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APRIL (TNFSF13) in Th1, Th2 and Th17 Responses

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UNIVERSITY OF MIAMI

APRIL (TNFSF13) IN TH1, TH2 AND TH17 RESPONSES

By
Yanping Xiao

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida
December 2009
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
Doctor of Philosophy

APRIL (TNFSF13) IN TH1, TH2 AND TH17 RESPONSES

Yanping Xiao

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The T cell function of a proliferation inducing ligand (APRIL or TNFSF13) remains unclear. By comparing APRIL−/− mice with wild type (WT) mice, we have investigated the roles of APRIL in Th1, Th2 and Th17 responses.

With regard to APRIL in Th1 responses, cultured APRIL−/− CD4+ T cells showed increased IFN-γ production under non-polarizing, but not under Th1 polarizing, conditions. No difference in antigen-specific IgG2a levels existed between APRIL−/− and WT mice immunized with ovalbumin (OVA) and complete Freund’s adjuvant (CFA) which induces Th1 polarization. Our data indicate that APRIL represses Th1 responses only under non-polarizing conditions.

As for APRIL in Th2 responses, cultured APRIL−/− CD4+ T cells exhibited enhanced Th2 cytokine production under non-polarizing conditions, and augmented IL-13 production under Th2 polarizing conditions. Upon immunization with OVA and aluminum potassium sulfate (alum) which induces Th2 polarization, APRIL−/− mice responded with an increased antigen-specific IgG1 response. In the OVA-induced allergic lung inflammation model which is mediated by Th2 responses, APRIL−/− mice had significantly aggravated allergic lung inflammation. Accordingly, a decoy receptor-Ig fusion protein, TACI-Ig, treatment to block APRIL in WT mice enhanced allergic lung inflammation. In agreement with the role of APRIL in CD4+ T cells, the transfer of
APRIL sufficient, OVA-specific, TCR transgenic CD4+ T (OT-II) cells to APRIL−/− mice restored the suppressive effect of APRIL on allergic lung inflammation. Mechanistically, the expression of c-maf, the IL-4 gene transcription factor, was markedly enhanced in APRIL−/− CD4+ T cells under non-polarizing and Th2 polarizing conditions. Our data suggest that APRIL inhibits Th2 responses and allergic lung inflammation by suppressing IL-4 production in CD4+ T cells via diminished c-maf expression, and by suppressing IL-13 production in CD4+ T cells via an IL-4 independent, IL-13 specific pathway.

Regarding APRIL in Th17 responses, the incidence of Th17-mediated collagen-induced arthritis (CIA) in APRIL−/− mice was reduced, in parallel with diminished levels of antigen-specific IgG2a autoantibody and IL-17 production. Our data indicate that APRIL promotes IL-17 production, and that APRIL-triggered signals contribute to arthritis.

Our data clearly show that APRIL is important in T cell immunity, inhibitory in Th2 responses and costimulatory in Th17 responses.
ACKNOWLEDGEMENTS

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Chapter 1 INTRODUCTION

A proliferation inducing ligand (APRIL or TNFSF13) is a member of the tumor necrosis factor (TNF) superfamily. APRIL binds receptors on B and T cells (Dillon et al. 2006).

A substantial body of evidence supports the B cell function of APRIL, such as promoting immunoglobulin (Ig) class switching, B cell proliferation and survival, autoimmunity, and B cell malignancy (Dillon et al. 2006; Kimberley et al. 2009). However, little is known about the T cell function of APRIL. Studies have been focused on the effects of APRIL on T cell activation, proliferation, survival and expansion, but data are controversial (Yu et al. 2000; Siegel et al. 2001; Stein et al. 2002; Castigli et al. 2004; Varfolomeev et al. 2004; Hendriks et al. 2005; Dillon et al. 2006; Mackay et al. 2006; Hardenberg et al. 2008). The two reports on APRIL\(^{-\Delta}\) mice also showed conflicting results (Castigli et al. 2004; Varfolomeev et al. 2004). Studies in greater depth on the role of APRIL in T cell immunity, such as T help (Th) cell responses, are needed.

The function of APRIL in allergic lung inflammation, which is mediated by Th2 responses, has not been reported. It is unclear whether APRIL participates in rheumatoid arthritis (RA) and its animal model collagen-induced arthritis (CIA), both of which are mediated by Th17 responses.

Using APRIL\(^{-\Delta}\) mice, we have investigated the roles of APRIL in Th1, Th2 and Th17 responses, in Th2-mediated allergic lung inflammation, and in Th17-mediated CIA.
1.1. The TNF superfamily of ligands and receptors

As reviewed by Aggarwal, over a century ago, physicians reported that human tumors regressed after bacterial infections or treatment with bacterial extracts (Aggarwal 2003). In 1962, O'Malley et al. showed that the tumor regression effects of lipopolysaccharide (LPS) from the bacterial extracts are mediated through the induction of a factor in the serum, and they named this factor tumour-necrotizing factor (Aggarwal 2003). In 1975, the name “tumor necrosis factor (TNF)” was first used for the endotoxin-induced serum factor that causes necrosis of tumors (Carswell et al. 1975). Lymphotoxin (LT) was described in 1968 by Williams et al. as a protein produced by lymphocytes and capable of killing tumor cells (Aggarwal 2003). In 1984, the cDNAs of LT and TNF were cloned and their protein sequences were compared. It was found that human TNF has about 30% homology in its amino acid sequence with LT (Gray et al. 1984; Pennica et al. 1984). A year later, Aggarwal et al. first reported the characterization of TNF receptors (TNFRs) indicating that LT and TNF have a common receptor (Aggarwal et al. 1985). Based on these two cytokines, more and more proteins were identified, and the TNF superfamily of ligands and receptors came into shape. Now the TNF superfamily consists of 19 ligands and 30 receptors (Table 1.1; Table 1.2) (Ware 2008). The interactions between ligands and receptors were summarized in Fig. 1.1 (Fang 2004). Of note, the receptor mTNFRH3 was not shown in Fig. 1.1. It was identified in 2003 and no corresponding ligand has been found so far (Schneider et al. 2003; Bossen et al. 2006).
### Table 1.1. TNF superfamily (Ware 2008)

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<tr>
<th>Gene name/alias</th>
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#### 1.1.1. Structure and forms of the ligands and receptors of the TNF superfamily

The ligands of the TNF superfamily belong to the type II transmembrane proteins (i.e. intracellular N terminus and extracellular C terminus without a signal peptide). They are characterized by a conserved extracellular C-terminal domain, the TNF homology domain (THD). This homo- or heterotrimeric domain is responsible for receptor binding and its sequence identity between family members is \(~20–30\%\) (Bodmer et al. 2002). The THD folds into an antiparallel \(\beta\)-sandwich that assembles into trimers and thus each ligand has three receptor binding sites, formed as a groove between adjacent subunits (Ware 2003). Therefore, during the interaction of ligand and receptor, three receptor monomers bind to a trimeric ligand (Croft 2009). Although most ligands are synthesized as membrane-bound proteins, soluble forms can be generated by proteolysis (Fig. 1.1). Distinct proteases are involved in this process, depending on the ligand: metalloproteases of the ADAM (a disintegrin and metalloproteinase domain) family act on TNF and...
RANKL, matrilysin acts on Fas ligand (FasL), and members of the subtilisin-like furin family act on BAFF, EDA, TWEAK and APRIL (Bodmer et al. 2002).

**Table 1.2. TNF receptor superfamily** (Ware 2008)

<table>
<thead>
<tr>
<th>Gene name/aliases</th>
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<td>2q11-q13</td>
<td>ch10</td>
<td>NM_022336</td>
</tr>
<tr>
<td>DR6</td>
<td>6p12.2-21.1</td>
<td>ch17</td>
<td>NM_014452</td>
</tr>
<tr>
<td>EDA2R</td>
<td>Xq11.1</td>
<td>Unknown</td>
<td>NM_021783</td>
</tr>
<tr>
<td>mTNFRH3</td>
<td>Unknown</td>
<td>ch7(69.9 cM)</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Generally, the receptors of the TNF superfamily are type I transmembrane proteins (i.e. extracellular N terminus and intracellular C terminus with a signal peptide). However, BCMA, TACI, BAFFR and XEDAR are type III transmembrane proteins (i.e. extracellular N terminus and intracellular C terminus without a signal peptide). TRAIL-R3 is anchored by a covalently linked C-terminal glycolipid, and OPG and DcR3 lack a membrane-interacting domain and are therefore secreted as soluble proteins. Soluble receptors can also be generated by proteolytic processing (CD27, CD30, CD40, TNF-R1...
and TNF-R2), or by alternative splicing of the exon encoding the transmembrane domain (Fas and 4-1BB) (Bodmer et al. 2002). The key feature of the extracellular domains of the TNF superfamily receptors is the presence of cysteine-rich domains (CRDs), which are pseudo-repeats, typically containing six cysteine residues engaged in the formation of three disulfide bonds (Bodmer et al. 2002). The CRDs are responsible for the ligand binding. Significant variation in the number of CRDs occurs among family members. Most of them have 3-4 CRDs. However, BAFF-R has only a partial CRD (Ware 2003) (Fig. 1.1). The intracellular domains of the TNF superfamily receptors are related to their signaling properties. They either contain a stretch of highly homologous amino acids termed the death domain (DD), or contain one or more TNF receptor associated factor (TRAF)-interacting motifs (TIMs), or do not contain functional intracellular signaling domains/motifs (Dempsey et al. 2003). Thus the TNF superfamily receptors can be classified into three major groups.
**Figure 1.1. Interacting proteins of the TNF-TNFR superfamily** (Fang 2004).

TNFR and TNF family members are shown on the left and right of the figure, respectively. Some of the TNF members can be released from membrane by proteolysis through distinct proteases.
1.1.2. Signaling pathways for the TNF superfamily

Generally, the signaling pathway that a certain TNF superfamily ligand-receptor interaction utilizes is determined by the type of the receptor. The TNF superfamily receptors can be classified into three major groups, as mentioned above. The first group, including TNF-R1, Fas, DR3, DR4, DR5, and DR6, contains a DD in the cytoplasmic tail. Binding of these DD-containing receptors to their ligands leads to the recruitment of intracellular DD containing adaptors, Fas associated DD (FADD) and TNFR associated DD (TRADD). These molecules in turn activate the caspase cascade and induce apoptosis. While FADD (recruited by Fas, DR4 and DR5) signals apoptosis solely, TRADD (recruited by TNF-R1, DR3 and DR6) can associate with FADD or recruit TRAFs, thereby activating both apoptosis and anti-apoptotic processes (Fig. 1.2). The second group includes TNF-R2, CD40, CD30, CD27, LTβR, OX40, 4-1BB, BAFF-R, BCMA, TACI, RANK, NGFR, HVEM, GITR, TROY, EDAR, XEDAR, RELT and Fn14. These receptors contain one or more TIMs in their cytoplasmic tails. Interaction of these TIM-containing receptors with their ligands leads to the recruitment of TRAFs, resulting in the activation of multiple signal transduction pathways such as nuclear factor-κB (NF-κB), Jun N-terminal kinase (JNK), p38, extracellular signal-related kinase (ERK) and phosphoinositide 3-kinase (PI3K). Thus they are important for cell survival, activation and differentiation (Fig. 1.2). The third group, including DcR1, DcR2, DcR3 and OPG, does not contain functional intracellular signaling domains/motifs. However, these “decoy receptors” can effectively compete with the other two signaling groups of receptors for their ligands, thus interfere with the activation of signal transduction pathways by other TNF receptors (Dempsey et al. 2003).
Seven TRAF-family members have been identified (Aggarwal 2003; Xu et al. 2004). The interactions of TRAFs with the TNF superfamily receptors are shown in Table 1.3 (Aggarwal 2003). TRAF4 and TRAF7 have so far not been shown to directly interact with TNFRs (Zapata et al. 2007; Mathew et al. 2009).
In summary, TNFR family members transmit signals through different cytoplasmic adaptor proteins: FADD, TRADD or TRAFs, thus activate different signal transduction pathways and play a variety of roles.

**Table 1.3. Interaction of TRAFs with the TNFR superfamily members (Aggarwal 2003)**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>TRAF1</th>
<th>TRAF2</th>
<th>TRAF3</th>
<th>TRAF5</th>
<th>TRAF6</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFR2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LTβR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N. D.</td>
</tr>
<tr>
<td>CD40</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>CD30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>N. D.</td>
</tr>
<tr>
<td>CD27</td>
<td>N. D.</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>OX40</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>N. D.</td>
</tr>
<tr>
<td>4-1BB</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>N. D.</td>
</tr>
<tr>
<td>GITR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HVEM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>RANK</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TACI</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BCMA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>TROY</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>DR6</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XEDAR</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>FN14</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BAFFR</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NGFR</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

*BAFFR, B-cell-activating factor receptor; BCMA, B-cell maturation antigen; DR6, death receptor 6; GITR, glucocorticoid-induced TNFR family receptor; HVEM, herpes-virus entry mediator; LTβR, lymphotxin-β receptor; N. D., not determined; NGFR, nerve growth-factor receptor; RANK, receptor activator of nuclear factor-κB; TACI, transmembrane activator and cyclophilin ligand interactor; TNFR, tumour-necrosis factor receptor; TRAF, TNFR-associated factor; XEDAR, X-linked ectodermal dysplasia receptor.*
1.1.3. Cellular expression of the TNF superfamily members

The members of the TNF superfamily are expressed by a wide variety of cells, with most of them expressed by cells of the immune system, including B cells, T cells, NK cells, monocytes and dendritic cells (Aggarwal 2003; Croft 2009). The expression patterns of the ligands and the receptors of the TNF superfamily are different. Almost all ligands are expressed by cells of the immune system (Aggarwal 2003). However, the receptors are expressed by a wide variety of cells. For example, no cell type in the body has yet been found that does not express TNFR1 (Aggarwal 2003).

1.1.4. Diverse functions of the TNF superfamily members

The functions of the TNF superfamily members are diverse and can be described as “double-edged swords” (Aggarwal 2003). They are implicated in the development, organization and homeostasis of lymphoid, mammary, neuronal and ectodermal tissues (Ware 2003). They either induce cell activation, proliferation, survival, differentiation or apoptosis. They are not only essential for regulating normal functions such as haematopoiesis, morphogenesis, immune responses, protection from bacterial infection, immune surveillance and tumor regression, but also involved in dysregulation leading to various diseases such as autoimmunity (rheumatoid arthritis, systemic lupus erythematosus and diabetes), allergic asthma, tumorigenesis, viral replication, transplant rejection, and bone resorption (Aggarwal 2003). It is an emerging phenomenon for a member of the TNF superfamily to have both positive and negative roles. Here, we discuss the effects of TNF superfamily members on the immune system.
1.1.4.1. Regulation of immune responses

Many TNF-TNFR interactions can provide costimulatory signals to B cells and especially T cells in cell activation, clonal expansion, differentiation, survival, and effector functions. As opposed to these positive costimulatory functions, some TNF-TNFR interactions can deliver inhibitory signals in immune responses.

1.1.4.1.1. Effects on B cells

The TNF superfamily members play important roles in B cell immunity. For example, CD40-CD40L is essential for germinal center formation and immunoglobulin isotype switch (Kawabe et al. 1994; Cerutti et al. 1998; Zan et al. 1998). CD30L-CD30, however, suppresses immunoglobulin class switch and antibody production (Cerutti et al. 1998; Cerutti et al. 2000). The BAFF/APRIL system is crucial for B cell development and humoral responses and the receptor TACI has both positive and negative functions in B cell immunity, which will be discussed in section 1.2.

1.1.4.1.2. Effects on T cells

Costimulatory receptors can be divided into two main groups: the immunoglobulin superfamily, such as CD28 and inducible T-cell costimulator (ICOS), and the TNF superfamily receptors (Croft 2003). For a long time, CD28-B7 interaction was considered to be the principle costimulatory signal. However, members of the immunoglobulin-like CD28–B7 costimulatory family cannot fully account for an effective long-lasting T-cell response or the generation of memory T cells (Croft 2003). Now it has been shown that the interactions of TNF-TNFR such as LIGHT-HVEM, CD70-CD27, 4-1BBL and 4-1BB,
OX40L-OX40, CD40L-CD40, GITR-L and GITR, CD30L-CD30, and TL1A-DR3 can provide costimulatory signals during early and/or late T cell responses (Croft 2003; Croft 2009; Vinay et al. 2009). In addition to these positive functions, 4-1BB and OX40 are also described to be instrumental in controlling the generation and activity of T regulatory cells (Treg), perhaps in both positive and negative manners (So et al. 2008). CD30L-CD30 has been reported to be important for the suppressive effect of Treg on allograft rejection mediated by memory CD8 T cells (Dai et al. 2004). The herpesvirus entry mediator (HVEM) exhibits both positive and negative effects on T cell activation depending on its ligands. The binding of LIGHT or LTα to HVEM delivers a costimulatory signal, whereas the binding of BTLA (B- and T-lymphocyte attenuator, an immunoglobulin family member) or CD160 (a weak homologue to killer cell immunoglobulin-like receptors) to HVEM delivers a coinhibitory signal (Cai et al. 2008; Cai et al. 2009). APRIL and BAFF may also have both positive and negative functions in T cell immunity, which will be discussed in section 1.2.

1.1.4.2. Mediation of apoptosis

Following a successful immune response, the majority of the effector cells should be terminated, a process known as activation-induced cell death (AICD). The immune system achieves this through apoptosis mediated by cytokine withdrawal or death receptor signals. Apoptosis mediated by death receptors is one of the key features of the TNF superfamily. For example, Fas-FasL, TRAIL-DR4/DR5 and TNF-TNFR1 have been shown to contribute to apoptosis (Aggarwal 2003).
1.1.4.3. Development of secondary lymphoid organs

For instance, RANKL-RANK and LTα1β2-LTβR are reported to be important in the proper development of peripheral lymph nodes (LNs) (Kim et al. 2000).

1.1.4.4. Protection from microbial infection

The TNFR signaling is also involved in an effective host response against infection with bacteria. TNF-TNFR1 is believed to have a critical role in protection from infection (Aggarwal 2003).

1.2. APRIL and its receptors

APRIL, a proliferation inducing ligand, is a member of the TNF superfamily (TNFSF13). It was first identified through searching the expressed sequence tag (EST) database in 1998 by Hahne et al. (Hahne et al. 1998), followed by similar discoveries with different names (Table 1.4) (Shu et al. 1999; Kelly et al. 2000). Among the TNF superfamily ligands, APRIL is most closely related to B-cell activation factor of the TNF family (BAFF or TNFSF13B). They share ~30% sequence identity in the TNF homology domain (THD) (Dillon et al. 2006). BAFF has also been discovered by different groups and given different names (Table 1.4) (Dillon et al. 2006).

The discoveries of APRIL and BAFF was closely followed by the identification of their receptors. In 2000, a previously orphaned TNF receptor family member, the transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI or TNFRSF13B) was shown to bind to BAFF and to mediate the activation of NF-κB pathway (Yan et al. 2000). Shortly thereafter, several studies demonstrated that APRIL
and BAFF share receptors, TACI and B cell maturation antigen (BCMA or TNFRSF17) (Dillon et al. 2006). In addition, BAFF receptor (BAFF-R or TNFRSF13C) specifically binds BAFF, but not APRIL (Dillon et al. 2006). The existence of a third APRIL-specific receptor was also proposed, based on the evidence suggesting that APRIL modestly stimulates the proliferation of Jurkat T cells, NIH-3T3 fibroblasts, HT29 colon carcinoma cells, and A549 lung epithelial cells which do not express TACI or BCMA, and that BAFF does not bind to APRIL-responsive tumor cells or induce their proliferation (Dillon et al. 2006). During the search for the additional possible APRIL-specific receptor, heparan sulfate proteoglycan (HSPG) was identified as an APRIL-specific binding partner by two research groups (Hendriks et al. 2005; Ingold et al. 2005). Whether HSPG represents the unidentified third receptor remains to be clarified, since HSPG may just function to accumulate APRIL on the cell surface to facilitate its binding to TACI or BCMA (Hendriks et al. 2005; Ingold et al. 2005). Interestingly, HSPG also binds TACI (Bischof et al. 2006). Recently, weak binding of a shorter variant of APRIL to BAFF-R was found in the murine system (Bossen et al. 2006). The various aliases and nomenclature associated with the APRIL/BAFF system are summarized in Table 1.4 (Dillon et al. 2006). The interactions between APRIL/BAFF and their receptors are shown in Fig. 1.3. Since the discoveries of APRIL and BAFF, much work has focused on BAFF. APRIL has received less attention. The following discussion will concentrate on APRIL.

