The Role of IL-27 and IL-35 in inflammatory diseases

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Abstract

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic inflammation within the synovial tissues in multiple joints leading to progressive, erosive destruction of cartilage and underlying joints. The severity of RA is associated with the overexpression of proinflammatory cytokines within the synovial tissue, such as TNF-α, IL-1 and IL-6. Recently, IL-17 is thought to play a critical role in maintaining the inflammatory processes within the arthritic joints. Although the etiology and pathogenesis of RA has not been completely elucidated, neutralizing Antibodies against the inflammatory components have been shown to successfully suppress joint inflammation, reduce the relapse rate and delay disease onset in RA patients. Therefore, the expression and regulation of cytokines produced during the disease progression has been the centre of interest in therapeutic studies. Cytokines are important mediators of immune functions in humans and animals. In this thesis, a murine model of RA has been used to investigate the roles of new cytokines Interleukin (IL)-27 and Interleukin (IL)-35.

Interleukin (IL)-27, is a heterodimeric cytokine comprised of an IL-12p40 related protein, Epstein-Barr virus-induced gene 3 (EBI3) and a unique IL-12p35 like protein p28. IL-27 is a member of IL-12 family, mainly generated by activated macrophages and dendritic cells. IL-27 binds a receptor composed of WSX-1/TCCR, a ligand-specific chain, and gp130, a signal-transducing molecule shared with other cytokines such as IL-6. IL-27 can promote both pro- and anti-inflammatory immune responses. A novel role of IL-27 regulating autoimmunity has been suggested by experiments on experimental autoimmune

encephalomyelitis (EAE) and central nervous system (CNS) inflammation when infected with *Toxoplasma gondii*. IL-27 suppresses these chronic diseases through inhibiting Th17 activity. Thus, IL-27 may have an important therapeutic potential for treatment of RA in humans. A major aim of this project has been to clone and express a recombinant murine IL-27 in sufficient quantities to study the role of IL-27 in a murine model of RA closely related to the human disease, collagen-induced arthritis (CIA). A short term administration of IL-27 to mice at the onset of the disease had a significantly suppressive effect on disease severity and incidence compared with untreated controls. Mice treated with the recombinant IL-27 also showed reduced serum IL-6, IL-17 and collagen-specific IgG2a. Spleen and lymph node cells from the IL-27-treated mice produced significantly less IFN-γ and IL-17 compared with cells from the control mice when cultured with collagen *in vitro*. In contrast, administration of IL-27 to mice during the late phase of CIA significantly exacerbated disease severity. IL-27-treated mice also showed elevated IFN-γ and IL-6 production by the lymphoid cells when compared to untreated mice. However, IL-17 synthesis was not affected between IL-27-treated mice and untreated mice. Consistent with this finding, *in vitro* IL-27 markedly inhibited the development of Th17 from naïve $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells, but had little or no effect on differentiated Th17 cells. Together, these results demonstrated that IL-27 had both pro-inflammatory and anti-inflammatory effects on chronic articular inflammation, mainly associated with Th17 functions.

Interleukin (IL)-35, another novel heterodimeric cytokine belonging to IL-12 family, is composed of EBI3 and the IL-12p35 subunit. Little is known about the

biological function of IL-35. To study the role of IL-35 in immune responses, murine recombinant IL-35 was cloned and expressed in a mammalian GS system to produce sufficient quantities. IL-35 induced proliferation of murine $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells when stimulated with immobilized anti-CD3 and anti-CD28 antibodies *in vitro*. The IL-35-expanded $CD4^+CD25^+$ T cell population expressed Foxp3 and produced elevated levels of IL-10, whereas the IL-35-induced $CD4^+CD25^-$ T cells produced IFN-γ but not IL-4. The IL-35-expanded $CD4^+CD25^+$ T cells maintained their suppressive functions against $CD4^+CD25^-$ effector cells. Furthermore, when cultured with soluble anti-CD3 and antigen-presenting cells, IL-35 directly suppressed the proliferation of $CD4^+CD25^-$ effector cells. Moreover, IL-35 inhibited the differentiation of Th17 cells *in vitro*. *In vivo*, IL-35 effectively suppressed established collagen-induced arthritis in mice with the suppression of IL-17 production but enhanced IFN-γ synthesis. Therefore, IL-35 is a novel cytokine suppressing the immune response through the expansion of regulatory T cells and suppression of Th17 cell development. For the future study of human IL-35, human EBI3 and p35 were cloned and linked together with an Fc fusion part. Human IL-35 was expressed in GS system and the function of the recombinant protein needs further study.

These data in this thesis provide direct evidence that IL-27 and IL-35 are important mediators in murine collagen-induced arthritis disease. This implicated that IL-27 and IL-35 could represent potential new targets for novel therapeutic agents in human RA. However, the findings on the dual role of IL-27 at the different disease process suggested that the involvement of IL-27 in the

pathogenesis of human RA should be carefully investigated before clinical therapy application.

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Abbreviations

Ab	antibody
AHR	airways hyper-responsiveness
APC	antigen presenting cell
BM	bone marrow
CD	clusters of differentiation
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein diacetate succinimidyl ester
CHO	Chinese Hamster Ovary
CIA	collagen- induced arthritis
Con A	Conncanavlin A
Cpm	counts per minute
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DC	dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
Foxp3	forkhead/winged helix family protein
GITR	Glucocorticoid-induced TNF receptor superfamily member 18
GM-CSF	granulocyte macrophage-colony stimulating factor
GS	glutamine synthetase

H&E	haematoxylin and eosin
HPRT	hypoxanthine phosphoribosyltransferase
IFN	interferon
Ig	immunoglobulin
IL-	interleukin
i.p.	intraperitoneal
IPEX	Immune dysregulation, polyendocrinopathy, entropathy, X-linked syndrome
KO	knockout
LAG-3	lymphocyte activation gene-3
Ln	lymph node
LPS	lipopolysaccharide
mAb	monoclonal antibody
MACS	magnetic cell sorting
MSX	Methionine Sulphoximine
mRNA	messenger ribonucleic acid
NK	natural killer
OD	optical density
PBS	phosphate buffered saline
PBMC	peripheral blood mononuclear cell
PCR	polymerase chain reaction
PE	phycoerythirin
PMA	phorbol 12-myristate 13-acetate
RA	rheumatoid arthritis
RoR	Retinoic orphan receptor

RPMI	Roswell Park Memorial Institue
RT-PCR	reverse transcriptase polymerase chain reaction
STAT	signal transducer and activator of transcription
s.c	subcutaneous
SD	standard deviation
T-bet	T-box expressed in T cells
TCR	T-cell receptor
TGF-β	transforming growth factor-b
Th	T helper
TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TMB	3,3',5,5'-tetramethylbenzidine peroxidase
TNF	Tumor necrosis factor
Tr1	T regulatory T cell type 1
Treg	regulatory T cells
WT	wild-type
M	molar
mM	millimolar
mg	milligram
ml	millilitre
µg	microgram
µl	microliter
µM	micromolar
nM	nanomolar
pg	picogram

Publications

1. Niedbala, W. Cai, B. Wei, X. Patakas, A. Leung, B.P. McInnes, I.B. & Liew, F.Y. (2008) Interleukin-27 attenuates collagen-induced arthritis. *Ann Rheum Dis*, **67 (10)**, 1474-9.

2. Niedbala, W. Wei, X.Q. Cai, B. Hueber, A.J. Leung, B.P. McInnes, I.B. & Liew, F.Y. (2007) IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. *Eur J Immunol*, **37(11),** 3021-9.

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Declaration

The study presented in this thesis represents original work carried out by the author. Work in molecular biology was in collaboration with Dr Xiao-Qing Wei, and work in FACS analysis was in collaboration with Dr Wanda Niedbala. This thesis has not been submitted in any form to any other University. Where reagents, materials or practical support has been provided by others, due acknowledgement has been made.

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Chapter 1
General Introduction

1.1 T helper (Th) lymphocytes

1.1.1 Introduction

Inflammation plays an important role in the host defense against pathogenic infection, tissue repair and regulation of innate and adaptive immune responses. However, the skewed regulation of immune responses can result in chronic inflammation, such as autoimmune diseases and cancer. The autoimmune diseases such as rheumatoid arthritis (RA) are the consequence of the action of a diverse cell population that includes B cells, macrophages and T cells. The major proportion of the inflammatory cells in RA is T cells. Activated $CD4^+$ T cells are the dominant subset of T cells in initiation and perpetuation of the chronic inflammation prevalent in RA (Feldmann et al., 1996; Namekawa et al., 1998). Classically, upon activation, $CD4^+$ T cells have been divided into Th1 or Th2 effector cells (reviewed in Mosmann & Sad., 1996; Murphy & Reiner, 2002). It has been known for many years that Th1 and Th2 cells provide immune responses to intracellular and extracelluar pathogens respectively, depending on their signature cytokine production. IFN-γ is the signature cytokine of Th1 subset, whereas IL-4, IL-5 and IL-13 are secreted by Th2 subset. Recently, a new lineage of effector $CD4^+$ T cell preferentially produces IL-17 (Th17) has been discovered. Th17 subset is distinct from Th1 and Th2 subsets, and provides diverse effector functions during immune responses. The importance of Th17 responses has been demonstrated in varieties of chronic autoimmune diseases including RA, which had been formally regarded as Th1 diseases. Another critical subset of $CD4^+$ T cells is regulatory T cells (Treg). There are two major types of Treg cells, the naturally occurring $CD4^+$ $CD25^+$ Tregs originated from the thymus and the

inducible Tregs generated in the periphery. Treg cells have important functions in the regulation of autoimmune diseases. Since Th1 and Th2 cells are well established, the following parts will focus on Th17 cells and Treg cells.

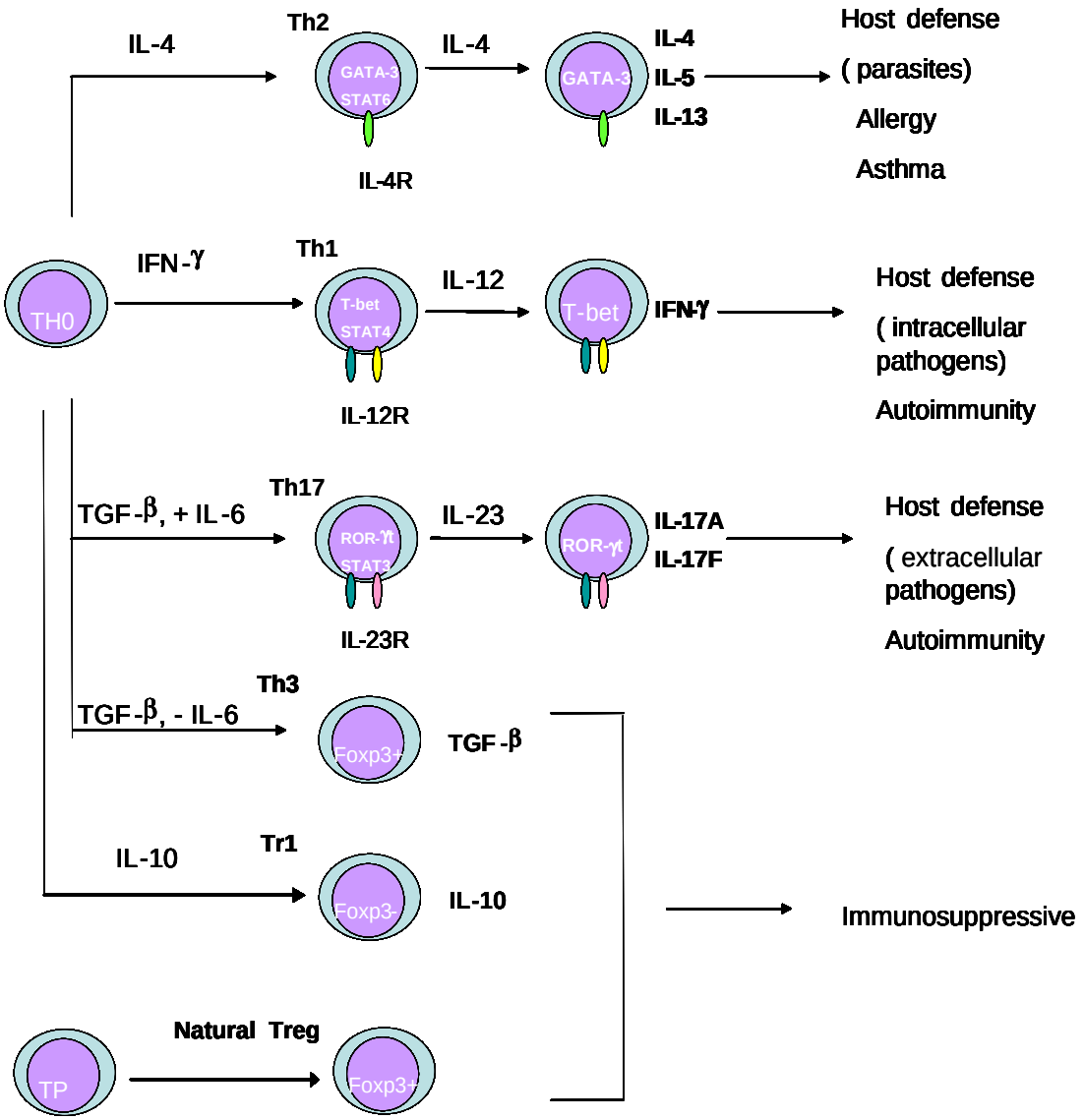


Figure 1.1 Differentiation of $CD4^+$ T cell lineages.

Naïve $CD4^+$ T cells can differentiate into three subsets of effector T cells (Th1, Th2, and Th17) and two subsets of Treg cells (Tr1 and Th3). Naturally occurring Treg cells are generated from $CD4^+$ thymic T cell precursors. The differentiation of these subsets is governed by selective cytokines and transcription factors, and each subset provides specialized functions. (Adapted from Bettelli et al., 2007)

1.1.2 Th2 cells

Th2 cells are characterised by the production of IL-4, IL-5, and IL-13, and these Th2 cytokines promote activation of innate cells and help B cells in IgG1 and IgE class switching (reviewed by Abbas, et al., 1996). The development of Th2 cells is initiated via TCR signalling acting in concert with IL-4 receptor through STAT6 (Paul, 1997). Signals derived from TCR and IL-4 receptor cooperatively upregulate the expression of GATA-3, a master regulator of Th2 differentiation (Zheng & Flavell, 1997). GATA-3 enhances its own expression, and drives epigenetic changes in the Th2 cytokine gene cluster (for IL-4, IL-5 and IL-13). Meanwhile, GATA-3 suppresses the dominant factors for Th1 polarization, including IL-12Rβ2 and STAT4 to block Th1 development (Ouyang et al., 1998). Furthermore, IL-4 is a negative regulator of Th17 effector cells (Harrington et al., 2005). Usually, the cytokines produced by a given Th lineage often act as potent self inducers of their own lineage, as well as inhibitors of other Th lineages. Thus, IL-4 produced by mature Th2 cells can reinforce Th2 development and inhibit Th1 and Th17 developments through positive and negative feedback loops.

Appropriate induction of Th2 effector cells promotes humoral immunity that is crucial for controlling infections by extracellular pathogens (Reiner, 2001). Furthermore, Th2 responses can down-regulate autoimmune diseases. It has been shown that IL-4 delays the onset of CIA, reduces the symptoms severity and prevents joints damage and bone erosion (Horsfall et al., 1997; Joosten et al., 1997). However, inappropriate action of Th2 cells can cause allergic reactions, such as asthma (Abbas, et al., 1996).

1.1.3 Th1 cells

In contrast to Th2 cells, Th1 cells came to be defined on the basis of their production of the signature cytokine, interferon (IFN)-γ. IFN-γ is important in the clearance of certain intracellular pathogens and promote IgG2a class switching in mice on B cells (reviewed in O' Garra, et al., 2000). The process of Th1 helper cells differentiation is initiated by TCR signalling in association with cytokine receptors via STAT1 (Gubler, et al., 1991; Murphy, et al., 2000). Type I IFNs, type II IFNs and the newly discovered IL-27 can activate STAT1, via their receptors on naïve $CD4^+$ T cells. STAT1 signalling sequentially up-regulates the expression of the transcription factor T-bet, a master regulator of Th1 differentiation. T-bet enhances the expression of IFN-γ and induces the IL-12 receptor β2 chain while suppressing GATA-3 and other Th2 differentiation factors (Kamiya, et al., 2004, Hibbert 2003). The induction of IL-12Rβ2 on developing Th1 cells allows IL-12 signaling via STAT4. This in turn leads to the maturation of Th1 effector cells that can produce IFN-γ. Furthermore, IL-12 induces IL-18 receptor, allowing IL-18 to synergize with IL-12 to enhance IFN-γ production from Th1 effector cells (Yoshimoto et al., 1998). It also has been revealed that IL-23 preferentially acts on the proliferation of memory T cells and the continuation of Th1 development (Oppmann, et al., 2000).

Appropriate induction of Th1 cells is required for effective cellular immunity to clear intracellular pathogens, such as *Leishmania genus, Mycobacteria* and *Salmonella*. Overactivation of Th1 cells have been thought to cause autoimmune inflammatory diseases, including RA, EAE, and certain allergic disorders, such

as delayed-type hypersensitivities (DTH) and contact hypersensitivity (Leonard et al., 1995; Kageyama et al., 1998; Doing & Flavell 2000). However, there is controversy over the role of Th1 cells in the onset phase of rheumatoid arthritis based on the observations that IFN-γ was not always highly expressed in the lesion (Husby et al., 1985; Kinne et al., 1997). Furthermore, mice deficient in IFN-γ or STAT1 were still susceptible to CIA and EAE (Ferber et al., 1996; Mattys 1998; Bettelli et al., 2004). CIA is exacerbated in mice lacking IFN-γ receptors (Manoury-Schwartz et al., 1997; Vermeire et al., 1997). These observations suggested that an additional Th cell type other than Th1 cells plays a more important role in these autoimmune diseases. This additional Th cell type was recently defined as Th17 cells.

1.1.4 Th17 cells

1.1.4.1 Introduction

Although the cytokine IL-17 was identified more than 10 years ago, Th17 cells as a new subset of $CD4^+$T cells was proposed from the recent observations on IL-23 and IL-12 deficient mice. It was demonstrated that IL-23 rather than IL-12 plays a critical role in the development of CIA and EAE (Cua et al., 2003; Murphy et al., 2003; Langrish et al., 2005; Chen et al., 2006). On gene expression profiles analysis, IL-23-cultured T cells preferentially express chronic inflammatory cytokines including IL-17, IL-6, and TNF-α, unlike that of IL-12-cultured Th1 cells. Furthermore, Langrish, et al. (2005) demonstrated that mice induced a more severe EAE upon the transfer of IL-17-producing $CD4^+$ T cells pre-cultured by IL-23, while mice did not induce severe EAE upon the transfer of Th1 cells.

Thus, IL-17-producing $CD4^+$ T cells are distinct from Th1 cells. It was also recognized that Th1 signaling (STAT1, T-Bet, STAT4) and Th2 signaling (STAT6) are not required by Th17 development (Harrington et al., 2005; Park et al., 2005) In contrast, IFN-γ and IL-4 actively suppress Th17 development, and neutralization of IFN-γ and IL-4 is important for Th17 development (Harrington et al., 2005; Park et al., 2005). Therefore, Th17 cells represent a distinct lineage of $CD4^+$ T cells.

1.1.4.2 Characteristics of Th17 cells

Th17 cells are characterized by their production of a distinct profile of cytokines, including IL-17 (or IL-17A) and IL-17F. Recently it has been shown that IL-21, a member of the common gamma chain family of cytokines, is secreted by Th17 cells in mouse (Korn et al., 2007; Nurieva et al., 2007). Instead of IL-21, human Th17 cells express IL-22 and IL-26; both belonging to IL-10 cytokine family (Acosta-Rodriguez et al., 2007; Wilson et al., 2007).

1.1.4.2.1 IL-17

IL-17, which was originally named as cytotoxic T lymphocyte-associated factor 8 (CTLA8), is a signature cytokine of Th17 lineage, first described as a gene product without clear functions by Rouvier et al in 1993. IL-17 gene is located in chromosome 6p12, and shows 58% homology with an open reading frame of the T lymphotrophic herpesvirus Saimiri gene 13. IL-17 is a disulfide-linked homodimeric glycoprotein consisting of 155 amino acids (Yao et al., 1995).

Human IL-17 displays close structural homology with mouse and rat IL-17, and they all have remarkably conserved glycosylation sites (Yao et al., 1995(2)). Besides being produced by Th17 cells, IL-17 is also expressed by $CD8^+$ T cells, γδ T cells, NK cells, eosinophils and neutrophils (summarized by Kawaguchi et al., 2004).

IL-17, also termed IL-17A, is a member of a newly identified cytokine family which comprises of six members. Five additional IL-17 cytokine family members have been identified and designated IL-17B, IL-17C, IL-17D, IL-17E (termed IL-25), and IL-17F. (Li et al., 2000; Lee et al., 2001; Hymowitz et al., 2001). The IL-17s are all expressed as homodimers with disulfide bridge link, except IL-17 B is expressed as a non-covalent dimer. IL-17 cytokine family members have a similar C-terminal region, with five well conserved cysteine residues accounting for a characteristic cysteine-knot formation. IL-17 family shows no resemblance with other known cytokines, thus it is a unique cytokine family. IL-17 B, C, D and E have low homology with IL-17A. In contrast, IL-17F has nearly 50% identity to IL-17A at the amino acid level (Moseley et al., 2003). *IL-17F* gene is located closely to *IL-17A* in all species (Moseley et al., 2003). Probably due to their close location, IL-17A and IL-17F are both expressed by Th17 cells.

Like other known cytokines, the biological activity of IL-17 is dependent on its receptors. There have been five IL-17 receptors described to date: IL-17RA, IL-17RB, IL-17RC, IL-17RD, and IL-17RE (Weaver et al., 2007). Despite extensive studies on IL-17, the studies of IL-17 receptor family have been limited. It has

been recently reported that IL-17 signaling requires a multimeric receptor complex consisting of IL-17RA and IL-17RC (Toy et al., 2006).

IL-17RA, the first identified member of the IL-17 receptor family, is a single-pass transmembrane receptor consisting of an extracellular amino acid domain and a long cytoplasmic tail (Yao et al., 1995). In contrast to relatively restricted expression of IL-17, IL -17RA has been found to be ubiquitously expressed at mRNA level in many cell types, including epithelial cells, neutrophils, fibroblasts, B and T lymphocytes, myelomonocytic cells, marrow stromal cells and vascular endothelial cells (Yao et al., 1995; Moseley et al., 2003). IL-17RA binds both IL-17 and IL-17F. However, in human, IL-17RA binds to IL-17 with 100-fold higher affinities than it binds to IL-17F (Hymowitz et al., 2001; Kuestner et al., 2007). IL-17RA deficient mice exhibit enhanced susceptibility to multiple infectious agents, which is consistent with the important role for IL-17A and IL-17F in host defence (Ye et al., 2001).

IL-17RC, also named IL-17RL, is another IL-17R family member and has recently been recognised as an essential component in IL-17-mediated signal transduction (Toy et al., 2006). IL-17 RC mRNA has been detected in human prostate cells, cartilage, heart, kidney, liver and muscle cells, and mouse tail fibroblast cells (Haudenschild et al., 2002; Moseley et al., 2003). It has been recently reported that human IL-17RC binds to IL-17A and IL-17F with similar high affinity, but m IL-17RC only binds m IL-17F (Kuestner et al., 2007). Like IL-17RA, IL-17RC is also a single-pass transmembrane receptor protein with a unique, long cytoplasmic tail (Haudenschild et al., 2002). IL-17RC shows 23%

homology with IL-17RA. However, IL-17RC has an alternative splice variant, which leads to over 90 splice isoforms of human IL-17RC. Some of the isoforms are secreted soluble proteins and they retain their ligand-binding properties (Haudenschild et al., 2002; 2006). It has been demonstrated that a soluble form of IL-17RC effectively inhibits the sigaling response of both IL-17A and IL-17F through blocking their bindings (Kuestner et al., 2007).

With co-immunoprecipitation, it was demonstrated that IL-17RA and IL-17RC have a physical interaction (Toy et al., 2006). IL-17RA was recently shown to be multimeric existing as a preformed complex in the absence of ligand binding, Upon binding to IL-17A and IL-17F, IL-17RA multimer complex undergo a major conformational alternation (Kramer et al., 2006).Therefore, it was suggested that this conformational alternation may induce a productive, heterotypic interaction with IL-17RC (Toy et al., 2006).

IL-17RA and IL-17RC contain a conserved cytoplasmic SEFIR (similar expression to fibroblast growth factor (FGF) genes, IL-17 receptors and Toll-IL-1R) domain (Novatchkova et al., 2003). The SEFIR domain provides an important interaction for downstream signalling molecules of IL-17. Initial studies indicated that IL-17 signaling may depend on the activation of mitogen-activated protein kinases and the transcription of NF-κB, two classical signalling pathways associated with proinflammatory signals (Shalom-Barak et al., 1998; Awane et al., 1999). However, the exact intermediate pathways between IL-17R and the downstream signals are still not fully elucidated. Recent studies suggested that the cytoplasmic adaptor protein Act1 is a crucial mediator of IL-

17 signalling (Chang et al., 2006). It seems that Act1 bridges IL-17R ligation by IL-17 and downstream signals. IL-17R-Act1-mediated signalling pathway is associated with IL-17- dependent autoimmune and inflammatory diseases in the intestine and central nervous system respectively (Qian et al., 2007).

IL-17, as an important inflammatory cytokine, acts on a large variety of cells, including epithelial cells, endothelial cells, fibroblasts, synoviocytes, and myeloid cells (Kolls et al., 2004). IL-17 induces the expression of proinflammatory cytokines, such as IL-6, TNF, IL-1β, GM-CSF, and CSF; chemokines, such as KC, MCP-1, and MCP-2, CXCL1, and IL-8; and matrix metalloproteases, which mediate tissue inflammation, tissue infiltration and destruction. Predominant cytokines and chemokines stimulated by IL-17 are especially involved in the proliferation, maturation, and chemotaxis of neutrophils, which features the coordination between innate and adaptive immunity (Kolls et al., 2004).

1.1.4.3 Cytokine Regulation of Th17 differentiation

It was initially thought that IL-23 induces the generation of Th17 cells. However, three independent groups simultaneously found that IL-23 failed to drive the differentiation of naïve $CD4^+$ T cells to Th17 cells in mice (Veldhoen et al., 2006; Mangan et al., 2006; Bettelli et al., 2006). This is consistent with the expression of IL-23R, as IL-23R is not expressed on naive T cells in mice (Parham et al., 2002). The further study suggested that IL-23 may provide a survival signal for already differentiated Th17 (Veldhoen et al., 2006). Subsequently, it has been

shown that TGF-β and IL-6 act cooperatively to induce the differentiation of Th17 commitment in mice. TGF-β, conventionally regarded as an anti-inflammatory agent, inhibits the differentiation of Th1 and Th2 cells (Gorelik et al., 2000; 2002). Previously it has been reported that TGF-β is an important factor in inducing Foxp3+ Treg cells *in vitro* (which will be further discussed in the following Treg cells section). During the generation of Th17 cells, TGF-β is also identified as a critical factor. The deviation is associated with the action of IL-6. TGF-β alone induces Foxp3+ Treg generation, while the presence of IL-6 directs TGF-β to drive Th17 cells generation rather than Treg cells (Bettelli et al., 2006). Furthermore, it has been reported that the combination of TGF-β and IL-6 up-regulates the expression of IL-23R on Th17 cells. Thereby, the expression of IL-23R allows IL-23 to amplify and stabilize the Th17 phenotype (Veldhoen et al., 2006).

Recent studies have revealed that IL-21 also can control the generation of Th17 cells in mice (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). They showed that IL-21, in combination with TGF-β, induces naïve $CD4^+$ T cells to produce IL-17. The ability of IL-21 to drive Th17 cells is independent of IL-6. Like IL-6, IL-21 also suppresses the induction of Foxp3+ Treg by TGF-β and upregulates the expression of IL-23R on Th17 cells accompanied by TGF-β. The combination of IL-6 and TGF-β can induce Th17 cells to produce IL-21, and then IL-21 with TGF-β can also induce Th17 cells themselves to produce IL-21, which shows like an autocrine loop to amplify Th17 response in mice (Zhou et al., 2007).

Several other cytokines have been shown to regulate the development or proliferation of Th-17 cells. IL-1 and TNF may play a role in Th17 cells expansion *in vitro* (Veldhoen et al., 2006). Neutralization of IFN-γ and IL-4 increases the expansion of Th17 cells driven by IL-23. In addition, type I and II IFNs, IL-12, and IL-27(which will be discussed in the following section) can suppress Th17 differentiation (Harrington et al., 2005; Murphy et al., 2003; Batten et al., 2006). IL-2, a cytokine important for growth and survival of Th1 and Th2 cells, has been found to strongly inhibit Th17 development in mice (Laurence et al., 2007).

Surprisingly, the generation of Th17 cells in human is different from that in mice. Despite the critical role of TGF-β in Th17 commitment in mice, human Th17 differentiation does not require TGF-β. In fact, TGF-β inhibits the production of IL-17 in human (Acosta-Rodriguez et al., 2007; Wilson et al., 2007; Chen et al., 2007). Interestingly, these studies have shown that IL-1β is a chief factor in induction of Th17 cells, and IL-6 acts to enhance IL-1β-induced Th17 differentiation (Acosta-Rodriguez et al., 2007; Wilson et al., 2007). However, there is debate about IL-23. The studies from Wilson et al (2007) and Chen et al (2007) showed that IL-23 is an effective inducer of human Th17 cells. Acosta-Rodriguez et al (2007) demonstrated that IL-23 is more likely to expand Th17 cells. Moreover, it has been shown that IL-2 plays a dual role in the regulation of human Th17 cells. Large doses of IL-2 at the early stage of Th17 differentiation can downregulate Th17 differentiation, whereas differentiated Th17 cells can be expanded by IL-2 (Acosta-Rodriguez et al., 2007; Wilson et al., 2007; Amadi-Obi et al., 2007). In addition, human memory $CD4^+$ T cells can rapidly express

high levels of IL-17 upon anti-CD3/anti-CD28 stimulation alone, while human naïve $CD4^+$ express low levels of IL-17 (Chen et al., 2007).

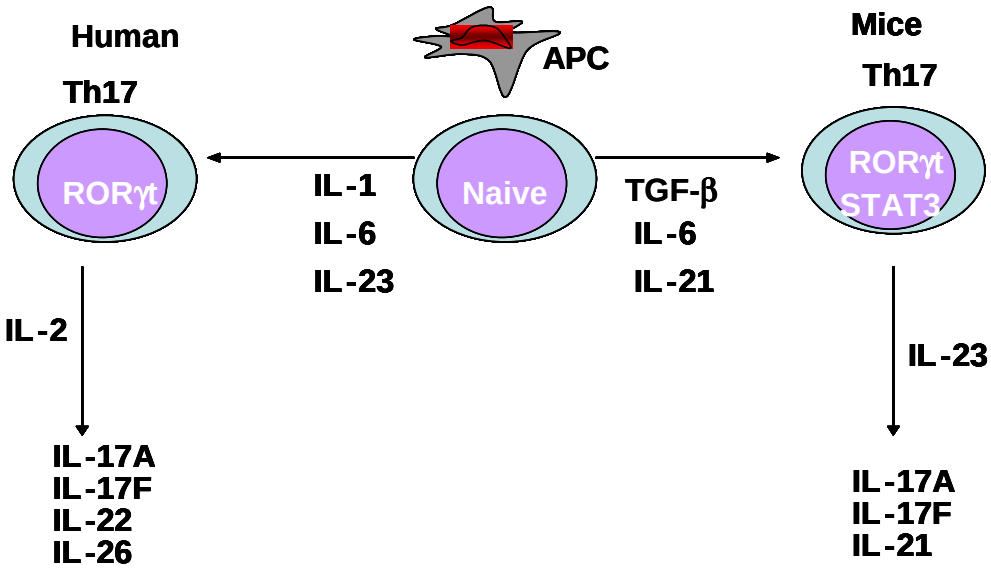


Figure 1.2 Th17 lineage commitments in humans versus mice. (Adapted from Laurence & J O'Shea, 2007)

1.1.4.4 Effect of Transcription factors on Th17 differentiation

1.1.4.4.1 STAT3

The transcription factors STATs family are key factors during Th effectors differentiation. As mentioned before, Th1 differentiation is through the activation of STAT1 and STAT4. On the other hand, Th2 differentiation is via selective STAT6 activation. It has been shown that Th17 differentiation is unimpaired in STAT4 or STAT6 deficient mice, which suggested that these transcription factors are not involved in the differentiation of Th17 lineage ((Harrington et al 2005; Park et al., 2005). Thus, Th17 differentiation is mediated by different transcription factors.

Recent studies have demonstrated that STAT3 is the essential regulator to mediate Th17 differentiation in mice (Yang et al., 2007; Nishihara et al., 2007). It has been observed that Th17 generation is greatly impaired in STAT3 deficient cells (Yang et al., 2006). Upon TCR stimulation, IL-6 and TGF-β rapidly activate STAT3 to direct the development of Th17 cells (Nishihara et al., 2007). It also has been found that IL-21 drives IL-17 production in a STAT3-dependent pathway (Wei et al., 2007). Furthermore, IL-6 and IL-21 can upregulate expression of the IL-23R through STAT3 signaling (Nurieva et al., 2007; Zhou et al., 2007).

In contrast to STAT3, STAT1 appears to be a negative regulator of Th17 generation (Harrington et al 2005; Kimura et al., 2007). Similarly, STAT5

activated by IL-2 inhibits Th17 cell differentiation in mice (Laurence et al., 2007). The STATs family in human Th17 cell development has not been clearly elucidated.

1.1.4.4.2 RORγt and RORα

It has been identified that every lineage of T cells has a special key transcription factor, like T-bet for Th1 development, GATA-3 for Th2 development, and Foxp3 for Treg development. The study by Ivanov and his colleages (2006) has identified the orphan nuclear receptor, retinoic acid-related orphan receptor (ROR) γt, as a transcription factor for Th17 lineage differentiation. Recently, Yang group (2008) has found that RORα is another specific transcription factor to direct Th17 differentiation.

RORγt is a specific isoform of RORγ that belongs to the retinoic acid-related orphan nuclear hormone receptor family. RORγt and RORγ are both encoded by Rorc locus and differ only in their terminal sequences through the utilization of different promoters (Dzhagalov, et al 2004). RORγ is broadly expressed, while RORγt is predominantly expressed in populations of intestinal lamina propria T lymphocytes, and many of these cells constitutively express IL-17 (Ivanov et al., 2006). In contrast, these expressing IL-17+ T cells are absent in RORγt deficient mice. These data proposed a strong correlation between RORγt and IL-17. *In vitro*, under Th17-polarizing conditions, RORγt-deficient T cells showed impaired Th17 development compared with wild-type control T cells. Importantly, the RORγt-deficient mice produce normal (or slightly higher) IFN-γ

under the Th1-polarizing conditions. Forced retrovirus-mediated expression of RORγt in naïve $CD4^+$ T cells resulted in the increased expression of IL-17 (Ivanov et al., 2006). This strongly indicated that the differentiation of Th17 cells requires the induction of RORγt. Murine $CD4^+$ T cells cultured with IL-6 and TGF-β express RORγt to become Th17 cells. It has also been reported that IL-21 with TGF-β induces the expression of RORγt (Zhou et al., 2007). These data together established that RORγt plays an important role in Th17 differentiation. In addition, it has been found that Retinoic Acid, Vitamin A metabolite, can reduce the expression of RORγt induced by TGF-β and inhibit the differentiation of Th17 cells (Mucida et al., 2007; Elias et al., 2008). In human, the combination of IL-6 and IL-1β promotes the expression of RORγt (Acosta-Rodriguez et al., 2007). Some studies also showed that IL-23 increases RORγt expression (Chen et al., 2007).

Another member of the ROR family, RORα has been found to be preferentially expressed in Th17 cells, but not in Th1 and Th2 cells (Yang et al., 2008). The combination of IL-6 and TGF-β are strong inducers of RORα in $CD4^+$ T cells. Overexpression of RORα could promote Th17 differentiation and upregulate IL-17 and IL-17F expression. Furthermore, coexpression of RORα and RORγt synergistically result in the increased expression of IL-17, IL-17F and IL-23R (Yang et al., 2008). Mice doubly deficient in RORα and RORγt function were profoundly impaired in Th17 generation and completely protected against EAE. In contrast, mice deficient only in RORα or RORγt developed moderate but less

severe diseases compared to wild-type mice (Yang et al., 2008). Therefore, RORα and RORγt together direct Th17 lineage differentiation.

1.1.4.4.3 Interferon-regulatory factor 4 (IRF4)

Interferon-regulatory factor 4 (IRF4) is a member of mammalian transcription factors that participate in the production of type 1 interferon, Toll-like receptor signalling and the differentiation of T helper cells (Negishi et al., 2005; Honda & Taniguchi 2006). IRF4 was originally known as a key inducer of GATA-3 and played an essential role in Th2 development (Hu et al., 2002; Rengarajan et al., 2002). Recently, a study has identified that the development of Th17 cells requires IRF4 (Brustle et al., 2007). *In vitro* under the Th17 polarization condition, T cells from IRF4 deficient mice fail to produce IL-17. *In vivo*, IRF4 deficient mice are completely protected from EAE. Notably, IRF4 deficient mice are far more resistant to this disease than those deficient in RORγt. Interestingly, further investigations found that IRF4 deficient mice showed a defective IL-6 response that was only confined to Th17 development. IL-6 decreases the ability to upregulate the expression of RORγt and downregulate the expression of Foxp3 for IRF4-deficient T helper cells in Th17 condition. Collectively, IRF4 is essential for Th17 development.

1.1.4.5 Role of Th17 in inflammation and autoimmune diseases

Th17 cells have been implicated to play an important role in host defence. IL-17 and IL-17F stimulate cytokines and chemokines, such as G-CSF and IL-8, to

generate neutrophils and attract these cells to the sites of inflammation, which might provide the early defence against trauma that would lead to tissue necrosis or sepsis (Mckenzie et al., 2006). IL-17 is also involved in protection against several bacteria, such as *Klebsiella pneumoniae* (Happel et al., 2005), *Streptococcus pneumoniae* (Malley et al., 2006) and *bacteroides species* (Chung 2003). Th17 cells also are important in protective response against fungus and yeast infection (Huang et al., 2004; Hohl et al., 2006). In addition, Th17 cells were shown to be indispensable against other pathogens, including *Mycobacterium tuberculosis* (Khader et al., 2007) or *Borrelia burgdorferi* (Burchill et al., 2003).

Apart from the beneficial role of Th17 in protection against microbes, dysregulated function of Th17 cells is clearly associated with chronic inflammatory diseases. The overexpression of IL-17 has been detected in sera and target tissues in various human autoimmune diseases, including multiple sclerosis (Matusevicius et al., 1999; Lock et al., 2002), systemic lupus erythematosus, inflammatory bowel diseases, asthma and rheumatoid arthritis (summarized by Bettelli et al., 2007). Consistent with those observations, Th17 cells play a pivotal role in murine autoimmune diseases models, including EAE, colitis, and CIA (Nakae et al., 2003; Langrish et al., 2005).

1.1.4. 6 Th17 in Rheumatoid Arthritis

RA is a common systemic autoimmune disease with a prevalence of approximately 1% of the population. The clinical symptoms are characterized by

chronic inflammation and cellular proliferation within the synovial lining of joints which ultimately result in erosive destruction of cartilage and underlying bones. Although there are various studies done in RA, the aetiology and pathogenesis of the diseases remain poorly understood. Although macrophages, B cells, mast cells, DCs, and fibroblast-like synoviocytes are involved in RA, the role of T cells infiltrating the synovium in RA has been emphasized.

RA was previously considered a Th1 mediated autoimmune disease because of a relative predominance of Th1 cells and IFN-γ in RA joints (Miltenburg et al., 1992; Dolhain et al., 1996; Smolen et al., 1996). The consequence of Th1 predominant response has been shown to contribute to induction and progression of chronicity. However, recent studies demonstrated that Th17 but not Th1 cells played a predominant pathogenic role in RA. Elevated levels of IL-17 expression have been detected in the synovial fluid from most RA patients (Kotake et al., 1999; Hwang et al., 2004; Hwang & Kim 2005). Th17 cells were found in T cell rich area of synovial tissue in RA patients (Chabaud et al., 1999). Then, T cell clones developed from RA patients were found to release IL-17 (Aarak et al., 1999). Furthermore, it has been reported that human RA T cells secreted IL-17 in response to IL-15, which can be found at a high level in the inflamed synovium (Ziolkowska et al., 2000).

Th17 promotes inflammation through enhancing the expression of TNFα and IL-1β by human macrophages (Jovanovic et al., 1998). TNFα and IL-1β have been demonstrated to be important mediators of RA. IL-17 has synergistic effects on TNFα on inducing IL-1, IL-6, and IL-8 synthesis in the synovial fibroblasts

(Katz et al., 2001). IL-17 also enhances the IL-1-induced synthesis of IL-6, leukaemia inhibitory factor (LIF) and MIP-3a by RA synoviocytes (Chabaud et al., 1998). IL-6 and LIF may contribute to cartilage destruction, while MIP-3a may recruit dendritic cells and T cells. IL-17 has a direct role in joint inflammation, cartilage damage, and bone erosion (Koenders et al., 2006). IL-17 stimulates IL-6 and IL-8 secretion in fibroblasts, endothelial and epithelial cells (Fossiez et al., 1996). IL-17 also induces CXC chemokines in the inflamed joint, which may attract neutrophils. Futhermore, IL-17 reduces proteoglycan synthesis and enhances collagen destruction in bone explants (Lubberts et al., 2000). In addition, IL-17 induces RANKL expression, which is essential for osteoclastogenesis and bone resorption (Kotake et al., 1999). Recently, it was found that Th17 cells express membrane bound RANKL and stimulate osteoclastogenesis (Sato et al, 2006). These observations mplicated that Th17 cells possibly play an important role in the bone destruction phase of autoimmune arthritis.

