cell-specific knockout of Efnb1 and Efnb2 results in less T cell number in the thymus as well as in the periphery (Jin, Luo, & Wu, 2014; Luo, Charpentier, et al., 2011). Using the Cre-LoxP model in mice, Cejalvo *et al.* reported that Efnb1 and Efnb2 on TECs affect the size of the thymus, especially the medullary compartment, starting at the perinatal stage (Cejalvo et al., 2015).

Several EPH/EFN family members are capable of interacting with TCR complex and playing a costimulatory role. Our earlier studies revealed that Efnb1, b2 and b3 are critical

costimulatory factors for T cell activation, which lead to increased cytokine secretion and T cell proliferation (Yu et al., 2003a; Yu, Luo, Wu, & Wu, 2003b, 2004). EFNB1 is also important for the control of lipid-rafts in malignant T cells (Jiang et al., 2008). Furthermore, Ephb6$^{-/-}$ mice show compromised T cell function manifested in lymphokine secretion, proliferation, as well as reduced EAE. Ephb6 clusters after TCR activation, and then regulates ZAP-70 activation, LAT phosphorylation and MAPK activation (Luo, Yu, Tremblay, & Wu, 2004). Besides, *in vitro* studies showed that from T cell-specific Efnb1 and b2 double knockout naive T cells are impaired in skewing into Th1 and Th17 subpopulations. In the same study, Efnb1 and b2 are revealed to modulate IL-6 signaling through the induction of STAT3 phosphorylation (Luo, Charpentier, et al., 2011). Lastly, EFNA1 expression on CD4$^+$ T cells from asthma patients is decreased, and EFNA1 suppresses Th2 cell activation (Wohlfahrt et al., 2004).

The EPHs/EFNs are known to control cell migration in different tissues, raising the possibility that they may similarly regulate lymphocyte migration. Stimamiglio *et al.* demonstrates that Ephb2 is essential for T cell progenitor migration during fetal thymus colonization (Stimamiglio et al., 2010). Ephrin A activation can alter T cell trafficking to peripheral lymph nodes *in vivo* (Sharfe et al., 2008). Further studies reveal that EFNA1 are important for both CD4$^+$ and CD8$^+$ T migration (Aasheim, Delabie, & Finne, 2005; Hjorthaug & Aasheim, 2007) and the activation Lck and Pyk2 are involved in the downstream signaling pathway. Also, CD45RO$^+$ T cells migrate towards EFNA1 better compared with CD45RA$^+$ cells (Holen et al., 2010). The signaling pathways downstream of EPHs/EFNs and chemokines can be regulated by adapter molecules including Grb2, Nck, and SLAP, which will ultimately influence cytoskeleton regulation through the members of Rho family proteins. For example, EFNA1 specifically inhibits CDC42 from the Rho family downstream of chemokine receptor CXCR4 but does not alter the receptor expression (Sharfe, Freywald, Toro, Dadi, & Roifman, 2002).

Recently, EPHs/EFNs are reported to interact with other molecules in the immune system. Maddigan *et al.* reported that EPHB3 is consistently expressed by malignant T lymphocytes and such expression lead to strong suppression of Fas-induced apoptosis (Maddigan et al., 2011). EPHB2 expressed on dendritic cells is also reported to be modulated by the ligation of TLR4 and TLR9 (Mimche et al., 2015). Moreover, our group has demonstrated that Efnb1/b2

expressed on T cells can physically interact with IL-7Rα and retard its internalization (Luo, Wu, et al., 2011).

When studying the role of Ephb6 in T cells activation/proliferation, we want to get a full picture of molecules that may be involved in T cell activation. Thus, an unbiased DNA microarray analysis was conducted. As a result, Stra6 (stimulated by retinoic acid gene 6) and Armc5 (Armadillo repeat-containing 5) were identified. We then generated mice with these two genes knockout to study their roles in T cell biology.

1.4 STRA6

Vitamin A is required throughout life and performs diverse functions in numerous cellular activities including vision, reproduction, growth and development.

1.4.1 Vitamin A homeostasis

Vitamin A is absorbed in enterocytes in the form of vitamin A precursors (i.e. β-carotene). Once in enterocytes, vitamin A precursors can be processed into retinyl esters, which are then transferred into the lymphatic system. Retinyl esters are then taken up and processed in hepatocytes and transferred to hepatic stellate cells (HSCs) for storage. In times of dietary retinoid-insufficiency, retinyl esters stored in HSCs will be hydrolyzed back to retinol and transferred to the hepatocyte, where it can bind to retinol binding protein (RBP) (D'Ambrosio, Clugston, & Blaner, 2011).

Retinol-bound RBP (holo-RBP) in blood is maintained stably and serves as a buffering system between dietary intake of retinoids and a stable supply for tissues that need vitamin A (Kawaguchi, Zhong, Kassai, Ter-Stepanian, & Sun, 2012). RBP receptors, such as STRA6, mediates cellular vitamin A uptake (Kawaguchi et al., 2007). Besides holo-RBP, retinoids in circulation can also present in other forms by binding to lipoproteins and albumin. However, up till now, how retinoids are transferred into cells remains elusive.

Once inside a cell, oxidization occurs, which will change retinol into retinal (ADH mediated). Further oxidation might happen, which results in the production of retinoic acid (RALDH mediated) (Mic, Molotkov, Benbrook, & Duester, 2003). There are two critical active derivatives of vitamin A to elicit its biological function, the aldehyde form and the acid form.

The aldehyde form is the chromophore for visual pigments in the eyes. The aldehyde form also modulates adipogenesis (von Lintig, Kiser, Golczak, & Palczewski, 2010; Ziouzenkova et al., 2007). The acid form, through its binding to the nuclear retinoic acid receptors, has the most diverse functions (Napoli, 1996; Stock, Napolitani, & Cerundolo, 2013).

1.4.2 STRA6 expression pattern and structure

The existence of a cellular receptor for RBP, which can transfer holo-RBP from the blood to the cytoplasm of target cells by crossing the cell membranes, was postulated as early as 1970's (Heller & Bok, 1976). Thirty years later, Kawaguchi *et al.* reveals that RBP bind to membrane protein STRA6, which mediates the cellular uptake of vitamin A (Kawaguchi et al., 2007). Accumulating evidence shows that STRA6 is expressed in a variety of tissues including the brain, testis, ovary, kidney and spleen (Bouillet et al., 1997). Recently, it is described that *STRA6* homologs are encoded in all known genomes of vertebrates (J. Wu et al., 2014).

STRA6 is a 74 kDa multi-transmembrane protein, which contains a short N-terminal tail in the extracellular space, nine transmembrane segments, and a C-terminal tail in the cytosol (Kawaguchi, Yu, Wiita, Honda, & Sun, 2008; Kawaguchi, Yu, Wiita, Ter-Stepanian, & Sun, 2008). Two large intracellular loops, as well as one large extracellular loop, are found with the presence of several smaller loops on each side of the membrane (Kawaguchi, Zhong, Kassai, Ter-Stepanian, & Sun, 2015). STRA6 binds to RBP with high affinity. Alanine-scanning mutagenesis reveals that the large extracellular loop between transmembrane segments 6 and 7 of STRA6 is the region for RBP binding (Kawaguchi, Yu, Wiita, Honda, et al., 2008).

1.4.2.1 STRA6 is a cellular transmembrane retinol binding protein for vitamin A transport

The mechanism of STRA6 in vitamin A exchange in and out of cells assembles the gate for ion channels (Kelly & von Lintig, 2015). Proteins such as lecithin retinol acyl transferase (LRAT), cellular retinol binding protein-1 (CRBP1), are important in assisting STRA6 functions. LRAT can enhance the retinol transfer into cells mediated by STRA6 (Isken et al., 2008). And the enhancement depends on the ratio of holo-RBP to STRA6. This phenomenon is possibly because of the accumulation of retinol in cells inhibits its further intake

(Kawaguchi et al., 2011). Moreover, retinol uptake in Lrat knockout mice is impaired (Amengual, Golczak, Palczewski, & von Lintig, 2012).

STRA6 also couples effectively with CRBP1 and CRBP2 for retinol efflux. In the presence of apo-RBP, STRA6 can promote the loading of retinol onto RBP (Kawaguchi et al., 2012). Pure apo-RBP can completely deplete retinol taken up by CRBP1 in an STRA6-dependent manner. STRA6 influx activity can be inhibited by free retinol outside the cells (Kawaguchi et al., 2011).

In 2012, the first Stra6 strain knockout mice were established (Ruiz et al., 2012). "Stra6 knockout mice are viable" (Amengual et al., 2014). When bred on diets repleted in vitamin A, Stra6 null mice have normal eye diameter, but display a reduction of retinal thickness. Also, in Stra6 KO mice, both retinol and its derivatives are decreased in both RPE and neurosensory retina (Ruiz et al., 2012). Also, most peripheral tissues do not display altered retinoid levels in Stra6 KO mice, indicating that Stra6 might not be the only vitamin A transport mode in these tissues (Amengual et al., 2014).

Mathew Wood Syndrome (MWS) is a rare clinical entity, which is mainly featured by "severe bilateral microphthalmia, pulmonary dysplasia, cardiac defects and diaphragmatic hernia" (Kelly & von Lintig, 2015). MWS is highly associated with mutations in STRA6 gene. Berry *et al.* reports that STRA6 can also bind to RBP-retinol and trigger the activation of JAK2/STAT5 signaling, indicating its potential role in type 2 diabetes (Berry, Jin, Majumdar, & Noy, 2011).

1.4.2.2 RBPR2 is a new retinol binding protein other than STRA6

In addition to STRA6, another RBP receptor was newly identified in 2013, namely RBPR2 (RBP4 receptor-2, RBPR2) (Alapatt et al., 2013). Overall, the amino acid sequence homology between murine Rbpr2 and Stra6 is 17.8%. Among them, some short fragments of the amino acid are more than 50% homology to Stra6. The expression of Rbpr2 is highest in liver, followed by small intestine, jejunum and ileum, and colon. Its expression is also inducible when preadipocytes are driving into adipocytes *in vitro*. RBPR2 is also a transmembrane receptor, which confers high RBP4 binding affinity and retinol transport, whereas Rbpr2 knockdown reduces both of these abilities (Alapatt et al., 2013). Further studies are needed for its role in vitamin A homeostasis.

1.4.3 Role of vitamin A and STRA6 in T cell function

Retinoic acid is the active form of vitamin A in the immune system. Retinoic acid can bind to retinoic acid receptors (RAR), retinoid X receptors (RXR) expressing on nuclei, and also, under certain conditions, PPARβγ (Schug, Berry, Shaw, Travis, & Noy, 2007). RXRs (RXRα, β and γ) can only bind to 9-*cis*-retinoic acid, whereas RARs (RARα, β and γ) bind and respond to both *all-trans*-RA and 9-*cis*-retinoic acid with high affinity (Allenby et al., 1993). RARs heterodimerizes with RXRs upon the ligation of a retinoic acid to modulate gene expression by binding to retinoic acid responsive elements located in the regulatory regions of target genes.

Recent evidence from studies utilizing retinol acid therapy or different animal models of retinoid receptor deficiency reveal that retinoic acid is critical for T cell activation, effector functions, and homing (Dawson et al., 2009; Hall, Cannons, et al., 2011; Yamada, Mizuno, Ross, & Sugawara, 2007). Retinoic acid stimulates human T lymphocytes proliferation through the IL-2-induced signaling pathway (Engedal, Gjevik, Blomhoff, & Blomhoff, 2006). Both RARα and γ are expressed in naïve $CD4^+$ T cells, whereas only RARα is critical for T cell activation (Dzhagalov, Chambon, & He, 2007; Hall, Cannons, et al., 2011). During infection with *Toxoplasma gondii*, a parasite inducing a strong Th1 response, vitamin A is critical for parasite clearance (Hall, Cannons, et al., 2011). Significant inhibition of Th17 cell differentiation is found in mouse small intestine in the absent of retinol acid (Cha et al., 2010). The addition of retinoic acid in culture dramatically enhances iTreg induction (Mucida et al., 2007) and retinoic acid acts as a cofactor for FoxP3 induction depending on TGF-β (Coombes et al., 2007).

Accumulating evidence demonstrates that the diet in developed countries fails to maintain homeostasis of the intestine, thus resulting in inflammatory intestinal disorders (Garrett, Gordon, & Glimcher, 2010; J. Wang & Sampson, 2011). Mice experiment support this with the fact that excess vitamin A in the diet is associated with a higher chance of inflammatory immune disorders (Hall, Grainger, Spencer, & Belkaid, 2011). Retinoic acid acts as a co-adjuvant in activating dendritic cells (DePaolo et al., 2011).

1.5 ARMC5

1.5.1 Characteristic of Armadillo repeat

Armadillo (ARM) was first found influencing the segmentation of Drosophila larva (Nusslein-Volhard & Wieschaus, 1980; Riggleman, Wieschaus, & Schedl, 1989). The core component of Armadillo protein is called "Armadillo repeat," which is a ~42-amino acid sequence motif (Peifer, Berg, & Reynolds, 1994). There are three alpha helices in each ARM repeat. More than one ARM repeats join tandemly to form ARM domain, which is a right-handed superhelix. ARM domains provide diverse protein-protein interactions in versatile cell biology process (Huber, Nelson, & Weis, 1997) (Figure 1.3). ARM domain-containing proteins are a large protein family, which can be found from yeast to humans. Armadillo protein family members are not necessarily identical in gene sequence at high percentages, but they share a similar structures (Tewari, Bailes, Bunting, & Coates, 2010). This family of proteins, like β-catenin, p120 catenin, etc., can bind to classical cadherins using their ARM domain, thus playing pivotal roles in controlling cell adhesion (Franz & Ridley, 2004; Gates & Peifer, 2005; W. Xu & Kimelman, 2007). The stability of β-catenin is the key to the canonical Wnt signaling pathway, which controls carcinogenesis and embryonic development (Clevers & Nusse, 2012). Importin-α, an ARM-domain-containing protein, serves as a binding scaffold for the transportation of hundreds of proteins into the nucleus (Goldfarb, Corbett, Mason, Harreman, & Adam, 2004). ARM-proteins, such as ARMC8, act as E3 ubiquitin ligases in protein degradation (Tomaru et al., 2010). Moreover, ARM-proteins can also form protein complexes, which act as adapters or molecular chaperones (D'Andrea & Regan, 2003; Szretter et al., 2010).

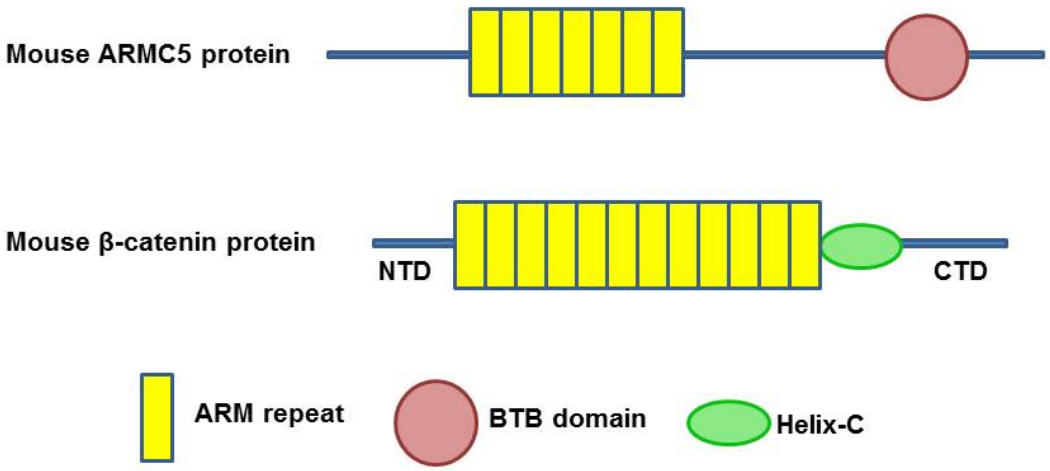

Figure 1.3 Diagram and structure of ARMC5 and β-catenin

1.5.2 Structure and function of β-catenin

The mouse β-catenin consists of 781 amino acids with twelve ARM-repeats. Distinct terminal domains can be found at both ends of its ARM domain, namely the amino-terminal domain (NTD) and the carboxyl-terminal domain (CTD) (Figure 1.3). NTD serves as the glycogen synthase kinase 3β (GSK3β) phosphorylation site, while CTD provides the binding sites for multiple proteins related to the function of β-catenin in transcription regulation. β-catenin is important in cell adhesion and plays a crucial role in the Wnt signaling pathway.

In cadherin-based adherent junctions, β-catenin uses its ARM domain to interact with the cytoplasmic part of cadherin, and also binds to α-catenin at the same time, to regulate the actin cytoskeleton. Moreover, this function is conserved among species.

β-catenin is a central molecule in the Wnt signaling pathway. With the activation of the Wnt signaling pathway, β-catenin will travel from cytoplasm to the nucleus, where it can bind to

multiple transcription factors to exert its function (Clevers & Nusse, 2012; Valenta, Hausmann, & Basler, 2012) .

Numerous evidence shows that the dysregulation of β-catenin signalling contributes to multiple malignancies, including colon cancer, prostate cancer melanoma, and endometrial cancer, *etc.* (H. Huang et al., 1999; Kaler, Augenlicht, & Klampfer, 2012; Miyaki et al., 1999). β-catenin mutations are frequently observed in these cancers. These mutations lead to increased stability of β-catenin. β-catenin knockout mice are embryonic lethal during the gastrulation stage, as no mesoderm formation is observed (Haegel et al., 1995). Tissue- or cell-specific knockout mice reveal that it is essential for tissue homeostasis and normal cell function (Behari et al., 2010; Gao, Arlotta, Macklis, & Chen, 2007; Kato et al., 2011).

1.5.3 Role of β-catenin and other ARM proteins in T cell function

The Wnt signaling pathway is critical for regulating of DC mutation, for lymphocytes development in the central lymphoid organs, and also for T cell activation in peripheral lymphoid tissues. However, recent studies have observed controversial results, especially in T cell biology. By Lck-Cre drive T cell-specific deletion of β-catenin, Xu *et al.* reports that T cell development in the thymus is impaired (Y. Xu, Banerjee, Huelsken, Birchmeier, & Sen, 2003). However, this is not supported by other studies (Cobas et al., 2004). Huang and colleagues report increased CD4 SP T cells in the thymus by forced expression of stabilized β-catenin (Z. Huang et al., 2006). TCF-1 and β-catenin are required to promote Th2 differentiation by stimulating GATA-3 expression. On the other hand, TCF-1 blocks Th1 differentiation by repressing IFN-γ. Moreover, TCF-1 is negatively regulated by T-bet (Oestreich, Huang, & Weinmann, 2011). By using retroviral constructs to express the stable form of β-catenin, Ding *et al.* reported that the stable β-catenin-expressing Treg cells show enhanced function in protecting mice against inflammatory bowel disease. T effector cells, on the other hand, become anergic with stable β-catenin expression (Ding, Shen, Lino, Curotto de Lafaille, & Lafaille, 2008). In contrast, a study from Zhao *et al.* does not support this (D. M. Zhao et al., 2010). They report that stabilized β-catenin expression is necessary for increased Ag-specific CD4 T cells in response to *Listeria monocytogenes* infection. In this study, enforced overexpression of β-catenin together with TCF-1 results in higher percentages and increased numbers of Ag-specific memory $CD8^+$ T cells. Heightened secondary expansion of

Ag-specific memory $CD8^+$ T cells and increased bacteria clearance are found after rechallenging in β-catenin and TCF-1 double transgenic mice. However, using genetic approaches of gain- and loss-of-function studies with adenoviral vectors or loxP-mediated T cell specific β-catenin KO mice, β-catenin is demonstrated to be dispensable for $CD8^+$ T cell memory function (Driessens, Zheng, & Gajewski, 2010; Prlic & Bevan, 2011).

Several explanations may be worth considering for the controversial results described above. Firstly, the different experiment conducted in different labs may result in different expression levels and different function levels of β-catenin in cells. β-catenin with partial deletion at the C-terminus can still interact with the histone acetylases CBP/p300 (De Vries et al., 2004). Secondly, at some stage of T cell development, alternative pathways are more easily triggered to compensate for the dysfunction caused by the gain- and loss-of-function studies. While in other stages or cells, this may not happen.

1.5.4 ARMC5 published papers mainly focus on the association of its mutations and PMAH

The mouse armadillo repeat containing 5 is an intracellular protein containing 926 amino acids (aa) with seven tandem copies of armadillo repeats from 139aa to 440aa, and 1 BTB domain lies in 745aa-813aa ("http://www.uniprot.org/uniprot/Q5EBP3,") (Figure 5).

Currently, all the publications related to ARMC5 are those describing its mutation being associated with human primary macronodular adrenal hyperplasia (PMAH), which is a rare cause of Cushing's syndrome (CS). Assie and colleagues first detect *ARMC5* mutations in tumors obtained from 18 of 33 patients (Assie et al., 2013). In this study, one germline mutation and one somatic *ARMC5* mutation are identified. The association of *ARMC5* mutations with PMAH is further confirmed by three independent studies in the US (Faucz et al., 2014), Brazil (Alencar et al., 2014), and Australia (Gagliardi et al., 2014). *ARMC5* mutations are detected in 4 of 5 familial PMAH cases (Gagliardi et al., 2014). Comparing to their wild-type counterparts, *ARMC5*-mutated PMAH patients show more frequent overt CS as well as bigger and higher number of nodules (Espiard et al., 2015).

How does *ARMC5* mutation lead to the development of PMAH? Transcriptome analysis of 10 tumor specimens from PMAH suggests a group of genes related to RNA processing is significantly enriched in the *ARMC5* mutated group comparing with nonmutated controls,

indicating the effect of ARMC5 in gene expression. Higher levels of apoptosis are found with the overexpression of nonmutated *ARMC5* gene in adrenocortical cells. In contrast, overexpression of ARMC5 mutations failed to do so (Assie et al., 2013). These findings could only partially explain the increased cell numbers in PMAH. The function of ARMC5 in adrenal gland and other organs remains obscure.

1.6 Hypotheses

Hypothesis 1. Efnb1/b2 is essential in 1) T-cell help for Ab production, and 2) T-cell chemotaxis, which will result in reduced CIA pathogenesis. The T cell-specific *EFNB1* and *EFNB2* expression might be used as a marker for the RA disease activity.

Hypothesis 2. Stra6 is critical in transporting vitamin A into lymphoid cells. Lack of *Stra6* may lead to impaired T cell function.

Hypothesis 3. Armc5 is critical in fetal development, and T-cell biology. ARMC5 functions depend on its interaction with molecules in different signaling pathways.

1.7 Research Objectives

1. To explore the mechanisms of Efnb1/Efnb2 expressed in T cells in the pathogenesis of CIA. To check whether the T cell specific *EFNB1* and *EFNB2* can be useful marker of RA disease activity.

2. To understand the potential job that Stra6 do in T cell function and anti-virus response.

3. To study the functional roles and molecular mechanisms of Armc5 in T cell biology.

Chapter 2 Article-1

The role of EFNB1 and EFNB2 in mouse collagen-induced arthritis and human rheumatoid arthritis

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2.1 The role of EFNB1 and EFNB2 in mouse collagen-induced arthritis and human rheumatoid arthritis

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Abstract

Objective EFNB1 and EFNB2 are ligands of EPH receptor tyrosine kinases. We investigated how their expression on T cells influences the pathogenesis of collagen-induced arthritis (CIA) in mice, and assessed correlations between the T-cell expression of these 2 molecules and rheumatoid arthritis (RA) disease activity.

Methods CIA was studied in mice with T cell-specific deletion of both Efnb1 and Efnb2 (dKO) mice. *Efnb1* and *Efnb2* mRNA expression in peripheral blood T cells from patients with rheumatoid arthritis was determined by reverse transcription/quantitative polymerase chain reaction.

Results In dKO mice, CIA clinical scores were reduced compared to wild type (WT) controls. Serum collagen-specific antibody (Ab) titres in dKO mice were lower than those in WT controls. On an equal-cell-number basis, dKO T cells provided vastly inferior help to B cells for collagen-specific Ab production *in vitro*. dKO T cells were compromised in their ability to migrate to arthritic paws *in vivo* and in their ability of chemotaxis towards CXCL12 *in vitro*. Deletion mutation of Efnb1 and Efnb2 intracellular tails revealed critical regions in controlling T-cell chemotaxis. T cells from RA patients expressed higher *EFNB1* mRNA levels which correlated with RA symptoms and laboratory findings.

Conclusions We have found that Efnb1 and Efnb2 in T cells were essential for pathogenic Ab production and for T-cell migration to inflammatory paws in mouse CIA. Our clinical study suggests that the expression of EFNB1 in T cells might be a useful parameter for monitoring RA disease activity and treatment responses.

Introduction

Rheumatoid arthritis (RA) is a polygenic autoimmune disease characterized by chronic, systemic inflammation that can affect a number of tissues and organs, but the joints and surrounding tissues are most frequently impacted. The pathogenesis of RA is not fully understood. Toll-like receptor agonists, such as proteoglycans and bacterial DNA, might play an etiological role in initiating the disease [1]. Some auto-Abs are RA pathogenic [2]. Various inflammatory cytokines, such as tumour necrosis factor-alpha, interleukin-1beta (IL-1β) [3], IL-6 [4] and IL-17 [5] are produced within the synovium by infiltrating leukocytes including T cells. Local macrophage-like synovial fibroblasts (SF) are key mediators of inflammation and tissue destruction in RA. In mice with severe combined immunodeficiency, activated SF are sufficient to invade and damage cartilage [6]. This indicates that, at a later stage in RA, inflammation and destruction can be mediated and sustained by SF.

EPHs comprise the largest family of cell surface receptor tyrosine kinases, representing about 25% of known receptor tyrosine kinases [7]. There is a total of 15 EPHs, classified by sequence homology into subfamilies A with 9 members, and B, with 6 members. EPHs bind to their EFN ligands, which are also cell surface molecules [7]. The 9 known EFNs are divided into A and B subfamilies based on the manner in which they anchor to the cell surface. Interactions between EPHs and EFNs are promiscuous. One EPH can interact with multiple EFNs and *vice versa*. In general, EPHA members preferentially interact with EFNA members, and EPHB members with EFNB members [8, 9].

EPHs can initiate signal transduction via their intracellular domain upon ligand binding (forward signaling). Also, EPHs can cross-talk with other receptor tyrosine kinases [10, 11], chemokine receptors [12] and adhesion molecules [13]. Their ligands, EFNs, can transduce signals into cells [8, 9] by a phenomenon called reverse signaling. Upon activation by EPHs, EFNs can rapidly undergo tyrosine phosphorylation and recruit adaptor proteins to start the signaling cascade [14, 15]. EFNBs can also transmit reverse signaling through the C-terminal PDZ domain-binding motif, which binds to PDZ domain-containing proteins [12, 16-18]. Like EPHs, EFNs can interact, directly or indirectly, with other cell surface molecules, such as chemokine receptors [19, 20].

EPHs and EFNs have crucial functions in many organs, systems and processes, such as the central nervous system [8, 9], angiogenesis [21], bone metabolism [22], intestinal epithelial cell maturation [23], kidney glomerular filtration [24], insulin secretion [25], blood pressure regulation [26, 27], etc.

We and others have reported that EPHs and EFNs, particularly their B family members as well as some A family members, are important in the immune system [28]. We have shown that EFNB1, EFNB2 and EFNB3 initiate signaling through their EPH receptors and can co-stimulate peripheral T cells [29-31]. We have reported that EPHB6 can transmit signals into T cells [32] and that its null mutation compromises T-cell responses in *vitro* and *in vivo* [33]. We have generated T cell-specific *Efnb1/Efnb2* double gene knockout mice and have observed that their T-cell development and function are compromised [19, 20].

In the present study, we investigated roles of EFNB1 and EFNB2 in the pathogenesis of RA using both animal models and human clinical studies.

Materials and Methods

Mouse CIA induction, clinical scoring and histology

The methodology for the mouse CIA model as well as its clinical and histology scoring has been described previously [34]. The mice were either in the C57BL/6 background or in the DBA/1LacJ background. Chick type II collagen (CTIIC; Chondrex, Redmond, WA) was used as an immunogen (with CFA on day 0, and with IFA on day 20 for boost) for the former strain, and bovine type II collagen (BTIIC; Chondrex), for the latter (with CFA on day 0).

Enzyme-linked immunosorbent assay (ELISA) for anti-CTIIC Ab titres

The method for andti-CTIIC Ab measurement was detailed in our previous publication [34].

Flow cytometry

Rat anti-mouse PD-1 monoclonal antibody (mAb), rat anti-mouse GL7 mAb, rat anti-mouse CD40L mAb and rat anti-mouse CXCR5 mAb were from BD Biosciences (Mississauga, ON, Canada). The remaining mAb used in flow cytometry are described in a previous publication [19].

T-B cell co-culture

Draining LN (dLN; inguinal and iliac) were harvested from WT and dKO mice 35 days after immunization with CCTII/CFA. T cells from dLN were enriched by Mouse T Cell Enrichment Kit (Stemcell Technologies, Vancouver, BC, Canada), and B cells, by Mouse B Cell Enrichment Kit (Stemcell Technologies). Purified T and B cells were mixed at different ratios and cultured in the absence or presence of T-cell proliferation grade CTIIC (50 μg/ml). The culture supernatants were harvested at different days for the measurement of CTIIC-specific IgG.

T-cell transfer and isolation from paws

C57BL/6 X B6.SJL F1 mice ($CD45.1^+CD45.2^+$) were immunized with CTIIC to induce CIA as described above. On day 35 after the first immunization, a mixture of (10-20) $x10^6$ T cells from non-immunized WT B6.SJL ($CD45.1^+$) mice and non-immunized EFNB1B2 dKO ($CD45.2^+$) mice at a 1:1 ratio was transferred to the immunized mice. Twelve hours after cell injection, the mice were perfused with 20 ml PBS under anesthesia to remove peripheral blood cells in paws. The paws were harvested, skinned and minced into small pieces, which were then digested with collagenase II (2 mg/ml, Chondrex) and dispase II (250 μg/ml, Roche

Diagnostics, Indianapolis, IN) in 15 ml Hank's balanced salt solution at 37°C in a shaker-incubator at 250 rpm for 40 min. The digested product was washed and passed through cell strainers (BD Bioscience) of 70-μm pore size. Cells isolated from paws as well as those from blood, spleen, and mesenteric, inguinal, and iliac lymph nodes were analyzed by flow cytometry.

Lentivirus preparation and infection

PCR-based deletion mutations of *Efnb1* and *Efnb2* intracellular tails were generated and were cloned into pLenti CMV/TO PGK GFP Destination vector (Addgene, Cambridge, MA). These constructs were transfected into HEK 293T cells along with packaging constructs pLp1, pLp2 and pLpSV. Viruses were isolated from the supernatants 72 h later. T cells from LN of non-immunized WT mice were isolated and stimulated with soluble anti-CD3 mAb (0.05 μg/ml; clone 145-2C11, BD Biosciences). After 12-16 h, the T cells were infected with lentiviruses in 96-well plates ($0.8x10^6$ cells/well) in the presence of 10 μg/ml polybrene infection/transfection reagent (Millipore, Billerica, MA) for the expression of full-length or deletion mutants of Efnb1 and Efnb2. They were cultured in the presence of 0.05 μg/ml anti-CD3 mAb for an additional 48 h and then underwent *in vitro* chemotaxis assay.

In vitro T-cell chemotaxis assay

LN T cells or spleen T cells ($0.7x10^6$) without or with lentivirus infection were placed on the top chamber of Transwell (pore size 5 μM; Corning, Mississauga, ON, Canada) in 100 μl RPMI 1640 medium containing 0.5% BSA and 10 nM HEPES. The bottom chamber was filled with 600 μl of the same medium but contained CXCL12 (200 ng/ml, R&D Systems). The Transwell plates were incubated for 2 h at 37°C. The numbers of total input cells and cells that migrated into the lower chamber were counted 3 times by flow cytometry. Mean percentages of migrated T cells were calculated according to the following formula:

% of migrated cells = 100 x cells in the bottom chamber at the end of assay/total input cells at the beginning of assay

Patients and healthy controls

Thirty-two patients diagnosed with RA, according to 2010 American College of Rheumatology against Rheumatism classification criteria for RA [35], were recruited from the Rheumatology Clinic of Sichuan Provincial People's Hospital, West China Hospital, and the

General Hospital of Chengdu Military Region in Chengdu, China. None of the patients was treated with disease modifying anti-rheumatic drugs at the time of the study. Patients with renal insufficiency or under corticosteroid treatment were excluded. Twenty-eight gender- and age-matched healthy volunteers were recruited as controls. The numbers of tender and swollen joints were ascertained after examination of 28 joints.

Reverse transcription-quantitative PCR (RT-qPCR) for human T cell EFNB1 expression

Peripheral blood mononuclear cells were isolated from heparinized blood by Ficoll density gradient (Axis-Shield, Oslo, Norway). T cells were then purified by T Cell-Negative Selection Kit (Miltenyi Biotec, Auburn, CA). *EFNB1* mRNA levels in T cells were assessed by RT-qPCR; the primers used were 5'-AAG AAC CTG GAG CCC GTA TC-3' and 5'-AAC ACG TTG GGG TCG AGA AC-3'. *GAPDH* mRNA expression served as internal control, using primers 5'-GTG AAC CAT GAG AAG TAT GAC AAC-3' and 5'-CAT GAG TCC TTC CAC GAT ACC-3'. The qPCR condition was: 10 min at 95℃, followed by 40 cycles of 3 steps: 10 s at 95℃, 15 s at 60℃, and 20 s at 72℃. mRNA expression was normalized to GAPDH expression levels, and relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method [36].

Results

Reduced CIA pathogenesis in dKO mice

We investigated whether T cell-specific Efnb1 and Efnb2 dKO would affect CIA pathogenesis. dKO in the C57BL/6 background showed delayed CTIIC-induced CIA onset as well as significantly lower clinical scores than WT (Fig. 2.1A, left panel). The CIA incidence of the dKO mice in this model was moderately lower than that of the WT mice. In the last 3 days of the experiment (days 57-60), there seems to be a significant drop of CIA incidence in the dKO mice (Fig. 2.1A, right panel). It is to be noted that the CIA scores in the dKO mice of the C57BL/6 background were very low (around 0.5). Therefore, a small variation will result in the disappearance of CIA manifestation, hence a drastic drop in disease incidence. Thus, the drop of the CIA incidence in the last 3 days, although seems quite big in the diagram, does not reflect a meaningful difference in CIA clinical scores. A more robust BTIIC-induced CIA using dKO mice in the DBA/1LacJ background was also employed with the clinical scores of WT mice reaching 14. In this CIA model, dKO mice also had significantly lower clinical scores than their WT counterpart, although the disease incidence of the dKO and WT mice was similar (Fig. 2.1B).

The CIA mice in the C57BL/6 background were sacrificed on day 60, and their paws underwent histopathological examination. As illustrated in Fig. 1C, dKO paws presented less synovial membrane hyperplasia (asterisks), less immune cell infiltration and mild cartilage erosion (arrowheads). Total pathological, lining hyperplasia, bone destruction and cell infiltration scores of dKO paws were significantly lower than those of WT paws (Fig. 2.1D). These results clearly show that EFNB1 and EFNB2 dKO in T cells leads to decreased CIA severity.

Lower collagen-specific Ab titres in dKO mice

Auto-Abs contribute significantly to RA pathogenesis [37, 38], and anti-collagen Ab are pathogenic in CIA. We assessed CTIIC-specific Ab titres in dKO and WT mice in the C57BL/6 background after CTIIC immunization. CTIIC-specific total IgG, IgG_1, IgG_3 and IgM titres in dKO sera on day 14 after immunization were significantly lower than in WT controls (Figs. 2.2A, 2B, 2C and 2D). Although CTIIC-specific IgG_{2a}, IgG_{2b} and IgG_{2c} titres in dKO mice were lower than those of WT controls, they did not reach statistical significance

(Data not shown). At the later time points than day 14, the CTIIC-specific IgG titres in the dKO and WT sera became similar (data available upon request).

Consistent with findings of lower CTIIC-specific Ab titres, on day 14 after initial CTIIC immunization, dKO mice presented significantly reduced percentages of $Fas^{+}GL7^{+}$ germinal centre (GC) B cells (Fig. 2.3A), and $B220^{low}CD138^{+}$ plasma cells (Data available upon request) in dLN, compared to their WT counterparts, although no consistent decrease of these cells was seen in either spleen (for both cell types) or bone marrow (for plasma cells).

dKO T cells failed to provide help to B cells

Follicle T helper cells (Tfh), a T-cell subset, are important in GC B cell differentiation to Ab-producing plasma cells [39, 40]. Tfh development was assessed in mice 14 days after CTIIC/CFA immunization in mice in the C57BL/6 background, a midpoint between time of immunization and full-fledged CIA. dKO dLN were smaller in size (data not shown), and the absolute numbers of T cells in dLN were significantly lower (Fig. 2.3B) than in their WT counterparts. The percentage of $CRCXR5^{+}$/$PD\text{-}1^{+}$ Tfh cells in dLN was significantly lower in dKO mice than in WT controls (Fig. 2.3C), although no such difference was discerned in the spleen (data not shown).

As dKO mice have fewer T cells in the spleen and LN [19], we wondered whether, on an equal-number basis, dKO T cells were compromised in providing help to B cells in Ab production. T and B cells were isolated from WT and dKO mice (in the C57BL/6 background) 35 days after the initial CTIIC/CFA immunization. WT or dKO T cells were cultured with B cells from WT or dKO mice at 1:1 and 1:5 T/B cell ratios in the absence or presence of CTIIC for 7 days. Collagen-specific IgG titres were measured in the supernatants. As seen in Fig. 3D, in the absence of CTIIC, little collagen-specific IgG was produced in all combinations. The presence of collagen boosted collagen-specific IgG production in WT T and WT B cell co-culture, but did not in dKO T and WT B cell co-culture. In these combinations, as the B cells in these combinations were all from immunized WT B cells, failed CTIIC-specific Ab production in dKO T and WT B cell co-culture was clearly due to lack of help from dKO T cells. Since the absolute input T-cell number of WT and dKO T cells was the same in this co-culture experiment, this result indicates that, on a per-cell basis, dKO T cells are compromised in providing help to B cells in arthrogenic Ab production, compared to WT T cells. When WT T cells were cultured with B cells from dKO mice, the CTIIC-specific Ab titres were very low

either in the presence of absence of CTIIC. This suggests that CTIIC-specific B cells from CTIIC-immunized dKO were not sufficiently primed and/or expanded *in vivo*. Since the EFNB1 and EFNB2 deletion was T-cell specific, the compromised priming/expansion of B cells from dKO mice is likely due to insufficient T-cell help received during *in vivo* immunization before the B cells were isolated. No CTIIC-specific Ab was detectable in the culture with T cells and B cells all from dKO mice, because the compromised *in vitro* help by dKO T cells is compounded by defective priming/expansion of B cells from the dKO mice during the immunization.

Reduced T-cell infiltration in arthritic joints of dKO mice

We isolated infiltrating cells from CIA paws, and flow cytometry found that dKO mice paws contained fewer T cells (Thy1.2$^+$; Fig. 2.4A). These findings corroborate our histologic observations (Fig. 2.1C).

Was the lower T cell number in dKO CIA paws due to reduced migration of dKO T cells into the paws, or simply reflected lower T cell numbers in dKO mice, which was demonstrated in our previous publication [19]? We conducted a cell transfer experiment to address this question. C57BL/6 x B6.SJL F1 mice (CD45.1$^+$CD45.2$^+$) were immunized with CTIIC/CFA on day 1 and CTIIC/IFA on day 21 to induce CIA. On day 35 after the initial immunization, T cells from non-immunized WT B6.SJL (CD45.1$^+$) and Efnb1/Efnb2 dKO in the C57BL/6 background (CD45.2$^+$) mice were co-transferred *i.v.* at a 1:1 ratio to F1 CIA mice. Sixteen hours after cell transfer, cells were collected from blood, spleen, LN (mesenteric, inguinal, iliac) and paws for flow cytometry. As illustrated in Fig. 4B, the CD45.1$^+$/CD45.2$^+$ (WT/KO) T cell ratio in CIA paws was ~4 (1.11% versus 0.29%), but the ratio in blood, spleen and LN was ~2 (Fig. 2.4B), indicating that dKO T cells were compromised in their ability to migrate to arthritic paws.