1.2.1. Structure and forms of APRIL and its receptors

The gene encoding human APRIL is located on chromosome 17p13, and mouse APRIL on chromosome 13 (Table 1.1). The APRIL gene contains six exons, encoding a
250-amino-acid protein for human APRIL and a 240-amino-acid protein for mouse APRIL. Like other ligands of the TNF superfamily, APRIL/BAFF is a type II transmembrane protein, and has an extracellular THD for receptor binding. Both APRIL and BAFF are homotrimers. However, they can also form APRIL/BAFF heterotrimers (Roschke et al. 2002). This heterotrimer is expected to bind only to TACI (Fig. 1.3), based on the observation that only TACI-immunoglobulin (TACI-Ig), but not BCMA-Ig or BAFF-R-Ig, can neutralize the heterotrimer (Roschke et al. 2002). Although BAFF and APRIL are synthesized as transmembrane proteins, they are proteolytically cleaved at a furin convertase cleavage site (Fig. 1.3). Whereas BAFF is cleaved from the cell surface and exists in both membrane-anchored and secreted forms, APRIL is processed in the Golgi apparatus and act solely as a secreted factor (Lopez-Fraga et al. 2001; Mackay and Leung 2006). However, a hybrid protein, TWE-PRIL, was reported to be composed of the TWEAK (TNFSF12) cytoplasmic and transmembrane domains and the APRIL extracellular domain (Pradet-Balade et al. 2002) (Fig. 1.3). This hybrid protein may exist as a membrane-bound form of APRIL. TWE-PRIL is predicted to bind to the receptors of APRIL (Fig. 1.3). In addition, APRIL trimers can bind HSPG to form oligomeric APRIL. Like the BAFF 60-mer, the HSPG-bound APRIL is required for optimal TACI signaling in B cells (Hendriks et al. 2005; Ingold et al. 2005; Bossen et al. 2008; Kimberley et al. 2009; Kimberley et al. 2009; Mackay et al. 2009). Moreover, a spliced variant form of BAFF (ΔBAFF) was identified to be able to associate with normal BAFF subunits to form heterotrimers which are non-cleavable and functionally inactive (Gavin et al. 2003; Gavin et al. 2005) (Fig. 1.3). A shorter variant of APRIL was also found to weakly bind BAFF-R in the murine system (Bossen and Schneider 2006).
The three receptors, TACI, BCMA and BAFF-R, are structurally unusual compared with other receptors of TNF superfamily. Generally, the receptors of the TNF superfamily are type I transmembrane proteins. However, BCMA, TACI, and BAFF-R are type III transmembrane proteins. Regarding the number of cysteine-rich domains (CRDs), most of the TNF superfamily ligands have 3-4 CRDs, whereas BCMA has one, TACI two, and BAFF-R only a partial CRD (Fig. 1.1). The intracellular domains of these three receptors contain TRAF-interacting motifs (TIMs) and no death domain (DD) (Fig. 1.1).

Whereas the THD of APRIL binds BCMA and TACI, a basic sequence (QKQKKQ) in the N-terminal part of the cleaved form of APRIL and outside the TNF fold was shown to be a HSPG binding site (Hendriks et al. 2005; Ingold et al. 2005). Consistently, APRIL can bind to BCMA and HSPG simultaneously (Hendriks et al. 2005). In addition, the interaction of APRIL with HSPG can be inhibited by heparin (Hendriks et al. 2005; Ingold et al. 2005).
Table 1.4. The aliases and nomenclature of APRIL, BAFF and their receptors (Dillon et al. 2006).

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full name</th>
<th>HUGO</th>
<th>CD</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>APRIL</td>
<td>A proliferation-inducing ligand</td>
<td>TNFSF13</td>
<td>CD256</td>
<td>2</td>
</tr>
<tr>
<td>TALL-2</td>
<td>TNF- and ApoL-related leukocyte-expressed ligand-2</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>TRDL-1</td>
<td>TNF-related death ligand-1</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>BLyS</td>
<td>B lymphocyte stimulator</td>
<td>TNFSF13B</td>
<td>CD257</td>
<td>5</td>
</tr>
<tr>
<td>BAFF</td>
<td>B-cell activating factor of the TNF family</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>TALL-1</td>
<td>TNF- and ApoL-related leukocyte-expressed ligand-1</td>
<td></td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>THANK</td>
<td>TNF homologue activates apoptosis, NF-κB, and JNK</td>
<td></td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>zTNF4</td>
<td>z-Tumour-necrosis factor-4</td>
<td></td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>TACI</td>
<td>Transmembrane activator and CAML interactor</td>
<td>TNFRSF13B</td>
<td>CD267</td>
<td>9</td>
</tr>
<tr>
<td>BCMA</td>
<td>B-cell maturation antigen</td>
<td>TNFRSF17</td>
<td>CD269</td>
<td>10</td>
</tr>
<tr>
<td>BAFF-R</td>
<td>BAFF-receptor</td>
<td>TNFRSF13C</td>
<td>CD268</td>
<td>11</td>
</tr>
<tr>
<td>BR3</td>
<td>BLyS receptor-3</td>
<td></td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Bcmd</td>
<td>B-cell maturation defect</td>
<td></td>
<td></td>
<td>13</td>
</tr>
<tr>
<td>HSPG</td>
<td>Heparan sulphate proteoglycan</td>
<td>N/A</td>
<td>N/A</td>
<td>15</td>
</tr>
</tbody>
</table>

CD, Cluster of Differentiation (see Further information); HUGO, Human Genome Organization (see Further information); NF-κB, nuclear factor-κB; TNF, tumour-necrosis factor. See REF. 22 for Further information.
Figure 1.3. Interaction and expression of ligands and receptors in the BAFF/APRIL system.

The top part of the figure shows the different types of ligands. From left to right: forms of BAFF/APRIL heterotrimers, a heterotrimer with ΔBAFF with red road signs indicating the impossibility of this ligand to be cleaved and have biological activity, APRIL cleaved from the Golgi then released as soluble ligand, the TWE–PRIL fusion protein, and BAFF homotrimers with a red arrow to indicate potential cleavage into a soluble ligand. A short variant form of APRIL is represented (right). Cell types that produce BAFF or APRIL are indicated in light blue boxes with an arrow. In the bottom part of the figure, receptors are represented from left to right: heparin sulfate proteoglycans (HSPG), TACI, BCMA and BAFF-R. Black solid arrows represent strong interactions, green solid arrows represent weak interactions, and broken lines represent interactions that have not been directly demonstrated. Below each receptor is a box that indicates cell types expressing these receptors (Mackay et al. 2007).
1.2.2. Binding affinity of APRIL/BAFF for their receptors.

The binding affinities of APRIL/BAFF for their receptors vary across reports, possibly due to differences in protein sources or in assay protocols (Day et al. 2005; Dillon et al. 2006). The values reported by several independent groups using analogous assay protocols of surface plasmon resonance were summarized in Table 1.5, showing that TACI-Ig/BCMA-Ig binds to APRIL and BAFF with similar affinities (Dillon et al. 2006).

The binding affinity of APRIL for HSPG was tested by flow cytometry-based saturation-binding studies. In these assays, APRIL, but not BAFF, bound in a heparin-sensitive manner to a variety of TACI- and BCMA-negative cell lines expressing HSPG endogenously. The $K_D$ for APRIL-HSPG interactions is 20–80 µM (Dillon et al. 2006).

1.2.3. Cellular expression of APRIL and its receptors

1.2.3.1. Cellular expression of ligands (Table 1.6)

1.2.3.1.1. Expression in monocytes, macrophages, dendritic cells and neutrophils

BAFF and APRIL are produced mainly by innate immune cells such as, monocytes, macrophages, dendritic cells (DCs), follicular DCs (FDCs) and neutrophils (Seyler et al. 2005; Dillon et al. 2006; Schwaller et al. 2007; Scapini et al. 2008; Mackay and Schneider 2009). TWE-PRIL is expressed in human monocytes (Pradet-Balade et al. 2002). The expression of APRIL/BAFF is increased in the presence of IFN-γ, IFN-α, CD40L, IL-10, granulocyte colony-stimulating factor (G-CSF), and by the activation of Toll-like receptors (TLRs) (Dillon et al. 2006; Mackay and Schneider 2009).
Table 1.5. Binding affinities of APRIL and BLyS for TACI-Ig and BCMA-Ig (Dillon et al. 2006)

<table>
<thead>
<tr>
<th>APRIL ($K_D$ nM)</th>
<th>BLyS ($K_D$ nM)</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TACI-Ig</td>
<td>BCMA-Ig</td>
<td></td>
</tr>
<tr>
<td>0.45</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>ND</td>
<td>0.63</td>
<td>ND</td>
</tr>
<tr>
<td>ND</td>
<td>4.9</td>
<td>ND</td>
</tr>
<tr>
<td>0.91</td>
<td>0.26</td>
<td>ND</td>
</tr>
<tr>
<td>0.60 (mu)</td>
<td>0.90</td>
<td>0.64</td>
</tr>
<tr>
<td>1.5 (muS)</td>
<td></td>
<td>1.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.15 (muS)</td>
</tr>
<tr>
<td>6.4</td>
<td>ND</td>
<td>0.16</td>
</tr>
</tbody>
</table>

Unless otherwise noted, the receptor–Fc fusion protein was immobilized to the Biacore sensor chip surface, the ligand flowed over the chip surface at varying concentrations, and $K_D$ values determined from the kinetic rate constants ($k_a$ and $k_d$). *Biacore studies were carried out using a solution assay format, performed by passing a pre-incubated mixture of ligand (at varying concentrations) and receptor over the receptor-derivated sensor chip. $K_D$ values were calculated from initial binding rates (Vi) and concentration of the varied component. ‡Ligands, rather than receptor–Fc fusion proteins, were immobilized to the Biacore sensor chip. §The soluble receptors were bound to microtitre wells coated with goat-anti-human Fc antibody and affinities to the ligands were assessed using an ELISA format. †Yu et al. tested both a full-length murine (mu) APRIL (residues 50–240) and a shortened version (muS; residues 101–240). APRIL, a proliferation-inducing ligand; BCMA-Ig, B-cell maturation antigen-immunoglobulin; ND, not determined; TACI, transmembrane activator and CAML interactor.

Table 1.6. The cellular expression pattern of ligands in the APRIL/BAFF system

<table>
<thead>
<tr>
<th>APRIL</th>
<th>BAFF</th>
<th>TWE-PRIL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monocytes</td>
<td>Monocytes</td>
<td>Monocytes</td>
</tr>
<tr>
<td>Macrophages</td>
<td>Macrophages</td>
<td></td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>Dendritic cells</td>
<td></td>
</tr>
<tr>
<td>Neutrofils</td>
<td>Neutrofils</td>
<td></td>
</tr>
<tr>
<td>T cells</td>
<td>T cells</td>
<td>T cells</td>
</tr>
<tr>
<td>B cells and malignant B cells</td>
<td>B cells and malignant B cells</td>
<td></td>
</tr>
<tr>
<td>Osteoclasts</td>
<td>Osteoclasts</td>
<td></td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>Epithelial cells</td>
<td></td>
</tr>
<tr>
<td>Non-lymphoid tumor cells</td>
<td>Fibroblast-like synoviocytes</td>
<td></td>
</tr>
<tr>
<td>“Nurse-like” cells</td>
<td>Astrocytes</td>
<td></td>
</tr>
<tr>
<td>Stromal cells</td>
<td>Mast cells</td>
<td></td>
</tr>
<tr>
<td>Megakaryocytes</td>
<td>Eosinophils</td>
<td></td>
</tr>
<tr>
<td>Mast cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophils</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
1.2.3.1.2. Expression in T cells

Like BAFF, APRIL is also expressed by T cells (Dillon et al. 2006; Mackay and Leung 2006). Among the APRIL positive cDNA clones used for the first identification of APRIL, one clone was present in the cDNA library derived from T cell lymphoma (Hahne et al. 1998). At the mRNA level, both APRIL and BAFF were expressed in mouse CD4\(^+\) T cells activated \textit{in vitro} under Th1 or Th2 conditions, but not in naïve T cells (Stein et al. 2002). However, resting human primary T cells produce APRIL and TWE-PRIL at the mRNA level and APRIL at the protein level, while activated human primary T cells express APRIL and TWE-PRIL at both the mRNA and protein level (Pradet-Balade et al. 2002).

1.2.3.1.3. Expression in B cells

APRIL and BAFF were originally considered only expressed by malignant B cells (Kern et al. 2004; Dillon et al. 2006), but not by normal B cells. However, there remains evidence suggesting that these two ligands are also produced by normal B cells. For example, BAFF and APRIL mRNAs and proteins were detected in human normal blood or tonsil-derived unstimulated B cells (Kern et al. 2004). Furthermore, another study demonstrated that in mice, BAFF mRNA is expressed in all B cell subsets from bone marrow, spleen, and peritoneal cavity. APRIL expression is restricted to B cells at early developmental stages in the bone marrow and the peritoneal B1 subset. Stimulation of B2 and B1 cells with LPS or CpG-oligodeoxynucleotides induced the intracellular expression of BAFF and APRIL (Chu et al. 2007).
1.2.3.1.4. Expression in other cells

Both BAFF and APRIL can be expressed in mast cells, eosinophils and osteoclasts as well as airway and intestinal epithelial cells (Ng et al. 2004; Dillon et al. 2006; Kato et al. 2006; He et al. 2007). BAFF is also detected in fibroblast-like synoviocytes and astrocytes (Dillon et al. 2006; Mackay et al. 2007). High levels of APRIL are detectable in transformed cell lines, human cancers of colon, cancers of the gastrointestinal tract, and the malignant glioblastoma cell lines (Dillon et al. 2006). Pancreatic, ovarian and uterine adenocarcinomas also express APRIL, indicated by analysis of the human EST database (Dillon et al. 2006). Moreover, APRIL is expressed by “nurse-like” cells from B-chronic lymphocytic leukaemia (B-CLL) patients, and osteoclasts and stromal cells in the bone marrow from multiple myeloma (MM) patients (Dillon et al. 2006). In addition, APRIL has been found in megakaryocytes (Bonci et al. 2004).

1.2.3.2. Cellular expression of receptors (Table 1.7)

<table>
<thead>
<tr>
<th></th>
<th>B cells</th>
<th>T cells</th>
<th>Other cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF-R</td>
<td>Yes</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>TACI</td>
<td>Yes</td>
<td>Yes/NO</td>
<td>Monocytes and DCs</td>
</tr>
<tr>
<td>BCMA</td>
<td>Yes</td>
<td>NO *</td>
<td></td>
</tr>
<tr>
<td>HSPG</td>
<td>Yes</td>
<td>Yes</td>
<td>Non-immune cells</td>
</tr>
</tbody>
</table>

*The possible exception is that BCMA is marginally expressed on T cells in the peritoneal cavity (Hardenberg et al. 2008).

1.2.3.2.1. Expression on B cells

BAFF-R, TACI and BCMA are primarily expressed in B cells. HSPG was also suggested to be expressed on B cells (Ingold et al. 2005). The expression levels of these
three receptors vary with the stages of B cell development and differentiation (Jelinek et al. 2005; Mackay et al. 2005) (Table 1.8). BAFF-R is predominantly expressed on mature, activated and memory B cells, but is barely detectable at the plasma-cell stage. TACI is mainly expressed by memory B cells, plasma cells, marginal zone B cells and B1 B cells, but not germinal center B cells (Dillon et al. 2006; Mackay and Schneider 2009). By comparison, BCMA is expressed strongly by plasma cells, plasmablasts and tonsillar germinal centre B cells (Dillon et al. 2006). Stringent regulation of the expression of these receptors may be an important mechanism that controls B cell development and differentiation (Dillon et al. 2006).

Table 1.8. Pattern of BAFF-R, TACI and BCMA expression on B cells

<table>
<thead>
<tr>
<th></th>
<th>Pro-B</th>
<th>Pre-B</th>
<th>Immature</th>
<th>Mature</th>
<th>Activated</th>
<th>Memory</th>
<th>Plasma cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAFF-R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>TACI</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>BCMA</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

1.2.3.2.2. Expression on T cells

BAFF-R has been shown to be expressed on activated, memory and regulatory T cells (Ng et al. 2004; Ye et al. 2004; Dillon et al. 2006; Mackay and Leung 2006). HSPG was also suggested to be expressed on T cells (Ingold et al. 2005). BCMA may not be expressed on T cells and appears to be a B cell-specific receptor. One possible exception is that BCMA was recently found to be marginally expressed on CD4⁺ and CD8⁺ T cells in the peritoneal cavity, by using different anti-BCMA monoclonal antibodies (Hardenberg et al. 2008). The possibility of BCMA expression on T cells remains to be confirmed by further study.
Whether TACI is expressed on T cells is controversial. TACI was first detected on a subset (equally distributed between CD4\(^+\) and CD8\(^+\) cells) of activated human circulating T cells by using a polyclonal anti-TACI antibody. The cells were activated by Phorbol 12-myristate 13-acetate (PMA) and ionomycin (von Bulow et al. 1997). However, another study showed weak TACI mRNA in human T cells which was unaltered by known modulators of the immune system, such as phytohaemagglutinin (PHA), IL-2, anti-CD3, PMA and ionomycin (Wu et al. 2000). Furthermore, the utilization of monoclonal anti-mouse and human TACI antibodies failed to detect TACI on mouse T cells (resting or activated at different time points) as well as in human peripheral blood and tonsil-derived activated T cells (Ng et al. 2004). The GeneChip results showed absence of the TACI transcript from all human T cell subsets assessed, including effector memory and central memory T cells, Th1 and Th2 cells generated in vitro, and specialized subsets such as T follicular-homing cells (Ng et al. 2004). Nevertheless, it seems there are more and more data supporting the expression of TACI on T cells. A RT-PCR analysis showed obvious TACI expression, though much weaker than that of BAFF-R, in murine purified CD4\(^+\) and CD8\(^+\) T cells (Ye et al. 2004). Another study clearly detected a small subset of TACI\(^+\) T cells in synovial tissue samples from patients with synovitis (Seyler et al. 2005). Recently, a detailed analysis of TACI expression on murine T cells in different immune compartments using different anti-TACI monoclonal antibodies demonstrated that TACI was expressed on a small percentage (<2.5%) of T cells in the spleen, mesenteric lymph nodes, and peripheral lymph nodes, but on ~19% of CD4\(^+\) T cells and 15% of CD8\(^+\) T cells in the peritoneal cavity (Hardenberg et al. 2008).
To verify whether this surface TACI expression is true, parallel analysis of TACI mRNA in the TACI+ T cells is needed (Mackay and Leung 2006).

1.2.3.2.3. Expression on other cells

The APRIL-specific binding partner, HSPG, is suggested to be expressed on non-immune tumor cell lines and nonhematopoietic cell lines, such as HT29 and SW480 colorectal adenocarcinomas, A549 lung carcinoma, 293 embryonic kidney cells, and NIH-3T3 murine fibroblasts (Hendriks et al. 2005; Ingold et al. 2005). TACI is also expressed by monocytes and DCs (Mackay and Schneider 2009).

1.2.4. Biological functions of APRIL

The biological functions of APRIL are much less defined than those of BAFF, and the roles of APRIL in T cell immunity are much less investigated than those in B cell immunity. *In vitro* and *in vivo* studies have been carried out to understand the biological activities of APRIL. Our knowledge of the *in vivo* functions of APRIL to date heavily relies on the studies of transgenic and knockout mice (Table 1.9; Table 1.10).

1.2.4.1. The B cell function of APRIL

BAFF is crucial for the generation and maintenance of mature B cells. B cell maturation beyond the immature transitional type 1 (T1) stage is impaired in BAFF−/− mice (Stein et al. 2002; Mackay and Leung 2006). APRIL does not have such a role in B cell development; instead, it shares the other functions of BAFF in B cell responses, such as promoting immunoglobulin (Ig) class switching, B cell proliferation and survival.
1.2.4.1.1. Promotion of Ig class switching

A key feature of the function of APRIL is promoting T cell-independent IgA class switching which has been well studied in humans and mice (Litinskiy et al. 2002; He et al. 2003; Castigli et al. 2004; Castigli et al. 2005; He et al. 2007; Sakurai et al. 2007). For example, in APRIL−/− mice, serum IgA levels were significantly decreased, and serum IgA antibody responses to mucosal immunization with T-dependent antigens and to type 1 T-independent antigens were impaired (Castigli et al. 2004). In humans, APRIL is required for IgA2 class switching in B cells triggered by intestinal epithelial cells (IECs), through a CD4+ T cell-independent pathway (He et al. 2007). APRIL also participates in inducing T cell-independent IgG and IgE class switching (Litinskiy et al. 2002; He et al. 2003; Castigli et al. 2004; Castigli et al. 2005).

1.2.4.1.2. Enhancement of B cell proliferation and survival

In vitro, APRIL can costimulate B cell proliferation. In vivo, APRIL increased spleen weight and the percentage of B cells in the spleen and peripheral lymph nodes of mice (Stein et al. 2002; Dillon et al. 2006). A wealth of data supports a role for APRIL in mediating plasmablast and plasma cell survival (Ingold et al. 2005; Dillon et al. 2006; Belnoue et al. 2008; Bossen et al. 2008; Cerutti 2008).

1.2.4.1.3. Augmentation of B-cell antigen presentation

APRIL was shown to up-regulate the expression of several key costimulatory molecules on B cells, and thus significantly enhance the function of B cells in antigen presentation (Dillon et al. 2006).
1.2.4.1.4. Negative role in germinal center formation

APRIL−/− mice have enlarged germinal centers, suggesting that APRIL is a negative modulator in germinal center formation (Castigli et al. 2004).

1.2.4.1.5. Regulation of antibody responses

The studies of APRIL knockout and transgenic mice suggested that APRIL promotes T-independent IgA, IgG and IgM and T-dependent IgA and IgM antibody responses, but diminishes T-dependent IgG antibody responses (Stein et al. 2002; Castigli et al. 2004).