Further evidence of Th17 involvement in the pathogenesis of RA came from animal studies. It has been shown that intraarticular injection of IL-17 in mice resulted in joint inflammation and cartilage destruction (Chabaud et al., 2001). Similarly, systemic as well as local overexpression of IL-17 via an adenoviral vector in the course of CIA was shown to accelerate synovial inflammation and joint destruction (Lubberts et al., 2002). In contrast, IL-17 deficient mice are resistant to CIA development (Nakae et al., 2003). It has also been reported that neutralization of endogenous IL-17 during the initial phase of arthritis suppressed the onset of experimental arthritis (Lubberts et al., 2001). Moreover,

neutralization of IL-17 in the established CIA can decrease the severity of CIA (Lubberts et al., 2004). These results suggested that Th17 is a potential novel target for the treatment of destructive arthritis.

1.1.5 Regulatory T cells

1.1.5.1 Introduction

The immune system is tightly regulated in response to self and non-self antigens. The fundamental mechanism of self-tolerance and how autoimmune diseases occur became a growing interest with the discovery of regulatory T cells. In 1995, Sakaguchi et al. identified and characterized a naturally occurring subset of $CD4^+CD25^+$ regulatory T cells, which are found to suppress the activation and expansion of self-active T cells, thereby controlling the development of autoimmune diseases. In contrast with naturally occurring $CD4^+CD25^+$ regulatory T cells, inducible $CD4^+$ Treg cells developed from conventional $CD4^+CD25^-$ T cells were identified. There are two well described types of inducible $CD4^+$ Treg cells: IL-10-secreting T regulatory type 1 cells (Tr1) and TGF-β–secreting T helper type 3 cells (Th3) (Groux et al., 1997; Chen et al., 2003). Tr1 cells were shown to be effective at controlling chronic intestinal inflammation, and Th3 cells were shown to maintain the tolerance of dietary antigen. Naturally Treg cells, Tr1 cells, and Th3 cells may cooperate to prevent autoimmune diseases *in vivo*.

1.1.5.2 Naturally $CD4^+CD25^+$ regulatory T cells (nTreg)

nTreg cells are produced in the thymus during ontogeny and constitute 5-10% of the total peripheral $CD4^+$ T cell population in normal human and mouse. Disruption of the thymic maturation or peripheral maintenance of nTreg cells results in severe autoimmune diseases (reviewed in Sakaguchi et al., 2004). nTreg cells are capable of suppressing a wide variety of immune cells from both the innate and adaptive immune systems, including effector $CD4^+CD25^-$ T cells (reviewed in Mills 2004; Von Boehmer 2005), $CD8^+$ T cells (Trzonkowski et al., 2004), NK cells (Azuma et al., 2003; reviewed in Ralainirina et al., 2007), B cells (Lim et al., 2005), or macrophages (Taams et al., 2005).

nTreg cells are anergic, and they poorly proliferate and produce cytokines in response to TCR stimulation. However, this anergy can be reversed by the addition of IL-2 (Fontenot et al., 2005). nTreg cells proliferate in the presence of higher than physiologically normal concentration of IL-2, and high concentration of IL-2 also abrogates the suppressive capacity of nTreg cells on the proliferation of effector $CD4^+CD25^-$ T cells *in vitro*. In contrast, the population of nTreg cells in the peripheral circulation is reduced in IL-2, IL-2Rα, or IL-2Rβ deficient mice (Papiernik et al., 1998; Furtado et al., 2002; Almeida et al., 2002; Bayer et al., 2005). These deficient mice died early due to severe lymphoproliferation and autoimmune diseases (Sadlack et al., 1993; Suzuki et al., 1995; Willerford et al., 1995). Correction or restoration of the production of nTreg cells can prevent lethal autoimmunity in these deficient mice (Wolf et al., 2001; Malek et al.,

2002). These results suggest that IL-2/IL-2R pathway is important in the development, expansion, and function of nTreg cells *in vivo*.

Various studies have been concentrated on identifying specific markers exclusively expressed by nTreg cells. Although no definitive markers have yet to be identified to date, the nTreg cells are currently characterized by the constitutive expression of several markers. The most widely recognized and useful marker is CD25, a component of the IL-2 receptor. CD25 is a highly specific marker constitutively expressed on the surface of nTreg cells. Depletion of $CD4^+CD25^+$ T cells results in spontaneous development of various autoimmune diseases, and reconstitution of normal $CD4^+CD25^+$ T cells prevents these disorders (Sakaguchi et al., 1995; Singh et al., 2001). Although CD25 is also expressed on activated T cells, the induction of CD25 on $CD4^+CD25^-$ T cells appears to have no suppressive function (Shevach et al., 2000). Thus, CD25 is a useful marker for separating nTreg cells from naïve $CD4^+CD25^-$ T cells. Besides CD25, there are other cell surface markers found on nTreg cells, including cytotoxic T lymphocyte-associated antigen (CTLA-4), and glucocortioid-induced tumor necrosis factor receptor family related gene (GITR), CD40, CD28 and OX40 (Reviewed in Yong et al., 2007). Furthermore, there are increasingly more findings on new molecule markers, like lymphocyte activation gene-3 (LAG-3), and programmed death receptor 1(reviewed in Liu & Leung 2006). These markers all may play a role in nTreg cells –mediated suppression.

The key finding that elucidates nTreg cells as a distinct lineage from other T cells is the specific transcription factor Foxp3, a member of the forkhead/winged-helix

family. The gene *Foxp3* was identified from a lethal X-linked lymphoproliferative disorder of the scurfy mouse strain and human disorder IPEX syndrome (immune dysregulation, polyendocrinopathy, X-linked syndrome), in which *Foxp3* gene is mutated (Lyon et al., 1990; Fontenot et al., 2003; Bennett et al., 2001). Foxp3 is required for the function of nTreg cells (Fontenot et al., 2003). Foxp3 is predominantly expressed within the $CD4^+CD25^+$ T cells population in both thymus and periphery, whereas activated $CD4^+CD25^-$ T cells, Th1 cells, Th2 cells, and Th17 cells do not express Foxp3 (Yagi et al., 2004; Sakaguchi et al., 2006). Forced expression of Foxp3 can convert $CD4^+CD25^-$ T cells to $CD4^+CD25^+$ T cells with a similar phenotype and function of nTreg cells (Fontenot et al., 2003; Hori et al., 2003). For example, Foxp3 transduction induces the expression of CD25, CTLA-4, GITR, and CD103, which are comparable with those found in nTreg cells (Hori et al., 2003). Foxp3-transduced $CD4^+CD25^-$ T cells are able to suppress the proliferation of other responder T cells *in vitro* and inhibit the development of autoimmune diseases *in vivo* (Hori et al., 2003). It has been shown that Foxp3 is an indispensable factor for the development of nTreg cells. Analysis of Foxp3-transgenic mice revealed the increased number of $CD4^+CD25^+$ T cells (Khattri et al., 2003). On the contrary, Foxp3-deficient mice exhibited reduced number of $CD4^+CD25^+$ T cells (Fontenot et al., 2003). However, a recent study has reported that only the function of nTreg cells is required for the expression of functional Foxp3 protein, but the expression of Foxp3 is not indispensable for nTreg cells development (Lin et al., 2007).

1.1.5.3 Tr1 cells

The definition of Tr1 cells refers to the IL-10 producing $CD4^+$ T cells population with regulatory function (Groux et al., 1997; Asseman et al., 1999). Tr1 cells are inducible cells, and they can be generated from naïve precursors stimulated with antigen in the presence of IL-10. It has also been reported that Tr1 cells can be generated from fully differentiated Th1 and Th2 cells under specific repeated stimulation (Hawrylowicz et al., 2005). Upon activation, Tr1 cells produce high levels of IL-10, moderate amounts of IL-5, low levels of IFN-γ and IL-2, but no detectable IL-4 levels (Wu et al., 2007). In some studies, Tr1 cells have also been shown to produce TGF-β. In contrast to nTreg cells, Tr1 cells do not express Foxp3 (Wakkach et al., 2003; Vieira et al., 2004). Tr1 cells can suppress the proliferation and cytokine production of naïve $CD4^+CD25^-$ T cells, Th1 and Th2 cells (Roncarolo et al., 2001). This regulatory activity of Tr1 cells can be reversed by neutralizing anti-IL-10 antibody or/and anti-TGF-β antibody, which indicated that Tr1-mediated suppression is mainly through the secretion of IL-10.

1.1.5.4 Th3 cells

Th3 cells are characterized by TGF-β. Th3 cells can be induced from naïve precursors under the influence of TGF-β (Chen et al., 2003; Fantini et al., 2004). In an autoregulatory loop, induced Th3 can trigger the secretion of TGF-β. Like nTreg cells, the expression of Foxp3 and CD25 is upregulated on induced Th3 cells (reviewed in Weiner 2001). Th3 cells can suppress both Th1 and Th2 cells

responses, and the suppressive mechanism is mainly dependent on the production of TGF-β (Fantini et al., 2004).

However, TGF-β is a critical factor for Th17 development. *In vitro* studies have shown that the addition of IL-6 suppressed TGF-β induced Th3 cells, while reciprocally promoting the generation of Th17 cells (Bettelli et al., 2006). Therefore, the cytokine environment may control the development of Th3 cells and Th17 cell, thereby balancing the host defence and autoimmunity.

1.1.5.5 Role of Regulatory T cells in rheumatoid arthritis (RA)

Regulatory T cells play a critical role in self-tolerance and autoimmune diseases. Autoimmune disorders are characterized by a breakdown in the mechanisms of tolerance to self-antigens. It has been shown that depletion of regulatory T cells in murine models leads to the spontaneous development of autoimmune diseases, such as colitis, gastritis, insulin-dependent autoimmune diabetes, and thyroiditis (Sakaguchi et al., 1995; Groux et al., 1997; McHugh & Shevach 2002; Mottet et al., 2003). In contrast, $CD4^{+}CD25^{+}$ T cells could prevent or ameliorate the development of experimentally induced autoimmune diseases (Sakaguchi et al., 1995; Kohm et al., 2002; Dipaolo et al., 2005). Human studies have shown that patients with autoimmune diseases have lower regulatory T cells counts or defective regulatory T cells (reviewed in Lan et al., 2005).

The role of regulatory T cells in RA has been analyzed in a series of reports using animal models of induced arthritis (Morgan et al., 2003; 2004; 2005; Frey et al.,

2005; Loughry et al., 2005; Kelchtermans et al., 2005; Nguyen et al., 2007). In all the cases, depletion of $CD4^+CD25^+$ T cells resulted in significantly increased incidence of arthritis diseases, worse severity such as paw swelling, and increased antigen (CII)-specific proliferation of splenocytes *in vitro*. These effects were reversed by adoptively transferring $CD4^+CD25^+$ T cells isolated from syngeneic naïve mice (Morgan et al., 2005; Frey et al., 2005). However, there is controversy in human RA studies. One study has shown that there were no significant difference in the frequency of regulatory T cells, between RA patients and normal controls (Ehrenstein et al., 2004). In contrast, another study has shown that the percentage of $CD4^+CD25^+$ T cells in the synovial fluid of RA patients was significantly higher than that of normal controls (Van Amelsfort et al., 2004). Furthermore, it appears that the number of regulatory T cells present in the inflamed synovial tissue of patients with RA is more than the number of regulatory T cells in their peripheral circulation (Cao et al., 2004). It has also been found that synovial regulatory T cells have the capacity to suppress the proliferation of autologous $CD4^+CD25^-$ responder T cells (Cao et al., 2004; Van Amelsfort et al., 2004). Of interest, another study has shown that $CD4^+$CD25high T cells isolated from patients with active RA, although anergic, were unable to regulate pro-inflammatory cytokines released by effector T cells and monocytes *in vitro*, when compared to cells isolated from healthy individuals (Ehrenstein et al., 2004).

1.2 Murine Collagen-Induced Arthritis (CIA) as a model for RA

Animal models of RA have provided basic pathogenesis of the disease as well as provided valuable research tools for testing new therapies. Among the variety of models reported, Collagen-induced arthritis (CIA) is the most commonly studied autoimmune models of RA. CIA shares many pathological and clinical characteristics of human RA. Like RA, CIA is characterized by mononuclear cell infiltration, synovial hyperplasia, and cartilage degradation.

CIA was first observed in rats following the immunization with type II collagen (Trentham et al., 1977), and further studies have found that a similar pathology could also be induced in primates and susceptible strains of mice (Courtenay et al., 1980). Of the susceptible strains of mice, DBA/1 mice are the standard mouse strain. The susceptibility of DBA/ 1 mouse to CIA is associated with specific major histocompatiblity complex (MHC) class II genes, especially H-2^q. Interestingly, male mice are more susceptible to CIA than female mice, which may be partly related to sex hormone. Another factor affecting the development of CIA is the age of mice. Mice are not susceptible to CIA until they reach 6-7 weeks old, and they become increasingly susceptible to CIA with the increasing age (Holmdahl et al., 2002).

CIA is induced in DBA/1 mice after the immunization of heterologous type II collagen (C II) with complete Freund's adjuvant (CFA). CII is a major protein in cartilage. Although the precise mechanisms how collagen II in CFA leads to chronic arthritis are not known, the development of CIA is dependent on both cellular and humoral responses in response to collagen II (Holmdahl et al., 2002).

Thus, both B cells and T cells play important roles in CIA. The major role of B cells is to produce anti-CII antibodies, which reacts with CII in cartilage and induces arthritis (Terato et al., 1992). The role of T cells is to help B cells produce anti-CII antibodies (Corthay et al., 1999). Also, T cells themselves probably play a role in joint inflammation. Furthermore, various cytokines are found to play crucial roles in the pathogenesis through the activation of immune system (Feldmann et al., 1996).

Following immunization, a moderate inflammatory reaction started at the injection site and lasted 1-2 weeks. The first signs of arthritis development in DBA/1 mice are visible around day 21 after immunization, and the disease became progressively worse for approximately 2-4 weeks before the slow resolution phase (Holmdahl et al., 2002).

Although CIA is widely accepted as an experimental model for RA, there are some differences between CIA and human RA. For example, mycobacteria used in CIA results in a dominant type I immune response. However, it is unknown whether RA is induced by an exogenous pathogen or a spontaneous autoimmune disease (Holmdahl et al., 2002).

1.3 IL-12 cytokine family

1.3.1 Introduction

Cytokines are small, secreted proteins released by many cells that can regulate an array of cellular processes, including altering the behaviour or properties of the cells and mediating the immune response to infection or injury (Nicola 1994). Cytokine environment present at the earliest stage of T cell priming can control the Th cell differentiation program. IL-4 appears to be a predominant early factor for determining the development of Th2 effector cells (Paul, 1997). In contrast, the dominant factor that drives the development of Th1 helper cells was identified as IL-12 (Murphy, et al., 2000). For many years, IL-12 was the only known heterodimeric cytokine before the discovery of IL-23. Later, on the basis of the advence of genomics and the development of bioinformatics, IL-27 and IL-35 (EBI3/p35) have been identified. IL-12 family cytokines started to receive attention.

Cytokines play a central role in regulating T cell expansion and differentiation. Cytokines also organize the balance between protection and immunopathology after infection. The etiology of RA is unknown, but proinflammatory cytokines play an important role in the pathogenesis of RA. Neutralizing Abs against the proinflammatory cytokines, including TNF-α, IL-1 and IL-6, have been shown to successfully suppress the joint inflammation (Kagari et al., 2002). Therefore, an understanding of the regulation of new cytokines may lead to novel therapeutic strategies. Initially, it was found that IL-12 played an important role in autoimmune diseases. Recently, IL-23 has been found to be more important than

IL-12 in the progression of T-cell dependent autoimmune diseases. However, the function of IL-27 and IL-35 remained unclear when I started the project. I was interested in how IL-27 and IL-35 can potentially regulate the autoinflammatory diseases, particularly in the CIA model.

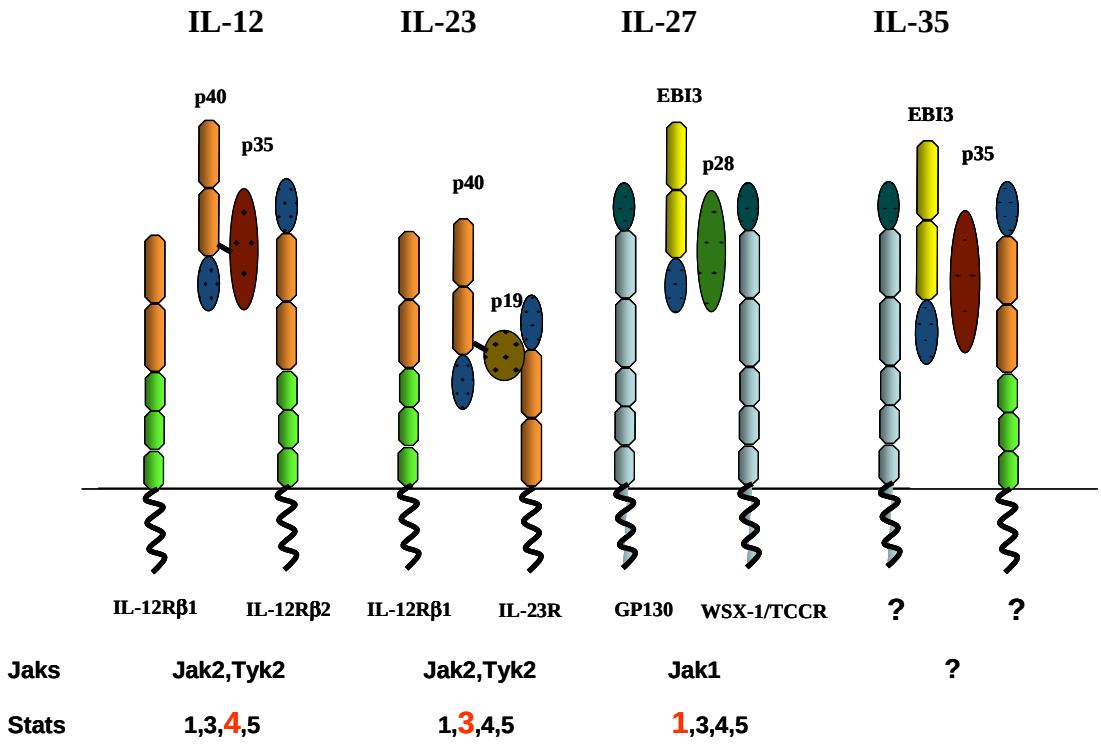


Figure 1.3 The structures of IL-12 family members and their receptors.

1.3.2 IL-12

IL-12 is the major cytokine that regulates the differentiation of naïve $CD4^+$ T cells to mature Th1 effector cells. IL-12 is secreted by a variety of cells, but most importantly by antigen presenting cells such as monocytes, macrophages, dendritic cells (DCs) and neutrophils. Numerous pathogenic organisms induce IL-12, including bacteria, parasites, viruses, and fungi (Ma & Trinchieri, 2001).

IL-12 is a heterodimeric 70-kDa glycoprotein composed of two disulfide-linked subunits designated p40 and p35. p35 shares homology with IL-6, granulocyte-specific colony-stimulating factor (GCSF), and is similar to other cytokines in the same class (Merberg, et al., 1992). p40 is homologous to the extracellular domain of the subunit of IL-6 receptor (Gearing & Coasman, 1991). The gene encoding p35 maps to chromosome 3 in human and chromosome 6 in mice, whereas the gene encoding p40 is located on chromosome 5 in human and chromosome 11 in mice (Sieburth, et al., 1992; Holscher, 2004). Therefore, the expression of the two genes is regulated independently. The p35 subunit is expressed in many cell types at constitutively low levels, while the p40 subunit expression is more limited but with higher levels (Agnello, et al., 2003). Also, free p35 is not secreted on its own, but p40 can be secreted with free monomer or homodimer ($p40_2$) (Watford, et al., 2003). The IL-12p40 homodimer shows antagonistic activity to IL-12, but does not mediate biological responses (Merberg, et al., 1992). The bioactive IL-12p70 is generated only when the two subunits are coexpressed in one cell (Gubler, et al., 1991).

The activities of IL-12 are mediated by a high-affinity receptor (IL-12R) consisting of two receptor subunits, β1 and β2. Both β1 and β2 are structurally related to the class I cytokine receptor superfamily and have strong homology with glycoprotein gp130 and leukaemia-inhibitory factor receptor (LIFR) (Presky, et al., 1996; Chua et al., 1994). It has been identified that IL-12 receptor subunits β1 and β2 have differing affinities for IL-12 in humans and mice. In humans, the individual binding affinity of IL-12Rβ1 and IL-12Rβ2 for IL-12 is low, however, the co-expression of β1 and β2 generate high-affinity binding of IL-12. In contrast, IL-12Rβ1 in mice mediates both high and low affinity binding of IL-12 and IL-12Rβ2 only has limited capacity for binding to IL-12 (Presky, et al., 1996).

The IL-12R β1 and β2 complex is expressed on activated T cells, NK cells, and DCs. Resting T cells do not express β1 or β2, but the expression of β1 and β2 are induced upon T cell activation. Following differentiation, Th1 cells express β1 and β2 subunits, but Th2 cells only express β1, not β2 (Rogge, et al., 1999). Therefore, IL-12Rβ2 expression is highly controlled on Th1 cells, and the control of IL-12Rβ2 expression is thought to regulate IL-12 response and Th1 phenotype. IFN-γ induces the expression of T-bet in Th1 cells, which in turn up-regulates IL-12R β2 expression. Whereas, IL-4 downregulates the expression of IL-12R β2 (Szabo, et al., 1997; Afkarian, et al., 2002).

The biological activity of IL-12 requires the interaction of IL-12p40 with IL-12Rβ1 and the interaction of IL-12p35 with IL-12Rβ2. The interaction of IL-12

with the IL-12 receptor complex initiates the signalling pathway through tyrosine phosphorylation of the Janus Kinase (JAKs), TYK2 and JAK2 and the activation of STATs, 1,3, 4 and 5 (Bacon, et al., 1995). However, STAT4 is the major specific mediator for IL-12 signalling (Bacon, et al., 1995; Kaplan, et al., 1996). IL-12Rβ2 subunit binds to STAT-4 to initiate IL-12 signal transduction. In contrast, IL-12Rβ1 has no STAT4 binding site and no reported signalling ability, but it is responsible for ligand binding (Wu, et al., 2000).

IL-12 is an especially important factor that plays a central role in regulating innate and adaptive immune response (reviewed in Trinchieri 2003). It acts on naïve $CD4^+$ T cells to induce their proliferation and differentiation to Th1 cells, which produce IFN-γ and promote adaptive immune responses. In contrast, IL-12 combination with IFN-γ antagonizes Th2 differentiation and downregulates the production of IL-4, IL-5, and IL-13 (Watford, et al., 2003). IL-12 induces IFN- γ production in T and NK cells (Trinchieri 1994). The production of IFN- γ stimulates bactericidal activity to phagocytic cells and enhances the innate immune response. Furthermore, IL-12 induces the expression of IL-18 receptor, and IL-18 synergizes with IL-12 to enhance the production of IFN- γ in a TCR independent pathway (Trinchieri 1994; 2003). In IL-12 deficient mice, Th1 differentiation and IFN- γ secretion are impaired. In contrast, Th2 development and IL-4 production appear to be enhanced (Mattner, et al., 1996, Magram, et al., 1996, Wu, et al., 1997; 2000). However, upon antigen stimulation, these deficient mice showed Th1 polarization and residual IFN-γ production. Therefore, IL-12 is not absolutely required for Th1 development. Recently, two new IL-12 family members, IL-23 and IL-27 have been discovered, and both IL-23 and IL-27 can

exert immunoregulatory effects on Th1 immune response. (Brombacher, et al., 2003; Watford, et al., 2003).

Uncontrolled self-reactive effector $CD4^+$ T cells results in a number of chronic inflammatory diseases. For many years, the IL-12-dependent Th1 cells were considered to be essential for the induction of autoimmune diseases, including collagen-induced arthritis (CIA), experimental autoimmune encephalitis (EAE), inflammatory colitis, and autoimmune uveitis (Leonard et al., 1995; Constaninescu et al., 1998; Segal et al., 1998). These opinions have been based on a number of studies, in which autoimmune disease was ablated in mice treated with neutralizing antibodies specific for IL-12p40 or mice deficient in the IL-12p40 subunit. Although IL-12 inducing IFN-γ was characterized in these autoimmune diseases, the discrepancy was that mice deficient in IFN-γ or IFN-γ receptors were found still susceptible to EAE and CIA (Ferber et al., 1996; Mattys 1998). Furthermore, a later study found that mice lacking IL-12 receptor complex are not resistant to EAE (Zhang et al., 2003). With the discovery of IL-23 sharing the p40 subunit of IL-12, it is suggested that IL-23, not IL-12, is critically associated with autoimmunity in these models.

1.3.3 IL-23

Oppmann et al. (2000) discovered that p40 can dimerize with p19, a novel protein identified by sequence database searching, to form a disulfide-linked herterodimeric cytokine designated IL-23. The structure of IL-23 p19 is closely related to IL-12p35 subunit. Like IL-12p35, IL-23p19 is expressed ubiquitously

and constitutively, but is not secreted by itself. Similar to IL-12, coexpression of p19 and p40 within the same cell is required to form biologically active IL-23. Like IL-12, IL-23 is also produced by activated APC such as DCs and phagocytic cells (Oppmann, et al., 2000).

As IL-23 shares the same IL-12p40 subunit, IL-23 also binds to IL-12Rβ1. However, it does not bind to IL-12Rβ2, but instead bind to IL-23R, a member of the hemopoietin receptor superfamily (Parham, et al., 2002). IL-23R shows 70% homology between human and mice, and the structure of IL-23R is very similar to IL-12R β2 and gp130. In mice, IL-23R is expressed by memory T cells, bone marrow macrophages, and LPS activated macrophages. In humans, IL-23R is detected on activated naïve T cells and memory T cells, NK cells, and is also expressed at low levels on macrophages, monocytes, and DCs. Only co-expression of IL-12R β1 and IL-23R on cells can confer IL-23 responsiveness (Parham, et al., 2002). Similar to IL-12 signalling, the binding of IL-23 to IL-23 receptor complex results in the activation of Tyk2, Jak2 and STAT1, 3, 4, and 5. STAT4 activation is much weaker in response to IL-23, when compared to the IL-12 response. Instead, STAT3 is dominant in the IL-23 signal-transduction cascade (Lankford & Frucht 2003, Watford, et al., 2004).

As a consequence of the structural similarity, IL-23 was predicted to have similar functions as IL-12. Initial studies have demonstrated that IL-23 stimulated IFN-γ production preferentially on memory/effector $CD4^+$ T cells and maintains Th1 development; while IL-12 is important for Th1 differentiation of naïve $CD4^+$ T cells (Oppmann, et al., 2000). Surprisingly, emerging studies have shown that IL-

23 plays an important role in Th17 cells, which is distinct from the role of IL-12 in T cell regulations. Aggarwal, et al. (2003) provided the first evidence that the addition of IL-23 in murine memory T cell cultures generated IL-17+ producing cells. Subsequent *in vivo* studies have shown a defect in Th17 subsets in IL-23 deficient mice (Cua et al., 2003; Langrish et al., 2005). Although it was thought that IL-23 was an important cytokine for the differentiation of Th17 cell, IL-23 is not required for the initial Th17 differentiation from naïve T cells in mice (Veldhoen et al., 2006; Bettelli et al., 2006). In fact, IL-23 is important to maintain and expand Th17 phenotype (Aggarwal et al., 2003; Langrish et al., 2005; Veldhoen et al., 2006).

The clear differences between IL-12 and IL-23 have been supported using gene disrupted mice models: p19-/-, p40-/- and p35-/- knock out mice (Cua et al. 2003; Murphy, et al., 2003). IL-23 p19-/ and p40-/- deficient mice are resistant to EAE and CIA, while IL-12p35-/- mice are highly susceptible to EAE and CIA. Thus, IL-23 rather than IL-12, is the critical cytokine in inflammatory autoimmune responses. Evidence of neutralizing antibodies against IL-23 effectively inhibiting the diseases process of EAE has again proved that IL-23 is associated with autoimmunity (Chen et al., 2006)

1.3. 4 IL-27

1.3.4.1 Historical perspective

In 1996, Epstein Barr virus-induced gene 3 (EBI3) was identified as an IL-12p40 homologue induced by EBV infection in B lymphocytes (Devergne et al., 1996).

In 1998, advances in the ability to search sequence databases with structure based alignment tools had led to the identification of a gp130-like protein, WSX-1 (named after the WSXWS protein motif that is found in the carboxyl terminus of many type I cytokine receptors), also called TCCR, which is described as an orphan receptor expressed on NK cells and T cells (Sprecher et al., 1998). In 2002, in search of proteins homologous to IL-12p35 and IL-6, Pflanz et al. (2002) group discovered p28 subunit, which can only partner with EBI3 to form a stable heterodimeric protein complex. This protein complex was designated IL-27, as a new member of the IL-12 family. Subsequently, it was established that WSX-1 partnered with gp130 to form heterodimeric receptors for IL-27 (Pflanz et al., 2004).

1.3.4.2 Characteristics of IL-27

Similar to other members of IL-12 family, IL-27 is a heterodimeric cytokine composed of two subunits: EBI3 and p28. EBI3, related to IL-12p40, lacks a membrane-anchoring motif and resembles a soluble cytokine receptor (Devergne et al., 1996). Human p28 gene is located on chromosome 16p11, and encodes a 243 amino acid polypeptide. Murine p28 gene encodes a 234 amino acid polypeptide, which is 78% identical to human p28. Like IL-12p35 and IL-23p19, IL-27p28 subunit is a member of the long-chain four-helical bundle cytokines. Interestingly, IL-27p28 displays a unique stretch of 13 glutamic acid residues, which has not been displayed in other helical bundle cytokines (Pflanz et al., 2002). EBI3 is secreted by itself; whereas p28 is poorly secreted by itself unless co-expressed with the partner EBI3. Although IL-27 shares a similar structural

makeup with IL-12 and IL-23, the subunits of IL-27 are linked by a covalent bond not a disulfide bond. Like IL-12 and IL-23, the biological activity IL-27 requires the expression of both EBI3 and p28 in the same cell (Pflanz, et al., 2002). The expression of IL-27 has been detected in monocytes, DCs derived from monocytes, endothelial cells, and trophoblast cells in human (Pflanz et al., 2002; Larousserie et al., 2004; 2006; Coulomb-L'Herrmine et al., 2007). The expression of murine IL-27 has been detected in activated macrophages and microglia cells (Pflanz et al., 2002; Sonobe et al., 2005).

1.3.4.3 Regulation of IL-27

Examination of cDNA libraries has indicated that both human and murine IL-27 are highly induced in activated antigen-presenting cells (Pflanz, et al., 2002). Although there are no sufficient studies in the regulation of IL-27 expression, it has been demonstrated that non-pathogenic Gram-negative bacteria, not Gram-positive bacteria, promotes a strong IL-27 expression. It is clear that LPS is the main immunostimulatory component within the cell wall of Gram-negative bacteria, and the effect of LPS is mediated through toll-like receptor 4 (TLR4). Thus, signalling via TLR4 activated by LPS is the key inducer of IL-27 (Schuetze et al., 2005; Schnurr et al., 2005). Besides TLR4, many other microbes via other TLRs can trigger the expression of IL-27, such as the engagement of TLR3 with polyinosinic-polycytidylic acid [Poly (I:C)], and ligation of TLR9 with double strand DNA. TLR7/8 ligand is also a strong inducer of IL-27 (Pirhonen et al., 2007). Furthermore, a variety of host derived factors, including CD40L, IL-1β, IFN-α, IFN-β and IFN-γ can upregulate the expression of IL-27

(reviewed in Villarino et al., 2004; Pirhonen et al., 2007). Conversely, the nucleotide ATP is the negative regulator of IL-27 expression (Schnurr et al., 2005).

When LPS stimulates monocyte-derived DCs, mRNA levels of IL-27p28 are induced rapidly and peak after 3 to 6 hr, then decline to the background levels after 24hr, whereas mRNA levels of EBI3 are induced more slowly and peak after 12 to 24hrs, and then drop but not reach the background levels even after 72 hr (Pflanz, et al., 2002). These observations indicated that different signalling pathways control the expression of EB3 and p28 subunits respectively. TLRs dependent EBI3 expression was shown to be induced in DCs via activation of the transcription factors NF-κB and PU-1(Wirtz et al., 2005).Toll/IL-1R-containing adaptor inducing IFN-β (TRIF) and its associated IFN regulatory factor (IRF)3 transcription factor are involved in TLR-inducible expression of human IL-27 p28 (Molle et al., 2007).

1.3.4.4 IL-27 Receptors

The IL-27 receptor is a heterodimer composed of WSX-1(TCCR, IL-27Ra) and a common gp130 chain (Pflanz et al., 2004). WSX-1 is a type I cytokine receptor with four conserved cysteine residues and a WSXWS protein motif in the carboxyl terminus (Sprecher et al., 1998; Chen, et al., 2000). WSX-1 shows homology to members of the IL-6 superfamily receptors, including the IL-12Rβ2 subunit, LIFR and gp130. WSX-1 is expressed mainly in lymphocytes (Pflanz, et al., 2004). Interestingly, naïve $CD4^+$ T cells and NK cells appear to express the

highest levels of WSX-1, but differentiated Th1 and Th2 cells show low expression of WSX-1(Pflanz, et al., 2004). gp130 is a shared receptor for IL-6 and other IL-6 family cytokines, and is widely expressed by a range of immune and non-immune cells (Reviewed in Taga & Kishimoto 1997).

WSX-1 is capable of binding to IL-27 with high affinity in the absence of gp130 (Pflanz et al., 2002), but WSX-1 requires gp130 to form IL-27 receptor complex that is responsible for IL-27 signal transduction (Pflanz, et al., 2004). In addition, gp130 does not bind to IL-27 alone (Schellel et al., 2005). WSX-1 and gp130 appear to be coexpressed on a variety of immune cell types, including $CD4^+$ T cells, $CD8^+$ T cells, NK cells, monocytes, mast cells, neutrophils and B cells (Pflanz, et al., 2004; reviewed in Batten & Ghilardi 2007).

1.3.4.5 Signalling through IL-27 receptors

The engagement of IL-27 with WSX-1/gp130 induces the activation of JAK1 and 2, Tyrosine kinase-2 (TYK2), STAT1, 2, 3, 4, and 5 in naïve $CD4^+$ T cells (Lucas et al., 2003). Furthermore, the activation of WSX-1 preferentially phosphorylates STAT1, while gp130 activation mainly contributes to a strong activation of STAT3 (Pflanz, et al., 2004; Kamiya, et al., 2004). Recently, it has been reported that IL-27 preferentially induces STAT3 in fully activated $CD4^+$ T cells, while both STAT1 and STAT3 are activated in early activated T cells upon IL-27 stimulation (Yoshimura et al., 2006).

1.3.4.6 The role of IL-27 in promoting Th1 response

Based on sequence and structure homology between IL-27 and IL-12, the function of IL-27 was initially investigated on Th1 immune response. As IL-27 is an early product of activated APCs, IL-27 drives rapid clonal expansion of naïve $CD4^+$ T cells and augments the secretion of IFN-γ by these $CD4^+$ T cells under non-polarizing culture conditions (Pflanz et al., 2002). Correspondingly, unpolarized naïve $CD4^+$ T cells from WSX-1 deficient mice produce less IFN-γ than wild-type counterparts (Chen et al., 2000; Yoshida et al., 2001). Also, IL-27 synergizes with IL-12 to trigger IFN-γ production of naïve $CD4^+$ T cells and NK cells (Pflanz et al., 2002). However, in the presence of high levels of IL-12, IL-27 is not able to further enhance Th1 development (Owaki et al., 2005). Accordingly, fully differentiated WSX-1-/- Th1 cells produce similiar levels of IFN-γ upon second stimulation compared to wild-type Th1 cells. Taken together, IL-27 is important for early initiation of Th1 response.

In agreement with the expected role of IL-27 in Th1 development *in vitro*, WSX-1 deficient mice showed an impaired Th1 response in *Listeria monocytogenes* and the early phase of *Leishmania major* (Chen et al., 2000; Yoshida, et al., 2001). Notably, normal Th1 response and IFN-γ production in WSX-1 deficient mice have been demonstrated at the late stage of *Leishmania major* (Yoshida, et al., 2001). These data supported that IL-27 is involved in the early phase of Th1 differentiation. In addition, EBI3 deficient mice display enhanced susceptibility to *Leishmania major* infection with defects in IFN-γ production in the early phase

compared to wild-type mice, emphasizing the importance of IL-27 at the initial stage of Th1 differentiation (Zahn et al., 2005).

Then, the molecular basis for IL-27 initiating Th1 responses has been elucidated. Upon IL-27 stimulation, WSX-1 activates the downstream of STAT1, leading to the induction of T-bet. T-bet enhancing the expression of IL-12Rβ2 is one of the critical events for Th1 differentiation (Lucas et al., 2003; Kamiya et al., 2004). Interestingly, it has also been found that IL-27 induces T-bet and IL-12Rβ2 at mRNA levels in a STAT-1- independent pathway (Lucas et al., 2003). More recently, it has been demonstrated that IL-27 can induce Th1 differentiation through ICAM-1/LFA-1 interaction in a STAT-1-dependent, but T-bet independent mechanism. Furthermore, IL-27 could induce another pathway, which is p38MARK /T-bet –dependent and STAT-1-independent pathway, and IL-12Rβ2 was shown to be upregulated in both pathways (Owaki et al., 2006).

In contrast to the early characterization of IL-27 as a pro-inflammatory cytokine that contributes to Th1 development, recently IL-27 is also recognized as an anti-inflammatory cytokine that limits immune responses.

1.3.4.7 IL-27 inhibits Th1 responses

Although IL-27 has been described to promote Th1 response, IL-27 also has effects in suppression of Th1 responses. The anti-inflammatory role of IL-27 has been clearly shown in several reports. WSX-1 deficient mice infected with *Toxoplasma gondii* remarkably develop a lethal $CD4^+$ T cell-dependent

inflammatory disease after two weeks of infection that displays enhanced proliferation of $CD4^+$ and $CD8^+$ T cells and excessive production of IFN-γ (Villarino et al., 2003). The involvement of IL-27 signaling in the induction of Th1 immune responses to reduce parasite burden has been further supported by WSX-1-/- mice infected with *Trypanosoma cruzii* or *Leishmania donovani* (Hamano et al., 2003; Rosas et al., 2006). Infected WSX-1 deficient mice showed higher sensitivity to infection and led to lethal liver necrosis compared to wild-type mice. Immune cells from infected WSX-1 KO mice produced more IFN-γ and other cytokines such as TNF-α compared to immune cells from infected wild-type mice. Likewise, in Con A-induced hepatitis model, WSX-1 deficient mice displayed enhanced liver diseases coupled with the elevated levels of IFN-γ and other inflammatory cytokines (Yamanaka et al., 2004).

Consistent with the observations above from *in vivo* work, $CD4^+$ T cells from WSX-1 -/- mice produce more IFN-γ than wild-type counterparts when stimulated with low doses of antigen in the presence of IL-12 (Villarino et al., 2003). IL-27 suppresses IFN-γ and other pro-inflammatory cytokine production from activated $CD4^+$T cells *in vitro* (Yoshimura et al., 2006). However, the molecular basis for the negative effect of IL-27 on Th1 response remains unclear. IL-27 suppresses IL-2 expression on $CD4^+$ T cells (Villarino et al., 2006). IL-2 is a potent growth factor for T cell development. The suppression of IL-2 expression by IL-27 may result in the regulation of excessive Th1 cell activation.

1.3.4.8 The role of IL-27 in suppressing Th2 response

It was originally thought that IL-27 promotes IFN-γ production, which could inhibit the development of Th2 cells. The first study which indicated that IL-27 has direct suppressive effects on Th2 response has shown that IL-27 inhibits $CD4^+$ T cell expression of GATA3 via STAT1-dependent pathway (Lucas et al., 2003). Subsequently, there has been increasing evidence of increased Th2 cell responses in IL-27 receptor deficient mice models. In particular, studies of WSX-1 deficient mice infected with the gastrointestinal nematode *Trichuris muris*, a well-characterized helminth removed by Th2-mediated immune responses, showed that these mice are resistant to infection (Artis et al., 2004). During infection, WSX-1 deficient mice displayed accelerated Th2 cell dependent intestinal goblet cell hyperplasia, mastocytosis, and produce more IL-5 and IL-13 compared to the wild-type counterparts. Furthermore, it has been reported that WSX-1-/- mice exhibited exacerbation of allergic asthmatic phenotypes, including airway hyper-responsiveness (AHR), eosinophilic infiltration, and mucus overproduction in response to OVA challenge (Miyazaki et al., 2005).

Recent studies have revealed that IL-27 can directly inhibit Th2 cell polarization, and simultaneously IL-27 induces Th1 cell differentiation in the absence of IFN-γ and IL-12 *in vitro* (Yoshimoto et al., 2007). Consistent with *in vitro* results demonstrating the ability of IL-27 to suppress Th2 response, *in vivo* studies showed that daily treatment with IL-27 for the first week after *Leishmania major* infection significantly decreased footpad swelling and parasite burden via the inhibition of Th2 cell development and induction of Th1 cell development.

Furthermore, IL-27 transgenic mice are not capable of mounting Th2 response against *Strongyloides venezuelensis* infection. The molecular mechanism has been explained that IL-27 downregulates GATA-3 expression and upregulates T-bet expression.

Remarkably, IL-27 can inhibit already differentiated Th2 cells to produce IL-5 and IL-13 via the activation of both STAT1 and STAT3 (Yoshimoto et al., 2007). In contrast, IFN-γ fails to suppress the production of IL-5 and IL-13 from already differentiated Th2 cells. Consistent with these *in vitro* results, intranasal administration of IL-27 decreases OVA-induced airway hyperresponsiveness and inflammation in OVA-sensitized mice *in vivo*. Therefore, IL-27 is a critical cytokine involved in the down-regulation of Th2 responses independently of IL-12 and IFN-γ.