We conducted *in vitro* cell migration assays to evaluate Efnb1 and Efnb2 function in CXCR4-mediated T-cell chemotaxis. dKO LN T cells showed reduced chemotaxis towards CXCL12, the CXCR4 ligand, compared to their WT counterparts (Figs. 2.5A). Conversely, when WT T cells were overexpressed with full-length *Efnb1* (Fig. 2.5B, left panel) or *Efnb2* (Fig. 2.5B, right panel) using a lentivirus-mediated expression system, the cells manifested increased migration towards CXCL12.

We generated deletion mutants of *Efnb1* and *Efnb2* with different lengths of deletions, as illustrated in Figure 2.5C. These mutants, as well as full-length *Efnb1* and *Efnb2*, were overexpressed in WT T cells using the lentivirus expression system. WT T cells overexpressing a mutant with deletion of C-terminal 16 aa (i.e., deletion of the sequence harbouring the PDZ domain-binding motif, and the last 2 Tyr residues, Y342 and Y343, of the Efnb1 intracellular tail, Efnb1-Δ2Y) did not reduce but enhanced T cell chemotaxis towards CXCL12 in *in vitro* Transwell assays, compared to T cells overexpressing full-length Efnb1 (Fig. 2.5D, left panel), suggesting the existence of a negative regulatory element in this segment. Additional deletion of a stretch harbouring Y328 and Y323 (Efnb1-Δ4Y, i.e., deletion of C-terminal 23 aa) resulted in drastically decreased chemotaxis, indicating that the Efnb1 intracellular sequence between aa 323 and aa 329 contains a critical positive element(s) controlling T cell chemotaxis towards CXCL12. A further deletion from aa 312 to aa 322 (including Y316 and Y312; EFNB1-Δ6Y; i.e., deletion of C-terminal 34 aa) did not cause an additional reduction of chemotaixis.

A similar experiment was conducted with lentiviruses expressing deletion mutants of *Efnb2* (Figure 2.5D, right panel). The results were similar, except that deletion of the sequence harbouring the PDZ domain-binding motif, Y333 and Y334 of the Efnb2 intracellular tail (Efnb2-Δ2Y, i.e., deletion of C-terminal 5 aa), did not enhance chemotaxis. Further deletion using Efnb2-Δ4Y lentivirus with deletions of C-terminal 24 aa (including Y314 and Y319 deletion) significantly reduced the chemotaxis, indicating the existence of a critical positive regulatory element between aa 313 and aa 331. A further deletion from aa 306 to aa 312 (including Y307; Efnb2-Δ5Y; i.e., deletion of C-terminal 31 aa) did not cause an additional reduction of chemotaxis.

EFNB1 expression on T cells correlates with arthritis severity in RA patients

We measured *EFNB1* and *EFNB2* expression at the mRNA level in T cells from RA patients and gender- and sex-matched healthy controls, to assess their correlation with the clinical findings and laboratory parameters. *EFNB1* (Fig. 2.6A) but not *EFNB2* (data available upon request) expression in T cells from RA patients was significantly higher than in T cells of healthy controls. *EFNB1* expression levels in RA T cells were significantly correlated with the clinical observations, such as numbers of swollen and tender joints (Figs. 2.6B). EFNB1

expression levels in RA T cells were also significantly correlated with 4 laboratory parameters (Fig. 2.6C), such as serum anti-cyclic citrullinated protein (CCP) Ab, rheumatic factors (RF), erythrocyte sedimentation rates (ESR) and plasma C-reactive protein (CRP) levels in RA patients.

Discussion

Efnb1/Efnb2 dKO mice have compromised T-cell development and present reduced numbers of peripheral T cells in lymphoid organs [19]. It was thus not surprising that dKO mice became resistant to CIA induction, as CIA is a T cell-dependent disease model. Novel findings in our present work are that, on a per-cell basis, dKO T cells are compromised in providing help to B cells for arthrogenic Ab (anti-CTIIC Ab) production, and in migrating to inflammatory joints. Previously, we also demonstrated that CD4 dKO T cells are defective in developing into Th17 cells [19], which are known to be involved in RA pathogenesis [5]. All these defects likely contribute to resistance to CIA pathogenesis in dKO mice, indicating that the role of EFNB1 and EFNB2 in RA/CIA pathogenesis is multifaceted. While deletion of the 2 genes leads to ameliorated CIA in mice, our clinical studies proved that the reverse is also true: increased *EFNB1* expression in T cells correlates with RA disease activity. The upregulation of EFNB1 is unlikely caused by the inflammatory environment in RA, because our mouse data showed that inflammatory cytokines such as IL-6, TNFα or IL-17 did not affect Efnb1 or Efnb2 expression in T cells (data available upon request). The causative role of high EFNB1 expression in T cells in RA pathogenesis in humans has yet to be validated.

We discovered that Tfh cell percentage in dLN from dKO mice is decreased. Such a decrease could lead to reduced T cell help to B cells in B-cell differentiation towards Ab-producing plasma cells. We assessed the functionality of dKO Tfh cells, but found that their expression of ICOS and CD40L, which play important roles in B-cell differentiation [41], was comparable to that in their WT counterparts (data available upon request). Also, *in vitro*-differentiated dKO and WT Tfh cells were all capable of producing high levels of IL-21 (data available upon request), a cytokine essential in B-cell differentiation and proliferation [41]. Admittedly, the dKO Tfh cell functionality tests were not exhaustive, and they could still have so-far undetected defects. It is likely that reduced dKO Tfh percentage, absolute number, and some general help of non-Tfh cells to B cells all contributed to decreased anti-CTIIC Ab titres in dKO mice.

It is noteworthy that reduced anti-CTIIC Ab titres in dKO mice appeared on day 14 after the initial immunization, which was corroborated by reduced GC B cell and plasma cell percentages in dLN at that time point. This was a week before the beginning of CIA symptom

manifestation, starting on day 21, and was probably a critical period during which arthrogenic Abs play a role in triggering CIA pathogenesis, which, at a later stage, could become a self-sustained, vicious circle locally in arthritic joints independently of T and B cells [37, 38]. The vital roles of T cell Efnb1 and Efnb2 in humoral immune responses are probably restricted to the early stage after immunization, as decreases of GC B cells (Fig. 3A) and plasma cells only appeared in dLN but did not propagate into the spleen and bone marrow, the latter harbouring long-lived plasma cells (data available upon request) capable of sustained Ab production. Indeed, after day 21, serum anti-CTIIC Ab titres in dKO and WT mice no longer presented significant differences (data available upon request).

In addition to the reduced production of pathogenic Ab, lower inflammatory cytokine production in CIA could also be a contributing factor to the reduced CIA severity in dKO mice, since we have evidence that serum IL-6 levels in the dKO mice were lower than those in their WT counterparts in CIA (data available upon request).

We revealed that Efnb1 and Efnb2 have critical functions in T-cell chemotactic migration to inflammatory joints in CIA. Such functions are not restricted to a single chemokine (CXCL12), but seem to be generally applicable to other chemokines, such as CCL5, CCL19, CCL20 and CCL21 (data not shown).

Efnb intracellular tails are about 82-aa long and lack any enzymatic activity. Efnb intracellular tails are characterized by 2 major features: the C-terminal PDZ domain-binding motif and 5-6 Tyr residues, which could be associated with PDZ-domain-containing proteins (e.g., PDZ-RGS3, GRIP1/2, TIAM1 and DISHEVELED [12, 17, 42-44]), and SH2-domain-containing proteins (e.g., GRB4, STAT3 and CRK, respectively [15, 45, 46]). These 2 features are located in the last 34 aa for Efnb1 and last 31 aa for Efnb2, which are highly conserved. Efnbs can also have PDZ- and SH2-independent functions [14], some of which presumably depend on the SH3 domain of associating proteins (e.g., CRK and GRB4 [15, 46]). By deleting different lengths of the *Efnb1* and *Efnb2* intracellular tail, we have identified critical intracellular tail regions (i.e., from aa 323 to aa 329 in Efnb1, and aa 313 to aa 331 in Efnb2) for T-cell chemotaxis enhancement. Elements in these critical regions, such as Y323 and Y328 in Efnb1 and Y314 and Y319 in Efnb2, are probably responsible for mediating signals related to T-cell chemotaxis. The exact associating proteins involved in this function are yet to be identified, but candidates include known EFNB-associating SH2-domain-containing proteins,

such as CRK [46], GRB4 [15] and STAT3 [45]. We have also determined that the last 16 aa in the C-terminus of Efnb1 (including Y342 and Y343 plus the PDZ-domain-binding motif), and the last 5 aa in the C-terminus of Efnb2 (including Y333 and Y334 plus the PDZ-domain-binding motif) are not important for promoting T-cell chemotaxis, as their deletion did not reduce Efnb1's and Efnb2's ability to promote T-cell chemotaxis. On the contrary, deletion of the region in Efnb1 but not Efnb2 augmented T-cell chemotaxis, suggesting the existence of a negative regulator in this region. Adaptor proteins containing the PDZ domain (such as GRIP1/2, DISHEVELED, TIAM1 and PDZ-RGS3) or SH2 could be candidates for such a function.

Our clinical study showed that EFNB1 expression in peripheral T cells was correlated with RA disease activities. Although such correlation was not found between EFNB2 expression and RA disease activities, it is not totally unexpected, given the fact that EFNB2 single gene KO in T cells in mice does not result in apparent compromise of T-cell function [47]; the defective immune response is only demonstrated when both EFNB1 and EFNB2 are deleted due to the possible redundancy of their functions [20]. From an application point of view, EFNB1 expression levels in T cells could be a new parameter for monitoring disease severity and possibly treatment responses in RA patients.

In summary, we have identified new functions of Efnb1 and Efnb2 in T cells in their contribution to CIA pathogenesis in animal models, and our clinical studies suggest that EFNB1 expression in T cells might be a useful parameter for monitoring RA disease activity and treatment responses.

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Figure legends

Figure 2.1 dKO mice were resistant to CIA induction

A. CIA clinical scores and incidence of mice in the C57BL/6 background

Mean CIA clinical scores for WT (n=19) and dKO (n=16) ± SEM were plotted. *indicates significant difference ($p<0.05$, Student's *t* test). The CIA incidence was also plotted.

B. CIA clinical scores and incidence of mice in the DBA/1LacJ background

Mean clinical scores for WT (n=12) and dKO (n=9) ± SEM were plotted. *indicates significant difference ($p<0.05$, Student's *t* test). The CIA incidence was also plotted.

C. Histology of arthritic paws of CIA mice in the C57BL/6 background

dKO and WT mice paws (60 days after the first immunization) were sectioned and stained with H&E or safranin O (SO; for cartilage staining) as indicated. Representative sections from WT and dKO are shown.

D. Histological scores of arthritic paws of CIA mice in the C57BL/6 background

Sections were prepared as described in C. The histology scores of all paws from 13 WT and 9 dKO mice were blindly evaluated for lining hyperplasia (1st panel), bone erosion (2nd panel) and cell infiltration (3rd panel). The overall scores for each animal were calculated and shown in the last panel. Student's *t* tests were performed, and *p*-values are indicated.

Figure 2.2 Reduced serum collagen-specific Ab titres in dKO mice

Arbitrary titres of CTIIC-specific IgG (A), IgG_1 (B), IgG_3 (C) and IgM (D) in dKO and WT mice on day 14 after immunization were analyzed by ELISA. Numbers of mice (n) per group are indicated. Data are expressed as means ± SEM. *p*-values are reported (Student's *t* test).

Figure 2.3 dKO T cells presented less help to B cells in mice of the C57BL/6 background

A. Reduced percentage of GC B cells in dKO dLN

The percentages of $B220^+GL7^+Fas^+$ GC B cells from the dLN and spleen of WT and dKO mice 14 days after CTIIC/CFA immunization were analysed by flow cytometry. The experiments were conducted 3-5 times and representative histograms are presented.

B. Reduced absolute T-cell number in dLN of dKO mice

Total dLN cells were obtained from WT (n=4) and dKO (n=4) mice on day 14 after the initial CTIIC/CFA immunization, and absolute numbers of dLN T cells per mouse are presented in a line graph. The *p*-value is indicated (Student's *t* test).

C. Reduced Tfh numbers in dLN from dKO mice after CTIIC/CFA immunization

Percentages of PD-1$^+$CXCR5$^+$ Tfh cells among effector CD4 cells (CD4$^+$CD44$^+$CD62L$^-$) in the dLN of WT and dKO mice on day 14 after CTIIC/CFA immunization were analyzed by flow cytometry. The experiments were conducted 3 times, and representative histograms are presented.

D. dKO T cells failed to help primed B cells to produce collagen-specific IgG in vitro

CD4$^+$ T cells and B220$^+$ B cells were isolated from dLN of WT and dKO mice 35 days after the first immunization. T and B cells were mixed at a ratio of 1:1 or 1:5 as indicated, and then co-cultured in the absence or presence of 50 μg/ml CTIIC. Collagen-specific IgG in the supernatants 7 days after culture was measured by ELISA. Data are representative of 3 independent experiments.

Figure 2.4 Compromised migration of dKO T cells to arthritic paws in mice of the C57BL/6 background

A. Reduced numbers of infiltrating T cells in dKO mice paws after CTIIC/CFA immunization

On day 14 after CTIIC/CFA immunization, cells infiltrating WT and dKO mice paws were isolated, stained and enumerated by flow cytometry. The numbers of infiltrating Thy1.2$^+$ T cells per paw are shown. Mouse numbers per group are indicated. Student's *t* test assessed the statistical significance of differences between WT and dKO mice, and *p*-values are reported.

B. Compromised migration of transferred dKO T cells into arthritic paws

T cells from the spleens of naïve WT (B6.SJL) and dKO (C57BL/6) mice were isolated through negative selection. WT and dKO T cells were mixed at a 1:1 ratio, and (10-20) x 10^6 mixed cells were injected i.v. into C57BL/6 x B6.SJL F1 mice which were immunized with CTIIC/CFA 35 days earlier. Twelve hours after the injection, CD45.1$^+$ (WT donor T cells), CD45.2$^+$ (dKO donor T cells) in blood, spleen, LN (mesenteric, inguinal, iliac), and paws were analyzed by flow cytometry. The experiments were conducted independently 3 times, and representative histograms are presented.

Figure 2.5 Efnb1 and Efnb2 expression affect T cell chemotaxis towards CXCL12 using mice in the C57BL/6 background

A. Impaired dKO T cell migration towards CXCL12 in vitro

LN T cells migrated into the lower chamber (containing CXCL12) in a Transwell system were counted by flow cytometry. The means ± SEM of total migrated T cells are shown. Data are representative of 3 independent experiments.

B. Enhanced T cell chemotaxis towards CXCL12 after Efnb1 and Efnb2 overexpression

WT spleen and mesenteric LN cells were infected with lentivirus encoding full-length *Efnb1* (left panel) or *Efnb2* (right panel), or control lentivirus. The means ± SE of percentages of T cells migrated toward 200 ng/ml CXCL12 in 7 independent Transwell experiments are reported. *p*-values are shown (Student's *t* test).

C. Illustration of Efnb1 and Efnb2 deletion mutants

The general structures of Efnb1 and Efnb2 are depicted. Different deletion mutants and their nomenclatures are illustrated.

D. Critical regions in the Efnb1 and Efnb2 intracellular tail for T-cell chemotaxis towards CXCL12

WT spleen and mesenteric LN cells were infected with lentiviruses encoding full-length or deletion mutants of Efnb1 (left panel) and Efnb2 (right panel). The percentages (means ± SE) of T cells with different infections migrating towards CXCL12 in Transwell assay are plotted. The numbers of independent experiments conducted for different lentiviruses are indicated. *p*-values between different infection groups are shown (Student's *t* test).

Figure 2.6 EFNB1 mRNA levels in T cells of RA patients

A. EFNB mRNA levels are higher in T cells of RA patients than in healthy controls

T cells from PBMC of RA patients and healthy controls were analyzed for *EFNB1* mRNA expression by RT-qPCR. Arbitrary EFNB1 expression levels are shown. Each symbol represents an individual. Sample numbers (n) are shown. The long and short horizontal bars represent means ± SE, respectively. Statistical significance was assessed by Student's *t* test, and the *p*-value is indicated.

B. Correlation of EFNB1 mRNA expression in T cells with RA disease activities in RA patients

Relative *EFNB1* mRNA expression levels in RA patients were plotted against RA symptoms (numbers of tender and swollen joints per patient). The numbers of patients (n) and correlation efficiency (r^2) are indicated. The significance of correlation was analyzed by Pearson's correlation test, and *p*-values are presented.

C. Correlation of EFNB1 mRNA expression in T cells with laboratory findings in RA patients

Relative *EFNB1* mRNA expression levels in RA patients were plotted against their serum anti-CCP Ab levels, serum rheumatic factor, erythrocyte sedimentation rate and plasma C-reactive protein levels. The number of patients (n) and correlation efficiency (r^2) are indicated. The significance of correlation was analyzed by Pearson's correlation test, and *p*-values are presented.

Figures

Figure 2.1 dKO mice were resistant to CIA induction

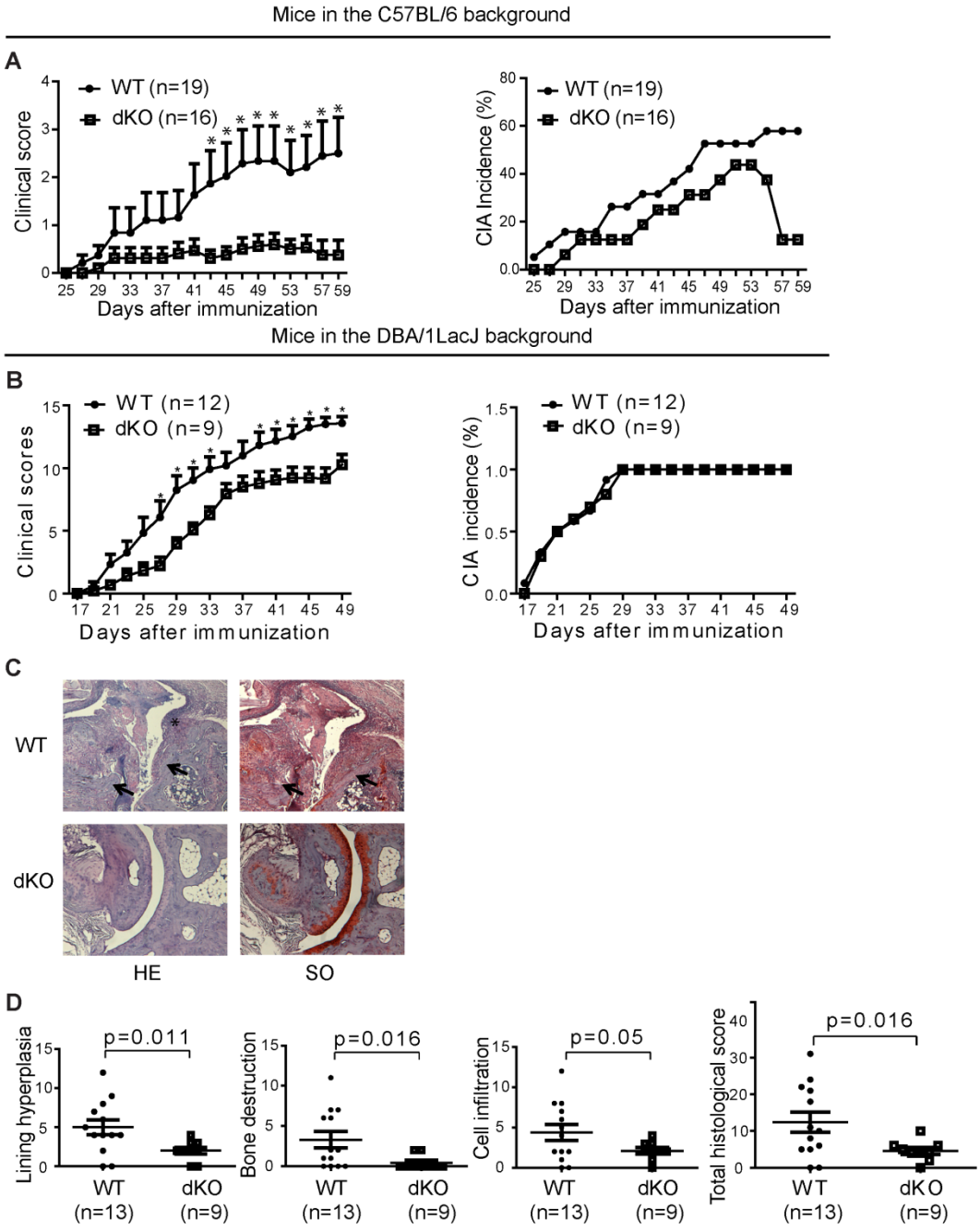


Figure 2.2 Reduced serum collagen-specific Ab titres in dKO mice

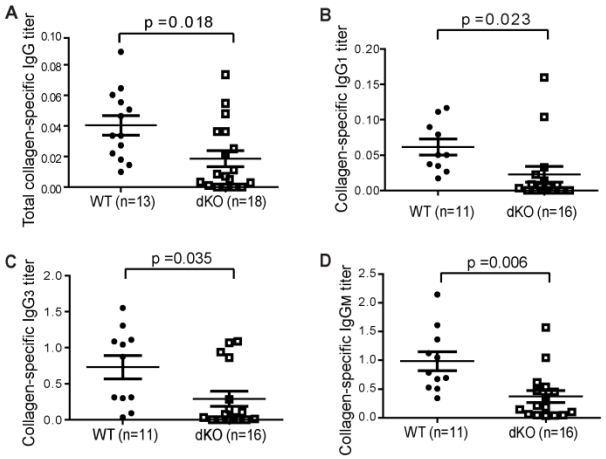


Figure 2.3 dKO T cells presented less help to B cells in mice of the C57BL/6 background

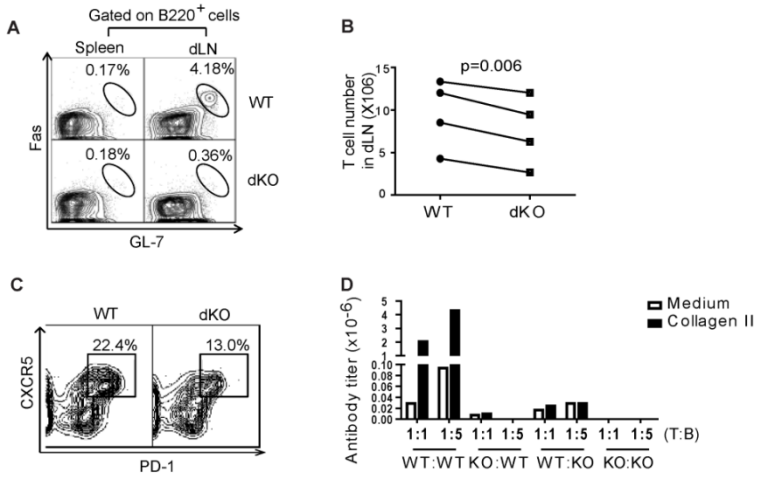


Figure 2.4 Compromised migration of dKO T cells to arthritic paws in mice of the C57BL/6 background

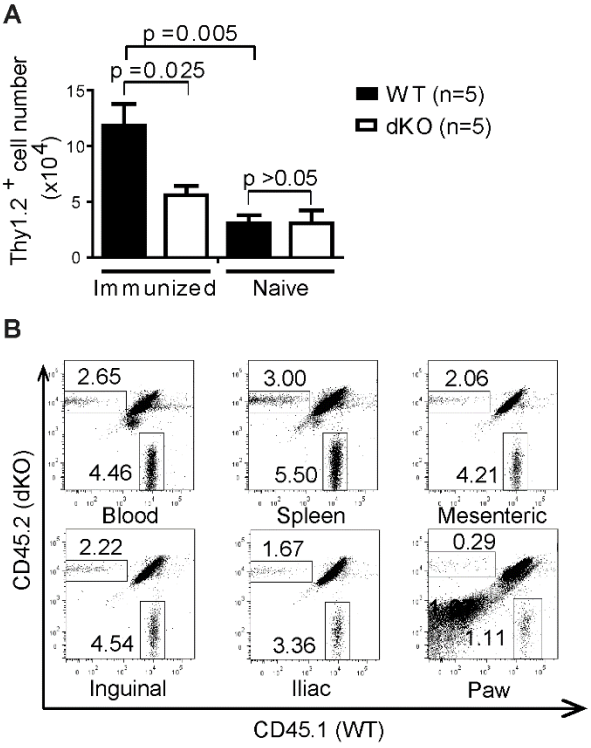


Figure 2.5 Efnb1 and Efnb2 expression affect T cell chemotaxis towards CXCL12 using mice in the C57BL/6 background

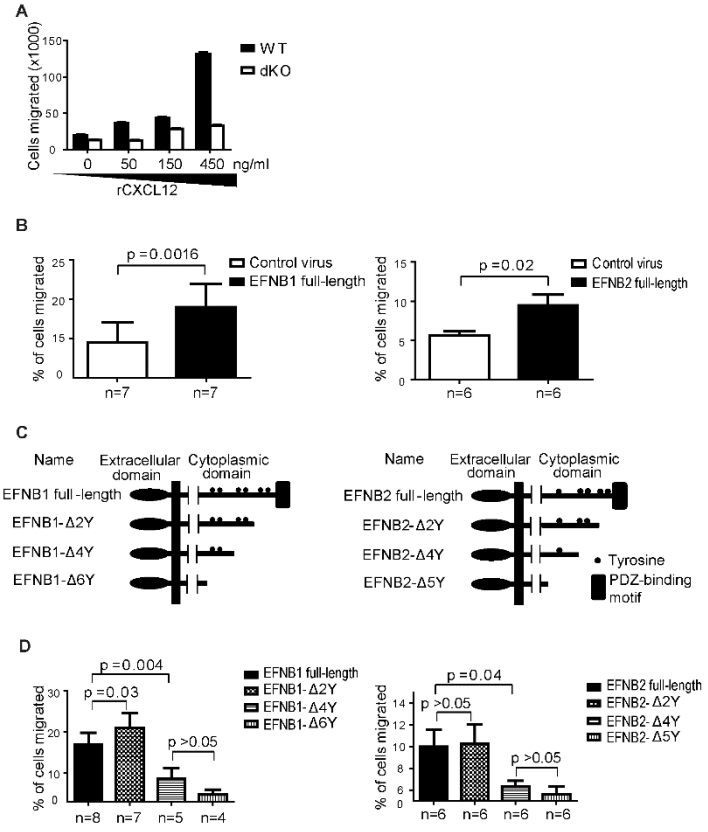
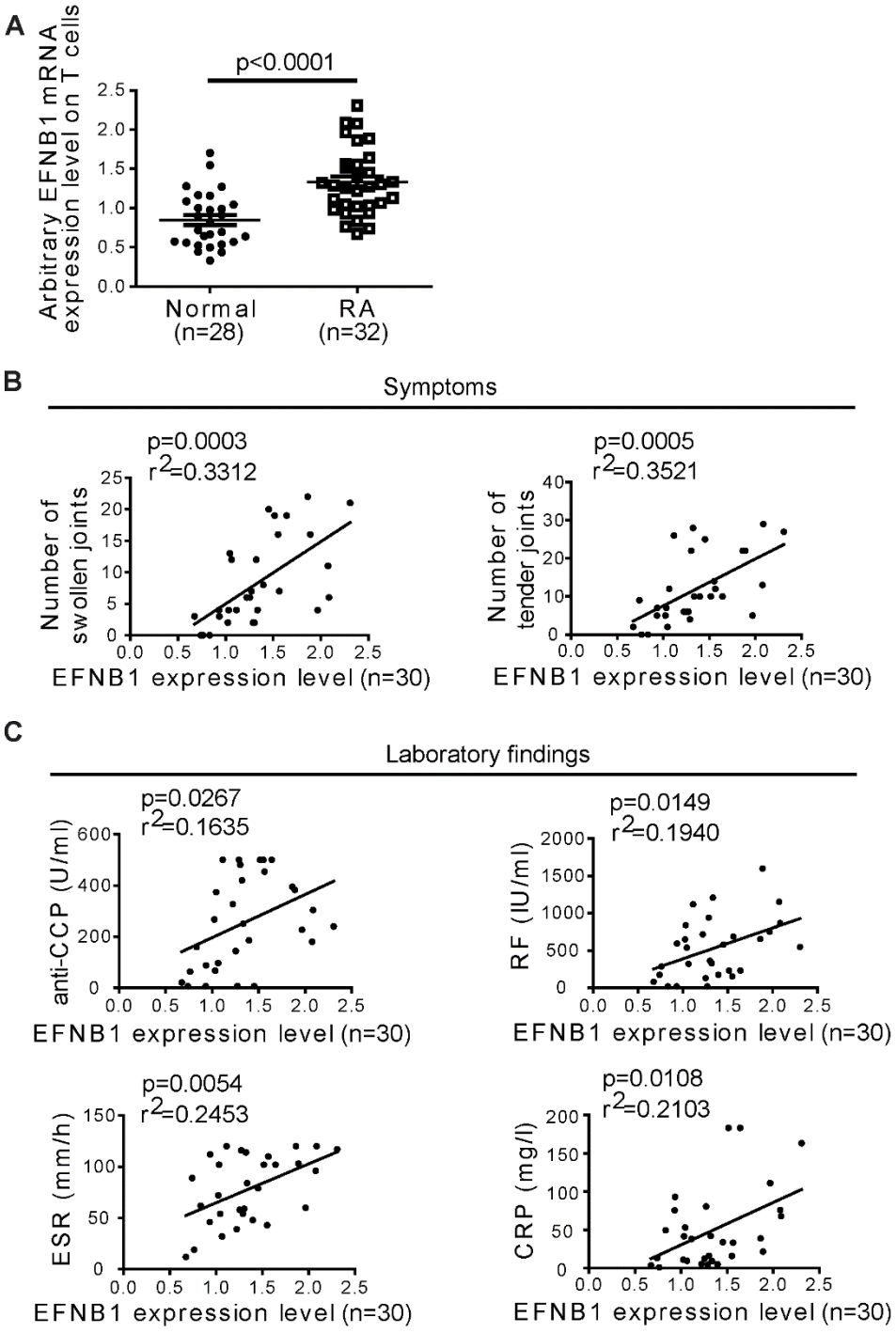


Figure 2.6 EFNB1 mRNA levels in T cells of RA patients



Chapter 3 Article-2

To investigate the necessity of STRA6 upregulation in T cells during T cell immune responses

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3.1 To investigate the necessity of STRA6 upregulation in T cells during T cell immune responses

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Abstract

Our earlier study revealed that *Stra6* (stimulated by retinoic acid gene 6) was up-regulated within 3 h of TCR stimulation. STRA6 is the high-affinity receptor for plasma retinol-binding protein (RBP) and mediates cellular vitamin A uptake. We generated Stra6 knockout (KO) mice to assess whether such up-regulation was critical for T-cell activation, differentiation and function. Stra6 KO mice under vitamin A sufficient conditions were fertile without apparent anomalies upon visual inspection. The size, cellularity and lymphocyte subpopulations of Stra6 KO thymus and spleen were comparable to those of their wild type (WT) controls. KO and WT T cells were similar in terms of TCR-stimulated proliferation *in vitro* and homeostatic expansion *in vivo*. Naive KO CD4 cells differentiated *in vitro* into Th1, Th2, Th17 as well as regulatory T cells in an analogous manner as their WT counterparts. *In vivo* experiments revealed that anti-viral immune responses to lymphocytic choriomeningitis virus in KO mice were comparable to those of WT controls. We also demonstrated that Stra6 KO and WT mice had similar glucose tolerance. Total vitamin A levels are dramatically lower in the eyes of KO mice as compared to those of WT mice, but the levels in other organs were not significantly affected after Stra6 deletion under vitamin A sufficient conditions, indicating that the eye is the mouse organ most sensitive to the loss of Stra6. Our results demonstrate that 1) in vitamin A sufficiency, the deletion of Stra6 in T cells does no affect the T-cell immune responses so-far tested, including those depend on STAT5 signaling; 2) Stra6-independent vitamin A uptake compensated the lack of Stra6 in lymphoid organs under vitamin A sufficient conditions in mice; 3) Stra6 is critical for vitamin A uptake in the eyes even in vitamin A sufficiency.

Introduction

During T-cell immune responses, naive T cells are activated by stimuli through TCR in the company of co-stimulation signals, and undergo multiple rounds of proliferation before entering the differentiation phase, after which they become effector T cells. The expression of many molecules is modulated during activation and differentiation stages, with some of them playing pivotal regulatory roles, while others exert support and house-keeping functions to cope with increased metabolic demands. We undertook unbiased exploration with DNA microarray analysis of molecules up- or down-regulated in T cells within the first 16 h after stimulation by anti-CD3 with a view to identifying those that are critical in the early T-cell activation stage. A group of molecules with the highest levels of altered expression in activated T cells was chosen, with resting T cells as reference, and verified by Northern blotting analysis. Stra6 (stimulated by retinoic acid gene 6) is among those that have been validated. We generated *Stra6* gene knockout (KO) mice to assess the significance of its up-regulation in T-cell activation and, consequently, T-cell immune responses.

At the outset of our investigation in 2004, no function was ascribed to STRA6, a 74-kDa protein with multiple transmembrane domains that was first identified in retinoic acid-stimulated P19 embryonic carcinoma cells upon retinoic acid stimulation [1]. In 2007, Kawaguchi et al. used an unbiased technique to identify STRA6 as a specific cell-surface receptor for plasma retinol binding protein (RBP) and showed that STRA6 mediates cellular vitamin A uptake from holo-RBP (RBP/vitamin A complex) in bovine retinal pigment epithelium cells [2]. STRA6-mediated vitamin A uptake from holo-RBP is coupled to intracellular proteins as confirmed by several independent studies [1-5], and its mechanism in coupling to specific intracellular proteins has been elucidated [4]. Pasutto et al. [6] observed that mutations in STRA6 correlated with many eye, heart, diaphragm and lung malformations as well as mental retardation in Matthew-Wood syndrome in humans, corroborating its reported roles in vitamin A uptake by cells as vitamin A is vital in organogenesis. Recent reports indicate that single nucleotide polymorphisms or mutations in STRA6 gene are correlated with the congenital eye malformations microphthalmia, anophthalmia and coloboma [7,8] as well as Matthew-Wood syndrome [9]. Genetic null mutation of *Stra6* in mice results in a significant retinoid reduction in the retinal pigment epithelium and

neurosensory retina, diminished visual responses and eye morphology, although the last-mentioned defect is not as serious as in patients with STRA6 mutations [10].

There is a report suggesting that STRA6 is not only a vitamin A transporter but can also function as a cytokine receptor. Upon binding with holo-RBP, STRA6 is phosphorylated at tyrosine residue 643, which, in turn, recruits and triggers JAK2 and STAT5 activation [11].

The ascribed roles of STRA6 in vitamin A transport and the STAT5 signalling pathway are certainly relevant to T-cell activation and function. Retinoids are known to modulate Th1 (T helper 1), Th2, Th17 and reglulatory T (T_{reg}) cell development and function [12-17]. At the molecular level, it has been demonstrated that retinoic acid opens up the FoxP3 promoter tertiary structure for activated FoxP3 transcription [18]. RARα can interact with STAT5a and b [19], which are critical molecules in the signaling pathway of a key T activation cytokine IL-2 [20].

Vitamin A is absorbed from dietary nutrients. There are several possible modes of vitamin A transport to cells in different organs. Vitamin A in the diet can be transported to liver cells and other cell types in the form of chylomicron-bound retinyl ester [21,22]. The liver is the primary storage site for vitamin A in the form of all-trans-retinyl ester, which can be reverted to vitamin A [22]. As alluded to above, vitamin A associates with RBP in blood, and such complexes can deliver vitamin A to cells via the RBP receptor STRA6 [2]. Transthyretin can associate with vitamin A-bound RBP, and such coupling serve to prevent renal filtration of the holo-RBP [23], Recently, Alapatt et al. [24] discovered a second RBP receptor, a STRA6 homologue called RBPR2. RBPR2 is expressed in the liver, intestines, fatty tissues, and spleen. Like STRA6, RBPR2 is fully capable of binding to RBP and transporting vitamin A into cells. As vitamin A is hydrophobic, it should also be able to diffuse through cell membranes without any specific receptors.

The relative contribution of STRA6 to vitamin A cellular import in lymphoid organs has not been evaluated and is a secondary goal of our study.

In this study, we demonstrated that Stra6 KO mice were vital and fertile, manifesting no apparent anomalies in their lymphoid organs and T cell-dependent immune responses under vitamin A sufficient conditions. Intracellular vitamin A concentrations in lymphoid organs, such as the thymus and spleen of the KO mice were comparable to those of WT controls,

although the vitamin A content in cells from KO eyes was significantly lower that that from the WT eyes. The implications of these data are discussed.

Materials and methods

RT-qPCR

Stra6 mRNA in cells and tissues from KO, heterozygous and WT mice was measured by RT-qPCR. Total RNA was extracted with TRIzol® (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed with Superscript II™ reverse-transcriptase (Invitrogen). The forward and reverse primers were 5'-AGG CAT CTG AGA ATG GAA GCC AGA-3' and 5'-AGC AGA ACC AGG AAC GAC AGT GAA-3', respectively. A 184-bp product was detected with the following amplification program: 95°C × 15 min, 1 cycle; 94°C × 15 s, 55°C × 30 s, 72°C × 30 s, 35 cycles. β-actin mRNA levels were measured as internal controls; the forward and reverse primers were 5'-TGG TAC CAC AGG CAT TGT GAT-3' and 5'-TGA TGT CAC GCA CGA TTT CCC T-3', respectively, with the same amplification program as for STRA6 mRNA. The data were expressed as ratios of STRA6 and β-actin signals.

Generation of Stra6 KO mice

A PCR fragment amplified with the Stra6 cDNA sequence served as a probe to isolate genomic BAC DNA clone 7O8 from the RPCI-22 129/sv mouse BAC genomic library. The targeting vector was constructed by recombination [25] and routine cloning methods using an 11-kb Stra6 genomic fragment from clone 7O8 as the starting meterial. A 2.7-kb MunI-XbaI genomic fragment containing exon 2 was replaced by a 1.1-kb Neo cassette from pMC1Neo-Poly A flanked by 2 diagnostic restriction sites, XbaI and ScaI, as illustrated in Figure 2A. The final targeting fragment was excised from its cloning vector backbone by Not I digestion and electroporated into R1 embryonic stem (ES) cells for G418 selection [26]. The targeted ES cell clones were injected into C57BL/6 blastocysts. Chimeric male mice were mated with C57BL/6 females to establish mutated Stra6 allele germline transmission.

Southern blotting with probes corresponding to the 5' and 3' sequences outside the targeting region, as illustrated in Figure 2A (red squares), were used to screen for gene-targeted ES cells and eventually to confirm gene deletion in mouse tail DNA. With the 5' probe, the targeted allele should present a 7.8-kb XbaI band, and the WT allele, a 5.2-kb XbaI band. With the 3' probe, the targeted allele should present a 6.9-kb ScaI band, and the WT allele, a 4.3-kb ScaI band (Fig. 3.2A).

PCR was adopted for routine genotyping of the targeted allele(s). The following PCR conditions were applied: 4 min at 94 °C, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, with a final incubation at 72 °C for 10 min. The KO forward primer 5'- GCG TCA CCT TAA TAT GCG AAG TG-3' and reverse primer 5'-CAA GAA GTC CGT GGC TGA GTC TA-3' detected a 400-bp fragment from the targeted allele. The WT forward primer 5'-TCT CCC AGG TCT GGT TTG AG-3' and reverse primer 5'-TTA GGG CAA CAC CCT ACT GG-3' detected a 197-bp fragment from the WT allele.