1.2.4.2. The T cell function of APRIL

BAFF has emerged as a regulator of T cell function. BAFF costimulates T cell activation, proliferation and cytokine production in vitro (Huard et al. 2001; Huard et al. 2004; Ng et al. 2004). BAFF-R mutant or BAFF deficient mice (Ye et al. 2004) showed impaired T cell-mediated allograft rejection. However, increased intragraft mRNA expression of Th2 cytokines (IL-4, IL-5 and IL-13) was also observed in BAFF-R mutant mice, suggesting an inhibitory role for BAFF in Th2 responses. Consistently, BAFF transgenic mice (Sutherland et al. 2005) exhibited subdued Th2 responses in a Th2-mediated model of allergic airway inflammation. BAFF transgenic mice also had enhanced cutaneous delayed-type hypersensitivity reaction, a typical Th1-mediated response. Recently, BAFF was shown to promote Th17 cell expansion (Lai Kwan Lam et al. 2008).
The role of APRIL in T cell biology remains unclear. The effects of APRIL on T cell activation, proliferation, survival and expansion have been investigated, but data are controversial. *In vitro* evidence supporting the T cell function of APRIL comes from studies showing that murine APRIL costimulates anti-CD3-induced T cell proliferation (Yu et al. 2000), anti-APRIL antibodies impair primary T cell activation (Siegel and Lenardo 2001), and MEGA-APRIL costimulates CD4\(^+\) T cell activation (Hendriks et al. 2005). *In vivo* findings in APRIL transgenic mice expressing human APRIL in T cells suggest that APRIL modulates T cell immunity (Stein et al. 2002). APRIL transgenic T cells showed increased proliferation after stimulation with anti-CD3 in combination with anti-CD28 *in vitro*. APRIL transgenic T cells also exhibited elevated survival *in vitro* and *in vivo*, correlating with increased Bcl-2 levels. In addition, APRIL transgenic mice had an accumulation of CD62L\(^-\) CD44\(^{\text{low}}\) T cells which did not correspond to classically activated T cells (CD62L\(^-\) CD44\(^{\text{hi}}\)). APRIL\(^{-/-}\) mice, in one study (Castigli et al. 2004), had increased numbers of CD44\(^{\text{hi}}\) CD62L\(^{\text{lo}}\) CD4\(^+\) effector/memory T cells and increased IgG responses to T-dependent antigens, though showed normal T and B cell development and proliferation. While these *in vitro* and *in vivo* data indicate a role of APRIL in T cell immunity, other studies failed to obtain consistent results. In another report on APRIL\(^{-/-}\) mice (Varfolomeev et al. 2004), APRIL\(^{-/-}\) T cells showed normal *in vitro* proliferation in response to anti-CD3 monoclonal antibody or concanavalin A (ConA). T cells from APRIL\(^{-/-}\) mice immunized with keyhole limpet hemocyanin (KLH) had normal proliferation and cytokine production after restimulation *in vitro* with KLH. In addition, human ZZ APRIL, a trimeric APRIL protein with high specific activity, consistently failed to costimulate normal resting human or mouse T cells *in vitro* (Dillon et al. 2006).
Moreover, a recent report by Hardenberg et al. did not reveal a role for APRIL in T cell responses, either (Hardenberg et al. 2008). They found no difference in the \textit{in vitro} and \textit{in vivo} expansion of T cells from OT-I (TCR transgenic CD8\(^{+}\)) APRIL transgenic and OT-II (TCR transgenic CD4\(^{+}\)) APRIL transgenic mice compared with OT-I and OT-II cells, respectively. Furthermore, after primary intramuscular immunization with adenovirus, APRIL transgenic and littermate mice did not show differences in CD8\(^{+}\) T cell expansion in blood, spleen and peritoneal cavity, nor in IFN-\(\gamma\) production. Upon both primary and secondary intranasal ovalbumin immunization, no difference was observed in the absolute numbers of T cells in spleen, lung, and draining lymph nodes between APRIL transgenic and littermate mice. To clarify the role of APRIL in T cell biology, an in depth analysis of the T cell function of APRIL is demanded. This is the focus of our study.
Table 1.9. APRIL function revealed by transgenic and knockout mice (Dillon et al. 2006)

<table>
<thead>
<tr>
<th>Mice</th>
<th>Phenotype</th>
<th>Refs</th>
</tr>
</thead>
</table>
| APRIL transgenic (human APRIL expressed in T cells) | Enhanced survival of transgenic T cells in vitro  
Enhanced survival of superantigen-reactive T cells in vivo, correlating with elevated BCL2 levels  
Increased IgM, but not IgG, response to TD antigen  
Enhanced Th-2 IgM and IgG responses  
Development of B-1 cell neoplasia in aged transgenic mice | 61 |
| APRIL knockout | Decreased circulating IgA  
Impaired IgA responses  
Increased number of effector/memory T cells  
Enhanced germinal centre reactions  
Normal peripheral B-cell populations  
No discernible phenotype  
Normal B- and T-cell development and in vitro function  
Normal Th and TD humoral responses | 62, 68 |
| TACI-Ig transgenic | Lack of B-1 cells  
Block of B-2 cells at the Th1 stage of development  
Severe depletion of marginal zone and follicular B-2 B cells  
Mild effect on peritoneal B-1 B cells | 70, 71 |
| BCMA-Ig transgenic (murine form of BCMA that binds APRIL well but Blys weakly) | No discernible phenotype | 71 |
| TACI knockout | Increased number of mature B cells  
Impaired Ig responses to Th2 antigens  
B-cell hyperresponsiveness  
SLE nephritis (development of circulating auto-antibodies), especially in aged knockout mice  
Premature death from IC nephritis and/or lymphoma  
Lymphadenopathy | 72, 73, 74 |
| BCMA knockout | Normal B- and T-cell development | 75, 76 |
| | Impaired survival of long-lived plasma cells | 64 |
| BCMA x TACI double knockout | Similar to TACI knockout | 77 |

APRIL, a proliferation-inducing ligand; BCMA, B-cell maturation antigen; Ig, immunoglobulin; TACI, transmembrane activator and CAML interactor; SLE, systemic lupus erythematosus; TD, thymus dependent; TI, thymus independent; Th, transitional 1.
Table 1.10 *

<table>
<thead>
<tr>
<th>Mice</th>
<th>T cell phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Knockout mice</strong></td>
<td></td>
</tr>
<tr>
<td>BAFF−/−</td>
<td>Normal Th1-mediated DTH</td>
</tr>
<tr>
<td></td>
<td>Normal response in Th1-mediated Experimental Autoimmune Encephalomyelitis (EAE)</td>
</tr>
<tr>
<td></td>
<td>Modest increased of allograft survival, significantly increased when combined with a non-effective low dose of cyclosporin A</td>
</tr>
<tr>
<td></td>
<td>Normal numbers and proportion of naive and effector/memory T cells</td>
</tr>
<tr>
<td>APRIL−/−</td>
<td>Normal T cell development and numbers</td>
</tr>
<tr>
<td></td>
<td>Normal T cell responses</td>
</tr>
<tr>
<td></td>
<td>Increased percentages of CD44high/CD62Llow effector/memory T cells</td>
</tr>
<tr>
<td>BAFF-R mutant (A/WySnJ line)</td>
<td>Anti-CD3-activated T cells do not respond to BAFF-induced co-stimulatory signals</td>
</tr>
<tr>
<td></td>
<td>Normal T cell response to anti-CD28-mediated co-stimulation of anti-CD3 activation</td>
</tr>
<tr>
<td></td>
<td>Normal T cell proliferation in response to antigens in vivo</td>
</tr>
<tr>
<td></td>
<td>CD4+ T cells provide normal T cell help to B cells</td>
</tr>
<tr>
<td>TACI−/−</td>
<td>Normal T cell proliferation to anti-CD3 in vitro and co-stimulation following addition of BAFF</td>
</tr>
<tr>
<td></td>
<td>Normal allograft rejection</td>
</tr>
<tr>
<td></td>
<td>Normal T cell numbers and responses</td>
</tr>
<tr>
<td></td>
<td>T cells hyper-proliferative in response to anti-CD3 + BAFF</td>
</tr>
<tr>
<td></td>
<td>Increased numbers of CD4+ T cells in Peyer’s Patches</td>
</tr>
<tr>
<td></td>
<td>Impaired CD8 T cell priming</td>
</tr>
<tr>
<td>BCMA−/−</td>
<td>Normal T cell numbers and function</td>
</tr>
<tr>
<td>BCMA−/−x TACI−/−</td>
<td>Same as TACI−/−</td>
</tr>
<tr>
<td>TACI−/−</td>
<td></td>
</tr>
<tr>
<td><strong>Transgenic mice</strong></td>
<td></td>
</tr>
<tr>
<td>BAFF Tg mice</td>
<td>Increased proportion of effector/memory T cells</td>
</tr>
<tr>
<td></td>
<td>Two fold increase in T cell numbers in the spleen and mesenteric lymph nodes</td>
</tr>
<tr>
<td></td>
<td>Enhanced Th1-mediated DTH responses</td>
</tr>
<tr>
<td></td>
<td>Suppressed Th2-mediated allergic airway inflammation</td>
</tr>
<tr>
<td>APRIL Tg mice</td>
<td>Increased survival of CD4+ and CD8+ T cells</td>
</tr>
<tr>
<td></td>
<td>Enhanced survival of superantigen-reactive T cells linked to increased of Bcl-2 expression</td>
</tr>
<tr>
<td></td>
<td>Increased T cell proliferation</td>
</tr>
<tr>
<td></td>
<td>Increased production of IL-2 by activated CD8+ T cells</td>
</tr>
<tr>
<td></td>
<td>Reduced percentages of T cells in peripheral lymph nodes</td>
</tr>
<tr>
<td>TACI Tg mice</td>
<td>No T cell phenotype described</td>
</tr>
<tr>
<td>mBCMA Tg mice (binds murine APRIL well but not mouse BAFF)</td>
<td>No T cell phenotype</td>
</tr>
</tbody>
</table>

*This table is derived from a previous review (Mackay and Leung 2006).*
1.2.4.3. The effects of APRIL on other cells

The functions of BAFF are essentially restricted to the immune system. In contrast, functions for APRIL outside of the immune system have been revealed. For instance, APRIL modestly stimulates the proliferation of NIH-3T3 fibroblasts, HT-29 colon carcinoma cells, and A549 lung epithelial cells (Dillon et al. 2006). APRIL was also suggested to serve as a growth factor for terminal megakaryocytopoiesis and may promote physiological platelet production (Bonci et al. 2004).

1.2.5. APRIL in immune disorders and malignancies

A substantial body of evidence has accumulated pointing to the roles of APRIL and BAFF in autoimmune disease and malignancies. Clinical trials aimed at neutralizing BAFF, APRIL, or both in autoimmune disorders and chronic B cell malignancies have been carried out (Dillon et al. 2006; Ferrer et al. 2009).

1.2.5.1. APRIL in immune disorders

Though less studied than BAFF, APRIL has been shown to be important in the establishment and/or maintenance of autoimmune diseases, including systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), Sjögren's syndrome and multiple sclerosis (Dillon et al. 2006; Ferrer et al. 2009). APRIL in rheumatoid arthritis (RA) will be discussed in section 1.5.

In contrast, APRIL is also suggested to be involved in common variable immunodeficiency (CVID) associated with mutations in the gene encoding TACI (Castigli et al. 2005; Salzer et al. 2005).
1.2.5.2. APRIL in malignancies

APRIL was originally described to stimulate tumor growth (Hahne et al. 1998). Current studies are focused on APRIL in B cell malignancies, though APRIL has also been shown to influence the development of T cell and other cell malignancies.

1.2.5.2.1. APRIL in B cell malignancies

The first evidence for the role of APRIL in B cell malignancies comes from a study of aging APRIL transgenic mice which develop a B1 B cell-associated neoplasm reminiscent of B cell chronic lymphocytic leukemia (B-CLL) (Planelles et al. 2004). So far, much attention has been paid to APRIL regarding B cell malignancies including B-CLL, non-Hodgkin’s lymphoma (NHL), and multiple myeloma (MM) (Dillon et al. 2006; Hardenberg et al. 2008; Kimberley et al. 2009). Data from these studies suggests that APRIL can sustain the survival of malignant B cells in both paracrine and autocrine manners. In addition, APRIL is also implicated in the paracrine promotion of Hodgkin’s lymphoma (HL), a B-cell derived tumor (Schwaller et al. 2007; Kimberley et al. 2009).

1.2.5.2.2. APRIL in T cell malignancies

Much like the T cell function of APRIL, little is known about the role of APRIL in T cell malignancies. One study showed that malignant T cells isolated from patients with Sezary syndrome bind APRIL and survive longer in culture in the presence of APRIL (Dillon et al. 2006). However, a recent study suggested that APRIL can hardly affect the development of retrovirally induced T cell lymphoma (Hardenberg et al. 2008).
1.2.5.2.3. APRIL in other cell malignancies

APRIL also enhances the survival of human glioblastoma cell lines, human hepatocellular carcinoma cell (HCC) lines, human lung carcinoma cell line (A549) and human colon carcinoma cell line (HT29), as well as the growth of solid tumors and the formation of erythroleukemia (Dillon et al. 2006; Hardenberg et al. 2008; Kimberley et al. 2009).

1.2.6. Signaling pathways

The study of APRIL/BAFF signaling is difficult due to the complexity of the APRIL/BAFF system. APRIL and BAFF bind to different receptors and exert different functions; further, signaling pathways may vary according to receptor. The receptors involved in the different functions of APRIL and BAFF are summarized in Table 1.11. So far, our knowledge about the signaling pathways activated by APRIL/BAFF is within the context of B cell function.

Table 1.11. The receptors involved in different functions of APRIL and BAFF

<table>
<thead>
<tr>
<th>B cell function</th>
<th>APRIL</th>
<th>BAFF</th>
</tr>
</thead>
</table>
| B cell maturation                   | B cell function
| B cell proliferation/survival       | TACI, HSPG     | BAFF-R, TACI (negative)               |
| Ig class switching                  | TACI, HSPG     | BAFF-R, TACI (negative)               |
| Plasma cell survival                | BCMA, HSPG, TACI | BCMA, TACI                            |
| T cell function                     | ?              | BAFF-R, TACI (negative)?              |
| The growth of certain tumor         | HSPG           |                                       |
| and cell lines                      |                |                                       |
1.2.6.1. Signaling in B cell responses

The major signaling pathway utilized by the APRIL/BAFF system in B cells is the NF-κB signaling pathway. This signaling pathway can be classified into two subsets, the classical/canonical NF-κB signaling pathway which involves the nuclear translocation of p50 (NF-κB-1)-RelA (p65) complex and the alternative/non-canonical NF-κB signaling pathway which is evidenced by the nuclear translocation of p52 (NF-κB-2)-RelB complex (Fig. 1.4). TACI can interact with TRAFs 2, 5, and 6, and BCMA with TRAFs 1, 2, and 3, activating the classical NF-κB signaling pathway (Fig. 1.4) (Mackay et al. 2003; Sakurai et al. 2007). BAFF-R can interact with TRAF 3, and preferentially activates the alternative NF-κB signaling pathway (Fig. 1.4) (Mackay and Ambrose 2003; Sakurai et al. 2007). TACI and BCMA do not activate the alternative NF-κB signaling pathway, whereas BAFF-R may also stimulate the classical NF-κB pathway (Mackay and Ambrose 2003). The study by Sakurai et al. suggested that the APRIL-HSPG binding is necessary for activation of the alternative NF-κB pathway and for induction of activation-induced cytidine deaminase (AID) (Fig. 1.4) (Sakurai et al. 2007). This study demonstrated that HSPG is essential for APRIL-induced B-cell responses such as B-cell proliferation, IgG and IgA production, the induction of AID and the non-canonical NF-κB-2 pathway. In contrast, TACI contributes to phosphorylation of physiological AID kinase and protein kinase A (PKA, critical in post-translational regulation of AID activity) (Sakurai et al. 2007). While this direct signaling from HSPG is possible, the general notion is that APRIL-HSPG interactions are used to provide a structural platform for the formation of effective signaling complexes (Kimberley et al. 2009) (Fig. 1.5). Studies showed that signaling through TACI in mature B cells or plasmablasts requires multimeric ligands
such as BAFF 60-mers, HSPG-bound APRIL (Fig. 1.5) or membrane-bound ligands, but not soluble, trimeric ligands (Hendriks et al. 2005; Ingold et al. 2005; Bossen et al. 2008; Kimberley et al. 2009; Kimberley et al. 2009; Mackay and Schneider 2009).

Figure 1.4. The NF-κB pathways used by the receptors for BAFF and APRIL in B cell responses.

APRIL signaling through TACI and BCMA utilizes the classical NF-κB pathway, while its binding to HSPG activates the alternative NF-κB pathway. BAFF-induced BAFF-R signalling is primarily via the alternative NF-κB pathway, while it may also activate the classical NF-κB pathway. This figure is derived from a previous review (Mackay and Ambrose 2003) with some modifications.
Figure 1.5. Possible APRIL signaling pathways in either malignant cells or normal B cells (Kimberley et al. 2009).
In addition to the NF-κB signaling pathway, a phosphatidylinositol 3-kinase (PI3K)/Akt pathway through TACI was reported recently (Gupta et al. 2009) (Fig. 1.6). This is the first study to characterize APRIL-TACI-specific signaling in inducing proliferation of human follicular lymphoma (FL) B cells. This study showed that APRIL expressed within the tumor microenvironment, upon engagement with TACI, activates the PI3K pathway. Activation of PI3K leads to phosphorylation of Akt and mammalian target of rapamycin (mTOR) and the mTOR specific substrates p70S6 kinase and 4E binding protein 1 (4E-BP1) in a TACI-dependent manner. Then APRIL-TACI signaling results in phosphorylation of Rb and upregulation of cyclin D1 which eventually induces cell proliferation.

Other possible signaling pathways activated by APRIL in either malignant cells or normal B cells have also been reported (Kimberley et al. 2009) (Fig. 1.5). In summary, APRIL may bind HSPG to form a structural platform for optimal signaling. After interacting with APRIL, BCMA or TACI recruits several distinct TRAFs and activates a number of transcription factors in different signaling pathways. Both BCMA and TACI can activate NF-κB, AP-1/JNK, MAPK, or PI3K/AKT, while TACI can signal through ERK-1/2, and BCMA through p38 and ELK-1. These primarily result in the upregulation of several anti-apoptotic proteins, the down-regulation of other pro-apoptotic molecules, or the increased expression of several oncogenes. The possible end results are B cell survival, proliferation, Ig class switching and antibody production (Kimberley et al. 2009).
Figure 1.6. Schematic diagram for APRIL-induced signaling through TACI in follicular lymphoma (FL) B cells.
Malignant cells from FL express TACI, which delivers a proliferative signal upon engagement with APRIL. APRIL TACI interactions activate the PI3K, Akt, and mTOR pathway, followed by phosphorylation of p70S6 kinase, 4E-BP1, and S6. Activation of mTOR results in upregulation of cyclin D1 expression and Rb phosphorylation. The hypothetical TRAF proteins involved in APRIL TACI–mediated PI3K and NFκB activation are indicated by dashed lines (Gupta et al. 2009).

1.2.6.2. Signaling in T cell responses

Whether the signaling pathways implicated in the B cell functions of APRIL and BAFF may also apply to their T cell functions has not been tested yet. BAFF-R, but not TACI, is shown to mediate the T cell function of BAFF (Ng et al. 2004; Ye et al. 2004).
However, the hyperproliferating of TACT− T cells in response to anti-CD3 antibody and BAFF (Seshasayee et al. 2003) suggests that TACI may be an inhibitory BAFF receptor in T cell immunity. In contrast, the receptor involved in the T cell function of APRIL is still unknown.

1.2.6.3. Signaling in stimulating certain tumor growth

Unlike malignant B cells that express APRIL receptors, solid tumors and some cell lines such as Jurkat T cells, NIH-3T3 fibroblasts, HT-29 colon carcinoma cells, and A549 lung epithelial cells respond to APRIL but do not express TACI or BCMA. They express HSPG and recruit APRIL via HSPG. It is not certain whether the interaction of APRIL with HSPG directly delivers signaling or just facilitates APRIL’s interaction with a receptor that promotes tumor growth (Hendriks et al. 2005; Dillon et al. 2006; Kimberley et al. 2009).

1.3. Th1, Th2 and Th17 responses

Naïve CD4+ T cells, upon contact with antigen-presenting cells (APCs), are activated in the presence of signals generated by the T-cell receptor (TCR) (signal 1) and co-stimulatory molecules (signal 2). Within specific cytokine milieu leading to the activation of distinct transcription factors, CD4+ T cells differentiate into one of the different T helper cell (Th) subsets with different cytokine profiles and distinct effector functions (Fig. 1.7).

Th subsets were first described by Mossman and Coffman in 1986 (Mosmann et al. 1986). They found that mouse T-cell clones can be segregated into two subsets according
to their mutually exclusive production of IFN-γ or IL-4, IL-5 and IL-13, and named these
two subsets Th1 and Th2 cells, respectively.