1.3.4.9 The role of IL-27 in suppressing Th17 response

Early studies showed that IL-27 neutralization is beneficial in autoimmune diseases, including adjuvant-induced arthritis in rats and experimental autoimmune encephalomyelitis (EAE) (Goldberg et al., 2004). However, those claims are contradicted with the intriguing evidence of IL-27 mediated Th17 suppression. Stumhofer et al. (2006) showed that WSX-1 deficient mice chronically infected with *Toxoplasma gondii* developed severe CNS inflammation associated with augmented Th17 responses. Batten et al. (2006) showed that WSX-1 deficient mice were hypersusceptible to EAE induced by immunization with myelin oligodendrocyte glycoprotein (MOG) in CFA, and

generated more Th17 cells. Consistent with the *in vivo* results, both groups demonstrated that IL-27 suppresses the development of Th17 driven by IL-6 and TGF-β *in vitro*. Furthermore, IL-27 inhibits IL-23-induced IL-17 on activated $CD4^+$ T cells (Yoshimura et al., 2006). Thus, IL-27 may be a potential target for treating inflammatory diseases and autoimmune diseases mediated by Th17. More recent studies have found the enhanced expression of IL-27 in the target tissues of autoimmune diseases, including CNS at the effector phases of relapsing-remitting EAE (Fitzgerald et al., 2007), and retinal ganglion and photoreceptor cells of uveitis and scleritis (Amadi-Obi et al., 2007). Mice treated with exogenous IL-27 had reduced CNS inflammatory infiltration and a lower proportion of Th17 cells (Fitzgerald et al., 2007).

The molecular mechanism for IL-27-mediated Th17 inhibition is still not clear. Considering IL-6 as an important factor for the development of Th17 cells, and the fact that IL-6 and IL-27 share the common receptor gp130, it was speculated that IL-27 may compete with IL-6 for receptor binding, which may result in the inhibition of Th17 cells. However, IL-27 appears to further reduce IL-17 production in the absence of IL-6, which indicated that IL-27 exerts its inhibitory effects beyond simply antagonizing IL-6-mediated signalling (Stumhofer et al., 2006). Based on STAT-1 deficient mice, it was established that the suppression of Th17 development by IL-27 is dependent on STAT1 pathway. T-bet has been found to be a negative regulator of Th17 cells, but IL-27 does not reduce the inhibition of Th17 cells in T-bet deficient cells. These data indicated that T-bet is not required for IL-27 mediated Th17 inhibition (Stumhofer et al., 2006). In addition, SOCS-3, an important regulator of Th17 generation, can be induced by

IL-27. Although it has been proposed that SOCS-3 may mediate the inhibitory effects of IL-27 in Th17 cells, studies have shown that Th17 generation using SOCS-3 deficient T cells was still suppressed by IL-27(Stumhofer et al., 2006). Therefore, IL-27 is an effective suppressor of Th17 development via a STAT1 dependent process, and the exact pathways remain to be identified.

1.3.4.10 IL-27 inhibits the development of TGF-β-induced Foxp3+ Treg cells (Th3), but induces IL-10-producing Tr1 cells

TGF-β can induce naïve T cells to Treg cells with the high expression of Foxp3. Recent studies have found that IL-27 inhibited the development of Tregs generated by TGF-β (Neufert et al., 2007; Huber et al., 2008). In contrast to these inducible Treg cells, IL-27 has not been found to have effect on naturally occurring Foxp3+ Tregs (Neufert et al., 2007).IL-27 suppressed TGF-β mediated Treg cells phenotype at the levels of Foxp3, CD25 and CTLA-4 expression (Huber et al., 2007). This inhibitory effect of IL-27 on the inducible Treg cells is dependent of STAT3 signaling, but independent of STAT1 signaling (Neufert et al., 2007; Huber et al., 2007).

Tr1 cells are another type of induced Treg that regulate T cell function by producing IL-10, but lack the expression of Foxp3. Although IL-27 inhibits the development of TGF-β-induced Foxp3+ Treg cells, recent studies have shown that IL-27 induced naïve $CD4^+$ T cells into IL-10-producing cells, and TGF-β further enhanced the generation of induced IL-10+ Tr1 cells by IL-27 (Awasthi et al., 2007; Fitzgerald et al., 2007 (2); Stumhofer et al., 2007). These IL-10-

producing cells are Foxp3 negative. Furthermore, it was found that IL-27 induces IL-10 in Th1 and Th2 but not Th17 conditions. Notably, the ability of IL-27 to induce IL-10 requires both STAT1 and STAT3 (Stumhofer et al., 2007).

1.3.4.11 The role of IL-27 on other cells

Besides the effects of IL-27 on $CD4^+$ T cells, the immunosuppressive effects of IL-27 also extend to other lymphocytes. IL-27 stimulates $CD8^+$ T cells and enhances cytotoxic activity, which plays an important role for protection against viral infection and cancer (Salcedo et al., 2004; reviewed in Shimizu et al., 2006). IL-27 enhances the proliferation of B cells and induces IgG2a class switching *in vitro* (Yamanaka et al., 2004). Although IL-27 acts synergistically with IL-2 and IL-12 to promote proliferation of NK cells and enhances the production of IFN-γ from these cells *in vitro* (Pflanz et al., 2002), NK cells from WSX-1-/- mice produced more IFN-γ and IL-4 compared to wild-type mice in response to con A stimulation (Yamanaka et al., 2004). These data suggested that IL-27 has a regulatory role in NK cell function. IL-27 also inhibits macrophages to produce cytokines, including IL-12 and TNF-α (Holscher et al., 2005). Furthermore, IL-27 can suppress endotoxin-induced production of reactive oxygen mediated by granulocytes and macrophages (Wirtz et al., 2006). In turn, lethal septic peritonitis was protected by the neutralization of IL-27. IL-27 also induces STAT1 and STAT3 in osteoblasts, but its effects have not been identified. This indicated that IL-27 may protect against bone destructive autoimmune diseases (Kamiya et al., 2007). Thus, IL-27 has broad immunosuppressive effects in

immune responses, although the underlying mechanisms for this immunosuppressive function are not fully understood.

1.3.4.12 The studies of IL-27 signaling deficient mice to immunological challenge

Mouse genotype	Challenge	T helper response	Outcome	Reference
EBI3$^{-/-}$	*Leishmania major*	Early Th1 defect	Delayed but effective response	Zahn et al., 2005
WSX-1$^{-/-}$	*Leishmania major*	Early Th1 defect	Delayed but effective response	Yoshida et al., 2001 Artis et al., 2004
WSX-1$^{-/-}$	*Mycobacteria bovis* BCG	Early Th1 defect	Delayed but effective response, increased granuloma number and size	Yoshida et al., 2001
WSX-1$^{-/-}$	*Mycobacterium tuberculosis*	Th1 reduced	Enhanced bacterial clearance	Pearl et al., 2004
WSX-1$^{-/-}$	*Mycobacterium tuberculosis*	Th1 enhanced	Enhanced bacterial clearance, lung immunopathology	Holscher et al., 2005
WSX-1$^{-/-}$	*Trypanosoma cruzi*	Th2 enhanced	Increased parasitemia, hepatic immunopathology	Hamano et al., 2003
WSX-1$^{-/-}$	*Leishmania donovani*	Th1 enhanced	Enhanced bacterial clearance, hepatic immunopathology	Rosas et al., 2006
WSX-1$^{-/-}$	Acute *Toxoplasma gondii*	No defect	Highly effective response, hepatic immunopathology	Villarino et al., 2003
WSX-1$^{-/-}$	Chronic toxoplasmic encephalitis	Th17 enhanced	Exacerbated encephalitis	Stumhofer et al., 2006
WSX-1$^{-/-}$	*Trichuris muris*	Th2 enhanced	Enhanced helminth clearance	Artis et al., 2004; Bancroft et al., 2004

Mouse genotype	Challenge	T helper response	Outcome	Reference
WSX-1$^{-/-}$	Concanavalin A-induced hepatitis	Th1,Th2 enhanced	Exacerbated hepatitis	Yamanaka et al., 2004
EBI3$^{-/-}$	ConA-induced hepatitis	Th1 reduced	Resistant to hepatitis	Siebler et al., 2007
WSX-1$^{-/-}$	DSS-induced colitis	Th1 reduced	Resistance to colitis	Honda et al., 2005
EBI3$^{-/-}$	Oxazolone-induced colitis	Th2 reduced	Resistance to Th2-mediated colitis	Nieuwenhuis et al., 2002
EBI3$^{-/-}$	Trinitrobenzene sulfonic acid-induced colitis	Th1 unaffected	Normal development of Th1-mediated colitis	Nieuwenhuis et al., 2002
EBI3$^{-/-}$	Caecal ligation and puncture (CLP) induced septic peritonitis	Not known	Enhanced granulocytes ability to bacterial clearance, resistance to sepsis	Wirtz et al., 2006
WSX-1$^{-/-}$	EAE	Th17 enhanced	Exacerbated encephalitis	Batten et al., 2006
WSX-1$^{-/-}$	Allergic asthma	Th2 enhanced	Exacerbated disease	Miyazaki et al., 2005
WSX-1$^{-/-}$	Proteoglycan-induced arthritis (PGIA)	Th1 reduced	Delayed in arthritis development and reduced diseases severity	Cao et al., 2007)

Table 1.1 List of the studies with IL-27 signaling deficient mice (adapted from Batten & Chilardi 2007)

1.3. 5 IL-35 (EBI3/p35)

EBI3 was originally identified as a transcriptionally activated gene in Epstein-Barr virus-infected human B lymphocytes. EBI3 encodes a 34-Da glycoprotein that lacks a membrane anchored motif. Structurally, EBI3 bears 27% homology to the subunit IL-12p40 at the amino acid sequence level (Devergne, et al., 1996). EBI3 can form heterodimers with IL-27 p28 subunit. However, EBI3 is expressed much more widely than p28. EBI3 is expressed by human B lymphoblast cell lines, macrophage like cells in the lamina propria of the human colon in patients with ulterative colitis (Christ 1998), human colon epithelial cell lines, and by placental syncytiotrophoblasts at very high levels (Devergne, et al., 2001). It raises possibility that EBI3 might associate with other partners and have functions other than IL-27. IL-12 was identified and purified from culture supernatant of EBV-transformed B cell lines and is expected to be closely associated with EBI3. IL-12 p35 is ubiquitously expressed whereas IL-12 p40 expression is inducible. The dissociation between p35 and p40 gene regulation suggests that either subunit may be associated with other partners. p40 associates with p19 to form IL-23. It has been demonstrated that EBI3 associates non-covalently with IL-12p35 to form a novel heterodimeric cytokine (Devergne, et al., 1997). Notably, coexpression of EBI3 and p35 facilitates their secretion, where they are not efficiently secreted when expressed alone. The expression of p35 was detected in the entire cell types positive for the expression of EBI3 in placental tissues (Devergne 2001). Additionally, intestinal epithelial cells were also shown to express EBI3 and p35 together (Masser, et al., 2004). Thus, EBI3-p35 heterodimer may represent a novel cytokine of the IL-12 family. This heterodimeric cytokine has been called IL-35 by the International Union of

Immunological Societies (IUIS) subcommittee on Interleukin Nomenclature and the HUGO Gene Nomenclature Committee (Schrader 2002).

IL-12p40 can form homodimers to compete with IL-12 and through binding to IL-12 receptors, and IL-23 receptors shares β1 subunit of IL-12 receptors. However, unlike IL-12p40 homodimer and IL-23, IL-35 does not bind to IL-12 β1 and β2 receptor (Devergne, et al., 1997). Therefore, IL-35 might have different receptor complex. It is known that EBI3 binds to gp130, but it is uncertain whether IL-35 interacts with gp130 to date.

As the survival of EBV-infected B lymphocytes and syncytial trophoblasts needs an effective NK and T cells response, it is likely that IL-35 might have an effect on NK and T cells, and downregulate the biological effects of IL-12. It was speculated that the association of EBI3 with p35 might inhibit the expression of IL-12 (Devergne, et al., 1997). However, little was known about the biological role of IL-35 in immune responses since this cytokine has been found. Although neither EBI3-/- or IL-12p35-/- mice displayed overt autoimmune diseases, EBI3-/- mice are more susceptible to leishmaniasis (Zahn et al., 2005), and IL-12p35-/- mice are more susceptible to *Leishmania major* infection (Mattner et al., 1996), EAE and CIA (Gran et al., 2002; Murphy et al., 2003). These phenomenon were always interpreted as a lack of IL-27 and IL-12. It is possible that the lack of IL-35 function was involved in the EBI3-/- and IL-12p35-/- mice. A very recent study has demonstrated that both *EBI3* and *IL-12p35* genes were highly expressed by mouse Foxp3+Treg cells, but not by resting or activated effector $CD4^+$ T cells (Collison et al., 2007). IL-35 heterodimer is preferentially

constitutively secreted by Treg cells but not effector T cells. It also has been shown that *EBI3* is a downstream target of Foxp3, indicating IL-35 might regulate function of Treg. EBI3-/- and IL-12p35-/- Treg cells showed significantly reduced abilities to suppress the proliferation of effector T cells *in vitro* compared to wild-type Treg cells. Furthermore, EBI3-/- and IL-12p35-/- Treg cells failed to cure inflammatory bowel diseases compared to wild-type Treg cells *in vivo* (Collison et al., 2007). Therefore, IL-35 is required by Treg cells for maximal regulatory function.

1.4 Specific aims of this thesis

Cytokines play a central role in regulating T cell expansion and differentiation, and an understanding of cytokines may lead to novel therapeutic strategies.

IL-27 initially has been characterized as a cytokine in the initiation of Th1 responses; however, subsequent work has provided evidence that IL-27 has broad anti-inflammatory effects on Th1, Th2, and Th17 subsets. My hypothesis was that IL-27 might play an anti-inflammatory role in RA since Th17 has been shown to be involved in the pathogenesis of RA. Thus, one part of this project was designed to elucidate the function of IL-27 in CIA, a model that closely resembles human RA.

IL-35 (EBI3/p35) is a naturally occurring herterodimeric cytokine, but the biological role of IL-35 was unknown when I started the project. The expression of EBI3 and p35 has been highly detected in placental tissues and intestinal epithelial cells. This indicated that IL-35 may be important in immune regulation. A very recent study has demonstrated that IL-35 is required for the regulatory function of Treg cells, which highlighted that IL-35 may be a inhibitory cytokine. Thereby, one part of this project was designed to elucidate the biological role of IL-35 in immune responses, and the possibility of using IL-35 as a therapeutic agent in inflammatory diseases, like CIA.

The objectives of this project were to solve these questions:

1. To clone and express murine IL-27 and IL-35 in order to provide enough materials for murine *in vitro* and *in vivo* study. In addition, to clone and express human recombinant IL-35 for the future human studies.
2. To investigate the potential role for recombinant IL-27 in murine model of inflammatory arthritis, CIA.
3. To characterize the role of recombinant IL-35 in different subsets of $CD4^+$ T cells *in vitro,* and study the effects of IL-35 in inflammatory response *in vivo,* using murine CIA model.

I will employ a range of *in vitro* and *in vivo* techniques to address the above questions. The project will not only provide dynamic and advanced training in immunology for a PhD program, but the results should also provide important information on the crucial area of immune regulation by cytokines, leading to potential cytokine therapeutic approaches to a range of inflammatory diseases.

Chapter 2

Material & Methods

2.1 Molecular Cloning

2.1.1 Generation and culture of Dendritic cells (DCs)

Human EBI3 mRNA and p35 mRNA were expressed by DC. DCs were generated from isolated Peripheral blood mononuclear cells (PBMC) from healthy volunteers. PBMC were incubated with RPMI1640 complete medium (supplemented 2mM L-glutamine, 100U/ml penicillin & 100 μg/ml streptomycin, 10% foetal calf serum (FCS), and 50 mM β-Mercaptoethanol) (Invitrogen) in 25 cm^2 flasks at 37^oC in a humidified incubator with 5% CO_2 for 2 h to adhere to the flask. Then the supernatant including non-adherent cells was removed, and the remaining adherent cells were cultured with RPMI1640 complete medium in the presence of 10 ng/ml IL-4 (R & D Systems) and 5 ng/ml GM-CSF (R & D Systems) for 6 days (Ranieri 1999), thus, monocytes were differentiated into mature DCs. The DCs were stimulated with 100 ng/ml lipopolysaccharide (LPS) (Sigma) and 100 ng/ml of interferon (IFN)-γ (R&D Systems). After 7 h incubation, the DCs were harvested (Johansson 2002) and the pelleted cells were lysed in buffer RLT (Qiagen) with 1% β-Mercaptoethanol (β-ME) (Sigma). The lysates were stored at –70^oC until required.

2.1.2 RNA purification

Total cellular RNA was extracted from the lysates that were prepared as described above using the RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. In brief, the lysate samples were homogenized using QIAshredder spin columns. 1 volume of 70% ethanol was added to the homogenized lysate and transferred to an RNeasy spin column and centrifuged

for 15 seconds at 10,000 rpm and the flow through the column was discarded. RW1 buffer (700 µl) was added to the column and spun for 15 seconds at 10,000 rpm. The column was washed twice with 550 µl RPE buffer. Finally, RNA was eluted in 40 µl of Rnase-free water. The amount and quality of purified RNA was determined by measuring the OD_{260} and OD_{280} in GeneQuant (Amersham pharmacia biotech).

2.1.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Cloned DNA (cDNA) was generated from the RNA prepared as described above using the superscript II RT system (Invitrogen). 1-5 µg of RNA in a volume of <11 µl was added to 1 µl of Random Primer (RP) (50 ng/µl) (Roche), and the total volume was made up to 12 µl with Rnase-free water. The mixture was heated at 70^{o}C for 10 minutes and then placed on ice for at least 1 minute. During this time, a mixture of 4 µl 5 x first strand buffer, 2 µl 0.1M DTT, and 1 ul 10 mM dNTP was prepared. This mixture (7 µl) was added into the tube containing the RNA and RP mixture. 1 µl of Superscript II (200 u /µl) was added to the sample and reverse transcribed using the following conditions: 25^{o}C for 10 minutes, 42^{o}C for 50 minutes, and 70^{o}C for 15 minutes in the Mastercycler gradient (Eppendorf).

2.1.4 Polymerase Chain Reaction (PCR)

The cDNA obtained above was used as a template in subsequent PCR reactions. All the primer- oligonucleotides (listed in table 2.1 below) were designed using Primer Express^{TM}v1.0 program (PE biosystems) and purchased from Sigma. 1 µl

cDNA was mixed with 5 µl 10 x *Pfu* Buffer (Invitrogen), 1 µl 10 mM dNTP, and 2.5 µl specific 5' and 3' primer- oligonucleotides (0.04 µg/µl). 1µl of *Pfu DNA polymerase* (5 unit/ µl) (Invitrogen) was added and the volume adjusted to 50 µl with DEPC H_2O. The mixed sample was amplified in a thermo-cycler (Sigma) under following reaction conditions: 35 cycles of denaturating at 95^{o}C for 1 minute, annealing at 56^{o}C for 2 minutes, and extending at 72^{o}C for 3 minutes, followed by a final extension step of 72^{o}C for 10 minutes. After the reaction, the PCR samples were cooled at 4^{o}C.

Table 2.1 List of PCR Primer- oligonucleotides used for cloning

Oligonucleotide	DNA sequences (5'-3')	Location
hEBI3-5'	ATGACCCCGCAGCTTCT CCTGGCCCTTGTCCT	Human EBI3-cDNA
hEBI3-3'	TTGCTACTTGCCCAGGC TCATTGTGGCAGTGG	Human EBI3-cDNA
hEBI3-SMP-1	GAAATCTTCTCACTGAA GTACTGGATCC	Human EBI3-cDNA in TA vector
hEBI3-SMP-2	GATTTCTGGGAAGGGC CAGGACCCGGGA	Human EBI3-cDNA in TA vector
hEBI3-5'-Bgl II	TCTGAGATCTCTGCCCG CCCTGCAGTGGAAGG	Human EBI3-cDNA
hEBI3-3'-Bgl II	CTTGAGATCTGCCCAGG CTCATTGTGGCAGTG	Human EBI3-cDNA
Hp35-5'-NP1	AGCAAGAGACCAGAGT CCCGGGAAAGTCCT	Human p35-cDNA
Hp35-3'-NP2	CCTAGTTCTTAATCCAC ATCCTATCAAAGT	Human p35-cDNA
Hp35-5'	CCTCGGGACAATTATA AAAATGTGGCCCCCTG	Human p35-cDNA
Hp35-3'	GACAACGGTTTGGAGG GACCTCGCTTTTTAGG	Human p35-cDNA
Hp35-SMP-P1	CAAATCTTTCTAGATCA AAACATGCTGG	Human p35-cDNA in TA vector
Hp35-SMP-P2	GATTTGCCTCTTAGGAT CCATCAGAAGC	Human p35-cDNA in TA vector
Hp35-Not I-P1	TTTGCGGCCGCACCTCC CCGTGGCCACTCCAG	Human p35-cDNA
Hp35-Not I-P2	TTTTGCGGCCGCATTCA GATAGCTCATCACTCT	Human p35-cDNA
McsP1-Bgl II	AGCTTAGATCTG	pEE14.4 vector
McsP2 -Bgl II	AATTCAGATCTA	pEE14.4 vector
Psec-P1-Bgl II	GCTAAGATCTATGGAG ACAGACACACTCCTGC	pSec-Linker-Fc vector
PFc-P2-Bgl II	CAAGATCTCTATTATTT ACCCGGGGACAGGAA	pSec-Linker-Fc vector

2.1.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyse or confirm the size of DNA fragments from PCR or restriction endonuclease digestion. Agarose gel (1%) was made with 1 g agarose (Sigma) into 100 ml 0.5 x TBE buffer (100 mM Tris.HCl, pH 8.0, 450 mM boric acid, and 12.5 mM EDTA), and Ethidium bromide (Sigma) was added at a final concentration of 10 ng/ml. The DNA products were mixed 1:5 with 5 x loading buffer (0.25% Bromophenol Blue, 80% Glycerol, 10 mM Tris.HCl pH 7.4, and 1 mM EDTA) and loaded onto the gel which was then electrophoresed at 1-5 V/cm and visualised under ultra-violet (UV) light.

2.1.6 PCR Fragment Purification

PCR products were purified using the Qiagen Gel extraction kit (Giagen). The bands of interest visualized under UV light were cut from the gel with a sterile scalpel blade, and purified from the agarose following the Gel extraction kit protocol. The concentration of the purified DNA fragment was measured with GeneQuant.

2.1.7 Addition of 3' A-overhangs on PCR products by Pfu polymerases

DNA amplified products by *Pfu polymerases* were blunt ended fragments. In order to ligate into the pCR 2.1 vector (Invitrogen), the purified DNA products were incubated with *Taq polymerase* (Invitrogen). 40 µl of the purified DNA fragments were mixed with 1 µl dNTP, 5 µl PCR buffer (10 x) (200 mM Tris HCl pH 8.4 and 500 mM KCl), 1µl 50 mM MgCl, 1 µl *Taq polymerase* (Invitrogen) and 2 µl DEPC H_2O. The reaction mixture was incubated at 72^{o}C for

10 minutes, and placed on ice for at least 1 minute. Then the DNA product was gel purified as described in section 2.1.6.

2.1.8 Restriction Endonuclease Digestion

Most of the enzymes used were purchased from Roche Biochemicals unless specified. Restriction endonuclease digestion was set up according to the manufacturer's protocols. Typically, 1 μg of DNA was digested using 20 units of enzyme and adjusted to a total volume of 20 μl in a recommended salt condition. The digests were incubated at 37^{o}C for at least 1 h and analysed by agarose gel electrophoresis (see section 2.1.5).

2.1.9 Dephosphorylation of linearised plasmid DNA

To clone the DNA fragments into a selected plasmid vector, the vector DNA was cut by restriction endonuclease (see section 2.1.8). To prevent self-ligation of the plasmid, the digested vector was incubated with calf alkaline phosphatase (Roche) following the manufacturer's instruction, and then gel purified.

2.1.10 Ligation of DNA fragments into plasmid DNA vector

Before ligation, the amount of vector DNA and the DNA fragment to be inserted were analyzed. Equal molar ratio of the insert DNA and vector DNA, were ligated with T4 ligase (Invitrogen) (50 ng DNA vector: 5 units ligase) according to the manufacturer's protocol. The ligation reaction was incubated at room temperature for a minimum of 30 minutes, and then incubated at 15^{o}C overnight.

2.1.11 Transformation of DH5α competent cells

An aliquot of 50 µl DH5α competent cells (Invitrogen) was thawed on ice, before adding 5 µl of ligation reaction or 1/1000 diluted plasmid. The competent cells were incubated on ice for 30 minutes, and heat shock treated at 37^{o}C for 40 seconds before cooling on ice for another 2 minutes. 1 ml of sterile 2-YT broth without ampiciline was added and the cells were incubated at 37^{o}C for 1 h in a shaking incubator. The cells were spun down at 13,000 rpm for 15 seconds, and the supernatant was removed. The cells were re-suspended before being plated on an LB-agar plate containing 100 µg/ml ampiciline. The agar plate was incubated in 37^{o}C oven overnight to obtain single colonies.

2.1.12 Purification of plasmid DNA

Individual colonies from LB-agar plates were picked using 100 µl size tips, and cultured in 3 ml of sterile 2-YT broth containing 100 µg/ml ampiciline overnight at 37^{o}C with vigorous shaking. Plasmid DNA was purified using QIAprep spin Miniprep Kit (Qiagen) according to the instructions. Positive clones were identified by restriction endonuclease digestion (see section 2.1.8). If necessary, 10 µl of DNA from a positive clone was sent to DNASHEF Company for sequencing. The rest was stored in -20^{o}C until required.

2.2 Expression of recombinant proteins with Glutamine Synthetase (GS) system

The Lonza Biologics' Glutamine Synthetase (GS) gene expression system produces high protein expression levels from Chinese Hamster Ovary cells

(CHO). The following procedures were carried out according to the Lonza Biologics' operating procedures for use with the CHO-K1 cell line. In brief:

2.2.1 Preparation of constructs

The constructs were prepared using methods described in above sections. The pEE 14.4 vector was obtained from Lonza Biologics.

2.2.2 Cell culture

CHO-K1 (Lonza Biologics) were grown in glutamine-free Dulbecco's Modified Eagle's Medium (DME) (JRH Biosciences) which contained 10% dialysed Foetal Bovine Serum (dFCS) (Invitrogen), 100 U/ml penicillin & 100 μg/ml streptomycin, and 10 ml GS supplement (JRH Biosciences), called GS medium. The cells were sub-cultured every 3-4 days.

2.2.3 Transfection

The construct was transfected into CHO-K1 cells using Genejuice Transfection Reagent (Novagen). The day before transfection, 8 $x10^5$ CHO-K1 cells per 10 cm Petri dish in GS medium were incubated at 37^oC and 5% CO_2 overnight to reach 50-80% confluency before transfection. Genejuice (9 μl) was added to 100 μl of serum free medium Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) and mixed thoroughly by vortexing. The mixture was then incubated at room temperature for 5 minutes. Then, 3 μg DNA was added to the Genejuice / serum free medium mixture, and shaken gently before incubating at room temperature for 10 minutes. The entire volume of the mixture was added onto the surface of

dish in a drop-wise manner. The cells were incubated for 5 h at 37^{o}C before replacing the medium with fresh GS medium.

2.2.4 Selection of GS-CHO transfectants

The high expressing cell lines were selected by Methionine sulphoximine (MSX) (Sigma). 24 h following transfection, a 25 μM final concentration of MSX was added to the dish. Colonies appeared about two weeks after transfection, and individual colonies were picked and grown in 96-well plates. The colonies with high protein expression levels were identified using ELISA (see section 2.4.1) and expanded with increasing concentrations of MSX from 50 μM to 500 μM to generate a cell line with a high protein expression level.

2.3 Purification of recombinant Fc-fusion proteins

2.3.1 Production of recombinant Fc- fusion proteins

The recombinant Fc proteins were expressed by the transfected cells. The established transfected cells were expanded from one 25 cm^2 flask to ten 75 cm^2 flasks with DMEM medium containing 10% FCS (Invitrogen) and 100 U Penicillin & 100 μg/ml streptomycin, and 2 mM L-glutamine culture medium. Then, the cells were passaged into ten 175 cm^2 flasks and cultured with DMEM medium containing 10% low IgG FCS (Invitrogen), 100 U/ml penicillin & 100 μg/ml streptomycin, and 2 mM L-glutamine. The supernatants were harvested after the cells were grown for 2 weeks.

2.3.2 Purification of recombinant Fc fusion proteins

The recombinant Fc fusion proteins were purified using Econo-Pac protein A cartridge (Bio-RAD) following the manufacturer's guidelines. The supernatants were spun down at 5000 rpm for 30 minutes at 4^{o}C to remove cell debris before being loaded onto an Econo-Pac protein A cartridge at 4^{o}C. The cartridge was washed with 20-30 ml Binding Buffer (20 mM Sodium Phosphate Buffer PH7.0) at room temperature to remove non-specific proteins. The recombinant Fc fusion protein was eluted with Elution buffer (0.1 M Glycine HCl pH 3.0) and 1 ml was collected for each fraction and neutralised immediately with 25 µl of Neutralisation Buffer (1 M Tris. HCl pH 9.0). The fractions containing the proteins required were identified using LP Data View program (Bio-RAD), and the purified recombinant protein was dialysed in dialysis buffer (2% Nacl, 0.02% KCl, 0.024% KH_2PO_4, and 0.144% Na_2HPO_4) overnight at 4^{o}C. The protein concentration of each aliquot was estimated using the Coomassie Protein Assay (Pierce).

2.4 Protein Analysis and Detection

2.4.1 Enzyme linked immunosorbent assay (ELISA)

The Fc fusion protein expression levels of the transfected CHO cells were identified using the human IgG Fc antibody (CALTAC). 96 well plates (Dynex Technologies) were coated overnight at 4 ^{o}C with the appropriate concentrations of capture antibody, anti-HIgG purified antibody (1:1000 dilution) in 0.1 M NaHCO3, pH 8.2. The working volume was 50 µl per well. The plates were washed twice with 1 x PBS containing 0.05% Tween 20 (washing buffer), and

blocked for 2 h at 37^{o}C with 100 µl/well of 10% FCS in PBS (ELISA buffer). After washing four times with washing buffer, 50 µl samples were added in triplicate to the plate. Serial double dilutions of hIgG in triplicate were also added as standards. The plates were then incubated for 2 h at 37^{o}C to allow binding between cytokine and antibody. 50 µl of detection antibody (biotin anti-hIgGFc 1:1000 dilution) (CALTAC) in ELISA buffer was added after six washes, and the plate was incubated at 37^{o}C. After 1 h incubation, the plate was washed 6 times before adding 50 µl of 1:1000 dilution of ExtrAvidin (Sigma) in ELISA buffer in each well. The plate was incubated for another hour at 37^{o}C and washed 8 times. Finally, 100 µl/well of Microwell Peroxidase Substrate was added and the reaction was allowed to develop at room temperature in the dark until a sufficient colour product was visible (TMB, Gaitherburg). The plate was then read at 630 nm on a MRX II microplate reader (Dynex Technologies) reader.

2.4.2 SDS- PAGE

The purity of the recombinant protein was analyzed by SDS-PAGE following the manufacturer's protocol. In brief, the protein was denatured by heating to 70^{o}C for 10 minutes and loaded onto a NuPAGE 4-12% Bis-Tris Gel (Invitrogen). The gel was electrophoresed at 200 V, 400 mA until the dye front was close to the bottom, and the gel was rinsed in sterilized water before staining with Bio-safe Coomassie staining (BIO-RAD). After 1 h staining, the gel was destained with sterilized water until the band of the interest was clearly visible.

2.4.3 Western blot analysis of proteins

After running the SDS-PAGE (see section 2.4.2), instead of staining, the gel was transferred to a nitrocellulose membrane (Trans-Blot, BIO-RAD) in transfer buffer (20 mM Tris, 40 mM glycine, and 20% v/v methanol) for 1 h at 30V, 400 mA. The transferred membrane was incubated in blocking buffer (5% non-fat milk in PBS) for 2 h with shaking at room temperature before the primary antibody α-HIgG purified antibody (1:2000 dilution in blocking buffer) was added. After 1 h incubation with the primary antibody, the membrane was washed 3 times in washing buffer, and was incubated in blocking buffer with the DONKEY anti-goat IgG-HRP conjugate (1:4000 dilution in Blocking buffer) (Sigma) for 1 h. The membrane was washed 3 times with washing buffer before being developed by enhanced chemiluminescence (Amersham Pharmacia Biotech) and visualised by exposing to X-ray film (Kodak).

2.5 *In vitro* studies

2.5.1 Cells

In all the *in vitro* studies described below, the mouse cells were harvested from female BALB/c (H-$2^{d\text{-}d}$, IgM^{a}) mice, which were purchased from Harlan Olac. All mice were housed and treated in the Biological Service facilities in the University of Glasgow according to the local UK home office guidelines under specific pathogens free condition.

2.5.2 Regents

Table 2.2 List of cytokines used for *in vitro* culture

Cytokines	Source	Source	Concentration	Usage
IL-27	R&D Systems	murine	10 ng/ml to 100 ng/ml	cell culture
IL-23	R&D Systems	murine	20 ng/ml	cell culture
IL-6	R&D Systems	murine	20 ng/ml	cell culture
TGF-β	R&D Systems	murine	1 ng/ml	cell culture
IL-1β	BD Bioscience	murine	10 ng/ml	cell culture
IL-2	GlaxoSmithKline	human	10 ng/ml	cell culture

Table2.3 List of antibodies used for *in vitro* culture and FACS analysis

Antibodies (Clone No.)	**Source**	**Isotype**	**Concentration**	**Usage**
CD3(145-2C11)	BD Bioscience	Hamster IgG1	1 μg/ml (soluble) 2.5 μg/ml (plate-bound)	T cells activations
CD28 (37.51)	BD Bioscience	Syrian Hamster IgG2	1 μg/ml	Co-stimulatory signal for T cell activations
IL-4 (30340.11)	R&D Systems	Rat IgG1	10 μg/ml	Cell culture
IL-2	R&D Systems	Rat IgG2a	10 μg/ml	Cell culture
IFN-γ (XMG1.2)	BD Bioscience	Rat IgG1	10 μg/ml	Cell culture
CD16/32 (2.4G2)	BD Bioscience	Rat IgG2b	0.5 μg/100μl	Fc receptor blocking
FITC CD4 (RM4-4/5)	BD Bioscience	Rat IgG2a	0.5 μg/100μl	FACS
PE-CD25 (PC61/7D4)	BD Bioscience	Rat IgG2b	3.6 μg/10^8 cells	T cell separation FACS
Alexa Fluor-IL-17	e Bioscience	Rat IgG1	0.5 μg/10^6cells	FACS
PE-IL-17A	e Bioscience	Rat IgG2a	0.5 μg/10^6cells	FACS
PE-Foxp3	e Bioscience	Rat IgG2a	1 μg/10^6cells	FACS
PE-IFN-γ	BD Bioscience	Rat IgG1	0.5 μg/10^6cells	FACS

Antibodies (Clone No.)	**Source**	**Isotype**	**Concentration**	**Usage**
FITC Rat IgG2a	BD Bioscience	Rat IgG2a	0.5 μg/100μl	FACS
PE Rat IgG2b	BD Bioscience	Rat IgG2b	3.6 μg/10^8 cells	FACS
Alexa Fluor-Rat IgG1	e Bioscience	Rat IgG1	0.5 μg/10^6cells	FACS
PE Rat IgG2a	e Bioscience	Rat IgG2a	0.5 μg/10^6cells	FACS
PE Rat IgG1	BD Bioscience	Rat IgG1	0.5 μg/10^6cells	FACS

2.5.3 Preparation of Lymphocytes

Single cell suspensions were obtained by forcing murine lymph nodes and spleens that were prepared in cold RPMI1640 medium through sterile Nitex (Cadisch & Sons). The cell suspensions were centrifugated at 400 g for 5 minutes and resuspended in 1 ml red cell lysis buffer (Sigma) for exactly 1 minute, 20 ml cold RPMI 1640 containing 100 U/ml penicillin & 100 μg/ml streptomycin was added to inhibit the lysis. The cell suspensions were washed 3 times with centrifugation, before being resuspended in RPMI 1640 Complete Medium.

2.5.4 T cell separation –autoMACS (Miltenyi Biotech)

2.5.4.1 Negative selection of $CD4^+$ T cells

The cell suspensions (see section 2.5.3) in RPMI 1640 Complete Medium were incubated in 75 cm^2 flasks at 37^oC in a humidified incubator with 5% CO_2 for 1 h, which allowed adherent cells to adhere to the flask. Then, non-adherent cells including the supernatant were collected and centrifuged at 400 g for 5 minutes and re-suspended in 20 ml cold MACS Buffer (1 x PBS without Calcium, Magnesium, Invitrogen, 2% FCS, 100 U/ml penicillin & 100 μg/ml streptomycin). 10 μl of cells were counted with a Neubauer haemocytometer (Weber Scientific International) by diluting in 10 μl of a cell counting fluid (0.01 M acetic acid, 0.1% trypan blue in PBS). The remaining cells were centrifuged at 400g for 5 minutes and incubated with the cocktail of biotin-conjugated monoclonal antibodies [against CD8a (Ly-2, Rat IgG2a), CD11b (Mac-1, Rat IgG2b), CD45R (B220, Rat IgG2a), DX5 (Rat IgM) and Ter-119 (Rat IgG2b)]

(Miltenyi Biotech), at a concentration of 10 μl antibody per 10^7 total cells in 40 μl MACS Buffer for 15 minutes at 4 ^oC. Anti-biotin microbeads (20 μl per 10^7 cells in 80 μl MACS Buffer) were added to the cells and incubated for another 15 minutes. The cells were then washed with 40 ml MACS Buffer, centrifuged at 300g for 10 minutes, and re-suspend in 6 ml MACS Buffer. $CD4^+$ T cells were then negatively selected by an AutoMACS machine according to Manufacture's protocol. The purity of $CD4^+$ T cells was determined by Flow Cytometry (see section 2.5. 6). Routinely the purity of $CD4^+$ T cells was ⩾95%.

2.5.4.2 Positive selection of $CD4^+CD25^+$ T cells and negative selection of $CD4^+CD25^-$ T cells

The selected $CD4^+$ T (see section 2.5.4.1) were incubated with PE-CD25 antibody (3.6 μg per 10^8 cells) in 1 ml MACS Buffer at 4^oC in the dark for 15 minutes, and then washed twice with 20 ml MACS Buffer. The cells were then incubated with anti-PE microbeads (36 μl per 10^8 cells) in 1 ml MACS buffer at 4^oC in the dark for another 15 minutes. Cells were washed by centrifugation at 300g for 10minutes and re-suspend in 4 ml MACS Buffer. Magnetically labelled $CD4^+CD25^+$ T cells were sorted on AutoMACS by positive selection according to Manufacture's protocol. The depleted cells were $CD4^+CD25^-$ T cells. The purity of $CD4^+CD25^+$ T cells were analysed by Flow Cytometry (see section 2.5. 6), routinely ⩾ 90%.

2.5.5 Culture and stimulation of murine $CD4^+$, $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells

2.5.5.1 T cells were cultured in two culture conditions

1). In U-bottom 96 well plates (Nunc), 3 x 10^4 of $CD4^+$, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells per well were cultured in triplicate with an equivalent number of mitomycin C (50 μg/ml Sigma) treated APC in 200 μl of RPMI1640 complete medium in the presence of 1 μg/ml of anti-CD3. Various concentrations of murine cytokines were added to cell culture as indicated in the text.

2). 1 x10^5 $CD4^+$, $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells per well were cultured in triplicate in anti-CD3 (2.5 μg/ml) coated 96-well flat bottom plates in 200 μl of RPMI1640 complete medium in the presence of 1 μg/ml of anti-CD28. Various concentrations of murine cytokines were added to cell culture as indicated in the text.

2.5.5.2 Established Th17 cell culture

$CD4^+CD25^-$ T cells (3x 10^4 cells/well) were cultured with anti-CD3 (1 μg/ml) and mitomycin C (50 μg/ml)-treated APC (3x 10^4 cells/well) under optimal Th17 culture conditions: IL-6 (20 ng/ml), IL-1β (10 ng/ml) and TGF-β (1 ng/ml), and the neutralizing antibodies anti-IL-4 (10 μg/ml), anti-IFN-γ (10 μg/ml) and anti-IL-2 (10 μg/ml). After 4 days, activated cells were washed and re-stimulated with anti-CD3 (1 μg/ml) and APC (3x 10^4 cells/well) in the presence of IL-23 (20

ng/ml) for another 3 days. Various concentrations of IL-27 were added to cell culture as indicated in the text.

2.5.5. 3 Regulatory T cell suppressive function assay

Regulatory T cell suppressive function assays were set up in 96-well U-bottom plates. In brief, $CD4^+CD25^-$ T cells (3x 10^4 cells/well used as responders) were co-cultured with an equivalent number of freshly purified or activated $CD4^+CD25^+$ T cells as regulatory T cells in the presence of anti-CD3 (1 μg/ml) and APC (3x 10^4 cells/well) in 200 μl of RPMI1640 complete medium.

2.5.6 Proliferation Assay

Proliferation assay was performed in 96-well U-bottom or 96-well flat bottom plates. Generally, after 3- 4 days cell culture, 100 μl/well of supernatant was harvested and 100 μl/ well of 1 μCi of [^{3}H]-thymidin (Amersham Pharmacia Biotech) was added and cultured for 8-12 hours. Plates were harvested onto a glass fibre filter mat (Packard) using a Micromate 196 Harvester (Packard) and ^{3}H -TdR uptake was counted on a Betaplate counter (Wallac).

2.5.7 Flow Cytometry

2.5.7.1 CFSE staining

Before setting up cell culture, $CD4^+CD25^-$ T cells were incubated in PBS containing 2 μM CFSE (Sigma) for 15 minutes at 37^{o}C in the dark. The cells

were then washed twice with RPMI1640 complete medium and pelleted by centrifugation at 400 x g for 5 minutes and re-suspended in a volume of 200 µl 1 x PBS Buffer (Invitrogen) containing 2% FCS and100 U Penicillin & 100 µg/ml streptomycin. The cells were checked for cell cycles by Flow Cytometry (FACScan, BD Bioscience) and analyzed using Cellquest software (BD Bioscience).

2.5.7.2 Surface staining

Cell surface markers were performed by surface staining. The cells were transferred to FACS Tube (BD Bioscience) and washed by MACS Buffer. After centrifugation (300 g, 5 minutes), the cells were incubated with anti-CD16/CD32 antibody (0.5 µg/100 µl) for 15 minutes to block Fc receptors and non-specific binding. Then, the appropriate antibodies were added as indicated in the text and incubated for 20 minutes at 4^{o}C in the dark. The cells were then washed twice with MACS Buffer, re-suspended in 200 µl of MACS buffer and analyzed by Flow Cytometry.