The KO mice were backcrossed to the C57BL/6 background for 8 generations and then used for experimentation. All mice were housed under specific pathogen-free conditions and fed with mouse chow (Teklad Global 2018, Teklan Diets, Madison, WI) containing 15 IU/g Vitamin A. The mice had access to water and chow *ad libitum*. The mouse organs and tissues were retrieved after the mice were euthanatized by i.p. injection of 100 µl of Euthanyl (pentobarbital sodium, 120 mg/ml containing 1% lidocaine). The same method of euthanasia was used for unwanted heterozygous mice or extra unused mice. The studies were approved by the Institutional Animal Protection Committees of the CRCHUM and INRS-IAF.

Flow cytometry

Single cell suspensions from the thymus, spleen and lymph nodes were prepared and stained immediately or after culture with antibodies (Abs) against CD4, CD8, CD25, CD19, B220, CD69 and STRA6. In some experiments, intracellular proteins, such as FoxP3, IFN-γ, IL-4, IL-17, and TNF-α, were detected after the cells were pre-stained with Abs against cell surface antigens fixed with BD Cytofix/Cytoperm™ solution (BD Biosciences, San Diego, CA) and then stained with monoclonal Abs (mAb) against intracellular antigens. The Abs deployed for flow cytometry are listed in Table 3.1. Flow cytometry analysis of the stained cells are described in our previous publications [27-30].

Flow cytometry was also employed to assess lymphocytic choriomeningitis virus (LCMV)-specific T cells. Synthetic peptides gp_{33-41}: KAVYNFATC (LCMV-GP, H-$2D^b$); $np_{396-404}$: FQPQNGQFI (LCMV-NP, H-$2D^b$); $gp_{276-286}$: SGVENPGGYCL (LCMV-GP, H-$2D^b$); and gp_{61-80}: GLNGPDIYKGVYQFKSVEFD (LCMV-GP, I-A^b) were purchased from Sigma-Genosys (Oakville, Ontario, Canada). PE-gp_{33-41}, PE-$np_{396-404}$ and PE-$gp_{276-286}$ H-$2D^b$ tetrameric complexes were synthesized in-house and used at 1/100 dilution as previously

described [26]. These MHC-tetramers were used to detect LCMV-specific $CD8^+$ T cells on day 8 post LCMV infection. Briefly, splenocytes were first stained with PE-gp_{33-41}, PE-$np_{396-404}$ or PE-$gp_{276-286}$ tetramers for 30 minutes at 37°C, followed by staining with FITC-rat anti-mouse CD8α and APC-rat anti-mouse CD62L mAbs at 4°C for another 20 minutes. 7-AAD was used for exclusion of dead cells. After washing, cells were fixed in 0.5% paraformaldehyde and samples were analyzed by flow cytometry. One million splenocytes from LCMV-infected mice were seeded in single wells of 96-well round-bottomed plates. They were maintained in 5% RPMI-1640 supplemented with 100 units/ml interleukin-2, 10 μg/ml brefeldin A, 10 μM gp_{33-41} or gp_{61-80} peptide. After 5 h of incubation at 37°C, the cells were stained with PE-conjugated rat anti-mouse CD8α or CD4 mAbs and 7-AAD. They were then fixed, permeabilized and stained with APC-labeled rat anti-mouse TNF-α and FITC-labeled rat anti-mouse IFN-γ mAbs. IFN-γ and TNF-α-secreting T cells were counted by flow cytometry [31].

T-cell proliferation in vitro and in vivo after being transferred to sub-lethally-irradiated mice

Spleen cells were loaded with carboxyfluorescein succinimidyl ester (CFSE; 5μM for 5 mins), and then cultured in the presence of soluble hamster against mouse CD3 mAb (clone 2C11; 0.5 μg/ml) [27, 28, 32, 33]. After 3 days, CFSE fluorescence of the CD4 and CD8 subpopulations was analyzed by flow cytometry for TCR-stimulated proliferation. T-cell homeostatic expansion was evaluated by i.v. injection of 5 x 10^6 CFSE-loaded spleen cells into C57BL/6 recipients 5 h after sub-lethal irradiation (650 Rad). On day 5, the CFSE fluorescence of CD4 and CD8 cells from the spleen and LN was studied by flow cytometry.

In vitro Th1, Th2, Th17 and T_{reg} cell polarization

In vitro Th and T_{reg} cell differentiation was conducted as follows [27, 34]. Naïve CD4 T cells ($CD4^+CD62L^+CD44^{low}$) were isolated from KO or WT mouse spleens with MagCellect Mouse Naïve $CD4^+$ T cell Isolation kits (R & D Systems). T cell-depleted WT spleen cells were irradiated at 3000 Rad and used as feeder cells. The naïve CD4 cells (0.1×10^6/ well) were mixed with the feeder cells (0.5×10^6/well) and cultured in 96-well plates in the presence of soluble anti-CD3ε mAb (clone 145-2C11, 2 μg/ml; BD Biosciences). Cultures were supplemented with recombinant mouse IL-12 (10 ng/ml; R & D Systems) and anti-IL-4 mAb (10 μg/ml; R & D Systems) for the Th1 condition; recombinant mouse IL-4 (20 ng/ml; R & D

Systems), and anti-IL-12 mAb (10 μg/ml; BD Biosciences) and anti-IFN-γ mAb (10 μg/ml; R & D Systems) for the Th2 condition; recombinant mouse IL-6 (20 ng/ml; R & D Systems), recombinant human TGF-β1 (5 ng/ml; R & D Systems) and anti-IL-4 and anti-IFN-γ mAbs (10 μg/ml for each; R & D Systems) for the Th17 condition; recombinant human TGF-β1 (5 ng/ml; R & D Systems), and anti-IL-4 and anti-IFN-γ mAb (10 μg/ml; R & D Systems) for the T_{reg} condition.

LCMV infection

LCMV clone 13 was obtained from Dr. R.M. Zinkernagel (University of Zurich, Zurich, Switzerland). Viral stock was propagated *in vitro,* and viral titers were quantified by focus-forming assay [31]. Mice were infected by the i.v. route with 2×10^6 focus-forming units of LCMV clone 13. They were sacrificed 8 days post-infection, and their spleens were harvested for primary immune response analysis.

Glucose tolerance tests

The KO and WT mice fasted for 16 h and injected i.v. with D-glucose (2 mg/g body weight) in PBS. Blood samples from the tail vein were taken at 5, 15, 30, 60, and 90 min after the injection for glucose measurements with a glucose meter (Bayer, Toronto, Ontario).

Measurement of serum and intracellular vitamin A and retinyl ester concentrations by high-pressure liquid chromatography (HPLC)

Serum and tissue samples, collected in a dark, cold room, were stored at -80^{o}C until their analysis. Retinoids were extracted by homogenizing tissues in a butanol-acetonitrile mixture (1:1) with a tissue/solvent ratio of 200 mg/700 μl, in Eppendorf tubes on ice by 5 30-s pulses with 1-min intervals. K_2HPO_4 solution (6.89M) was added to the tubes in proportion to the homogenized mixture (20 μl for 900 μl homogenized mixture). For retinoid extraction from sera, 200-μl butanol-acetonitrile mixture (1:1) was added to 200-μl serum, and the mixture was vortexed for 1 min; 20 μl K_2HPO_4 solution (6.89M) was then added to the mixture before 30-s vortexing. The tissue and serum samples thus prepared were centrifuged for 20 min at 14,000*g* at 4^{o}C. Cleared supernatants were passed through Spin-X filters (0.45μm pore size; Costar, Batavia, Illinois, USA) at 14,000*g* for 10 min at 4^{o}C. For retinyl ester measurement, the samples prepared as aforementioned before the step of filtration were vacuum-dried and

re-dissolved in 100% methanol, followed by centrifugation at 14,000 g for 10 min. The supernatants were then analyzed by HPLC.

Vitamin A in extracts was quantified by HPLC in an ÄKTA Purifier (Model UPC10; GE Healthcare, Baie d'Urfé, Quebec, Canada) and reverse-phase column (μ-RPC C2/C18 ST 4.6/100; GE Healthcare). Samples (100 μl) were eluted with a linear gradient from 100% eluent A (acetontrile: water = 65:35) to 100% eluent B (acetontrile:water = 90:10) in 5-column volumes at a flow rate of 1 ml/min. Both eluates contained 10 mM ammonium acetate. Vitamin A was detected at 313 nm wave-length. Its characteristic retention volume was identified with pure Vitamin A from Sigma (Oakville, ON, Canada) as a standard. Areas under the curves were computed by UNICORN5.11 software (GE Healthcare). The sensitivity of the assay was 250 ng.

Retinyl ester in extracts was quantified by HPLC in an Eclipse XDB-C18 reverse-phase column (4.6 X150 mm, 5 μm, Agilent, Santa Clara, CA). Samples (200 μl) were eluted with a linear gradient from 100% methanol to 100% ethyl acetate in 5 column volumes at a flow rate of 1 ml/min. Retinyl ester was detected at 324 nm wavelength. Its characteristic retention volume was identified with retinyl palmitate (Sigma) as standard. Areas under the curves were computed by Agilent LC software. Sensitivity of the assay was 1.5 ng.

Statistics

Student's t tests were employed to analyze statistical differences between WT and KO mice for their lymphoid organ weight and cellularity, and for their retinoid contents (retinol and retinyl esters) in different organs. ANOVA was used to compare the glucose tolerance between WT and KO mice.

Results

Stra6 expression in different organs and activated T cells

Stra6 mRNA expression was assessed by RT-qPCR. Among the organs and tissues examined, the thymus had the highest expression level, followed by the heart and kidneys (Fig. 3.1A). Stra6 expression in the spleen was moderate. The skeleton muscles and liver had barely detectable *Stra6* mRNA. The *Stra6* mRNA expression levels in the lung, liver, spleen and kidney assessed by our RT/qPCR was consistent with Northern results reported previously by Bouillet [1]. High STRA6 expression in the thymus suggested that it might have some critical functions in T-cell development and T-cell function. As depicted in Figure 3.1B, *Stra6* expression in resting spleen T cells (0 h) was modest, consistent with values of the whole spleen. The expression was augmented with in 3 h after T-cell activation by TCR cross-linking and reached a peak at 48 h. This result corroborates our initial DNA microarray data, through which *Stra6* was found upregulated during T-cell activation.

Generation of Stra6 KO mice

To evaluate the roles of Stra6 in the immune system in general and T cell-mediated immune responses in particular, we produced Stra6 KO mice. The targeting strategy is illustrated in Figure 3.2A. Germline transmission was confirmed by Southern blotting of tail DNA (Fig. 3.2B). With the 5' end probe, the WT allele after XbaI digestion presented a 7.8-kb band, and the KO allele, a 5.2-kb band (Fig. 3.2B, upper panel). With the 3' end probe, the WT allele after ScaI digestion presented a 6.9-kb band, and the KO allele, a 4.3-kb band (Fig. 3.2B, lower panel). WT (mice 3, 4, and 5) and heterozygous mice (mice 1, 2 and 6) were thus identified. Mouse 1 was backcrossed to the C57BL/6 background for 8 generations and then used in the experiments described hereafter.

To ascertain whether *Stra6* gene deletion results in its lack of expression, we measured *Stra6* mRNA in spleen cells by RT-qPCR. STRA6 mRNA was detectable in WT but not in KO spleen cells (Fig. 3.2C). The lack of *Stra6* expression in KO cells at the protein level was confirmed by flow cytometry, as Stra6 was detectable in WT but not KO thymocyte surface (Fig. 3.2D).

Normal lymphoid organs and lymphocyte subpopulations in Stra6 KO mice

Stra6 KO mice were viable and fertile with no apparent anomalies upon visual inspection. Weight and cellularity of the KO thymus and spleen were comparable to those of WT mice (Fig. 3.3A). T-cell ($CD4^+$ plus $CD8^+$ versus non T-cell ($CD4^-CD8^-$) subpopulations and CD4 versus CD8 T-cell subpopulations in the spleen and LN of WT and KO mice showed no consistent differences (Fig. 3.3B). The percentages of B cells ($CD19^+B220^+$) in the spleen and lymph nodes in WT and KO mice were also similar (Fig. 3C). In KO thymi, the percentages of CD4 single-positive and CD8 single-positive, CD4CD8 double-positive cells and $CD4^+$/$FoxP3^+$ T_{reg} cells were comparable to those in WT thymi (Fig. 3.3D). The comparable percentages of T_{reg} cells in the thymi of WT and KO mice were confirmed by the measurement of $FoxP3^+$ cells among $CD4^+CD25^+$ thymocytes (Fig. 3.3E).

In the periphery, the percentages of $FoxP3^+$ T_{reg} cells among CD4 cells in the spleen (Fig. 3.3F) and lymph nodes (Fig. 3.3G) from WT and KO mice were also similar.

These results show that Stra6 KO mice have normal lymphoid organ and T-cell development.

Normal activation, proliferation and differentiation of Stra6 KO T cells

KO and WT T cells were stimulated by solid-phase anti-CD3 mAb for 16 h. The activation markers CD25 and CD69 in CD4 and CD8 T cells were quantified by flow cytometry. KO and WT T cells showed similar up-regulation of these markers (Fig. 3.4A). To assess T-cell proliferation, KO and WT T cells in total spleen cells were loaded with CFSE and stimulated by soluble anti-CD3 mAb. After 3 days, their proliferation was assessed by flow cytometry. CD4 and CD8 KO T cells proliferated like their WT counterparts, as shown in Figure 4B. To measure T-cell homeostatic expansion, spleen T cells were loaded with CFSE and then injected into sub-lethally-irradiated syngeneic recipients. The proliferation of these transferred KO CD4 and CD8 cells in recipient spleens and LN during the 5 days after the injection was measured based on their CFSE content according to flow cytometry. As shown in Figure 3.4C, the cells from WT and KO mice proliferated similarly in vivo. Therefore, KO T-cell proliferation, whether caused by TCR stimulation in vitro or homeostatic expansion in vivo, was not defective.

When KO and WT naïve CD4 cells were cultured under Th1, Th2, Th17 and T_{reg} conditions, they achieved comparable Th1, Th2, Th17 and T_{reg} cell percentages (Fig. 3.5), indicating normal differentiation of naïve KO CD4 cells into these subpopulations.

The effect of Stra6 deletion in anti-LCMV immune responses in vivo

The function of Stra6 KO T cells *in vivo* was evaluated in the LCMV infection model. As illustrated in Figure 3.6A, the number of total splenocytes, and CD4 and CD8 cells on day 8 post-infection (8 dpi) presented no significant differences in WT and KO mice (Fig. 3.6A). The absolute numbers (Fig. 3.6B) and relative percentages (Fig. 6C) of LCMV-specific tetramer-positive ($gp33^+$, $np396^+$ and $gp276^+$) CD8 cells in virus-infected mice were all increased in comparison to uninfected control C57BL/6 mice (data not shown), but there were no significant differences between KO and WT mice with regard to these parameters. The absolute numbers and relative percentages of LCMV-specific TNF-α-producing CD4 (gp61) and CD8 cells (gp33) (Figs. 3.6D and 6E), and LCMV-specific IFN-γ-producing CD4 and CD8 cells (Figs. 3.6F and 6G) in KO mice were comparable to those in WT controls. These results indicate that the STRA6 deletion had no discernable effect on anti-LCMV immune responses.

Normal glucose tolerance in Stra6 KO mice

One report suggests that Stra6 stimulation by RBP induces the expression of SOCS3, which inhibits insulin signaling [12]. We assessed the glucose tolerance of KO mice and found that KO and WT mice showed no significant difference in glucose tolerance (Fig. 3.7), suggesting that in the absence of Stra6, the insulin signaling of the KO mice on a normal diet is not enhanced.

Organ retinyl ester and retinol levels in Stra6 KO mice

As vitamin A has been reported to play an important role in immune regulation [12-17], a lack of immunological phenotype so-far tested in the KO mice prompted us to examine vitamin A contents of lymphoid organs as well as several other organs including the eyes. Vitamin A is stored in organs predominantly in the form of retinyl ester, which is a lipid and can reach high concentrations. A minor stored form is retinol bound to CRBP, and the retinol content in the cells is limited by the availability of CRBP. Retinyl esters and retinol and can be quickly converted to each other inside the cells. We thus measured the contents of both retinyl esters and retinol in these organs.

As shown in Figure 3.8A, the WT and KO spleen and thymus had no significant difference in their retinyl ester contents, nor did the brains and kidneys. The retinyl ester contents in WT

eyes were much higher than that of other organs, and the contents in the KO eyes were significantly lower compared to those of the WT counterparts. WT or KO blood had no detectable retinyl ester (data not shown). Retinyl ester is known to be high in the blood only right after a meal enriched in vitamin A.

The retinol levels in these WT and KO organs were of the same pattern as retinyl ester, although at much lower levels (Fig. 3.8B; note the scale difference). The KO spleen, thymus, kidney and brain had no significant difference in retinol contents compared to their WT counterparts. Unlike retinyl ester, the retinol was detectable in the sera, but was of similar levels in WT and KO sera. The eyes contained the highest levels of retinol. KO eyes presented significantly lower levels of retinol than the WT counterparts.

These data indicate that under a vitamin A sufficient condition, lymphoid organs still take up vitamin A without Stra6 in mice. This explains the lack of immunological phenotype in the KO mice. However, even under such a vitamin A sufficient condition, the eyes still heavily depend on Stra6 for vitamin A uptake, as they are the organs with the highest vitamin A demand.

Discussion

STRA6 is a receptor of holo-RBP (i.e., vitamin A-bound RBP) for cellular vitamin A uptake. *Stra6* is up-regulated after T-cell activation. In this study, we generated Stra6 KO mice to assess whether such up-regulation was essential for T cell-mediated immune responses and Stra6's role in vitamin A uptake. Under a vitamin A sufficient condition, Stra6 KO mice developed normally and were fertile. Their T cells presented no signs of abnormality in terms of development, activation marker up-regulation, proliferation, and Th and T_{reg} cell differentiation. KO mice also had normal anti-LCMV immune responses. There was no significant difference in intracellular vitamin A content, in the forms of both retinyl ester and retinol, in lymphoid organs from WT and KO mice. However, even under the vitamin A sufficient condition, the KO eyes contained significantly lower amounts of retinyl ester and retinol, indicating a critical role of vitamin A uptake in this organ.

A caveat of whole organ retinoid analysis is that contribution of retinoid in the blood can affect the total retinoid levels. This is especially true if the organ is rich in blood, which contains RBP-bound vitamin A. Despite this caveat, whole organ retinoid analysis can be used as an approximation of cellular retinoid uptake. This is especially true as several of the organs we tested (i.e., the thymus, brain and eyes) are not blood rich. Moreover, sera had no detectable retinyl ester (data not shown); so the retinyl ester levels of the organs tested will not be upward influenced by the blood retinyl ester levels. The reduced vitamin A contents in the eyes of Stra6 KO mice is not unexpected, as the eyes have the highest concentrations of vitamin A among all the organs (Fig. 3.8) due to its heavy reliance on vitamin A for vision, and probably need all the capacities of vitamin A transport including the pathway of RBP/STRA6 to achieve this high vitamin A content, even in vitamin A sufficiency. Consistent to our findings, RBP KO mice have normal vitamin A levels in most of their organs, but a reduced one in the eyes [35].

Vitamin A and its metabolites – retinoic acids – are clearly required in immune responses [13-18, 36]. It is reported that in hepatocytes, holo-RBP triggers STRA6, leading to the activation of JAK2/STAT5 signaling pathway, which is also essential in the activation and function of immune cells. *Stra6* is up-regulated within 24 h of T-cell activation (Fig. 3.1B). Is such up-regulation, or more fundamentally, the existence of Stra6, essential for T cell-mediated

immune responses? We demonstrated that in Stra6 KO mice, a lack of Stra6 did not affect T-cell activation/proliferation/differentiation *in vitro* and anti-viral immune responses *in vivo* under a vitamin A sufficient condition. These observations suggest following possible and not necessarily mutually exclusive explanations: 1) STRA6 up-regulation/existence only becomes important for T-cell functions during vitamin A deficiency, when all capacities of vitamin A import to immune cells are required; 2) STRA6 homologue RBPR2 can compensate for STRA6 function in the immune cells, as this homologue is expressed in the spleen; also, retinyl esters bound to lipoproteins secreted by the small intestine can deliver vitamin A to peripheral organs under vitamin A sufficient conditions; 3) STRA6 plays a minimal role in modulating the JAK2/STAT5 signalling pathway in immune cells, and its upregulation after T cell activation has nothing to do with JAK2/STAT5 signaling; 4) We cannot exclude the possibility that Stra6 deletion might still affect certain T cell-mediated immune responses to some extent, but they have not been assessed in our experiments, or their magnitude was too small to be discerned by current assays; 5) Such up-regulation might be a parallel and irrelevant event during T-cell activation.

There is little systemic documentation on vitamin A sufficiency status in wild mammals in today's world. However, it is well-documented that vitamin A deficiency is prevalent in African and Southeast Asian populations, particularly affecting children and pregnant women, according to the World Health Organization [37], and such deficiency predisposes them to infectious diseases [38]. It is conceivable that, during evolution, mammals might have experienced vitamin A deficiencies in certain periods or regions in the world. Better cellular vitamin A transport will confer an evolutionary advantage to these animals with regard to but not restricted by their ability to cope with infectious diseases. If STAR6 is universally critical in all cell types for vitamin A uptake, its function should be revealed in immune responses in vitamin A deficiency. Experiments addressing this possibility are in progress.

STRA6 point mutations are found in some patients, with microphthalmia, anophthalmia, coloboma [7] and Matthew-Wood syndrome [referring to combinations of microphthalmia/anophthalmia, cardiac malformations, pulmonary dysgenesis, and diaphragmatic hernia; ref. 9]. In a study of 2 unrelated consanguineous families with malformation syndromes sharing anophthalmia and distinct eyebrows as common signs, homozygous mutations were identified in STRA6 [6]. Our Stra6 KO mice and those generated

by Ruiz *et al.* [10] did not have dramatic phenotypes, such as a total absence of eyes, as seen in humans with STRA6 mutations.

Why cannot Stra6 KO in mice reproduce human disease phenotypes caused by STRA6 mutations? A simple explanation is that this is due to species differences. It is not unprecedented that gene mutations in mice and humans have very different phenotypes. For example, partial or complete loss of ABCA4 functions cause many blinding diseases in humans including retinitis pigmentosa, cone-rod dystrophy and Stargdardt macular dystrophy, but ABCA4 KO in mice does not cause blindness unless combined with a deletion of other genes such as RDH8 [39-41]. On the other hand, disease loci of microphthalmia and anophthalmia have been mapped to multiple chromosomes [42-45]. Patients with Matthew-Wood syndrome or malformation syndromes have quite large phenotype variations in terms of organ affliction and disease severity. Such observations suggest that these syndromes are not monogenic, and STRA6 mutation alone is not sufficient to evoke all such phenotypes. It could explain why no serious ophthalmic [13] or other organ malformations are apparent in Stra6 null mutation mice. If STRA6's major function is cellular vitamin A uptake, and human organ malformation syndromes are mainly caused by a lack of available intracellular vitamin A, it would support the notion that STRA6 only plays a minor role in cellular vitamin A uptake in vitamin A sufficiency, especially in organs other than the eyes. Unless other routes of cellular vitamin A uptake such as those mediated by RBPR2 or by retinyl esters bound to lipoproteins are simultaneously compromised, vitamin A in the cells of most, if not all, organs vitamin A contents will remain in the normal range, and the organs will develop and function normally in vitamin A sufficiency. However, significant phenotype might be revealed in vitamin A deficiency. This hypothesis is supported by results from RBP KO mice. These KO mice are fertile and have no organ abnormality other than the vision phenotype [46-48], as is the case of Stra6 KO mice when fed with a vitamin A sufficient diet. However, they manifest severe systemic phenotype of embryonic lethality under a vitamin A deficient condition [49,50]. Consistently, in mouse embryo culture where is no retinyl ester pathway, RBP knockdown also causes severe developmental defects [51].

In summary, we conclude that, under normal dietary conditions, mouse lymphoid organ development, T-cell activation and differentiation, including T_{reg} cell development, and anti-

LCMV responses, could proceed normally in the absence of Stra6 under vitamin A sufficient conditions.

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Tables

Table 3.1 Antibodies for flow cytometry

Antibody	*Supplier*
PE-donkey anti-goat IgG	R & D Systems
APC-rat anti-mouse CD25 (clone PC61)	BD Biosciences
FITC-rat anti-mouse CD25 (clone 7D4)	BD Biosciences
PE-rat anti-mouse CD4 (clones GK1.5 and H129.19)	BD Biosciences
PerCP-rat anti-mouse CD4 (clone RM4-5)	BD Biosciences
biotin-rat anti mouse CD8b (clone 53-5.8)	BD Biosciences
APC-Cy7- anti-mouse B220 (clone RA3-6B2)	BD Biosciences
PE- or APC-hamster anti-mouse CD3ε (clone 145-2C11)	BD Biosciences
biotin- or FITC-rat anti-mouse CD44 (clone 1M7)	BD Biosciences
FITC- or PE-rat anti-mouse CD8α (clone 53-6.7)	BD Biosciences
APC-rat anti-mouse CD8α (clone H57-597)	BD Biosciences
PE-rat anti-mouse IL-17A	BD Biosciences
PE- and APC-rat anti-mouse IFN-γ	BD Biosciences
PE- and APC-rat anti-mouse IL4 mAbs	BD Biosciences
PerCP-streptavidin and 7-Amino-actinomycin D (7-AAD)	BD Biosciences
APC-Cy7-Streptavidin™	BioLegend
APC-rat anti-mouse TNF-α (clone MP6-XT22)	eBioscience (San Diego, CA)
FITC-rat anti-mouse IFN-γ (clone XMG1.2)	eBioscience
APC-rat anti-mouse IL17A (clone eBio17B7)	eBioscience
APC-rat anti mouse/rat Foxp3 (clone FJK-16s) mAbs	eBioscience
PE-Cy7-streptavidin	eBioscience
intracellular antigen fixation buffer	eBioscience
10X permeabilization buffer	eBioscience
APC-Cy™ PE-rat anti-mouse CD25 (clone PC61)	Cedarlane Laboratories Ltd (Burlington, Ontario, Canada)
Goat anti-mouse STRA6 Ab	Everest Biotech (Upper Heyford, Oxfordshire, UK)

Figure legends

Figure 3.1. Stra6 mRNA expression in organs and activated T cells

Stra6 mRNA in organs (A) and activated total spleen T cells (B) was measured by RT-qPCR. WT spleen total T cells were cultured in wells coated with solid-phase anti-CD3 mAb and anti-CD28 mAb (0.5 μg/ml and 4 μg/ml, respectively, for coating) for the durations indicated. The cells were then harvested and their STRA6 mRNA levels measured by RT-qPCR. Samples were in triplicate for RT-qPCR and means ± SD of ratios versus β-actin signals are reported. Experiments were conducted twice, and representative data are illustrated.

Figure 3.2. Generation of Stra6 KO mice

A. Targeting strategy for generating Stra6 KO mice

The red squares on the 5' and 3' sides of the mouse Stra6 WT genomic sequence represent the sequences serving as probes for genotyping by Southern blotting.

B. Genotyping of Stra6 mutant mice

Tail DNA was digested with XbaI, and analyzed by Southern blotting (top panel), with the 5' probe whose location is indicated in A. A 7.8-kb band representing the WT allele and a 5.2-kb band representing the recombinant allele are indicated by arrows. Similarly, tail DNA was digested with ScaI and analyzed with the 3' probe (bottom panel). A 6.9-kb band representing the WT allele and a 4.3-kb band representing the recombinant allele are indicated by arrows.

C. Absence of Stra6 mRNA expression in Stra6 KO splenocytes

Stra6 mRNA levels from WT and KO splenocytes were analyzed by RT-qPCR. The RT-qPCR samples were in triplicate and the results are expressed as ratios of *Stra6* versus β-actin mRNA signals with means ± SD indicated. The experiments were conducted twice and representative data of one experiment are shown.

D. Absence of Stra6 protein expression in KO thymocytes

WT and KO thymocytes were stained with goat anti-mouse Stra6 Ab and analyzed by flow cytometry. The shaded area is the isotypic Ab staining control using WT thymocytes. The thick line represents WT thymocytes stained with anti-Stra6 Ab, and the dotted line, KO thymocytes stained with anti-Stra6 Ab. The experiments were carried out three times and a representative histogram is shown.

Figure 3.3. Stra6 KO mice presented normal lymphoid organs and lymphocyte subpopulations

A. Weight and cellularity of WT and KO thymi and spleens

Mouse number (n) in each group is shown. No significant difference is detected in weight and cellularity between KO and WT organs (p>0.01, paired Student's t test).

B. CD4 and CD8 T-cell subpopulations in WT and KO spleens and LN

Splenocytes and LN cells were analyzed by 2-color flow cytometry for percentages of CD4 and CD8 T cells.

C. B cell populations in the spleen and LN of WT and KO mice

Splenocytes and LN cells were analyzed by 2-color flow cytometry for percentages of $CD19^+/B220^+$ B cells.

D and E. Normal thymocyte subpopulations and endogenous T_{reg} cells in KO thymi

CD4/CD8 double-negative, CD4/CD8 double-positive, CD4 single-positive and CD8 single-positive cells and $CD4^+CD8^-FoxP3^+$ T_{reg} cells in KO and WT thymi were analyzed by 3-color flow cytometry (D). $CD4^+CD25^+FoxP3^+$ T_{reg} cells in the WT and KO thymi were also analyzed by 3-color flow cytometry (E).

F and G. $CD4^+CD8^-FoxP3^+$ T_{reg} cells in KO and WT spleens and LN

$CD4^+CD8^-FoxP3^+$ T_{reg} cells in KO and WT spleens (F) and LN (G) were analyzed by 3-color flow cytometry.

The experiments in B through G were conducted more than 3 times, and representative histograms are presented. Percentages of relevant populations are indicated.

Figure 3.4. Normal activation and proliferation of KO T cells

A. Normal activation marker CD69 and CD25 expression on KO T cells

Total spleen cells were stimulated overnight by soluble anti-CD3 mAb (0.5 μg/ml). CD69 and CD25 expression on CD4 (left panel) and CD8 (right panel) T cells was measured by 3-color flow cytometry.

B. Normal proliferation of KO CD4 and CD8 cells upon TCR activation in vitro

Total spleen cells were loaded with CFSE and then stimulated with soluble anti-CD3 mAb (0.5 μg/ml). The cells were harvested after 72 h, and stained for CD4 and CD8; CFSE levels in these cells were analyzed by 3-color flow cytometry.

C. KO CD4 and CD8 cells present normal homeostatic expansion in vivo

Five million CFSE-loaded spleen cells were injected i.v. into sub-lethally irradiated (650 Rad) C57BL/6 recipients 5 h after the irradiation. On days 6, the CFSE fluorescence of CD4 and CD8 cells from the spleen and LN was analyzed by flow cytometry.

All experiments in this figure were conducted twice or more, and representative histograms are shown.

Figure 3.5. Normal differentiation of Stra6 KO CD4 cells in vitro

Naïve CD4 cells were cultured under conditions favouring Th1 (A), Th2 (B), Th17 (C) and T_{reg} (D) cell differentiation. Their intracellular cytokine or FoxP3 expression was quantified by flow cytometry on day 3 for Th1, Th17 and T_{reg} cells, and on day 5 for Th2 cells. Experiments were repeated more than 3 times, and representative histograms are shown.

Figure 3.6. Normal in vivo anti-LCMV immune responses of Stra6 KO mice

A. Spleen cell numbers on day 8 after LCMV infection

Means $\pm$ SD of absolute numbers of total splenocytes, $CD4^+$ cells, and $CD8^+$ cells in spleens of WT littermate control (n=4) and KO (n=4) mice on day 8 post-LCMV infection are presented.

B and C. LCMV-specific CD8 cells on day 8 post-LCMV infection

On day 8 post-infection, the absolute numbers of gp33, np396 and gp276 tetramer-positive CD8 T cells per spleen (B) and the percentage of gp33, np396 and gp276 tetramer-positive cells among CD8 cells (C) were measured by flow cytometry. Means $\pm$ SD of data from 4 pairs of WT littermate control and Stra6 KO mice are presented.

D and E. LCMV-specific TNF-α-producing CD4 and CD8 cells on day 8 post-LCMV infection

The absolute number of TNF-α-producing LCMV-specific CD4 cells (gp61-specific) and CD8 cells (gp33-specific) per spleen (D) and percentage (E) of these cells among total spleen cells of KO and WT mice on day 8 post-LCMV infection. Means $\pm$ SD of data from 4 pairs of Stra6 KO mice and WT littermate controls are shown.

F and G. Virus-specific IFN-γ-producing CD4 and CD8 cells on day 8 post-LCMV infection

The absolute number of TNF-α-producing LCMV-specific CD4 cells (gp61-specific) and CD8 cells (gp33-specific) per spleen (D) and percentage (E) of these cells among total spleen cells of KO and WT mice on day 8 post-LCMV infection. Means $\pm$ SD of data from 4 pairs of Stra6 KO mice and WT littermate controls are shown.

The results in this figure are analyzed by Student's t test. No significant difference was found between WT and groups.

Figure 3.7. Glucose tolerance of KO and WT mice

WT (n=5) and KO (n=7) mice were fasted for 16 h, and then injected i.p. with D-glucose (2 mg/g body weight). Blood glucose was measured at different time points from the time of injection until 120 min. Means $\pm$ SD of glucose levels (mg/dL) are reported. No statistical significant difference is observed between the KO and WT groups (ANOVA).

Figure 3.8. Intracellular retinoid contents in lymphoid and other organs of Stra6 KO mice were comparable to those of WT mice

Retinoid (retinyl ester, A; retinol, B) contents (nmol/gram tissue or nmol/ml serum) of the eyes, brain, kidney, spleen, thymus, spleen, thymus and sera from KO and WT mice were measured by HPLC. The mouse numbers (n) per group are indicated. The results are expressed as means + SD. The *p*-values are indicated when significant (Student's *t* test).