Over the years, this Th1/Th2 paradigm of CD4⁺ T cell differentiation helped explain
many phenomena in immunity. Th1 responses have been found to be required for the
induction of organ-specific autoimmunity, such as experimental autoimmune
encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (Korn et al. 2009).
However, IFN-γ- and IFN-γ receptor–deficient mice did not ameliorate but rather
exacerbate EAE and CIA (Chu et al. 2003; Korn et al. 2009). Mice that lack other
molecules involved in Th1 differentiation such as IL-12p35, IL-12 receptor-β2, and IL-18,
also developed more severe EAE (Korn et al. 2009). This raised the possibility of
existence of another subset of T cells which is responsible for the induction of these
autoimmune diseases. In 2000, a novel p19 protein was found to engage IL-12p40 to
form a cytokine, IL-23 (Oppmann et al. 2000), and IL-23 is essential for the expansion of
IL-17-producing T cells capable to induce EAE (Langrish et al. 2005). This and other
studies demonstrated that CD4⁺ T cells producing IL-17 are associated with
autoimmunity and can be considered a novel distinct Th subset (Harrington et al. 2005;
Langrish et al. 2005; Park et al. 2005). In 2005, Harrington et al. named these IL-17
producing CD4⁺ T cells “TH-17” cells (Harrington et al. 2005). During these five years,
overwhelming studies have been carried out to address the Th17 responses.

The differentiation of Th1, Th2 and Th17 cells is considered exclusive (Korn et al.
2009; O'Shea et al. 2009; Wilson et al. 2009). For example, Th1 transcriptional factor T-
bet antagonizes IL-4 expression and Th2 transcriptional factor GATA-3 counteracts INF-
γ production (O'Shea et al. 2009). Th17 transcriptional factor, retinoic-acid-receptor-
related orphan receptor-γt (RORγt), inhibits INF-γ and IL-4 production, while INF-γ and IL-4 antagonize the signaling pathway for Th17 differentiation (O'Shea et al. 2009). However, recent studies argue for more complexity and flexibility than was previously assumed. For instance, TH17 cells can also produce IFN-γ, so the relationship between Th1 and Th17 cells is currently contentious (O'Shea et al. 2009). It was suggested that Th1 and Th17 cells function in autoimmunity in a sequential manner. Th17 cells orchestrate tissue inflammation by inducing pro-inflammatory cytokines (IL-6, IL-1 and TNFα) and chemokines (IP-10 and MCP-1) which recruit Th1 cells and mononuclear cells to the target tissue (Dardalhon et al. 2008).

The discussion here will be focused on the signaling pathways, the cytokines and the transcription factors involved in Th cell differentiation as well as the functions of Th responses.
Figure 1.7. Cytokines and transcription factor networks regulate Th cell differentiation.

This figure is derived from a previous review (Wilson et al. 2009) with the positions of labels CD28 and CD80/CD86 corrected.
1.3.1. Th1 responses

1.3.1.1. Th1 cell differentiation (Fig. 1.7) (Korn et al. 2009; Wilson et al. 2009)

Th1 cell differentiation is initiated by the activation of signal transducer and activator of transcription 1 (STAT1) by IFN-\(\gamma\) and/or IL-27, both of which upregulate T-bet. T-bet, which antagonizes GATA-binding protein 3 (GATA-3), induces the expression of H2.0-like homeobox (HLX). Cooperating with HLX, T-bet induces IFN-\(\gamma\) production. Then the expression of IL-12 receptor-\(\beta_2\) (IL-12R\(\beta_2\)) is initiated, and it pairs with IL-12R\(\beta_1\) to form the IL-12 receptor (IL-12R). IL-12, secreted by APCs, binds IL-12R to activate STAT4, which is essential for the maintenance of Th1 responses. T-bet also induces the activation of runt-related transcription factor 3 (RUNX3), and along with STAT4, drives Th1 cell differentiation. IFN-\(\gamma\) induces Th1 cell differentiation and feeds back to amplify the frequency of Th1 cells. IL-2-induced STAT5 activation has a permissive role in the initial stages of Th1 cell differentiation.

1.3.1.2. Transcriptional factors

T-bet, a transcription factor of the T box family, is largely expressed in Th1 but not in Th2 cells. T-bet is the master regulator of Th1 differentiation. It binds to the IFN-\(\gamma\) promoter and induces the expression of this cytokine (Korn et al. 2009). It also antagonizes GATA-3 and represses the expression of the IL-4 gene to inhibit Th2 cell differentiation (Wilson et al. 2009). RUNX3 represses IL-4 production by binding to the IL-4 gene silencer in the Th2 cytokine locus while cooperates with T-bet to promote IFN-\(\gamma\) production in Th1 cells (Collins et al. 2009).
1.3.1.3. Effector functions

The master effector cytokine produced by Th1 cells is IFN-γ, while Th1 cells also produce other effector cytokines such as TNF and lymphotoxin. Th1 cells are important for cell-mediated immunity against intracellular pathogens. Type I hypersensitivity is a typical Th1 response (Sutherland et al. 2005). The IgG2a antibody response is mediated by Th1 cells. Moreover, Th1 cells play an important role in autoimmune disease such as T-cell mediated colitis.

1.3.2. Th2 responses

1.3.2.1. Th2 cell differentiation (Fig. 1.7) (Ho et al. 2009; Wilson et al. 2009)

Th2 cell differentiation is initiated by IL-4 signaling through STAT6 to up-regulate GATA3 production. In turn, GATA3 induces the expression of c-maf, the transcription factor of the IL-4 gene, and together GATA3 and STAT6 induce the production of IL-4, IL-5 and IL-13. IL-4 drives Th2 cell differentiation in a positive-feedback loop. In addition to this STAT6 dependent signaling pathway, STAT-6 independent pathways are also involved in the initiation of Th2 cell differentiation. Notch signaling, through recombination-signal-binding protein for immunoglobulin-J region (RBPJ) which recruits mastermind-like 1 (MAML1) and p300 to form intracellular Notch–RBPJ–MAML1–p300 complex, transactivates the distal promoter of the GATA3 gene. This leads to the production of IL-4 by newly activated CD4⁺ T cells. IL-2 signaling can activate STAT5 which binds to the IL-4 gene, thus also resulting in IL-4 expression in newly activated T cells. Although IL-2 signaling does not up-regulate GATA-3 expression, it does require the presence of GATA-3 for its action (Zhu et al. 2006). Signals through the TCR can
also induce early IL-4 production, probably mediated by NFκ-B. In addition, STAT6-independent GATA-3 autoactivation reinforces the expression of GATA3 and directs IL-4-independent Th2 development and commitment (Ouyang et al. 2000). The cis-acting element responsible for GATA3 autoactivation remains to be determined.

1.3.2.2. Transcriptional factors

GATA-3 is a master transcriptional factor for Th2 cell differentiation. It directly transactivates the IL-5 and IL-13 genes, facilitates the conversion of the IL4–IL5–IL13 locus to an open conformation, and inhibits IFN-γ expression (Zhang et al. 1998; Kishikawa et al. 2001; Ho et al. 2009). Both STAT6-dependent and STAT6-independent pathways of IL-4 production require GATA3 (Ho et al. 2009).

C-maf, a member of the basic leucine zipper factor AP-1 family and the cellular homolog of the avian viral oncogene v-maf, is critical for IL-4 production (Ho et al. 1996; Kim et al. 1999). It directly transactivates the IL-4 gene, promotes the differentiation of Th2 cells mainly by an IL-4-dependent mechanism, and attenuates Th1 differentiation (Ho et al. 1996; Ho et al. 1998; Hwang et al. 2002). Overexpression of c-maf in transgenic mice (Ho et al. 1998) increased the production of Th2 cytokines (IL-4, IL-5 and IL-10) and the IL-4-dependent immunoglobulins (IgG1 and IgE). A gene-dose effect of c-maf for Th2 cytokine production is inferred from less Th2 cytokine production in transgenic mouse lines expressing lower levels of c-maf. The Th2 biased responses of c-maf transgenic mice were IL-4 dependent, and were not observed in c-maf transgenic mice bred onto an IL-4-deficient background. Spontaneously increased percentage of eosinophils in the bronchoalveolar lavage fluid (BALF) was also observed in c-maf
transgenic mice (Hausding et al. 2004). In contrast, T cells from c-maf<sup>−/−</sup> mice (Kim et al. 1999) were markedly deficient in IL-4 production. The expression of other Th2 cytokines, IL-5, IL-6 and IL-10, were also reduced in c-maf<sup>−/−</sup> T cells under unskewed conditions, but were almost fully restored under Th2 skewed conditions in the presence of exogenous IL-4. C-maf<sup>−/−</sup> mice had a modestly impaired production of IgG1 antibody. However, IL-13 production was normal in c-maf<sup>−/−</sup> T cells under unskewed and Th2 skewed conditions and the levels of IgE antibodies were normal in c-maf<sup>−/−</sup> mice. In addition to its role in Th2 cell differentiation, c-maf can also promote IL-17 production (Bauquet et al. 2009), regulate IL-10 expression during Th17 polarization (Xu et al. 2009), and participate in promoting the differentiation of IL-10-producing regulatory type 1 (Tr1) T cells (Pot et al. 2009).

Naive Th cells express negligible levels of c-maf (Hwang et al. 2002). The expression of c-maf can be substantially induced in developing Th2 but not Th1 cells as early as 72 h after initial stimulation (Ho et al. 1996; Hwang et al. 2002). IL-4/Stat6 signaling contributes to c-maf up-regulation (Zhu et al. 2001). Interaction between inducible T cell costimulator (ICOS) and its ligand B7.1 and B7.2 homolog (B7h) also promotes c-maf expression via IL-4 regulation (Nurieva et al. 2003; Nurieva et al. 2003). GATA-3 and IL-6 are involved in c-maf up-regulation, independent of IL-4/STAT6 signals (Ouyang et al. 2000; Yang et al. 2005). Moreover, recent studies showed that c-maf production was markedly increased under Th17 polarizing conditions induced by IL-6 plus TGF-β (Bauquet et al. 2009; Xu et al. 2009). Other factors, such as the type 2 G protein-coupled receptor (VPAC2), truncated c-maf inducing protein (Tc-mip), Vav-1, and IL-27 are also
reported to induce c-maf expression (Grimbert et al. 2003; Voice et al. 2004; Tanaka et al. 2005; Huang et al. 2006; Pot et al. 2009).

Other transcriptional factors, the nuclear factor of activated T cells (NF-AT), NF-AT interacting protein (NIP-45), IFN regulatory factor 4 (IRF4) and JunB can cooperate with c-maf to transactivate the IL-4 gene (Hodge et al. 1996; Ho et al. 1998; Voice et al. 2004). In addition, growth factor independent-1 (Gfi-1), induced by IL-4 signaling in activated T cells through STAT6, plays an important physiologic role in Th2 cell expansion (Zhu et al. 2006). In contrast, RUNX3 suppresses the production of IL-4 by binding to the IL-4 gene silencer in the Th2 cytokine locus (Collins et al. 2009).

1.3.2.3. Effector functions

Th2 cells mediate the humoral immunity against extracellular pathogens such as helminths. IgG1 and IgE antibody responses are Th2 responses. Th2 cells also contribute to the pathogenesis of allergic diseases such as allergic asthma. The major effector cytokines that mediate the functions of Th2 cells include IL-4, IL-13, IL-5, and IL-10. IL-4 is not only a critical cytokine in the induction of Th2 differentiation, but also the class switch factor of IgG1 and IgE. IL-4 plays a central role in the pathogenesis of allergic inflammation by inducing IgE class switching. IL-13 is a major effector molecule mediating several pathological features of allergic asthma, and also plays key roles in parasite immunity (Kasaian et al. 2008). IL-13 shares approximately 20% amino acid sequence identity and a range of biological activities with IL-4 (Kasaian and Miller 2008). Both IL-4 and IL-13 promote IgE class switching and down-regulate inflammatory responses (Kim et al. 1999; Lieberson et al. 2001; Kasaian and Miller 2008). It is also
known that IL-4 and IL-13 share the IL-4 receptor α chain (IL-4Rα) which is a component of two heterodimeric IL-4 receptors: the type I receptor composed of IL-4Rα and γ common chain (γc), and the type II receptor composed of IL-4Rα and IL-13Rα1. IL-4 signals through both IL-4Rs whereas IL-13 signals only through the type II IL-4R (Kasaian and Miller 2008; Wills-Karp et al. 2008). The finding that IL-4 and IL-13 share type II IL-4R may partially explain the overlap of biological activities of these two cytokines (Kim et al. 1999). IL-5 was initially identified to support the growth and differentiation of activated B cells (Takatsu et al. 2008). Now it is known that IL-5 is a key cytokine responsible for eosinophil differentiation and survival, thus plays a crucial role in eosinophilia which is associated with a wide variety of conditions, including asthma and atopic diseases, helminth infections, drug hypersensitivity, and neoplastic disorders (Fang et al. 2008; Takatsu and Nakajima 2008). In contrast, the function of IL-10 is inhibitory. It is a key anti-inflammatory immunoregulator during infection with viruses, bacteria, fungi, protozoa, and helminths, thus impedes pathogen clearance and ameliorates immunopathology (Couper et al. 2008). Moreover, IL-10 inhibits eosinophilia in asthma (Ogawa et al. 2008).

It has been well-established that type-2 immunity characterized by eosinophilia, goblet cell hyperplasia, mucus production and IgE class switching, is highly dependent on IL-4, IL-5, IL-13 and another cytokine produced by Th2 cells, IL-9 (Barlow et al. 2009). IL-9 induces mast cell proliferation and B cell infiltration of the lungs, which may be a direct effect, whereas the other effects of IL-9 on type-2 immunity appear to be mediated by IL-13 (Steenwinckel et al. 2007; Barnes 2008). IL-25, also produced by Th2
cells, can induce type-2 cytokine effector response and directly control type-2 immunity via IL-4, IL-5, and IL-13 (Barlow and McKenzie 2009).

1.3.3. Th17 responses

1.3.3.1. Th17 cell differentiation (Fig. 1.7) (Korn et al. 2009; Wilson et al. 2009)

Th17 cell differentiation is initiated by IL-6 induced activation of STAT3. STAT3 induces IL-21 expression and cooperates with TGF-β signaling to induce the expression of retinoic-acid-receptor-related orphan receptor-γt (RORγt), and then the production of IL-17, IL-21, IL-23R and IL-22. STAT3 activation is attenuated by IL-2-induced STAT5. IL-21 induces the Th17 cell differentiation and feeds back to amplify the frequency of Th17 cells, as do IFN-γ and IL-4 in the differentiation of Th1 and Th2 cells, respectively. IL-23 is not involved in the initial differentiation of Th17 cells. Similarly to IL-12 for Th1 cells, IL-23 serves to expand and stabilize Th17 responses. In addition, IL-27 dampens Th17 responses depending on STAT1 signaling but independent of enhanced Th1 commitment. Moreover, a recent report showed that IL-9 is predominantly produced by Th17 cells and synergizes with TGF-β1 to induce Th17 cell differentiation (Elyaman et al. 2009).

1.3.3.2. Transcriptional factors (Weaver et al. 2007; Collins et al. 2009; Korn et al. 2009)

The steroid receptor–type nuclear receptor RORγt, a splice variant of RORγ expressed in T cells, is selectively expressed in Th17 cells. RORγt has been shown to be a master transcriptional factor in Th17 cell differentiation, just like T-bet and GATA3 in the differentiation of Th1 and Th2 cells, respectively. However, although reduced, IL-17-
producing cells are not absent in RORγt-deficient mice. Another member of the retinoid nuclear receptor family, RORα, is also selectively expressed in Th17 cells and plays a similar but not identical role to RORγt in the differentiation of Th17 cells (Yang et al. 2008). The mechanisms by which RORγt and possibly RORα regulate Th17 cell differentiation have not yet been fully elucidated. It is unclear whether RORγt binds directly to the IL-17 promoter, although a potential ROR-binding site exists in this promoter. RORγt and RORα are both strongly induced by TGF-β plus IL-6 or TGF-β plus IL-21. The induction of RORγt is dependent on STAT3. It is likely that RORγt must cooperate with other transcription factors, such as interferon regulatory factor 4 (IRF4) and RUNX1 in the differentiation of Th17 cells.

1.3.3.3. Effector functions (Weaver et al. 2007; Korn et al. 2009)

Th17 cells are important effector cells in host defense against certain pathogens such as specific extracellular bacteria and fungi. Th17 cells also play critical roles in mediating tissue inflammation in autoimmune disease such as experimental arthritis/rheumatoid arthritis, EAE/multiple sclerosis, inflammatory bowel disease, and inflammatory skin disease in mice and psoriasis in humans. Th17 cells exert their functions by producing effector cytokines including IL-17, IL-21, IL-22, and IL-23. IL-17 is the founding member of the IL-17 family of cytokines, which consists of IL-17A (also called IL-17), IL-17B, IL-17C, IL-17D, IL-17E (also called IL-25), and IL-17F. IL-17E (or IL-25) is not produced by Th17 cells, but produced by Th2 cells. Both IL-17A and IL-17F are expressed by Th17 cells, γδ T cells, NKT cells, NK cells, neutrophils, and eosinophils. The cellular sources of IL-17B, IL-17C and IL-17D are unknown. The IL-17 receptors also constitute a distinct family of cytokine receptors which includes IL-17RA, IL-17RB,
IL-17RC, IL-17RD, and IL-17RE. IL-17RA and IL-17RC are the receptors for IL-17A and IL-17F, respectively. The receptors of IL-17 and IL-22 are broadly distributed. IL-17RA is highly expressed on hematopoietic cells, and, at lower levels, on osteoblasts, fibroblasts, endothelial cells, and epithelial cells. In contrast, IL-17RC is expressed at low levels on hematopoietic cells, but is highly expressed on nonhematopoietic cells. The receptor for IL-22, a heterodimer of the specific IL-22R and the IL-10R2, is also widely expressed, including in epithelial and endothelial cells. However, IL-22R is not expressed on immune cells, thus is used by Th17 cells to communicate with tissues but not with other immune cells. This broad receptor distribution results in broad tissue responses to Th17-related cytokines, which may explain the prominent capability of Th17 cells to induce tissue inflammation and autoimmunity.

1.4. Allergic asthma and allergic lung inflammation

The incidence of asthma has increased rapidly over the last two decades, now affecting nearly one in eight individuals in the developed countries (Meyer et al. 2008). This rapid change may be related to the reduced exposure to infectious pathogens during childhood, which is a direct result of improved hygiene, frequent use of antibiotics and vaccination. This concept is referred as the hygiene hypothesis (Strachan 1989; Fang 2004). Among the different forms of asthma, e.g. allergen-induced, infection-induced, exercise-induced, and aspirin-associated asthma, allergic asthma is the most common form, representing 70%–80% of cases (Meyer et al. 2008). Allergic asthma is characterized by allergic lung inflammation and airway hyperreactivity (AHR). IL-13, IL-4, IL-5 and IL-9 produced by Th2 T cells and NKT cells are the main pathogenic factors implicated. The characteristics of allergic lung inflammation include elevated
allergen-specific IgE levels, airway eosinophilic infiltration, and exaggerated mucus production (Fang et al. 2008).

Mouse models for allergic asthma have been developed by sensitizing and challenging mice with allergens. The “classical” ovalbumin (OVA) challenged mouse model of allergic asthma is frequently used. In this model, OVA derived from chicken egg induces a robust, allergic pulmonary inflammation (Kumar et al. 2008). OVA, however, is seldom implicated in human asthma. Therefore, alternative allergens with possible greater clinical relevance, such as house dust mite (HDM) and cockroach extracts, have also been used (Sarpong et al. 2003; Johnson et al. 2004; Nials et al. 2008). In an attempt to reproduce all features of asthma in mouse models, both acute and chronic challenge mouse models have been investigated. Mouse models of the acute allergic response to inhaled allergens have been widely used to elucidate the mechanism of allergic asthma and to identify novel therapeutic targets. The acute challenge mouse models reproduce many key features of clinical asthma, such as elevated IgE levels, airway inflammation, goblet cell hyperplasia, epithelial hypertrophy, AHR to specific stimuli and, in some models, early- and late-phase bronchoconstriction in response to allergen challenge (Nials and Uddin 2008). Chronic allergen challenge mouse models have been developed by increasing the number of allergen challenges in order to reproduce more of the features of clinical asthma, such as airway remodelling and persistent AHR, and to enable new therapies to be evaluated in a therapeutic setting rather than a prophylactic setting (Nials and Uddin 2008).
1.4.1. Mechanism of allergic asthma

Although not fully understood, the mechanism that controls the development of allergic asthma has been extensively studied and much has been elucidated (Fig. 1.8) (Barnes 2008; Ryanna et al. 2009). During the sensitization phase of allergic asthma induction, allergens are presented to naïve CD4$^+$ T cells by dendritic cells, which results in the differentiation of Th2 cells and the production of Th2 cytokines. IL-4 and IL-13 can stimulate B cells to produce allergen-specific IgE. During challenge phase, the same allergens are inhaled and bind to IgE engaged to FceRI receptors on mast cells, which activates mast cells and leads to degranulation and release of histamine and leukotrienes. These events result in AHR and airway constriction (early allergic response). A few hours after the initial allergen challenge, Th2 cytokines, such as IL-4, IL-13, IL-5 and IL-9, exert their effects on eosinophils, B cells, mast cells, airway epithelium and smooth muscle, leading to airway eosinophil infiltration, elevated serum IgE levels, exaggerated mucus production, AHR, and bronchoconstriction (late allergic response). There are three effector pathways for Th2 cytokines to induce the features of allergic asthma (Fig. 1.8), as described below.