2.5.7.3 Intracelluar Cytokine Staining

IL-17-producing cells, IFN-γ-producing cells and Foxp3 expressing cells were determined by intracellular sytokine staining. In general, cells were stimulated for 5 hours with phorbol myistate acetate (BD Bioscience) and ionomycin (BD Bioscience) with Golgistop (BD Bioscience) added after 1hr. Cell stimulation was terminated by fixing in freshly prepared Fixation/Permeadbilization working

solution (BD Bioscience). Fixed cells were stained with fluorescent-conjugated antibodies in Permeabilization Buffer for 20minutes (4^{o}C, Dark), and then washed, and analyzed with Flow Cytometry.

2.5.8 Measurement of Cytokine Production

Cytokine production was measured by ELISA as described in section 2.4.1. Most of the paired capture and biotinylated detection antibodies were purchased from BD Bioscience unless specified. Details of the concentrations of antibody used and the lower limit of detection for each assay are given in Table 2.4. IL-1Ra production was measured using Quantikine IL-1Ra Immunoassay Kit (R&D Systems) according to the manufacturer's protocol.

Table 2.4 List of cytokine antibodies used for ELISA

Cytokine	**Capture Antibody (μg/ml)**	**Detection Antibody (μg/ml)**	**Lower Limit of Detection of Assay (pg/ml)**
IL-2	1	1	10
IFN-γ	1	0.5	40
IL-17	0.5	0.5	10
IL-6	0.5	0.5	10
IL-4	1	1	10
IL-10 (Biosource)	1.25	0.06	10
IL-12	2	0.5	20
TNF-α	1:250 (Volume)	1:250 (Volume)	5

2.5.9 Real-Time PCR

To quantify message levels, Real-time PCR was performed using an ABI prism 7700 sequence detector (Applied Biosystems) according to the manufacturer's instruction. All the primers and probes were purchased from Applied Biosystems (Listed in the table 2.5).

1 μl cDNA template converted from mRNA was mixed with 12.5 μl Master mix (10 mM dNTP mix, 5 mM $MgCl_2$, 0.625 U AmpliTaqGold^{TM}, 0.25 U AmErase N-Glycosylase, 10 x qPCR^{TM} buffers), 1 μl probe, and 0.75 μl of each of the primers. The total volume of each sample was adjusted to 25 μl with DEPC H_2O. The mixed sample was loaded to a Thermo-Fast 96 semi-skirt plate (ABgene) and amplified on an ABI prism 7700 sequence detector under following reaction cycles: the first cycle was 50^{o}C for 2 minutes and followed at 95^{o}C for 10 minutes, and the remaining 45 cycles were 95 ^{o}C for 15 seconds and 60 ^{o}C for 1 minute. Data analysis was performed using Sequence Detector Software (Applied Biosystems). The cDNA levels during the liner phase of amplification were normalized with respect to the levels of 'housekeeping' gene encoding HPRT (hypoxanthine phosphorbosyltransferase). The results were presented as relative mRNA expression level.

Table 2.5 List of Primers and Probes used for Real time PCR

Gene	Forward Primer	Reverse Primer	Probe (5'-FAM&3'-TAMRA)
Foxp3	5'-CCCAGGAAAG ACAGCAACCTT-3'	5'-TTCTCACAAC CAGGCCACTTG-3'	5'-ATCCTACCCACTGC TGGCAAATGGAGT-3'
HPRT	5'-GCAGTACAGC CCCAAAATGG-3'	5'-AACAAAGTCTGG CCTGTATCCAA-3'	5'- TAAGTTGCAAGCT TGCTGGTGAAAAGGA -3'
T-bet	5'-GCCAGGGAACC GCTTATATG-3'	5'-AACTTCCTGGCG CATCCA-3'	5'-CCCAGACTCCCCC AACACCGGA-3'
GATA-3	5'-TCCTCCTCTACG CTCCTTGCTA-3'	5'-ACACTGATTCCT TGGCGCTC-3'	5'-TCGTGATCGGAAG AGCAACCGTCTC-3'

2. 6 *In Vivo* studies: Collagen- induced Arthritis (CIA)

2.6.1 Animal

All the *in vivo* studies described below were carried out under a project licence provided by UK home office and was performed to address scientific questions that could not be investigated by *in vitro* studies alone. The mice used were male DBA/1 mice purchased from Harlan Olac. All mice were kept in the Biological Service facilities in the University of Glasgow according to the local UK home office guidelines under specific pathogens free condition.

2.6.2 Induction and treatment of CIA

CIA was induced in mice as previously described (Ruchatz 1998). Briefly, male DBA/1 mice (6-8 weeks) were immunised with 100 μg of Bovine type II collagen (MD Bioscience) in Freund's complete adjuvant (MD Bioscience) by intradermal injection (day 0), and boosted intraperitoneally (i.p.) on day 21 with collagen II (100 μg in PBS). Daily injections of IL-27-Fc (2 μg/mouse/day), IL-35-Fc (2 or 1 μg/mouse/day), IL-23-Fc (2 μg/mouse/day), EBI3-Fc (2 μg/mouse/day) or PBS were administered i.p. for 10 days starting at different time points (day 21 or 27) as indicated in the text.

2.6.3 Clinical assessment of CIA

Mice were monitored every other day for signs of arthritis, and the disease severity was recorded following a scoring system for each limb: 0 = normal, 1 =

erythema, 2 = erythema plus swelling, 3 = extension/loss function and total score = sum of four limbs. Paw thickness was measured with a dial-caliper (Kroeplin).

2.6.4 Histological assessment

Mice were sacrificed and the hind limbs removed, fixed in 10% formalin for 3 weeks, and decalcified with 35% (v/v) formic acid and 13% (w/v) sodium citrate for another 2-3 weeks. Then, the tissues were fixed in wax and cut in to 5 μm sections and stained with haematoxylin and eosin. Quantification of arthritis in each joint were scored blindly, based on the degree of inflammation, synovial hyperplasia and erosion as described previously (Joosten 1997).

2.6.5 *Ex Vivo* cell culture

Splenocyte were harvested from immunized mice at the indicated time-points, and single suspension of spleen cells were prepared as described in section 2.5.3). Spleen cells were cultured at 2 $x10^6$ cells per ml for up to 96 hours in RPMI 1640 Complete Medium with graded concentrations of type II collagen at 37°C in 5% CO_2. Proliferation assays were performed in triplicate in U-bottom 96-well plates as previously described in section 2.5.6. Supernatants from parallel triplicate cultures were estimated for cytokine contents by ELISA.

2.6.6 Serum anti-Collagen Antibodies ELISA

Anti-collagen Abs titers in serum obtained at the end of treatment of immunized mice were measured by ELISA. Briefly, 96-well plates (Dynex Technologies) were coated overnight at 4°C with 4 μg/ml bovine type II collagen in 0.1M

NaHCO3, pH8.2, blocked, and serially-diluted serum were added. Total IgG was detected with horseradish peroxidase-conjugated goat anti-mouse IgG (Genzyme), and biotin-conjugated anti-mouse IgG1 and IgG2a (BD Bioscience). The plates were developed as described in section 2.4.1 and read at 630nm.

2.6.7 Multiplex Bead Assay-10 plex luminex

Serum cytokine production was also analysed by Multiplex Bead Assay (Biosource) according to the manufacturer's instructions. The Assay contained 10 cytokines: GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40 and TNF-α. The principle of this method is a sandwich immunoassay where specific antibodies have already been coated to the surface of fluorescently encoded microspheres. Each microsphere is labelled with a distinguishable fluorphore that allows it to be assigned or gated to a particular region by the scanner. Briefly, cytokine capture microspheres were first incubated with serum samples and followed by biotinylated detection antibodies. Relevant standards used for quantitative analysis were added at the same time as serum samples. Finally, streptavidin-RPE was added and the fluorescence bound to the microspheres was analysed using Luminex XMAP^{TM} system. The fluorescence intensity was proportional to the concentration of cytokines present in the samples.

2.7 Statistical analysis

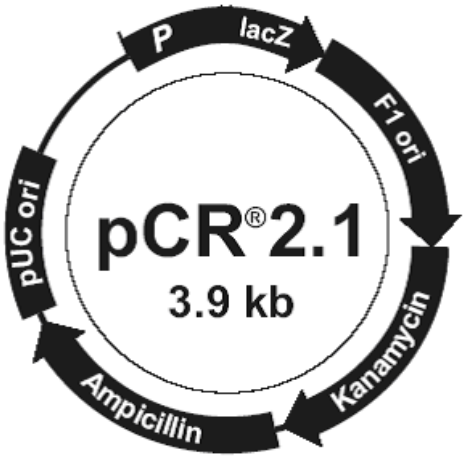
Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's Test, or Mann-Whitney *U* test as indicated in the text. Statistical significance was defined as *$p<0.05$, or **$p<0.01$.

2.8 List of plasmid vectors

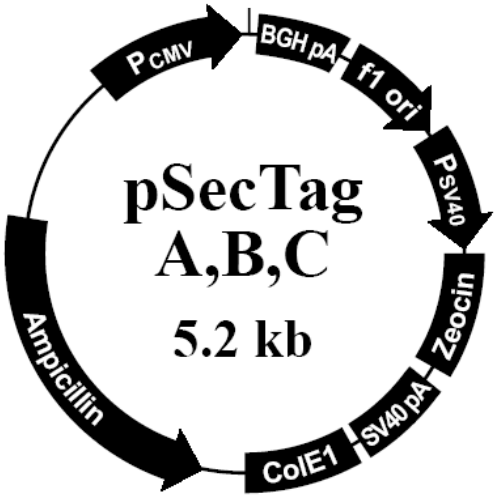
Plasmid vectors used for cloning, transfection or expression of recombinant proteins are listed in Figure 2.1.

Figure 2.1 List of Plasmid vectors

pCR2.1 vector



pSecTag2A



pEE14.4-1

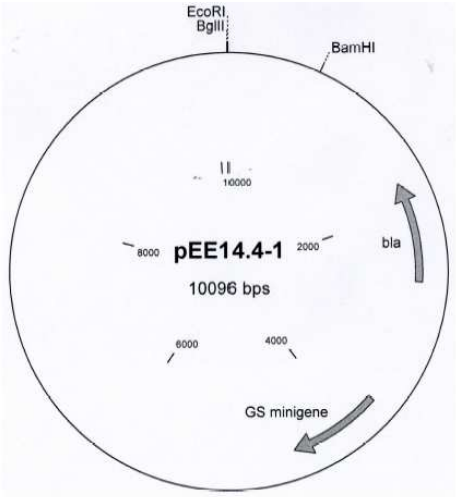


Table 2.6 List of suppliers' details

Company	**Address**
ABgene Ltd	Abgene House, Blenheim Road, Epsom KT19 9AP, UK
Amsersham Pharmacia Biotech Ltd	Amersham Place, Little Chalfont, Buckinghamsire HP7 9NA, UK
Applied Biosystems	850 Lincoln Centre Drive, Foster City, CA 94404, USA
BD Bioscience Ltd	21 Between Town Road, Cowley, Oxford OX4 3LY,UK
BioSource ^{TM}	Rue de L'industrie, 8B-1400 Nivelles, Belgium
Bio-RAD Labtoratories	Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX
Carisch & Sons company	745 High Rd, London, N12 0BP UK
Chondrex Inc C/o MD Biosciences	Morwell Diagnostics GmbH, Gewerbestrasse 9, Postfach 8132, Zuriich Switzerland
DNASHEF Technologies	Edinburgh Royal Infirmary, 1 Lauriston Place, Edinburgh EH3 9YW, UK
Dynex Technologies	Columbia House, Columbia Drive, Worthing, West Sussex, BN13 3HD, UK
eBiosciences C/o Insight Biotechnology Ltd	Wembley Commercial Centre, East Lane, Wembley HA9 7XX
Eppendorf UK Ltd c/o Helena Biosciences Europe Ltd	Queensway South, Team Valley Trading Estate, Gateshead, Tyne & Wear NE11 0ZF, UK
Genzyme ltd	12 Rookwood Way, Haverhill, Suffolk, CB9 8PU, UK

Company	Address
Harlan Olac	Shaw's Farm, Bicester, Oxon Ox25 1TP, UK
Invitrogen Life Technologies	3 Fountain Drive, Inchinnan Business Park, Paisley, UK
JRH Biosciences	West Portway Industrial Estate, Andover, Hempshire, SP10 3LF
Kodak Ltd	Kodak House, Sation Road, Hemel Hempstead, Hertfordshire, HP 1 1JU,UK
Lonza Biologics Ltd	228 Bath Road, Slough, Berkshire SL1 4DX, UK
Miltenyi Biotec Ltd	Almac House, Church Lane, Bisley, Surrey GU24 9DR
Novagen Merck Biosciences Ltd	Boulevard Industrial Park, Padge Road, Beeston, Nottingham, NG9 2JR
Packard Instrument Company	800 Research Parkway Meriden, CT 06450 USA
Pierce c/o Perbio Science UK Ltd	Unit 9, Atley Way, North Nelson Industrial Estate Cramlington, Northumberland NE231 WA
Qiagen Ltd	QIAGEN House, Fleming Way, Crawley, West Sussex, RH10 9NQ, UK
Roche Diagnostics Ltd	Bell Lane, Lewes, East Sussex BN7 1LG, UK
R&D Systems Europe Ltd	19 Barton Lane, Abingdon Science Park, Abingdon, Oxon OX14 3NB

Company	Address
Sigma-Aldrich Company Ltd	Fancy Road, Poole, Dorset BH12 4QH
VWR International Ltd	Haasrode Researchpark Zone 3, Geldenaaksebaan 464 Leuven B309
Wallac Oy	Mustionkatu 6, 20101 Turku, Finland

Chapter 3

Cloning and expression of murine IL-27-Fc, IL-35-Fc and human IL-35-Fc with GS system

3.1 Cloning and expression of murine IL-27-Fc and IL-35-Fc with GS system

3.1.1 Introduction

The murine cDNA encoding IL-27-Fc and IL-35-Fc were successfully cloned in our lab and constructed into pSecTag2A vector (Invitrogen). Schematic representation of recombinant murine IL-27-Fc and IL-35-Fc are shown in Figure 3.1. However, the production of these recombinant proteins expressed from that vector was low. In order to produce large quantities of IL-27-Fc and IL-35-Fc for *in vivo* studies, it was necessary to choose a new mammalian expression system. GS system, as described in previous material and method, is a highly efficient mammalian expression system, and provides a way to produce recombinant proteins in high quantities. Therefore, the general approach underlying the cloning of IL-27-Fc and IL-35-Fc from the previous expression vector into GS vector was to get the higher amount of the expression of recombinant proteins with GS system. As a comparison, IL-12-Fc and IL-23-Fc previously constructed in pSecTag2A vector were also transferred into GS system.

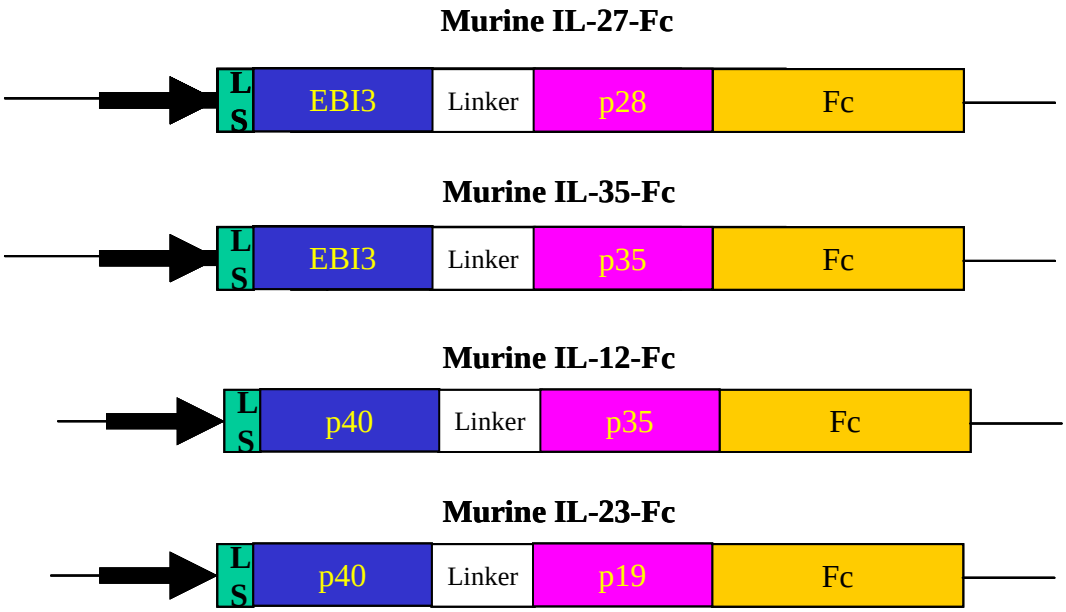


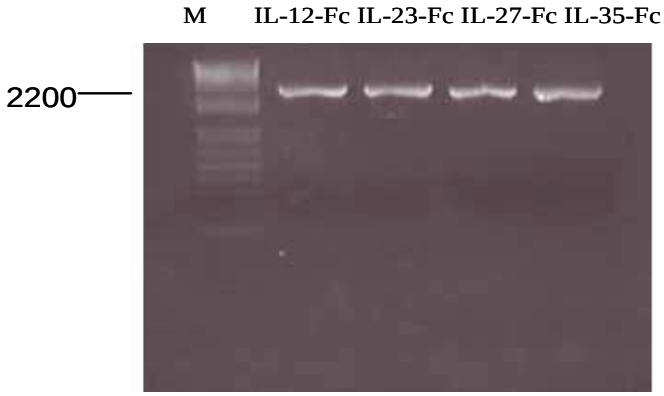
Figure 3.1 Schematic representation of recombinant murine IL-27-Fc, IL-35-Fc, IL-12-Fc, and IL-23-Fc.

The fusion protein includes the leader sequence, Pro/Thr rich regions of the native IL-12p40, IL-27 EBI3, linker, Pro/Thr region of IL-12p35, IL-23p19, IL-27p28, and the Fc part of human IgG1 at their C-terminus.

3.1.2 Cloning of the cDNA insert encoding IL-27-Fc, IL-35-Fc, IL-12-Fc, and IL-23-Fc

PCR amplification, using the pSecTag2A vectors encoding IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc as templates respectively, resulted in around 2200 bp PCR fragments containing the cDNA encoding IL-35-Fc, IL-12-Fc, IL-27-Fc, and IL-23-Fc with the Bgl II site insertion on both ends (Figure 3.2 A). These above 2200 bp PCR fragments were cloned into the PEE14.4-1 GS vector via the Bgl II site in the polylinker respectively. The sequence identities of these cloned products were confirmed by restriction enzyme mapping respectively (Figure 3.2 B, C, D).

A.



B. C. D.

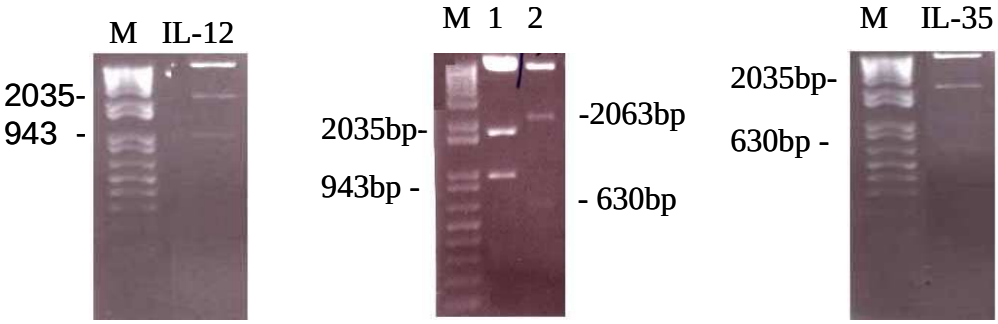


Figure 3.2 Cloning of the cDNA insert encoding murine IL-12-Fc, IL-23-Fc, IL-27-Fc and IL-35-Fc.

(A) PCR was performed on pSecTag2A vector with interest inserts to obtain PCR fragments encoding murine IL-12-Fc, IL-23-Fc, IL-27-Fc, and IL-35-Fc, and a Bgl II site was inserted at both ends of these fragments. (B) BamH I digestion of mIL-12-pEE14.4 cDNA resulted in 2035 bp, 943 bp, and 273 bp fragments, but 273 bp fragment was too small to be visualized on gel. (C) BamH I digested mIL-23-pEE14.4 cDNA (1) and mIL-27-pEE14.4 cDNA (2) to obtain 2035 bp and 943 bp, 2063 bp and 630 bp fragments. (D) BamH I digestion of m IL-35-Fc-pEE14.4 DNA resulted in 2035 bp and 630 bp fragments. (The ladder used is 1kb+ DNA ladder)

3.1.3 Expression of IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc with GS system

These constructs of IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc within GS vector were transfected into CHO-K1 cells, expression colonies were selected out by detecting the Fc fusion part from the cell culture supernatants using hIgG Fc ELISA, which is described in material and methods. With the increasing concentrations of MSX, the expressing cell lines were educated to produce higher and stable recombinant protein production. The expression level in the culture supernatants from GS system was 10 times higher compared to the expression level from the previous cloning (Figure 3.3). However, the protein expression from IL-27-Fc and IL-35-Fc colonies was still much lower than the expression from IL-12-Fc and IL-23-Fc colonies. The expressing cells were washed and lysated, and the lysates proteins were checked for levels of recombinant proteins which were maintained inside the cells by western blot. As shown in Figure 3.4, there were no proteins recognized by human IgG antibody. This indicated that the recombinant proteins with Fc fusion part were all secreted outside CHO-K1 cells.

The recombinant fusion proteins IL-23-Fc, IL-27-Fc, IL-35-Fc were purified using Econo-Pac protein A cartridge. The purity of the recombinant proteins were analysed by SDS-PAGE (Figure 3.5). The recombinant proteins were also analysed by western blot. Monoclonal antibody directed against human IgG resulted in IL-23-Fc band of 87.3 kd, IL-27-Fc of 78.8 kd, IL-35-Fc band of 80 kd, and IL-12 band of 88.9 kd (Figure 3.6). Monoclonal antibody against murine IL-12 p40 recognized the same protein bands of IL-12-Fc and IL-23-Fc, but not

IL-27-Fc and IL-35-Fc (Figure 3.6). No additional bands were visible using these two detection systems.

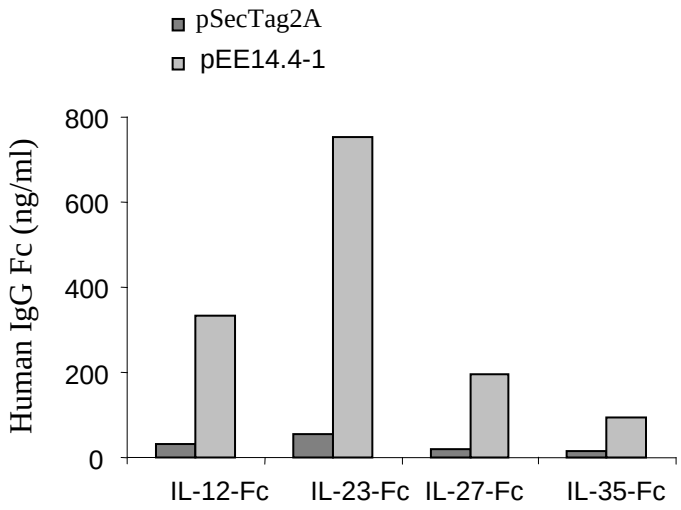


Figure 3.3 Comparison of the levels of Fc fusion proteins expression from pSecTag2A vector and pEE14.4-1 vector.

The selected stable high-yielding IL-12-Fc, IL-23-Fc, IL-27-Fc and IL-35-Fc cell lines from pSecTag2A vector and pEE14.4-1 vector were cultured in the 175 cm^2 size of flasks for 5 days, and 150 μl of supernatants were removed and analyzed for Fc fusion protein expression by human IgG Fc ELISA. The cell line transfected with pEE14.4-1 vector encoding the insert genes yielded 10 times high levels of Fc fusion protein compared with the cell line transfected with pSecTag2A vector encoding the insert genes.

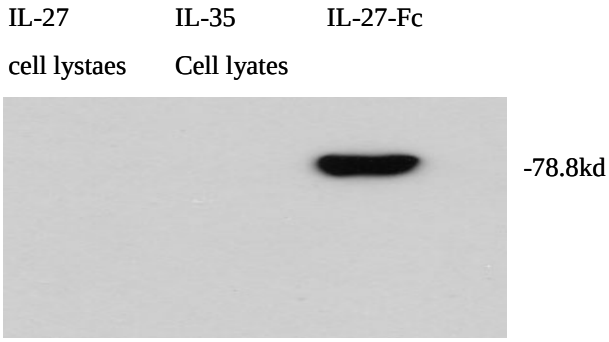


Figure 3.4 Western Blot analysis of cell lysates.

CHO-K1 cells respectively expressing IL-27-Fc and IL-35-Fc were harvested, washed and lysated. Cell lysates were detected by human IgG-Fc antibody. There were no proteins recognized by human IgG antibody. This indicated that the recombinant proteins with Fc fusion part were all secreted outside CHO-K1 cells. IL-27-Fc protein was used as a positive control.

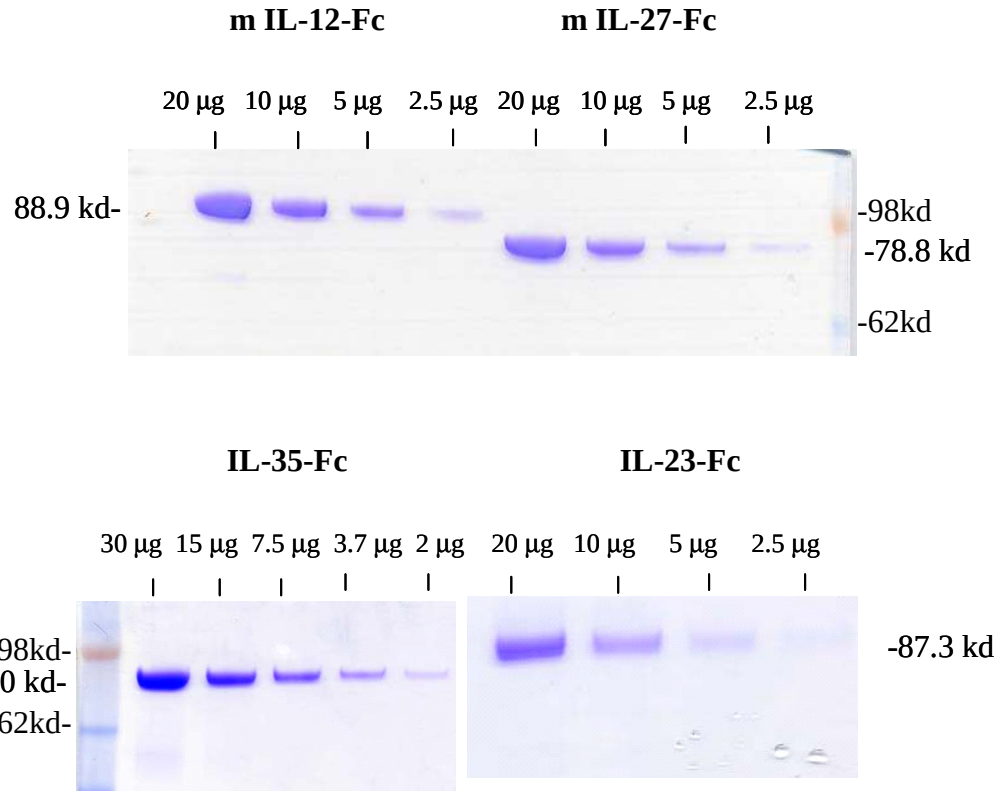


Figure 3.5 SDS-PAGE analysis of purified recombinant proteins.

Various amounts of purified recombinant proteins IL-12-Fc, IL-27-Fc, IL-35-Fc and IL-23-Fc were run on SDS-PAGE, and stained with Coomassie blue to demonstrate their purities. The molecular weights of these products were the same as they were designed (The ladder used is SeeBlue Plus2 from Invitrogen).

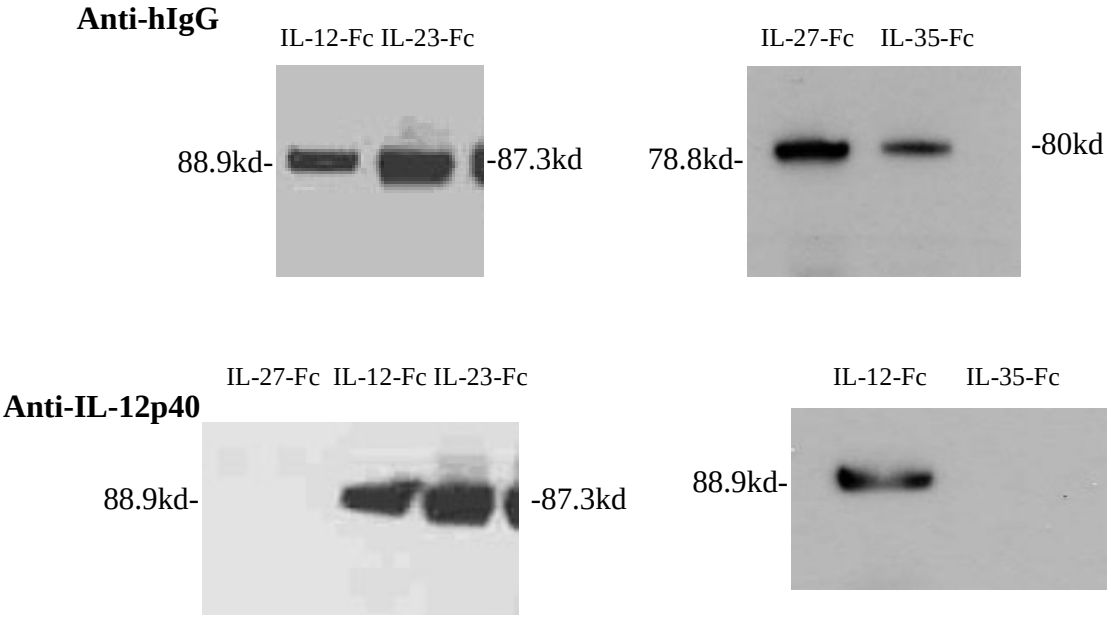


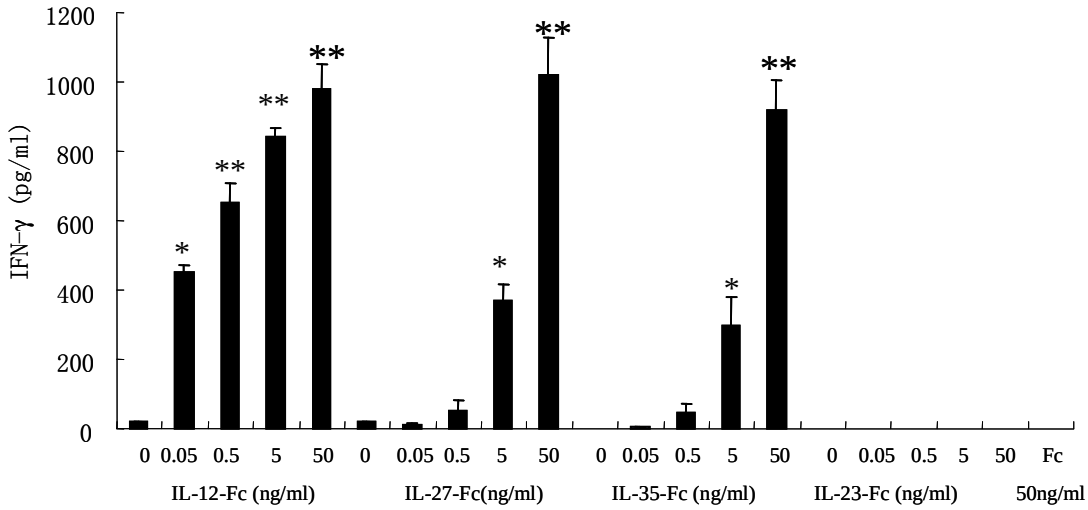
Figure 3.6 Western blot analyses of purified recombinant proteins.

The purified recombinant proteins mIL-12-Fc, m IL-35-Fc, mIL-27-Fc and mIL-23-Fc were demonstrated in western blot by two detecting antibodies: anti-hIgG and anti-IL-12p40. Both antibodies detected the same protein bands of 88.9 kd of mIL-12-Fc and 87.3 kd of mIL-23-Fc. 78.8 kd size of m IL-27-Fc and 80 kd size of IL-35-Fc were only detected by anti-hIgG.

3.1.4 Bioactivity of the recombinant proteins IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc

The recombinant proteins IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc were assessed for their biological activities *in vitro*. $CD4^+$ T cells isolated from BALB/c mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28, and the increasing concentrations of recombinant proteins IL-35-Fc, IL-27-Fc, IL-12-Fc and IL-23-Fc were added to the beginning cell culture. After 72 h, the culture supernatants were harvested and analyzed for IFN-γ by ELISA. As expected, IL-12-Fc and IL-27-Fc stimulated the production of IFN-γ from $CD4^+$ T cells in a dose dependent manner (Figure 3. 7). Also, IL-35-Fc stimulated $CD4^+$ T cells to produce IFN-γ in a dose dependent manner (Figure 3.7), which will be discussed in chapter 5. IgG Fc did not induce IFN-γ on $CD4^+$ T cells, which indicated that the Fc part on the recombinant proteins did not play any role to affect the biological function of these recombinant proteins. As expected, IL-23-Fc did not enhance the production of IFN-γ, because IL-23 does not act on naïve murine $CD4^+$ T cells to induce IFN-γ (Figure 3.7 A). To further test the activity of IL-23, the culture supernatants were analyzed for IL-17 by ELISA. IL-23 enhanced the secretion of IL-17 （ Figure 3.7 B）.

A



B

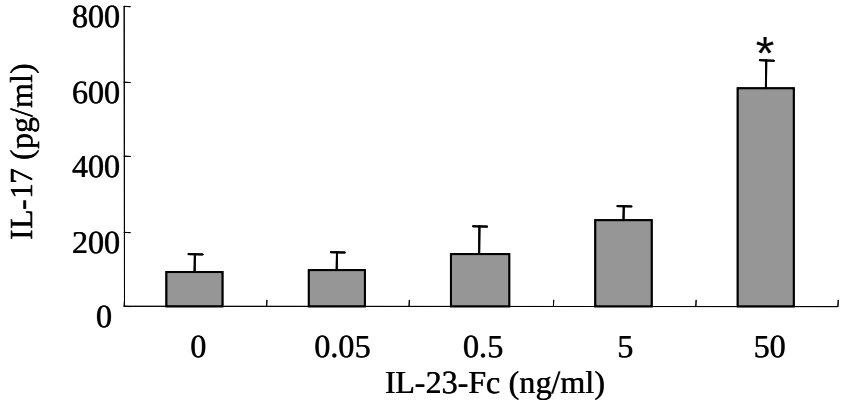


Figure 3.7 Effect of purified recombinant proteins on $CD4^+$ T cells.

1 $x10^5$ $CD4^+$ T cells isolated from BALB/c mice were stimulated with plated-bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (1 μg/ml), and the increasing concentrations of recombinant proteins IL-35-Fc, IL-12-Fc, IL-27-Fc, and IL-23-Fc (0.05, 0.5, 5, 50 ng/ml) were added to the cell culture. After 72 h, the culture supernatants were harvested and analyzed for IFN-γ (A) and IL-17 (B) by ELISA. Data are Mean ± SD, ** $p< 0.01$, *$p< 0.05$ compared to control, by student's *t* test.

3.2 Cloning and expression of human IL-35-Fc

3.2.1 Introduction

The biological role in mice might be different from the biological role in humans. The human recombinant IL-35-Fc fusion protein was designed to investigate the biological effects of the human IL-35 heterodimer in humans. The cloning and construction of the IL-35-Fc gene was to express the two soluble fragments of EBI3 and p35 fused with a flexible linker and tagged with human IgG-hinge Fc (Figure 3.8).

Figure 3.8 Schematic representation of recombinant human IL-35-Fc.

This recombinant protein includes the leading sequence, encoding the protein rich region of EBI3 and p35 linked with a flexible linker sequence, and the Fc part of human IgG at the C-terminus.

3.2.2 Cloning of the cDNA insert encoding human IL-35-Fc

Human cDNA encoding EBI3 was prepared by RT-PCR from mRNA that was extracted from LPS and IFN-γ stimulated human DCs. Using specific primers, PCR amplification resulted in a PCR product of 690 bp (Figure 3.9 A). This band was extracted from the gel and subsequently cloned into the pCR2.1 TA vector (Figure 3.9 B). A Bgl II site silent mutation was introduced into the TA vector carrying the EBI3 insert by PCR. The template vector without the silent mutation site was digested by Dpn I, and the remaining amplified DNA vector was sequenced using M13R and T7 primers to confirm the sequence with silent mutation (Figure 3.10). Then, using this DNA vector as a template, another round of PCR amplification was performed to yield a 665 bp fragment with a Bgl II site insertion on both ends (Figure 3.9 C). This product encoding EBI3 with the Bgl II enzyme site on both ends was transfered into the BamH I site of vector pSecTag2A containing the insert of Fc and a flexible linker (named as vector pSec-Linker-Fc). This was confirmed by restriction enzymes mapping (Figure 3.9 D, E).

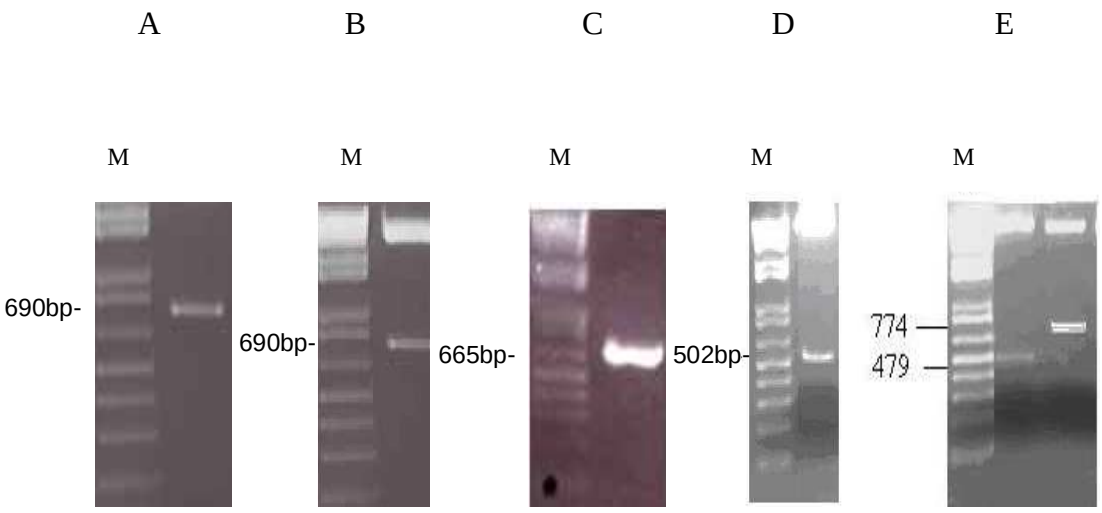


Figure 3.9 Cloning of the cDNA encoding human EBI3.

cDNA encoding EBI3 derived from DC was obtained by RT-PCR (A). The PCR fragment encoding EBI3 was cloned into the TA pCR2.1 vector (B). The PCR fragment was amplified from cloned EBI3 TA vector containing the silent mutation, and a Bgl II site was introduced at both ends (C). Pst I digested vector pSec-Linker-Fc inserted with EBI3-Fc to obtain a 502 bp fragment (D). Bam HI and Hind III digested pSec-Linker-Fc vector inserted with DNA fragment encoding EBI3 to obtain a 479 bp fragment, and Not I and Hind III together digested pSecTag2A vector inserted with EBI3 to obtain a 774 bp fragment (E). (The ladder used is 1kb+ DNA ladder).

Cloning
ATGACCCCGCAGCTTCTCCTGGCCCTTGTCCTCTGGGCCAGCTGCCCGCCCTGCAGTG
ATGACCCCGCAGCTTCTCCTGGCCCTTGTCCTCTGGGCCAGCTGCCCGCCCTGCAGTG
Genebank
GAAGGAAAGGGCCCCCAGCAGCTCTGACACTGCCCCGGGTGCAATGCCGAGCCTCTC
GAAGGAAAGGGCCCCCAGCAGCTCTGACACTGCCCCGGGTGCAATGCCGAGCCTCTC

GGTACCCGATCGCCGTGGATTGCTCCTGGACCCTGCCGCCTGCTCCAAACTCCACCA
GGTACCCGATCGCCGTGGATTGCTCCTGGACCCTGCCGCCTGCTCCAAACTCCACCA

GCCCCGTGTCCTTCATTGCCACGTACAGGCTCGGCATGGCTGCCCGGGGCCACAGCT
GCCCCGTGTCCTTCATTGCCACGTACAGGCTCGGCATGGCTGCCCGGGGCCACAGCT

GGCCCTGCCTGCAGCAGACGCCAACGTCCACCAGCTGCACCATCACGGATGTCCAGC
GGCCCTGCCTGCAGCAGACGCCAACGTCCACCAGCTGCACCATCACGGATGTCCAGC

TGTTCTCCATGGCTCCCTACGTGCTCAATGTCACCGCCGTCCACCCCTGGGGCTCCAG
TGTTCTCCATGGCTCCCTACGTGCTCAATGTCACCGCCGTCCACCCCTGGGGCTCCAG

CAGCAGCTTCGTGCCTTTCATAACAGAGCACATCATCAAGCCCGACCCTCCAGAAGG
CAGCAGCTTCGTGCCTTTCATAACAGAGCACATCATCAAGCCCGACCCTCCAGAAGG

CGTGCGCCTAAGCCCCCTCGCTGAGCGCCAGCTACAGGTGCAGTGGGAGCCTCCCGG
CGTGCGCCTAAGCCCCCTCGCTGAGCGCCAGCTACAGGTGCAGTGGGAGCCTCCCGG

GTCCTGGCCCTTCCCAGAA**A**ATCTTCTCACTGAAGTACTGGATCCGTTACAAGCGTCAG
GTCCTGGCCCTTCCCAGA**G**ATCTTCTCACTGAAGTACTGGATCCGTTACAAGCGTCAG

GGAGCTGCGCGCTTCCACCGGGTGGGGCCCATTGAAGCCACGTCCTTCATCCTCAGG
GGAGCTGCGCGCTTCCACCGGGTGGGGCCCATTGAAGCCACGTCCTTCATCCTCAGG

GCTGTGCGGCCCCGAGCCAGGTACTACGTCCAAGTGGCGGCTCAGGACCTCACAGAC
GCTGTGCGGCCCCGAGCCAGGTACTACGTCCAAGTGGCGGCTCAGGACCTCACAGAC

TACGGGGAACTGAGTGACTGGAGTCTCCCCGCCACTGCCACAATGAGCCTGGGCAAG
TACGGGGAACTGAGTGACTGGAGTCTCCCCGCCACTGCCACAATGAGCCTGGGCAAG

TAG
TAG

Figure 3.10 Comparison of Nucleotide sequence of EBI3 from cloning and gene bank.