Figures

Figure 3.1 *Stra6* mRNA expression in organs and activated T cells

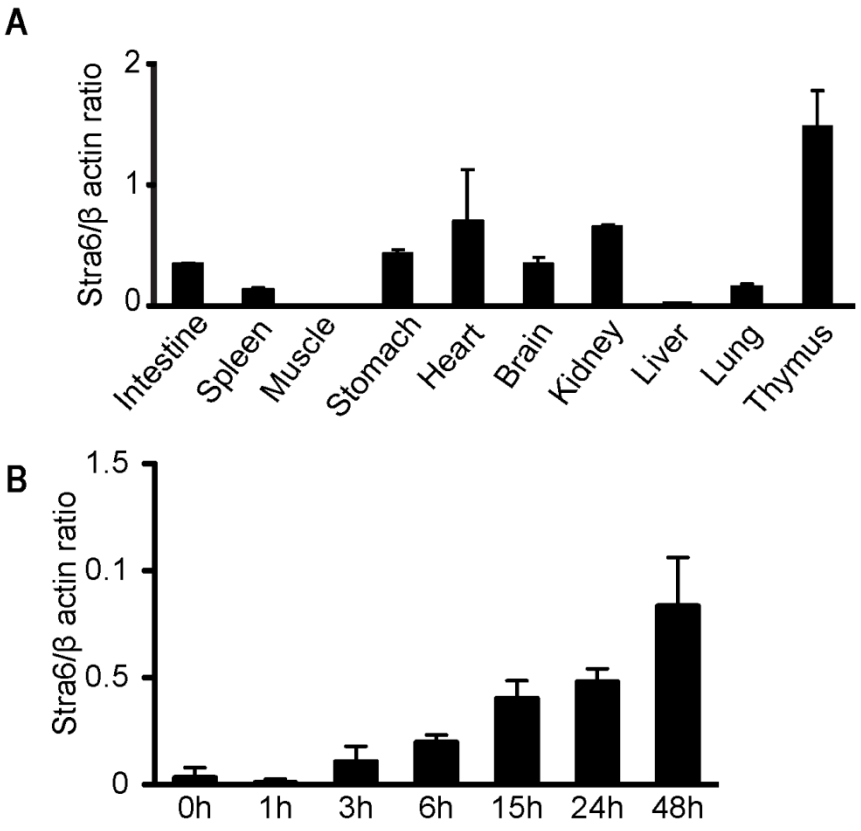


Figure 3.2 Generation of Stra6 KO mice

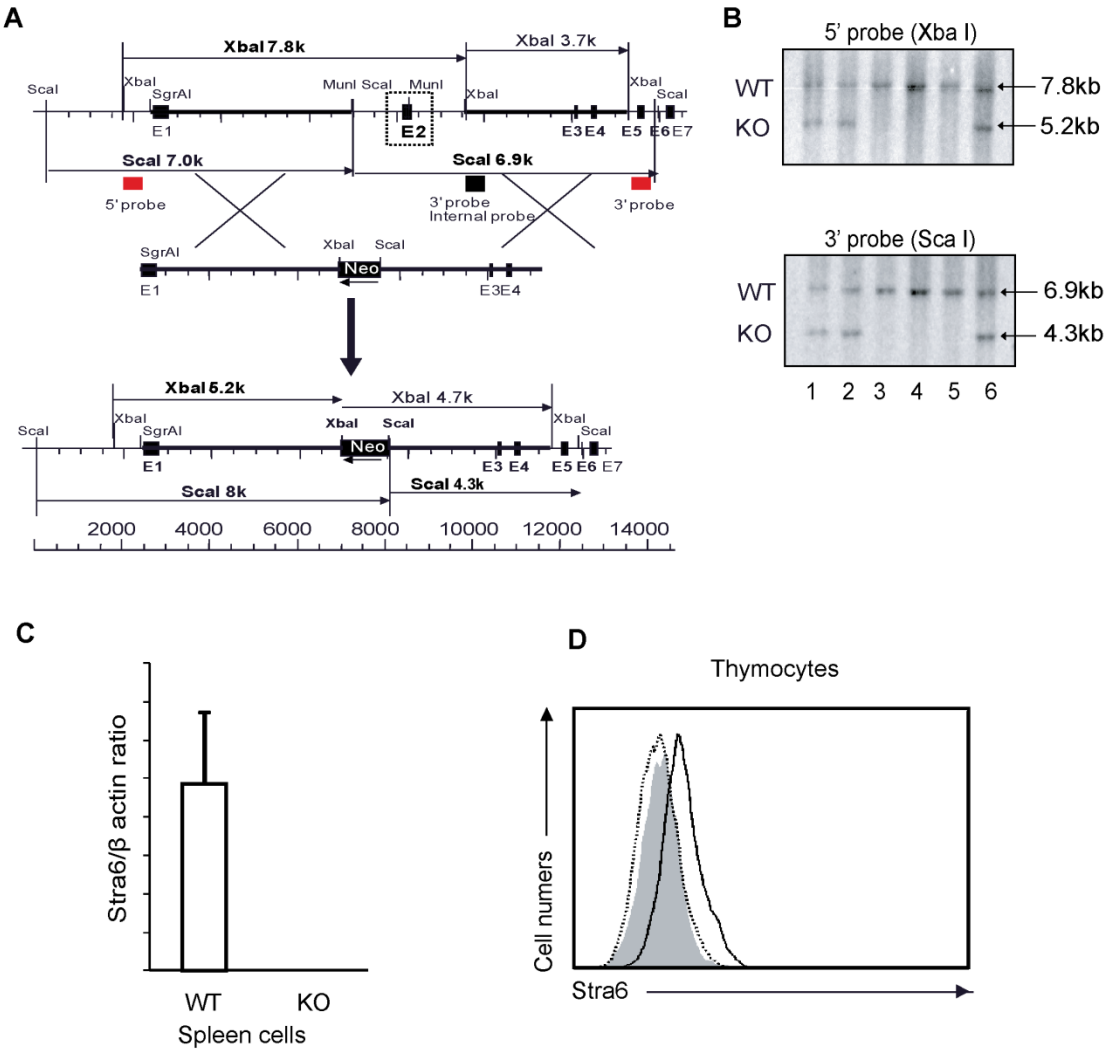


Figure 3.3 Stra6 KO mice presented normal lymphoid organs and lymphocyte subpopulations

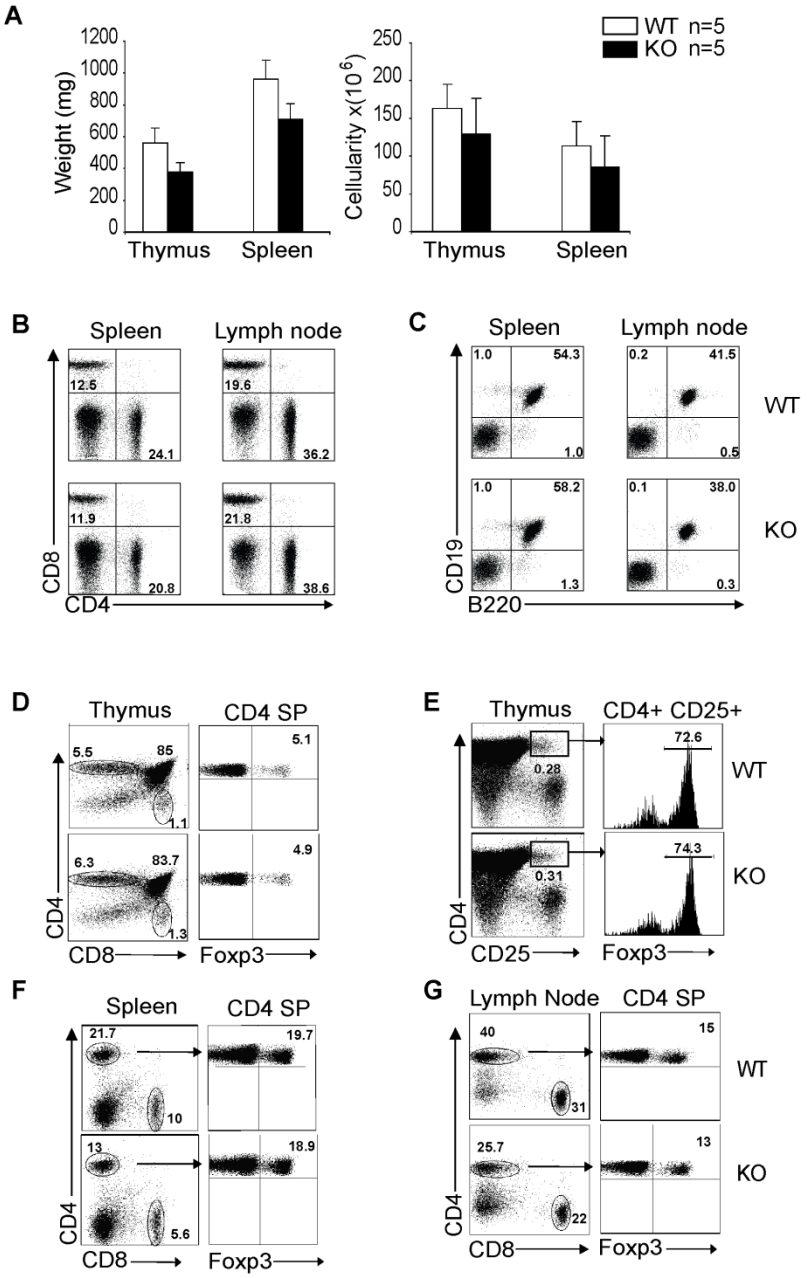


Figure 3.4 Normal activation and proliferation of KO T cells

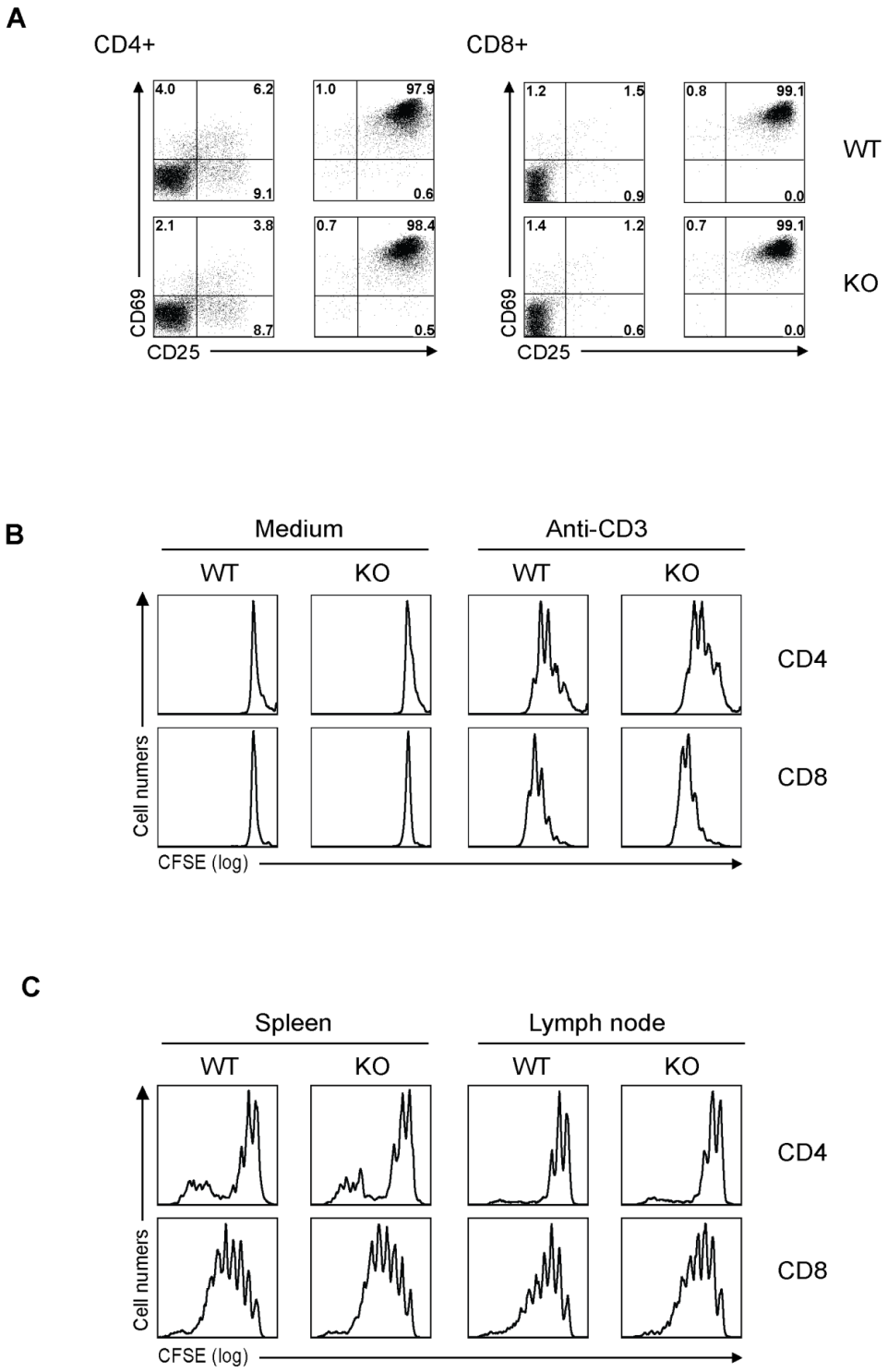


Figure 3.5 Normal differentiation of Stra6 KO CD4 cells in vitro

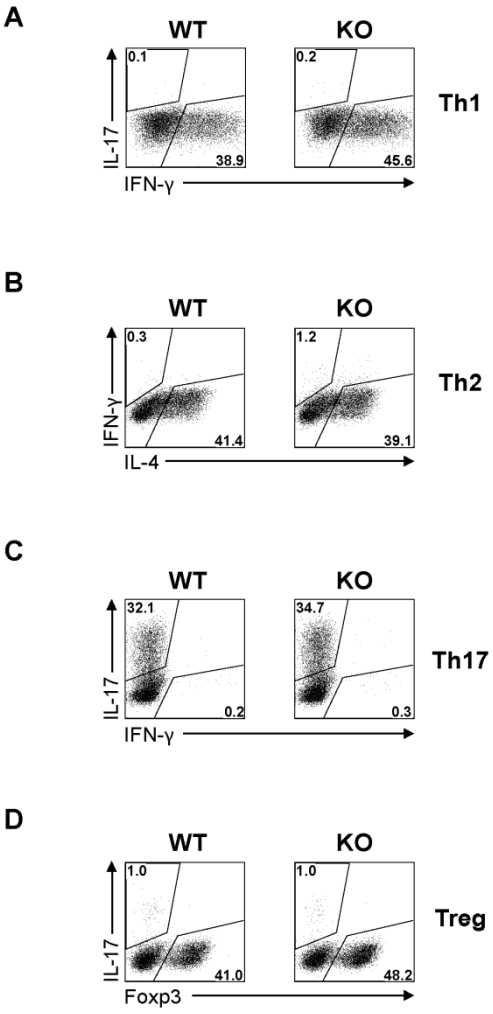


Figure 3.6 Normal in vivo anti-LCMV immune responses of Stra6 KO mice

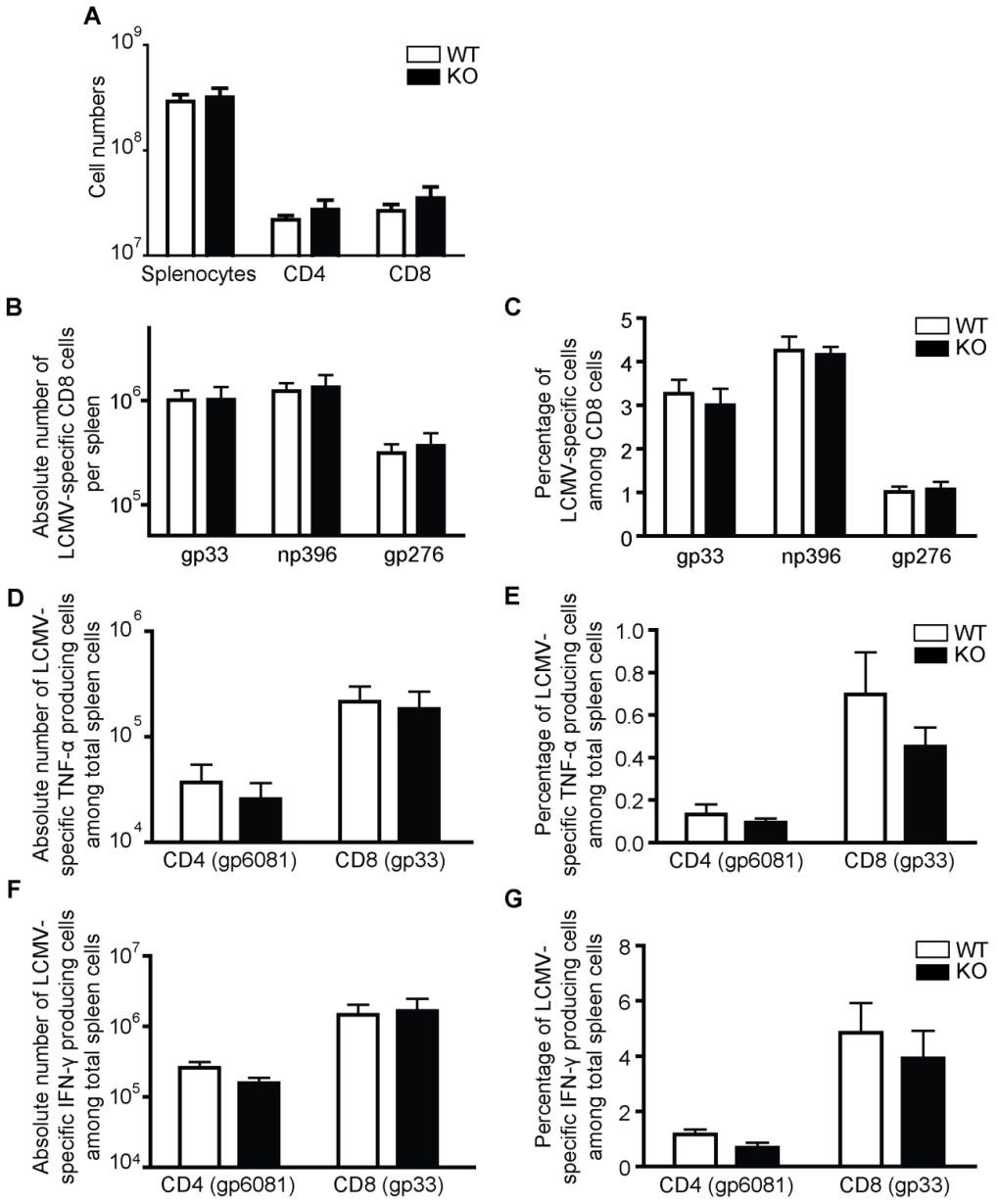


Figure 3.7 Glucose tolerance of KO and WT mice

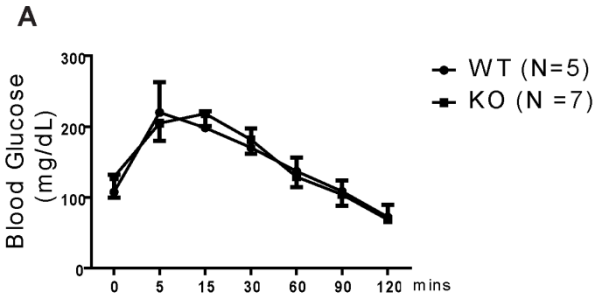
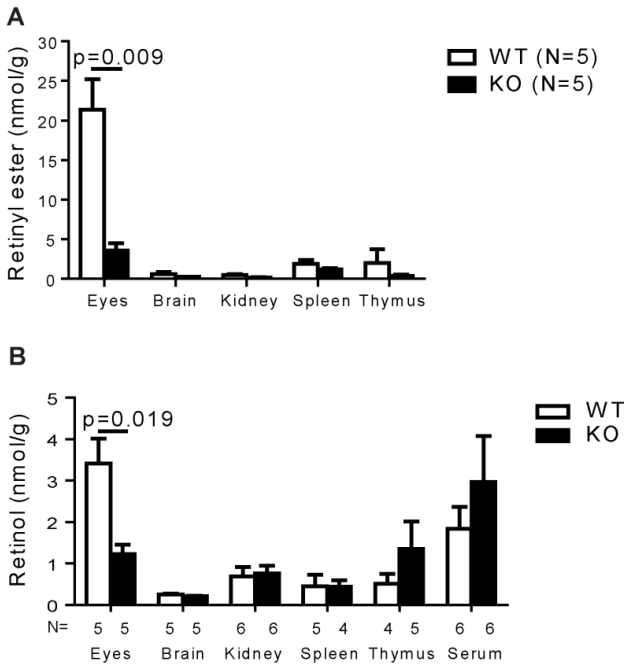


Figure 3.8 Intracellular retinoid contents in lymphoid and other organs of Stra6 KO mice were comparable to those of WT mice



Chapter 4 Article-3

Armc5 deletion causes developmental defects and compromises T cell immune responses

This work has been accepted by **Nature Communications**.

Yan Hu, Linjiang Lao, Jianning Mao, Wei Jin, Hongyu Luo, Tania Charpentier, Alain Lamarre, Shijie Qi, Junzheng Peng, Martin Marcinkiewicz, and Jiangping Wu. *Armc5* deletion causes developmental defects and compromises T cell immune responses.

4.1 *Armc5* deletion causes developmental defects and compromises T cell immune responses

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Short title: **ARMC5 is essential for development and T cell function**

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Abstract

Armadillo repeat containing 5 (ARMC5) is a cytosolic protein with no enzymatic activities. Little is known about its function and mechanisms of action, except that gene mutations are associated with risks of primary macronodular adrenal gland hyperplasia. We have mapped *Armc5* expression by *in situ* hybridization, and generated *Armc5* knockout (KO) mice, which are small in body size. Here we show that these KO mice present with compromised T cell proliferation and differentiation into Th1 and Th17 cells, increased T cell apoptosis, reduced severity of experimental autoimmune encephalitis, and defective immune responses to lymphocytic choriomeningitis virus infection. KO mice develop adrenal gland hyperplasia in old age. Yeast 2-hybrid assays identify 16 ARMC5-binding partners. These data indicate that ARMC5 is crucial in fetal development, T cell function and adrenal gland growth homeostasis, and that the functions of ARMC5 likely depend on its interaction with molecules involved in multiple signaling pathways.

Introduction

The gene Armadillo was first identified in the fruit fly *Drosophila melanogaster* as a gene controlling larval segmentation with morphological similarity to armadillos[1, 2]. β-Catenin is the human and mouse orthologue of fruit fly Armadillo[3]. Armadillo/β-catenin protein contains 13 and 12 conserved armadillo (ARM) repeats, respectively**:** each repeat is about 40 amino acid (aa) long and consists of 3 α-helices[4]. Multiple repeats form an ARM domain which has a groove for binding various other proteins in its tertiary structure[5]. More than 240 proteins, from yeasts to humans, are known to contain an ARM domain[6, 7]. Although β-catenin is believed to interact with and regulate cytoskeleton function, its roles and those of ARM domain-containing proteins, in general, are very versatile in cell biology, including cytoskeleton organization[8], cell-cell interactions[9], protein nuclear import[10], degradation[11] and folding[12], cell signaling/sensing[13, 14, 15], molecular chaperoning[16], cell invasion/mobility/migration[17], transcription control[18], cell division/proliferation[19], and spindle formation[20], to name some of them.

At the tissue and organ levels, ARM domain-containing proteins are involved in T cell development[21], lung morphogenesis[22], limb dorsal-ventral axis formation[23], neural tube development[24], osteoblast/chondrocyte switch[25], synovial joint formation[26], adrenal gland cortex development[27], and tumor suppression[28].

Due to the very diverse functions of ARM domain-containing proteins, it is challenging to predict their mechanisms of action. Indeed, these aspects of many ARM domain-containing

proteins remain undeciphered, and quite a number of them are given the name ARMC (ARM repeat-containing), followed by Arabic numbers (*e.g.,* ARMC1, 2, 3 and so on). ARMC5 is one such protein.

Human and mouse ARMC5 proteins share 90% aa sequence homology and have similar structures[29, 30]. Mouse ARMC5 is 926 aa in length and contains 7 ARM repeats. A BTB/POZ domain towards its C-terminus is responsible for dimerization or trimerization[31, 32, 33]. Several groups reported in 2013 and 2014 that *ARMC5* gene mutations are associated with primary macronodular adrenal hyperplasia (PMAH) and Cushing's syndrome[34, 35, 36]. Assié *et al.*[34] demonstrated that no viable HeLa cells could be obtained when they were stably transfected with *ARMC5*-expressing vectors. They suggested that the default function of wild type (WT) ARMC5 is the suppression of cell proliferation or promotion of apoptosis, which might explain the adrenal cortex hyperplasia seen in patients with ARMC mutations. No other reports on ARMC5 function and mechanisms of action are available in the literature.

In the present work, we have studied the tissue-specific expression of *Armc5*. We have generated *Armc5* gene knockout (KO) mice, and revealed that ARMC5 is vital in development and immune responses. We also show that aged KO mice develop adrenal gland hyperplasia. We have identified a group of ARMC5-interacting proteins by yeast 2-hybrid (Y2H) assay, paving the way for further mechanistic and functional investigations of ARMC5.

Materials and Methods

ISH

To localize *Armc5* mRNA, 1526-bp (starting from GATATC to the end) mouse *Armc5* cDNA (GenBank: BC032200, cDNA clone MGC: 36606) in pSPORT1 was employed as template for S and AS riboprobe synthesis, with SP6 and T7 RNA polymerase for both ^{35}S-UTP and ^{35}S-CTP incorporation[37].

Tissues from WT mice were frozen in -35˚C isopentane and kept at -80˚C until they were sectioned. ISH, X-ray and emulsion autoradiography focused on 10-μm thick cryostat-cut sections. Briefly, overnight hybridization at 55 ˚C was followed by extensive washing and digestion with RNase to eliminate non-specifically bound probes. Anatomical level images of ISH were generated using X-Ray film autoradiography after 4 days' exposure. Microscopical level ISH was produced by dipping sections in NTB-2 photographic emulsion (Kodak). The exposure time was 28 days. The autoradiography labelling was revealed by D19 Developer (Kodak) and fixation with 35% sodium thiosulphate. Slides were left unstained or slightly stained with haematoxylin/eosin[37].

RT-qPCR

Armc5 mRNA in thymocytes, T cells, B cells and tissues from KO and WT mice was measured by RT-qPCR. Total RNA was extracted with TRIzol® (Invitrogen, Carlsbad, CA, USA) and then reverse-transcribed with Superscript II™ reverse-transcriptase (Invitrogen). Thymocytes were stained with anti-CD4 (1:400, Clone RM4-5, BD Bioscience), anti-CD8 (1:400, Clone 53-6.7, BioLegend), anti-CD25 (1:200, Clone PC61.5, eBioscience) and anti-

CD44 (1:200, Clone IM7, BioLegend) Abs. $CD4^+$ cells, $CD8^+$ cells, DP cells, DN cells and DN cells in different stages were sorted by flow cytometry. T cells and B cells were isolated by magnetic beads (EasySep™, Stem Cell Technology, Vancouver, BC, Canada). For RT-qPCR measurement of *Armc5* expression during T cell activation, mouse T-activator CD3/CD28 Dynabeads (ThermoFisher Scientific, Burlington, ON, Canada) were used for T cell activation *in vitro*, to avoid introducing Ag-presenting cells into purified CD4 or CD8 cells.

Forward and reverse primers were 5'-CAG TTA TGT GGT GAA GCT GGC GAA-3' and 5'-ACC CTC AGA AAT CAG CCA CAA CCT-3', respectively. A 139-bp product was detected with the following amplification program: 95˚C×15 min, 1 cycle; 95˚C×10 s, 59˚C×15 s, 72˚C×25 s, 35 cycles. *β-actin* mRNA levels were measured as internal controls. Forward and reverse primers were 5'-TCG TAC CAC AGG CAT TGT GAT GGA-3' and 5'-TGA TGT CAC GCA CGA TTT CCC TCT-3', respectively, with the same amplification program as for *Armc5* mRNA. The data were expressed as ratios of *Armc5* versus *β-actin* signals.

Armc5 overexpression in L cells

L cells (ATCC CRL-2648, ATCC) were transiently transfected with pReceiver-Lv120 plasmid expressing mouse *Armc5* with HA tag (EX-Mm23477-LV120, GeneCopoeia, Rockville, MD, USA) for 2 days, and fixed with 4% paraformaldehyde. Subcellular Armc5 localization in L cells was detected by immunofluorescence with biotinylated rat anti-HA Ab (1:500, 12158167001, Roche, Laval, QC, Canada), followed by FITC-conjugated streptavidin

(1:2000, S11223, ThermoFisher, Burlington, ON, Canada). The L cells were not authenticated and possible mycoplasma contamination was not tested.

Generation of Armc5 KO mice

A PCR fragment amplified from the *Armc5* cDNA sequence served as probe to isolate genomic BAC DNA clone 7O8 from the RPCI-22 129/sv mouse BAC genomic library. The targeting vector was constructed by recombination and routine cloning methods, with a 15-kb *Armc5* genomic fragment from clone 7O8 as starting material. A 2.7-kb HindIII/EcoRV genomic fragment containing exon 1-3 was replaced by a 1.1-kb Neo cassette from pMC1Neo-Poly A flanked by 2 diagnostic restriction sites, EcoRV, and HindIII, as illustrated in Figure 2a. The final targeting fragment was excised from its cloning vector backbone by NotI/EcoRI digestion and electroporated into R1 embryonic stem (ES) cells for G418 selection. Targeted ES cell clones were injected into C57BL/6J blastocysts. Chimeric male mice were mated with C57BL/6 females to establish mutated *Armc5* allele germline transmission.

Southern blotting with probes corresponding to 5' and 3' sequences outside the targeting region, as illustrated in Figure 2a (red squares), screened for gene-targeted ES cells and eventually confirmed gene deletion in mouse tail DNA. With the 5' probe, the targeted allele presented a 6.6-kb EcoRV band, and the WT allele, a 9.3-kb EcoRV band. With the 3' probe, the targeted allele presented an 8.7-kb HindIII band, and the WT allele, a 12.5-kb HindIII band (Supplementary Fig. 3).

Heterozygous mice were backcrossed to the C57BL/6J background for 8 generations and then crossed with 129/sv mice. WT and KO mice in the C57BL/6J×129/sv F1 background were studied. All animals were housed under specific pathogen-free conditions and handled in accordance with a protocol approved by the Institutional Animal Protection Committees of the CRCHUM and INRS-IAF.

Serum total IgG measurement

Flat bottom 96-well plates (Costar EIA/RIA, No. 3369, Fisher Scientific) were coated with goat anti-mouse IgG (100 µl/well, 1 µg/ml in PBS) and incubated overnight at 4°C. After five times of washings with PBS containing 0.05% Tween 20, the plates were blocked with PBS containing 3% BSA and 5% FBS for 1.5 h at room temperature. After 5 washings, diluted serum samples ($1:10^5$) and serially-diluted standard mouse IgG (sc-2025, Santa Cruz) were added to the wells (100 µl/well) and incubated at 37°C for 1 h. The plates were then washed 10 times, and diluted (1:4000) horse radish peroxidase-conjugated horse anti-mouse IgG (#7076S, Cell Signaling Technology) was added (100 µl/well) to the wells. The plates were incubated for 1 h at 37°C. After another 10 washings, 1-Step™ Ultra TMB-ELISA Substrate Solution (#34028, Thermo Scientific) was added to the wells (100 µl/well). The plates were incubated in the dark at room temperature for 20-30 min, and the reaction was stopped by 2M sulfuric acid (100 µl/well). Optical density at 450 nm of reactants was measured. Samples were assayed in duplicate. Mouse total IgG concentrations were calculated according to a standard curve established by serial dilutions of standard mouse IgG. Assay sensitivity was in the 0.39 and 6.25 ng/ml range.

Enzyme-linked immunosorbent assay (ELISA)

Glucocorticoid levels in WT and KO mouse sera were quantified by ELISA, detecting mouse glucocorticoids according to the manufacturer's instructions (MBS028416, MyBioSource, San Diego, CA, USA).

Flow cytometry

Single cell suspensions from the thymus, spleen and LN were prepared and stained immediately or after culture with Abs against CD3 (1:200, Clone 145-2C11, BD Bioscience), CD4 (1:400, Clone RM4-5, BD Bioscience), CD8 (1:400, Clone 53-6.7, BioLegend), CD25 (1:200, Clone PC61.5, eBioscience), CD44 (1:200, Clone IM7, BioLegend), CD45.1 (1:200, Clone A20, BD Bioscience), CD45.2 (1:200, Clone 104, BD Bioscience), CD62L (1:200, Clone MEL-14, BD Bioscience), CD107a (1:200, Clone 1D4B, BD Bioscience), CD127 (1:200, A019D5, BioLegend), KLRG1 (1:200, Clone 2F1/KLRG1, BioLegend), Thy1.2 (1:1000, Clone 30-H12, BioLegend), B220 (1:200, Clone RA3-6B2, BD Bioscience), 7AAD (1:25, 51-68981E, BD Bioscience), Annexin-V (1:50, 550474, BD Bioscience). In some experiments, intracellular proteins, such as IFN-γ (1:200, Clone XMG1.2, BD Bioscience), IL-17 (1:200, Clone TC11-18H10, BD Bioscience), FoxP3 (1;200, Clone 150D, BioLegend), T-bet (1:200, Clone 4B10, BioLegend), RORγt (1:200, Clone B2D, eBioscience) and TNF-α (1;200, Clone MP6-XT22, BD Bioscience) and Granzyme B (1;200, Clone GB11, BioLegend), were detected after the cells were pre-stained with Abs against cell surface Ag, permeabilized with BD Cytofix/Cytoperm^{TM} solution (BD Biosciences), and then stained with Abs against intracellular Ag [38, 39].

Flow cytometry was also employed to assess LCMV-specific T cells. The synthetic peptides gp_{33-41}: KAVYNFATC (LCMV-GP, H-2D^b), $np_{396-405}$: FQPQNGQFI (LCMV-NP, H-2D^b) and $gp_{276-286}$: SGVENPGGYCL (LCMV-GP, H-2D^b) were purchased from Sigma-Genosys (Oakville, ON, Canada). PE-gp_{33-41}, PE-$np_{396-405,}$ and PE-$gp_{276-286}$ H-2D^b tetrameric complexes were synthesized in-house and applied at 1/100 dilution [39]. These MHC-tetramers served to detect LCMV-specific CD8$^+$ T cells on day 8 post-LCMV infection. Briefly, splenocytes were first stained with PE-gp_{33-41}, PE-$np_{396-405}$ or PE-$gp_{276-286}$ tetramers for 30 min at 37°C, directly followed by surface staining (CD3, CD8, CD44, and CD62L) and dead cell exclusion (7AAD) for another 20 min at 4°C. The cells were then fixed with 1% paraformaldehyde, and samples were analyzed by flow cytometry.

For intracellular cytokine staining, 10^6 splenocytes from LCMV-infected mice were maintained for 5 h at 37° C in RPMI-1640 with 10% FCS and 55 μg/mL β-ME, supplemented with a final concentration of 50 U/ml IL-2, 5 μg/ml Brefeldin A, 2 μM Monensin, 2.5 μg/mL FITC-labeled anti-mouse CD107a and 5 μM gp_{33-41} or gp_{61-80} GLNGPDIYKGVYQFKSVEFD (LCMV-GP, I-Ab) synthetic peptide from Sigma-Genosys. After *ex vivo* incubation, surface staining and cell viability were verified with anti-mouse CD8a, CD4 and CD62L mAbs and 7AAD. The cells were then fixed, permeabilized and stained with anti-mouse TNF-α, IFN-γ and Granzyme B mAbs. Cytokine-producing T cells were analyzed by flow cytometry [40].

Lymphocyte proliferation and apoptosis in vitro

Spleen cells were loaded with carboxyfluorescein succinimidyl ester (CFSE: 5 μM for 5 min). After washing, they were stimulated with soluble hamster anti-mouse CD3ε mAb (clone 145-

2C11, 2 μg/ml; BD Biosciences) for T cell proliferation assays. This protocol allows best long-term T cell proliferation over a 4-day period, for clear demonstration of multiple cell proliferation rounds according to CFSE staining. In B-cell proliferation assays, CFSE-loaded Spleen cells were stimulated with goat anti-mouse IgM (5 μg/ml; Jackson ImmunoResearch), IL-4 (10 ng/ml) and goat-anti-mouse CD40 (2 μg/ml, Jackson ImmunoResearch). After 3-4 days, the cells were gated on CD4-, CD8- or B220-positive cells, and their CFSE intensity was ascertained by flow cytometry.

To assess Th1 and Th17 cell proliferation, naïve CD4 cells were loaded with CFSE, and cultured under Th1 and Th17 conditions for 4 days (detailed below). $CD4^+$ or intracellular IFN-γ^+ or IL-17^+ cells were then gated, and their CFSE intensity was assessed by flow cytometry.

For cell cycle analysis, total spleen cells were stimulated with anti-CD3ε mAb, as described above, and stained with anti-Thy1.2 mAb and propidium iodide (PI: 20 μg/ml) on days 0, 1 and 2. Thy1.2^+ T cells were gated and their PI signal strength was measured by flow cytometry.

For T cell apoptosis analysis, spleen cells were stimulated with anti-CD3ε mAb (2 μg/ml) plus crosslinked human FasL-FLAG (0.133 μg/ml; FasL–FLAG was pre-incubated for 24 hours at 4°C at a 1:1 ratio with 0.133 μg/ml mouse monoclonal Ab against FLAG (F1804, Sigma); the

final concentration of crosslinked FasL-FLAG for culture was 0.6 μg/ml [41]) and cultured for 4 h. Cells positive for CD4 or CD8 were gated and analyzed for annexin V expression.

Th1 and Th17 cell differentiation in vitro

T cell differentiation *in vitro* was undertaken as follows [38, 39]. Naïve $CD4^+$ T cells ($CD4^+$ $CD62L^+CD44^{low}$) were isolated from KO or WT mouse Spleen with EasySep™ mouse naïve $CD4^+$ T cell isolation kits (19765, Stem Cell Technology). Naïve $CD4^+$ T cells from WT and KO mice (0.1×10^6 cells/well) were mixed with feeder cells (0.5×10^6 cells/well) and cultured in 96-well plates in the presence of soluble anti-CD3ε Ab (2 μg/ml). Feeder cells plus anti CD3ε Ab were used, as in our hands, they achieved the most consistent Th1 and Th17 differentiation conditions. Cultures were supplemented with recombinant mouse IL-12 (10 ng/ml; 419-ML, R & D Systems, Minneapolis, MN, USA) and anti-IL-4 mAb (5 μg/ml; MAB404, R & D Systems) for the Th1 condition, with recombinant mouse IL-6 (20 ng/ml; 406-ML, R & D Systems), recombinant human TGF-β1 (5 ng/ml; 240-B, R & D Systems) and anti-IL-4 (5 μg/ml) and anti-IFN-γ mAbs (5 μg/ml; MAB485, R & D Systems) for the Th17 condition. The cells were stimulated with PMA (10μM) and ionomycin (100 μg/ml) in the presence of 5 μg/ml Brefeldin A for the last 4 h of culture, and their intracellular IFN-γ, T-bet, IL-17, and RORγt were analyzed by flow cytometry.

Chimera generation

Eight- to 10-week-old C57BL/6J ($CD45.2^+$) × C57B6.SJL ($CD45.1^+$) F1 mice were irradiated at 1,100 rads. Twenty-four h later, they received *i.v.* 2×10^6 fetal liver cells from WT or KO mice) in the C57BL/6J×129/sv ($CD45.2^+$) F1 background. Peripheral white blood cells of

recipients were analyzed by flow cytometry 8 weeks after fetal liver cell transplantation. Twelve weeks after transplantation, chimeras with successful implantation of donor-derived white blood cells were studied in *in vitro* T cell function experiments and for EAE induction.

EAE induction and assessment

EAE was induced in 8- to 12-week-old female WT and KO mice[42]. Briefly, mice were immunized with 300 μg MOG_{35-55} peptide (Biomatik, Wilmington, DE, USA) emulsified in complete Freund's adjuvant, followed by *i.p.* injection of 400 ng pertussis toxin (List Biological Laboratories, Campbell, CA, USA) on days 0 and 2. EAE development was scored daily between days 0 and 35 according to a scale ranging from 0 to 5, as follows: 0, no sign of paralysis, 1, weak tail; 2, paralyzed tail; 3, paralyzed tail and weakness of hind limbs; 4, completely paralyzed hind limbs; 5, moribund. Scores were assigned in 0.5 unit increments when symptoms fell between 2 full scores.

Female chimeras with successful donor cell implantation (verified according to CD45.2 single- positive cells in peripheral blood) 12 weeks after fetal liver transplantation were also used for EAE induction. The same protocol described above was followed, except that 200 μg MOG_{35-55} peptide for immunization and 200 ng pertussis toxin/injection were administered to each chimeric mouse.

EAE histology

To assess the degree of inflammation and CNS demyelination, EAE mice were euthanized on day 30 and perfused by intra-cardiac injection of PBS. Spinal cord sections were stained with

H/E or NovaUltra™ Luxol Fast Blue Staining Kit (IHC World, Woodstock, MD, USA), according to the manufacturer's instructions. Each SC section was subdivided into 4 regions: 1 anterior, 1 posterior, and 2 lateral. Each region was scored on a scale of 0 to 3 for lymphocyte infiltration and demyelination in a 1-way blinded fashion. Thus, each animal had a potentially maximal score of 12 points for lymphocyte infiltration and demyelination, respectively [43, 44]. Total pathological scores were the sum of these 2 parameters.

Isolation of mononuclear cells from the spinal cord and brain

Peripheral blood was removed from the spinal cord and brain by intra-cardiac perfusion through the left ventricle with heparinized ice-cold PBS. The spinal cord and brain were harvested, ground and then passed through a 70-μm mesh screen. The cells were centrifuged through a 40%-60%-90% discontinuous Percoll gradient. Mononuclear cells at the 40% to 60% Percoll interface were collected and stained for phenotype analysis.

Differentiation/characterization of mouse Th1 and Th17 cells

Cells from draining LN and mononuclear cells from the spinal cord and brain were further stimulated with PMA (5 nM) and ionomycin (500 ng/ml) for 4 h in the presence of Golgi Stop (554724, BD Biosciences), before being harvested. They were stained with Abs against cell surface Ag, fixed with Cytofix/Cytoperm™ solution (555028, BD Biosciences), and then stained with mAbs against intracellular IFN-γ (1:200, Clone XMG1.2, BD Bioscience) and IL-17 (1:200, Clone TC11-18H10, BD Bioscience). Stained cells were analyzed by flow cytometry.

LCMV infection

LCMV-WE was obtained from Dr. R.M. Zinkernagel (University of Zurich, Zurich, Switzerland). Viral stock was propagated *in vitro*, and viral titers were quantified by focus-forming assay [40]. Mice were infected by the *i.v.* route with 200 focus-forming units (ffu) of LCMV-WE. They were sacrificed 8 days post-infection, and their spleens were harvested for primary immune response analysis. $CD8^+$ T cells were isolated from the spleen of naïve or infected WT mice, with EasySep™ mouse $CD8^+$ T cell isolation kits (19853, Stem Cell Technology). RNA from isolated cells was extracted with TRIzol® (Invitrogen), followed by RT-qPCR.

Y2H assay

Y2H screening was performed by Hybrigenics Services (Paris, France). The coding sequence for human *ARMC5* cDNA (aa 30-935) (GenBank accession number GI: 157426855) was PCR-amplified and cloned into pB29 as a N-terminal fusion protein to LexA (N-ARMC5-LexA-C). The construct was verified by sequencing the entire insert and served as bait to screen a random-primed human thymocyte cDNA library constructed in the pP6 plasmid. pB29 and pP6 vectors were derived from the original pBTM116 [45, 46] and pGADGH [47] plasmids, respectively.

Eighty million yeast clones (8-fold the complexity of the library) were screened via a mating approach with YHGX13 (Y187 ade2-10: loxP-kanMX-loxP, matα) and L40Gal4 (mata) yeast strains [48]. One hundred and sixty-five His^+ colonies were selected on medium lacking tryptophan, leucine, and histidine, and supplemented with 5.0 mM of 3-aminotriazole to

quench bait auto-activation. Prey fragments of positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were considered to identify corresponding interacting proteins in the GenBank database via a fully-automated procedure. A Predicted Biological Confidence score was attributed to each interaction [36].

Statistics and general methods

For *in vivo* animal studies, the sample size was determined by estimation, based on our past experience and literature. No formal randomization was used, but littermates or age and sex matched WT and KO mice were used. In general, two-tailed Student's *t* tests were used. *Chi*-square test was used to compare the difference between two proportions. One-way ANOVA followed with Bonferroni's multiple comparisons test was used in data from more than three groups. For the histology experiments, one-way blind examination was performed.

Data availability

The data that support the findings of this study are available in the text and the supplementary data files, or from the corresponding author upon request.

Results

Armc5 expression in mice and T cells

Armc5 mRNA expression was analyzed by *in situ* hybridization (ISH) in adult WT mice. Hematoxylin/eosin (HE) staining of a consecutive sagittal whole body section preceded ISH (Fig. 1a, upper panel). *Armc5* expression, based on anti-sense riboprobe hybridization (Fig. 1a, middle panel), was high in the thymus, stomach, bone marrow, and lymphatic tissues (including lymph nodes and intestinal wall). The hybridization was also apparent in the adrenal gland, and skin. Some hybridization occurred in brain structures, with noticeable levels found in the cerebellum. Control hybridization with sense (S) riboprobes revealed a faint nonspecific background (Fig. 1a, bottom panel).