The first effector pathway involves IgE-mediated events. IL-4 and IL-13 drive IgE production by B cells, leading to activation of mast cells, while IL-9 induces mast cell proliferation (Steenwinckel et al. 2007; Barnes 2008). Then activated mast cells release histamine, leukotrienes and prostaglandin D2, all which have effects on airway epithelium and smooth muscle, and cause AHR and mucus overproduction (Wills-Karp 1999; Barnes 2008; Ryanna et al. 2009). Mast cells also release cytokines associated with allergic inflammation, including IL-4, IL-5, and IL-13 (Barnes 2008).
The second effector pathway involves eosinophil-mediated responses. IL-5 is a key cytokine responsible for eosinophil differentiation, survival, and accumulation (Hamelmann et al. 2001; Barnes 2008; Fang et al. 2008), thus contributes to airway eosinophilia. Eotaxin, produced by lung epithelial, fibroblast, and smooth muscle cells stimulated by IL-4 and IL-13 as well as produced by eosinophils, also can recruit eosinophils into the lung independently of IL-5 (Conroy et al. 2001; Mattes et al. 2002). In addition, IL-4/IL-13 may control eosinophil influx by regulating the expression of vascular cell adhesion molecule-1 (VCAM-1) (Wills-Karp 1999). Once recruited into the respiratory tract, eosinophils secrete the eosinophil-specific proteins, including major basic protein (MBP), eosinophilic cationic protein (ECP), and eosinophilic peroxidase (EPO); lipid mediators, such as leukotrienes and prostaglandin E2; cytokines, including TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-13, and IL-5; and chemokines, such as receptor activator of NF-κB ligand (RANKL) and eotaxin. These factors directly or indirectly affect airway epithelial cells and smooth muscle, and induce mucus oversecretion, fibrosis, and AHR (Wills-Karp 1999; Hamelmann and Gelfand 2001).

The third effector pathway is that Th2 cytokines act directly on airway epithelium and smooth muscle to induce AHR and mucus overproduction. Via this direct action, IL-13 can induce AHR and mucus overproduction, and IL-4 AHR (Kuperman et al. 2002; Whittaker et al. 2002; Wills-Karp et al. 2003; Meyer et al. 2008; Ryanna et al. 2009).

Among the Th2 cytokines, IL-13 is the most critical cytokine in allergic asthma. Overexpression of IL-13 in the lungs of transgenic mice leads to airway inflammation, AHR, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and
eotaxin production (Zhu et al. 1999). In contrast, blockade of IL-13 markedly suppresses allergen-induced AHR, mucus production and eosinophilia (Kuperman et al. 2002). IL-13 is thus both necessary and sufficient for the induction of experimental allergic asthma. Whereas IL-4, though crucial in driving Th2 cell differentiation, is not required for the induction of AHR and mucus production in allergic asthma (Wills-Karp 1999; Walter et al. 2001). IL-9 has also emerged to be an important cytokine in allergic asthma. IL-9 overexpression in mice results in airway eosinophilic infiltration, mucus hyperplasia, mastocytosis, AHR, and increased expression of other Th2 cytokines and IgE. In contrast, IL-9 blockade represses lung eosinophilia, mucus hypersecretion, and AHR in experimental mice (Steenwinckel et al. 2007; Barnes 2008). While the promotion of mast cell proliferation and B cell infiltration by IL-9 appears to direct, the other effects of IL-9, mucus hyperplasia and airway eosinophilia, are mediated by IL-13 (Steenwinckel et al. 2007; Barnes 2008).

Th2 cells play an essential role in orchestrating the inflammatory responses in allergic asthma, specifically, through producing Th2 cytokines. CD1d-restricted, invariant TCR-positive, natural killer T (iNKT) cells are also required for allergen-induced airway inflammation and AHR (Lisbonne et al. 2003; Fang et al. 2008; Meyer et al. 2008). Upon activation, iNKT cells can rapidly produce large quantities of cytokines, including IL-4 and IL-13 (Meyer et al. 2008). In contrast, regulatory T cells (Tregs) may limit Th2 cell proliferation, thus suppress the development of allergic asthma (Barnes 2008; Ryanna et al. 2009). In addition, epithelial cells release stem-cell factor (SCF) which is important for maintaining mucosal mast cells at the airway surface, and CC-chemokine ligand 11 (CCL11) which recruits eosinophils via CC-chemokine receptor 3 (CCR3) (Barnes 2008).
Moreover, epithelial cells and mast cells secrete thymic stromal lymphopoietin (TSLP) which induces dendritic cells to release chemokines CCL17 and CCL22 to attract Th2 cells via CCR4 (Barnes 2008).

1.4.2. APRIL/BAFF in allergic asthma

The role of APRIL in allergic asthma has not been reported. BAFF has been shown to attenuate Th2 cytokine production (Ye et al. 2004) and suppress allergic airway inflammation (Sutherland et al. 2005).

Figure 1.8. Mechanism of allergic asthma.
This figure is derived from a previous review (Ryanna et al. 2009) with modifications.
1.5. Rheumatoid arthritis (RA) and collagen-induced arthritis (CIA)

Rheumatoid arthritis (RA) is a very common systemic autoimmune disease, affecting ~1% of the world-wide adult population (Nakae et al. 2003; Mackay et al. 2005). The etiology of human RA is unclear. The disease can be started under different conditions, such as mechanical stress, viral or bacterial infection, and senescence (Cho et al. 2007). RA is a chronic disease characterized by synovial inflammation and joint damage (Lai Kwan Lam et al. 2008). Although RA targets the joint synovial membrane, cartilage, and bone, it can also involve inflammation of the tissue around the joints and other organs, therefore is considered a systemic disease (Dillon et al. 2006). Both B and T cells mediate the pathogenesis of RA, and proinflammatory cytokines are critically involved (Lai Kwan Lam et al. 2008).

Antigens involved in RA have been proposed to be viral proteins, such as cytomegalovirus (CMV) and Epstein-Barr Virus (EBV), as well as several autologous proteins normally expressed in the joints, including gp39, proteoglycan, and type II collagen (CII) (Brand et al. 2003). CII has received considerable attention as an autoantigen in RA because it is a major component of and is expressed exclusively in the articular cartilage of joints, the site of inflammation in RA (Brand et al. 2003; Cho et al. 2007). The high prevalences of CII-specific antibodies and T cells in RA patients, especially during the early phase of RA, indicate that CII is one of the major autoantigens of human RA and CII-specific immunity plays an important role in the initiation of joint inflammation (Brand et al. 2003; Cho et al. 2007). Arthritis induced with CII in animals, collagen-induced arthritis (CIA), is the most widely used, valid animal model of RA for the study of autoimmune arthritis pathogenesis and for testing new therapeutic methods.
Similar to human RA, autoimmunity to CII in the CIA model can generate autoimmune arthritis, including inflammation of synovial joints, destruction of cartilage, and bone erosion; Disease susceptibility is clearly associated with the expression of specific class II molecules of the major histocompatibility complex (MHC II); And both B and T cell immune responses are required in CIA (Campbell et al. 2000; Wang et al. 2001; Brand et al. 2003; Cho et al. 2007).

1.5.1. Mechanism of RA/CIA

The mechanism of human RA has not been fully elucidated. However, the accumulating knowledge about the pathogenesis of CIA (Fig. 1.9) (Cho et al. 2007) has provided great insight into the mechanism of RA.

1.5.1.1 Autoantibodies in RA/CIA

The CII-specific autoantibody of IgG2 isotype is crucial to initiate CIA (Brand et al. 2003; Nandakumar et al. 2004). Within a given strain, the levels of antibody correlate well with the presence or absence of arthritis. Passive transfer of anti-CII sera induces an inflammatory arthritis in strains genetically susceptible or nonsusceptible to CIA (Brand et al. 2003). Moreover, collagen antibody-induced arthritis (CAIA) has emerged to be a simple animal model for RA (Khachigian 2006; Nandakumar et al. 2006).

In CIA (Cho et al. 2007), CII-specific antibodies of the IgG2 isotype, produced by mice immunized with heterologous CII protein and CFA, bind to the CII of cartilage in joints and activate the complement system. This results in the recruitment and activation of neutrophils and macrophages via C5a, a cleavage product of complement component 5. The activated leukocytes secrete chemokines and proinflammatory cytokines, such as IL-
1β, TNF-α, IL-8, IL-6, nitric oxide (NO), prostaglandin E2, and macrophage inflammatory proteins (MIPs). These factors recruit diverse immune cells, including natural killer (NK) cells, dendritic cells, T cells, and B cells, and activate infiltrating immune cells, synovial fibroblasts, and synovial macrophages. IL-1β and TNF-α are crucial cytokines during CIA onset and IL-1β is also critical during CIA progression. In ongoing inflammation, T cells, B cells, dendritic cells, and synovial macrophages proliferate and act in concert to secrete more cytokines, chemokines and enzymes. This leads to the degradation of the extracellular matrix (ECM) and cartilage by matrix metalloproteinases (MMPs), elastase, and cathepsin G, the hyperplasia of the synovial fibroblasts and macrophages, and sometimes the formation of ectopic germinal centers.

In human RA, autoantibodies, including rheumatoid factor (RF or RhF), anti-citrullinated protein/cyclic citrullinated peptide (CCP) antibodies, and anti-CII antibodies, are useful in the diagnosis and prognosis of RA (Cho et al. 2007; Imboden 2009). These autoantibodies may also influence the inflammatory response via complement activation in the joint, especially in the early stages of RA, a similar manner to the CIA or CAIA model (Cho et al. 2007).

1.5.1.2 T cells in RA/CIA

T cells are clearly required for the development of CIA, though they are not sufficient to induce full-scale of CIA since passive transfer of CII-specific T cells to susceptible strains induces only mild joint inflammation, while severe inflammation is induced in transfer of CII-specific antibodies (Brand et al. 2003).

Regulatory T cells (Tregs), whose function may be impaired, also infiltrate the synovium to protect against tissue destruction and secrete TGF-β and IL-10. The
simultaneous presence of inflammation and Tregs may create a condition with higher levels of TGF-β and IL-6 and lower levels of IFN-γ and IL-4, which induces Th17 cell differentiation (Cho et al. 2007). IL-17, IL-23 and IL-21 produced by Th17 cells are pathogenic (Korn et al. 2009). IL-17 activates NF-κB and stimulates the production of several inflammatory mediators, including TNF-α, IL-1, receptor activator of NF-κB ligand (RANKL), IL-6, IL-8, macrophage colony stimulating factor (M-CSF), and prostaglandin E2 (Cho et al. 2007). RANKL expression on the surface of Th17 cells induces osteoclastogenesis, which promotes cartilage and bone destruction/resorption independently of TNF and IL-1 (Korn et al. 2009). Furthermore, Th17 cells may in turn provide help to B cell to produce more CII-specific autoantibodies (McInnes et al. 2007), which is supported by the previous finding that IL-17 deficiency suppressed anti-CII IgG2a levels and CIA (Nakae et al. 2003). This may form a positive feedback loop to amplify the joint inflammation and damage.

Th1 cells were originally considered to mediate CIA. However, IFN-γ- and IFN-γ receptor–deficient mice did not ameliorate but rather exacerbate CIA (Chu et al. 2003). Later it was found that IFN-γ regulates susceptibility to CIA through suppression of IL-17 (Chu et al. 2007). Regarding the relationship between Th1 and Th17 cells, it was proposed that Th17 cells orchestrate tissue inflammation and induce the secretion of the chemokine which recruits Th1 cells to the target tissue (Dardalhon et al. 2008).
Figure 1.9. Schematic diagram of inflammation and bone erosion in the arthritic joints of CIA mice (Cho et al. 2007).

PGE2: prostaglandin E2; NO: nitric oxide; MIPs: macrophage inflammatory proteins; RANKL: receptor activator of NF-κB ligand; M-CSF: macrophage colony stimulating factor; MMPs: matrix metalloproteinases.

1.5.2. APRIL/BAFF in RA/CIA

Both APRIL and BAFF protein levels are increased in RA patients, with significantly higher levels in synovial fluid than in the serum (Tan et al. 2003; Dillon et al. 2006; Lai Kwan Lam et al. 2008; Ferrer et al. 2009). A TACI-Ig fusion protein, blocking both BAFF and APRIL, substantially inhibited mouse CIA (Wang et al. 2001), indicating that APRIL and/or BAFF contribute to CIA. Comparing the effects of BCMA-Ig (blocking both BAFF and APRIL) and BAFF-R-Ig (blocking only BAFF), BAFF appeared to be a key factor for the progression of CIA in mice (Mackay et al. 2005). A recent study using
local BAFF gene silencing approach revealed a role of BAFF in promoting Th17 cell expansion and arthritic development in CIA (Lai Kwan Lam et al. 2008). Accumulating evidence showing elevated APRIL or APRIL/BAFF heterotrimers in sera and elevated APRIL in joints of RA patients suggests that APRIL may also play a role in RA (Roschke et al. 2002; Tan et al. 2003; Seyler et al. 2005; Vallerskog et al. 2006; Nagatani et al. 2007; Gabay et al. 2009). Clearly, to better distinguish the function of APRIL and BAFF in RA/CIA, CIA studies under APRIL deficient conditions are needed, which is a part of our study.
2.1. Generation of APRIL \( ^{-/-} \) mice (Xiao et al. 2008)

APRIL deficient mice were created by replacing exons 2-5 and the majority of exon 1 and 6 of the APRIL gene with a neomycin cassette (Fig. 2.1A) by homologous recombination in embryonic stem (ES) cells and blastocyst injection. Transfected ES cell colonies were analyzed by Southern blotting (Fig. 2.1B). Tail DNA samples from APRIL \( ^{-/-} \) mice showed no APRIL at the DNA level, as determined by Southern blotting and PCR (Fig. 2.1C, D). APRIL \( ^{-/-} \) mice also showed complete absence of APRIL mRNA in splenocytes while mRNA for TNF-related weak inducer of apoptosis (Tweak, or TNFSF12) and Sentrin specific peptidase 3 (Senp3), two genes located 794 bp and 731 bp up- and down-stream, respectively, of APRIL were expressed normally (Fig. 2.1E). APRIL \( ^{-/-} \) mice appeared normal in growth, weight, and breeding, consistent with a previous report (Castigli et al. 2004).

2.2. APRIL promotes serum IgA production (Xiao et al. 2008)

As reported previously (Castigli et al. 2004), we confirmed that naïve APRIL \( ^{-/-} \) mice have reduced serum IgA levels (Fig. 2.2A) while other immunoglobulin isotype levels are normal when compared to APRIL \( ^{+/+} \) littermates (Fig. 2.2B-G). A gene dose effect of APRIL for IgA production is inferred from the intermediate IgA levels in heterozygous mice.
Figure 2.1. Generation of APRIL−/− mice

A. Upper panel: Genomic locus of murine APRIL. 1-6: Exons of APRIL gene. TW7: Exon 7 of Tweak. S1-S4: Exons of Senp3. Lower panel: APRIL gene targeting construct. H: Hind III, E: EcoR I, B: BamH I. The positions of probes used for Southern blots are indicated by black and gray bars. The positions of the PCR primer sets for genotype analysis are indicated by black (WT) and gray (deletion mutant) arrow pairs. B. Southern blot analysis of DNA derived from several transfected ES cell clones, digested by Hind III. C. Southern blot analysis of tail-derived genomic DNA digested by BamH I. D. PCR analysis of tail-derived genomic DNA. E. Real time RT-PCR analysis of APRIL, Tweak and Senp3 cDNA from splenocytes. The APRIL++ sample was used as the calibrator (relative mRNA expression =1).
Figure 2.2. Unimmunized APRIL−/− mice have reduced serum IgA levels.

Antibody isotype levels in sera of unimmunized mice were determined by ELISA. * p=0.0171, APRIL−/− vs APRIL+/+, n=4; unpaired t test.

2.3. APRIL suppresses T cell proliferation (Xiao et al. 2008)

APRIL−/− mice exhibited moderately increased T cell proliferation which was significant only at a concentration of 2.5µg/ml of anti-CD3 stimulation (Fig. 2.3), compared with WT mice. The increased T cell proliferation in APRIL−/− mice is consistent with a previous finding that APRIL−/− mice have increased numbers of CD44hiCD62Llo CD4+ effector/memory T cells (Castigli et al. 2004).
Figure 2.3. APRIL−/− mice show increased T cell proliferation.

Splenocytes were cultured at 1×10^6 cells/ml and stimulated with 0-5μg/ml of anti-CD3 for 3 days. T cell proliferation was measured by [3H] thymidine incorporation. Representative data represent 7 pairs of mice from 5 independent experiments; * p=0.0401; n=7; paired t test. −/−: APRIL−/−; +/+: WT.

2.4. APRIL represses Th1 responses only under non-polarizing conditions (Xiao et al. 2008)

Cultured APRIL−/− CD4+ T cells showed increased IFN-γ production under Th neutral (ThN), non-polarizing conditions, but not under Th1 polarizing conditions (Fig. 2.4A, B; Table 2.1). Increased IFN-γ production was also observed in naive APRIL−/− splenocytes stimulated with anti-CD3 (data not shown). To analyze the effect of APRIL on the Th1 mediated antibody response (IgG2a), we immunized mice with ovalbumin (OVA) emulsified in complete Freund’s adjuvant (CFA) which induces Th1 polarization. No difference in OVA specific IgG2a levels was observed between APRIL−/− and WT mice (Fig. 2.5A, B), indicating that APRIL does not affect Th1 antibody responses. Taken together, our data suggest that APRIL represses Th1 responses only under non-polarizing conditions.
Figure 2.4. APRIL−/− mice have increased cytokine production.

Cytokine production by CD4+ T cells under non-polarizing (ThN), Th1, Th2 and Th17 polarizing conditions upon primary (1°) and secondary (2°) stimulation was measured by ELISA. For details see Material and Methods. *, p<0.05; **, p<0.01; ***, p<0.001; n=10; paired t test. Data from 6 independent experiments (n=1-3 each) were combined. n.d.- Not done.
Table 2.1. Effect of APRIL deficiency on cytokine production by unpolarized (ThN), Th1, Th2 or Th17 polarized CD4+ T cells after primary (1°) and secondary (2°) stimulation

<table>
<thead>
<tr>
<th>Polarization</th>
<th>Cytokine regulation in APRIL-/- versus APRIL+/+ CD4+ T cellsa</th>
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a) ‘Up’ and ‘Down’ indicates statistically significantly higher or lower cytokine levels, respectively, secreted by APRIL-/- CD4+ T cells compared to APRIL+/+ CD4+ T cells. 1° or 2° indicates change only after primary or secondary stimulation, respectively; 1° + 2° indicates change after either stimulation.

2.5. APRIL inhibits Th2 responses and allergic lung inflammation

2.5.1. APRIL-/- mice have enhanced Th2 cytokine production (Xiao et al. 2008)

Cytokine production by purified, unpolarized and Th2 polarized CD4+ T cells showed significant changes in APRIL-/- mice (Fig. 2.4; Table 2.1). Under ThN conditions, IL-4 was upregulated in primary (1°) stimulation (Fig. 2.4E), while all Th2 cytokines were increased upon secondary (2°) stimulation (Fig. 2.4I-L) of APRIL-/- CD4+ T cells. Under Th2 polarizing conditions, APRIL-/- CD4+ T cells secreted increased IL-13 in 1° and 2° stimulation (Fig. 2.4H, L) and significantly more IL-17 (Th17 cytokine) in 2° activation (Fig. 2.4D) than APRIL+/+ CD4+ T cells.
Figure 2.5. APRIL\textsuperscript{\textminus}/\textminus\ mice show increased IgG1 levels with immunization.

A, B. Normal IgG1 or IgG2a levels upon immunization with OVA/CFA on day 0 and OVA/IFA on day 14. n=12, data from 4 independent experiments (n=1-6 each) were combined. C, D. Increased antigen specific IgG1 levels in APRIL\textsuperscript{\textminus}/\textminus\ mice upon immunization with OVA/alum on day 0 and day 14. *, p<0.05; **, p<0.01; n=5-6, data from 2 independent experiments (n=2-3 each) were combined; unpaired t test. \(-/-\): APRIL\textsuperscript{\textminus}/\textminus\; ; +/-: WT.

2.5.2. APRIL\textsuperscript{\textminus}/\textminus\ mice show increased Th2 antibody responses (Xiao et al. 2008)

To assess the role of APRIL on the Th2 mediated antibody response (IgG1), we immunized mice with OVA precipitated with aluminum potassium sulfate (alum) which induces Th2 polarization. A significant increase in the OVA-specific IgG1 levels was
apparent in APRIL⁻/⁻ mice (Fig. 2.5C-D), indicating that the presence of APRIL suppresses Th2 antibody responses.