The upper sequence was obtained from cloning, and the lower sequence was from genebank. The highlighted point indicates the silent mutation.

The cDNA encoding human p35 fragment was cloned using a nested PCR. The first amplification round products were used as a template in a second PCR reaction, which resulted in a PCR product of 762 bp (Figure 3.11 A). This PCR fragment was cloned into the pCR2.1 TA vector (figure 3.11 B), and site directed mutagenesis was performed to insert the Bgl II site silent mutation into TA vector carrying p35 fragment part. The template vector without the silent mutation site was digested by Dpn I, and the remaining amplified DNA vector with silent mutation was confirmed by sequencing using M13R and T7 primers (Figure 3.12). Then, using this DNA vector as a template, another round of amplification was performed with the insertion of a Not I site at both ends of the PCR fragment (Figure 3.11 C). This PCR product encoding p35 with the Not I on both ends were transfered into the Not I site of vector pSec-Linker-Fc, already containing the cDNA encoding EBI3 (Figure 3.11 D, E).

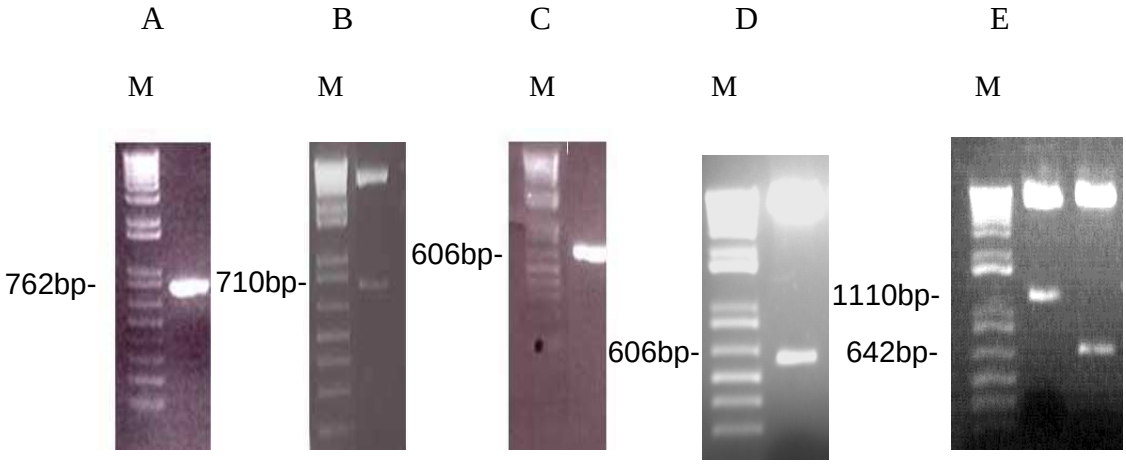


Figure 3.11 Cloning of the cDNA encoding human p35.

cDNA encoding p35 derived from DC was obtained by RT-PCR (A). The PCR fragments encoding p35 was cloned into the TA pCR2.1 vector (B). The PCR fragment was amplified from cloned p35 TA vector containing silent mutation, and a Not I site was introduced at both ends (C). 606 bp fragment encoding p35 was cloned into the vector pSec-L-Fc containing the EBI3 fragment (D). Hind III digestion of this construct resulted in an 1110 bp fragment, and BamH I digestion of this construct resulted in a 642 bp fragment (E). (The ladder used is 1kb+ DNA ladder).

Cloning

ATGTGGCCCCCTGGGTCAGCCTCCCAGCCACCGCCCTCACCTGCCGCGGCCACAGGT
ATGTGGCCCCCTGGGTCAGCCTCCCAGCCACCGCCCTCACCTGCCGCGGCCACAGGT
Genebank

CTGCATCCAGCGGCTCGCCCTGTGTCCCTGCAGTGCCGGCTCAGCATGTGTCCAGCGC
CTGCATCCAGCGGCTCGCCCTGTGTCCCTGCAGTGCCGGCTCAGCATGTGTCCAGCGC

GCAGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAA
GCAGCCTCCTCCTTGTGGCTACCCTGGTCCTCCTGGACCACCTCAGTTTGGCCAGAAA

CCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAAC
CCTCCCCGTGGCCACTCCAGACCCAGGAATGTTCCCATGCCTTCACCACTCCCAAAAC

CTGCTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTAC
CTGCTGAGGGCCGTCAGCAACATGCTCCAGAAGGCCAGACAAACTCTAGAATTTTAC

CCTTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCAC
CCTTGCACTTCTGAAGAGATTGATCATGAAGATATCACAAAAGATAAAACCAGCAC

AGTGGAGGCCTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAG
AGTGGAGGCCTGTTTACCATTGGAATTAACCAAGAATGAGAGTTGCCTAAATTCCAG

AGAGACCTCTTTCATAACTAATGGGAGTTGCCTGGCCTC CAGAAAGACCTCTTTTAT
AGAGACCTCTTTCATAACTAATGGGAGTTGCCTGGCCTC CAGAAAGACCTCTTTTAT

GATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAAGATGTACCAGGTGGAGTT
GATGGCCCTGTGCCTTAGTAGTATTTATGAAGACTTGAAGATGTACCAGGTGGAGTT

CAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAGA**A**GCAGATCTTTCTAGATCA
CAAGACCATGAATGCAAAGCTTCTGATGGATCCTAAGA**G**GCAGATCTTTCTAGATCA

AAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGA
AAACATGCTGGCAGTTATTGATGAGCTGATGCAGGCCCTGAATTTCAACAGTGAGA

CTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAAAATCAAGC
CTGTGCCACAAAAATCCTCCCTTGAAGAACCGGATTTTTATAAAACTAAAATCAAGC

TCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGATGAG
TCTGCATACTTCTTCATGCTTTCAGAATTCGGGCAGTGACTATTGATAGAGTGATGAG

CTATCTGAATGCT TCCT AA
CTATCTGAATGCT TCCT AA

Figure 3.12 Comparison of Nucleotide sequence of p35 from cloning and gene bank.

The upper sequence was obtained from cloning, and the lower sequence was from genebank. The highlighted point indicates the silent mutation.

PCR amplification, using the pSec- EBI3-linker-p35-Fc as a template, resulted in a 2270 bp PCR fragment containing the cDNA encoding EBI3, p35 and the hinge IgG Fc with the Bgl II site insertion on both ends (Figure 3.13 A). This PCR product was cloned into the PEE14.4 GS vector via the Bgl II site in the polylinker (Figure 3.13 B). The reading frame of EBI3-p35-Fc was sent for sequenceing to confirm the correct identity. Also, the sequence identity of the cloned product was confirmed by restriction enzyme mapping (Figure 3.13 C, D).

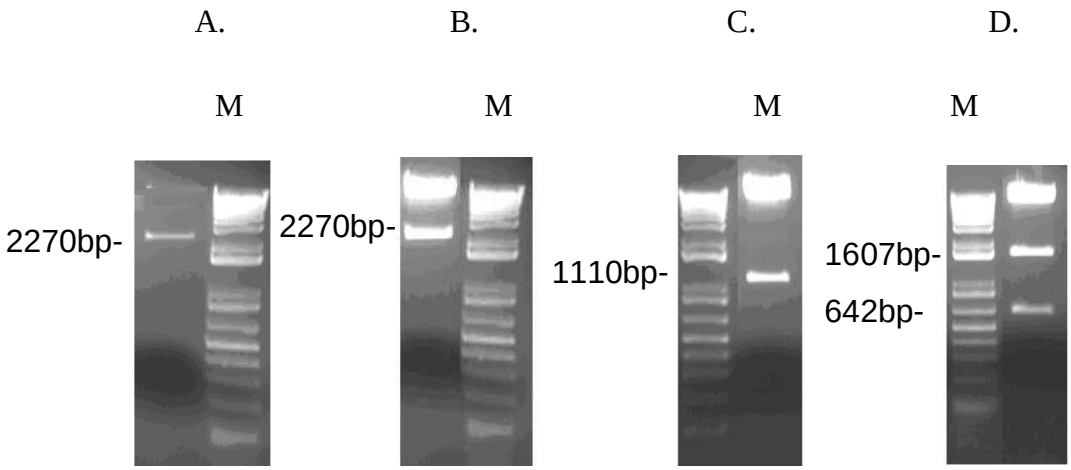


Figure 3.13 Cloning of human EBI3-L-p35-Fc cDNA into pEE14.4 vector.

PCR was performed on the pSec-EBI3-L-p35-Fc construct to obtain a PCR fragment encoding EBI3-Linker-p35-Fc, with a Bgl II site inserted at both ends of the fragment (A). This 2270bp PCR fragment was cloned into pEE14.4 vector (B). The enzyme digest was to confirm the correct insertion. Hind III digestion of the pEE14.4 vector construct confirmed the presence of an 1110 bp fragment (C). BamH I digestion of this construct resulted in two fragments: 642 bp and 1607 bp (D). (The ladder used is 1kb+ DNA ladder).

3.2.3 Expression of human IL-35-Fc in CHO-K1 cells

The PEE14.4 vector carrying the cDNA insert encoding EBI3-linker-p35-Fc was transfected into CHO-K1 cells, and the expressing colonies were detected by hIgG Fc ELISA. With the gradually increasing concentration of MSX from 50 μM to 500 μM, the highest stable expressing colonies were selected out and analyzed by hIgG Fc ELISA (3.14 A)，and confirmed by IL-12 p35/ IgG Fc ELISA (3.14 B) which is described in material and method. Then, a large-scale of cell colonies with the highest protein expression were cultured, and the supernatants were harvested. Using Protein A affinity purification, the recombinant fusion proteins of human IL-35-Fc were purified. The purity of the recombinant proteins were analysed by SDS-PAGE (Figure 3.15) and detected by western blot with anti-human IgG (Figure 3.16)

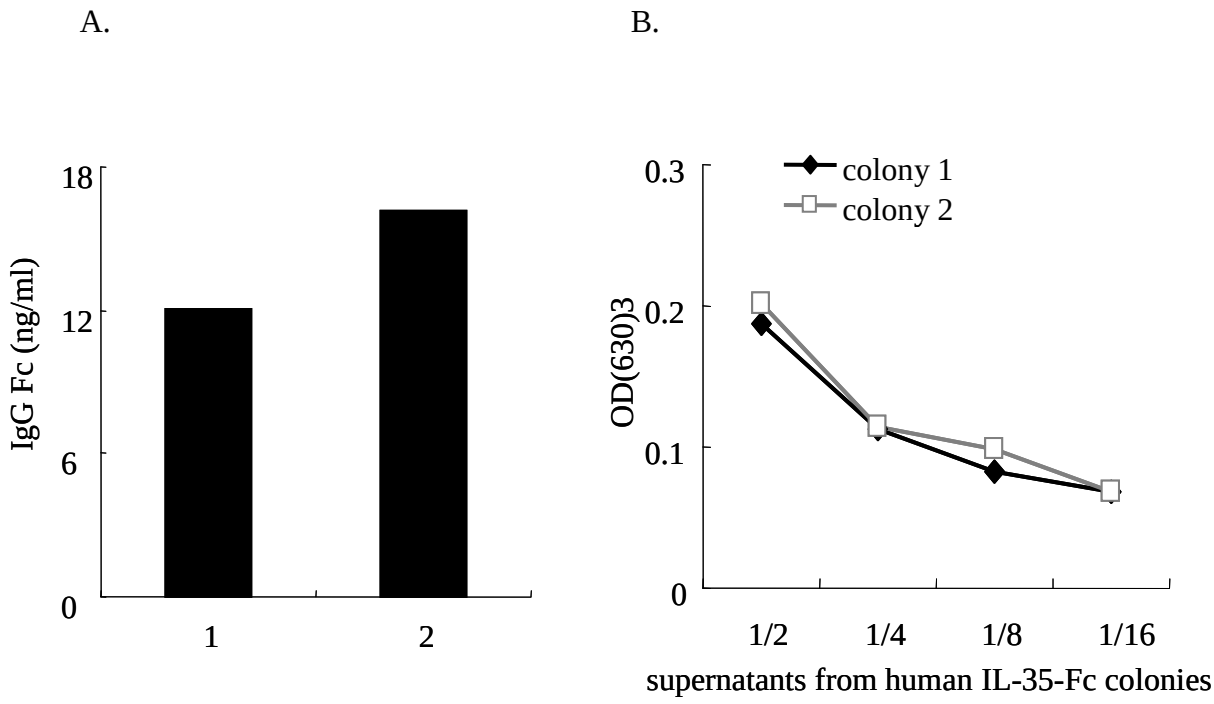


Figure 3.14 The levels of recombinant human IL-35-Fc protein expression.

Two highest stable expressing colonies were selected out, cultured in 24 well plates for 48 hours, and the supernatants were harvested, analyzed by hIgG Fc ELISA (A)，and confirmed by IL-12 p35/ IgG Fc ELISA (B).

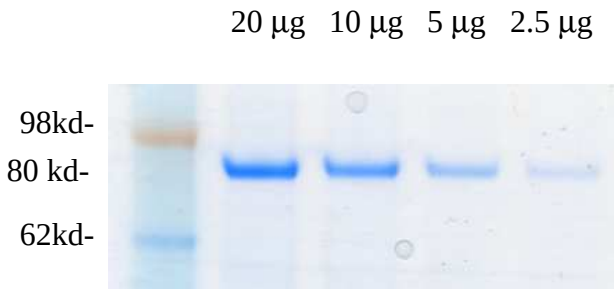


Figure 3.15 SDS-PAGE analysis of purified recombinant protein human IL-35-Fc.

Various amounts of purified recombinant protein human IL-35-Fc were run on SDS-PAGE, and stained with Coomassie blue to demonstrate their purities. The molecular weight was the same as it was designed. (The marker used is SeeBlue Plus2 protein marker).

Figure 3.16 Western blot analyses of purified recombinant protein human IL-35-Fc.

The purified recombinant protein human IL-35-Fc was demonstrated in western blot by anti-hIgG.

3.3 Summary

The murine cDNA encoding IL-27-FC, IL-35-Fc, IL-12-Fc and IL-23-Fc were constructed from pSecTag2A vector to PEE14.4-1 GS vector, and expressed in GS system. Using elevated levels of MSX, GS vector copy numbers were amplified multiple numbers, which resulted in a 10 times higher production of recombinant proteins than the previous vector pSecTag2A. Also, the recombinant proteins retained the expected bioactivities.

The human cDNA fragments encoding EBI3 and p35 were cloned from human DC by RT-PCR. The cloned cDNA EBI3 and p35 have been joined together by a flexible linker, and linked to the cDNA fragment encoding human IgG Fc. The resulting cDNA construct was cloned into PEE14.4-1GS vector and used to express human IL-35-Fc in CHO cells. The recombinant protein has a molecular weight of 80 kd and could be detected by antibody against human IgG, and the purity could be found in SDS-PAGE coomassie blue staining.

Chapter 4

IL-27 and Collagen-induced arthritis

(Some of results in this chapter already have been in publication as shown in Appendix 1)

4.1 Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease characterized by chronic inflammation of the synovial tissues in multiple joints and by subsequent progressive destruction of articular joints (Firestein & Zvaifler, 1991; Kouskoff, et al., 1996). Although it has been well described that synoviocytes, macrophages, DCs, and B cells contributed to RA, the prominent T cells infiltration suggested that RA is a T cell mediated disease (Van Boxel & Paget 1975; Yocum 1999). During the previous decade, RA was considered as a systemic Th1-driven disease with a relative predominance of IFN-γ production and a lack of Th2 cytokines (Miltenburg et al., 1992; Dolhain et al., 1996; Feldmann et al., 1996). However, recent studies demonstrated that Th17 cells played a predominant pathogenic role in RA (Lubberts et al., 2005, Chu et al., 2006). $CD4^+$ T cell- produced IL-17 promotes inflammation through enhancing the expression of TNF-α and IL-1β by monocytes. IL-17 has a synergistic effect on TNF-α inducing IL-1, IL-6, and IL-8 in the skin and synovial fibroblasts. IL-17 also enhances the IL-1-induced synthesis of IL-6 and LIF by RA synoviocytes (Jovanovic et al., 1998, Katz et al., 2001). Furthermore, IL-17 has a direct role in joint inflammation, cartilage damage, and bone erosion (Lubberts et al., 2001, Cai et al., 2001). It has been demonstrated that IL-17 neutralization could suppress arthritis in several experimental animal models (Nakae et al., 2003, Lubberts et al., 2005).

Although the etiology and pathogenesis of RA has not been completely elucidated, neutralizing Abs against the inflammatory components, particularly TNF-α, IL-1, and IL-6 have been shown to successfully suppress joint

inflammation, reduce the relapse rate and delay disease onset in RA patients (Kagari et al., 2002). Therefore, the expression and regulation of cytokines that are produced during the disease progression have been the major interest in therapeutic studies.

To understand the immune mediated events in RA, various disease models have been established. Collagen induced arthritis (CIA) is one of the well-developed models and closely resembles the features of human RA (Trentham et al., 1977). The progression of CIA is relatively dependent on both cellular and humoral immune response to collagen type II (CII), and various cytokines are involved in the pathogenesis of the disease development (Feldmann et al., 1996).

The heterodimeric cytokine IL-27， a member of the IL-12 family, is comprised of an IL-12p40 related protein, EBI3 and a unique IL-12p35 like protein p28 (Pflanz et al., 2002). Currently, the role of IL-27 in the regulation of immune responses is quite controversial. Some studies have demonstrated that IL-27 promotes naive T cells proliferation and initiates Th1 immune responses (Pflanz et al., 2002; Chen et al., 2000). However, other studies have shown that IL-27 suppresses the expansion of effector and memory T cells and inhibits several different cytokines including IL-2, IL-4, IFN-γ and IL-17 suggesting anti-inflammatory functions of IL-27 (Artis et al., 2004; Batten et al., 2006; Villarino et al., 2006). The dual role of IL-27 *in vitro* has also been demonstrated on *in vivo* infectious and autoimmune inflammatory models. IL-27 neutralization suppressed inflammation in rodent adjuvant arthritis (Goldberg et al., 2004). In contrast, IL-27R knockout mice displayed enhance CNS inflammation when

infected with Toxoplasma gondii, or are hypersusceptible to EAE (Batten et al., 2006, Stumhofer et al., 2006). However, at the present moment, our understanding of the role of IL-27 in RA is limited. It was therefore the objective of this chapter to explore the role of IL-27 in RA.

In this study, we found that IL-27 was able to attenuate CIA when administrated at the onset of disease. Reduced disease development was associated with down regulation of IL-17 and IL-6 productions. In contrast, when IL-27 was administered late in disease development, it exacerbated disease progression. Disease exacerbation was accompanied by elevated IFN-γ and IL-6 production. Consistent with this, we observed that *in vitro* IL-27 was able to inhibit Th17 differentiation from naïve $CD4^+$ T cells, but had little or no effect on IL-17 production by polarised Th17 cells.

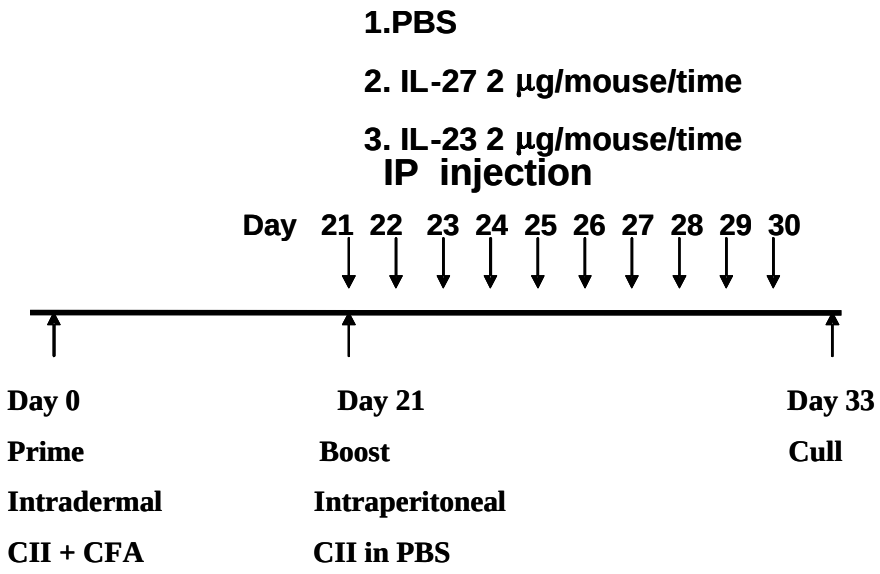
4.2 Results

4.2.1 Administration of IL-27 prevents the development of CIA at the early stage of disease

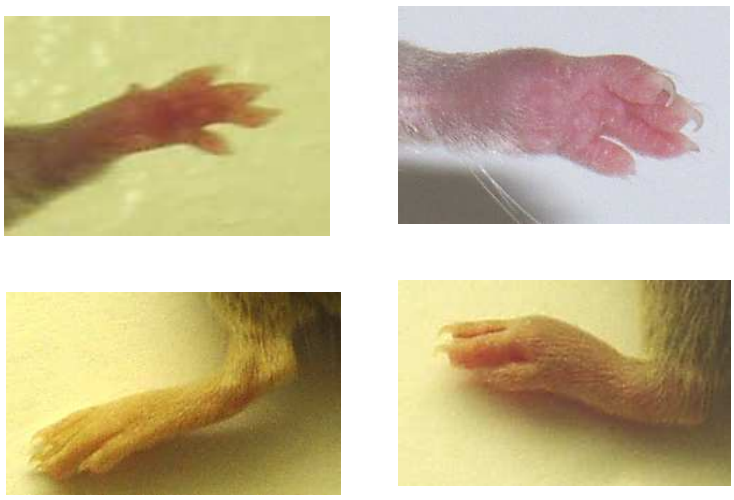
Since the immunopathogensis of CIA closely resembles RA, the effect of IL-27 administration on CIA in susceptible male DBA/I mice was monitored. Mice immunized by intradermal injection of type II collagen in Freund's complete adjuvant began to show clinical sign of arthritis after challenge i.p. with collagen II on day 21. Mice were given 10 daily i.p. injections of IL-27 (2 μg/dose), IL-23 (2 μg/dose) or PBS starting on day 21 (Figure 4.1). As expected, IL-23 group and PBS control group developed severe arthritis. In contrast, mice treated with IL-27 developed significantly attenuated disease in the mean number of arthritic paws

(Figure 4.2A) and mean clinical score (Figure 4.2B). Also, mice treated with IL-27 had much reduced incidence of developing arthritic diseases compared to PBS control goup and IL-23 treated mice group (Figure 4.2C). Mean paw thickness of mice treated with IL-27 was lower compared to PBS control group (Figure 4.2D). In contrast, IL-23 treated mice developed significant higher mean paw thickness compared to PBS control mice and IL-27 treated mice (Figure 4.2D). To examine whether IL-27 administration modified articular destruction, cartilage and bone integrity were evaluated histologically. Histology examination of the hind limb joints from IL-23 and PBS control treated mice revealed massive cell infiltration of the joint compartment with synovial hyperplasia and bone erosion (Figure 4.3A, Figure 4.3B). Administration of IL-27 markedly suppressed each of these parameters (Figure 4.3 C). The histological scores were summarized in Figure 4.3D. Together these data clearly indicated that IL-27 potently suppressed the development of CIA.

A.



B.



Normal paws Arthritic paws

Figure 4.1 The schedule of Collagen-induced arthritis.

DBA/1 mice were primed intraermally with 100 µg of bovine collagen type II (CII) in complete Freund's adjuvant (CFA) at the base of tail on day 0, and boosted intraperitoneally with 100 µg of CII in PBS on day 21. 10 times of 200 µl PBS, 2 µg/ 200 µl of IL-27, 2 µg/ 200 µl of IL-23 were given i.p. starting on day 21 (A). The mice were observed for clinical sign of arthritis (B), and the footpads were measured by callipers every other day. Mice were culled on day 33.

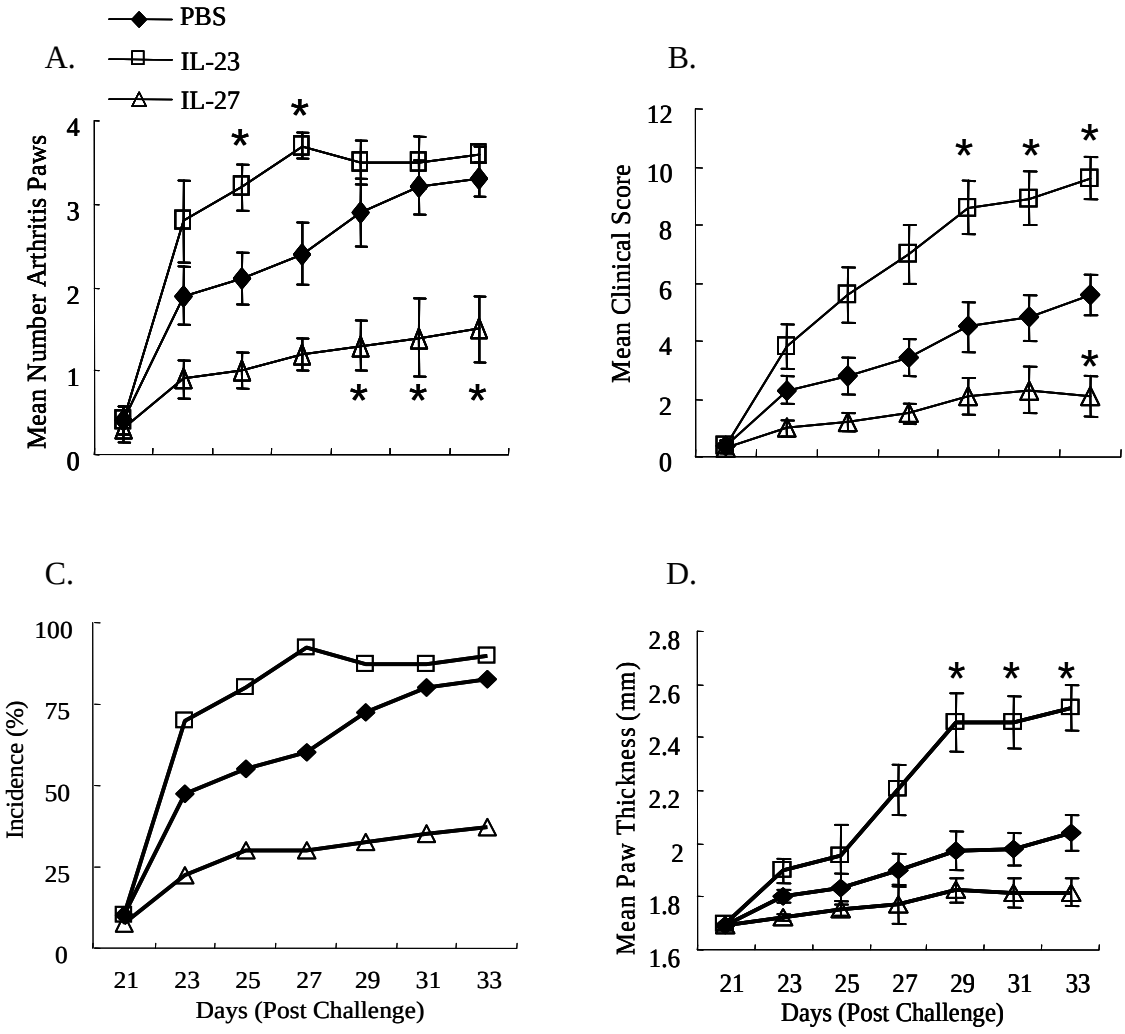


Figure 4.2 IL-27 inhibited the development of CIA.

Collagen-primed DBA/I mice were randomly divided into groups of 10, challenged on day 21, and given 10 daily i.p. injections of 2 μg/200 μl of IL-27, 2 μg /200 μl of IL-23, or 200 μl of PBS starting on day 21. Mice were monitored for disease progression as indicated by (A) Mean number of arthritic paws, (B) Mean clinical score, (C) incidence, and (D) Mean Paw Thickness (mm). Values are mean ± SEM.*, $p < 0.05$. IL-27 treated mice developed significantly less severe disease compared with IL-23 treated mice and control PBS group.

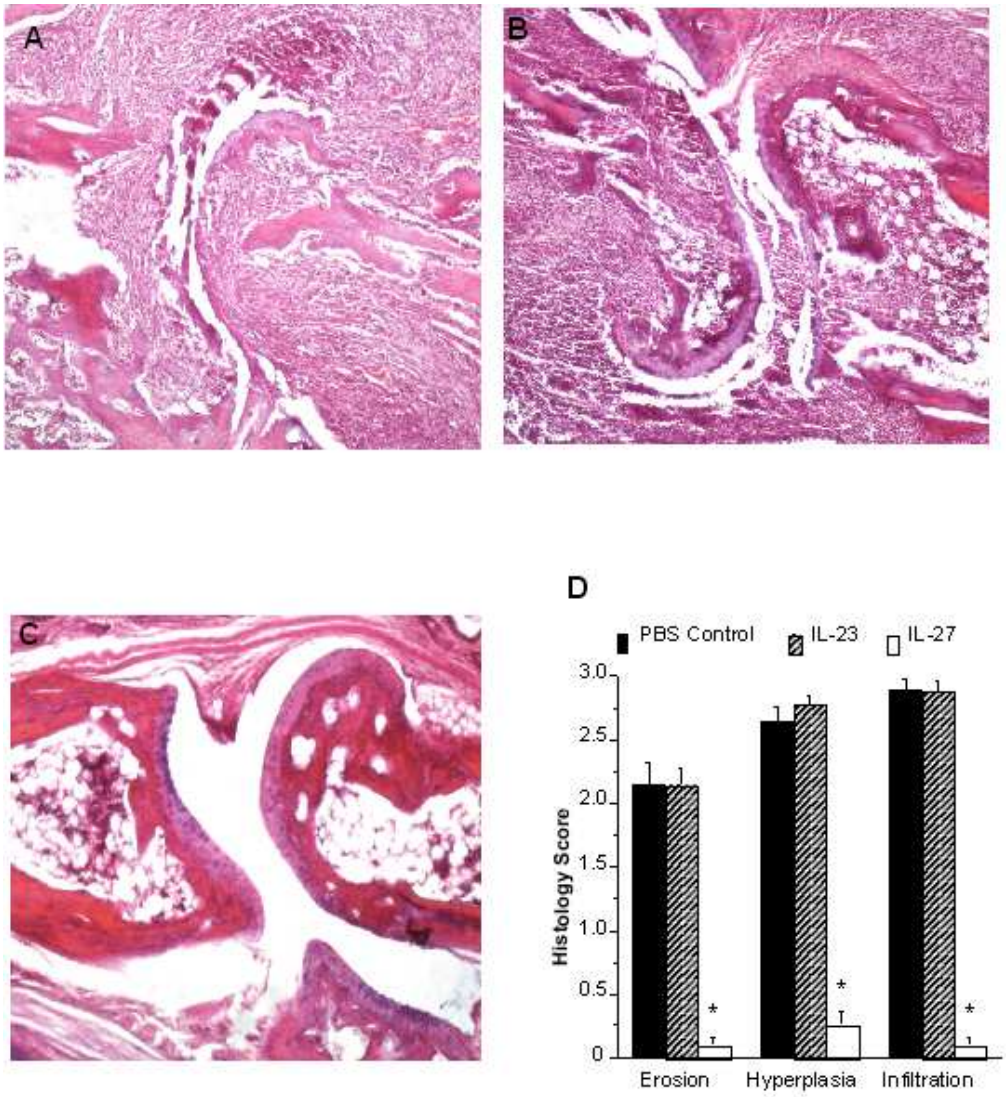


Figure 4.3 Administration of IL-27 significantly reduced joint pathology.

On day 33, mice were administrated with 10 times of IL-27, IL-23, or PBS were culled. Hind limbs (five mice /group) were removed, formalin-fixed, decalcified, and stained with H&E. Profound cartilage and bone erosion, hyperplasia and cellular infiltration were observed in the control PBS group (A) and IL-23-treated mice goup (B). IL-27-treated mice exhibited reduced histological evidence of destruction (C). Histological appearances were scored (0-3) for the presence of bone erosion, synovial hyperplasia, and cellular infiltration (D). Data are mean ± SEM.*, $p < 0.05$, compared to PBS control group, and original magnification (A, B, and C) is x 50.

4.2.2 Serum cytokines *in vivo*

To gain insight into the mechanism of how IL-27 could suppress articular inflammation, serum samples in the arthritic mice were taken on day 33 when they were culled, and serum cytokines were analyzed by multiple bead assay-10 plex luminex or ELISA. Since the role of IL-23 in CIA has been published (Murphy et al., 2003), the following studies were focused on the mice treated with IL-27 comparing with control mice treated with PBS. Although IL-1β, IL-4, IL-5, and GM-CSF were undetectable in serum from both PBS treatment group and IL-27 treatment group, TNF-α, IL-10, IL-2 and IFN-γ levels were similarly low in both groups (Figure 4.4.A). There was no difference in IL-12 level between PBS treatment group and IL-27 treatment group (Figure 4.4 A); however, IL-6 (Figure 4.4 B) and IL-17 (Figure 4.4 C) levels were significantly reduced in the mice treated with IL-27 when compared with mice treated with PBS. Interestingly, sera from mice treated with IL-27 contained slightly higher IL-1Rα than those from control mice (Figure 4.4 A).

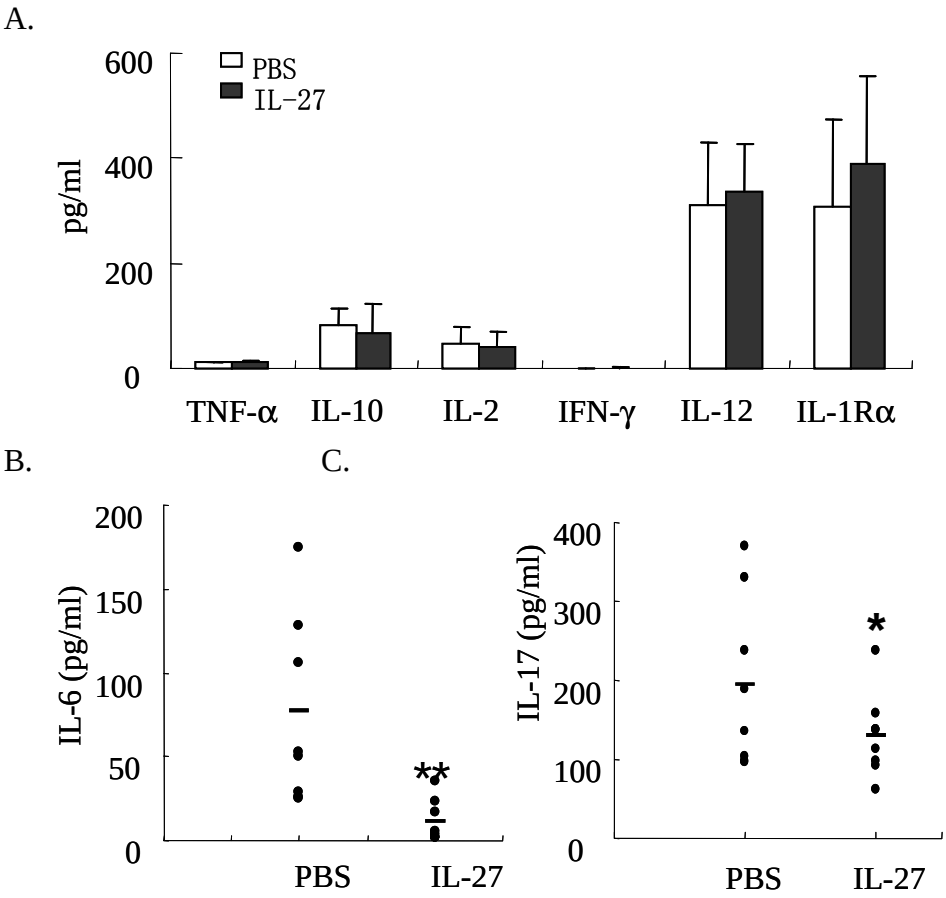


Figure 4.4 Serum cytokines levels of IL-27 and PBS treated mice.

DBA/1 mice treated with either IL-27 or PBS control mice were sacrificed on day 33 and serum was collected from seven mice in each group. Levels of TNF-α, IL-10, IL-2, IFN-γ, IL-12, IL-1Rα (A), IL-6 (B), and IL-17 (C) were determined by ELISA of individual samples. Data are mean ± SEM. representative of 2 experiments. There was no significant difference in TNF-α, IL-10, IL-2, IFN-γ, IL-12, and IL-1Rα between two groups, but IL-6 and IL-17 were significantly reduced in the IL-27-treated mice. Each dot represents an individual mouse, and representative of 2 experiments; *, $p< 0.05$, **, $p< 0.01$ compared to PBS control group.

4.2.3 IL-27 treated mice showed reduced anti-collagen Ab production

Because Ab levels against Collagen II correlate well with the development of arthritis, CII specific antibodies IgG1 and IgG2a levels were measured by ELISA (Figure 4.5). Collagen-specific IgG2a levels in sera from the mice receiving IL-27 were significantly lower than control mice, but IgG1 antibody was not different between two groups.

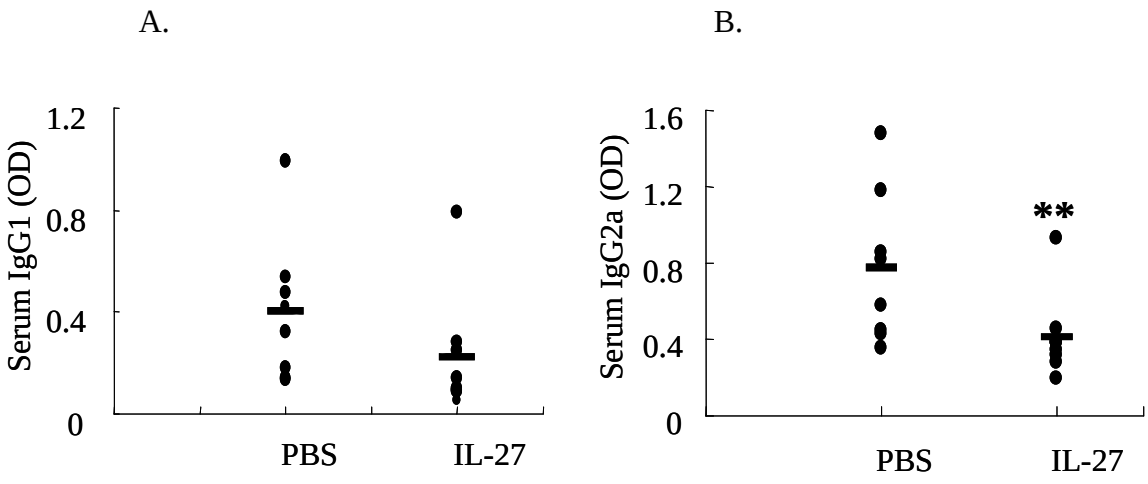


Figure 4.5 Assessment of serum anti-collagen Ab responses in IL-27 and PBS treated mice.

At the end of treatment with IL-27 or PBS, serums were collected on day 33 and collagen specific antibodies IgG1 (A) and IgG2a (B) in serum were determined by ELISA. IL-27-treated mice produced significantly lower IgG2a but not IgG1 antibody. Serum samples were diluted 4000-fold. Each dot represents an individual mouse, and data are expressed as mean absorbance (OD 630). n=7 and are representative of 2 experiments; **, $p < 0.01$, compared to PBS control group.

4.2.4 IL-27 suppresses CII-specific proinflammatory immune response *ex vivo*

To further explore the effect of IL-27 mediated suppression during CIA development, CIA was induced in mice and the administration of PBS or IL-27 was exactly the same as previously described in Figure 4.1. However, mice were culled at two different time points (as shown in Figure 4.6) to examine CII-specific T cell immune responses.

Spleen cells from mice that had been treated with IL-27 or control PBS were harvested after 6 times of treatment (day 27) and they were cultured with type II collagen *in vitro*. Cells from the IL-27 treated mice produced significantly less IL-17 (Figure 4.7) compared with parallel cultures from control PBS group, which indicated a suppression of Th-17 response. However, T cell proliferation, IL-6, TNF-α, IFN-γ, and IL-1Rα production remained similar between the two groups (Figure 4.7).

The above observations from the first time point (day 27) were supported by the second time point, in which inguinal lymph nodes and spleen cells from mice that had been treated with IL-27 or control PBS were harvested after 10 times treatment (day 33). Inguinal lymph nodes and spleen cells were stimulated with PMA and ionomycin and analysed by intracellular staining. Control mice induced higher proportion of IL-17 and IFN-γ –producing spleen cells and lymph nodes cells compared with IL-27 treated mice (Figure 4.8). The remaining spleen cells were cultured with type II collagen *in vitro*. Cells from the IL-27 treated mice

produced significantly less IL-17 than cells from control PBS group (Figure 4.9), while other cytokines production showed no difference between two groups (Figure 4.9). Furthermore, the T cell mitogen, ConA, stimulated equally high levels of IL-17, IL-6, TNF-α, and IFN-γ by spleen cells from both groups of mice, indicating that IL-17 suppression of the immune response was Ag-specific (Figure 4.10).