At the anatomical level, *Armc5* expression was high in the thymus cortex (Fig. 1b, upper left panel). This was confirmed at the microscopic level (Fig. 1b, bottom left panel). The higher *Armc5* signals in the cortex than in the medulla were due to higher cell density in the former. Sense riboprobes detected little background noise (Figs. 1b, right column). Based on reverse transcription-quantitative polymerase chain reaction (RT-qPCR) results, thymic stroma cells (including epithelial cells) had *Armc5* expression similar to that of thymocytes (Supplementary Fig. 1a). Further, there was no significant difference in *Armc5* expression among thymocyte subpopulations (CD4/CD8 double-negative (DN) 1-4, CD4/CD8 double-positive (DP), and CD4- or CD8- single-positive (SP); Supplementary Figs. 1b and 1c; gating strategy: Supplementary Fig. 2a), and between naïve and memory spleen T cells (Supplementary Fig. 1d; gating strategy: Supplementary Fig. 2b).

Moderately intense *Armc5* labeling was apparent in spleen white pulp but not in red pulp (Fig. 1c, upper panel). At the microscopic level, small groups of cells in WP displayed *Armc5* signals (Fig. 1c bottom panel). Sense riboprobes detected no signals (Fig. 1c middle panel).

Armc5 mRNA expression was induced rapidly in CD4 cells in 2 h after CD3ε plus CD28 stimulation, then subsided and remained low between 24 to 72 h post-activation (Fig. 1d, upper row). CD8 T cells had less *Armc5* induction and the levels remained low between 24 to 72 h (Fig. 1d, lower row).

ARMC5 was mainly a cytosolic protein, as it was detected in the cytoplasm of L cells transiently transfected with a mouse ARMC5 expression construct (Figs. 1e and 1f).

Generation of Armc5 KO mice

We generated *Armc5* KO mice to understand the biological roles of ARMC5 in general and T cell-mediated immune responses in particular. Our targeting strategy is illustrated (Fig. 2a). Germline transmission was confirmed by Southern blotting of tail DNA (Supplementary Fig. 3). With the 5' end probe, the WT allele after EcoRV digestion gave a 9.3-kb band, and the KO allele, a 6.6-kb band (Supplementary Fig. 3, upper panel). With the 3' end probe, the WT allele after HindIII digestion presented a 12.5-kb band, and the KO allele, an 8.7-kb band (Supplementary Fig. 3, lower panel). WT (mice 3 and 7) and heterozygous mice (mice 1, 2, 4,

5 and 6) were thus identified. Mouse 1 in the original 129/sv×C57BL/6J background was backcrossed to different genetic backgrounds for experimentation, as detailed below.

Armc5 deletion of KO mice at the mRNA level in spleen T cells, thymocytes, lymph nodes, brain and adrenal glands was confirmed by RT-qPCR (Fig. 2b).

General phenotype of Armc5 KO mice

When *Armc5* KO mice were in the C57BL/6J×129/sv F1 background, only about 10% live KO pups were delivered in a heterozygous × heterozygous mating strategy, below the expected 25% Mendelian rate. After F1 mice were backcrossed to C57BL/6 for 5 or more generations, no KO pups could be produced, nor were live KO pups born after the mice were backcrossed 8 generations to the 129/sv background. This suggested that *Armc5* deletion caused embryonic lethality, with its severity depending on genetic background of the mice: embryonic lethality became more severe with higher degrees of genetic background purity. KO mice in the C57BL/6J×129/sv F1 background were studied in subsequent experiments.

KO embryos were smaller than WT controls at embryonic day 14 (Fig. 3a). These KO pups were smaller at age 8-12 weeks (Fig. 3b). Body weight was significantly lower in KO and WT mice at age 4 and 8 weeks than in their WT littermates (Fig. 3c). Both male and female KO mice weighed only about 60% as much as WT controls.

We examined serum growth hormone levels because of growth retardation in KO mice, but no significant difference was found between them and their WT counterparts (Supplementary Fig. 4).

ARMC5 gene mutations have been reported to be linked with PMAH and Cushing's syndrome[34]. However, KO mice presented normal adrenal gland size and histology (Supplementary Fig. 5) and serum glucocorticoid levels (Supplementary Fig. 6) in young age (less than age 5 months). In old age (>15 months), grossly, KO mice showed enlarged adrenal glands without apparent nodular structure, and histologically, there is no identifiable nodular hyperplasia (Fig. 3d). Serum glucocorticoid levels were significantly increased in aged KO mice (Fig. 3e), supporting the notion that the adrenal gland hyperplasia is of cortex in nature. It is to be noted that the mice were sacrificed between 12:30-1:30pm, and their blood was harvested for the measurement of glucocorticoids, whose secretion is at the nadir at this time point. The moderate but significant increase of glucocorticoid levels in the KO mice is reminiscent of human PMAH, in which the increase of glucocorticoid levels is not drastic and is caused by the large mass of the adrenal gland, while on a per cell basis, the secretion is reduced[34].

Armc5 KO phenotype in lymphoid organs and T cells

Thymus (Supplementary Fig. 7a) and spleen (Supplementary Fig. 7b) weight and cellularity were not significantly different in KO and WT mice. Moreover, thymocyte sub-populations ($CD4^+CD8^+$ double-positive, $CD4^+$ single-positive and $CD8^+$ single-positive cells) in the KO

and WT thymus were comparable (Supplementary Fig. 7c), as were spleen lymphocyte subpopulations (Thy1.2$^+$ T cells versus B220$^+$ B cells; CD4$^+$ versus CD8$^+$ T cells; Supplementary Fig. 7d).

Despite seemingly normal T cell development in KO mice, T cell proliferation triggered by anti-CD3ε was compromised in both CD4 and CD8 cells (Fig. 4A, left and middle panels; gating strategy: Supplementary Fig. 2c). It is to be noted that activation markers CD25 and CD69 shortly after CD3 stimulation were drastically upregulated and were always comparable between WT and KO T cells (Supplementary Fig. 8). The proliferation rate of KO B cells was also lower (Fig. 4a, right panel; gating strategy: Supplementary Fig. 2d). Cell cycle analysis revealed that G1/S progression was compromised in KO T cells (Fig. 4b; gating strategy: Supplementary Fig. 2e). We also demonstrated that KO T cells (gated on CD4$^+$ plus CD8$^+$ cells) presented increased FasL-triggered apoptosis (Fig. 4c; gating strategy: Supplementary Fig. 2f).

Naïve KO CD4 cells cultured under Th1 and Th17 conditions manifested reduced proliferation, as expected (Fig. 5a; gating strategy: Supplementary Fig. 2g). The differentiation of naïve CD4 cells into Th1 and Th17 cells was defective (Fig. 5b; gating strategy: Supplementary Fig. 2g), since the percentages of Th1 or Th17 cells were decreased among CD4 cells, which had already proliferated. The expression of transcription factors T-bet and RORγt - essential for Th1 and Th17 differentiation, respectively - were normal in KO CD4 cells cultured under Th1 and Th17 differentiation conditions (Figs. 5c and 5d), when gated on

either total CD4 cells or on those already differentiated cells (IFN-γ^+ or IL-17^+ cells), suggesting that the defective differentiation is not caused by a lack of these transcription factors.

As for humoral immune responses, KO serum IgG levels were comparable to those of WT controls (Supplementary Fig. 9).

We generated chimeric mice by transplanting KO and WT fetal liver cells in the C57BL/6J×129/sv F1 background ($CD45.2^+$ single-positive) into lethally irradiated C57BL/6J×C57B6.SJL F1 mice ($CD45.1^+CD45.2^+$ double-positive). Peripheral white blood cells of the recipients were analyzed by flow cytometry 8 weeks after transplantation, and recipients of similar degrees of KO and WT chimerism were paired for experimentation. Typically, about 80-85% of peripheral white blood cells were of donor origin ($CD45.2^+$ single-positive), and 12-15%, of recipient origin ($CD45.1^+CD45.2^+$ double-positive). In spleen $Thy1.2^+$ total T cells, $CD4^+$ T cells and $CD8^+$ T cells, 60-70% were of donor origin, and 30-35%, of recipient origin (Supplementary Fig. 10). Unlike in *Armc5* KO mice, KO T cells in chimeras were developed in a WT environment, devoid of influence by putatively unknown factors which might exist in the total KO environment and have aberrant effects on T cell development.

We showed that donor-derived KO naïve CD4 cells were defective in differentiating into Th1 cells (Fig. 5e; gating strategy: Supplementary Fig. 2h), similar to CD4 cells from

unmanipulated, naïve KO mice (Fig. 5b). The KO Th17 cell differentiation in this model was also compromised, although did not reach statistical significance, probably due to an inadequate sample size (Fig. 5e).

Experimental autoimmune encephalomyelitis (EAE) in KO mice

To understand the role of ARMC5 in *in vivo* T cell immune responses, particularly CD4-mediated immune responses, we induced experimental autoimmune encephalomyelitis (EAE) in *Armc5* WT and KO mice. As shown in Figure 6a, WT mice started to manifest clinical signs of EAE on day 13.2 ± 1. 30 (means ± SEM) after immunization, and their symptoms peaked on day 23. The onset of clinical symptoms in KO mice was delayed by about 7 days, and their maximum disease score was significantly lower than that of WT controls ($p<0.01$, two-tailed Student's *t* test) after day 18. Disease incidence was lower in KO mice between days 15 to 18, although it reached 100% in both KO and WT groups after day 28 (Fig. 6b). A trend toward less body weight loss in KO mice was noted after EAE induction compared to WT controls, but statistically significant difference was reached only on day 22 (Fig. 6c).

KO mice had significantly fewer cells in their draining LN and fewer infiltrating mononuclear cells in the brain and spinal cords on day 14 after MOG immunization compared to WT controls (Fig. 6d). After *ex vivo* PMA/ionomycin stimulation, the percentage of interferon-gamma (IFN-γ^+) CD4 cells among total CD4 cells from the LN of KO mice was significantly lower than that of WT mice (6.2% versus 15.4%), although the percentage of IL-17^+ cells among CD4 cells was similar in KO and WT draining LN (Fig. 6e). The percentages of IFN-γ^+

and IL-17$^+$ populations in CD4$^+$ T cells from the central nervous system (CNS) of KO mice were significantly lower after *ex vivo* PMA/ionomycin stimulation than in WT mice (Fig. 6f).

Histologically, spinal cords from KO animals on day 30 after MOG immunization showed less severe mononuclear cell infiltration and demyelination, according to hematoxylin/eosin and Luxol Fast Blue staining, respectively, compared to their WT counterparts (Fig. 6g). Histological data from 4 KO and 5 WT spinal cords are summarized (Fig. 6h). Mononuclear cell infiltration in KO spinal cords was significantly lower than in WT controls. Although demyelination in the former was also lower, it did not reach statistical significance. However, combined pathological scores, which included degrees of both mononuclear cell infiltration and demyelination, were significantly lower in KO mice. We did not observe changes in the percentages of Treg cells in the spleen of naïve KO mice or in the draining LN of KO mice on day 17 during EAE induction, compared to WT controls (Fig. 6i; gating strategy: Supplementary Fig. 2i). Therefore, it is unlikely that Treg cells are implicated in reduced EAE severity in KO mice.

To exclude the possible influence of the *Armc5* KO background on the immune system of KO mice, EAE was also induced in chimeras transplanted with fetal liver cells from *Armc5* WT and KO embryos on day 13-15. Overall, KO chimeras still displayed a lower degree of EAE than WT chimeras, but the difference was not as dramatic as in real KO versus WT mice. The onset of clinical symptoms in KO chimeras occurred 2.5 days (mean) later than in WT chimeras. KO chimera clinical scores tended to be lower than those of WT mice, but were

only significantly different between day 12 and 14 (Fig. 6j). EAE incidence was significantly lower on days 11, 12 and 14 after immunization (Fig. 6k). A trend of less body weight loss was noted in KO chimeric mice, although no statistical difference was apparent between the KO and WT groups (Fig. 6l). When stimulated *ex vivo* by PMA/ionomycin, the percentage of KO donor-derived IFN-γ^+ CD4 cells among total KO donor-derived CD4 cells from the CNS was significantly lower than in WT controls (Fig. 6m; gating strategy: Supplementary Fig. 2h), as was the case in real KO mice. However, there was no significant difference between the percentage of KO donor-derived IL-17^+ CD4 cells among total KO donor-derived CD4 cells and that of WT mice. The reduced degree of difference in EAE manifestation in KO versus WT chimeras, compared to that in real KO versus WT mice, was not unexpected, as KO chimeras contained about 30% recipient-derived T cells, which were fully immuno-competent WT T cells.

Antiviral immune responses in KO mice

CD8 T cell-mediated immune responses play a critical role against lymphocytic choriomeningitis virus (LCMV) infection. Therefore, we assessed KO $CD8^+$ T cell functions in LCMV infection. Eight days after mice were infected with LCMV (strain WE), absolute numbers of WT $CD8^+$ T cells but not $CD4^+$ T cells increased significantly (Fig. 7a; gating strategy: Supplementary Fig. 2j). LCMV tetramer staining showed that both the number of gp_{33-41}-, $np_{396-405}$- and $gp_{276-286}$-specific CD8 T cells per spleen and their percentage among total spleen $CD8^+$ T cells were significantly lower in KO than in WT mice (Fig. 7b, 7c and 7d; gating strategy: Supplementary Fig. 2k), suggesting compromised CD8 cell clonal expansion after viral Ag stimulation in KO mice.

After infection, CD8 cells develop into KLRG1^{hi}CD127^{lo} short-lived effector cells (SLEC) and KLRG1^{lo}CD127^{med} memory precursor effector cells (MPEC)[35]. In KO mice, 8 days after LCMV infection, the percentage of SLEC among CD8 T cells was significantly lower (Fig. 7e and 7f; gating strategy: Supplementary Fig. 2k), indicating defective anti-virus effector cell development. At the same time, MPEC percentage among CD8 T cells was increased in KO mice. The significance of this finding is not clear at present, although the percentage of CD62L^{lo}CD44^{hi} effector memory cells among total CD8 cells (Supplementary Fig. 11a) and LCMV subdominant epitope ($np_{396-405}$ and $gp_{276-286}$)-specific CD8 T cells (Fig. 7g; gating strategy, Supplementary Fig. 2k) in KO mice was reduced.

We next examined the presence of LCMV-specific, cytokine-producing splenic T cells in virus-infected mice. As seen in Figure 7h (gating strategy: Supplementary Fig. 2k), the absolute number of gp_{33-41}-specific TNF-α-positive CD8$^+$ T cells, IFN-γ-positive CD8$^+$ T cells, and IFN-γ/TNF-α double-positive CD8 T cells per spleen was significantly lower in KO than in WT mice 8 days post-infection. Significantly lower percentages of gp_{33-41}-specific, IFN-γ-positive CD8 T cells and IFN-γ/TNF-α double-positive CD8$^+$ T cells, but not TNF-α-positive CD8 T cells, among total spleen CD8 T cells, were found in KO spleens (Fig. 7i, representative dot plots shown in Supplementary Fig. 11c). Similarly decreased numbers and percentages of LCMV-specific cytokine-producing cells were observed in the CD4 cell population, although the reduction was of lower magnitude compared to those in the CD8 cells (Fig. 7j; representative dot plots shown in Supplementary Fig. 12d).

In addition, lower percentages of gp_{33-41}-specific $CD107a^+GranB^+$ T cells among total $CD8^+$ T cells were observed in KO spleen (Fig. 7k; representative dot plots shown in Supplementary Fig. 11f), implying the presence of fewer functional virus-specific cytotoxic $CD8^+$ T cells in KO mice. Virus titers in the kidneys, liver and spleen were significantly higher in KO mice 8 days post-LCMV infection, suggesting compromised virus clearance (Fig. 7l).

Identification of ARMC5-binding proteins by Y2H assay

ARMC5 has no enzymatic activity: its functions depend on interaction with molecules involved in different signaling pathways. To identify ARMC5-binding proteins, we conducted Y2H assays with human ARMC5 protein (Glu30-Ala935) as bait, and a human primary thymocyte cDNA expression library as prey. The binding proteins were given Predicted Biological Confidence (PBC) scores[36], and 16 proteins with scores between A and D ("A" having the highest confidence of binding) are found (Table 1), if their coding sequences are in-frame and have no in-frame stop codons. A complete list of binding proteins identified by Y2H assay and a map showing the interaction regions between ARMC5 and its binding partners are provided in the Supplementary materials section (Supplementary Data 1 and Supplementary Fig. 12).

Discussion

Our study demonstrated that *Armc5* mRNA was highly expressed in the thymus and adrenal glands. Its deletion led to small body size in mice and compromised T cell proliferation and differentiation. KO mice presented defective induction of EAE and anti-LCMV immune responses. KO mice developed adrenal gland hyperplasia in old age. ARMC5 is a protein without enzymatic activity. Our Y2H assays identified 16 candidate ARMC5-binding proteins potentially capable of linking ARMC5 to different signaling pathways involved in cell cycling and apoptosis.

Armc5 expression at the mRNA level was upregulated immediately (within 2 h) after CD4 T cell activation by TCR ligation and less so and at a slower pace in CD8 cells. Its expression level then declined in the following days (Fig. 1d). *Armc5* expression in CD4 cells cultured under Th1 or Th17 conditions after 1 day remained low (Supplementary Fig. 13), and was not influenced by the presence of different lymphokines, such as IL-2, IL-6, or TGF-β1 (Supplementary Fig. 14). *Armc5* mRNA expression in CD8 cells 8 days after LCMV infection was significantly lower than in naïve CD8 cells (Supplementary Fig. 15). These data suggest that this molecule is probably important in the early stage of TCR-triggered T cell activation to prepare cells for entry into the cell cycle. This notion is supported by cell cycle analysis, which revealed that KO T cells were compromised in G1/S progression (Fig. 4b).

We found reduced numbers of infiltrating T cells as well as Th1 (IFN-γ^+) and Th17 (IL-17^+) cells in the CNS of KO EAE mice compared to WT EAE controls. Such decreases were likely responsible for the diminished EAE manifestations in KO mice. Reduced CNS lymphocyte

infiltration could be caused by compromised clonal expansion/differentiation of T cells in the periphery, defective migration of such cells into the CNS, reduced expansion/differentiation of these cells in the CNS, decreased apoptosis of cells in the periphery and CNS, or all of the above. Defective KO T cell clonal expansion/differentiation in the periphery was apparent according to our *in vitro* and *in vivo* results (Figs. 4 and 7b), but whether this is also the case in the CNS remains to be studied.

We demonstrated that ARMC5 deletion resulted in comprised TCR-stimulated proliferation of both $CD4^+$ and $CD8^+$ T cells *in vitro* and LCMV-specific $CD8^+$ T cell clonal expansion *in vivo*. Moreover, we observed a significant reduction in SLECs in KO mice following LCMV infection while MPECs were increased. Taken together, these results suggest a function for ARMC5 in promoting T cell growth following TCR engagement possibly by regulating activation threshold levels; this provides a potential explanation for the observed increase in T cell death following FasL engagement (Fig. 4c) in KO mice. It is possible that augmented apoptosis also plays a role in compromised Th1 and Th17 development from naïve KO CD4 cells. However, *ARMC5* mutations lead to PMAH in humans and diffuse adrenal gland hyperplasia in mice (Fig. 3d), indicating that it has a default function of repressing adrenal cell proliferation, or a default pro-apoptotic function, or both. Indeed, an *in vitro* study of the human adrenal gland cell line H295R revealed that ARMC5 overexpression culminates in apoptosis[34], supporting its putative default anti-apoptotic function in adrenal glands. Dichotomous functions of ARMC5 in T cells versus adrenal glands indicate its tissue- or context-specificity, likely due to ARMC5's association with different binding partners. In different types of cells, ARMC5 might preferentially bind to a certain partner, depending on

its relative abundance in a given cell type or cell status. Consequently, the default function of ARMC5 in certain types of cells or cells with a given status could be either pro- or anti-proliferation, pro- or anti-apoptosis, or neutral. It could explain the different phenotypes seen in T cells versus adrenal glands, in terms of proliferation and apoptosis. It could also explain the obvious dilemma that KO T cell development in the thymus, which involves fast thymocyte proliferation, is normal, but TCR-stimulated T cell proliferation/differentiation and virus-induced T cell clonal expansion are defective in KO mice.

Although B cells were not the focus of this study, we did demonstrate that KO B cells were compromised in proliferation triggered by BCR ligation. Although KO mice had normal serum IgG levels, it is possible that, under strenuous conditions, KO mice might manifest defective humoral immune responses.

Based on the functional results of our ARMC5 study, those from PMAH investigations, and protein association information from Y2H assays, we propose the following speculative model of ARMC5 mechanisms of action. ARMC5 transcription and protein expression are increased when the cells are activated. Induced ARMC5 forms dimers (or multimers) in cytosol. Such dimers are able to interact with different molecules in pathways regulating cell cycling and apoptosis, *e.g.,* CUL3 for cell cycling, and DAPK1 for apoptosis. Therefore, depending on the relative abundance of binding proteins in different cell types and cells in different states, ARMC5 may interact preferentially with one or the other, leading to opposite functions in regulating cell proliferation and apoptosis. It should be noted that list of associating proteins might expand, pending further verification.

In summary, we demonstrated that ARMC5 has vital functions in fetal development, T cell biology, immune responses and adrenal gland biology. We have created *Armc5* KO mice as the first animal model of a rare human disease: PMAH. Our mechanistic study to identify ARMC5-binding partners has laid the groundwork for further elucidation of ARMC5's mechanisms of action. With a better understanding of these mechanisms, this molecule may be deployed as a therapeutic target in immune and endocrine disorders.

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Tables

Table 4.1 ARMC5-binding proteins identified by Y2H assay

Gene name	**PBC score**	**Binding clones**	**Different clones**	**Major known function**
DAPK1	A	13	4	Tumor suppressor, apoptosis, autophagy
ARMC5	B	2	2	Self- dimerization
STK24	B	3	3	Apoptosis, upstream of MAPK, acts on Tao
TTF1	B	3	2	Transcription terminator, apoptosis, tumor risk
POLR2A	B	3	2	DNA-directed RNA polymerase II subunit RPB1
CUL3	C	2	2	E3 component, WNK degradation, BTB domain, cell cycle, cyclin E degradation
CDCA7L	D	1	1	Cell cycle, transcription co-activator, c-Myc interactor, FoxP3-binding
C10orf46 (CACUL1)	D	1	1	CDK2-associated, cell cycle, promotes prolifereation
E2F2	D	1	1	Cell cycle, transcription factor, T cell quiescence
FAM65B	D	1	1	Skeletal muscle development, hearing
FLJ20105 (PICH)	D	1	1	cell division
HUWE1	D	6	1	Ubiquitination and proteasomal degradation, Base-excision repair, neural differentiation and

				proliferation
KIF11	D	1	1	ATP-dependent microtubule motor activity
PCBP1	D	2	1	Cadherin binding, involved in cell-cell adhesion, Burkitt lymphoma
RPN2	D	1	1	Endopeptidase activity, ubiquitin-dependent protein catabolic process
TCF12	D	1	1	Immune response, regulation of transcription
ZBTB40	D	2	1	Bone mineralization, cellular response to DNA damage stimulus

Y2H assays were performed by Hybrigenics Services (Paris, France). The coding sequence for human *ARMC5* cDNA (aa 30-935) served as bait to screen a random-primed human thymocyte cDNA library. Eighty million yeast clones (8-fold the complexity of the library) were screened. One hundred and sixty-five His^+ colonies were selected. The prey fragments of positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify corresponding interacting proteins in the GenBank database via a fully-automated procedure. A Predicted Biological Confidence (PBC) score (from A-F; A being of very high confidence in the interaction and F being experimentally-proven artifacts) was attributed to each interaction. Sixteen proteins with PBC scores between A and D are listed, if their coding sequences are in-frame and have no in-frame stop codons. Binding clones: number of total clones interacting with the bait. Different clones: number of

different clones of the same cDNA interacting with the bait. Known functions of the prey proteins are described.

(Notes on supplementary information)

Figure legends

Figure 4.1 Armc5 tissue-specific expression

Armc5 mRNA expression in mice is assessed by ISH. (**a**) *Armc5* expression in adult mouse using whole-body sections. Upper panel: H/E staining; middle and bottom panels: dark field X-ray film autography with anti-sense (AS) cRNA or sense (S) cRNA as probes, respectively. Bar = 1 cm. AG: adrenal gland; B: bone; BM: bone marrow; Cb: cerebellum; K: kidney; Lint: large intestine; LT: lymphatic tissue; Sk: skin; ST: stomach; Th: thymus; VB: vertebrae. (**b**) *Armc5* expression in the adult thymus. Upper row: dark field X-ray film autography; lower row: bright field emulsion autoradiography; left column: anti-sense probe; right column: sense probe. Bars = 2 mm and 20 μm. Cx: cortex; Me: medulla. (**c**) *Armc5* expression in the adult spleen. Upper and middle panels: dark field X-ray film autography, with anti-sense and sense probes, respectively; bottom panel: bright field emulsion autoradiography. Bars=2 mm and 20 μm. WP: white pulp; RP: red pulp; CAr: central artery; Cp: capillary. (**d**) *Armc5* mRNA in mouse spleen CD4 and CD8 cells measured by RT-qPCR. Experiments were performed 3 times. The results of representative experiments are shown. To facilitate comparison, normalized ratios of *Armc5* versus β-actin signals (means ± SEM) are presented; the 0-h signal ratio of each experiment is considered as 1. (**e**) ARMC5 subcellular localization in L cells was detected immufluorescence. L cells were transfected with HA-tagged mouse ARMC5-expressing construct or an empty vector, as indicated. (**f**) Phase contract micrographs of views in (**e**). The experiments were conducted 3 times, and micrographs of a representative experiment are shown. Scale bar: 5 μm.

Figure 4.2 Generation of Armc5 KO mice

(**a**) *Armc5* KO mice were generated by targeted gene deletion. The targeting strategy is depicted. Red squares on 5' and 3' sides of the mouse *Armc5* WT genomic sequence represent sequences serving as probes for genotyping by Southern blotting. (**b**) *Armc5* mRNA deletion in KO mice was confirmed by RT-qPCR. The results are expressed as normalized ratios (means ± SEM) of *Armc5* versus *β-actin* mRNA signals. The values from WT mice are considered as 1. Experiments were conducted more than 3 times, and representative results are reported.

Figure 4.3 General phenotype of KO mice

(**a**) Representative photos of WT, HT, KO fetuses on embryonic day 14. (**b**) Representative photos of adult KO and WT littermates. Left panel: males (8 weeks old); right panel: females (12 weeks old). (**c**) Body weight (means ± SEM) of *Armc5* KO and WT littermates at age 4 and 8 weeks. Mouse numbers (n) per group are indicated. **p*<0.001 (two-tailed Student's *t* test). (**d**) Morphology (upper panel) and histology (lower panel, HE staining) of adrenal glands from old KO mice (19 months old). Magnification: 5X. Scale bar: 500 μm. (**e**) Serum glucocorticoid levels in old KO mice. Means ± SEM of serum glucocorticoids in old KO and WT mice are shown. Age of each group (means ± SEM) and mouse number per group are indicated. Two-tailed Student's *t* test was used for statistical analysis.

Figure 4.4 KO T cell proliferation and apoptosis

(**a**) Proliferation of spleen $CD4^+$ and $CD8^+$ T cells and $B220^+$ B cells from WT and KO mice according to CFSE staining. CFSE intensity was ascertained by flow cytometry. Experiments were conducted independently 4-6 times. Representative histograms are shown. (**b**) Cell cycle

progression of spleen T cells from WT and KO mice. The percentages of cells in G_1, S and G_2 phases are indicated. Experiments were conducted independently 3 times. Representative histograms are shown. (**c**) Apoptosis of WT and KO spleen T cells (gated on $CD4^+$ plus $CD8^+$ cells) upon FasL stimulation was determined by their annexin V expression according to flow cytometry. Experiments were conducted independently 3 times. Representative histograms are shown.

Figure 4.5 Proliferation and differentiation of naïve KO CD4 cells into Th1 and Th17 cells

(**a**) Proliferation of WT and KO naïve spleen CD4 cells under Th1 and Th17 conditions was assessed based on CFSE content according to flow cytometry. Experiments were conducted 3 times, and representative histograms are shown. Grey peaks represent the CFSE content of CD4 cells at day 0. (**b)** These cells' differentiation into Th1 and Th17 cells was also determined by flow cytometry according to intracellular IFN-γ and IL-17 positivity (gated on total $CD4^+$). Representative dot plots are shown in the left panel. Means ± SEM of data from 3 experiments are presented as bar graphs in the right panel. Mouse numbers (n) per group are indicated. *p*-values are reported in the bar graphs (two-tailed Student's *t* test). (**c** and **d**) T-bet and RORγt expression in CD4 cells cultured under Th1 and Th17 conditions or in $IFN\gamma^+$ or $IL\text{-}17^+$ cells was determined by flow cytometry. Experiments were conducted 3 times. Representative histograms are shown. (**e**) Th1 and Th17 differentiation of naïve spleen CD4 cells (CD45.2 single-positive) derived from WT and KO donors in chimeric mice was analyzed by flow cytometry based on their intracellular IFN-γ and IL-17 expression. Representative dot plots are shown in the left panel. Means ± SEM of data from 3 experiments

are presented as bar graphs in the right panel. Mouse numbers (n) per group are indicated. *p*-values are reported in the bar graphs (two-tailed Student's *t* test).

Figure 4.6 EAE induction in KO mice

(**a**) Means ± SEM of EAE clinical scores of KO and WT mice. **p*<0.05 (two-tailed Student's *t* test). (**b**) EAE incidence in KO and WT mice. **p*<0.05 (chi-square test). (**c**) Means ± SEM of body weight of KO and WT mice during EAE induction. Body weight of mice on day 10 post-immunization was considered as 100%. **p*<0.05 (two-tailed Student's *t* test). (**d**) Means ± SEM of cellularity in draining LN and of cells infiltrating the CNS of mice 14 days after MOG immunization. Mouse numbers (n) and *p*-values (paired two-tailed Student's *t* test) are indicated. (**e** and **f**) Cytokine-producing cells among CD4 cells from draining LN (**e**) and CNS (**f**) on days 13-18 after MOG immunization. Left panels: representative dot plots; right panel: bar graphs (means +SEM) summarizing all the results, with mouse numbers and *p*-values (two-tailed Student's *t* test) indicated. (**g**) HE (left column) or Luxol Fast Blue (right column) staining of spinal cords 30 days after MOG immunization. Asterisks indicate cell infiltration. Arrows point to demyelination. (**h**) Means ± SEM of mononuclear cell infiltration scores, demyelination scores, and total pathological scores, which is the sum of the first 2 scores. Mouse numbers (n) and *p*-values (two-tailed Student's *t* test) are indicated. (**i**) Treg cells in naïve KO mice on day 17 during EAE induction. Left panel; representative dot plots; right panel: means + SEM of data from 3 experiments. NS: not significant (two-tailed Student's *t* test). (**j**) Means ± SEM of EAE clinical scores of chimeric mice. **p*<0.05 (two-tailed Student's *t* test). (**k**) EAE incidence in chimeric mice. **p*<0.05 (chi-square test). (**l**) Means ± SEM of body weight of chimeric mice with body weight on day 10 after MOG immunization

considered as 100%. No significant difference is found (two-tailed Student's *t* test). (**m**) Cytokine-producing donor-derived CD4 cells in the CNS of chimeric mice on day 14 after MOG immunization. Left panel: representative dot plots; right panel: summary (means ± SEM) of all the results, with mouse numbers (n) and *p*-values (paired two-tailed Student's *t* test) indicated.

Figure 4.7 Anti-LCMV immune responses in KO mice

(**a**) Spleen CD8 cell numbers in KO mice on day 8 after LCMV infection as determined by flow cytometry. Mice number (n), means ± SEM and *p*-values (two-tailed Student's *t* test) are indicated. (**b**) Virus-specific spleen CD8 cells in KO mice on day 8 post-LCMV infection according to flow cytometry. Representative dot plots are shown. (**c**) Means ± SEM of percentages of gp_{33-41}, $np_{396-405}$ and $gp_{276-286}$ tetramer-positive cells among spleen CD8 cells from all the results are presented. Numbers (n) of mice per group and *p*-values (two-tailed Student's *t* test) are indicated. (**d**) Means ± SEM of absolute numbers of gp_{33-41}, $np_{396-405}$ and $gp_{276-286}$ tetramer-positive CD8 cells in the KO and WT mouse spleens on day 8 post-infection. Numbers (n) of mice per group and *p*-values (two-tailed Student's *t* test) are indicated. (**e and f**) Memory and effector CD8 cell maturation in LCMV-infected WT and KO mice on day 8 post-LCMV infection. $KLRG1^{lo}CD127^{hi}$ cells are considered as memory precursor effector cells (MPEC), and $KLRG1^{hi}CD127^{lo}$ cells as short-lived effector cells (SLEC). Means ± SEM are presented. Numbers (n) of mice per group and *p*-values (two-tailed Student's *t* test) are indicated (**e**). Representative dot plots are shown (**f**). (**g**) On day 8 post-infection, total gp_{33-41} $np_{396-405}$ and $gp_{276-286}$ tetramer-positive CD8 cells in KO and WT mouse spleen were assessed for activation markers. Means ± SEM are presented. Numbers (n) of mice per group and *p*-values (two-tailed Student's *t* test) are indicated. (**h**) Absolute number of virus-specific, cytokine-producing CD8 cells. (**i and j**) Percentages of virus-specific, cytokine-producing cells among CD8 cells (**i**) and CD4 cells (**j**) on day 8 post-LCMV infection. Means ± SEM of data are shown. Mouse numbers (n) per group and *p*-values (two-tailed Student's *t* test) are indicated. (**k**) Means ± SEM of percentages of gp_{33-41}-specific $CD107a^{+}GranB^{+}$ CD8

T cells on day 8 post-LCMV infection. Mouse numbers (n) per group and *p*-values (two-tailed Student's *t* test) are indicated. **(I)** Means + SEM of viral titers in the kidney, liver and spleen on day 8 post-LCMV infection. Mouse numbers (n) per group and *p*-values (two-tailed Student's *t* test) are indicated.