2.5.3. APRIL⁻/⁻ mice have significantly aggravated allergic lung inflammation

Since Th2 cytokines, especially IL-13, are essential for the development of allergic asthma, we hypothesized that APRIL inhibits allergic lung inflammation and that APRIL deficient mice would have exaggerated allergic lung inflammation. To test this hypothesis we induced lung inflammation in APRIL⁻/⁻ and WT mice using the classical OVA model. Mice were sensitized on day 0 and boosted on day 5 by i.p. injection of OVA in alum, then aerosol challenged with OVA on day 12, and lung inflammation was analyzed on day 15 (Fig. 2.6A). We found that APRIL⁻/⁻ mice had significantly aggravated lung inflammation compared with WT mice, as documented by increased total cell numbers and elevated numbers and percentage of eosinophils and lymphocytes in the bronchoalveolar lavage fluid (BALF) (Fig. 2.6B-C), by augmented inflammatory cell infiltration in the lung detected by H&E staining (Fig. 2.7A), by excessive mucus secretion detected by PAS staining (Fig. 2.7B), by elevated serum OVA-specific IgE production (Fig. 2.8A), and by enhanced Th2 cytokine (IL-13, IL-5 and IL-6) production by bronchial lymph node (LN) cells (Fig. 2.8B). Due to the large increase in the percentage of eosinophils, the macrophage percentage was decreased in the BALF of APRIL⁻/⁻ mice (Fig. 2.6B). IL-4 and INF-γ production by bronchial LN cells was undetectable in both APRIL⁻/⁻ and WT mice.
Figure 2.6. APRIL−/− mice have increased airway eosinophil infiltration.

A. The scheme for induction of allergic lung inflammation in the murine model of allergic lung inflammation. APRIL−/− and WT mice were sensitized on day 0 and boosted on day 5 by i.p. injection of 66μg OVA coprecipitated with 6.6mg alum. On day 12, mice were aerosol challenged with 0.5% OVA in PBS for 1h. On day 15, allergic lung inflammation was evaluated. B. Inflammatory cell number and percentage in the BALF. *, p<0.05; **, p<0.01; unpaired t test; n=7-9. Data from 5 independent experiments were combined. C. BALF cells on cytospin slides stained with Geimsa-Wright (original 40x). The eosinophils are stained blue in nuclei and red in cytoplasm, indicated by arrows. −/−: APRIL−/− ; +/+: WT.
Figure 2.7. APRIL⁻/⁻ mice have augmented inflammatory cell infiltration and mucus secretion in the lung.

A. Histological analysis of lung - H&E staining. Lung inflammation is indicated by an increased presence of the perivascular infiltration of eosinophils and lymphocytes (original 40x). B. Histological analysis of lung - PAS staining to detect mucus production. Mucus produced by goblet cells is stained as purple, indicated by arrows (original 40x). 

-/-: APRIL⁻/⁻; +/+: WT.
Figure 2.8. APRIL−/− mice have elevated serum OVA-specific IgE production and enhanced Th2 cytokine production by bronchial LN cells.

**A.** OVA-specific IgE antibody titers detected by ELISA. The serum titration shown was within the linear range. *, p<0.05; unpaired t test; n=7-9. Data from 5 independent experiments were combined. 

**B.** Cytokine production by OVA restimulated bronchial LN cells. Bronchial LN cells were cultured in the presence or absence of OVA (100μg/ml) for 4 days. Cytokine production in the supernatant was determined by ELISA. *, p<0.05; unpaired t test; n=4-6. Cells from 2 mice were pooled in the culture, if necessary. Data from 5 independent experiments were combined. -/-: APRIL−/−; +/+: WT.

### 2.5.4. Generation of TACI-Ig

To confirm the role of APRIL in allergic lung inflammation, we generated a decoy receptor-Ig fusion protein, TACI-Ig, to block APRIL in WT mice to analyze its effect on allergic lung inflammation.

TACI-Ig was constructed by fusing the extracellular domain of mouse TACI cDNA (Met 1 - Thr 129, 387bp) (Xia et al. 2000) to an Igκ chain signal peptide (92 bp) at the 5’ end and to the mouse IgG1-Fc sequence (680bp) at the 3’ end (Fig. 2.9A). TACI is a type-III transmembrane protein without a signal peptide. The purpose for the addition of an Igκ chain signal peptide was to make TACI-Ig secreted. ELISA analysis showed that TACI-Ig in the supernatant of the TACI-Ig transfected 3T3 cell culture bound mouse
APRIL and IgG1. Western blots using anti-mouse IgG1 antibody showed that TACI-Ig in the cell lysate and the supernatant was of correct size, ~40 kDa, according to the analysis using Vector NTI.

Since APRIL induces IgA class switching (Litinskii et al. 2002; Castigli et al. 2004), the function of TACI-Ig can be verified by testing its ability to block APRIL-induced IgA production in IgD+ B cells. Purified mouse IgD+ B cells were cultured with anti-IgM, IL-5 and APRIL in the presence or absence of 35 μg/ml of TACI-Ig or control mouse IgG for 5 days. Supernatant samples were analyzed by ELISA. IgA production was undetectable in the supernatant with TACI-Ig treatment, whereas it was detected in the supernatants with mouse IgG treatment and without TACI-Ig/IgG treatment, with comparable concentrations (Fig. 2.9B). These data suggest that our newly generated TACI-Ig is functional.

2.5.5. TACI-Ig treatment in WT mice enhances allergic lung inflammation

To analyze the effect of TACI-Ig on allergic lung inflammation in WT mice, we treated WT mice with TACI-Ig or the control mouse IgG at 100 μg/mouse by i.p. injection, on each day from day 11 to 14 (day -1 to +2 relative to aerosol) during allergic lung inflammation induction (Fig. 2.10A). Consistent with the finding in APRIL−/− mice, TACI-Ig treatment enhanced allergic lung inflammation, as indicated by elevated total cell numbers and eosinophil numbers and percentage in the BALF (Fig. 2.10B-C), by exacerbated inflammatory cell infiltration and mucus secretion in the lung (Fig. 2.11), by enhanced serum ovalbumin-specific IgE levels (Fig. 2.12A), and by augmented cytokine (IL-13, IL-5 and IL-6) production by bronchial LN cells (Fig. 2.12B). It is important to
note that TACI-Ig can also bind BAFF with comparable affinity (Dillon et al. 2006), and BAFF was also reported to be a negative regulator in Th2 cytokine production and allergic lung inflammation (Ye et al. 2004; Sutherland et al. 2005). Therefore, the effect of TACI-Ig treatment on lung inflammation may reflect the effects of both APRIL and BAFF blockade. Accordingly, we observed that the differences between TACI-Ig and IgG treatment in BALF eosinophil number and percentage (~5 fold) (Fig. 2.10B) were bigger than those between APRIL/− and WT mice (3-4 fold) (Fig. 2.6B). The effects of TACI-Ig treatment and genetic deletion of APRIL on serum OVA-specific IgE levels were comparable (Fig. 2.12A; Fig. 2.8A). It has been shown that BAFF does not inhibit serum OVA-specific IgE levels but suppresses allergic lung inflammation (Sutherland et al. 2005). However, the difference between TACI-Ig and IgG treatment in Th2 cytokine production by bronchial LN cells (~2 fold) (Fig. 2.12B) was smaller than that between APRIL/− and WT mice (~5 fold for IL-13, ~4 fold for IL-5 and ~3 fold for IL-6) (Fig. 2.8B). When bronchial LN cells from TACI-Ig and IgG treated mice were restimulated in vitro with OVA, no TACI-Ig was added to the culture, possibly explaining the smaller difference in Th2 cytokine production compared with APRIL/− versus WT mice. The presence of APRIL in the bronchial LN cell culture might partially restore the inhibitory function of APRIL.
**Figure 2.9. Functional assay for TACI-Ig**

Purified mouse IgD\(^+\) B cells at 2×10\(^6\) cells/ml were cultured with anti-IgM, IL-5 and APRIL in the presence or absence of 35\(\mu\)g/ml of TACI-Ig or control mouse IgG. After 5 days, supernatants were collected for ELISA to determine IgA production. n=2; representative of two independent experiments.
Figure 2.10. TACI-Ig treatment in WT mice increases airway eosinophil infiltration.

A. The scheme for TACI-Ig treatment in the allergic lung inflammation model. During allergic lung inflammation induction, on each day from day 11 to 14, WT mice were given TACI-Ig or the control mouse IgG (mIgG) at 100 μg/mouse by i.p. injection. B. Inflammatory cell number and percentage in the BALF. *, p<0.05; paired t test; n=4. Data from 2 independent experiments were combined. C. BALF cells on cytoospin slides stained with Geimsa-Wright (original 40x). The eosinophils are stained blue in nuclei and red in cytoplasm, indicated by arrows.
Figure 2.11. TACI-Ig treatment in WT mice augments inflammatory cell infiltration and mucus secretion in the lung.

A. Histological analysis of lung - H&E staining. Lung inflammation is indicated by an increased presence of the perivascular infiltration of eosinophils and lymphocytes (original 40x).

B. Histological analysis of lung - PAS staining to detect mucus production. Mucus produced by goblet cells is stained as purple, indicated by arrows (original 40x).
Figure 2.12. TACI-Ig treatment in WT mice enhances serum OVA-specific IgE production and Th2 cytokine production by bronchial LN cells.

A. OVA-specific IgE antibody titers detected by ELISA. The serum titration shown was within the linear range. B. Cytokine production by OVA restimulated bronchial LN cells. Bronchial LN cells were cultured in the presence or absence of OVA (100μg/ml) for 4 days. Cytokine production in the supernatant was determined by ELISA. *, p<0.05; **, p<0.01; paired t test; n=4. Data from 2 independent experiments were combined.

2.5.6. APRIL sufficient, antigen-specific CD4+ T cells restore the suppressive effect of APRIL on allergic lung inflammation in APRIL−/− mice

Elevated Th2 cytokine production by APRIL deficient CD4+ T cells was observed in vitro when purified CD4+ cells were stimulated with anti-CD3 antibody (Fig. 2.4). This observation suggests that the suppressive signal of APRIL is intrinsic to CD4+ T cells. Therefore, the transfer of antigen-specific WT CD4+ T cells to APRIL−/− mice should be able to restore the suppressive effect of APRIL on allergic lung inflammation and abolish the difference between APRIL−/− and WT mice in the severity of allergic lung inflammation. To test this hypothesis, APRIL sufficient, OVA-specific, TCR transgenic CD4+ T (OT-II) cells at 1x10⁶ cells/mouse were transferred to APRIL−/− and WT mice by i.v. injection, two days before the first OVA/alum injection for allergic lung
inflammation induction (Fig. 2.13A). As expected, APRIL sufficient OT-II cell transfer abolished the difference between APRIL−/− and WT mice in the severity of allergic lung inflammation as indicated by comparable BALF eosinophil numbers and percentage, lung inflammatory cell infiltration, mucus secretion, serum OVA specific IgE production and Th2 cytokine production (Fig. 2.13B-D and data not shown). Taking advantage of GFP expression in OT-II cells, we monitored OT-II (CD4+GFP+) cell expansion in the recipients following immunization to make sure the cell transfer was successful. In peripheral blood, two days after OT-II cell transfer, OT-II cells were detectable only at very low frequency. Following OVA/alum sensitization on day 0, OT-II cell expansion went up, and then decreased after OVA/alum boosting on day 5. On day 12, OT-II cell frequency in blood was at a very low level. After OVA aerosol challenge, there was still a slight drop. On day 15, OT-II cells were barely detected in blood. In contrast, a high percentage of GFP+ OT-II cells were found in the BALF, bronchial LNs and lungs (Fig. 2.14), indicating that OT-II cells are recruited to the lung after aerosol challenge. Again no difference was detected between APRIL−/− and WT mice in OT-II cell expansion (Fig. 2.14). Our data suggest that the effect of APRIL is CD4+ T cell autonomous and does not require APRIL production by other cells. In support of this, we and others observed that APRIL is expressed by CD4+ T cells (Pradet-Balade et al. 2002; Stein et al. 2002) (Data not shown), and APRIL is a secreted soluble ligand (Lopez-Fraga et al. 2001) which enables it to function in autocrine and/or paracrine ways.
Figure 2.13. The transfer of OT-II cells abolishes the difference between APRIL−/− and WT mice in the severity of lung inflammation.

A. The scheme for OT-II cell transfer in the allergic lung inflammation model. Two days before the first OVA/alum injection to induce allergic lung inflammation, GFP+ OT-II cells at 1x10^6 cells/mouse were transferred to mice by tail i.v. injection. B. Inflammatory cell number and percentage in the BALF. C. OVA-specific IgE antibody titers detected by ELISA. The serum titration shown was within the linear range. D. Cytokine production by OVA restimulated bronchial LN cells. Bronchial LN cells were cultured in the presence or absence of OVA (100μg/ml) for 4 days. Cytokine production in the supernatant was determined by ELISA. n=14-15; data from 6 independent experiments were combined. −/−: APRIL−/− ; +/+: WT.
To examine the kinetics of OT-II cell expansion in peripheral blood, mice were bled on day 0, 5, and 12 via tail vein before OVA/alum i.p. injection or OVA aerosol challenge, and on day 15 via the orbital sinus. Cells isolated from the blood samples, bronchial LNs, BALF, and lungs were stained with CD4-PE for FACS. CD4+GFP+ cells were identified as OT-II cells. n=8-15; data from 6 independent experiments were combined.

-/-: APRIL-/- ; +/+: WT.

2.5.7. C-maf expression is enhanced in cultured APRIL-/- CD4+ T cells

To investigate the mechanism by which APRIL down-regulates Th2 cytokine production, we analyzed the expression of the two most important Th2 cytokine transcription factors, GATA-3 and c-maf, in cultured CD4+ T cells from APRIL-/- and WT mice. We found that c-maf expression was significantly enhanced in CD4+ T cells from APRIL-/- mice at both the RNA and protein level and under both ThN and Th2 conditions. GATA-3 expression was unchanged (Fig. 2.15A-C). Our data demonstrate that APRIL suppresses c-maf expression. It is noteworthy that IL-4 can induce c-maf expression (Zhu et al. 2001), thus the augmented c-maf expression could be caused by the increased IL-4 production in APRIL-/- CD4+ T cells. Nevertheless, the increased c-maf level in APRIL-/- CD4+ T cells under Th2 conditions even in the presence of excess
exogenous IL-4 suggests that the inhibition of c-maf expression by APRIL is direct, not secondary to repression by IL-4.

Figure 2.15. C-maf, not GATA-3, expression is enhanced in cultured CD4\(^+\) T cells from APRIL\(^{-/-}\) mice.

Purified CD4\(^+\) cells were cultured under ThN and Th2 conditions for 4 days. Upon secondary stimulation with anti-CD3 antibody for 2 days, cells were harvested for RNA isolation and cell lysate preparation. A. C-maf and GATA-3 expression detected by quantitative Taqman real-time RT-PCR. RNA isolated from unstimulated and stimulated CD4\(^+\) cells was analyzed. Relative expression was calculated relative to the unstimulated WT samples, normalized to \(\beta\)-actin. *, \(p<0.05\); **, \(p<0.01\); paired t test; \(n=8\) (CD4\(^+\) cells from 2 mice were pooled, 16 mice per group). Data from 5 independent experiments were combined. B. C-maf and GATA-3 expression detected by Western blots. Whole cell lysate was analyzed. C. Quantification of Western Blot bands by using Scion Image, alpha 4.0.3.2. Relative intensity was calculated relative to \(\beta\)-actin. *, \(p<0.05\); **, \(p<0.01\); paired t test; \(n=5\) (CD4\(^+\) cells from 2 mice were pooled, 10 mice per group). Data from 2 independent experiments were combined. \(-/-\): APRIL\(^{-/-}\); +/-: WT.
2.5.8. TACI-Ig and APRIL have no effect on c-maf expression in the CD4⁺ T cell culture

To confirm the observation that APRIL inhibits c-maf expression, we conducted assays to analyze c-maf expression in the CD4⁺ T cell culture with TACI-Ig or APRIL treatment. TACI-Ig at 30µg/ml was added to the APRIL⁺/+ CD4⁺ T cell culture to block APRIL, with mouse IgG as a control. APRIL at 1µg/ml was added to the APRIL⁻/- CD4⁺ T cell culture to restore APRIL function, with PBS as a control. The APRIL concentration in the supernatants of APRIL⁺/+ CD4⁺ T cell culture was lower than 60ng/ml and undetectable by ELISA using TACI-Ig as trapping reagent and anti-APRIL-Biotin as detection antibody (data not shown). The amount of TACI-Ig or APRIL added to the culture was well above that level in excess. No difference in c-maf expression at the RNA level was observed between TACI-Ig or APRIL treated and control treated mice (Fig. 2.16). The increased c-maf expression in cultured APRIL⁻/- CD4⁺ T cells thus could not be mimicked by adding TACI-Ig to block APRIL in cultured WT CD4⁺ T cells, or restored by adding APRIL to cultured APRIL⁻/- CD4⁺ T cells.
Figure 2.16. TACI-Ig and APRIL have no effect on c-maf expression in the CD4⁺ T cell culture.

C-maf expression was analyzed by quantitative Taqman real-time RT-PCR after TACI-Ig or APRIL treatment. During the 6 days of culture as mentioned in Figure 2.15, TACI-Ig at 30µg/ml was added to the APRIL⁺/⁺ CD4⁺ T cell culture to block APRIL, with mouse IgG (mIgG) as a control. APRIL at 1µg/ml was added to the APRIL⁻/⁻ CD4⁺ T cell culture to restore APRIL function, with PBS as a control. Fold change was calculated over control samples, normalized to β-actin. n=5 (CD4⁺ cells from 2-4 mice were pooled, 17 mice per group). Data from 5 independent experiments were combined.

2.6. APRIL promotes Th17 responses and collagen-induced arthritis (CIA) (Xiao et al. 2008)

Our data showed that APRIL affected cytokine production in vitro (Fig. 2.4; Table 2.1). The IL-17 production by CD4⁺ T cells under ThN and Th1 conditions was very low, upon 1° or 2° stimulation, and showed no difference between APRIL⁻/⁻ mice and WT mice (Fig. 2.4 C, D; Table 2.1). Under Th2 conditions, IL-17 expression was increased in APRIL⁻/⁻ CD4⁺ T cells upon 2° stimulation (Fig. 2.4 C, D; Table 2.1). Under Th17 conditions in the presence of polarizing cytokines, IL-17 expression showed no difference between APRIL⁻/⁻ mice and WT mice, upon 1° or 2° stimulation. However, IL-10 production was impaired in APRIL⁻/⁻ CD4⁺ T cells under Th17 conditions, upon 1° stimulation (Fig. 2.4 G; Table 2.1).
To determine whether the effect of APRIL on cytokines was of patho-physiological importance, especially whether APRIL affects IL-17 production \textit{in vivo}, we subjected APRIL$^{-/-}$ mice to collagen-induced arthritis (CIA), a classical animal model for rheumatoid arthritis (RA). RA and CIA are mediated by Th17 responses (Nakae et al. 2003; Chu et al. 2007; Furuzawa-Carballeda et al. 2007), and the role of APRIL in RA/CIA is unclear.

\textbf{2.6.1. APRIL$^{-/-}$ mice exhibit decreased incidence of CIA}

Upon immunization with chick type II collagen (CII) in the presence of CFA, APRIL$^{-/-}$ showed a significantly decreased incidence of arthritis when compared to APRIL$^{+/+}$ or heterozygous littermates (Fig. 2.17A). The induction of arthritis was dependent on CII, since no arthritis occurred in the group injected only with adjuvant (Fig. 2.17A). Analysis of the percentage of mice becoming newly arthritic at different periods shows a sharp decline of disease onset in APRIL$^{-/-}$ mice during the 22-33 day period after initial challenge (Fig. 2.17B). However, the clinical scores (Fig. 2.17C) of APRIL$^{-/-}$ mice that did become arthritic were the same as that of arthritic APRIL$^{+/+}$ littermates, suggesting that disease severity is identical, once the threshold of disease induction has been passed. Likewise, disease pathology, as determined by decalcification and histopathology of affected and unaffected joints, was identical in APRIL sufficient mice (Fig. 2.17D-G) and deficient mice (data not shown).
Figure 2.17. APRIL<sup>−/−</sup> mice exhibit decreased incidence of CIA.

A. Incidence: APRIL<sup>−/−</sup>: 56.3% (n=16), APRIL<sup>+/−</sup>: 90.0% (n=20), APRIL<sup>+/+</sup>: 92.3% (n=13), and adjuvant controls: 0% (n=9). p=0.044 (−/− vs +/+) and p=0.049 (+/− vs −/−) and p=1.000 (+/− vs +/+), Fisher’s exact test;

B. Percentage of newly arthritic mice during different onset days.

C. Clinical scores (disease severity) of affected mice. APRIL<sup>−/−</sup>: n=10, APRIL<sup>+/−</sup>: n=18, and APRIL<sup>+/+</sup>: n=12 (C); Data from 4 independent experiments were combined (A-C).

D, E. Normal and CIA paws of APRIL<sup>+/+</sup> mice.