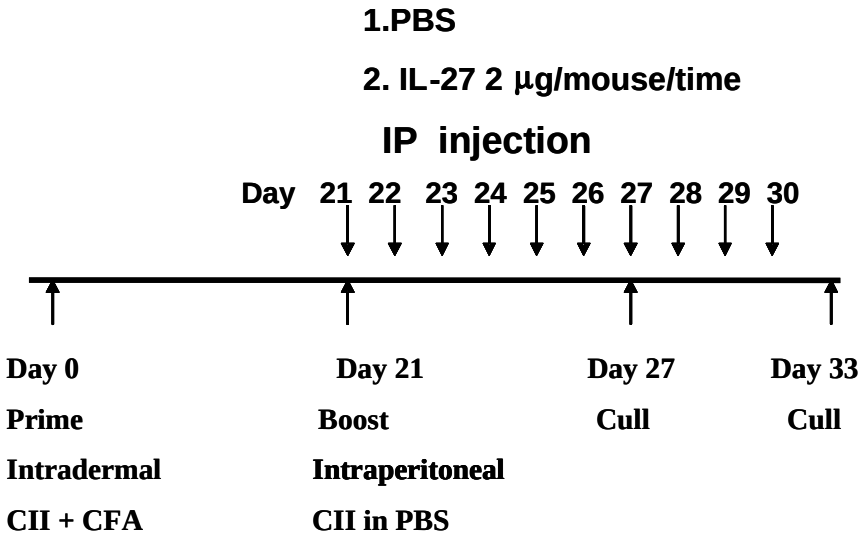


Figure 4.6 The schedule of Collagen-induced arthritis.

DBA/1 mice were primed intraermally with 100 μg of bovine collagen type II (CII) in complete Freund's adjuvant (CFA) at the base of tail on day 0, and boosted intraperitoneally with 100 μg of CII in PBS on day 21. 10 times of 200 μl PBS, 2 μg/ 200 μl of IL-27 were given i.p. starting on day 21. The mice were observed for clinical sign of arthritis, and the footpads were measured by callipers every other day. Mice were culled at two time points on day 27 and day 33.

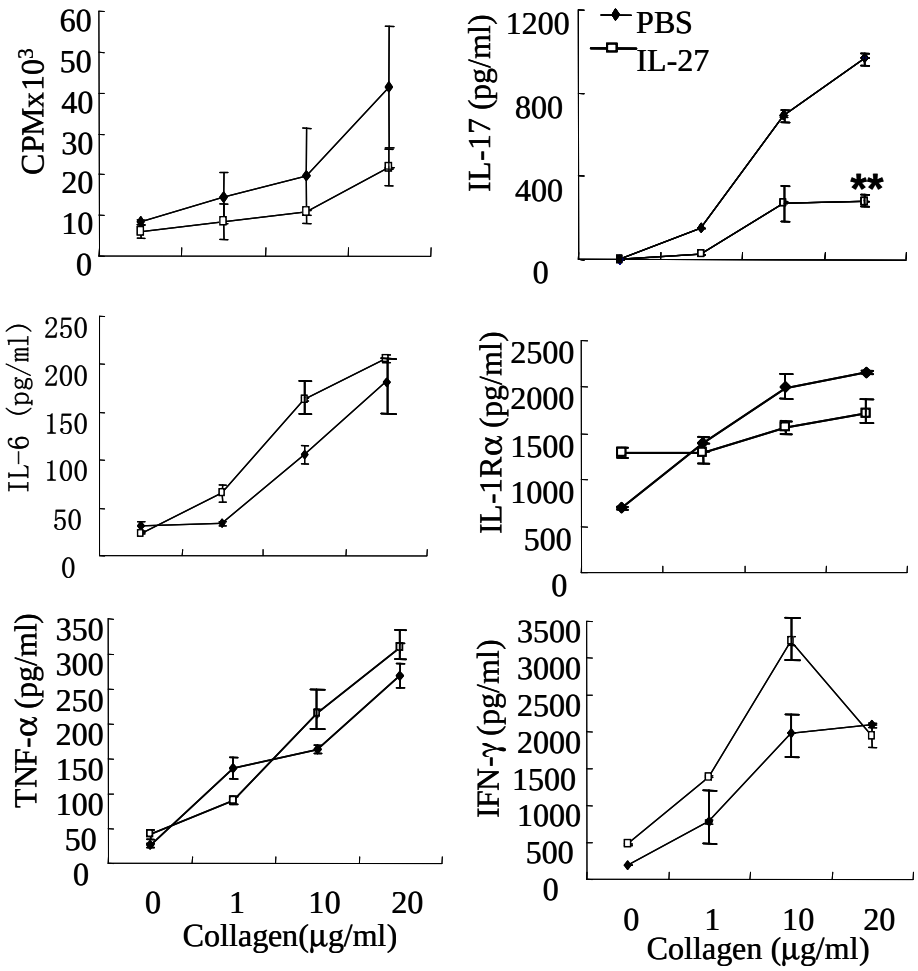


Figure 4.7 Assessment of *in vitro* responses against collagen from mice treated with IL-27 or PBS.

Spleen cells (pooled from 4 mice per group) were collected from IL-27 treated or PBS treated mice after 6 times of injections (day 27) and cultured with titrated concentrations of collagen for 96 h. T cell proliferation (A) was determined by [^{3}H] thymidine uptake. Supernatants from parallel cultures were collected after 72 h. IL-17, IL-6, TNF-α, IL-1Rα, and IFN-γ levels were measure by ELISA. There was no significant difference in T cell proliferation, IL-6, TNF-α, IL-1Rα, and IFN-γ concentrations between two groups, but cells from the IL-27- treated mice produced significantly less IL-17. Data are expressed as mean ± SEM of triplicate cultures. **, $p < 0.01$, compared to PBS control group.

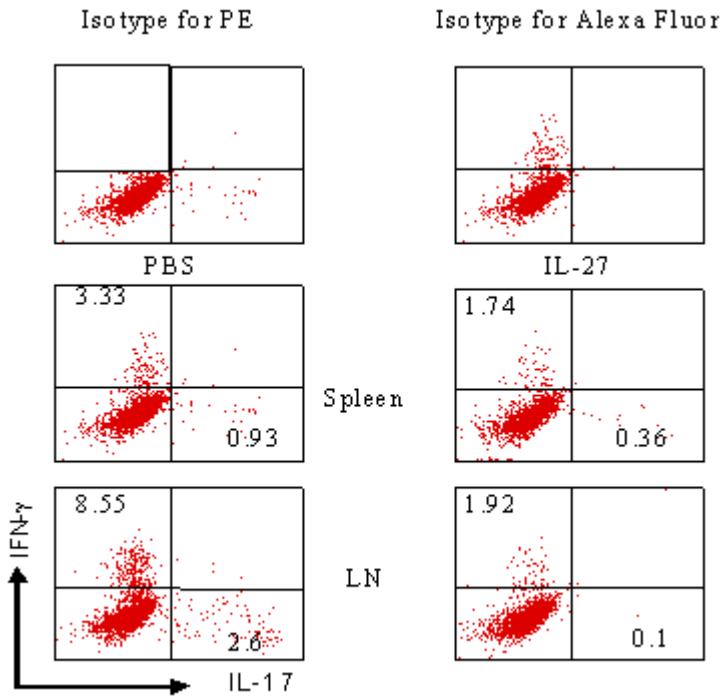


Figure 4.8 Intracelluar cytokine analysis of IFN-γ and IL-17 secretion.

Spleen cells and LN cells were harvested (n=6) from mice treated with IL-27 or PBS on day 33, stimulated with PMA and ionomycin and stained intracellularly for IL-17 and INF-γ. The percent of IL-17^{+} and IFN-γ^{+} cells were reduced by IL-27 treatment. The number in quadrants represent the percentage of IL-17- or IFN-γ expressing cells.

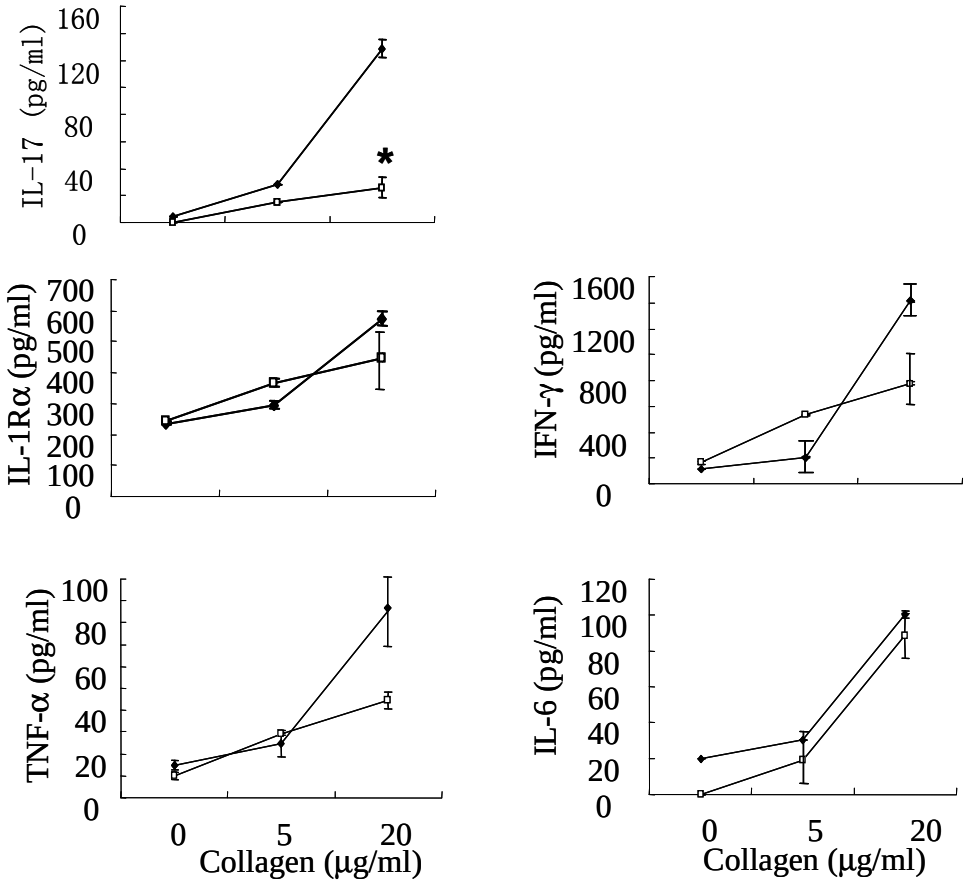


Figure 4.9 Assessment of *in vitro* responses against collagen from mice treated with IL-27 or PBS.

Spleen cells (pooled from 6 mice per group) were collected from IL-27 treated or PBS treated mice after 10 times injections (day 33) and cultured with collagen as described before. Supernatants from parallel cultures were collected after 72 h. IL-17, IL-6, TNF-α, IL-1Rα, and IFN-γ levels were measure by ELISA. Cells from the IL-27- treated mice produced significantly less IL-17, but there was no significant difference in IL-6, TNF-α, IL-1Rα, and IFN-γ concentrations between two groups. Data are expressed as mean ± SEM and pooled of triplicate cultures. *, $p < 0.05$, compared to PBS control group.

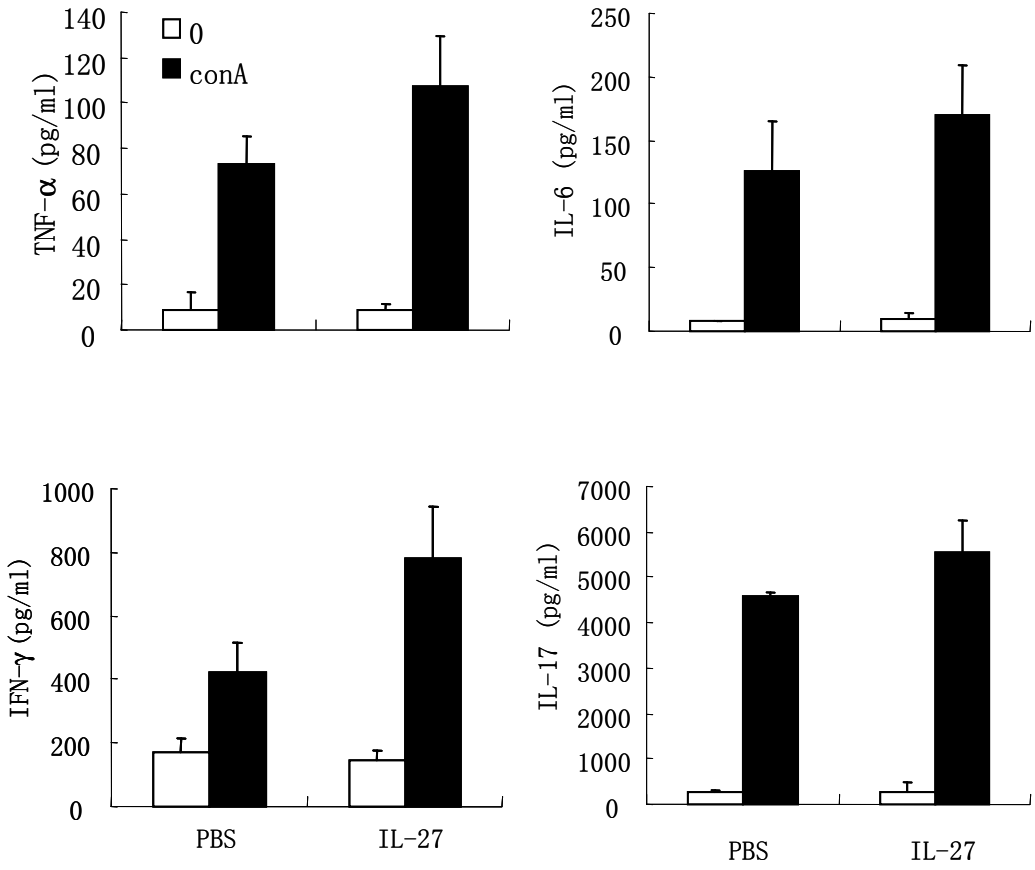


Figure 4.10 Stimulation of spleen cells with ConA from IL-27 treated or PBS treated mice.

Spleen cells (pooled from 6 mice per group) from IL-27 treated mice or PBS treated mice were cultured with con A (1μg/ml) for 96 h. Supernatants from cultures were collected, and IL-17, IL-6, TNF-α, and IFN-γ levels were measure by ELISA. Equally high levels of IL-17, IL-6, TNF-α, and IFN-γ production were produced between two groups. Data are expressed as mean ± SEM. There is no statistical difference between PBS control mice and IL-27 treated mice.

4.2.5 Administration of IL-27 at the later stage of CIA enhances the disease expression

To determine the role of IL-27 during joint inflammation and tissue destruction at a later stage of CIA, administration of IL-27 was started on day 27 after CII immunization. On day 27, about 50% of CIA mice paws began to show clinical sign of arthritis. Mice were injected i.p. daily with IL-27 (2 μg/dose) or PBS from days 27 to 36, for a total of ten administrations. Surprisingly, mice treated with IL-27 developed significantly more severe diseases in the mean paw thickness compared to PBS control CIA mice (Figure 4.11A). However, there was no difference in the mean number of arthritic paws; arthritic incidence and mean clinical score (Figure 4.11 B C). Similarly, there was no difference in serum cytokines between PBS and IL-27 treated group (Figure 4.12). This observation was also reflected in immune responses *in vitro* (Figure 4.13). Spleen cells from IL-27 recipients produced slightly higher concentrations of IL-6 and IFN-γ, and slightly lower concentrations of TNF-α and IL-17, in response to collagen compared to spleen cells from control group, while there is no significant difference between the two groups.

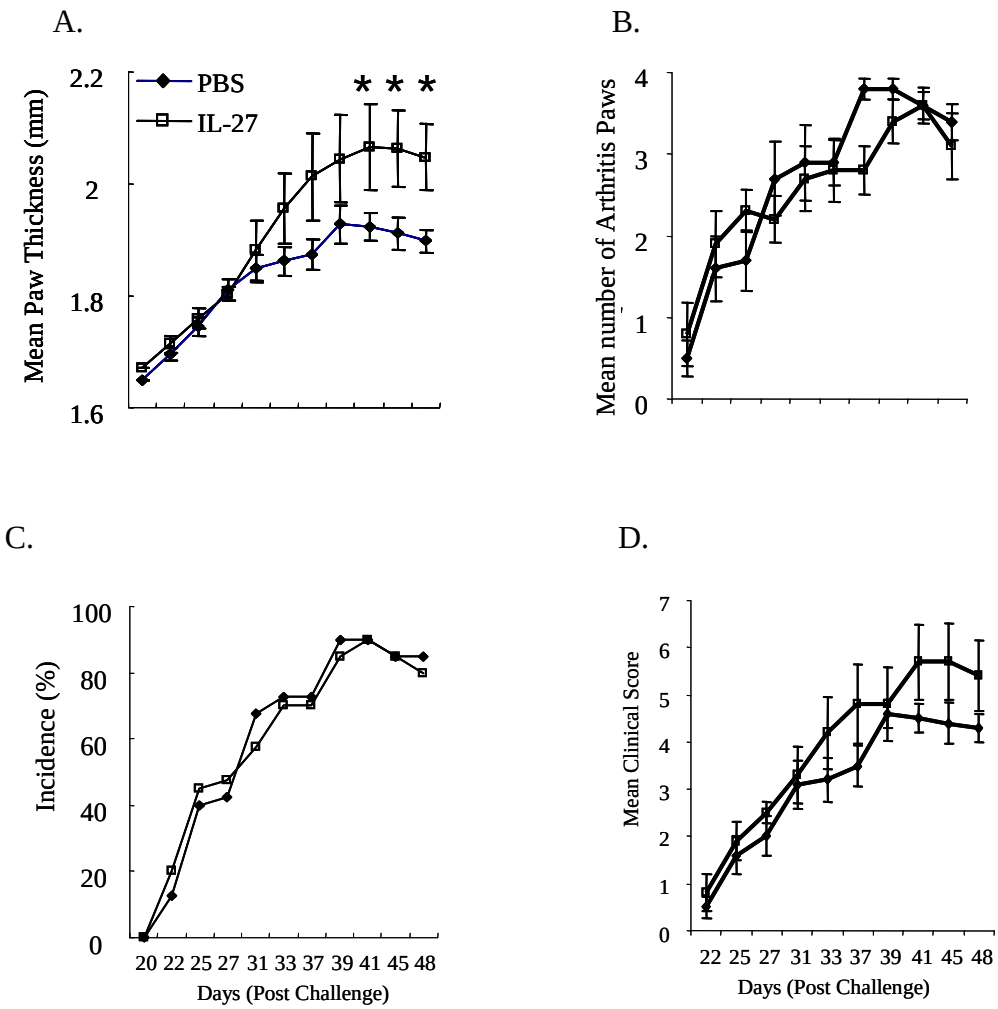


Figure 4.11 IL-27 enhanced the development of CIA at the later stage.

Collagen-primed DBA/1 mice were randomly divided into groups of 10, challenged on day 21, and given 10 daily i.p. injections of 2 μg/200 μl of IL-27, or 200 μl of PBS starting on day 27. Mice were monitored for disease progression as indicated by (A) Mean paw thickness, and (B) Mean Number of Arthritic Paws, (C) incidence and (D) Mean Clinical Score. Values are mean ± SEM.*, $p < 0.05$. IL-27 treated mice developed more severe disease compared to control PBS group.

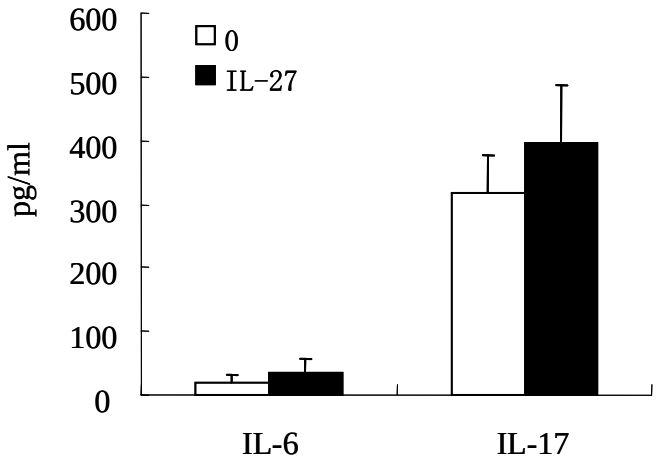


Figure 4.12 Serum cytokine levels of IL-27 and PBS treated mice.

DBA/1 mice treated with either IL-27 or PBS control mice (n=5 in each group) were sacrificed on day 36 and serum was collected. Levels of IL-6 and IL-17 were determined by ELISA of individual samples. There was no difference in serum cytokines between two groups. Data are mean ± SEM. There is no statistical difference between two groups.

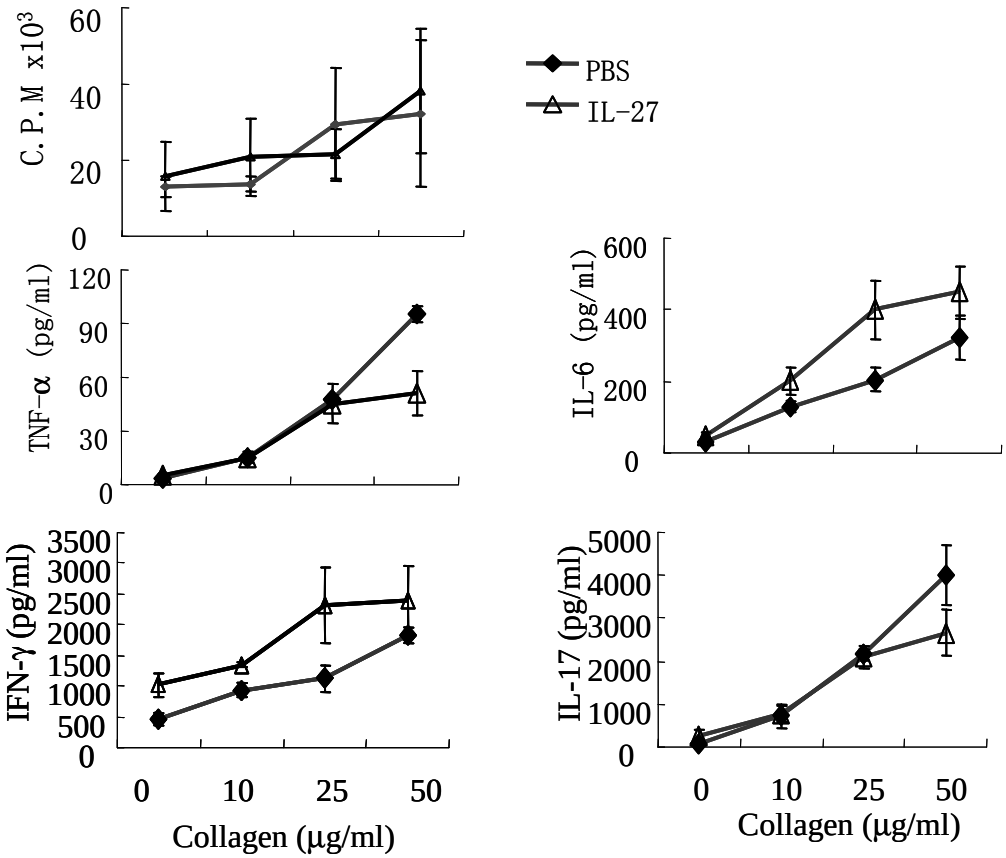


Figure 4.13 Assessment of *in vitro* responses against collagen from mice treated with IL-27 or PBS.

Spleen cells (pooled from 5 mice per group) were collected from IL-27 treated or PBS treated mice after 10 times injections (day 36) and cultured with collagen as described before. T cell proliferation (A), which was determined by [3H] thymidine uptake, is expressed as mean stimulation index ± SEM of triplicate cultures. Supernatants from parallel cultures were collected after 72 h. IL-17, IL-6, TNF-α, and IFN-γ levels were measure by ELISA and expressed as mean ± SEM. There were no significant differences in cell proliferations, IL-17, IL-6, TNF-α, and IFN-γ concentrations between two groups.

4.2.6 IL-27 suppresses the development of Th17 cells

Because of the critical importance of IL-17 in the pathogenesis of CIA, and the above data suggested that IL-27 down-regulated IL-17 *in vivo*, it is important to determine the direct effect of IL-27 in the Th-17 cell differentiation pathway i*n vitro*. When $CD4^+$ T cells isolated from the spleens of wild-type DBA/1 mice were activated with immobilized anti-CD3 plus anti-CD28, moderate amount of IL-17 were produced; and the addition of IL-27 inhibited the production of IL-17 (Figure 4.14 A). Also, according to Figure 4.14A, it was found that 25 ng/ml of IL-27 was the optimal concentration to inhibit IL-17 production. Therefore, the following experiments were done using 25 ng/ml of IL-27. According to the published reports (Veldhoen et al., 2006, Mangan et al., 2006), the efficiency of *in vitro* IL-17 production requires the combination of TGF-β, IL-1β and IL-6. When the cell culture medium was supplemented with TGF-β, IL-1β and IL-6, robust secretion of IL-17 occurred, but IL-27 still efficiently suppressed IL-17 production (Figure 4.14 B). Furthermore, to exclude artificially skewed development towards Th1 or Th2 helper lineage, the neutralizing antibodies anti-IL-4, anti-IFN-γ, and anti-IL-2 were added to the above cell culture, high amount of IL-17 was produced. The presence of IL-27 still efficiently suppresses Th17 cells differentiation (Figure 4.14 C).

Similar results were obtained with $CD4^+$ T cells isolated from BALB/c mice. $CD4^+$ T cells from BALB/c mice, labelled with CFSE, were cultured with APC, anti-CD3, and the combination of TGF-β, IL-1β and IL-6. In the presence of IL-27, $CD4^+$ T cells stained much less positive for IL-17 compared with the $CD4^+$ T cells without the presence of IL-27 (Figure 4.15 Aa). This reduction was

supported by the paralleled ELISA results (Figure 4.15 Ab). This generally confirmed those results in the published reports (Stumhofer et al., 2006). IL-27 can trigger signalling in a variety of cell types, therefore, the experiments were repeated using $CD4^{+}CD25^{+}$ T cells and $CD4^{+}CD25^{-}$ T cell from BALB/c mice, treatment with IL-27 markedly reduced Th17 cells development (Figure 4.15 B, C). Meanwhile, IL-27 had no effect on T cell proliferation (Figure Figure 4.15 Aa) or the percent of $Foxp3^{+}$ Treg cells (Figure 4.15 Ca).

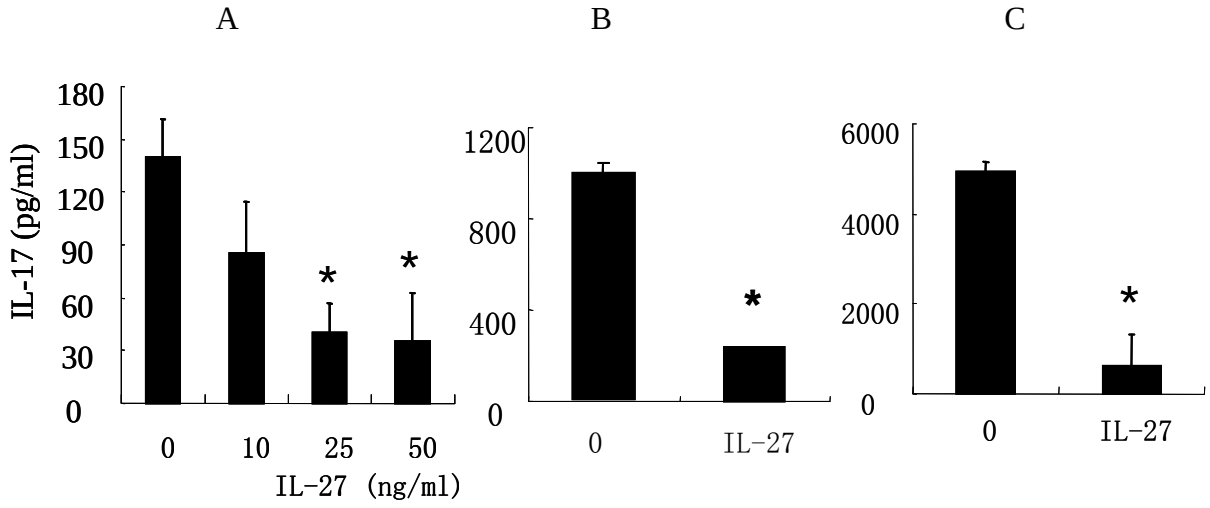


Figure 4.14 IL-27 suppression of IL-17 production by $CD4^+$ T cells.

$CD4^+$ T cells from DBA/I mice were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab in the presence of graded concentrations of IL-27 for 3 days, and then the culture supernatants were analyzed for IL-17 by ELISA (A). $CD4^+$ T cells from DBA/I mice were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab, and TGF-β, IL-1β and IL-6, in the absence or presence of IL-27 (25 ng/ml) for 3 days, and then the culture supernatants were analyzed for IL-17 by ELISA (B). $CD4^+$ T cells from DBA/I mice were stimulated with plate-bound anti-CD3 Ab plus soluble anti-CD28 Ab, and TGF-β, IL-1β, IL-6, anti- IL-4, anti- IFN-γ, and anti-IL-2, in the absence or presence of IL-27 (25 ng/ml) for 3 days, and then the culture supernatants were analyzed for IL-17 by ELISA (C). IL-27 efficiently suppresses Th-17 cells differentiation in all A, B and C conditions. Data are expressed as mean ± SEM, representative of 3 experiments, *, $p < 0.05$ compared to control.

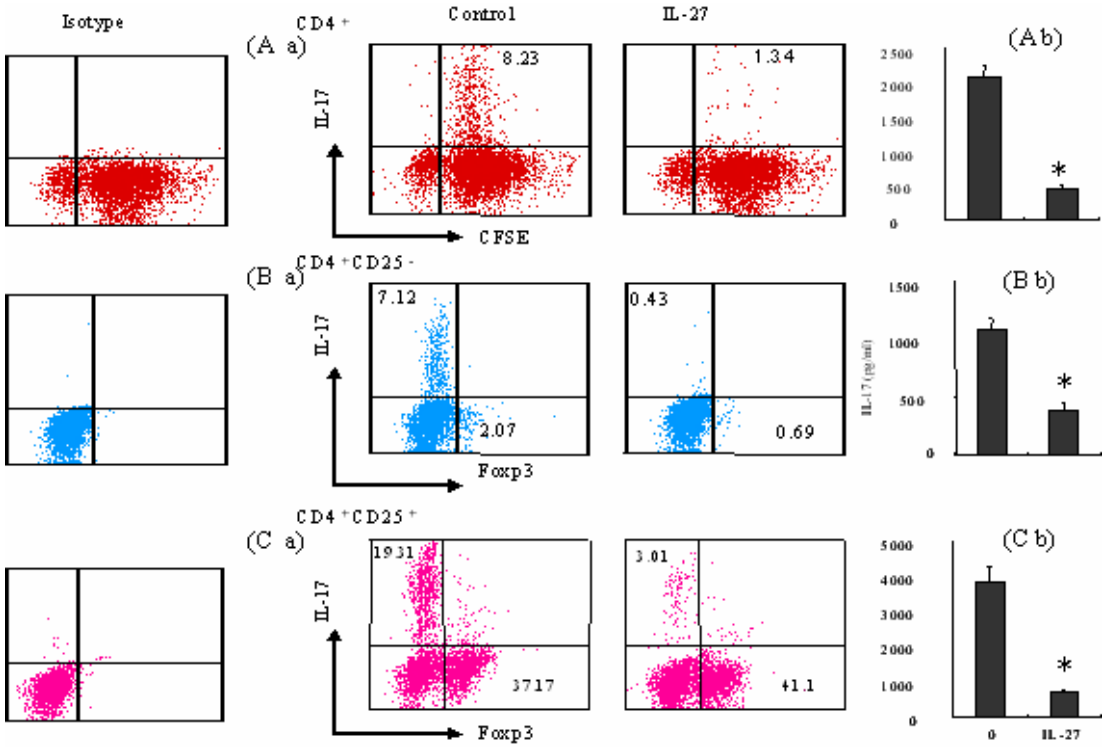


Figure 4.15 IL-27 mediated the inhibition of Th-17 development.

$CD4^+$ T cells (A), $CD4^+CD25^+$ T cells (B) and $CD4^+CD25^-$ T cell (C) were isolated from BALB/c mice, and the $CD4^+$ T cells were labeled with CFSE, then the three groups of cells respectively were activated with APC, anti-CD3, and TGF-β, IL-1β and IL-6, in the absence or presence of IL-27 (25 ng/ml) for 3 days. After that, the cells, respectively, were stimulated with 4 h with PMA and ionomycin, stained for intracellular IL-17 (Aa, Ba, Ca), or Foxp3 (Ba, Ca) and analyzed on flow cytometry. The parallel culture supernatants were analyzed for IL-17 by ELISA (Ab, Bb, and Cb). IL-27 had no effect on cell proliferation (CFSE) or Foxp3 expression but significantly reduced the expression of IL-17. Data are representative of three independent experiments. Plots are gated on T cells; numbers in quadrants indicate the frequency of cells each. ELISA data are expressed as mean ± SEM, *, $p< 0.05$ compared to control group.

4.2.7 IL-27 does not suppress IL-17 production on established Th-17 cells

Those above data presented has demonstrated that IL-27 negatively regulates Th-17 cell development. It is also important to examine the effect of IL-27 on established Th-17 producing cells. $CD4^+$ T cells isolated from BALB/c mice were cultured with APC, anti-CD3, the combination of TGF-β, IL-1β and IL-6, and the neutralizing antibodies anti-IL-4, anti-IFN-γ and anti-IL-2. Under the optimal Th-17 inducing condition, naïve $CD4^+$ T cells were polarized to Th-17 cells. After 4 days of cell culture, cells were washed and re-stimulated with APC and anti-CD3, in the presence of IL-23 which is an important factor to maintain established Th-17 cells. The established Th-17 cells produced high amount of IL-17^+ cells, but the addition of IL-27 showed no suppressive effect on IL-17^+ cells (Figure 4.16A). It was further confirmed by the parallel ELISA result (Figure 4.16B). Similar results were also observed on $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cell by ELISA (Figure 4.16C, D).

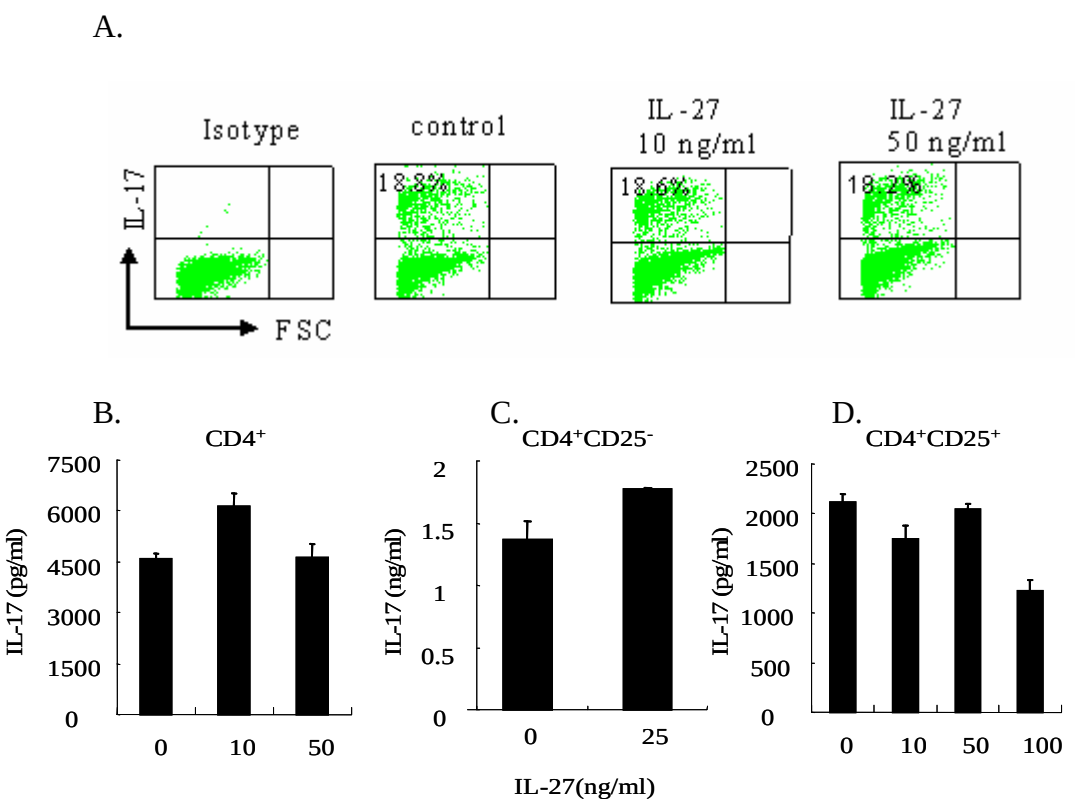


Figure 4.16 IL-27 does not suppress IL-17 production on established Th17 cells. $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cell isolated from BAB/c mice were respectively cultured for 4 days under the optimal Th17 inducing condition, washed and restimulated with APC, anti-CD3 and IL-23, in the graded concentrations of IL-27 for 3 days. The re-stimulated Th17 cells generated from $CD4^+$ T cells were analyzed for intracellular IL-17 staining by flow cytometry (A). The established Th17 cells produced high amount of IL-17^+ cells, but IL-27 showed no suppressive effect on IL-17^+ cells. The culture supernatants from restimulated Th17 cells generated from $CD4^+$ T cells, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cell, respectively were analyzed for IL-17 production by ELISA (B, C, and D). Again, IL-27 showed no effect on IL-17 production. Data are representative of three independent experiments. Plots are gated on T cells; numbers in quadrants indicate the frequency of cells each. ELISA data are expressed as mean ± SEM, and there is no statistical significance among them.

4.3 Conclusion and discussion

The data presented in this chapter demonstrate that IL-27 can play a dual role in murine model of CIA. The administration of IL-27 at the onset of CIA dramatically attenuated disease severity, but the administration of IL-27 at the later stage of CIA did not inhibit the disease development, but even exacerbated the disease severity.

A short term administration of IL-27 at the onset of the disease markedly suppressed the disease development compared with untreated PBS controls (Figure 4.2). Histological examination revealed that while untreated mice developed severe cellular infiltration in the joints, synovial hyperplasia and joint erosion, this pathology was profoundly reduced in IL-27-treated animals (Figure 4.3). Treatment of mice with IL-27 also decreased the amounts of serum IL-17, IL-6 and collagen-specific IgG2a (Figure 4.4B, 4.5). There were lower frequency of IFN-γ^+ and IL-17^+ expressing cells from spleen and lymph nodes of the IL-27-treated mice compared with the control mice (Figure 4.8). Also, the spleen and lymph node cells from the IL-27-treated mice produced less IL-17 compared with cells from the control mice when cultured with collagen *in vitro* (Figure 4.7, 4.9). These results indicated that the effect of IL-27-mediated inhibition of disease development was probably mainly due to the inhibition of IL-17 production. Consistent with the *in vivo* finding, *in vitro* data presented that IL-27 markedly inhibited the differentiation of Th-17 from naïve $CD4^+$, $CD4^+CD25^-$ and $CD4^+CD25^+$ three T cells populations under different Th-17 inducing conditions (Figure 4.14, 4.15). These results confirmed previous observations that IL-27 can suppress the development of Th-17, thus attenuating the development of

autoimmune inflammation, such as experimental autoimmune encephalomyelitis, chronic neuroinflammation, and human uveitis and scleritis (Yoshimura et al., 2006; Batten et al., 2006; Stumhofer et al., 2006; Amadi-Obi et al., 2007). The mechanism in which IL-27 inhibits the generation of Th17 has been mainly implicated through a STAT-1-dependent pathway (Batten et al., 2006; Stumhofer et al., 2006), and STAT-3 signalling has also been considered to be partially involved (Yoshimura et al., 2006). However, it is unclear whether STAT-1 and STAT-3 signalling are involved in IL-27 inhibited the production of IL-17 in CIA here. Further investigations would elucidate the potential pathways involved in the regulatory role of IL-27 on Th-17 development during CIA diseases process.

Currently, the role of IL-27 in various models of inflammatory diseases is still controversial. In some disease models IL-27 had been found to suppress the inflammation as mentioned above, but in other disease models, IL-27 was found to exacerbate inflammation (Goldberg et al., 2004; 2004 (2)). This controversy was also shown in the results in this chapter. In contrast to the inhibitory role of IL-27 administrated at the early stage of CIA, IL-27 did not inhibit the disease development, but in fact exacerbated the disease severity when administered during the late phase of CIA (Figure 4.11). There were no difference in serum IL-17 and IL-17 synthesis by the lymphoid cells between IL-27-treated mice and control mice (Figure 4.12, 4.13). This indicated that Th-17 cells were not affected by the IL-27 at the later phase of CIA. This can probably be explained by the fact that the CIA disease already progressed and Th17 cells were already differentiated one week after CII challenge. The data here showed that IL-27 had

little or no effect on pre-polarised Th17 cells *in vitro* (Figure 4.16). Furthermore, at the advanced disease stage of CIA, the lymph node and spleen cells from the mice with the administration of IL-27 produced elevated IFN-γ and IL-6 production when cultured with collagen *in vitro*, compared to the cells from untreated mice. The elevated pro-inflammatory cytokines IL-6 and IFN-γ induced by Il-27 may be a consequence of the disease exacerbation. The inhibitory effect on IL-6 and IFN-γ synthesis by IL-27 administrated at the early phase of CIA, and the accelerated effect on these cytokines synthesis when IL-27 administrated at the later phase of CIA, demonstrated that IL-27 might have the opposite effect on the inflammatory process depending on the timing of administration.

Together, these results demonstrated that IL-27 had both pro-inflammatory and anti-inflammatory effects. Any potential therapeutic use of IL-27 in modulating inflammatory diseases has to be approached with careful analysis.

Chapter 5

The role of a novel cytokine IL-35

(Some of results in this chapter already have been in publication as shown in Appendix 2)

5.1 Introduction

Cytokines play pivotal roles in the regulation of immune response and inflammation. Epstein-Barr virus-induced gene 3 (EBI3), first identified in B lymphoblastoid cell lines during EBV infection, encodes a 34-kDa glycoprotein with 27% amino acid identity to the IL-12 p40 subunit (Devergne, 1996). Both EBI3 and p40 are encoded by mRNAs with a 3' untranslated Alu repeat sequence, lacking a membrane anchoring motif, and are predicted to be secreted (Devergne, 1996). IL-12, composed of p40 and p35 subunits, was identified and purified from culture supernatant of EBV-transformed B cell lines and would be expected to be closely associated with EBI3 (Schoenhaut et al., 1992). IL-12 p35 is ubiquitously expressed whereas IL-12 p40 expression is inducible (Schoenhaut et al., 1992). The dissociation between p35 and p40 gene regulation suggests that either subunit may be associated with other partners. It has been known that p40 associates with p19 to form IL-23 (Oppmann et al., 2000). Recently discovered IL-27 is made with EBI3 and p28 protein (Pflanz et al., 2002). The association of EBI3 with IL-12 p35 to form a heterodimeric hematopoietin was discovered by Devergne's group in 1997, and this EBI3-p35 heterodimer may represent a novel cytokine of the IL-6 family which includes IL-12, IL-23 and IL-27. This novel heterodimeric cytokine EBI3-p35 has been officially designated interleukin-35 (IL-35) in 2002 (Schrader, 2002).