Figures

Figure 4.1 Armc5 tissue-specific expression

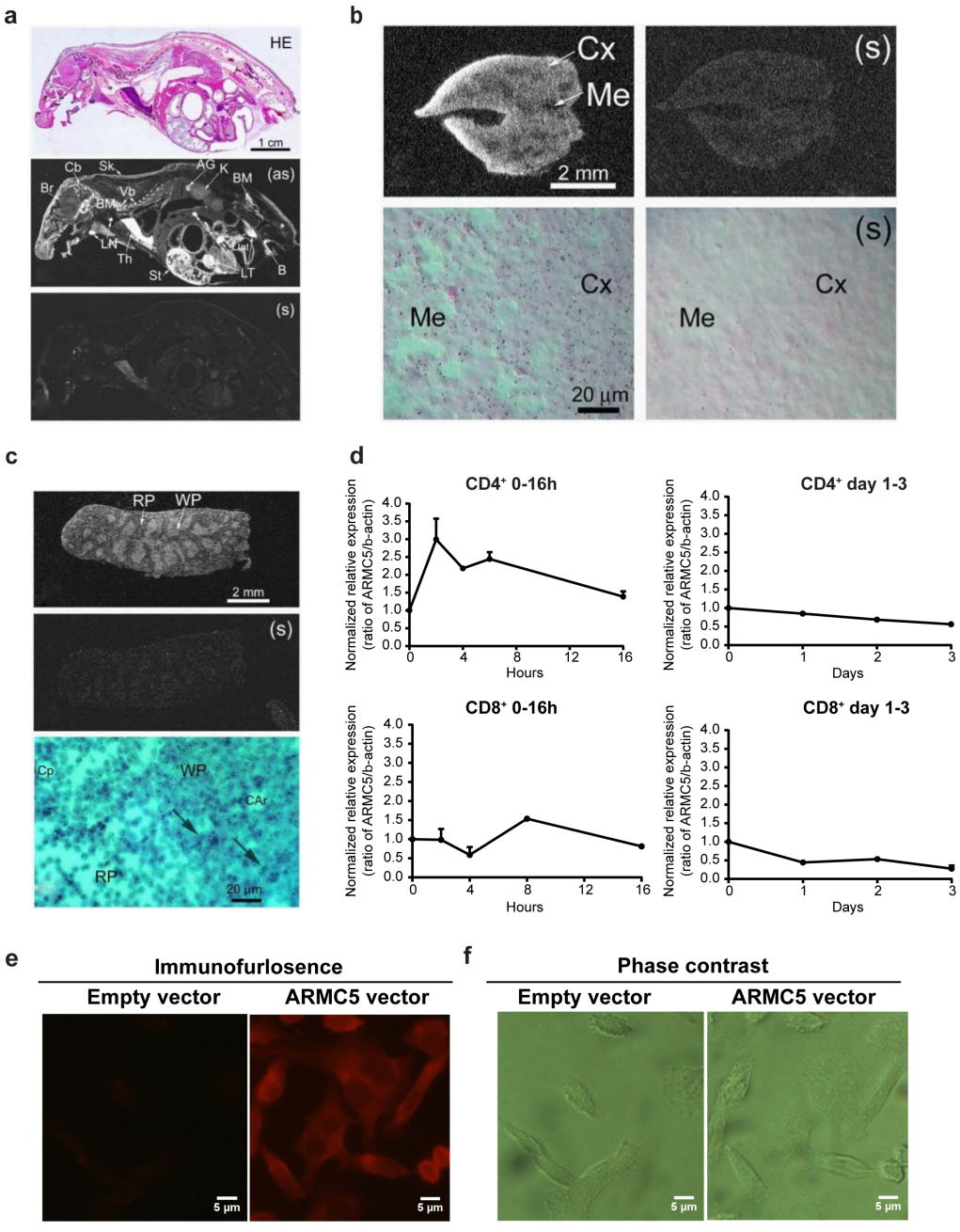


Figure 4.2 Generation of Armc5 KO mice

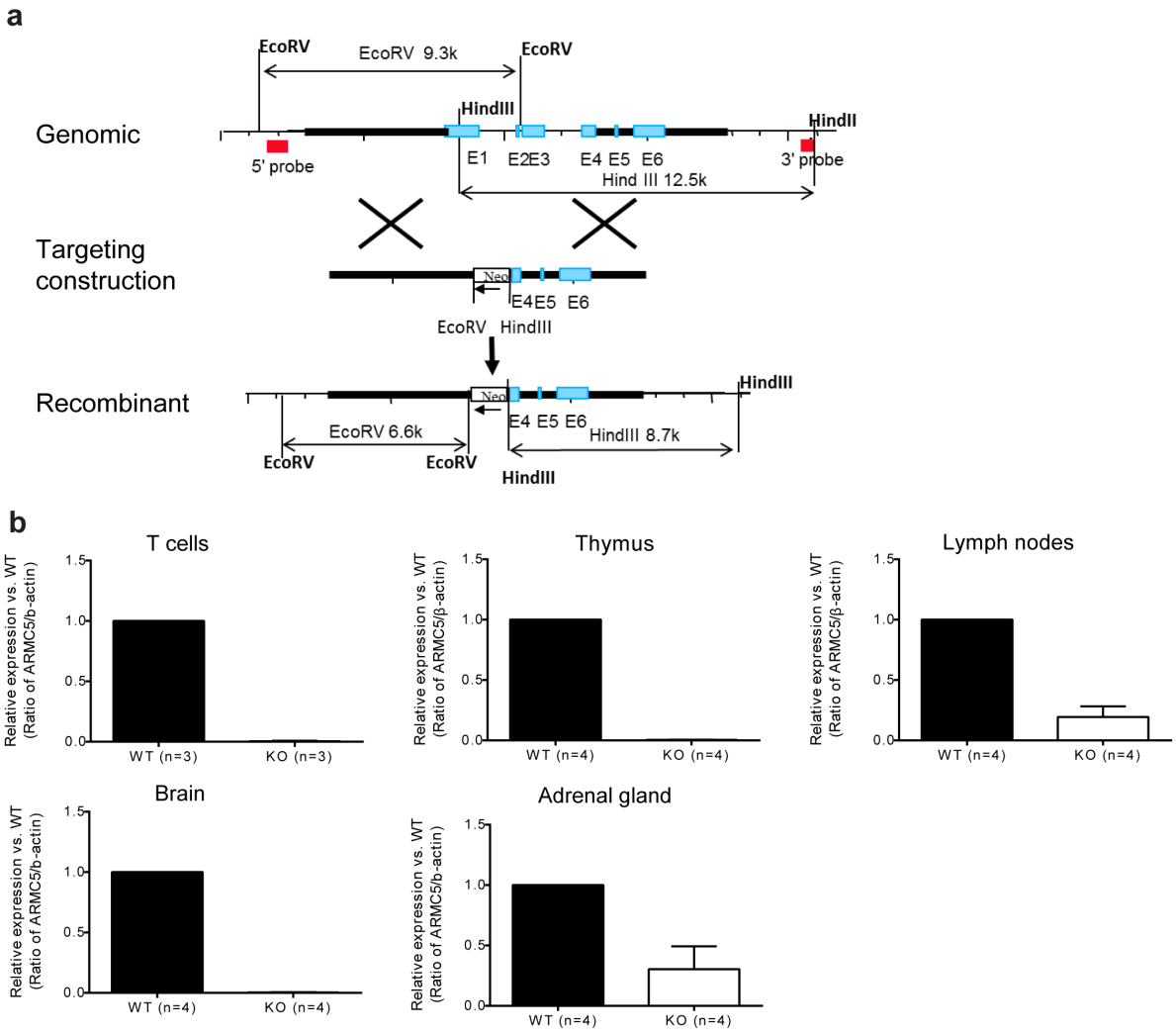


Figure 4.3 General phenotype of KO mice

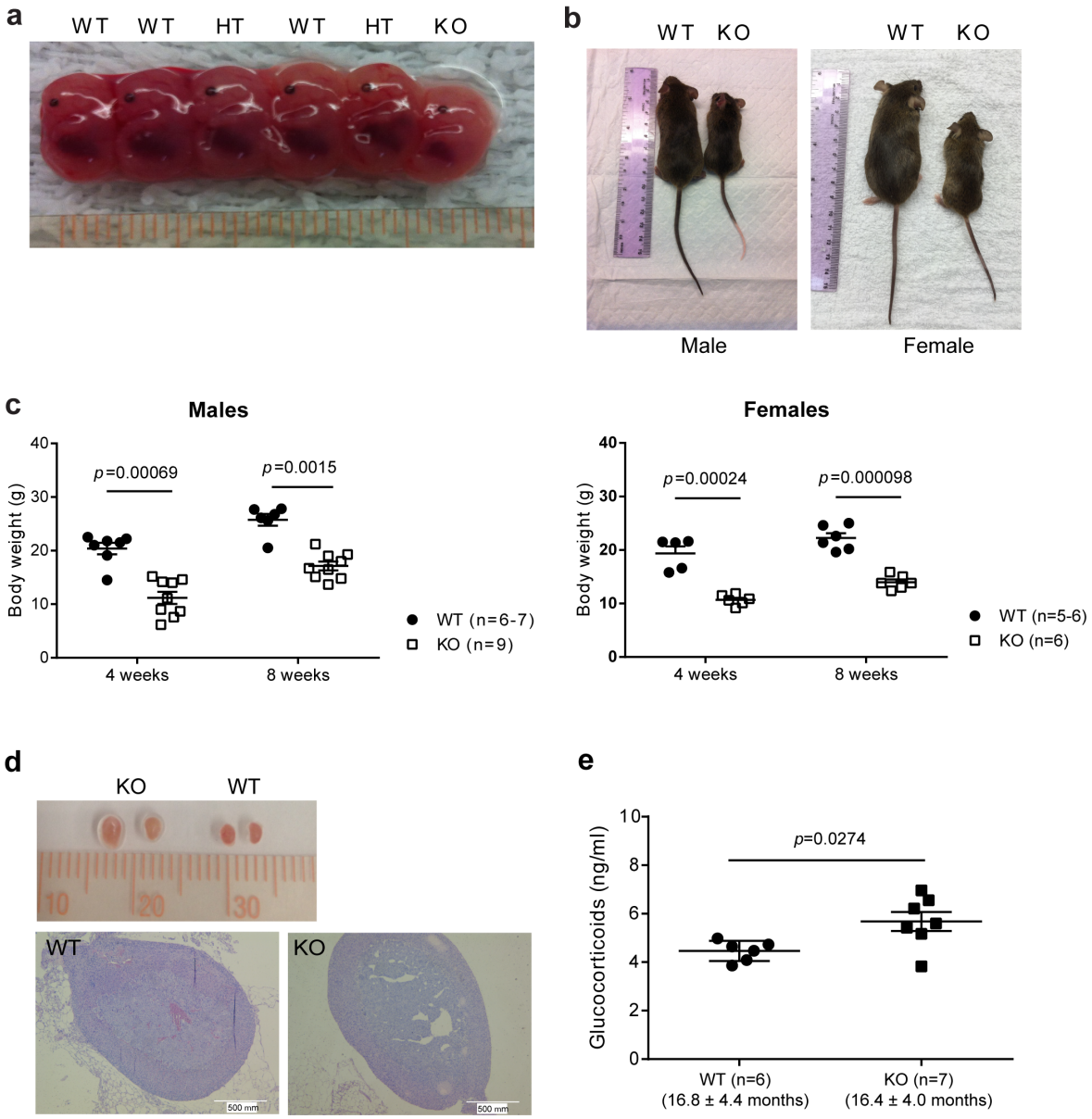


Figure 4.4 Armc5 KO mice presented normal thymus and spleen weight, cellularity and cell subpopulations

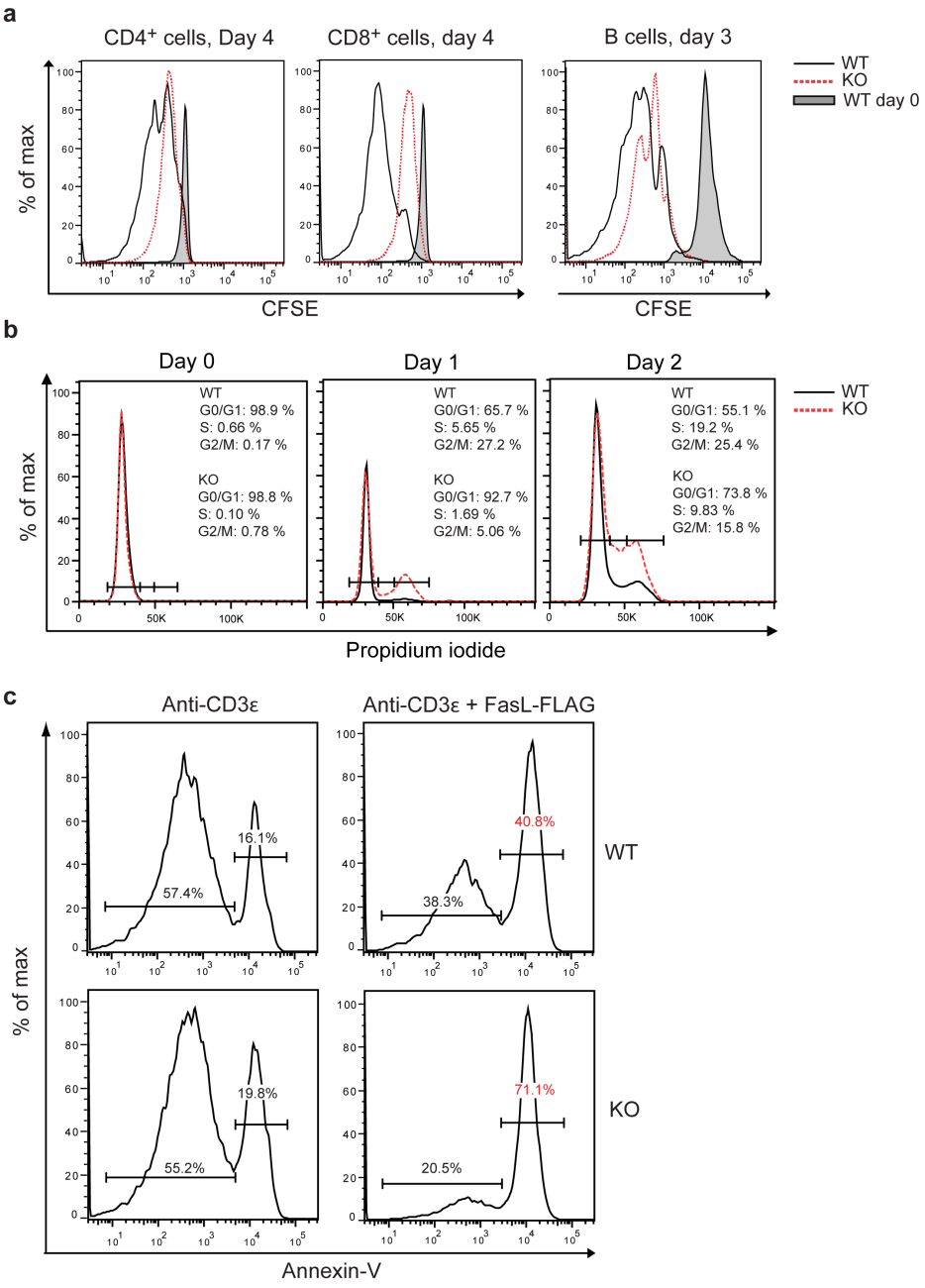


Figure 4.5 KO T cells are compromised in proliferation and differentiation

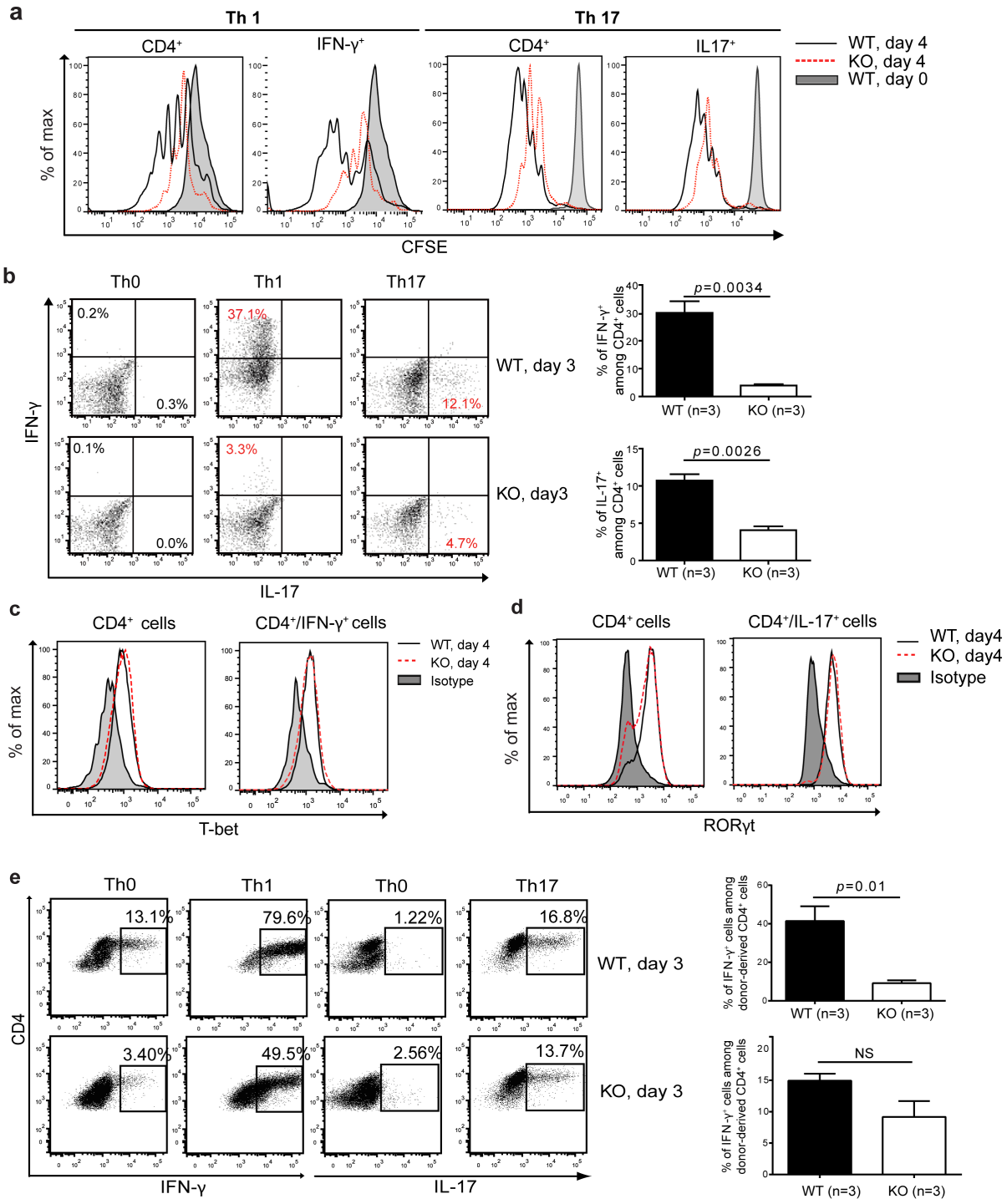


Figure 4.6 KO T cells developed from the chimeric mice were compromised in proliferation and differentiation

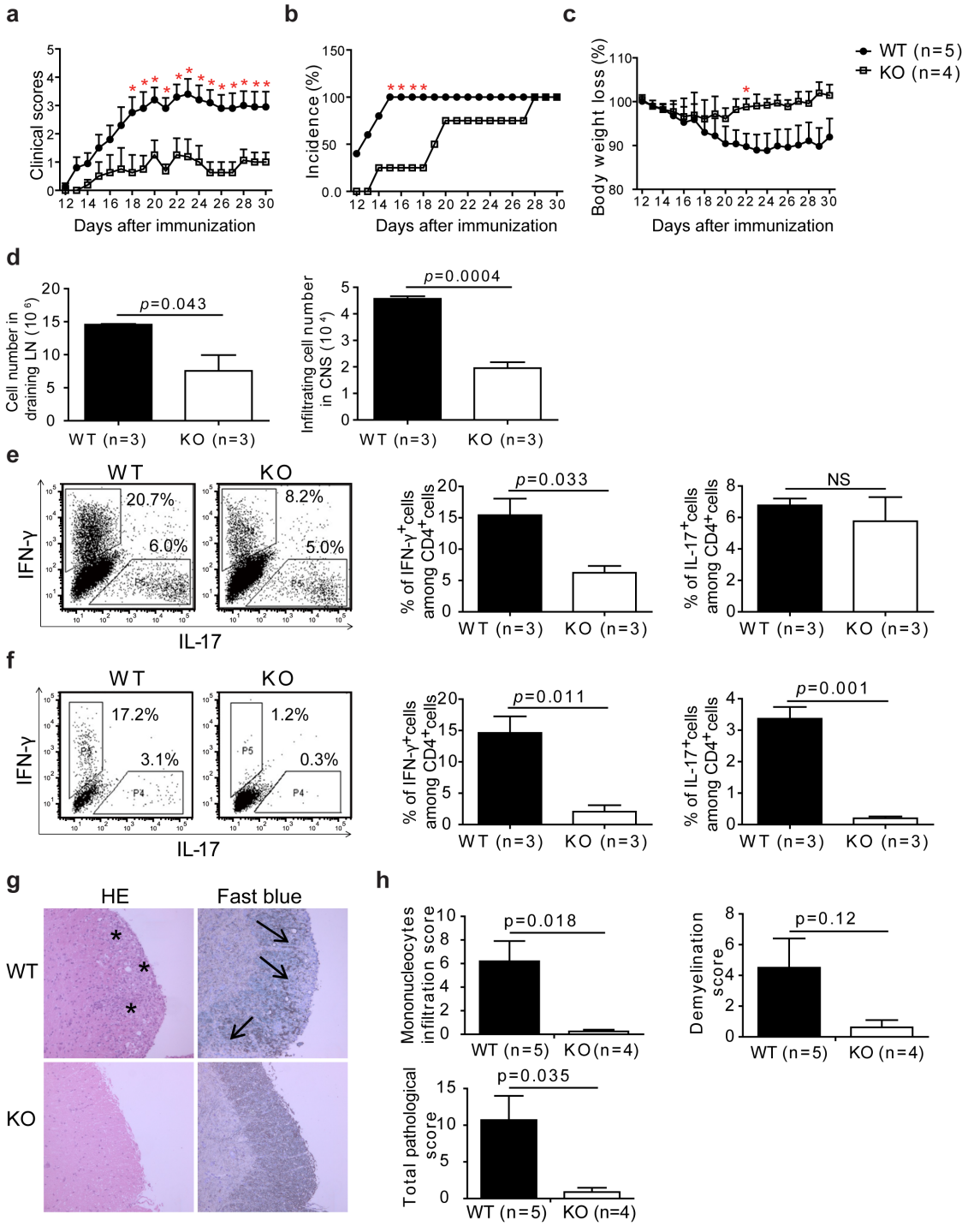


Figure 4.6 continued

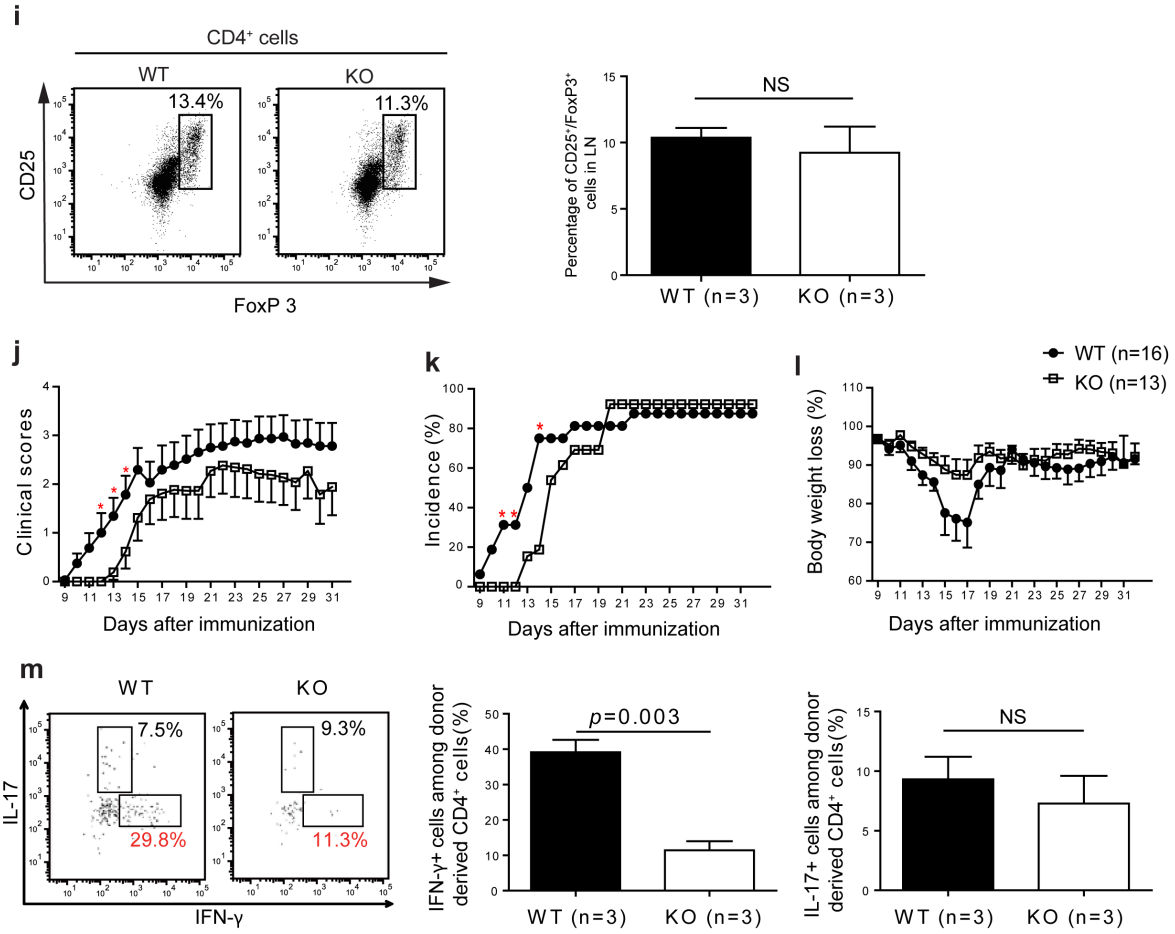


Figure 4.7 KO mice are resistant to EAE induction

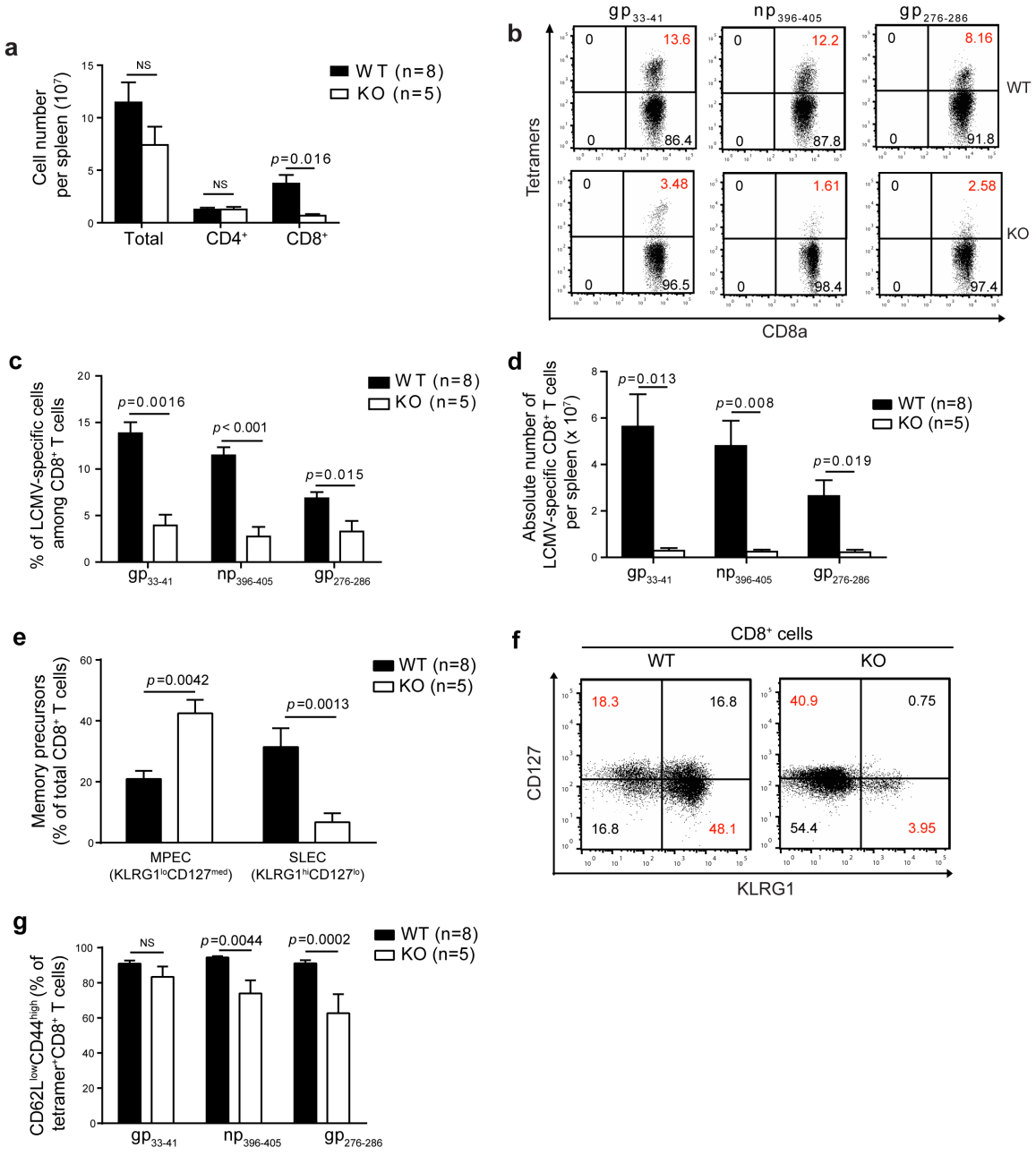


Figure 4.7 continued

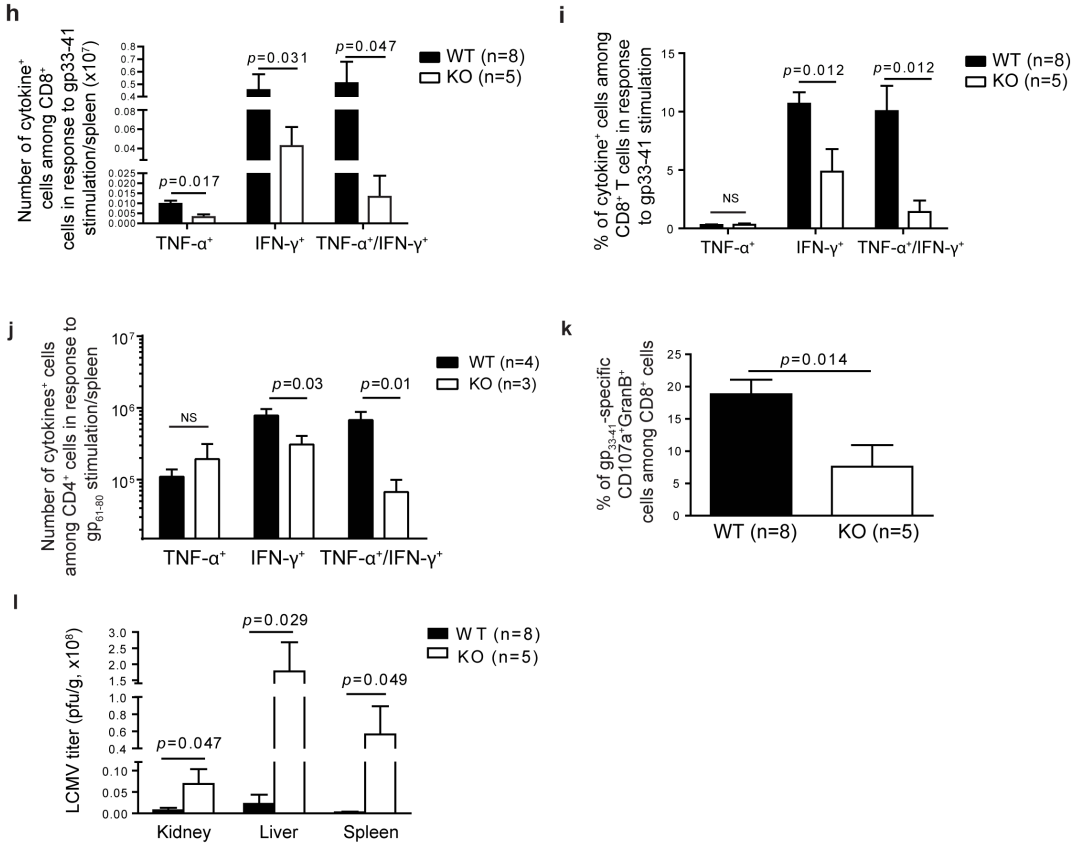


Figure 4.8 KO mice present compromised anti-LCMV immune response

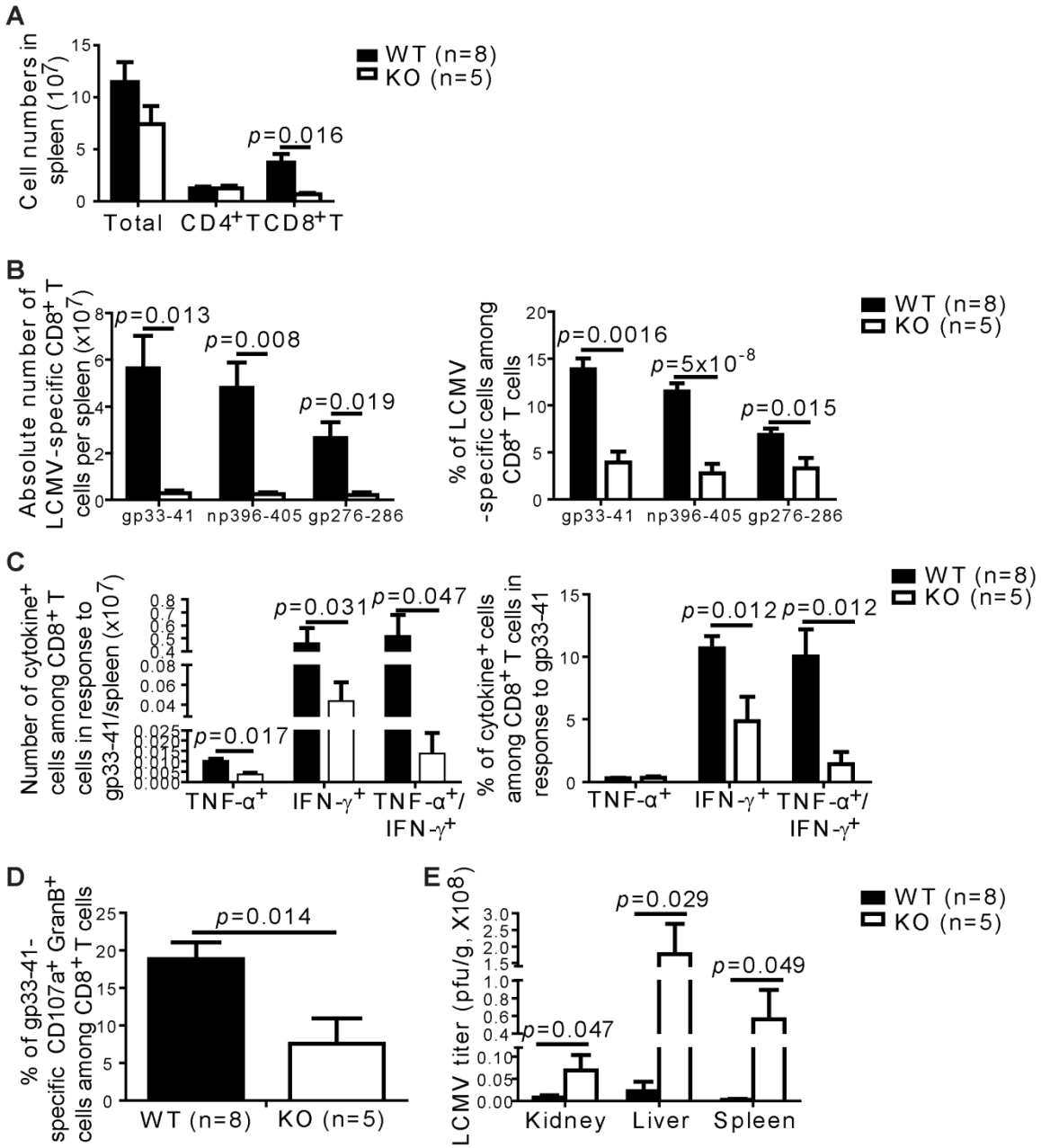
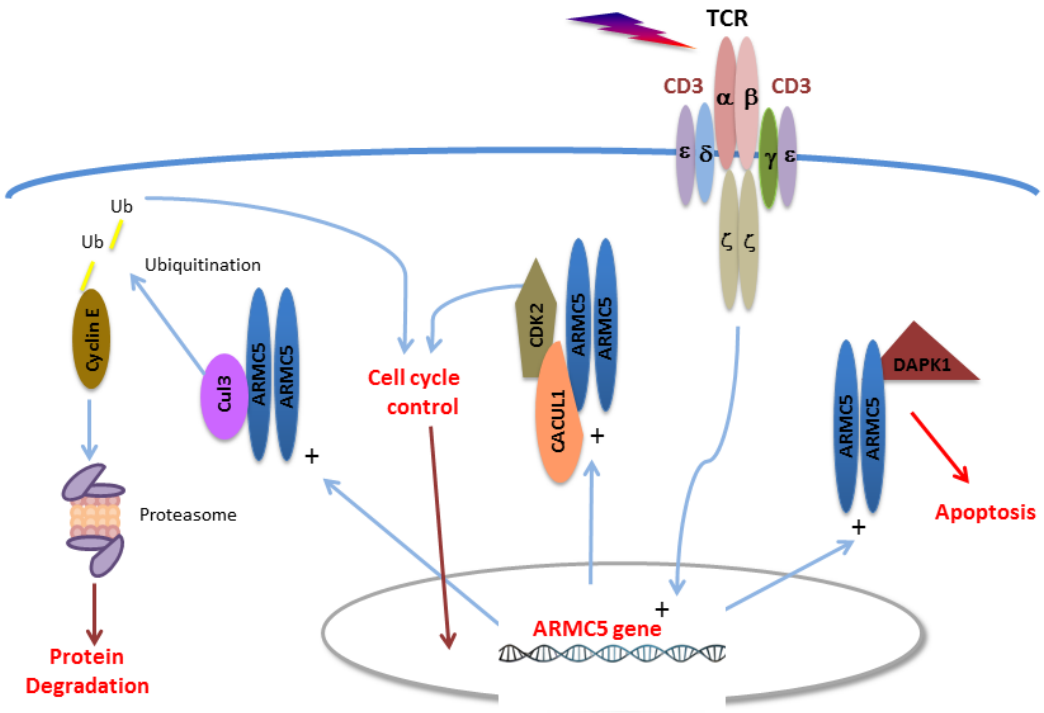


Figure 4.9 Hypothetical model of ARMC5 action mechanisms



Chapter 5 Discussion

Receptor tyrosine kinases are a group of key signaling molecules, which have dual functions: sensing the environmental stimuli outside the cells and transmitting them into the cells. They are particularly important in the immune cells according to the existing literature. Based on this conviction, in the past 20 years, our laboratory has elected to study the function of T cell abundant receptor tyrosine kinase, Ephb6. A large number of publications result from this endeavor. As both Efnb1 and Efnb2 are the ligands for Ephb6, we then proceeded to study their roles in T cell biology in multiple ways. We created single gene KO of *Efnb1*, *Efnb2* in T cells in mice, as well as *Efnb1/Efnb2* double KO. We found that only the double KO mice showed the phenotype in T cell biology.

Naïve T cells have the ability to expansion and differentiation into effector cells once they encounter foreign antigens. Stimuli can trigger T lymphocytes proliferation and differentiation signaling pathways only when both the TCR activation and co-stimulation signals are triggered. During T cell activation, a huge number of molecules are modulated. Some of these molecules play essential regulatory roles, while others exert house-keeping functions and/or act as supporters to cope with increased or changed metabolic demands. As Ephb6 deletion in T cells causes compromised T cell activation/proliferation, we speculated that molecules with altered expression during T cell activation in WT versus *Ephb6* gene KO are critical in T cell activation. We thus did DNA microarray analysis to identify potential targets that were differentially expressed in WT versus Ephb6 KO T cells in the early T-cell activation stage. About 30 molecules were up- or down-regulated more than three folds in WT T cells compared with KO T cells shortly after TCR stimulation. *Stra6* and *Armc5* were among those that had been validated for their altered expression. We generated mice with these two genes deleted to study their roles in T cell biology. Stra6 KO mice were normal in their immune responses, while Armc5 KO resulted in resistance to autoimmune disease and compromised anti-virus immune responses.

Several issues arising from our studies are worth discussing.

The role of Efnb1/b2, Stra6 and Armc5 in $CD4^+$ T cell activation

$CD4^+$ T cell activation triggers T cell function in terms of proliferation, differentiation, migration, as well as cytokine production. As mentioned earlier in this thesis, lots of molecules participate in $CD4^+$ T cell activation at multiple levels. Efnb1/b2, Stra6 and Armc5 are proteins of different families with distinct functions. Even though their expression levels can be changed upon anti-CD3 stimulation, their potential roles in regulating T cell activation are totally different.

A decade ago, our group found that solid-phase EFNB1 and EFNB2 act as costimulatory factors in T cell activation. Receptors of ephrin B1 and ephrin B2 can co-localize with TCR ten minutes after anti-CD3 crosslink, which augments the MAPK/ERK pathway (Yu et al., 2003a, 2004). This indicates that forward signaling is essential for $CD4^+$ T cell activation. *Efnb1/Efnb2* double KO mice were then generated. We found that the Efnb1 and Efnb2 reverse signaling are also critical for T cell homeostatic expansion, for T cell differentiation into Th1 and Th17, as well as for IL-6 signaling (Luo, Charpentier, et al., 2011). Based on these, in this thesis, using lentivirus containing truncated *Efnb1* and *Efnb2* intracellular tail regions, we confirmed that reverse signaling regulates T cell chemotaxis. Moreover, several tyrosine residues in the intracellular region of Efnb1 and Efnb2, which are just beneath the cell surface, are the major players in regulating T cell migration. Whether the same mechanism applies to T cell activation remains unknown. However, based on these results, we postulate that Efnb1 and Efnb2, as cell surface receptors, may enhance $CD4^+$ T cell activation signal on the cell surface or intracellularly just beneath the cell membrane, even though detailed mechanisms are poorly understood.

Comparing with EFNB1 and EFNB2, ARMC5 is mainly a cytosolic protein, as it was detected in the cytoplasm of L cells transiently transfected with a human ARMC5 expression construct. Our study found *Armc5* mRNA expression was rapidly induced in T cells within 1-2 h after anti-CD3 plus anti-CD28 stimulation, and then subsided. Most importantly, the T cell proliferation and differentiation in *Armc5* KO mice are impaired. This means that the change at the mRNA level for T cell activation might be the first trigger for T cell proliferation and

differentiation. We thus postulate that Armc5 participate in T cell activation by regulating its downstream signaling pathways in the cytosol or even in the nucleus. Further studies are required to address questions like the Armc5 expression pattern at the protein level after activation, the part of the TCR stimulation/co-stimulatory pathway that actually links to Armc5, and the underlying mechanisms of ARMC5 in T cell activation, *etc*.

Vitamin A and its metabolites are needed for $CD4^+$ T cell activation (Benson, Pino-Lagos, Rosemblatt, & Noelle, 2007; Hall, Grainger, et al., 2011). However, in this study, we found that *Stra6* KO mice does not contribute to T cell activation as far as we can detect. This indicates that Stra6 might not be necessary for the vitamin A transport in T cells. Other compensate pathways may exist. However, this is the issue yet to be solved (Terra et al., 2013).

Redundancy

Our previous *in vitro* study showed that solid phase EFNB1 or EFNB2 was capable of increasing T cell response to TCR stimulation (Yu et al., 2004). However, neither Efnb1 nor Efnb2 single deletion in mice showed apparent impact on T cell immune responses. For STRA6, in spite of published articles claiming that this molecule is absolutely necessary for cellular vitamin A import, its deletion did not lead to any discernable changes in mouse development, physiology, and cellular vitamin A content, or to any abnormalities in the immune system. For Armc5 deletion, although the KO mice showed a strong phenotype in terms of body size, the lymphoid organ size, cellularity and lymphocyte subpopulations were still normal. However, we found that in KO mice, the T cell proliferation/differentiation and anti-virus immune responses, were compromised.

Throughout the evolutionary history of life, our biological systems have developed high redundancy to overcome possible aberrant mutations that might lead to disastrous consequences. Genes of the same family or genes with similar functions are usually involved in such compensative mechanism. Efnb1 and Efnb2 are cases in the former point. Deletion of either of them shows no immunological phenotype, but if both of them are deleted, their importance is revealed. This type of redundancy guarantees that the functional roles of this family in T cell biology. Secondly, the biological system might use a totally different

mechanism of redundancy for certain functions. This is evidenced in the case of STRA6. Although this molecule has been shown to facilitate cellular vitamin A import, for most cell types, passive vitamin A diffusion is probably sufficient for the need. Our results also demonstrate that claims in the published articles, even those in high-impact journals such as Nature, could be flawed, as STRA6 was dubbed as an indispensable molecule from vitamin A cellular import, but obviously, this is not the case based on our gene KO study. Thirdly, other genes might partially compensate for the function of a certain molecule, as in the case of ARMC5. Such compensation seems to be sufficient for T cell development, which also needs proliferation, but it is not sufficient when there are strong provocations such as EAE induction or virus infection.