D. Normal paw of an adjuvant control mouse. E. CIA paw with erythema and swelling of a CIA injected mouse.

F, G. Histological assessment of paw joints of APRIL<sup>+/+</sup> mice by H&E staining (original 20x). F shows an adjuvant control joint with even and clear joint space and smooth articular cartilage. G shows a CIA joint with infiltration of inflammatory cells, cartilage destruction and bone erosion.
2.6.2. APRIL−/− mice show diminished IL-17 production in *in vivo* CII primed T cells

Arthritis in the CIA model is known to be associated with increased IL-17 production. Accordingly, inguinal LN cells isolated from APRIL−/− mice one week after a single chick CII immunization, prior to disease onset, showed significantly diminished IL-17 production upon anti-CD3 restimulation *in vitro* in comparison to immunized APRIL+/+ mice (Fig. 2.18A). The IL-17 production by T cells was induced by CII immunization, since splenocytes from mice immunized with OVA and CFA/IFA or from naïve mice, stimulated with anti-CD3, produced low levels of IL-17 which showed no difference between APRIL−/− and APRIL+/+ mice (Fig. 2.18B and data not shown). While T cell proliferation of CII primed inguinal LN cells was enhanced in APRIL−/− mice (data not shown), INF-γ production was very low and showed no significant difference between APRIL−/− and APRIL+/+ mice, and IL-4 was undetectable (Fig. 2.18C). Our data show that CII/CFA induced a Th17 dominated immune response. In contrast, OVA/CFA/IFA induced a Th1 dominated immune response, according to the relatively high levels of INF-γ, relatively low levels of IL-17, and undetectable IL-4 production (Fig. 2.18B, D). TGF-β, IL-6, TNF-α and IL-1β are important for Th17 induction and IL-23 for maintenance. We also analyzed the *in vitro* production of these cytokines in CII primed inguinal LN cells. IL-6 and TNF-α showed a trend to diminution in APRIL−/− mice (Fig. 2.18E-F) while TGF-β, IL-23 and IL-1β were not detectable by ELISA (data not shown). The values for IL-17, IL-6 and TNF-α showed similar pattern (Fig. 2.18A, E-F) and were significantly correlated to each other as indicated by linear regression analysis (Fig. 2.18G-I). The values of APRIL−/− mice tended to be clustered towards the lower end of
the scale while the values for APRIL\(^{+/+}\) mice clustered towards the higher end, supporting the finding of attenuated production of inflammatory cytokines in APRIL\(^{-/-}\) mice.

2.6.3. APRIL\(^{-/-}\) mice have reduced CII-specific IgG2a autoantibody levels

Cross reacting IgG2 autoantibody against mouse CII, induced by chicken CII immunization, has been shown to be essential in CIA induction in addition to the disease association of IL-17. Comparing anti-mouse collagen IgG2a, IgG2b and IgG1 autoantibody levels in healthy and arthritic mice, we found that increased IgG2a (Fig. 2.19A) and to a lesser extent IgG2b (Fig. 2.19B) levels were correlated with clinical disease while anti-mouse CII IgG1 levels were not (Fig. 2.19C). APRIL\(^{-/-}\) mice had significantly lower anti-mouse CII autoantibody levels of the IgG2a isotype (Fig. 2.19A) than APRIL\(^{+/+}\) mice, correlating with and explaining decreased disease incidence. As in APRIL\(^{+/+}\) mice, arthritic APRIL\(^{-/-}\) mice tended to have higher IgG2a levels than healthy APRIL\(^{-/-}\) mice (Fig. 2.19A, left columns). Since the antibody levels were very low, it is difficult to detect significant difference. The IgG2a levels of arthritic APRIL\(^{-/-}\) mice was about twice of the level of non-arthritic, but challenged APRIL\(^{+/+}\) mice, suggesting that the threshold for CIA induction by IgG2a autoantibody is relatively low.
Figure 2.18. Diminished IL-17 production is associated with reduced arthritis in APRIL−/− mice.

A, C, E, F. Inguinal LN cells, isolated one week after a chick CII/CFA immunization, were cultured at 2x10^6/ml in the presence or absence of denatured chick CII (100μg/ml) or anti-CD3 (2.5μg/ml) for 4 days. Cytokine production in the supernatant was determined by ELISA. *, p=0.0291; n=7; unpaired t test. Data from 4 independent experiments (n=1-2 each) were combined.

B. Splenocytes, isolated on day 17 after OVA/CFA immunization on day 0 and OVA/IFA immunization on day 14, were cultured at 5x10^6/ml in the absence or presence of OVA (100μg/ml) or anti-CD3 (2.5μg/ml) for 3 days. Cytokine production in the supernatant was determined by ELISA. n=6, from the mice in Fig. 2.5 A and B with serum IgG1 and IgG2a tested.

G – I. Correlation between each two of the cytokine production of IL-17, IL-6 and TNF-α. n=14, linear regression: r^2=0.7155, p=0.0001 (G), r^2=0.5585, p=0.0021 (H), r^2=0.8425, p<0.0001 (I).
Reduced IgG2a autoantibody production is associated with decreased arthritis in APRIL$^{-/-}$ mice.

Serum antibody titers to mouse CII on day 34 were determined by ELISA. The serum titer shown was within the linear range of antibody dilution; $n=5-6$ ($n=3$ for non-arthritis APRIL$^{+/+}$ mice). *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA followed by Bonferroni post-tests.
Chapter 3 DISCUSSION

3.1. APRIL represses Th1 responses only under non-polarizing conditions

We showed that APRIL suppressed Th1 cytokine production by cultured CD4\(^+\) T cells under non-polarizing, but not under Th1 polarizing, conditions. Furthermore, APRIL had no effect on Th1 antibody responses under \textit{in vivo} Th1 polarizing conditions induced by Freund’s adjuvant. Our data suggest that cytokines, added to achieve Th1 polarization \textit{in vitro}, and the Freund’s adjuvant, administrated to induce Th1 polarization \textit{in vivo}, can override the absence of APRIL.

3.2. APRIL inhibits Th2 responses and allergic lung inflammation

We found that cultured APRIL\(^{-/-}\) CD4\(^+\) T cells exhibited enhanced Th2 cytokine production under non-polarizing conditions, and augmented IL-13 production under Th2 polarizing conditions. Upon immunization with OVA and alum which induces Th2 polarization, APRIL\(^{-/-}\) mice responded with an increased antigen-specific Th2 antibody (IgG1) response. Consistently, APRIL\(^{-/-}\) mice had significantly augmented lung inflammation compared with WT mice in the OVA-induced allergic lung inflammation model, which is mediated by Th2 polarized CD4 cells. To confirm the role of APRIL in allergic lung inflammation, WT mice were treated with TACI-Ig to block APRIL, and enhanced allergic lung inflammation was observed. Furthermore, corresponding to our \textit{in vitro} study suggesting that the suppressive signal of APRIL is intrinsic to CD4\(^+\) T cells, the transfer of APRIL sufficient, OVA-specific, TCR transgenic CD4\(^+\) T (OT-II) cells to APRIL\(^{-/-}\) mice restored the suppressive effect of APRIL on allergic lung inflammation,
which suggests that APRIL expressed by antigen-specific CD4+ T cells is responsible for suppression of allergic lung inflammation. Taken together, our data strongly support the finding that APRIL inhibits Th2 responses and allergic lung inflammation by suppressing Th2 cytokine production by CD4+ T cells.

To investigate the mechanism by which APRIL signaling down-regulates Th2 responses and allergic lung inflammation, we analyzed the expression of the two most important Th2 cytokine transcription factors, GATA-3 and c-maf, in cultured CD4+ T cells from APRIL−/− and WT mice. We found that c-maf, but not GATA-3, expression was markedly enhanced in APRIL−/− CD4+ T cells at both the RNA and protein level and under both ThN and Th2 conditions, in comparison to those from WT mice. It is known that c-maf directly and specifically transactivates the IL-4 gene and promotes Th2 cell differentiation mainly in an IL-4-dependent mechanism (Ho et al. 1996; Ho et al. 1998; Kim et al. 1999; Hwang et al. 2002). Overexpression of c-maf in transgenic mice (Ho et al. 1998) increased the production of Th2 cytokines (IL-4, IL-5 and IL-10) and the IL-4-dependent immunoglobulins (IgG1 and IgE). A clear gene dosage effect of the c-maf transgene was apparent, because splenocytes derived from the c-maf transgenic line expressing a moderately high level of transgenic c-maf, differentiated into classical Th2 cells when stimulated in vitro with plate-bound anti-CD3 and produced high levels of Th2 cytokines and significantly lower levels of IFN-γ than those derived from WT C57BL/6 mice. In contrast, splenocytes derived from the transgenic line expressing a lower level of c-maf matured into Th1-like cells secreting high levels of IFN-γ and very low levels of IL-4 and IL-5. The Th2 biased responses of c-maf transgenic mice were IL-4 dependent, and were not observed in c-maf transgenic mice bred onto an IL-4-deficient
background. In contrast, T cells from c-maf−/− mice (Kim et al. 1999) were markedly deficient in IL-4 production. The expression of other Th2 cytokines, IL-5, IL-6, IL-10, was also reduced in c-maf−/− T cells under unskewed conditions, but was almost fully restored under Th2 skewed conditions in the presence of exogenous IL-4. C-maf−/− mice had a modestly impaired production of both IgG1 and IgG2a antibodies. However, IL-13 production was normal in c-maf−/− T cells under unskewed and Th2 skewed conditions.

Based on these previous findings, the increased c-maf expression in cultured APRIL−/− CD4+ T cells readily explains our observation (Fig. 2.4) that, under ThN conditions, IL-4 expression was up-regulated and followed by an increase of the expression of other Th2 cytokines (IL-5, IL-10 and IL-13) upon secondary stimulation of APRIL−/− CD4+ T cells. Under Th2 conditions, although c-maf levels were also upregulated, the addition of IL-4 abolished the difference in Th2 cytokine production by CD4+ T cells between APRIL−/− and WT mice upon secondary stimulation. The exception was IL-13 production which remained increased in APRIL−/− CD4+ T cells even under Th2 conditions. Increased IL-13 expression under Th2 conditions suggests that APRIL is required for suppressing IL-13 production in an IL-4 independent, IL-13 specific pathway. In addition, the increased c-maf production also explains our observation that IL-17 production was enhanced in APRIL−/− CD4+ T cells under Th2 conditions, upon secondary stimulation (Fig. 2.4D), since it was found recently that c-maf promotes IL-17 production (Bauquet et al. 2009).

Furthermore, c-maf participates in the development of allergic lung inflammation, as evidenced by the up-regulated c-maf expression in human asthmatic airways after allergen challenge and the spontaneously increased percentage of eosinophils in the BALF of c-maf transgenic mice (Hausding et al. 2004). These findings directly support
our conclusion that the increased c-maf expression in cultured APRIL^{−/−} CD4^{+} T cells results in the augmented allergic lung inflammation in APRIL^{−/−} mice. Collectively, our data suggest that APRIL inhibits Th2 responses and allergic lung inflammation by suppressing IL-4 production in CD4^{+} T cells via diminished c-maf expression, and by suppressing IL-13 production in CD4^{+} T cells via an IL-4 independent, IL-13 specific pathway.

An IL-4 independent, IL-13 specific pathway could also be postulated by the previous observation that IL-13 levels, unlike other Th2 cytokines, were not reduced in c-maf^{−/−} T cells in the absence of exogenous IL-4 (Kim et al. 1999). So far, there are no reports about an IL-4 independent, IL-13 specific pathway for IL-13 production. The known IL-4 independent signaling pathways for Th2 cytokine production, such as Notch signaling and IL-2 receptor signaling, are not IL-13 specific (Ho et al. 2009). A part of the IL-13 specific mechanism for IL-13 production was suggested by a study showing that protein inhibitor of activated STAT1 (Pias1) mostly affected IL-13 expression by facilitating the recruitment of GATA-3 to the IL-13 promoter (Zhao et al. 2007). According to this finding, APRIL may affect the binding of GATA-3 to the IL-13 promoter via an IL-4 independent pathway. In addition, it was shown that IL-13 is not always coexpressed with other Th2 cytokines in normal Th cells on a single cell basis (Kishikawa et al. 2001). Taken together, these data raise the intriguing possibility that subsets of IL-13-producing Th2 cells exist whose formation is IL-4 independent (Kim et al. 1999).

Our study showed that the increased c-maf expression in cultured APRIL^{−/−} CD4^{+} T cells could not be mimicked by adding TACI-Ig to block APRIL in cultured WT CD4^{+} T cells, or rescued by adding APRIL to cultured APRIL^{−/−} CD4^{+} T cells. In contrast, the
enhanced severity of allergic lung inflammation observed in APRIL−/− mice was mimicked in APRIL+/+ mice by TACI-Ig treatment to block APRIL in vivo. These data suggest that the suppressive signal delivered by APRIL already exists in CD4+ T cells before cell isolation and can only be inducedblocked in vivo but not in vitro.

Both APRIL and BAFF have suppressive effects on allergic lung inflammation, suggesting that APRIL and BAFF mediated effects on T cells. In a previous study, μMT−/− X BAFF transgenic mice and BAFF transgenic mice showed similar suppression of allergic airway inflammation, demonstrating that the suppressive function of BAFF is B cell independent (Sutherland et al. 2005). Our study showed that APRIL-mediated suppression of allergic lung inflammation is CD4+ T cell autonomous and does not require APRIL production by other cells. Accordingly, TACI-Ig treatment blocking APRIL and BAFF enhanced allergic lung inflammation in our study. However, both APRIL and BAFF also have positive effects on the function of B cells, such as promoting Ig class switching, B cell activation and survival (Dillon et al. 2006). And it is well known that BAFF is essential for the generation and maintenance of mature B cells. Therefore, the outcome of TACI-Ig treatment may be determined by the balance of blocking T and B cell functions. In contrast to our results, two previous studies aiming at blocking the B cell functions of BAFF and/or APRIL showed that TACI-Ig treatment alleviates allergic lung inflammation (Moon et al. 2007; Bilsborough et al. 2008). One possible factor accounting for the opposite results is the mouse strain. BALB/c mice were used in the two studies mentioned above (Moon and Ryu 2007; Bilsborough et al. 2008), while mice on a C57BL/6 background were used in our study and the other previous study demonstrating the suppressive function of BAFF in allergic lung inflammation.
(Sutherland et al. 2005). However, more likely, the opposite findings may be due to the regimen of TACI-Ig treatment. In the first study, mice were sensitized on day 0 and boosted on day 14 by i.p. injection of 20µg OVA emulsified in 2mg alum in 100µl PBS. On days 28, 29, and 30, mice were aerosol challenged with 1% OVA in PBS for 30 min, and i.p. injected for three consecutive days with various doses of 100, 200, 400mg/kg TACI-Ig protein, 30min prior to OVA challenge. On day 31, mice were sacrificed for analysis. The study found that TACI-Ig treatment alleviated asthmatic symptoms, associated with a reduced BAFF protein level in alveolar-associated cells, decrease of non-lymphoid cell populations and the increase of hypodiploid cell formation in the BALF (Moon and Ryu 2007). In the second study, epicutaneous sensitization, instead of classical i.p. sensitization, to OVA was used, which skew towards higher IgE production and lower eosinophilia compared with classical i.p. sensitization, thus emphasizing the role of IgE in disease. Mice were sensitized on days 14-21 and boosted on days 35-42 by application of a patch of sterile gauze treated with 500µg OVA in 100µl PBS to the skin. On days 48 and 49, mice were challenged intranasally with 10µg OVA in 50µl PBS on 2 consecutive days. TACI-Ig was delivered at a dose of 4mg/kg by i.p. injection, three times per week, during days 0-48 (the prophylactic treatment regimen) and days 21-48 (the therapeutic treatment regimen). On day 51, mice were used for analysis. This study observed that TACI-Ig treatment reduced circulating mature B cell levels in the blood, and decreased allergic lung inflammation and airway hyperreactivity (AHR) (Bilsborough et al. 2008). This is the effect of BAFF blocking because mature B cells are almost completely lost in BAFF-deficient mice (Stein et al. 2002), but are normal in APRIL-deficient mice (Varfolomeev et al. 2004). In our study, mice were sensitized on
day 0 and boosted on day 5 by i.p. injection of 66μg OVA with 6.6mg alum in 200μl PBS. On day 12, mice were aerosol challenged with 0.5% OVA in PBS for 1 h. On each day from day 11 to 14, mice were given TACI-Ig or control mouse IgG at 100μg/mouse (4-5mg/kg) by i.p. injection. On day 15, mice were sacrificed for analysis. Compared with our study, the first study (Moon and Ryu 2007) used 25-100 fold of TACI-Ig and the second study (Bilsborough et al. 2008) used 3-5 times more days for TACI-Ig treatment, which may interfere with BAFF functions on B cells.

APRIL−/− mice have reduced levels of serum total IgA (Castigli et al. 2004; Xiao et al. 2008). And it was reported that mice with a targeted disruption of the α-switch region and 5’ H chain gene (IgA−/− mice), which lack total IgA, developed significantly reduced allergic lung inflammation compared with IgA+/+ controls, following allergen sensitization and challenge. This defect was attributable to fewer B cells in the lungs of IgA−/− mice (Arnaboldi et al. 2005). Our data indicate that the reduced levels of serum total IgA in APRIL−/− mice is not impaired enough to alleviate the development of allergic lung inflammation.

The receptor that mediates the inhibitory function of APRIL in Th2 responses and allergic lung inflammation remains to be investigated. TACI-Ig treatment alone could not determine whether TACI mediates the suppressive function of APRIL, due to the complicated APRIL/BAFF signaling system which involves two ligands (APRIL and BAFF), in secreted and membrane-bound forms, three receptors (TACI, BCMA and BAFF-R) and one binding partner (HSPG). It is appreciated that, according to our study and previous reports (Ye et al. 2004; Sutherland et al. 2005; Lai Kwan Lam et al. 2008; Xiao et al. 2008), both APRIL and BAFF promote IL-17 production and collagen-
induced arthritis, and repress Th2 cytokine production and allergic lung inflammation, which may lead to the conclusion that they also share receptors in T cell functions. However, whether BAFF shares receptors with APRIL in T cell functions is contentious. In one study, T cells from BAFF-R mutant mice fail to respond to BAFF, suggesting that the effect of BAFF on T cells occurs through BAFF-R (Ng et al. 2004). The possibility that BAFF shares receptors with APRIL in T cell functions makes the receptor study for APRIL more difficult. So far, APRIL is known to bind TACI, BCMA, BAFF-R and HSPG. BCMA appears to be only expressed on B cells. The interaction between a shorter variant of APRIL and BAFF-R is weak binding (Bossen and Schneider 2006). HSPG is expressed on B and T cells, but may only facilitate APRIL’s binding to receptors (Hendriks et al. 2005; Ingold et al. 2005; Kimberley et al. 2009). TACI is expressed on B cells. Though controversial, increasing evidence indicates that TACI is expressed also on T cells (Ye et al. 2004; Mackay and Leung 2006; Hardenberg et al. 2008). Furthermore, studies on TACI-/- mice suggest an inhibitory role for TACI in T cell function, based on the evidence that Peyer’s Patches of TACI-/- mice showed a slight but discernable increase in CD4+ cells (Yan et al. 2001), and that TACI-/- T cells were observed to hyperproliferate when stimulated with anti-CD3 antibody and BAFF (Seshasayee et al. 2003). Taken together, TACI could be the candidate receptor for the inhibitory function of APRIL in Th2 responses. To test this hypothesis, studies on Th2 responses and allergic lung inflammation in TACI-/- mice or using TACI blocking antibodies could be helpful.

The mechanisms utilized by APRIL to inhibit Th2 responses and allergic lung inflammation are summarized in Fig. 3.1. In CD4+ T cells, c-maf transactivates the IL-4 gene and induces IL-4 production. IL-4 goes to the positive feedback loop to induce
STAT6 and GATA-3 expression, thus promoting other Th2 cytokine production. APRIL, secreted by CD4+ T cells or other cells (though not required), possibly binds to TACI on CD4+ T cells. With the possible help from in vivo signals, APRIL diminishes c-maf expression, thus suppresses IL-4 expression first, followed by the repression of other Th2 cytokines. As for IL-13, APRIL suppresses its expression via an IL-4 independent, IL-13 specific pathway. Therefore, APRIL deficiency causes the increased Th2 cytokine production, hence the augmented Th2 responses and allergic lung inflammation in APRIL-/- mice.

Figure 3.1. The mechanisms utilized by APRIL to inhibit Th2 responses and allergic lung inflammation.
In conclusion, APRIL was identified as a novel negative regulator in Th2 responses and allergic lung inflammation. APRIL or its agonist may be valuable in the treatment of asthma. Moreover, when APRIL blocking therapy is utilized for B cell malignancy or autoimmunity, enhanced Th2 responses and allergic lung inflammation should be considered as adverse effects.