EBI3 is expressed at a high level in human B lymphoblast cells, tonsil and spleen (Devergne, 1996). Notably co-expression of EBI3 and p35 facilitates their secretion. They are not efficiently secreted when expressed alone. It has been demonstrated that p35 was detected in the entire EBI3 positive cell types,

especially in placental tissue throughout pregnancy and intestinal epithelial cells (Devergne, 2001; Masser, 2004). More importantly, a very recent paper found a high expression of *EBI3* and *IL-12p35* mRNA in mouse Foxp3$^+$ Treg cells, but not in resting or activated effector CD4$^+$ T cells (Collison et al., 2007).

When the project was designed, there has no report to suggest that IL-35 had any biological functions. To investigate the biological effects of IL-35, we constructed a soluble Fc fusion protein by flexibly linking the EBI3 chain to the p35 polypeptide, as described in the chapter 3. In this chapter, I will present data from the characterisation of this IL-35-Fc fusion protein in the murine model.

5.2 Results

5.2.1 IL-35 induces the proliferation of naïve CD4$^+$CD25$^-$ T cells with plate-bound anti-CD3 and soluble anti-CD28 stimulation

Naïve CD4$^+$CD25$^-$ T cells were purified from spleen and lymph nodes of BALB/c mice, and the cells were stimulated with immobilized anti-CD3 for 4 days, and IL-35 was titrated into the beginning culture. T cells proliferation was dose dependent in response to IL-35 (Figure 5.1). However, in the absence of CD3 activation, there was no cell proliferation observed. This indicated that IL-35-induced proliferation was dependent on CD3/TCR stimulation. In similar experiments, naïve CD4$^+$CD25$^-$ T cells were activated with immobilized anti-CD3 and soluble anti-CD28 for 4 days, the graded concentrations of IL-35 or EBI3-Fc were added in the beginning of the culture. The proliferation of naïve CD4$^+$CD25$^-$ T cells driven by IL-35 was profoundly enhanced. Thus, IL-35-

mediated proliferation was improved by co-stimulatory signal through CD28. (Figure 5.1).

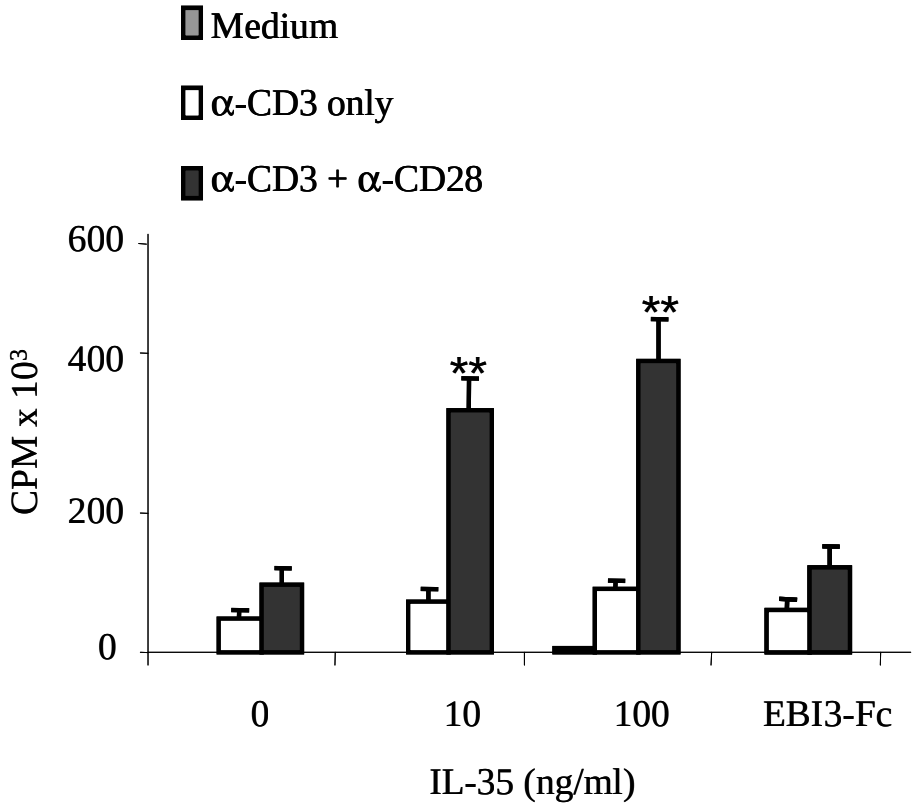


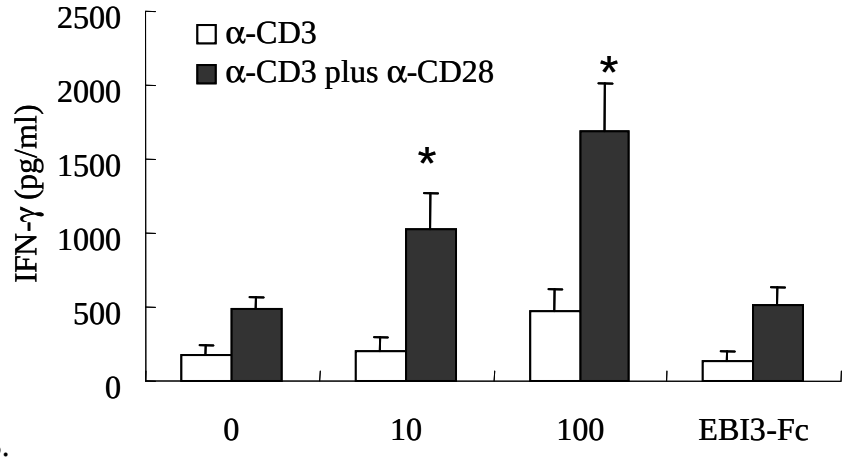
Figure 5.1 IL-35 induces $CD4^+CD25^-$ T cells proliferation under polyclonal TCR activation and costimulation.

$CD4^+CD25^-$ T cells purified from spleen and lymph nodes of BALB/c were stimulated for 4 days in the medium alone, with plate-coated anti-CD3 alone, or plate-coated anti-CD3 plus soluble anti-CD28, in the presence of graded concentrations of IL-35 or EBI3-Fc (100ng/ml) or medium alone. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are shown as mean ± SEM. n=4, and are representative of at least three independent experiments. **, $p < 0.01$ compared to control.

5.2.2 IL-35 triggers naïve $CD4^+CD25^-$ T cells to produce IFN-γ, but not IL-4

The structure of IL-35 is closely tied to Th1 cytokine family, IL-12 and IL-27. Therefore, it was of interest to investigate the effect of IL-35 in IFN-γ synthesis. Naïve $CD4^+CD25^-$ T cells isolated from BALB/c spleen and lymph nodes were stimulated for 4 days with plate-coated anti-CD3 alone or plate-coated anti-CD3 plus soluble anti-CD28. IL-35 itself induced low levels of IFN-γ production in anti-CD3 activated assay. With costimulation of anti-CD28, IL-35 alone was capable of inducing significant amount of IFN-γ (Figure 5.2A). EBI3-Fc had no effect on IFN-γ synthesis. In contrast, IL-35 had little effect on IL-4 synthesis, and the slightly enhanced production of IL-4 compared to control was due to the higher proliferation from IL-35 cultured $CD4^+CD25^-$ T cells (Figure 5.2B).Thus, IL-35 appeared to trigger naïve $CD4^+CD25^-$ T cells to produce IFN-γ, but not IL-4.

A.



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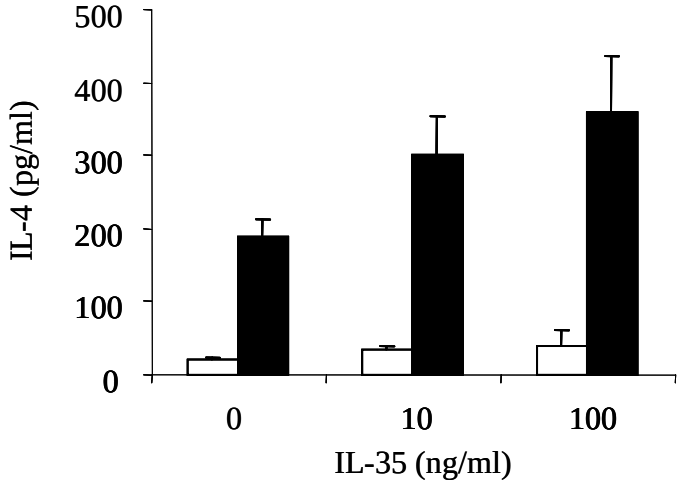


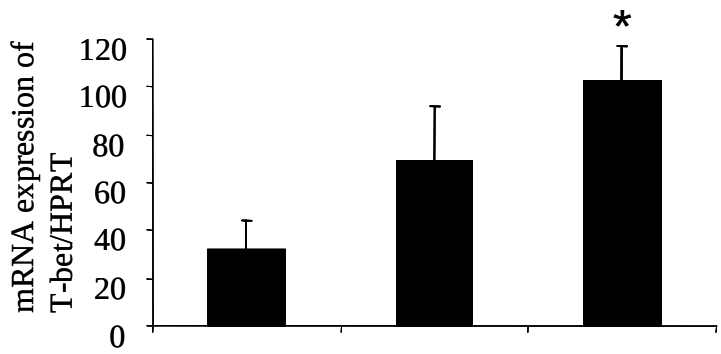
Figure 5.2 IL-35 induces $CD4^+CD25^-$ T cells to produce IFN-γ, but little IL-4.

Purified $CD4^+CD25^-$ T cells from BALB/c spleen and lymph nodes were stimulated for 4 days with plate-coated anti-CD3 alone or plate-coated anti-CD3 plus soluble anti-CD28, in the presence of graded concentrations of IL-35, or EBI3-Fc (100ng/ml), or medium alone. IFN-γ (A) and IL-4 (B) concentrations in the parallel culture supernatants were measured by ELISA. Data are shown as mean ± SEM. n=4, and are representative of at least three independent experiments. *, $p < 0.05$ compared to control by student's *t* test.

5.2.3 IL-35 induces the expression of T-bet

Th1 differentiation requires the expression of T-bet, a master transcription regulator, by directly activating Th1 associated genetic process and repressing the production of Th2 cytokines (Hibbert et al., 2003). Therefore, these activated $CD4^{+}CD25^{-}$ T cells from the above costimulation culture conditions were analysed for T-bet expression by RT-PCR. IL-35 greatly enhanced T-bet expression compared to control (Figure 5.3 A). As a comparison, the effect of IL-35 on the expression of GATA-3, which is a master transcription factor for Th2 development, was also examined. As shown in Figure 5.3B, GATA-3 mRNA expression was not affected, but even slightly repressed by IL-35.

A.



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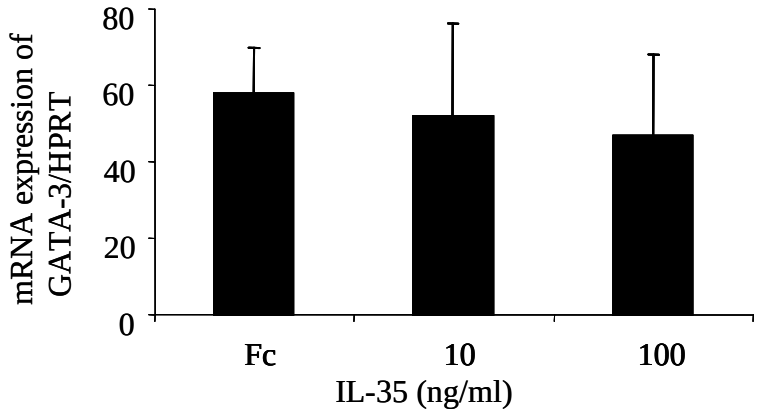


Figure 5.3 IL-35 induces the expression of T-bet, but not GATA-3.

Purified $CD4^{+}CD25^{-}$ T cells from BALB/c mice were stimulated with plate-bound anti-CD3 and soluble anti-CD28 in the graded concentrations of IL-35 or human Fc (100ng/ml). After culture for 48 h, total mRNA were isolated and analyzed by Taqman-PCR for the mRNA expression of transcription factors T-bet (A) and GATA-3 (B). Data are represented as the percentage of HPRT expression, n=4, and are representative of at least three independent experiments. *, $p < 0.05$ compared to control.

5.2.4 IL-35 induces expression of STAT-1 phosphorylation

As is known that T-bet is induced early in T cell development by a STAT-1 dependent mechanism (Hibbert et al., 2003), the next experiment was to check whether IL-35 was responsible for the induction of STAT1. Purified naïve $CD4^{+}CD25^{-}$ T cells were activated with plate-coated anti-CD3 plus soluble anti-CD28 for 24 h, in the presence or absence of IL-35. As shown in the Figure 5.4, STAT1 was strongly phosphorylated in response to IL-35. This data indicated that IL-35 might exert its effect on the up-regulation of T-Bet expression through STAT-1 activation.

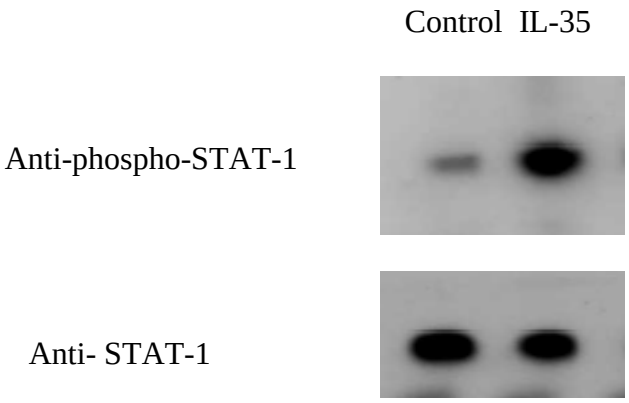


Figure 5.4 IL-35 induces the expression of STAT-1 phosphorylation.

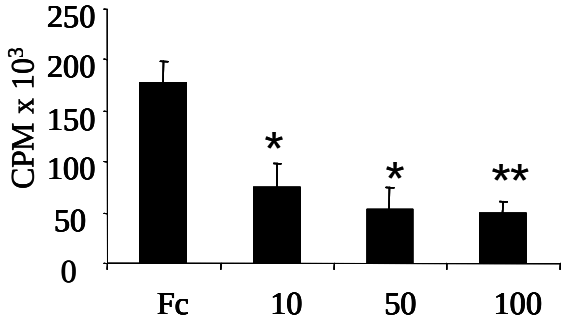
Purified naïve $CD4^{+}CD25^{-}$ T cells from BALB/c mice were activated with plate-coated anti-CD3 plus soluble anti-CD28 for 24 h, in the absence or presence of IL-35 (100ng/ml). The cells were then washed and lysated. Total cell lysates were prepared and subjected to Western Blotting with antiphosphotyrosine antibodies.

5.2.5 IL-35 inhibits the proliferation of naïve $CD4^+CD25^-$ T cells with APC and soluble anti-CD3

The next studies were performed to determine whether IL-35 regulates the proliferation of naïve T cells under different culture condition using mitomycin C- treated antigen-presenting cells (APCs) from spleen cells and soluble anti-CD3 antibody. Upon encounter between mitomycin C- treated APC and soluble anti-CD3, purified $CD4^+CD25^-$ T cells became activated and proliferated at a similar level as when they were activated with plate bound anti-CD3 and anti-CD28. It was surprising that IL-35 significantly suppressed the proliferation of $CD4^+CD25^-$ T cells in a dose dependent manner (Figure 5.5 A).

Furthermore, the supernatants were harvested and the cytokines were analysed. IFN-γ (Figure 5.5 B) and IL-4 (Figure 5.5 C) production were markedly inhibited in the cell culture in the presence of IL-35 compared to the control culture with human IgGFc. These results therefore demonstrated that IL-35 is an inhibitory cytokine under more “physiological” conditions.

A.



B. C.

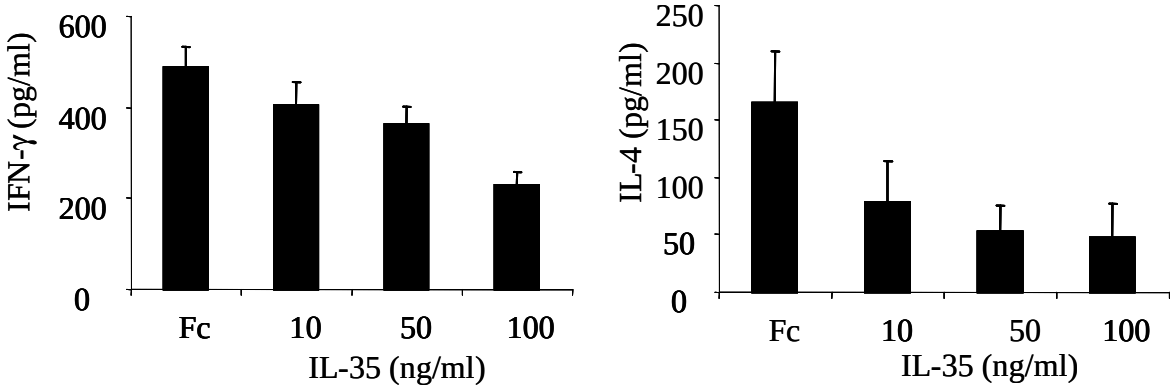


Figure 5.5 IL-35 suppresses the proliferation of $CD4^+CD25^-$ T cells and inhibited the cytokine productions of $CD4^+CD25^-$ T cells.

$CD4^+CD25^-$ T cells were purified from spleen and lymph nodes of BALB/c mice and cultured for 4 days with mitomycin C- treated APCs and soluble anti-CD3 antibody in the presence of graded concentrations of IL-35 or human Fc. IL-35 markedly suppressed the proliferation (A), IFN-γ (B) and IL-4 (C) production of $CD4^+CD25^-$ T cells. Cell proliferation was determined by [^{3}H] thymidine uptake. Data are mean ± SEM. n=4, and are representative of three independent experiments, *, $p< 0.05$, **, $p< 0.01$ compared to Fc control by student's *t* test.

5.2.6 IL-35 expands $CD4^+CD25^+$ T cells with co-stimulation

Regulatory T cells mediate significant protection against autoimmune diseases by suppressing the proliferation of auto-reactive T cells (Sakaguchi et al., 2004). To determine whether IL-35 have effects on Treg cell, $CD4^+CD25^+$ T cells were purified from spleen and lymph nodes of BALB/c mice and cultured *in vitro* with IL-35 in the presence of plate-bound anti-CD3 for 3 days, and anti-CD28 antibody plus IL-2 were added at 24 h interval after the start of the culture. Under these culture conditions, IL-35 markedly induced proliferation of $CD4^+CD25^+$ T cells (Figure 5.6 A), with a significant elevated level of IL-10 in the culture supernatants compared to Fc control (Figure 5.6 B).To further identify the effect of IL-35 on Treg cells, the characteristics of Treg cells were examined when they were expanded by IL-35. The expression of Foxp3, a transcript factor specifically expressed in Tregs cells, was detected by Taqman and intracellular staining. The majority of $CD4^+CD25^+$ T cells from wild- type BALB/c mice expressed high levels of Foxp3. Although there are slightly higher levels of Foxp3 mRNA in $CD4^+CD25^+$ T cells expanded in the presence of IL-35 compared to control (Figure 5.6.C), no significant difference for Foxp3 expression was observed intracelluarly (Figure 5.6. D). Thus, these data showed that IL-35 expanded $CD4^+CD25^+$ T cells, but did not induce additional Foxp3 expression of Treg cells.

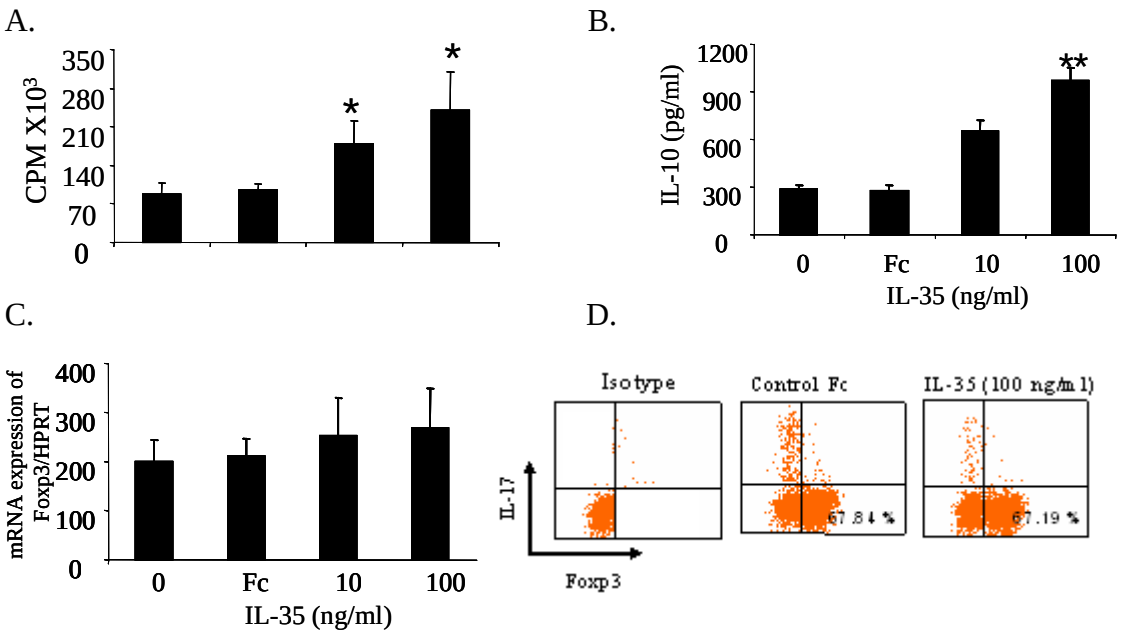


Figure 5.6 IL-35 expands $CD4^+CD25^+$ T cells under polyclonal TCR activation and co-stimulation.

$CD4^+CD25^+$ T cells were purified from spleen and lymph nodes of BALB/c mice and cultured *w*ith the different concentrations of IL-35 or human Fc in the presence of plate-bound anti-CD3 for 3 days, and anti-CD28 antibody and IL-2 were added at 24 h interval after the start of the culture. IL-35 induced $CD4^+CD25^+$ T cells to proliferate (A), and to produce IL-10 (B). There was no difference in Foxp3 expression (C and D). Cell proliferation was determined by [^{3}H] thymidine uptake. IL-10 concentrations in the culture supernatants were measured by ELISA. Total mRNA was isolated and analyzed by taqman for the mRNA expression of transcription factor Foxp3. Data are representative as the percentage of HPRT expression. For intracelluarlly staining, the cells were stained for Foxp3 and analyzed on a flow cytometer. The number in quadrants represent the percentage of $Foxp3^+$ expressing cells. Data are shown as mean ± SEM. n=4, and are representative of three independent experiments, *, $p< 0.05$; **, $p< 0.01$ compared to Fc control.

5.2.7 The IL-35- expanded $CD4^+CD25^+$ T cells maintain the suppressive function against the effector $CD4^+CD25^-$ T cells

Regulatory $CD4^+CD25^+$ T cells have the capability to suppress effector $CD4^+CD25^-$ T cells *in vitro* (Sakaguchi et al., 2004). In the presence of IL-35, the capability of Treg to suppress the proliferation of effector T cells was enhanced compared to the culture in the absence of IL-35 (Figure 5.7A). Notably, under mitomycin C- treated APC and soluble anti-CD3 antibody culture condition, IL-35 had little or no effect on $CD4^+CD25^+$ T cells (Figure 5.7.A). However, consistent with data presented in Figure 5.5 A, IL-35 directly suppressed the proliferation of effector $CD4^+CD25^-$ T cells in the absence of Treg cells (Figure 5.7.A). To rule out the direct suppressive effect of IL-35 on effector T cells, purified $CD4^+CD25^+$ T cells ware expanded with plate-bound anti-CD3 in the presence of IL-35 for 4 days, and soluble anti-CD28 plus IL-2 were supplemented after 24 hours interval at the beginning of cell culture. Then, the IL-35- expanded $CD4^+CD25^+$ T cells were washed and the suppressive function was checked. Expanded $CD4^+CD25^+$ T cells by IL-35 maintained the normal capacity to suppress $CD4^+CD25^-$ effector T cells (Figure 5.7.B).

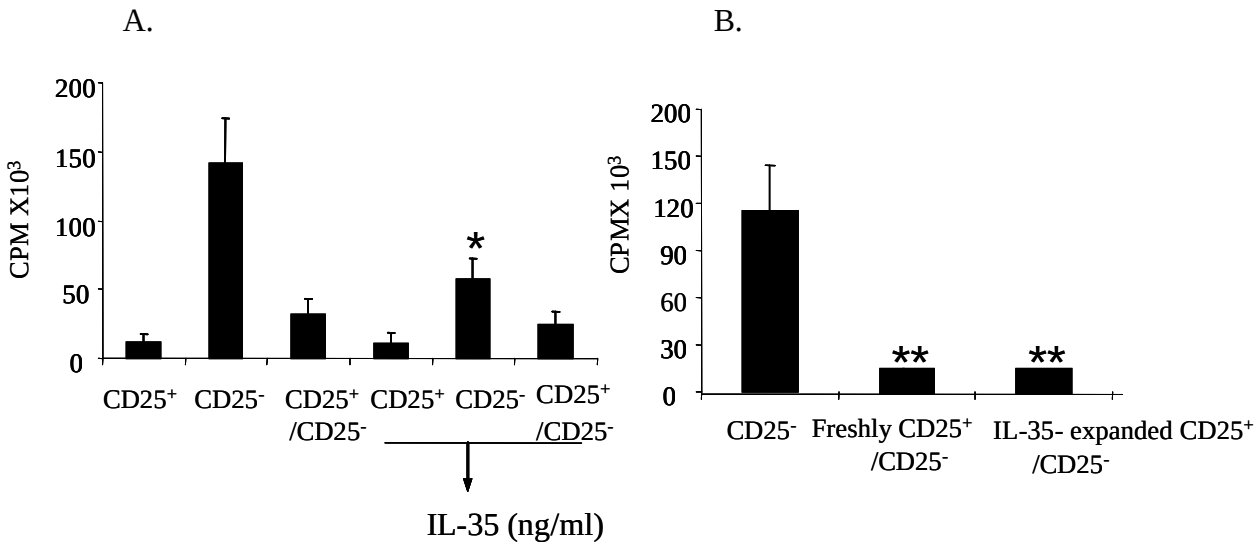


Figure 5.7 The IL-35- expanded $CD4^+CD25^+$ T cells maintain suppressive function against the effector $CD4^+CD25^-$ T cells.

A. Under mitomycin C- treated APCs and soluble anti-CD3 antibody culture condition, $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were separated from spleen and lymph nodes of BALB/c mice, and were cultured individually or mixed together (1:1 ratio). IL-35 (100 ng/ml) were added in some culture. After 3 days culture, cell proliferation was determined by [^{3}H] thymidine uptake. Data are shown as mean ± SEM. n=4, and are representative of at least three independent experiments,*, $p< 0.05$ compared to control.

B. Purified $CD4^+CD25^+$ T cells was expanded with plate-bound anti-CD3 in the presence of IL-35 (100ng/ml) for 4 days, and soluble anti-CD28 plus IL-2 were supplemented after 24 hours interval at the beginning of cell culture. Then, the IL-35 -expanded $CD4^+CD25^+$ T cells were washed and cultured with freshly purified $CD4^+CD25^-$ T cells for 3 days. Cell proliferation was determined by [3H] thymidine uptake. Data are shown as mean ± SEM. n=4, and are representative of three independent experiments, **, $p< 0.01$ compared with $CD4^+CD25^-$ T cells proliferation alone.

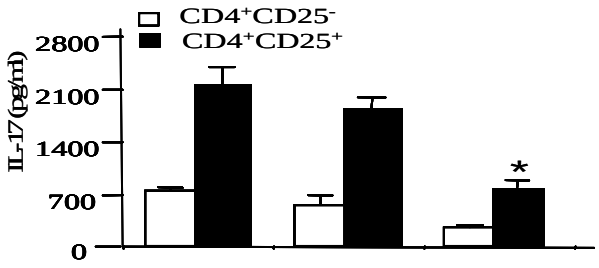
5.2.8 IL-35 inhibits Th-17 development

As described in chapter 4, IL-27 is found to inhibit IL-17 production, which raises the question about the effect of IL-35 on Th-17 development. Purified $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from BALB/c mice were stimulated for 4 days with plate-coated anti-CD3 and anti-CD28 in the absence or presence of IL-35 or EBI3-Fc, and additional IL-2 was added after 24 h in the $CD4^+CD25^+$ T cell culture. Activated $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells acquired the ability to produce IL-17, in the absence of exogenous IL-35. Activated $CD4^+CD25^+$ T cells spontaneously induced nearly 3 times more IL-17 compared to activated $CD4^+CD25^-$ T cells. IL-35 strongly suppressed the production of IL-17 in both activated T cells (Figure 5.8 A). In contrast, EBI3-Fc failed to reduce IL-17 production.

IL-1, TGF-β, and IL-6 are essential for Th-17 differentiation, whereas blockade of IFN-γ, IL-2, and IL-4 amplifies their development (Veldhoen et al., 2006, Mangan et al., 2006). Therefore, we tested the ability of IL-35 to directly affect Th17 cells. Purified $CD4^+$ T cells isolated from the spleens and lymph nodes of BALB/c mice were stimulated with anti-CD3 plus anti-CD28 for 4 days. Some cultures received IL-1, TGF-β, and IL-6 (a Th-17 cytokine mixture), and others received the Th-17 cytokine mixture with the combination of blocking antibodies: IFN-γ, IL-2, and IL-4 (an optimal Th-17- inducing conditions). In both culture conditions, analysis of supernatants showed that IL-35 significantly suppressed the production of IL-17 (Figure 5.8B). In a parallel study, these cultured cells were restimulated with PMA and ionomycin, then analysed for IL-17 by flow cytometry. Consistent with the supernatants data presented above, the

addition of IL-35 efficiently inhibited the differentiation of Th17 producing cells (Figure 5.8C). Notably, IL-35 seemed less effective in inhibiting Th-17 production in the culture supplemented with blocking antibodies compared to the culture without blocking antibodies. Thus, IFN-γ production was analyzed from the Th-17 cytokine mixture culture only. IL-35 significantly enhanced the production of IFN-γ compared to the control culture (Figure 5.8D). These results collectively suggested that IL-35 is a potent antagonist of Th-17 activity, and the inhibitory ability of IL-35 might be partly through enhancing IFN-γ synthesis.

A.



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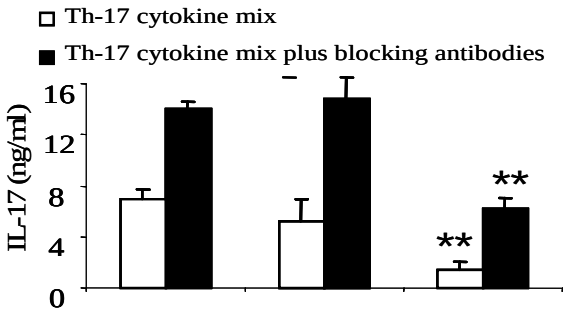

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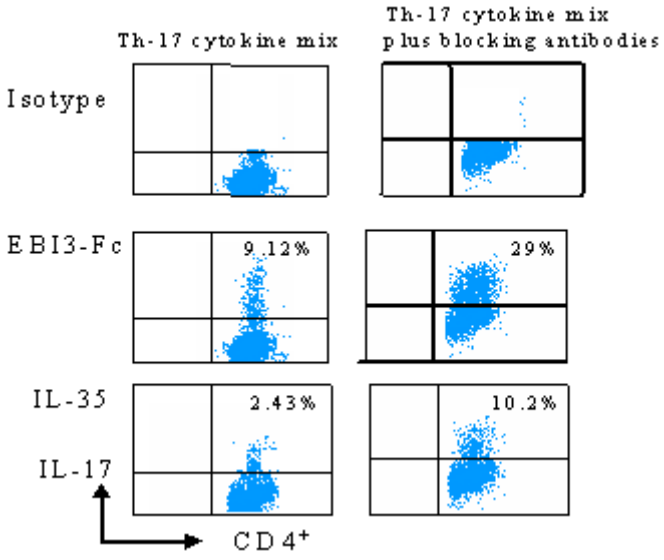

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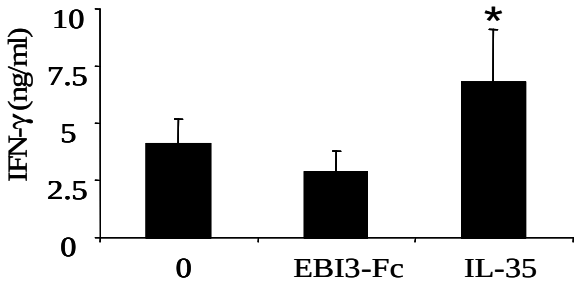


Figure 5.8 IL-35 inhibits Th-17 development, but promotes Th1 cell differentiation.

(A.) Highly purified $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells from BALB/c mice were stimulated for 4 days with plate-coated anti-CD3 and anti-CD28 in the absence or presence of IL-35 (100 ng/ml), or EBI3-Fc (100 ng/ml), and additional IL-2 was added after 24 h later in the $CD4^+CD25^+$ T cell culture. After 3 days culture, the supernatants were harvested and analyzed for IL-17 by ELISA. Data are shown as mean ± SEM. n=4, and are representative of three independent experiments. *, $p< 0.05$.

(B, C.D) Purified $CD4^+$ T cells isolated from the spleens and lymph nodes of BALB/c mice were stimulated with anti-CD3 plus anti-CD28 for 4 days, under two culture conditions: a Th-17 cytokine mixture condition (IL-1, TGF-β, and IL-6), and a optimal Th-17- inducing condition (IL-1, TGF-β, and IL-6, and blocking antibodies IFN-γ, IL-2, and IL-4). IL-35 or EBI3-Fc was added to some samples. After 3 days culture, the supernatants were harvested and analyzed for IL-17 (B) and IFN-γ (D) by ELISA. Data are shown as mean ± SEM. n=4, and are representative of three independent experiments. *, $p< 0.05$; **, $p< 0.01$ compared to control. Then, the cultured cells were restimulated with PMA and ionomycin, after that the cells were stained for intracellular IL-17, and analyzed by flow cytometry. The numbers in quadrants represent the percentage of IL-17-expressing $CD4^+$ T cells.

5.2.9 Treatment with IL-35 reduced the severity of CIA

In the previous sections I have shown that IL-35 played certain roles *in vitro*. This raises the question about the *in vivo* biological function of IL-35. CIA, as described before, is an animal model for RA. IL-12 and IL-35 share the same p35 subunit, and studies have defined that IL-12 regulates the inflammatory progression of CIA (Joosten 1997). These raised our interest to examine the process of CIA administrated with IL-35。

CIA was induced as described in the material and methods. As expected, the mice began to develop the clinical sign of arthritis on day 24. From then, the mice were given 10 daily i.p. injections of IL-35 (2 μg/dose) or PBS. The mice were culled after IL-35 administration (day 34) to investigate the kinetics of the response (Figure 5.9).

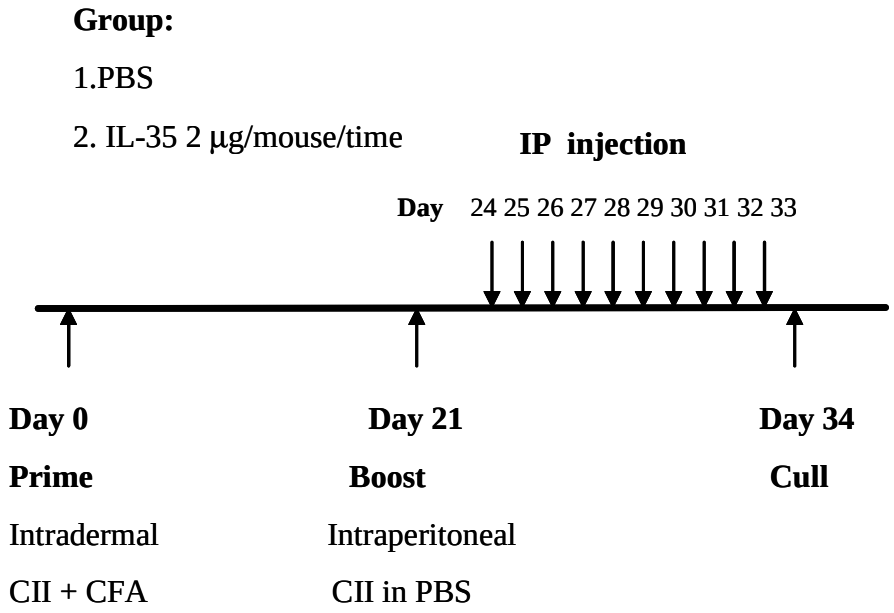


Figure 5.9 The schedule of Collagen-induced arthritis.

DBA/1 mice (7 week old) were primed intradermally with 100 μg of bovine collagen type II (CII) in complete Freund's adjuvant (CFA) at the base of tail on day 0, and boosted intraperitoneally with 100 μg of CII in PBS on day 21. 10 times of 200 μl PBS, 2 μg/ 200 μl of IL-35 were given i.p. starting on day 24. The mice were observed for clinical sign of arthritis, and the footpads were measured by callipers every other day. Mice were culled on day 34.

Control mice treated with PBS developed the expected disease progression. In contrast, mice treated with IL-35 displayed a significant reduction in arthritic incidence (Figure 5.10A) and in the number of arthritic paws (Figure 5.10B). Likewise, the thicknesses of the paw swelling and mean clinical score in IL-35 treated mice were also milder, relative to PBS treated mice (Figure 5.10C, D). Histological analysis of joints revealed that mice treated with PBS exhibited cell infiltration into joint compartment, synovial hyperplasia and bone erosion (Figure 5.11A). Each of these parameters was markedly suppressed in the mice treated with IL-35 (Figure 5.11B). The histological scores are summarized in Figure 5.11C. These results indicated that IL-35 potently down-regulate the development of CIA and prevent the progression of articular damage.

To gain insight into the mechanism of IL-35- mediated suppression, serum levels of certain cytokines were measured at the end of the treatment. TNF-α, IFN-γ, IL-1Ra, and IL-6 were similar, IL-17 and IL-12 were slightly reduced, whereas IL-10 was greatly enhanced in the mice treated with IL-35 compared with control mice (Figure 5.12A). In addition, serum anti-CII Ab was analyzed, but there was no significant difference in the levels of anti-CII IgGs, IgG1, or IgG2a between the two groups of mice (Figure 5.12B).

To determine the effect of *in vivo* administration of IL-35 on the IFN-γ and IL-17-producing lymphocytes, spleen cells were isolated on day 34 after treatment, and stimulated with PMA and ionomycin and analysed by intracellular staining. Spleen cells from IL-35-treated mice produced a lower percentage of IL-17-secreting cells compared to that of the PBS-treated control mice. Interestingly,

the percentage of IFN-γ expressing cells in IL-35 treated mice was modestly enhanced compared to control mice (Figure 5.13).

To elucidate the role of IL-35 in T cell function, a T cell Ag-recalling assay against CII was conducted. Proliferative response against CII of mixed splenocyte cell culture was observed, but there was no difference between IL-35 treated mice and control mice (Figure 5.14A). Likewise, there was also no difference between the two groups in the pattern of cytokines consisting of TNF-α, IL-6, IL-12, and IL-17, secreted in the supernatants of cell culture (Figure 5.14 C.D.E). However, there was a slight enhancement of IFN-γ detected in cell culture from IL-35 treated mice compared with control mice (Figure 5.14 B).

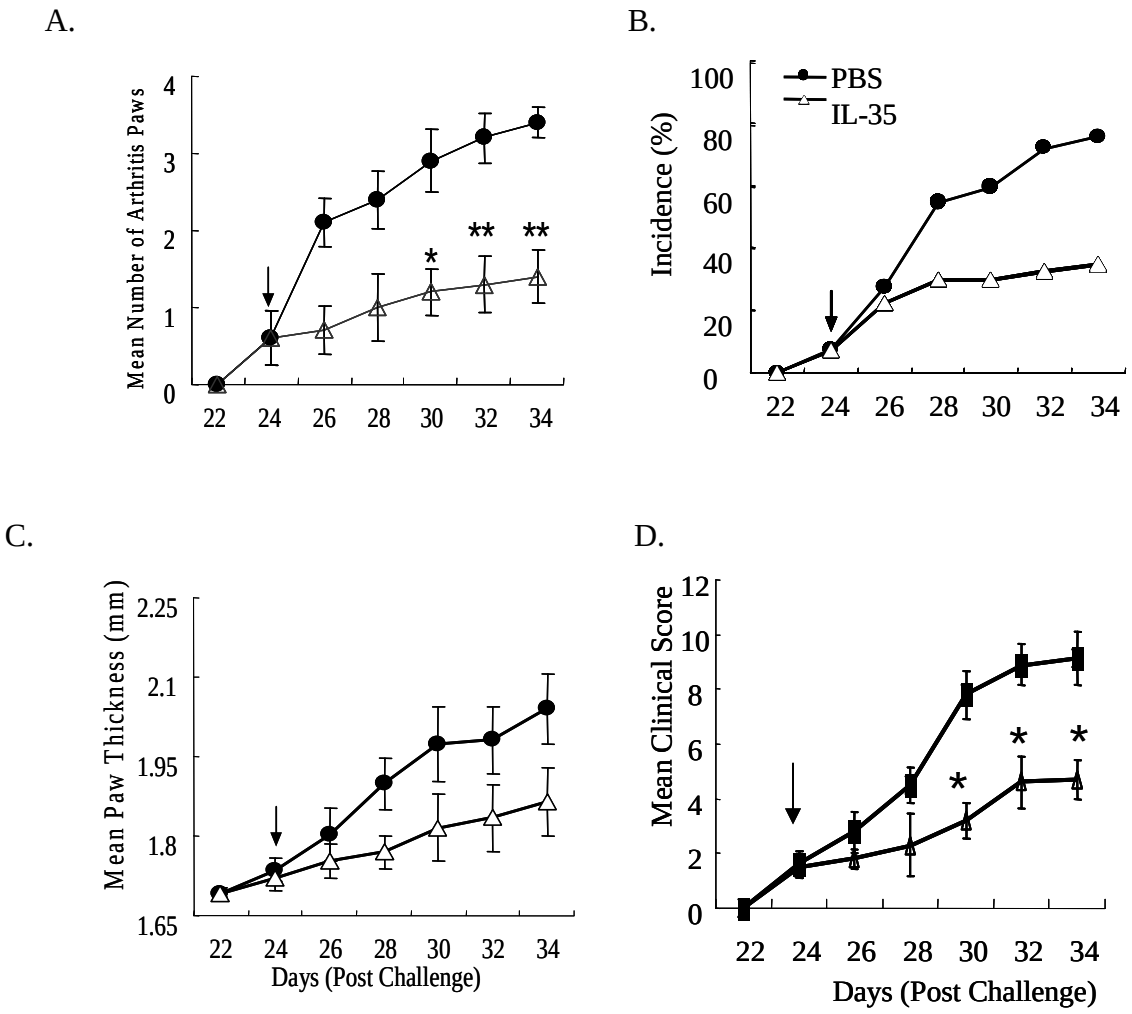
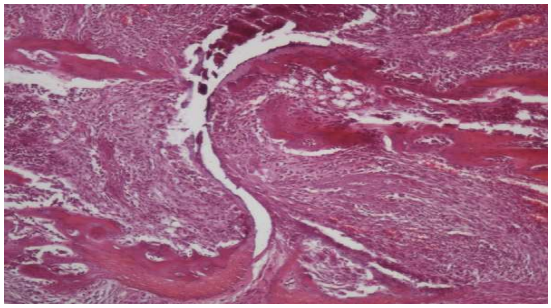


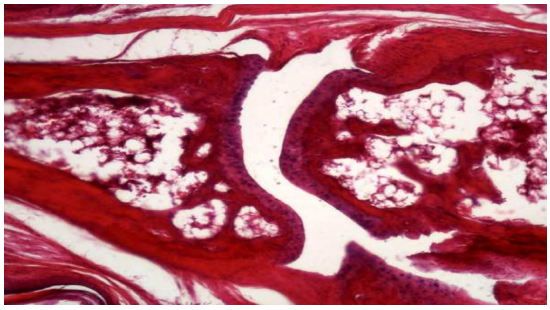
Figure 5.10 IL-35 suppressed the disease progress of CIA in DBA/1 mice.