Spinal Bifida phenotype

We were not able to get live *Armc5* KO mice in either the pure C57BL/6 or 129/sv background. In an attempt to get live KO mice, we crossed heterozygous *Armc5* mice in the 129/sv background with CD-1 IGS outbred mice. We observed a higher percentage (42%) of KO mice with kinky tails in this mixed background comparing with their WT littermates (16%) (Fig. 5.1). Further study revealed that a higher percentage of *Armc5* KO fetuses on embryonic day 11.5 presented anencephaly (Data not shown). Both kinky tail and anencephaly are the clinical signs of neural tube defects, which are congenital malformations of the brain and spinal cord. The neural tube development can be regulated by multiple factors related to the proliferation and migration of neuroectodermal cells during embryonic development (Padmanabhan, 2006) (Pulikkunnel & Thomas, 2005). Our *in situ* hybridization also revealed that on embryonic day 11, *Armc5* was highly expressed in the neural tube (data not shown), supporting its potential role in neural tube development. Since this phenotype is prominent only in the 129/sv × CD-1 IGS F1 mice, we postulate that some contributions from the CD1 IGS genetic background act in concert with Armc5 for the observed neural tube defects; these concerted effects might cause defective neural cell proliferation or migration, leading to neural tube defects.

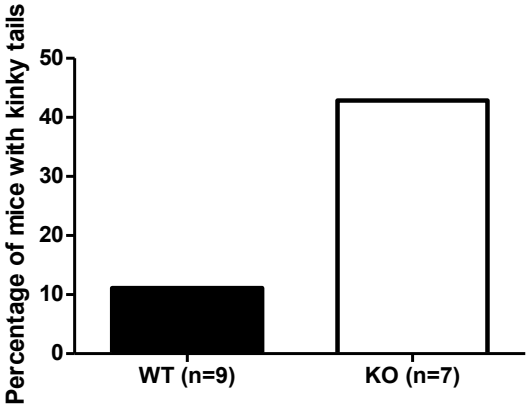


Figure 5.1 Percentage of mice with kinky tails in 129/sv × CD-1 IGS F1 background

ARMC5 in primary macronodular adrenal gland hyperplasia (PMAH)

During our investigation on the functions of ARMC5, several authors have reported that *ARMC5* mutations in human patients are associated with PMAH. Assié G *et al.* found that ARMC5 expression was lower in the tumor mass from macronodular adrenal gland hyperplasia individual carrying *ARMC5* mutations (Assie et al., 2013). In the same study, siRNA knockdown of ARMC5 in adrenocortical H295R cells reduced gene expression of several steroidogenic enzymes and adrenal transcription factors at the mRNA levels (Assie et al., 2013). However, in our study, Armc5 KO mice did not show any abnormality in adrenal histology or serum glucocorticoid titers. These may be due to several reasons. Firstly, it is possible that the discrepancy is caused by species difference between humans and mice (Terra et al., 2013). The second possibility is that Armc5 has different functions in different types of tissues. Thirdly, PMAH is typically diagnosed in patients between 40-60 years of age, due to the slow progress of the adrenal gland hyperplasia (Assie et al., 2013; Bourdeau et al., 2016). However, in our study, both the adrenal histology and serum glucocorticoid titers are measured from mice less than 16 weeks old, which is equivalent to 20-30 years of age in human beings. It is worth examining the adrenal glands of KO mice when they are at old ages. However, we think that it is unlikely that aged KO mice will develop adrenal gland abnormality for the following reasons. Not all individuals with *ARMC5* mutations develop PMAH, and only 25% PMAH patients have *ARMC5* mutations (Hsiao et al., 2009), suggesting that PMAH is not a monogenic disease, and it takes time to accumulate the second or third hit of mutation in the tissue to alter the function or expression of ARMC5 to allow the hyperplasia

to occur. That is probably the reason why PMAH is developed at a later part of the life in humans. However, our KO mice already have the gene completely deleted, but they do not manifest adrenal gland phenotype. In this case, waiting for a longer time will not contribute to the alteration or dysfunction of ARMC5, as it is already deleted. We are more inclined to believe that in humans, *ARMC5* mutations leads to altered or gain-of-function of ARMC5, which causes the proliferative phenotype of the adrenal gland. Mutations of *ARMC5* gene in humans are not equivalent to loss-of-function, as in the case of *Armc5* gene KO in mice. In fact, there is no solid evidence showing that PMAH patients have ARMC5 loss-of-function occurring in their adrenal glands. Although Assie *et al.* showed that the PMAH adrenal nodules had reduced ARMC5 expression according to immunoblotting, these data are not convincing, as none of the commercial anti-ARMC5 Abs is specific according to our assessment.

The role of ARMC5 in cell proliferation versus apoptosis

We demonstrated that the knockout of Armc5 resulted in comprised TCR-stimulated proliferation of both $CD4^+$ and $CD8^+$ T cells *in vitro*. Such a handicap contributed to diminished *in vivo* anti-virus responses in KO mice, particularly in the form of CD8 clonal expansion. Taken together, these indicate Armc5 may function as a positive regulator of T cell growth. However, mutations of *ARMC5* results in adrenal hyperplasia in PMAH, suggesting that it may function as a negative regulator of tumor cell growth. Further, *in vitro* study in H295R cell line showed that overexpression of ARMC5 results in cell apoptosis (Assie et al., 2013). Cell proliferation and cell death are such diametrically opposed cellular events, making us wondering how Armc5 accomplishes these two opposing tasks. There are several possible explanations for this.

First of all, the most well known molecules that have mechanistic overlap between cell proliferation and apoptosis are probably the proto-oncogenes. Proto-oncogenes have their roles both during normal cell physiological processes such as cell growth, proliferation, survival, as well as during tumor cell malignant transformation. c-Myc is a two-edged sword in oncogenesis, which is capable of concomitantly inducing cell proliferation and apoptosis (Shortt & Johnstone, 2012). On one hand, c-Myc is a central molecule in regulating cell proliferation. This involves multiple signaling pathways triggered by nutrients, growth factors

and mitogenic stimuli, resulting in the stabilization of Myc protein for optimal growth condition under a physiological condition. Also, the *c-myc* gene promoter is capable of receiving signals from distinct transcription factors to regulate multiple events in cell cycling. For example, the general transcription factor IIH (TFIIH) regulates c-Myc transcription at multiple points between initiation and promoter escape (Weber, Liu, Collins, & Levens, 2005). Under certain conditions, transcription factor NFAT1 (nuclear factor of activated T cells 1) can directly bind to the c-Myc promoter and also interact with a variety of transcriptional cofactors (Mognol, de Araujo-Souza, Robbs, Teixeira, & Viola, 2012). On the other hand, c-Myc can prevent inappropriate cell growth, making it a safeguard against malignant transformation (Lowe, Cepero, & Evan, 2004). Clinical evidence supports this as more Myc is expressed in some cancer, especially in late-stage cancer (Terunuma et al., 2014; Wolfer et al., 2010). The apoptotic machinery of c-Myc is not limited to its ability to amplify both known pathways of apoptosis, but can interact with the tumor suppressor gene product p53 at different molecular levels (Hoffman & Liebermann, 2008). Therefore, based on our results and the current understanding of proto-oncogenes, we postulate that ARMC5 may play important roles in both cell expansion and apoptosis, depending on the cellular context.

Mechanistically, both the ARM domain and the BTB domain of ARMC5 molecule provide interaction surface, which permits dimerization and the ability to bind to other molecules. Binding with versatile molecules leads to the activation of different signaling pathways involving distinct biological functions. The ARM repeat-containing proteins are known to be crucial for different cell functions such as cell adhesion, cell fate specification, cell migration and cell proliferation, depending on what proteins they associate with. The BTB domain controls a wealth of cell functions, including modulate transcription in the nuclei, regulate cytoskeleton dynamics in the cytoplasm, control ion channel assembly and gating in the cell membrane, again depending on the proteins it interacts with.

As mentioned in the Introduction, the positively charged ARM repeat region of β-catenin is particularly critical for interaction with its binding partners. Even though these binding partners have no apparent evolutionary relationship (Fig. 5.2), they share several peculiar characteristics. First, they usually contain ~30 amino acids and contact the ARM domain on an excessively large surface area. Second, these binding motifs frequently have phosphorylated Ser/Thr residues, which greatly enhanced their binding to the ARM domain (Ha, Tonozuka,

Stamos, Choi, & Weis, 2004). Third, some of them have same motifs contain Asp and Glu residues to form salt bridges with Lys435 and Lys 312 located in the binding groove of β-catenin (Graham, Weaver, Mao, Kimelman, & Xu, 2000).

Among the 12 armadillo repeats in the β-catenin binding groove, repeats 5-9 is the essential sites that intact with Tcf, cadherin, APC and ICAT (W. Xu & Kimelman, 2007). Also, the groove in ARMC repeats 3-4 is also critical for binding with Tcf, axin, and part of APC and cadherin (Graham, Ferkey, Mao, Kimelman, & Xu, 2001; Xing, Clements, Kimelman, & Xu, 2003) (Xing et al., 2004). The NTD and CTD at both end of β-catenin also have binding partners of their own, but they could function as a regulator for the association of ARM repeat and their binding partners.

The BTB domain usually contains ~120 aa. It is a structurally conserved domain for protein-protein interactions. The variation of highly variable residues buried within the core domain scaffold and specific domains at the both end of the each BTB-containing proteins contribute to different behaviors of the BTB domain.

Some proteins are simply made up of BTB domain. However, more frequently as it is in ARMC5, the BTB-containing proteins typically host a second protein-protein interaction domain. This allows the BTB domain-containing proteins to carry out multiple functions, depending on which proteins they associate with. For example, the BTB domain in BTB-ZF transcription factors provides a dimerization interface, which could promote DNA binding ability. Several BTB-Kelch proteins are known for the regulation of actin function because of the Kelch domain they bear.

Thus, as both ARM repeats and the BTB domain provide a versatile protein-protein interacting surface, the functions of the ARM repeat-containing and BTB domain-containing proteins are very diverse because a variety of proteins could bind to them. This provides a molecular base for the possible divergent functions of ARMC5.

Y2H data

Y2H assays are efficient for mapping the whole protein-protein interactomes. Using ARMC5 protein from Glu30-Ala935 as bait, a human primary thymocyte cDNA library was screened

for potential ARMC5-binding partners. Eight proteins including ARMC5 itself lie within the confidence category "D" or above. Several potential binding regions of bait and preys are consistent with existing literature. For example, in our Y2H, ARMC5 bait was found to interact with two CUL3 prey fragments at 3-1130 nt and 54-1556 nt. Both encode a peptide sequence at the N-terminal domain of CUL3, and this peptide sequence includes a 388-amino acid segment which is known to be associated with the BTB domain of BTB-Kelch family of E3 ubiquitin ligases (Canning et al., 2013). Needless to say, further validation of the association between ARMC5 and binding proteins found in Y2H is warranted.

Based on the functional results of ARMC5 from our study and those from PMAH studies, and the protein association information from Y2H, we propose the following model for the mechanisms for the action of ARMC5, as illustrated in Figure 3. When cells are activated, there is increased ARMC5 transcription and protein expression. The upregulated ARMC5 forms dimers in the cytosol. Such dimers will bind to a variety of molecules to regulate cell cycling and apoptosis. Therefore, ARMC5 may function as cell proliferation or apoptosis promoters or suppressors.

Summary and Future Directions

In this thesis study, the potential functions of EFNB1, EFNB2, STRA6 and ARMC5 in T cell biology were investigated. Using *in vitro* and *in vivo* approaches, such as primary cell culture and animal disease models like CIA, EAE, the underlying molecular mechanisms were investigated. We have following essential findings.

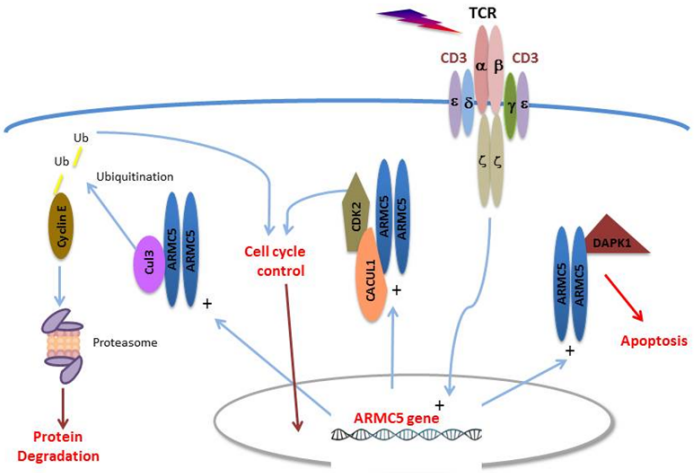


Figure 5.3 Putative function of ARMC5

1. By studying T cells specific Efnb1 and Efnb2 double KO mice, their new roles in T cells function were studied in the CIA model.

2. We proved that T cell-specific EFNB1 expression level can be a potential marker for monitoring RA disease activity.

3. The deletion of STRA6 does not affect the T-cell immune responses, nor does the cellular vitamin A import.

4. We investigated the expression patterns and function of *Armc5* by *in situ* hybridization and gene knockout mice.

5. Armc5 is critical for T cell proliferation and differentiation *in vitro*.

6. Armc5 is essential for the pathogenesis of EAE and anti-virus immune response in mice.

Some interesting questions remain to be further investigated in the future.

1. To validate the interaction between ARMC5 and the Y2H hits

   a) To confirm the Y2H results by co-immunoprecipitation.

   b) To dissect which part of ARMC5 interacts with the true binding partner using deletion mutations.

   c) To knockdown the expression of the potential binding partner of ARMC5 using siRNA, thus, to confirm the functional significance of the binding.

2. To study the potential function of ARMC5 in cell apoptosis and cell growth

As ARMC5 might be involved in multiple processes of cell biology such as apoptosis, cell cycle regulation, and protein degradation, we want to find out the mechanism which ARMC5 exerts its roles in these processes.

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Appendix

Article: TNF-like ligand 1A (TL1A) gene knockout leads to ameliorated collagen-induced arthritis in mice: implication of TL1A in humoral immune responses

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TNF-like ligand 1A (TL1A) gene knockout leads to ameliorated collagen-induced arthritis in mice: implication of TL1A in humoral immune responses

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Abstract

TNF-like ligand 1A (TL1A), also known as TNFSF15, is a member of the TNF superfamily. Its known receptor is death receptor 3 (DR3). In humans, TL1A also binds to a secreted TNF family member called decoy receptor 3, which interferes with the interaction between TL1A and DR3. TL1A/DR3 signal has been implicated in several autoimmune diseases in animal models as well as in clinical conditions. We generated TL1A gene knockout (KO) mice to assess its role in collagen-induced arthritis (CIA), a mouse model of human rheumatoid arthritis. The KO mice were fertile and had no visible anomalies. Their lymphoid organ size and cellularity, T and B cell subpopulations, helper T cell and regulatory T cell development *in vivo* and *in vitro* and anti-viral immune responses were comparable to those of wild type mice. However, the KO mice presented ameliorated CIA in terms of clinical scores, disease incidence and pathological scores. The KO mice had reduced titres of pathogenic anti-collagen Abs in the sera. No apparent defect was found in the function of follicular helper T cells. We revealed that plasma cells but not B cells expressed high levels of DR3, and were direct targets of TL1A. In the presence of TL1A, they survived better and produced more pathogenic Ab. This study presented novel knowledge about the role of TL1A in humoral immune responses and its mechanism of action in CIA pathogenesis.

Introduction

TNF-like ligand 1A (TL1A), also called TNFSF15, is a member of the TNF superfamily. It was identified during homology searches in an endothelial cell cDNA library. It is produced by the endothelial cells(1), monocytes, dendritic cells, T cells, natural killer T cells (NKT), synovial fibroblasts and chondrocytes, either upon stimulation or *in situ* in inflammatory sites(2-6). Like other TNF superfamily members, TL1A can either be membrane-bound or soluble after being cleaved from the cell surface, but the cleavage happens on dendritic cells (DC) and not on T cells(1, 7). It was also discovered that the soluble form of TL1A was more potent in inducing IFN-γ production by T cells(8).

The receptor of TL1A death receptor 3 (DR3) is also called TNFRSF25, Apo3, WSL-1, TRAMP or LARD(9). In addition to the full length isoform, murine DR3 was reported to have two truncated variants: one secreted form displaying no transmembrane region and another lacking the fourth CRD in its extracellular sequence (10). A repertoire of all DR3-expressing cells has not yet been completed. DR3 is detectable on T cells, NK cells, NKT cells, osteocytes, renal tubule epithelial cells and *in vitro* plasma cells, but primary B cells express very low levels of DR3(3, 11-13). Previous studies of the immunological functions of TL1A and DR3 primarily focused on T cells, NK cells and NKT cells(3, 12, 14-16). In addition to DR3, decoy receptor 3 could also bind to TL1A in humans, and blocks the interaction between TL1A and DR3(1). Mice do not have a human decoy receptor 3 ortholog.

Earlier *in vitro* studies have shown that TL1A promotes T cell proliferation, Th17 differentiation and the production of pro-inflammatory cytokines by T cells (9), indicating its potential role in inflammatory autoimmune diseases. The presence of TL1A in diseased tissues, fluids and sera from patients of chronic colitis and rheumatoid arthritis (RA) corroborates the findings in animal models (2, 5, 17-19). *In vivo* studies of different animal models have provided direct evidence supporting the role of TL1A in autoimmune diseases. We have demonstrated that the administration of recombinant TL1A to mice with collagen-induced arthritis (CIA) aggravates the disease (5). Furthermore, overexpression of TL1A in transgenic mice results in spontaneous inflammatory bowel disease (IBD) as well as increased number of T helper cells (9, 20, 21). On the other hand, DR3 gene knockout (KO) mice have reduced disease severity of allergic lung inflammation and antigen-induced arthritis and

present less effective anti-viral and anti-bacterial immune responses(15, 22-25). Blocking DR3-TL1A interaction by TL1A-neutralizing Ab would partially protect the KO mice from CIA, lung inflammation, and 2,4,6-trinitrobenzene sulfonic acid (TNBS)- and dextran sodium sulphate (DSS)-induced colitis(3, 4, 9, 23). TL1A KO mice are less susceptible to experimental autoimmune encephalomyelitis (EAE) induction(22).

Rheumatoid arthritis (RA) is a chronic polygenic autoimmune disease characterized by chronic systemic inflammation that may affect various tissues and organs. Joints and their surrounding tissues are most frequently affected. Often, there is persistent synovitis and presence of auto-Abs leading to progressive destruction of cartilage and bone in the joints (26). The pathogenesis of RA is not fully understood with three primary, outstanding questions to be fully answered: (1) How is the immune response initiated by genetic and/or environmental factors? (2) How does the immune response lead to local joint inflammation? (3) How does the inflammation cause bone destruction? There are some findings from patients and animal models that have provided some partial answers to these questions(27-36).

In the current study, we generated *TL1A* knockout (KO) mice to study the role of TL1A in CIA pathogenesis. We found TL1A KO mice had significantly lower CIA clinical scores and incidence, and lower serum anti-collagen Ab titres than their wild type (WT) counterparts. Deletion of TL1A minimally affected T cell functions; however, TL1A could directly deliver survival signals to plasma cells as a way to promote pathogenic collagen-specific Ab production. Our findings revealed previously undocumented functions of TL1A in RA pathogenesis.

Materials and methods

Generation of TL1A KO mice

A polymerase chain reaction (PCR) fragment amplified from the *TL1A* cDNA was used as a probe to isolate genomic BAC DNA clone 112H6 from the 129/sv mouse BAC genomic library RPCI-22. The targeting vector was constructed by recombination and routine cloning methods using an 11-kb *TL1A* genomic fragment from clone 112H6 as the starting material (Fig. 1A) (37). The 2.05-kb ScaI-XhoI genomic fragment containing exon 1 was replaced by a 1.1-kb Neo cassette from pMC1Neo Poly A (Fig. 1A). The final targeting fragment was excised from its cloning vector backbone by Not I digestion and electroporated into R1 embryonic stem (ES) cells for G418 (38). The targeted ES cell clones were injected into C57BL/6 blastocysts. Chimeric male mice were mated with C57BL/6 females to establish mutated *TL1A* allele germline transmission.

Southern blotting with a probe corresponding to the 5' sequence outside the targeting region, as illustrated in Figure 1A (hatched rectangle) was used to screen gene-targeted ES cells and eventually for the confirmation of the gene deletion in mouse tail DNA. The targeted allele showed an 11.7-kb StuI/StuI band, and the WT allele, a 5.4-kb StuI/StuI band.

The *TL1A*-KO mice were subsequently backcrossed to the DBA/1LacJ for 8 generations for experimentation in this study. The littermate WT mice served as controls. In the beginning of the experiments, mice were on average 8 to 12 weeks of age.

PCR was adopted for routine genotyping of the targeted allele(s). The following PCR conditions were applied: 4 min at 94 ^{o}C, followed by 35 cycles of 30 s at 94 ^{o}C, 30 s at 68 ^{o}C, and 30 s at 72 ^{o}C with a final incubation at 72 ^{o}C for 10 min. One forward primer and two reverse primers (forward primer 5'- TCC ACA GCC AAC ATA GGC AAG GA-3'; reverse primers 1: 5'- GTG TGG CTT GCA ACA GGA AAT GGA -3'; reverse primer 2 5'- ACC TGC GTG CAA TCC ATC TTG TTC-3') were included in the PCR which amplified a 581-bp fragment from the targeted allele and a 335-bp fragment from the WT allele. All mice were housed under specific pathogen-free conditions and studied according to protocols approved by the Institutional Animal Protection Committees of the CRCHUM and INRS-IAF.

Reverse transcription-quantitative PCR (RT-qPCR)

Murine TL1A and DR3 mRNA was measured by RT-qPCR. Total RNA was isolated from cells or tissues with Trizol reagent (Invitrogen, Carlsbad CA). For TL1A mRNA measurement, the forward primer 5'-TCA TTT CCC ATC CTC GCA GGA CTT-3' and reverse primer 5'-TAA TTG TCA GGT GTG CTC TCG GCT-3' were used to generate a 166-bp fragment. For measurement of mRNA of all 3 DR3 isoforms (the full length one, the one with transmembrane domain deletion and the one with deletion of the extracellular CRD domain), the forward primer 5'-AGA GGT ATG GCC CGT TTT G-3' and reverse primer 5'-AAG TGG TTG TCT CTG GTC AAG-3' were used to generate a 133-bp fragment. For mRNA of the full-length DR3 isoform, the forward primer 5'-TGC CTG GCT GGC TTC TAT ATA CGT G -3' and reverse primer 5'-ACA GAC AGC AGT GCA AGC CTT A-3' were used to generate a 92-bp fragment. The PCR conditions for both reactions were as follows: 2 min at 95^{o}C followed by 45 cycles of 10 s at 95^{o}C, 15 s at 56^{o}C and 20 s at 72^{o}C. Samples were in triplicates. β-actin mRNA levels were measured as described in our previous publication and taken as internal controls(5). The data were expressed as signal ratios of either TL1A mRNA/β-acting mRNA or DR3 mRNA/β-actin mRNA.

Flow cytometry

Single cell suspensions from the thymus, spleen and lymph nodes (LN) were prepared and stained immediately or after culture for 2-, 3-, 4- or 5-color flow cytometry. Mouse anti-mouse TL1A mAb (Tandys1a) was from eBioscience (San Diego, CA). Anti-PD-1 mAb; anti-GL7 mAb, anti-ICOS mAb, anti-CD40L mAb, anti-CXCR5 mAb, and anti-DR3 mAb were from BD Biosciences (Missisauga, ON, Canada) and eBioscience. The rest mAbs used in flow cytometry are described in our previous publications(39).

Cell culture and in vitro T help cells (Th) differentiation

In general, total spleen or LN cells, T cell subpopulations, B cell subpopulations or bone marrow cells were prepared, isolated with Miltanyii beads or sorted by flow cytometry as indicated and then cultured in RPMI1640 medium containing 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin G, 1× nonessential amino acids, 1 μM sodium pyruvate, 2.5 μM β-mercaptoethanol and other supplements as indicated. In some cases, recombinant mouse TL1A (aa76-252; R & D systems, Minneapolis, MN) was added to the culture.

In vitro Th differentiation was conducted as follows. Naïve CD4 T cells ($CD4^{+}CD62L^{+}$ $CD44^{low}$) were isolated from TL1A KO or WT mouse spleens with MagCellect Mouse Naïve

$CD4^+$ T cell Isolation kits (R & D Systems). T cell-depleted TL1A KO or WT spleen cells were irradiated at 3000 Rad and used as feeder cells. The naïve CD4 cells (0.1×10^6/ well) were mixed with the feeder cells (0.5×10^6/well) and cultured in 96-well plates in the presence of soluble anti-CD3ε mAb (clone 145-2C11, 2 μg/ml; BD Biosciences). Cultures were supplemented with recombinant mouse IL-12 (10 ng/ml; R & D Systems) and anti-IL-4 mAb (10 μg/ml; R & D Systems) for the Th1 condition; recombinant mouse IL-4 (20 ng/ml; R & D Systems), anti-IL-12 mAb (10 μg/ml; BD Biosciences) and anti-IFN-γ mAb (10 μg/ml; R & D Systems) for the Th2 condition; recombinant mouse IL-6 (20 ng/ml; R & D Systems), recombinant human TGF-β1 (5 ng/ml; R & D Systems) and anti-IL-4 and anti-IFN-γ mAb (10 μg/ml; R & D Systems) for the Th17 condition; recombinant human TGF-β1 (5 ng/ml; R & D Systems), anti-IL-4 and anti-IFN-γ mAb (10 μg/ml; R & D Systems) for the Treg condition; and recombinant mouse IL-6 (20 ng/ml; R & D Systems), anti-IL-4 and anti-IFN-γ mAb (10 μg/ml) for the Tfh-like cell condition, without any supplement for the Th0 condition.

Mouse CIA model

Eight- to 12-week old TL1A KO mice and their WT littermates in the DBA/1LacJ background were used in the experiments. Details of the induction of CIA in these mice were previously described (5). Briefly, mice were immunized with single intradermal injection at the base of the tail with 100 μg of bovine type II collagen (BTIIC; Chondrex, Redmond, WA), which was emulsified in equal volumes of complete Freund's adjuvant (CFA; 4 mg/ml Mycobacterium tuberculosis, strain H37Ra; Difco, Detroit, MI) on day 0. The mice were examined for their development and severity of arthritis from day 20 to 50. Disease severity was scored on a scale from 0 to 4 by visual inspection of each paw according to methodology of Brand et al. with some modifications as follows: 0 = normal paw; 1 = erythema and mild swelling confined to two or more digits, or the tarsal, or the ankle joint; 2 = erythema and mild swelling of any two regions of the metatarsal, tarsal or ankle joint; 3 = erythema and moderate swelling extending from the ankle to metatarsal joints; 4 = erythema and severe swelling encompassing the ankle, foot and digits, or ankylosis of the limb (40). The scores for each of four paws were added to give a final score, such that the maximal severity score was 16 for each mouse. The mice were considered as having arthritis, if their clinical score was equal to or above 1.

Histology

Mice immunized with BTIIC/CFA were sacrificed on day 50, and then their paws including ankles were surgically removed and fixed in 10% buffered formalin. Following decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Tissue sections (7 μm) that were stained with hematoxylin/eosin and Safranin O (for cartilage staining) were scored according to three parameters, i.e., lining hyperplasia, bone erosion destruction and cell infiltration, in one-way blind fashion with the examiner not knowing the identity of the sections. Each parameter was scored on a 0–3 scale, as previously described, and all four paws (one section per paw) of each animal were scored. The overall score of an animal was the sum of the three parameters of the four paws(41).

Isolation of infiltrating cells of hematopoietic origin in paws

The mouse vascular system was perfused with 20 ml PBS under anaesthesia. The paws were then harvested, skinned and minced into small pieces, which was then digested with collagenase II (2 mg/ml, Chondrex, Redmont, WA) and dispase II (250 μg/ml, Roche Diagnostics, Indianapolis, IN) in 5 ml Hank's balanced salt solution at 37 ^{o}C for 40 min. The digested product was washed and passed through cell strainers (BD bioscience, San Jose, CA) of 70 μm in pore size, consecutively. Cells in the passing-through liquid were enumerated and stained with anti-CD45.2, CD11c, CD11b, Thy1.2 and B220 mAbs, and analysed and counted with flow cytometry.

Enzyme-linked immunoabsorbent assay (ELISA) for the titres of anti-BTIIC Abs

ELISA was used to measure the titres of mouse serum anti-BTIIC Abs. Flat-bottom 96-well plates (Costar EIA/RIA plate #3590, Fisher Scientific, Pittsburgh, PA) were coated with BTIIC in PBS (100 μl/well) overnight at 4 ^{o}C. After blocking for 1 h at room temperature with 3% BSA in PBS, the plates were washed with PBS containing 0.05% Tween 20. Samples in duplicate were added to the wells (50 μl/well) and incubated overnight at 4 ^{o}C. After extensive washes, 50 μl horse radish peroixdase-conjugated goat anti-mouse IgG_1, IgG_{2a} and IgG_{2b} antisera (1:5000 dilution; Southern Biotechnology, Birmingham, AL) or rabbit anti-mouse IgG (1:5000 dilutions; GE healthcare life sciences, Little Chalfont, Bucks, United Kindom) were added to each well. Two hours later, the plates were washed, and tetramethylbenzidine (50 μl/well; BD Biosciences, San Jose, CA) was added followed by 10 min incubation at room temperature in the dark. Finally, 2N H_2SO_4 (50 μl/well) was added to

the wells to stop the reaction, and optical densities were determined at 450 nm. The serum anti-BTIIC Ab titre of a WT with the highest level of anti-BTIIC Ab on day 28 after the BTIIC/CFA immunization was arbitrarily designated as a titre of one and used as a standard to determine the titres of all the serum samples.

ELISPOT for enumeration of anti-BTIIC Ab-producing cells

Multiscreen® filter plate (Millipore, Billerica, MA) was coated with BTIIC as described for ELISA. The wells were blocked with culture medium (RPMI1640 medium containing 10% FCS) for 2 h at 37 ^{o}C and then washed. Cells from draining LN (1×10^{5} cells/100 μl/well) or bone marrow (BM; 5×10^{5} cells/100 μl/well) in culture medium were added to the wells and incubated for 4 h at 37 ^{o}C. The plates were then washed, and biotin-Streptavidin conjugated AffiniPure F(ab')2 fragment of goat anti-mouse IgG Ab (Jackson ImmunoResearch, West Grove, PA) was applied. After overnight incubation at 4 ^{o}C, the plates were extensively washed. Alkaline-phosphatase conjugated-streptavidin (1:60 R&D systems) was added to the wells and incubated for 2 h at room temperature. The spots were visualized by adding 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrate (R&D systems). Pictures of each well were taken with an ELISPOT reader (C.T.L., Shaker Heights, OH) and spots were counted visually.

Results

Generation of TL1A KO mice and general features of their immune system

To understand the role of TL1A in immune responses, we generated TL1A KO mice. The targeting strategy was illustrated in Figure 1A. Germ-line transmission of targeted TL1A was confirmed by Southern blotting using tail DNA (Fig. 1B), and PCR was used for routine genotyping of KO, WT and heterozygous pups (Fig. 1C). TL1A deletion of KO mice at the mRNA level was verified by RT/qPCR as shown in Figure 1D. The deletion of TL1A protein in T cells from KO mice was demonstrated in Figure 1E according to flow cyotmetry. The KO mice were fertile and had no visible anomaly. Lymphoid organs such as the thymus, spleens and lymph nodes were of normal sizes and cellularity (data not shown). The CD4CD8 double-negative (DN), CD4CD8 double-positive, CD4 single-positive and CD8 single-positive subpopulations in the TL1A KO thymi were comparable to those in the WT thymi (Fig. 2A). The CD4 and CD8 T cell percentages (Fig. 2A) and B (Fig. 2B) cell percentages in the spleen and lymph nodes of TL1A KO mice were in the normal ranges. These results indicate that TL1A KO does not significantly affect T and B cell development.

We examined Th1, Th17 and Treg cell populations in the spleen and lymph nodes of TL1A KO and WT mice ex vivo, but no apparent differences were found (Fig. 2C). Th2 cells in the KO and WT spleens were below the reliable detection level (data not shown). We next assessed whether a lack of TL1A affected Th1, Th2, Th17 and Treg cell development in vitro. We compared WT and KO T cell-depleted splenocytes in their capacity to support the development of the said T cell populations, using naïve KO CD4 T cells as starting cells, which were used because they did not produce TL1A to confound result interpretation. The KO non-T splenocytes were capable of supporting the naïve KO CD4 T cells differentiating into Th1, Th2, Th17 and Treg cells in a similar fashion as were their WT counterparts in supporting of naïve KO CD4 T cells (Fig. 2D). This suggests that a lack of TL1A minimally affects Th1, Th2, Th17 and Treg development in vitro.

The anti-viral immune responses of TL1A KO mice were evaluated in a lymphocytic choriomeningitis virus (LCMV) infection model. As illustrated in Supplementary Figure 1A, the number of CD4 cells and CD8 cells in the spleens on day 8 post-infection (8 dpi) presented no significant differences in WT and TL1A KO mice. The absolute numbers and relative

percentages of LCMV-specific, tetramer-positive (gp_{33-41}^{+} and $np_{396-404}^{+}$) CD8 cells in virus-infected mice were all increased in comparison to uninfected control C57BL/6 mice (data not shown), but there were no significant differences between TL1A KO and WT mice with regard to these parameters (Supplementary Figs. 1B and 1C). The absolute number and relative percentages of LCMV-specific TNF-α-producing CD4 (gp_{61-80}^{+}) and CD8 cells (gp_{33-41}^{+}) (Supplementary Figs. 1D and 1E), and LCMV-specific IFN-γ-producing CD4 (gp_{61-80}^{+}) and CD8 cells (gp_{33-41}^{+}) (Supplementary Figs. 1F and 1G) in TL1A-KO mice were comparable to those in WT controls. This result indicates that TL1A is not essential in anti-LCMV immune responses.

TL1A KO mice presented ameliorated CIA

Our previous study demonstrated that administering recombinant TL1A aggravates CIA (5). Bull et al. demonstrated that neutralizing Ab against TL1A partially blocks antigen-induced arthritis (23). We wondered whether a total lack of TL1A would affect RA pathogenesis in the CIA model. TL1A KO and WT mice in the DBA/1LacJ background were immunized with BTIIC/CFA to induce CIA. The draining lymph nodes (dLN) of the immunized mice were assessed on day 14 after BTIIC/CFA immunization, a mid-term point between the starting of the immunization and a full-fledged CIA. The KO dLN were smaller in size (Fig. 3A, 1st panel) and lower in cellularity (2nd panel) compared to their WT counterparts. The absolute numbers of T cells and B cells in these KO dLN were significantly lower than those in the WT dLN (3rd and 4th panels). We assessed the size of the germinal centres (GC) and the percentage and an absolute number of GC B cells in the dLN. As shown in Supplementary Figure 2A, TL1A KO dLN showed no abnormality in term of the size of the GC. This is consistent with the fact that the percentages of GC B cells among the total dLNs cells were comparable between WT and KO (Supplementary Figure 2B). However, the absolute number of GC B cells in the dLNs was significantly lower in KO than in WT mice (Supplementary Figure 2C), because of a reduction of the cellularity of the KO dLNs.

The TL1A KO mice displayed slower onset of CIA and significantly lower clinical scores (Fig. 3B). The incidence of CIA in the TL1A KO mice was also lower compared to that amongst the WT controls before day 31 after the immunization, although all TL1A KO mice eventually developed CIA (Fig. 3C). At the end of the experiment, on day 50, the arthritic paws of the TL1A KO and WT groups were collected and then assessed histologically. As

illustrated in Fig. 3D, the TL1A KO paws had less synovial membrane hyperplasia (asterisks), less immune cell infiltration and milder cartilage erosion (arrow heads). The pathological scores of the TL1A KO groups were significantly lower compared to the WT controls' (Fig. 3E). We isolated cells from paws on day 19 after BTIIC/CFA immunization, which was chosen because it was shortly before the onset of the disease and paws were still normal in appearance, so that the selection of the paws was in no way biased. The number of cells of the hematopoietic origin isolated from the paws was significantly smaller in TL1A KO mice than in WT mice, but the percentages of DC, neutrophils/macrophages/monocytes, T cells, and B cells among the total isolated cells from TL1A KO and WT paws showed no statistically significant differences (Fig. 3F).

These results clearly show that a lack of TL1A diminishes the severity of CIA.

TL1A KO mice had lower collagen-specific Ab titres after BTIIC/CFA immunization

The non-immunized TL1A KO and WT mice had comparable levels of total serum IgG, IgM, IgA, and IgG subtypes IgG_1, IgG_{2a} and IgG_{2b}, IgG_3 (Supplementary Fig. 3). Auto-Abs are pathogenic in RA (42, 43), and anti-collagen Ab is especially so in CIA. We measured the BTIIC-specific Ab during the course of CIA from day 14 to day 50 after BTIIC/CFA immunization. The BTIIC-specific total IgG titres in the KO mice were significantly lower than their WT counterparts at all time points starting from day 21 (Fig. 4A). Among the IgG isotypes, BTIIC-specific $IgG2_a$ and $IgG2_b$ but not IgG_1 titres in the TL1A KO mice were significantly lower than in the WT mice (Figs. 4B-D). The reduced titres of collagen-specific Abs titres, especially the $IgG2_a$ and $IgG2_b$ isotypes, which are major pathogenic Ab isotypes in CIA (44), is likely a relevant factor for the observed reduced CIA severity in the TL1A KO mice.

CIA-related T cell functions in TL1A KO mice

T cells are a major type of target cells of TL1A, and play a vital role in RA pathogenesis including producing inflammatory lymphokines such as IFN-γ and IL-17 and providing help to B cells for pathogenic Ab production. We assessed the antigen-specific dLN T cells for their IFN-γ- and IL-17-production and proliferation upon *in vitro* collagen restimulation shortly before the onset of CIA on day 14 after the immunization, a time point these cells reportedly become detectable in this location (45, 46). However, no significant difference between TL1A KO and WT dLN was found (Supplementary Figures 4A, 4B). This finding is

in agreement with a previous study concerning the role of TL1A in antigen-specific responses. In the OT-II system, deficiency of TL1A/DR3 signal does not affect naïve T cells differentiation under antigen-specific stimulation, and DR3 KO dLN cells from EAE mice proliferate normally to the stimulation of MOG peptides *in vitro*(15). It is also compatible with the data of polyclonal T cell activation as shown in Figure 2.

CRCXR5$^+$/PD-1$^+$ follicle T help cells (Tfh) express CD40L and ICOS on the cell surface and produce IL-21, all of which are essential in B cell survival, differentiation and eventually Ab production. We examined Tfh in the dLN on day 18 after the immunization, shortly before the lower titres of BTIIC-specific Abs in the KO CIA mice became apparent. The TL1A KO and WT dLN harboured similar percentages of CRCXR5$^+$/PD-1$^+$ Tfh (Fig. 5A, left column); TL1A KO and WT Tfh cells expressed comparable levels of CD40L and ICOS (Fig. 5A, right column). Probably as a consequence, the Fas$^+$GL7$^+$ germinal centre B cells percentages in the dLN of TL1A KO and WT were not different (Fig. 5B). With that said, since the absolute number of T cells in the KO dLN was reduced (Fig. 3A), the absolute number of Tfh in the KO dLN was still smaller than that of WT dLN. We confirmed that undifferentiated CD4 T cells (Th0) did not expression DR3, but Th1, Th17 and Tfh cells all expressed DR3, providing a basis for them to respond to TL1A (Fig. 5C). *In vitro* differentiated Tfh cells from naïve TL1A KO and WT CD4 cells were all capable of produce high levels of IL-21, compared to KO Th0, Th1 and Th17 cells (Fig. 5D). Exogenous TL1A did not enhance IL-21 production by KO Tfh cells (Fig. 5D). The results of this section suggest that the deletion or exogenous TL1A does not have significant effect on Tfh cells with regard to their B cell helper functions, such as their CD40L/ICOS expression and IL-21 production, although the diminished absolute number of Tfh in the KO dLN might be a factor contributing to the reduced level of collagen-specific Abs in KO CIA mice.

Plasma cells are direct target cells of TL1A

It is puzzling that in TL1A KO CIA mice, there was an obvious decrease in antigen-specific Ab production, yet T cell's B-cell-facilitating function showed no signs of compromise, with the exception of a decrease of the absolute number of Tfh. To date, B cells and their derivative plasma cells are not known target cells of TL1A, as an earlier study demonstrate that B cells do not express or express very little DR3 (3, 13), although Pelletier et al. did reveal that in vitro generated human plasma cells express DR3 at the protein level(47). We confirmed that

indeed, $B220^{+}CD138^{-}$ B cells did not express DR3, nor did $B220^{+}CD138^{+}$ plasmablasts (Fig. 6A, left and middle panel). However, we discovered that plasma cells from dLN of BTIIC/CFA-immunized WT mice expressed very high levels of DR3 (Fig. 6A, right panel). DR3 has a full-length isoform and also truncated isoforms (10). According to RT/qPCR, T cells and plasma cells expressed similar levels of the full-length DR3 isoform Fig. 6B, left panel), while the former expressed more isoforms with deletions (Fig. 6B, middle and right panels). The expression of the full length DR3 isoform on plasma cells provided a basis for them to respond to TL1A. We then interrogated the effect of TL1A on plasma cells. B cells and plasma cells from draining LN of KO mice 21 days after BTIIC/CFA immunization were negatively selected using B cell negative selection kit (STEMCELL Technologies) by eliminating T, NK, monocyte/macrophages and dendritic cells. The remaining B cells and plasma cells were cultured for six days in the presence of exogenous TL1A, as there was no endogenous TL1A in this system. In the presence of TL1A, significantly more plasma cells survived after the six-day culture (Fig. 6C, left panel) and there were higher levels of collagen-specific IgG secreted into the supernatants (Fig. 6C, right panel), compared to the culture without TL1A. In support of such in vitro results, on day 28 after the BTIIC/CFA immunization, when the anti-BTIIC Ab production was actively produced and WT mice had significantly higher titres of serum anti-BTIIC Abs, draining LN cells and bone marrow cells from WT mice presented significantly higher numbers of anti-BTIIC Ab-secreting plasma cells compared to those from TL1A KO draining LN, according to ELISPOT (Figs. 6D-6F). Collectively, the data from this section suggest that TL1A can directly promote plasma cell survival and function in terms of Ab production, and this effect in turn contributes to the elevated pathogenic Ab production in CIA. Conversely, in the absence of TL1A, the well-being of the plasma cells is compromised, and this is one of the factors leading to decreased levels of pathogenic anti-collagen Ab in TL1A KO mice.

Discussion

In this study, we revealed that TL1A KO mice had ameliorated CIA compared to WT mice. No significant T cell dysfunction including Th17 and Treg differentiation was apparent in the absence of TL1A, and the TL1A KO mice had normal anti-LCMV immune responses. The KO mice presented reduced production of pathogenic anti-collagen Ab. A novel finding in this study is that plasma cells were a direct target of TL1A, that these cells expressed high levels of the full-length isoform of TL1A receptor DR3. TL1A promoted plasma cell's survival and Ab-production.

The results of our current study and several previous studies support the general concept that TL1A can intensify inflammatory response. For example, DR3 KO mice present reduced EAE and antigen-induced arthritis (15). The administration of exogenous TL1A aggravates CIA, while TL1A neutralizing Ab reduces the severity of CIA(5, 23). Transgenic overexpression of TL1A worsens DSS-induced colitis and causes intestinal mucosal inflammation(9, 20, 21, 48). Several mechanistic aspects of these disease models corroborate each other. For example, our data using TL1A KO mice showed that TL1A was not required for constitutive or induced Th1, Th2, Th17 and Treg cell development; studies using deletion of DR3 reached the same conclusion(15). However, there are also discrepancies between the conclusions obtained from DR3 KO mice versus TL1A KO mice, and from TL1A administration/overexpression vs. TL1A/DR3 deletion studies. The discrepancies and possible explanations are discussed as follows.

It seems that excessive TL1A, either from exogenous recombinant protein or endogenous transgenic over-expression often results in enhanced IFN-γ and IL-17 expression, but TL1A KO or DR3 KO has minimal effect on constitutive or inducible Th1 and Th17 cell development (5, 9, 15, 48). A possible explanation for this discrepancy is that such Th cell development is vital to the biological system, and there is sufficient redundancy to compensate for the missing DR3 or TL1A in the KO mice. As a consequence, in the absence of DR3 or TL1A, Th cells could still be developed to near-normal levels. Alternatively, DR3 and TL1A are not utterly essential in Th cell development; the enhanced Th cell development in the presence of non-physiological high doses of exogenous or transgenic TL1A is due to its cross-reaction with other TNFR superfamily members (12). The results from CIA mice treated with

TL1A neutralizing Ab favour the first explanation (23). In this model, there is no excessive exogenous TL1A, and the reduction of the bio-active TL1A occurs at the adult stage and is not complete, less likely triggering a drastic compensation. Such a neutralizing Ab treatment can hamper the development of Th1 and Th17 cells, which are reported essential in CIA pathogenesis, and results in reduced CIA severity (49).

We demonstrated that TL1A KO mice showed no abnormality in anti-viral immune responses against LCMV. However, DR3-KO mice manifest compromised anti-MCMV immunity (24). Although TL1A is a ligand of DR3, there are around 20 other TNF superfamily members that share some degrees of homology with TL1A. In the absence of TL1A, other members of the TNF superfamily might individually or collectively bind to DR3, albeit at a lower affinity, and such binding might trigger some low level DR3 signalling, which could be sufficient to compensate for the missing TL1A. On the other hand, a missing DR3 will totally eliminate the DR3 signalling, resulting more drastic phenotype including compromised anti-virus immune responses. Although we favour this hypothesis, we cannot exclude the possibility that the observed difference in DR3 KO and TL1A KO regarding the anti-virus immune responses is caused by different viruses, i.e., MCMV vs. LCMV.

Another interesting point is the role of TL1A in Treg development. In our TL1A KO and WT mice, no difference was found in constitutive or induced Treg populations. Not surprisingly, there was no aggravation but amelioration of CIA in the TL1A KO mice. In mice with transgenic TL1A expression or mice administered with exogenous recombinant TL1A, the immune/inflammation responses were aggravated but not ameliorated (5, 9, 21, 48). In all the studies using either TL1A or DR3 KO mice models or TL1A neutralizing Abs(3, 15, 23-25), the immune responses were abated. Although the Treg status of the above mentioned studies are not always assessed, at least we could conclude that excessive TL1A does not lead to reduced immune response, a possible functional consequence of Treg upregulation. Conversely, TL1A or DR3 deletion does not lead to exuberant immune responses, which are a possible functional consequence of Treg downregulation.

Our ex vivo data indicated that the percentages of Th1 and Th17 cells in draining LN were comparable between CIA WT and CIA KO mice. However, since there was a drastic reduction of cellularity and absolute T cell number in the draining lymph nodes of KO mice, the

absolute numbers of these effector cells were also reduced. Such reduction could likely cause a compromised CIA development in the TL1A KO mice.

Several members of TNF family are involved in regulating the humoral immune responses. They could either affect the B cells directly or indirectly through T cells. A well-studied member is CD40L, which is essential to the generation of plasma cells (50). CD40/CD40L signalling initiates GC responses, GC B cell proliferation, isotype switching and differentiation of B cells into plasma cells (51). Since DR3 was reportedly expressed mainly on T cells in the immune system, we assessed the effect of TL1A on Tfh cells. Our *ex vivo* data indicated that the percentages of Tfh cells in draining LN were comparable between WT and KO mice with CIA. The functions of KO Tfh cells, in terms of CD40L expression and IL-21 production, were not compromised, neither. However, because there was a significant reduction of absolute T cell number in the draining LN of KO mice, comparing to that of WT mice, it is conceivable that the absolute number of Tfh and GC B cells in the draining LN of KO mice was reduced. Such reduction might contribute to compromised collagen-specific Ab production in CIA TL1A KO mice.

We found that the plasma cells also expressed functional DR3. This finding pin-pointed such plasma cells as a target cell population for TL1A's effect. We demonstrated that TL1A could promote draining LN plasma cell survival in vitro. The direct effect of TL1A on plasma cell survival might be one of the contributing mechanisms for the following observation: 1) TL1A KO results in reduced number of collagen-specific Ab producing plasma cells ex vivo, and reduced collagen-specific Ab titers in vivo; 2) recombinant TL1A enhanced collagen-specific Ab production *in vitro*. However, we noticed that in unimmunized KO mice, their total serum IgG levels, and plasma cell population were comparable to those of WT mice. It is possible that the benefit of TL1A to plasma cells is only for a limited time during a humoral response, and perhaps even specific to a restricted stage of the plasma cells.

We demonstrated that reduced pathogenic Ab production is a mechanism contributing to milder CIA in the TL1A-KO mice. However, it is probably not the only mechanism responsible for this phenotype. We have assessed several T cells function in the absence of TL1A, and did not find any anomaly, but such assessment is by no means exhaustive. We did find that there was a significant reduction of inflammatory immune cell infiltration in the CIA paws, and this phenomenon is consistent with the findings with several disease models in that

there is always a reduction of inflammatory cells in the diseased organ or tissues when DR3 or TL1A is missing (15, 22). This is certainly an additional mechanism by which TL1A contributes to the pathogenesis of CIA. We tested TL1A KO T cell chemotaxis toward several CIA-related chemokines *in vitro* using Transwell, but no significant defect was detected. More comprehensive investigations in this aspect including assessing local chemokine secretion by T cells, monocyte/macrophages, and DC, and the endothelium permeability in the excessive or absence of TL1A are warranted.

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Figure legends

Figure 1. Generation of TL1A KO mice

A. Targeting strategy to generate TL1A KO mice. The shaded rectangle on the 5' side of the mouse TL1A WT genomic sequence represents the sequence serving as a probe for genotyping by Southern blotting.

B and C. Genotyping of TL1A mutant mice. Tail DNA was digested with StuI, and analyzed by Southern blotting, with the 5' probe whose location is indicated in A. Arrows indicate 11.7-kb bands representing the WT allele and a 5.4-kb band representing the recombinant allele (B). PCR was used for routine genotyping with ear-lobe tissue DNA. The 335-bp bands representing the WT allele and the 581-bp bands representing the recombinant allele are indicated by arrows (C). HET: heterozygous.

D. Absence of TL1A mRNA expression in TL1A KO T cells. Spleen T cells from WT and TL1A KO mice were stimulated with solid anti-CD3 (2μg/ml) and anti-CD28 (2μg/ml) for 24 h. Cell RNA was extracted and analyzed by RT-qPCR for TL1A expression. The results are expressed as ratios of TL1A versus β-actin signals with means $\pm$ SD indicated.

E. Absence of TL1A protein expression in KO T cells. WT and TL1A KO spleen T cells were stimulated as in (D) and stained with anti-CD4 mAb and anti-TL1A Ab and analyzed by 2-color flow cytometry. The shaded lines represent the isotypic Ab control, and the thick lines represent TL1A staining of $CD4^+$-gated T cells of WT (left panel) and KO (right panel) mice. Experiments in D and E were repeated at least twice and representative data are shown.

Figure 2. TL1A KO mice presented normal lymphocyte subpopulations

Experiments in this figure were repeated more than three times, and representative data are presented (A, B and D).

A. T-cell populations in WT and TL1A KO LN, spleens and thymus. Thymocytes, mesenteric LN cells, splenocytes were analyzed by 2-color flow cytometry for percentages of different T cell subpopulations.

B. B-cell populations in the LN and spleen of WT and KO mice. Mesenteric LN cells and splenocytes were analyzed by 2-color flow cytometry for percentages of $CD19^+/B220^+$ B cells.

C. Normal Th1, Th17 and Treg populations in TL1A KO mice ex vivo. WT or TL1A KO spleen cells were stimulated with PMA (50ng/ml) and ionomycin (1μg/ml) for 4 h in the presence of BD Golgistop, and analyzed by flow cytometry *ex vivo* for Th1, Th17 and Treg cell populations based on intracellular IFN-γ, IL-17 and FoxP3 staining. The bar graphs represent the summary of data from 3-4 experiments, with Mean + SD indicated.

D. Normal differentiation of TL1A-KO CD4 cells in vitro. Naïve TL1A-KO CD4 cells were cultured under conditions favouring Th1, Th2, Th17 and Treg cells, using WT or TL1A KO feeder cells, as indicated. Their intracellular cytokine or FoxP3 expression was quantified by 3-color flow cytometry on day 3 for Th1, Th17 and Treg cells, and on day 5 for Th2 cells after 4-h PMA (50ng/ml) and ionomycin (1μg/ml) stimulation in the presence of BD Golgistop before the assay.

Figure 3. TL1A-KO mice manifested less severe CIA

A. Reduced numbers of immune cells in KO draining lymph node during CIA induction. Mice were immunized with BTIIC/CFA at the tail base on day 0. On day 14 after the immunization, draining lymph nodes (dLNs) were collected and photographed (1^{st} panel), cells from dLN of WT (n=10) and TL1A KO (n=11) mice were isolated, enumerated and analysed by flow cytometry for subpopulations. The numbers of total dLN cells per mouse (2^{nd} panel), CD4 T cells ($CD4^+$) of the dLNs per mouse (3^{rd} panel) and B cells ($B220^+$) of the dLNs per mouse (4^{th} panel) are shown. The horizontal bars indicate the means. Student's *t* test was used to assess the statistical significance of the difference between WT and TL1A KO mice and the *p*-value is indicated.

B. CIA clinical scores of WT and KO mice after BTIIC/CFA immunization. WT (n=8) and TL1A KO (n=9) mice were immunized with BTIIC/CFA on day 0, and scored for their RA symptoms daily starting from day 21 until day 50. Mean ± SEM scores are plotted. Scores for

the WT and TL1A KO mice were evaluated statistically by 2-Way ANOVA. *P*-values are indicated.

C. CIA incidence in WT and TL1A KO mice. The CIA incidence of the WT and TL1A KO groups in (A) is plotted.

D and E. Histology of mouse joints. WT and TL1A KO Mice from (B) were sacrificed on day 50, and all the paws were sectioned and stained with haematoxilin/eosin (H&E) or safranin O (for cartilage staining) as indicated. Representative histology images from each group are shown (D). Asterisks indicate synovial hyperplasia and arrow heads indicate cartilage damage. Naïve: joints from normal unimmunized DBA/1 mice; WT-CIA: joints from WT mice immunized with BTIIC/CFA; KO-CIA: joints from KO mice immunized with BTIIC/CFA. The histology of each paw (one section per paw) was evaluated by blinded raters and scored semi-quantitatively based on lining hyperplasia, bone erosion and cell infiltration. Each parameter was scored on a 0-3 scale, and all four paws of each animal were scored. The overall score for each animal, which is the sum of the three parameters of the four paws, is shown in the plot and the horizontal bars represent the mean scores (E). Student's *t* test was performed to assess the statistical significant of the difference between WT and TL1A KO mice and the *p*-value is indicated.

F. Reduced numbers of infiltrating immune cells in KO paws during CIA induction. On day 19 after BTIIC/CFA immunization, before the onset of CIA symptoms, cells infiltrating the paws of WT and TL1A KO mice were isolated, enumerated and analysed by flow cytometry for subpopulations. The total numbers of infiltrating cells of hematopoietic origin ($CD45.2^+$) per paw is shown in the left panel; percentage of dendritic cells ($CD11c^+$), in the second panel; percentage of neutrophils/monocytes/macrophages ($CD11b^+$), the third panel; percentage of T cells ($Thy1.2^+$), in the fourth panel; and percentage of B cells ($B220^+$), in the last panel. Mouse numbers per group are indicated. Student's *t* test was used to assess the statistical significance of the difference between WT and TL1A KO mice and the *p*-value is indicated.

Figure 4. Reduced collagen-specific Ab production in KO mice with CIA

Sera were obtained from WT (n=7) and TL1A KO (n=7) mice on 14, 21, 28, 35, 42 and 50 days after BCTII/CFA immunization. Arbitrary titres of collagen-specific total IgG (A), IgG_1

(B), IgG_{2a} (C) and IgG_{2b} (D) were determined by ELISA. Serum from a WT mouse exhibiting typical arthritis was arbitrarily given a titre of 1 and used as a reference in each assay. For A, B, C, D, mean ± SEM are presented and evaluated statistically by 2-Way ANOVA. *P*-values are indicated.

Figure 5. TL1A T cells presented normal help to B cells

The experiments in this figure were repeated three times and representative data are shown.

A. Flow cytometry analysis of Tfh in draining LN of mice after BTIIC/CFA immunization. WT or TL1A KO mice were immunized with BCTII/CFA and then sacrificed on day 18. $CD4^+$ T cells from dLN were purified and stimulated with solid anti-CD3ε (2μg/ml) and anti-CD28 (2μg/ml) Ab for 30 min. The percentage of $CD4^+PD\text{-}1^+CXCR5^+$ Tfh cells among $CD4^+$ cells were determined by flow cytometry and shown (right column). The gated Tfh were further analyzed for their CD40L and ICOS expressions (left column), and the percentages of $CD40L^+$ and $ICOS^+$ cells among Tfh ($PD\text{-}1^+/CXCR5^+$) cells are indicated.

B. Flow cytometry analysis of germinal centre (GC) B cell populations. $B220^+$ B cells from the draining LN of WT and TL1A KO mice on day 18 after BTIIC/CFA immunization were analysed for GL7 and Fas expression. The percentages of $B220^+GL7^+$ Fas^+ GC B cells among $B220^+$ B cells are indicated.

C. DR3 expression on Th and Tfh cells. Naïve $CD4^+$ T cells were cultured on anti-CD3ε (2μg/ml for coating) and anti-CD28 (2μg/ml for coating) Ab-coated wells under Th1, Th17 and Tfh-like cell differentiation conditions. After 3 days, DR3 expression on $CD4^+$ cells was assessed by flow cyotmetry. Shaded area: isotypic controls. Thick lines: DR3 signals.

D. IL-21 production by in vitro differentiated WT and KO Tfh cells. WT or TL1A KO naïve $CD4^+$ T cells were cultured in anti-CD3 and anti-CD28 Ab-coated wells under the Tfh-like cell differentiation condition. They were also cultured under Th0 (without reagents for Th differentiation except anti-CD3ε, anti-IFN-γ and anti-IL-4 Abs), Th1 or Th17 conditions as controls. TL1A at 10 and 50 ng/ml was added to some cultures as indicated. On day 3, the cells were re-stimulated with PMA/ionomycin for 9 hours and IL-21 in the supernatants was measured by ELISA. Samples in ELISA were in duplicate and means ± SD are shown.

Figure 6. The effect of TL1A on plasma cells

The experiments in A, B and C were repeated three times and representative data are shown.

A. DR3 expression in plasma cells. dLN cells from WT mice on day 15 after BTIIC/CFA immunization were gated on $B220^{+}CD138^{-}$ regular B cells (left panel), $B220^{+}CD138^{+}$ plasmablast and $Dump^{-}B220^{-}CD138^{+}$ plasma cells and analyzed for their DR3 expression by three-color or four-color flow cytometry. Shaded areas: isotypic controls. Thick lines: DR3 signals. Dump staining used anti-CD8, anti-CD11b, anti-CD11c, anti-F4/80, anti-IgM, anti-IgD and anti-CD4 mAbs.

B. DR3 isoform expression in CD4 cell and plasma cells. RT-qPCR was used to detect the mRNA of full-length DR3 and all DR3 isoforms (including the full-length and all truncated isoforms) in flow cytometer-sorted $CD4^{+}$ T cells (as controls) and $Dump^{-}B220^{-}CD138^{+}$ plasma cells. The results are expressed as ratios of DR3 (full-length or all isoforms) versus β-actin signals with means $\pm$ SD indicated. Left panel: full-length DR3 isoform expression in CD4 T and plasma cells; middle panel: the expression all DR3 isoforms (full-length plus all truncated isoforms) in CD4 T and plasma cells; right panel: ratios of full-length DR3 versus all isoforms of DR3.

C. Higher plasma cell numbers in and collagen-specific Ab production by WT dLN cells cultured in the presence of TL1A. dLN cells from WT mice 21 days after BTIIC/CFA immunization were cultured in the absence or presence of TL1A (50 ng/ml) for 6 days. The number of $Dump^{-}B220^{-}CD138^{+}$ plasma cells were counted by flow cytometry four times on day 6 and the plasma cell numbers per 1×10^{6} total dLN cells (means ± SD) are shown (left panel). The culture supernatants were harvested on day 6 and collagen-specific IgG levels in the supernatants were determined by ELISA, which was conducted in triplicate samples. Arbitrary titres of anti-collagen IgG Abs (means ± SD) are shown (right panel). Experiments were repeated at least twice and representative data are shown. Student's *t* test was used to assess the statistical significance of the difference and the *p*-value is indicated.

D-F. Reduced collagen-specific IgG-producing cells (CSIGGPC) in the dLN and bone marrow (BM). Twenty-eight days after BTIIC/CFA immunization, CSIGGPC in the dLN and BM were enumerated by ELISPOT. Representative images of spots in wells were showed in D. The spots in 8 replicate wells of each experiment were counted, and the data of three

similar experiments were pooled and summarized bar graphs (E for dLN and F for BM). Mean ± SEM are presented. *p<0.05; **p<0.01 (Student's *t* test).

Figures

Figure 1 Generation of TL1A KO mice

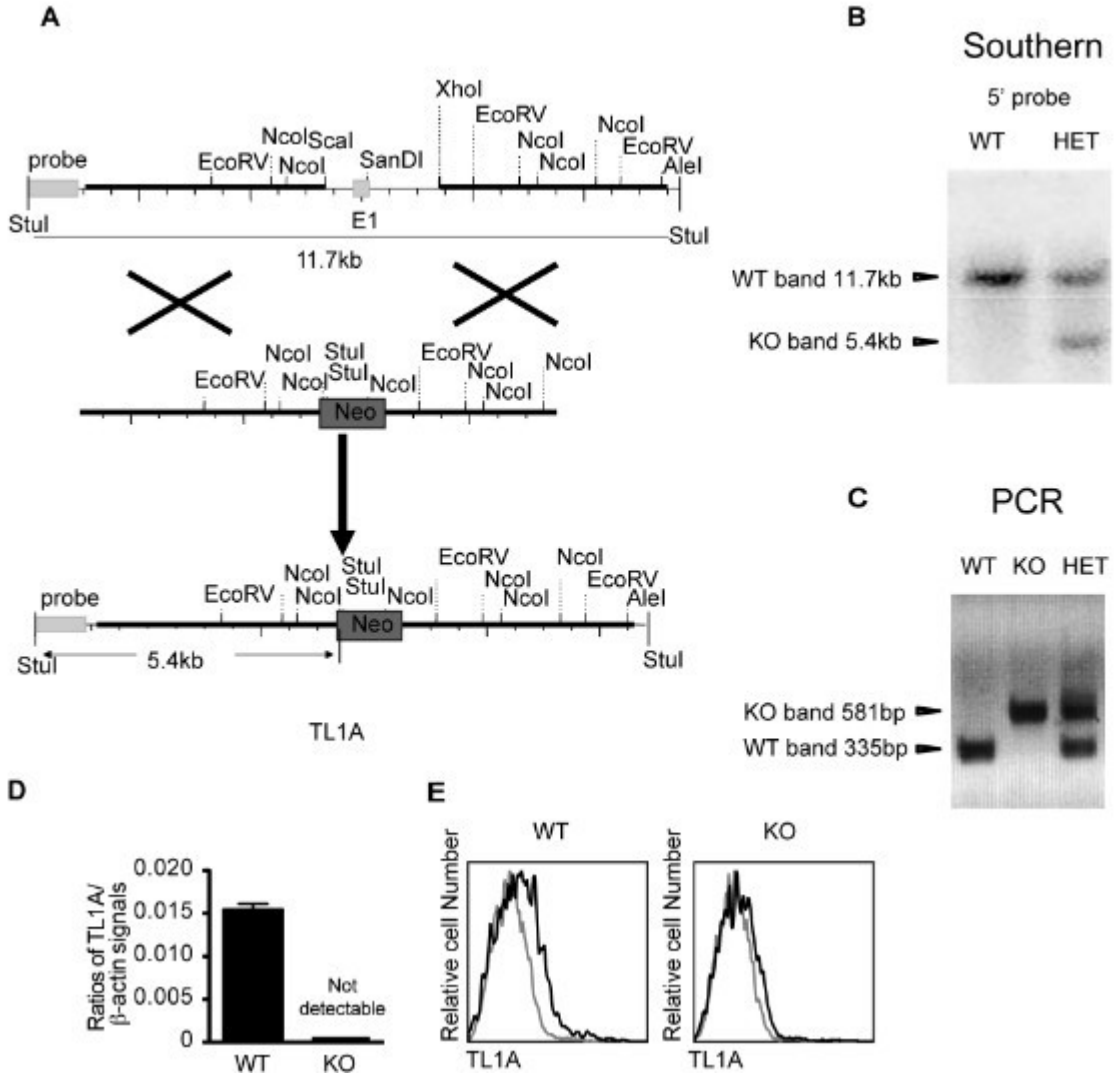
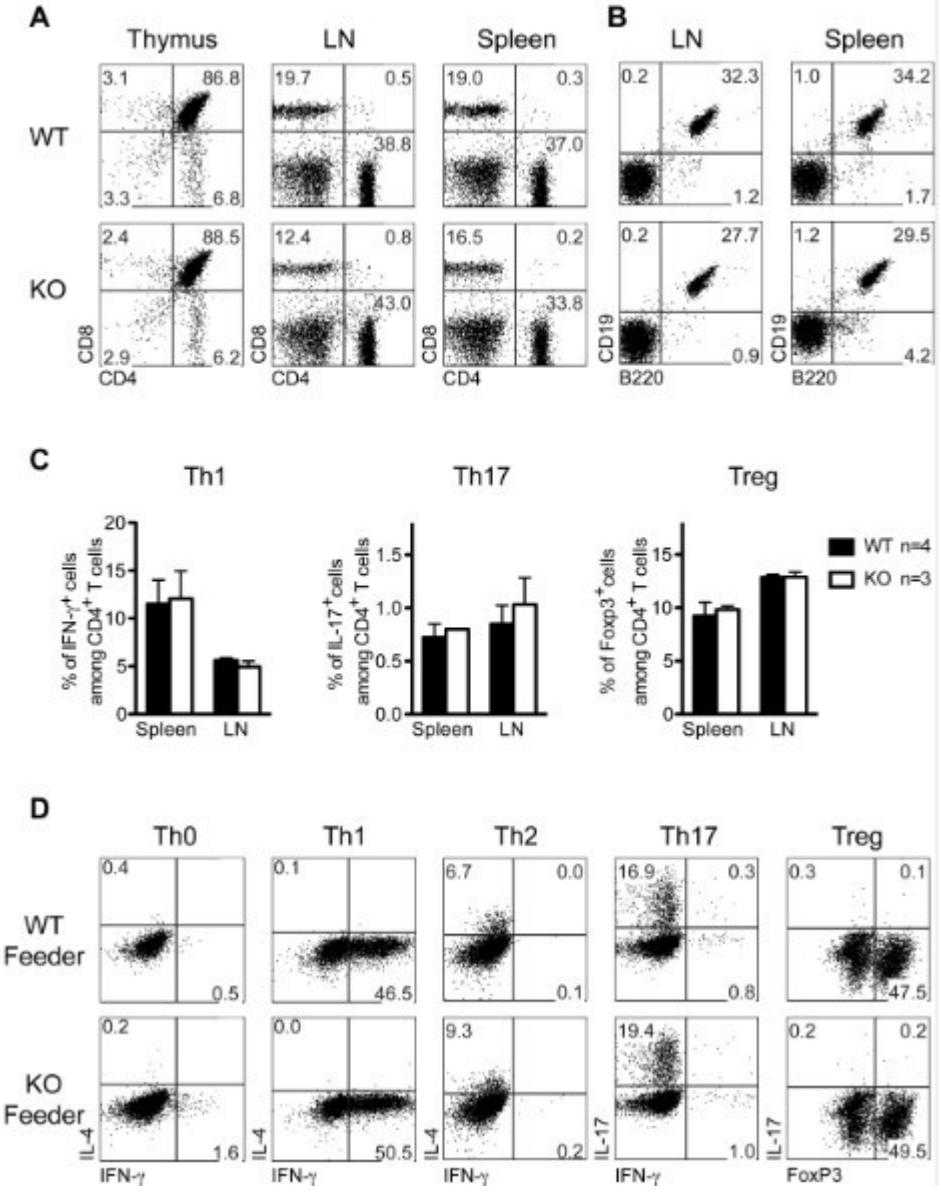


Figure 2 TL1A KO mice presented normal lymphocyte subpopulations



Fugure 3 TL1A-KO mice manifested less severe CIA

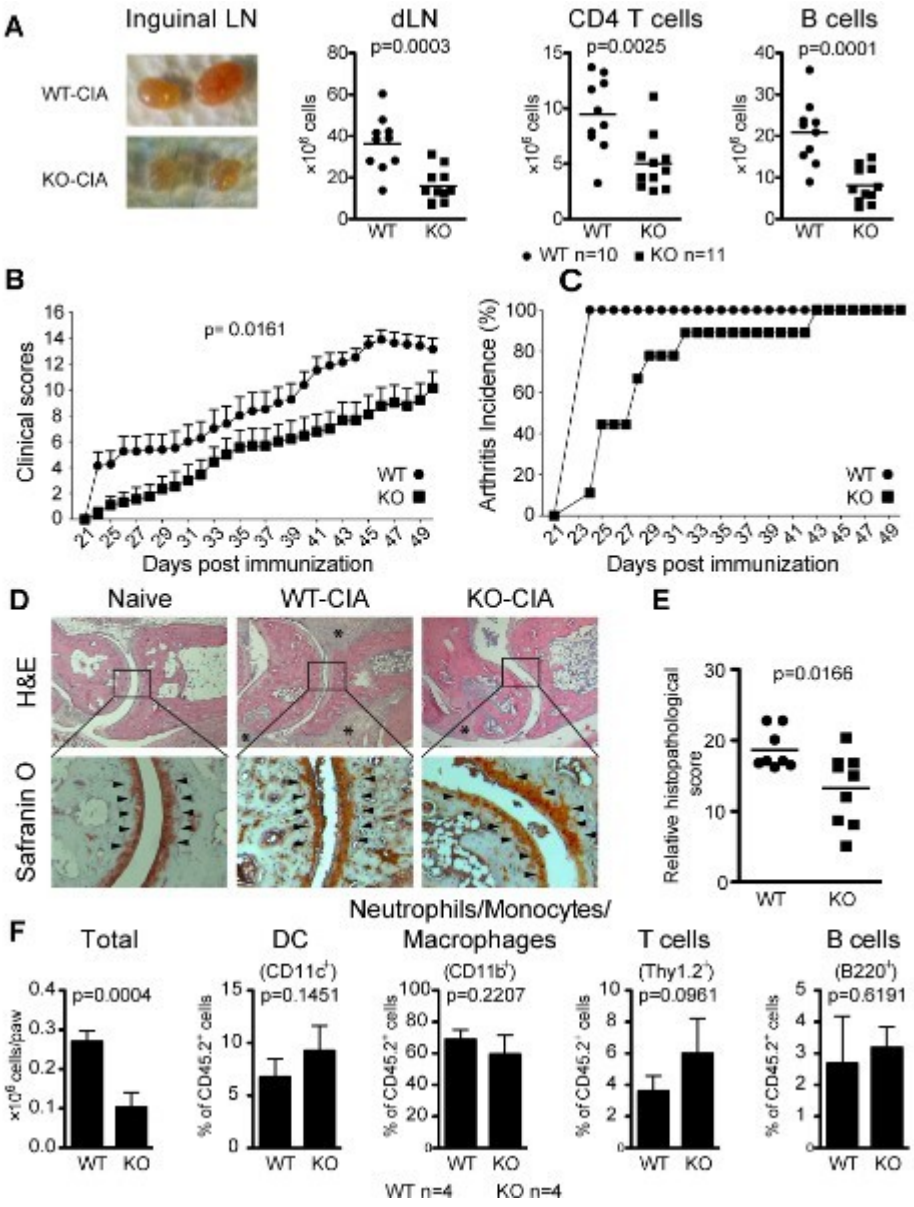


Figure 4 Reduced collagen-specific Ab production in KO mice with CIA

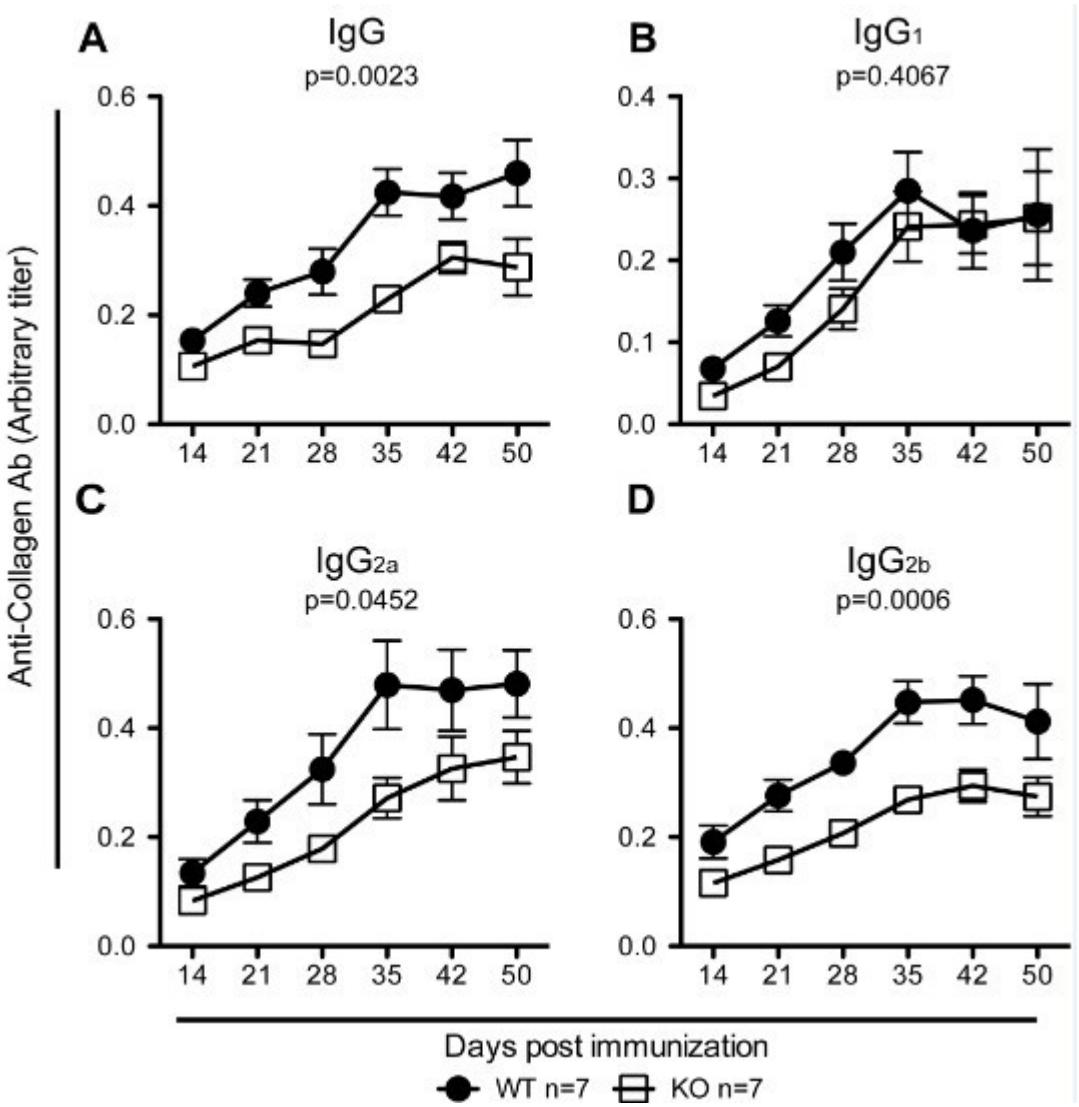


Figure 5 TL1A T cells presented normal help to B cells

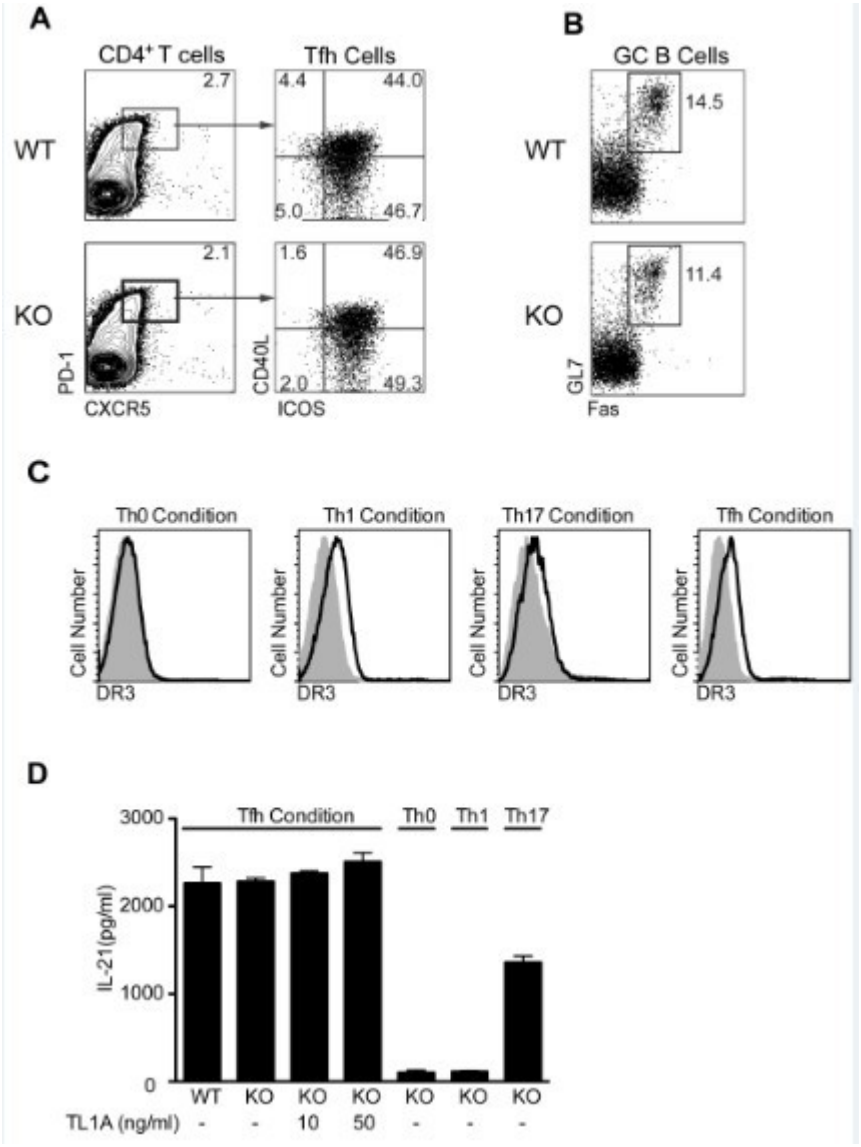


Figure 6 The effect of TL1A on plasma cells