Collagen-primed DBA/I mice were randomly divided into groups of 10, challenged on day 21, and given 10 daily i.p. injections of 2 μg/200 μl of EBI3/p35-Fc, or 200 μl of PBS starting on day 24 (Shown in arrow). Mice were monitored for disease progression as indicated. IL-35-treated mice developed significantly less arthritic incidence (A), less number of arthritic paws (B), less severe paw thickness (C) and lower clinical score (D) compared to control mice treated with PBS. Values are mean ± SEM. n=10, and are representative of two independent experiments, *, $p< 0.05$; **, $p< 0.01$ compared to PBS group.

A. **PBS**



B. **IL-35**



C.

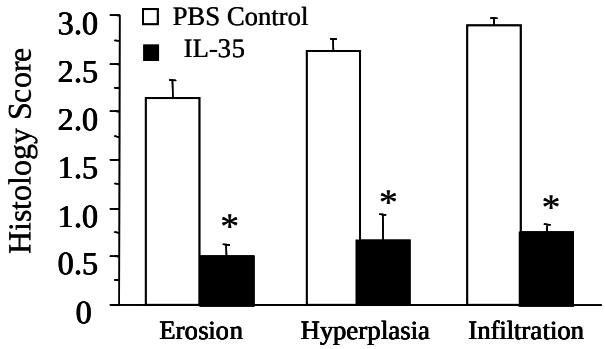
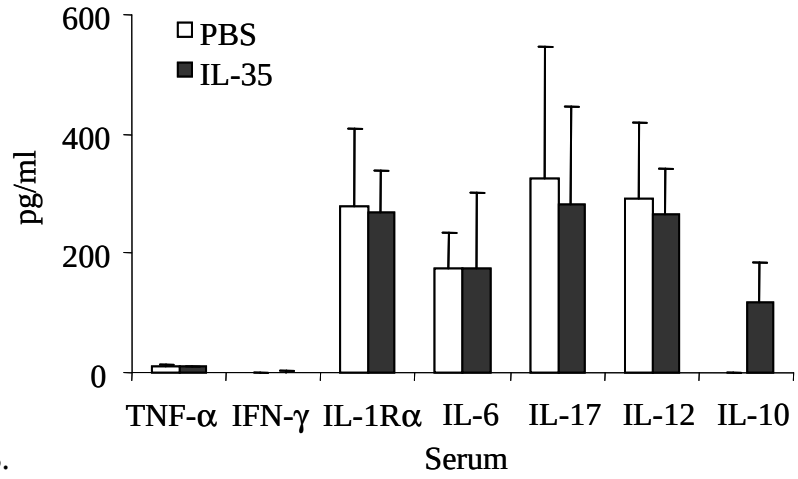


Figure 5.11 Administration of IL-35 significantly reduced joint pathology.

At the end of IL-35 or PBS administration, hind limbs (Six mice /group) were removed, formalin-fixed, decalcified, and stained with H&E. Profound cartilage and bone erosion, hyperplasia and cellular infiltration were observed in the control PBS group (A) whereas those of the IL-35-treated group exhibited reduced histological evidence of destruction (B). Original magnification (A and B) is x50. (C) Histological appearances were scored (0-3) for the presence of bone erosion, synovial hyperplasia, and cellular infiltration (D). Data are mean ± SEM.*, $p < 0.05$ compared to PBS group. (This figure has already been published in Niedbala et al., 2007, as shown in Appendix 2)

A.



B.

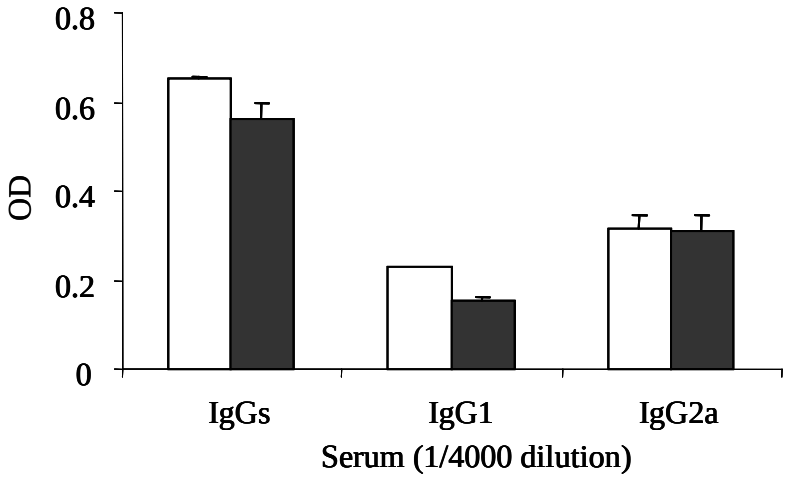


Figure 5.12 Effect of IL-35 or PBS treatment on serum cytokines and serum anti-collagen Abs.

Serum were collected after DBA/I mice were treated with either IL-35 or PBS on day 34. Levels of TNF-α, IFN-γ, IL-1Rα, IL-6, IL-17, IL-12, and IL-10 were determined by Luminax (A). Data are mean ± SEM of individual mouse (n=6). (B) Collagen-specific total IgGs, IgG1 and IgG2a in serum were determined by ELISA. Serum samples were diluted 4000-fold, and data are expressed as mean absorbance (OD 630) ± SEM of individual mouse (n=6). There is no significance between two groups.

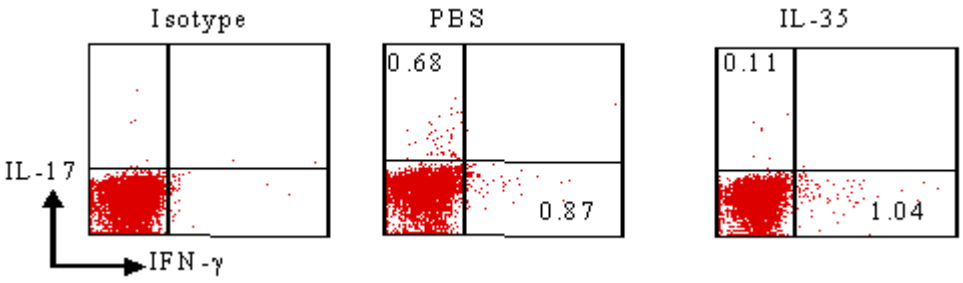


Figure 5.13 Intracelluar cytokine analysis of IFN-γ and IL-17 secretion.

Spleen cells from mice treated with IL-35 or PBS were stimulated with PMA and ionomycin and stained intracellularly for IL-17 and IFN-γ. The spleen cells from the IL-35-treated mice show a reduced frequency of IL-17-producing cells but a higher frequency of IFN-γ expressing cells. The number in quadrants represent the percentage of IL-17- or IFN-γ expressing cells.

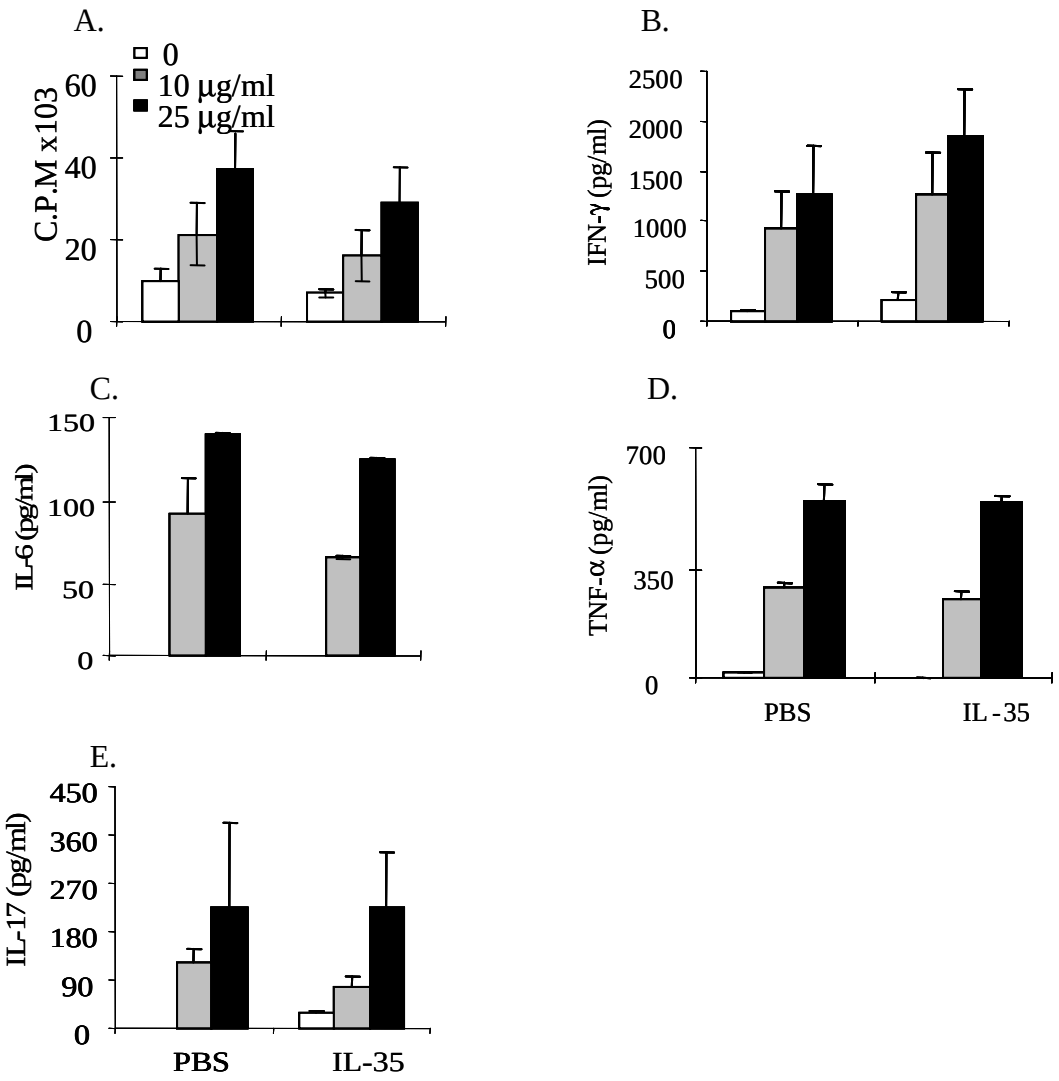


Figure 5.14 Assessment of *in vitro* responses against collagen from mice treated with IL-35 or PBS.

Spleen cells (pooled from 6 mice per group) were collected from IL-35 treated or PBS treated mice after 10 times of injections (day 34) and cultured with titrated concentrations of collagen for 96 h. T cell proliferation (A), which was determined by [^{3}H] thymidine uptake, is expressed as mean cpm ± SEM of triplicate cultures. Supernatants from parallel cultures were collected after 72h. IFN-γ (B), IL-6 (C), TNF-α (D), and IL-17(E) levels were measure by ELISA and expressed as mean ± SEM. There was no statistical difference between PBS and IL-35 treated group.

The therapeutic effect of IL-35 on CIA was further characterized. Firstly in order to exclude the possibility that the effect of IL-35 was due to the effect of the Fc fragment of the molecule, DBA/1 mice primed and boosted with CII were treated i.p. daily for 10 days with IL-35, EBI3-Fc, or PBS from day 24. Mice treated with IL-35 developed significantly less severe disease compared to the PBS-treated mice, but mice treated with EBI3-Fc developed diseases indistinguishable from PBS-treated mice (Figure 5.15 A). These data indicated that the effect of IL-35 was not due to the Fc fragments. Consistent with *in vitro* results which showed that EBI3-Fc had no effect on Th-17 cell differentiation.

Next, a dose response study on the therapeutic effect of IL-35 on CIA was carried out. DBA/1 mice primed and boosted with CII were treated i.p. daily for 10 days with graded doses of IL-35. A daily dose of 2 μg per mouse of IL-35 was found to produce the best therapeutic effect; although the daily low dose of 0.4 μg of IL-35 was also effective (Figure 5.15 B).These results therefore demonstrated that IL-35 may be a new effective therapeutic agent against clinical rheumatoid arthritis.

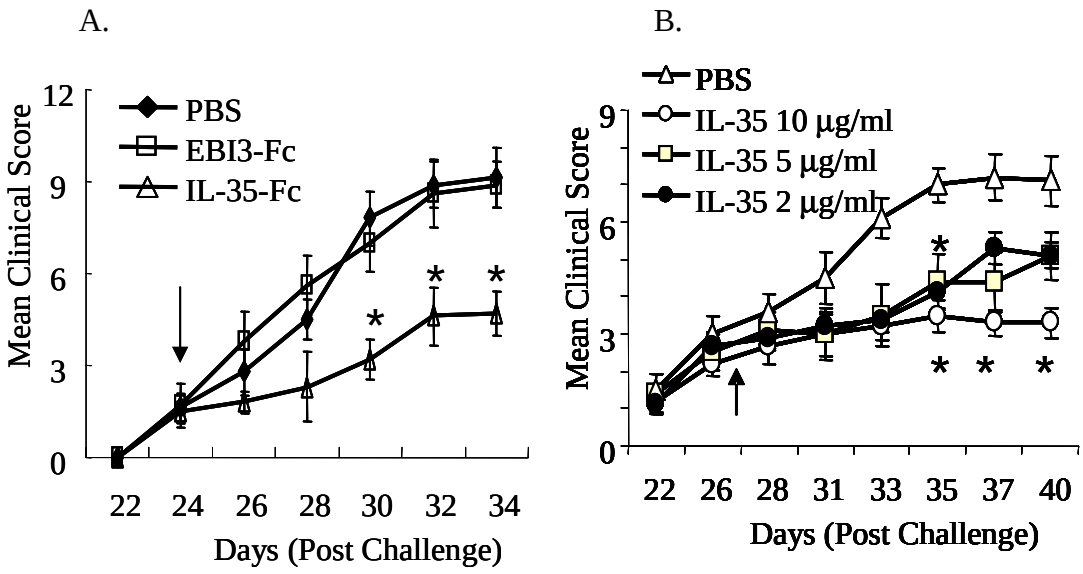


Figure 5.15 Further characterization of the therapeutic effect of IL-35 on CIA in DBA/1 mice.

A. DBA/1 mice were primed and boosted with CII as shown in Figure 5.10. Mice were treated i.p. daily with IL-35 (2 μg per mouse), EBI3-Fc (2 μg per mouse), or PBS for 10 days form day 24 as indicated by the arrows. Mice were monitored for disease progression. IL-35 but not EBI3-Fc suppressed CIA. Values are mean ± SEM. n=10,*,$p< 0.05$. ; **, $p< 0.01$ compared to PBS group.

B. DBA/1 mice were primed and boosted with CII as in Figure 5.10 and treated i.p. daily with graded concentrations of IL-35 for 10 days starting from day 27 (indicated by arrow). Mice were monitored for disease progression. The suppression of CIA by IL-35 was dose dependent. Values are mean ± SEM. n=10,*,$p< 0.05$ compared to PBS group. (This figure has already been in publication as shown in appendix 2).

5.3 Conclusion and discussion

EBI3 was reported in 1997 by Devergne group to associate non-covalently with IL-12 p35 to form a novel heterodmeric cytokine, and this novel cytokine has been officially designated as IL-35 in 2002 by the Interleukin Nomenclature and the HUGO Gene Nomenclature Committee. IL-12 p35 is constitutively expressed in most tissues, while EBI3 is mainly expressed in haematopoietic cells. The up-regulated expression of EBI3 and IL-12 p35 were found in placental trophoblasts (Devergne et al., 2001). Trophoblasts are known to play an important role in maternal tolerance to the semi-allogeneic foetus (Jokhi et al., 1997; Le Bouteiller et al., 1999), which indicated that IL-35 may be involved in the immunosuppressive activity.

IL-35 is a new member of the IL-12 family which includes IL-23 and IL-27. IL-35 shares EBI3 with IL-27, and the data in this chapter demonstrated that IL-35 has similarity with IL-27. However, this is not due to EBI3, because EBI3 on its own has no effect both *in vitro* and *in vivo* (Figure 5.1; 5.8 and 5.15 A). Like IL-27, IL-35 can induce the proliferation as well as anti-proliferation of $CD4^+$ T cells *in vitro*, and the dual functional role of IL-35 may depend closely on the mode of T cell activation. With the *in vitro* combination of plate-bound anti-CD3 and anti-CD28 co-stimulation, IL-35 induced the proliferation of effector T cells (Figure 5.1), and these activated T cells produced a high amount of IFN-γ (Figure 5.2 A). However, under *in vitro* APC and soluble anti-CD3 activation condition, IL-35 profoundly suppressed effector $CD4^+$ T cells and inhibited proinflammatory cytokine productions including IFN-γ (Figure 5.5). Furthermore, IL-35 induced the proliferation of $CD4^+CD25^+$ T cells with a

significant elevation of the IL-10 cytokine productions under the costimulation of anti-CD3 and anti-CD28 plus IL-2 condition (Figure 5.6), and these IL-35-expanded $CD4^+CD25^+$ T cells remained highly suppressive against the effector $CD4^+CD25^-$T cells (Figure 5.7). These results demonstrated that IL-35 may play a regulatory role in immune response. During strong antigenic challenge such as acute infections, IL-35 might be formed by the association of EBI3 and IL-12 p35. IL-35 might preferentially activate Th1-like cells which help clear the infection. Meanwhile, IL-35 might also expand Treg cells under acute infection. The Treg cells would prevent collateral damage by suppressing the residual effector cells during the ensuing chronic infective phase. Also, IL-35 might directly suppress effector T cells under chronic infections or inflammatory conditions.

Recently discovered Th-17 cells, a unique subset of T cells, has been associated with the development of the pathology noted in a variety of autoimmune diseases, including inflammatory bowel disease, rheumatoid arthritis, autoimmune encephalomyelitis, and multiple sclerosis (Lubberts 2004; Bettelli et al., 2007). IL-1β, IL-6 and TGF-β promote the development of Th-17. In contrast, IFN-γ, IL-4 and IL-2 can antagonize Th-17 cells. As emphasized in chapter 4 and other publications, IL-27 mediates the inhibitory effect on Th-17 cell activity (Stumhofer 2006; Yoshimura 2006). Data reported here demonstrated that IL-35 markedly inhibited the generation of IL-17 from $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells (Figure 5.8). It is of interest to note that the inhibitory activity of IL-35 on Th-17 development might partly depend on its stimulatory effect on the production of IFN-γ. Again, EBI3 alone does not inhibit

Th17 differentiation. The mechanism, which IL-27 mediates suppression of Th17 cell development, is mainly through STAT1 dependent pathway. To investigate the mechanism how IL-35 inhibits Th17 development, the receptors of IL-35 needs to be identified.

Since both Treg cells and Th17 cells are closely associated with the control of rheumatoid arthritis, the effects of IL-35 in murine collagen induced arthritis was investigated in this chapter. Administration of IL-35 effectively suppressed an established CIA in male DBA/1 mice (Figure 5.10). This suppressive effect was not due to Fc fusion part (Figure 5.15A). When compared to control mice, IL-35-treated mice showed less cell infiltration into the synovium and reduced articular erosion (Figure 5.10). The inhibitory effect of IL-35 on CIA was reflected by the immunological responses of spleen cells in terms of the reduced frequency of IL-17 postive cells and the increased frequency of IFN-γ positive cells (Figure 5.13). This is consistent with recent reports that Th17 rather than Th1 is the key pathogenic driver of arthritic diseases. Together, these results suggested that IL-35 may represent an alternative effective therapeutic agent against inflammatory diseases. The key physiological and pathophysiological roles of IL-35 in health and disease remain to be explored.

Chapter 6

General Discussion

6.1 Cloning and expression of murine IL-27-Fc, IL-35-Fc, and human IL-35-Fc with GS system

Murine IL-27-Fc, IL-35-Fc, IL-12-Fc and IL-23-Fc have been constructed in pSecTag2A expression vector in our lab before I started the project, but these recombinant proteins production quantities are quite low. In order to obtain sufficient quantities of murine IL-35-Fc and IL-27-Fc for *in vivo* model studies, a new highly efficient mammalian recombinant expression system called GS system was introduced. PCR was employed to obtain the cDNA fragments encoding IL-35-Fc, IL-12-Fc, IL-27-Fc, and IL-23-Fc from pSecTag2A constructed vector。The restriction enzyme sites were introduced into both ends of the cDNA fragments that allowed them to clone into the PEE14.4-1 GS vector. Restriction enzyme mapping showed that the cloned cDNA fragments had correct identities in the right orientations. The IL-35-Fc, IL-12-Fc, IL-27-Fc and IL-23-Fc expression constructs were transfected to CHO cells, and the permanently transfected cell lines were selected using increasing concentrations of MSX in the glutamine free medium. Glutamine is an essential amino acid, and CHO cells growing in culture require glutamine for their growth and survival. GS is the only enzyme responsible for the biosynthesis of glutamine in CHO cells. MSX selectively inhibits the activity of the GS enzyme. It has been observed that the low concentration of MSX (3μM) is lethal to normal CHO cells in the glutamine free medium. Interestingly, some CHO cells can be primed to live in the glutamine free medium with high concentrations of up to 5000μM MSX due to endogenous GS gene amplification (Sanders & Wilson 1984). Based on this principle, the GS vector was designed to contain GS gene. With the increased

concentration of MSX, the GS gene alone with the gene of interest would be amplified. In our study, the maximum concentration of MSX of 500μM led to the highest number of amplified vector expression. The cells started to die when the concentration of MSX exceeded 500μM. The clones with the highest expression were screened using a specific human IgG1 Fc ELISA. The expression level from the GS system was 10 times higher than the expression level from the pSecTag2A system. However, IL-27-Fc and IL-35-Fc colonies had a lower expression level compared to that of IL-12-Fc and IL-23-Fc colonies. Probably the low yields of IL-35-Fc and IL-27-Fc were due to their physiological structures and synthesis, or that the GS system still has not efficiently increased their protein expression. The use of different expression vector system or different promoters may increase the yield of murine IL-35-Fc and IL-27-Fc production. Purification of the recombinant proteins was facilitated by the Econo-Pac protein A column. The molecular weight and purity of the murine recombinant proteins IL-35-Fc, IL-12-Fc, IL-27-Fc and IL-23-Fc were examined by SDS-PAGE and Coomassie blue gel staining. *In vitro* and *in vivo* studies have shown that these recombinant fusion proteins were effective in immune responses.

The study of murine IL-35 has demonstrated that IL-35 is a novel anti-inflammatory cytokine suppressing the immune response. As a consequence for future exploration, the human IL-35 protein was constructed. Human cDNA fragments encoding EBI3 and p35 were cloned from human DC by RT-PCR. Silent mutation in both EBI3 and p35 cDNA fragments was obtained to avoid enzyme restriction sites when inserted into a vector. DNA sequencing was

performed to confirm the sequences identities of EBI3 and p35. It has been found that the fusion of cytokines to immunoglobulin Fc regions can prolong the biological half-life of the protein *in vivo* (Wooley et al., 1993). Also, a mammalian expression system was chosen for the production of recombinant protein human IL-35-Fc because it possesses the proper protein-folding and post-translational modification to express a functional Fc-fusion protein. Therefore, the cloned cDNA EBI3 and p35 have been joined together by a flexible linker, and linked to the cDNA fragment encoding human IgG Fc in pSecTag2A vector. The resulting cDNA construction of EBI3-p35-Fc was amplified by PCR and cloned into PEE14.4-1GS expression vector. DNA sequencing was performed to confirm the reading frame of EBI3-p35-Fc without any signal mutation during PCR amplification. Human IL-35-Fc expression construct vector was transfected into CHO cells, and the permanently transfected cell lines were selected using increasing concentrations of MSX in the glutamine free medium. The highest expression clone was screened using a specific human IgG1 Fc ELISA. The recombinant protein human IL-35-Fc has a molecular weight of 80 kd and could be detected by Western Blot analysis. The purity of human IL-35-Fc could be found in SDS-PAGE coomassie blue staining. The functional activities of human IL-35 are still unknown and need further studies.

6.2 IL-27 and collagen-induced arthritis

IL-27 is a heterodimeric cytokine belonging to a structurally related family that includes IL-12, IL-23, and IL-6. Major advances in understanding the biology of IL-27 have been achieved since its discovery in 2002.

IL-27 is mainly produced by activated macrophages and dendritic cells. The expression of IL-27 has been associated with autoimmune diseases, such as patients with Crohn's disease (Schmidt et al., 2005). The up-regulation of IL-27 expression has been observed in sites of inflammation, such as the CNS resident cells during EAE and chronic T.*gondii* infection, the retina tissue of uveitis and multiple sclerosis (Li et al., 2005; Stumhofer et al., 2006; Fitzgerald et al., 2007; Amadi-Obi et al., 2007). IL-27p28 expression has been detected in synovial tissues of RA patients in our laboratory (Niedbala et al., 2008), suggesting that IL-27 may be involved in chronic immunopathology of RA. However, p28 and EBI3 are not always expressed coordinately (Pflanz et al., 2002), and the detection of IL-27p28 expression alone is not proof of the expression of EBI3 in RA synovial tissues. Moreover, there are no synovial tissue samples from healthy control or osteoarthritis patients to compare the expression levels of IL-27 in RA patients. Due to the difficulties in human studies, the experiments in this thesis were designed to investigate the role of IL-27 in CIA, a murine model of RA.

Initial studies focused on the role of IL-27 on the pro-inflammatory activities, but more recent studies have demonstrated that IL-27 mediates anti-inflammatory responses (summarized in Batten & Ghilardi 2007). The results in this thesis provided evidence *in vivo* for the pro- and anti-inflammatory properties of IL-27 in the development of inflammatory arthritis. IL-27 effectively suppressed the disease development when exogenous IL-27 was administered at the onset of CIA, but IL-27 did not inhibit the disease development, and even exacerbated the disease severity when exogenous IL-27 was administered to mice with established CIA.

CIA model studies have demonstrated that several proinflammatory cytokines, including TNF-α, IL-1, and IL-6 play key roles in the disease development (Feldmann et al., 1996; Wooley et al., 1993; Takagi et al., 1998). Recent studies have revealed that a critical role of IL-17 in the destructive pathogenesis of arthritis (Lubberts et al., 2002; Nakae et al., 2003). In the studies presented in chapter 4, diseases reduction by IL-27 administrated at the onset of CIA was associated with significantly reduced IL-17, IL-6 and IFN-γ productions, while disease exacerbation by IL-27 administrated on established CIA was associated with elevated IL-6 and IFN-γ production. However, it is unclear how these opposite effects of IL-27 happened. Moreover, the endogenous levels of IL-27 during CIA development have not been examined yet. Probably the detection of endogenous IL-27 at various phases of CIA would provide important information on the mechanism how IL-27 modulates inflammatory responses. The availability of IL-27 knockout mice or transgenic mice might also provide useful tools to investigate the precise role of IL-27 in the development of CIA.

Th17 cells play a crucial role in the inductive phase of CIA development (Nakae et al., 2003), and anti-IL-17 treatment can suppress the disease development (Lubberts et al., 2004). Recent studies have found that IL-27 can antagonize the development of Th17 cells. In the murine models of EAE and chronic infection with T.*gondii*, the lack of IL-27 signaling resulted in an increased number of Th17 producing $CD4^+$ T cells, associated with enhanced CNS inflammation (Batten et al., 2006; Stumhofer et al., 2006). Exogenous IL-27 administered during the onset phase of disease significantly suppressed the development of EAE, associated with a decreased number of Th17 cells in the CNS (Fitzgerald

et al., 2007).Consistent with these studies, the studies have been done in this thesis have shown that IL-27-treatment at the onset of CIA suppressed the disease development and inhibited Th17 responses. Levels of IL-17 were significantly reduced in the serum of IL-27-treated mice, and there were reduced number of IL-17-producing cells in the lymph nodes and spleens of IL-27-treated mice compared to PBS-treated mice. Moreover, IL-27 administration effectively suppressed collagen-specific response corresponding with the inhibition of IL-17 production *in vitro* by lymph nodes and spleen cells. These results indicated that IL-27 probably systemically inhibits Th17 development during the diseases process. Th17 cells are mainly induced by IL-6 and TGF-β (Veldhoen et al., 2006). My studies also showed that IL-27 treatment at the onset of CIA reduced serum IL-6 production. The reduction of IL-6 production by IL-27 may indirectly inhibit the development of Th17. Furthermore, IL-6 is known to efficiently block TGF-β-induced regulatory T cells induction (Kimura et al., 2007), and it seems that the lower levels of IL-6 produced by mice treated with IL-27 at the onset of CIA may enhance the development of TGF-β inducible regulatory T cells. Furthermore, IL-27-treated mice at the early phase of CIA displayed lower collagen-specific IgG2a production in serum. This is in contradiction with the reports that IL-27 induced IgG2a switching in B cells (Yoshimoto et al., 2004). However, this is in agreement with recent studies that IL-17-deficient mice markedly suppressed CIA associating with a lower collagen-specific IgG2a production (Nakae et al., 2003). Thus, Collagen-specific B cell function during CIA may be due to the role of IL-17, not IL-27. These findings suggested that IL-27 may mainly inhibit the development of Th17

development to limit collagen-specific immune response and the destructive process in the early phase of CIA.

Unexpectedly, my studies have shown that IL-17 production was not affected when exogenous IL-27 was administered to mice with established CIA. This is consistent with *in vitro* finding that IL-27 has no effect on differentiated Th17 cells. It is possible that Th17 cells may be already polarized in the established CIA, and IL-27 may have no ability to regulate differentiated Th17 cells at the advanced disease stage of CIA. However, the clinical disease was exacerbated in the mice treated with IL-27 at the later phase of CIA. It is likely that IL-27 may induce different pathogenesis at the later phase of CIA. The elevated levels of IL-6 and IFN-γ production in response to activation with collagen were observed in the spleen cells from IL-27-treated mice. The elevated pro-inflammatory cytokine IL-6 may be a result of the effect of IL-27 on diseases exacerbation. Although IFN-γR deficient mice results in exacerbation of disease in CIA, the role of IFN-γ in chronic articular inflammation is still not clear (Chu et al., 2007). Thus, further studies are required to elucidate the role of the elevated production of IFN-γ in the late phase of CIA. Besides T cells, IL-27R is expressed on monocytes, NK cells, mast cells, activated B cells and dendritic cells (Pflanz et al., 2002; 2004). Consistent with the broad expression profile of its receptor, IL-27 has effects on non-T cells (Pflanz et al., 2002; Larousserie et al., 2006; Wirtz et al., 2006). It is possible that the IL-27 may influence on non-T cells to regulate CIA development. It would be important to further investigate the activities of IL-27 on established CIA.

Besides CIA model in our studies, IL-27 in arthritis has been studied in two other murine models. One study has shown that neutralization of IL-27p28 is beneficial for ongoing adjuvant-induced arthritis (Goldberg et al., 2004). It was suggested that IL-27 can play a pro-inflammatory role in adjuvant-induced arthritis. Another recent study has shown that the development of Proteoglycan (PG)-induced arthritis (PGIA) in IL-27R-/- mice was delayed and the severity was reduced in comparison with wild-type controls (Cao et al., 2008). IL-27 had little inhibitory activity on IL-17 production in PGIA model. Diminished arthritis on IL-27R-/- mice was mainly associated with a decreased Th1 response (Cao et al., 2008). These studies and our findings demonstrated that the role of IL-27 in arthritis is pleiotropic. Depending on the context and the cytokine milieu, IL-27 may play either a pro-inflammatory or an anti-inflammatory role in the regulation of inflammatory responses. Currently, most of the studies have been done on murine IL-27; few studies have focused on human IL-27. Although animal models resemble human diseases, there is no animal model identical with RA in humans. The studies presented in chapter 4 provide important information on the role of IL-27 in arthritis, and whether IL-27 can be used as a therapeutic target in RA remains to be explored.

6.3 IL-35 and inflammation

IL-35 is a new member of the IL-12 family, as it shares EBI3 subunit with IL-27, and it also shares p35 subunit with IL-12. Like IL-27, IL-35 has both immune stimulatory and inhibitory effects on T cell response *in vitro*, depending on the cell activation condition. When the naïve $CD4^+$ T cells were activated with immobilized anti-CD3 and anti-CD28, the presence of IL-35 enhanced T

cell proliferation. In contrast, when naïve $CD4^+$ T cells were activated with APCs and soluble anti-CD3, IL-35 profoundly inhibited the T cell proliferation. These opposite results suggested that IL-35 probably induced different signal pathways or costimulatory events, which influenced the different outcome of immune response. A recent report has demonstrated that IL-35 contributes to Treg cell function. Treg cells can suppress the homeostatic expansion of effector T cells. The suppressive capacity of EBI3-/- and IL-12p35-/- Treg cells was significantly reduced (Collison et al., 2007). The data presented in chapter 5 has shown that IL-35 expanded $CD4^+CD25^+$ T cells and maintained their capacities to suppress effector T cells *in vitro*. These results indicated that IL-35 represents a novel potential modulator for T cell response. IL-12 cytokine family members usually induce JAK/STAT signalling pathway to mediate biological responses. STAT3 has been shown to be indispensable to IL-27-mediated proliferation of $CD4^+$ T cells (Pflanz, et al., 2004; Kamiya, et al., 2004). Whether JAK/STAT pathway deliver the signalling for IL-35 on T cell response remains further investigation.

Th1 immune response is essential for host defence against pathogens including some bacterial, parasites, and tumours. In contrast, excess of Th1 immune response may cause some autoimmune diseases (Brombacher et al., 2003). Proper initiation and maintenance of Th1 response during infection is tightly regulated by related cytokines. Published reports have shown that early expression of EBI3 is critical for the induction phase of Th1 immunity. Th cells profile from EBI3-/- mice are significantly skewed toward Th2 (Zahn 2005). However, the results presented in chapter 5 have shown that EBI3 alone has no

influence on Th1 differentiation *in vitro*. This indicated that EBI3 may associate with other partners to play an important role in initiating Th1 response. Since IL-27 induces Th1 differentiation on naïve CD4+ T cells, previous publications always interpreted that the enhanced Th2 response from EBI3-/- mice was related to the lack of IL-27 function. My studies have shown that IL-35 can also induce IFN-γ production of naïve $CD4^+$ T cells. This suggested that IL-35 might play a potential role associated with EBI3 deficiency, and the role of IL-27 might be overestimated. It is possible that IL-35 and IL-27 together sensitize the naïve T cells to Th1 differentiation during the early phase of infection. IL-27 induces Th1 differentiation mainly through T-bet / STAT-1 dependent signalling (Kamiya 2004). The data presented in chapter 5 has provided evidence that IL-35 induced the T-bet expression and the expression of STAT-1 phosphorylation. It will therefore be important to determine whether IL-35- induced T-bet expression correlates with the phosphorylation of STAT-1. This also raises the question of the expression of IL-35 receptors. The p35 subunit of IL-35 is a component of IL-12, but IL-35 does not share the same receptor subunit IL-12Rβ2 as described in previous studies (Devergne et al., 1997). The identification of IL-35 receptors would greatly improve our understanding of the biological behaviour of IL-35.

Data presented in chapter 5 demonstrated that IL-35 could suppress Th17 differentiation *in vitro*. This is consistent with the current finding on EBI3 deficient mice (Yang et al., 2008). EBI3 deficient Th17 cells produce significantly higher levels of IL-17 than wild-type Th17 cells. However, our data has shown that Th17 development was not affected when EBI3 alone was added

to *in vitro* culture. Recent studies also have demonstrated that IL-27 p28 alone can partially inhibit IL-17 *in vitro*, and that dimerization with EBI3 forming IL-27 has a stronger ability to decrease Th-17 activity (Stumhofer et al., 2006). Although very little is known about the function of IL-12 p35 alone, the inhibition of Th17 development by IL-35 probably depends on EBI3 and p35 as a combined component. The mechanism, by which IL-27 mediates suppression of Th17 cell development, is mainly through STAT1 dependent pathway. The mechanism of IL-35 inhibiting Th17 development is still unclear. The induction of IFN-γ by IL-35 might partly be involved in the ability of IL-35 to inhibit Th17 activity.

IL-12 p35-/- mice have been reported to have enhanced joint swelling and joint pathology in CIA compared to wide-type mice, and the diseases exacerbation in IL-12p35-/- mice were correlated with the lack of function of IL-12 (Murphy et al., 2003). However, the results in chapter 5 presented the potential role of IL-35 to down-regulate the progress of arthritis in CIA. Our studies indicated that the enhanced arthritic destruction in IL-12p35 deficient mice may be partly due to the lack of IL-35. In our studies, IL-35-treated arthritic mice showed a decreased number of IL-17-producing $CD4^+$ T cells but an increased number of IFN-γ-producing $CD4^+$ T cells. IL-35 is probably an important inhibitor of Th-17 cell differentiation *in vivo*. Interestingly, serum IL-10 was up-regulated in IL-35-treated mice. *In vitro* data has shown that IL-35 enhanced IL-10 production produced by $CD4^+CD25^+$ T cells, which probably explained that IL-35 might induce Treg cells to produce IL-10 *in vivo*. Although IL-35 and IL-27 has similarities, there is a crucial difference between IL-35 and IL-27 in CIA. While

IL-35 was effective in suppressing an established CIA, IL-27 was effective only at the onset of the disease. Further investigation would elucidate the differences between IL-27 and IL-35 in CIA model.

Thus, IL-35 is a pro-inflammatory factor driving Th1 response in the initial phases of an immune response and helps clear the acute infection to prevent the onset of excessive autoimmune response. On the other hand, IL-35 is an anti-inflammatory factor maintaining Treg cells function and inhibiting Th17 development to prevent chronic inflammatory damage. It is also important to note that IL-35 can suppress CIA, which may provide a novel therapeutic target in the treatment of human RA.

6.4 Conclusion

The present study is separated into three major components. Firstly, Murine IL-27, IL-35 and human IL-35 were cloned and expressed. The second part of this project had focused on the role of IL-27 in CIA. The third part was the exploration of the role of murine IL-35 *in vitro* and *in vivo*.

Using GS mammalian expression system, recombinant murine IL-27-Fc, IL-35-Fc, IL-23-Fc, and IL-12-Fc were successfully produced in sufficient quantities, and the biological activities of IL-27-Fc, IL-23-Fc, and IL-12-Fc were functionally well. Human recombinant IL-35-Fc was successfully cloned and expressed in mammalian system. The production of human IL-35-Fc is needed for further study.

Using murine collagen induced arthritis model, IL-27 was found to attenuate the disease development when administrated at the onset of the disease, but exacerbated the disease progression when administered in the established disease phase. These *in vivo* results were supported by *in vitro* results that IL-27 was able to inhibit Th17 differentiation from naïve $CD4^+$ T cells, but had little or no effect on IL-17 production by polarised Th17 cells. The conflicting roles of IL-27 would mean that the use of IL-27 as an effective therapeutic agent against inflammatory diseases has to be careful.

IL-35 was found to play an important role in immune regulation. IL-35 could expand Treg cells, but also directly suppress the proliferation of effector T cells. IL-35 could induce Th1 cell polarization, but inhibit Th17 cell differentiation. Furthermore, mice treated with IL-35 developed markedly less severe collagen induced arthritis. These results suggest that IL-35 may be a novel reagent against inflammatory diseases.

6.5 Future studies

Results from the studies presented in this thesis suggest a number of key areas which merit future investigation.

- Identification of CIA phenotypes in IL-27 knockout mice and IL-35 knockout mice to analyse the role of endogenous IL-27 and IL-35 in CIA development.

- Identification of the receptors of IL-35 and subsequent analysis of the molecular mechanism of IL-35 in T cell immune response, including Treg cells, Th1 cells, and Th17 cells.

- Identification of the function of IL-35 in other chronic inflammatory diseases, such as EAE, colitis and diabetes.

- Identification of the expression levels of IL-27 and IL-35 in synovial tissues in RA patients and comparison to the expression levels of IL-27 and IL-35 in normal synovial tissues or OA patients.

- Identification of the function of human IL-35 in T cell responses *in vitro* to find out whether the function of human IL-35 is similar to murine IL-35, and subsequent study of human IL-35 in inflammatory diseases, such as RA.

7.0 Reference

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Appendix 1

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Appendix 2

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