3.3. APRIL promotes Th17 responses and collagen-induced arthritis (CIA) (Xiao et al. 2008)

Our data show that APRIL deficiency is associated with a significantly decreased incidence of arthritis and diminished IgG2a autoantibody levels to murine CII. Since the pathogenesis of arthritis in the murine CIA model is critically dependent on IgG2 autoantibodies to CII, and within a given strain, the levels of antibody correlate well with the presence or absence of arthritis, our data readily explain the diminished incidence of arthritis in APRIL−/− mice as being due, at least in part, to diminished IgG2a autoantibodies. APRIL−/− inguinal LN cells from CII immunized mice, in addition, secreted less IL-17 than LN cells from APRIL sufficient mice upon restimulation with anti-CD3 in vitro, indicating that Th17 polarization is diminished or that IL-17 production is dependent on APRIL costimulation. Since Th17 T cells are known to be very important in the pathogenesis of arthritis, diminished IL-17 production in APRIL−/− mice is also likely to contribute to diminished incidence of arthritis. The decreased IL-17 production and reduced CII-specific IgG2a antibody level associated with decreased CIA incidence in APRIL−/− mice, fit well with a previous study showing that in IL-17-deficient mice, CIA was suppressed in parallel with reduced anti-CII IgG2a levels while other Ig isotypes were unaffected (Nakae et al. 2003).
It seems puzzling that after CII or OVA immunization in Freund’s adjuvant only IgG2a specific for CII, but not for OVA, was decreased in APRIL−/− compared to APRIL+/+ mice. These two antigens induce very different immune responses. CII/CFA induces a Th17 dominated immune response while OVA/CFA/IFA induces a Th1 dominated immune response. In contrast to OVA, chick CII has cross reacting epitopes with mouse CII. CII-specific autoantibodies thus bind to CII of cartilage in joints and activate the complement system resulting in recruitment of neutrophils and macrophages and in the release of chemokines and proinflammatory cytokines that cause joint inflammation. Under certain conditions, such as low levels of IFN-γ and IL-4 and high levels of TGF-β and IL-6, Th17 cells are induced and osteoclastogenesis occurs, which causes bone erosion (Cho et al. 2007). This pathway appears to be costimulated by APRIL. Th17 cells may in turn provide help to B cell to produce more CII-specific autoantibodies (McInnes and Schett 2007), which is also supported by the previous finding that IL-17 deficiency suppressed anti-CII IgG2a levels and CIA (Nakae et al. 2003). Therefore, APRIL deficiency may firstly lead to a defect in IL-17 production, followed by a decreased CII-specific IgG2a autoantibody response.

APRIL deficiency does not affect Th1 responses driven by Freund’s adjuvant. Therefore, it is likely that the effect of APRIL in CIA is primarily mediated by autoantibody binding to CII in the joint, resulting in secondary responses that require APRIL support to maintain IgG2a levels, but in APRIL deficiency lead to lower IgG2a levels and diminished disease.

Further investigation is required to determine at which point APRIL intervenes as costimulus for IL-17 production. The following possibilities could be considered. First,
IL-17 production is suppressed by both IFN-γ and IL-4 (Chu et al. 2007), thus it is possible that increased IL-4 production observed in APRIL−/− mice under non-polarizing, homeostatic condition contributes to diminished IL-17 production after collagen challenge. Second, APRIL may regulate Th17 polarization, maintenance or Th17 related cytokine production. Analysis of the in vitro production of TGF-β, IL-6, IL-23, IL-1β and TNF-α of in vivo CII-primed inguinal LN cells showed a trend to diminution of IL-6 and TNF-α in APRIL−/− mice, whereas TGF-β, IL-23 and IL-1β were not detectable by ELISA. A significant correlation was found in production of IL-17, IL-6 and TNF-α, tending to be high in APRIL+/+ and low in APRIL−/− mice. IL-6 is one of the Th17 polarizing cytokines, and TNF-α is a crucial cytokine during CIA onset. Th17 cells can be induced in vitro in the presence of inflammatory cytokines, such as IL-1β, IL-6, and TNF-α, together with TGF-β. IL-17 in turn can stimulate the production of inflammatory mediators including TNF-α, IL-6. IL-17 in vitro and in vivo has additive or even synergistic activity with IL-1 and TNF-α in inducing proinflammatory cytokine expression and joint damage (Cho et al. 2007). A study using tumor necrosis factor receptor I (TNFRI) and IL-6 double knockout mice showed that blocking of TNFRI and IL-6 synergistically attenuated CIA (Yamaguchi et al. 2003).

The effect of APRIL on IL-17 production was different in vivo from its effect in in vitro assays. In CIA in vivo it is likely that CII-specific autoantibodies bind to CII of the cartilage in joints and cause the secretion of inflammatory cytokines, such as TGF-β and IL-6, which in turn induce IL-17 production. Under in vitro Th17 polarizing conditions induced with TGF-β and IL-6, no significant IL-17 decrease was observed in APRIL−/−
mice, suggesting that the function of APRIL may have been overridden by added TGF-β and/or IL-6 in the \textit{in vitro} polarization assay.

Our data demonstrate that APRIL has significant effects on the pathogenesis of arthritis by modulating autoantibody and cytokine production. Previous studies (Wang et al. 2001; Mackay et al. 2005) using TACI-Ig, BCMA-Ig and BAFFR-Ig as ligand blockers have not been able to distinguish between the effects of BAFF and APRIL on CIA. The new data allow us to conclude unambiguously that APRIL is a significant factor in the pathogenesis of RA in the murine model, and provide an explanation for the increased APRIL levels observed in human disease.

3.4. Concluding remarks

Our findings reveal novel important functions of APRIL in T cell immunity, i.e. inhibiting Th2 responses and promoting Th17 responses. The underlying mechanistic details of these findings require further studies. The elucidation of the role of APRIL in the cytokine networks that control Th1, Th2 and Th17 responses will be of great interest in explaining the pathogenesis of autoimmune or allergic diseases and of potential clinical importance for treatment.

The future studies are proposed as follows,

(1) To investigate the receptor involved in the inhibitory function of APRIL in Th2 responses. As discussed above, TACI could be the candidate receptor. Studies using TACI\textsuperscript{−/−} mice or TACI blocking antibodies could be helpful.
(2) To investigate the mechanisms for APRIL to costimulate IL-17 production. The Th17 related cytokines, the transcription factors and the receptor implicated could be investigated.

(3) To study how APRIL exerts inhibitory function in Th2 responses but costimulatory function in Th17 responses. Different receptor binding or the antagonism between cytokines could be the mechanism.

(4) To investigate why the suppressive signal delivered by APRIL can only be induced_blocked in vivo but not in vitro. One hypothesis is that signals from other cells are required for APRIL to suppress c-maf production. To test this, isolated total LN cells or LNs cut in half, instead of purified CD4^+ T cells, could be used for the blocking or restoring assays. Furthermore, isolated total LN cells or LNs from specific cell deficient mice could be used to identify the specific cell type required for APRIL to exert its function.

(5) To study the effect of APRIL on CD4^+ T cell expansion in allergic lung inflammation. The transfer of APRIL^−/− CD45.1 OT-II cells isolated from APRIL^−/− CD45.1 OT-II mice could be used for the study.
Chapter 4 MATERIALS AND METHODS

4.1. Generation of APRIL−/− mice (Xiao et al. 2008)

APRIL−/− mice were generated by Dr. Seiichi Motomura. An 8.4kb genomic DNA fragment including the mouse APRIL gene, exon 7 of the upstream gene for TNF-related weak inducer of apoptosis (Tweak, or TNFSF12), and the exons 1-4 of the downstream gene for sentrin specific peptidase 3 (Senp3), was used to insert a neomycin cassette (Fig. 2.1A). The targeting construct replaced part of APRIL’s exon 1, exons 2-5 and part of exon 6 with the neomycin cassette (Fig. 2.1A). Blastocyst injection and generation of founder mice were done in the transgene core facility of University of Miami. DNA samples derived from several transfected ES cell clones (digested by Hind III) and from tails (digested by BamH I) were analyzed by Southern blotting for APRIL DNA deficiency. PCR analysis of tail-derived genomic DNA was used for routine screening.

4.2. Mice

APRIL−/− mice for experiments were backcrossed onto C57BL/6 background for 7-14 generations. Control APRIL+/+ C57BL/6 mice were either APRIL+/+ littermates born in our facility or APRIL+/+ mice purchased from Charles River Laboratories. GFP+ OT-II mice were obtained by mating GFP mice with OT-II mice (OVA-specific TCR transgenic). OT-II mice were kindly provided by Dr. Rosenblatt’s laboratory at University of Miami.
4.3. **Real-time RT-PCR for APRIL expression** (Xiao et al. 2008)

Total RNA was extracted from splenocytes of naïve mice by using RNeasy Mini Kit (QIAGEN) plus an on-column DNase digestion with RNase-Free DNase (QIAGEN). First Strand cDNA Synthesis was performed by using SuperArray ReactionReady First Strand cDNA Synthesis Kit C-01 (SuperArray Bioscience Corporation). APRIL, Tweak and Senp 3 primer sets (SuperArray Bioscience Corporation) were used for SYBR Green real-time PCR. Standardization was performed with β-actin as the endogenous control and the APRIL+/+ sample as the calibrator. Real-time PCR was analyzed with 7300 Real-Time PCR System (Applied Biosystems).

4.4. **Culture medium**

The culture medium used in this study was Iscove's Modified Dulbecco's Minimal Essential Medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 10μg/ml gentamycin (Invitrogen), and 50μM β-mercapto-ethanol (Bio-Rad).

4.5. **T cell proliferation assay** (Xiao et al. 2008)

Splenocytes were cultured at 1×10^6/ml (200μl/well) in 96-well round-bottom plates and stimulated with 0-5μg/ml of anti-CD3 mAb (2C11) for 3 days. [³H] thymidine (Perkin Elmer) was added at 0.5μCi/well during the last 6-hour incubation, and ³H incorporation was quantitated by using a scintillation counter.
4.6. *In vitro* cytokine production under ThN, Th1, Th2 and Th17 conditions (Xiao et al. 2008)

CD4\(^+\) cells were purified from the spleen plus LN\(s\) of APRIL\(^{-}\) and WT mice using mouse CD4 T cell isolation kit (Miltenyi Biotec Inc.). Purified CD4\(^+\) T cells at 1x10\(^6\)/ml (200\(\mu\)l/well) were activated in 96-well flat-bottom plates with 2\(\mu\)g/ml coated anti-CD3 (2C11) and 1\(\mu\)g/ml soluble anti-CD28 (37.51, eBioscience). Cells were polarized under the following conditions. ThN: 10ng/ml IL-2 (eBioscience). Th1: 10ng/ml IL-2, 10ng/ml IL-12 (eBioscience), and 20\(\mu\)g/ml anti-IL-4 (11B11). Th2: 10ng/ml IL-2, 30ng/ml IL-4 (eBioscience), 10\(\mu\)g/ml anti-INF-\(\gamma\) (XMG1.2), and 2\(\mu\)g/ml anti-IL-12 (C17.8, eBioscience). Th17: 10ng/ml IL-6 (BD Pharmingen), 5ng/ml human TGF-\(\beta\)1 (R&D), 10\(\mu\)g/ml anti-IL-4 (11B11), and 10\(\mu\)g/ml anti-INF-\(\gamma\) (XMG1.2). After 4 days, cells were washed and re-cultured for 2 additional days at 1x10\(^6\)/ml with 2\(\mu\)g/ml plate-bound anti-CD3. Supernatants were collected on day 4 (primary stimulation) and day 6 (secondary stimulation) for ELISA to detect cytokine production (see section 4.18).

4.7. OVA immunization with alum or Freund’s adjuvant as well as antibody and cytokine production assays (Xiao et al. 2008)

Ovalbumin (OVA, Grade V) and aluminum potassium sulfate (alum) were purchased from Sigma. For OVA/alum immunization, each mouse was intraperitoneally (i.p.) injected with 200\(\mu\)l of OVA (66\(\mu\)g) coprecipitated with alum (6.6mg) on day 0 and day 14. For OVA/CFA/IFA immunization, each mouse was i.p. injected with 100 \(\mu\)l of OVA (66\(\mu\)g) emulsified with 100\(\mu\)l of CFA on day 0 and OVA (66\(\mu\)g) emulsified with IFA on day 14. On day 17, mice were sacrificed for antibody and cytokine production assays. Sera were collected from the orbital sinus for ELISA to detect OVA specific IgG1 and
IgG2a (see section 4.18). In this study, we analyzed IgG2a instead of IgG2c in C57BL/6 mice, since the cross-reactivity with the anti-IgG2a antibodies reflects most of the IgG2c in sera (Pan et al. 2004). Splenocytes were isolated and cultured at 5x10^6/ml (200µl/well) in 96-well round-bottom plates in the presence or absence of OVA (100µg/ml) or anti-CD3 (2.5µg/ml) for 3 days. Cytokine production in the supernatant was determined by ELISA (see section 4.18).

4.8. Induction of allergic lung inflammation in the classical OVA model

The allergic lung inflammation animal protocol was approved by the University of Miami Institutional Animal Care and Use Committee (ACUC). APRIL^-/- and WT mice were sensitized on day 0 and boosted on day 5 by intraperitoneal (i.p.) injection of 66µg OVA coprecipitated with 6.6mg alum in 200µl PBS. On day 12, mice were aerosol challenged with 0.5% OVA in PBS for 1h using an Ultrasonic Nebulizer (MABIS Healthcare). On day 15, allergic lung inflammation was evaluated (Fig. 2.6A).

4.9. Evaluation of allergic lung inflammation

4.9.1. Assessment for eosinophilic infiltration in the bronchoalveolar lavage fluid (BALF)

After cannulation of the trachea, the lung was lavaged for 4 times with a total of 2ml PBS. Cells recovered from the BALF were counted and used for cytopsin preparations (≤ 50,000 cells/slide). The slides were stained with Wright-Giemsa stain (Sigma), and more than 200 cells per slide were counted to determine differential cell counts for macrophages, eosinophils, and lymphocytes. Images were photographed.
4.9.2. Lung histology

Lungs were removed from mice after the bronchial lavage procedure and fixed in 10% neutral buffered formalin. Samples were submitted to the Histopathology Core of the Sylvester Cancer Center at the University of Miami, Miller School of Medicine, where specimens were embedded, sectioned, and stained with H&E to examine lung inflammation and with Periodic acid-Schiff (PAS) to determine mucus production. Images were photographed.

4.9.3. Analysis of serum OVA-specific IgE production

Blood samples were collected from the orbital sinus. Serum OVA-specific IgE was determined with ELISA (see section 4.18).

4.9.4. *In vitro* restimulation of bronchial lymph node (LN) cells for cytokine production

Bronchial LN cells were isolated and cultured at 1x10^6 cells/ml (200µl/well) in 96-well round-bottom plates in the presence or absence of OVA (100µg/ml) for 4 days. Supernatants were collected for cytokine ELISA (see section 4.18).

4.10. Generation of TACI-Ig

TACI-Ig was constructed by fusing the extracellular domain of mouse TACI cDNA (Met 1 - Thr 129, 387bp) (Xia et al. 2000) to an Igκ chain signal peptide (92 bp) at the 5’ end and to the mouse IgG1-Fc sequence (680bp) at the 3’ end (Fig. 2.9A). The extracellular domain of mouse TACI cDNA was obtained from the full-length TACI cDNA clone (BC141867, Open Biosystems). The TACI-Ig construct in the pBMG-neo
expression vector was transfected into 3T3 cells, and cultured in AIM-V medium (Invitrogen) with 10ng/ml of recombinant human EGF (R&D). The supernatant was collected, precipitated with ammonium sulfate (Sigma), and dialyzed in PBS. Then TACI-Ig was purified by protein A affinity chromatography.

Sandwich ELISA was used to verify the binding of TACI-Ig to mouse APRIL or mouse IgG1. In brief, 96-well plates were coated with mouse APRIL (PeproTech) (2-fold serial dilution starting at 300ng/ml) or mouse IgG1 (BD Pharmingen) (1:250), followed by adding diluted supernatant samples of TACI-Ig transfected 3T3 cells and then biotin-conjugated anti-mouse IgG1 antibody (BD Pharmingen) (1:250).

To verify the size of TACI-Ig, Western Blots using HRP-anti mouse IgG1 antibody (Jackson ImmunoResearch Laboratory) (1:1000) were carried out to analyze the supernatants and cell lysates of TACI-Ig transfected 3T3 cells under reducing conditions.

4.11. Functional assay for TACI-Ig

The function of TACI-Ig was tested by its ability to block APRIL-induced IgA production in IgD+ B cells. Mouse IgD+ B cells were purified from splenocytes by using FITC anti-mouse IgD (eBioscience) and the FITC selection kit, EasySep (StemCell Technologies). Purified IgD+ B cells at 2×10^6 cells/ml were cultured with 4μg/ml of anti-mouse IgM (Jackson ImmunoResearch Laboratory), 25ng/ml of mouse IL-5 (BD Biosciences), and 1μg/ml of mouse APRIL (PeproTech) in 96-well flat-bottom plates in the presence or absence of 35μg/ml of TACI-Ig or control mouse IgG (Sigma). After 5 days, supernatants were collected for ELISA to determine IgA production (see section 4.18).
4.12. TACI-Ig treatment in the allergic lung inflammation model

Allergic lung inflammation was induced in WT mice as described in section 4.8. On each day from day 11 to 14, mice were given TACI-Ig or control mouse IgG at 100μg/mouse by i.p. injection. Allergic lung inflammation was evaluated on day 15 (Fig. 2.10A).

4.13. GFP+ OT-II cell transfer in the allergic lung inflammation model

GFP+ OT-II cells were obtained by purifying CD4+ cells from the splenocytes of GFP+ OT-II mice using the mouse CD4 selection kit, EasySep (StemCell Technologies). Allergic lung inflammation was induced in APRIL-/- and WT mice as described in section 4.8. Two days before the first OVA/alum injection, OT-II cells at 1x10⁶ cells/mouse were transferred to mice by tail intravenous (i.v.) injection. Allergic lung inflammation was evaluated on day 15 (Fig. 2.13A).

4.14. Examination of the OT-II cell expansion in peripheral blood, bronchial LNs, BALF and lungs

To examine the kinetics of OT-II cell expansion in peripheral blood, mice were bled on day 0, 5, and 12 via tail vein before OVA/alum injection or OVA aerosol challenge, and on day 15 via the orbital sinus before sacrifice. Blood samples were processed with ACK lysing buffer to remove red blood cells. The remaining blood cells and the cells from the BALF, bronchial LNs and lungs were stained with PE conjugated anti-mouse CD4 antibody (BD Biosciences). After lung perfusion with ice cold PBS, lung cells were isolated by gently passing lung tissue through a cell strainer (BD Falcon). Samples were analyzed using a FACS LSR instrument (Becton Dickinson) and CellQuest software.
4.15. Cell culture, real-time RT-PCR and Western blots to determine the expression of GATA-3 and c-maf

CD4⁺ cells were purified from the LNs of APRIL⁻/⁻ and WT mice using the mouse CD4 selection kit, EasySep (StemCell Technologies). Purified CD4⁺ cells were cultured under ThN and Th2 conditions, as described in section 4.6. Upon secondary stimulation, cells were harvested for RNA isolation and cell lysate preparation.

For some experiments, during primary and secondary stimulation, TACI-Ig at 30µg/ml was added to the APRIL⁺/⁺ CD4⁺ T cell culture to block APRIL, with mouse IgG (Sigma) as a control. Mouse APRIL (PeproTech) at 1µg/ml was added to the APRIL⁻/⁻ CD4⁺ T cell culture to restore APRIL function, with PBS as a control.

Quantitative Taqman real-time RT-PCR was used to detect GATA-3 and c-maf expression at the RNA level. Total RNA samples were isolated from unstimulated and stimulated CD4⁺ cells by using the QIAcube instrument (QIAGEN) and the RNeasy mini kit (QIAGEN) plus an on-column DNase digestion with RNase-Free DNase (QIAGEN). Reverse transcription was performed using the Quantitect reverse transcription kit (QIAGEN). Gene expression assays (Applied Biosystems) for mouse GATA-3 (Mm01337569_m1), mouse c-maf (Mm01546091_s1), and mouse β-actin endogenous control (Part# 4352933E) were used for real-time PCR carried out in a 7300 Real-Time PCR system (Applied Biosystems). Relative expression or fold change was calculated using the ΔCt method, normalized to β-actin.

Western blots were performed to detect GATA-3 and c-maf expression at the protein level. Whole cell lysate samples were prepared. Western blotting was carried out under
reducing conditions. The amount of cell lysates loaded were 25µg/lane for GATA-3 and 50µg/lane for c-maf. For GATA-3 expression analysis, purified mouse anti-mouse/human GATA-3 (BD Pharmingen) was used as primary antibody (1:1000) and donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology) as secondary antibody (1:1000). For c-maf expression analysis, rabbit polyclonal to mouse c-maf (Abcam) was used as primary antibody (5ug/ml) and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) as secondary antibody (1:500). Protein bands on the membranes were visualized using standard chemiluminescent techniques. Since the size of the GATA-3 and c-maf band is very close to that of the loading control β-actin band, the antibodies for GATA-3 and c-maf detection were removed from the membranes with Restore Plus Western Blot Stripping Buffer (Pierce). Then the loading control was blotted by using mouse anti-mouse beta-actin (Abcam) as primary antibody (1:2000) and donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology) as secondary antibody (1:2000). Western blot bands on the films were scanned and quantified by using Scion Image, alpha 4.0.3.2. Relative intensity was calculated relative to β-actin.

4.16. CIA induction (Xiao et al. 2008)

Mice at 7–9 weeks of age were used in a CIA protocol approved by the University of Miami Animal Care and Use Committee (ACUC). CIA induction was carried out as previously described (Campbell et al. 2000). Chick CII (Sigma) at 2 mg/ml in 50 µl of 10 mM acetic acid was emulsified in an equal volume of CFA, and injected intradermally (i.d.) into the base of the tail on day 0 and day 21. For controls, CII was substituted by 10 mM acetic acid.
4.17. Evaluation of CIA (Xiao et al. 2008)

4.17.1. Clinical assessment of arthritis

Mice were assessed for redness and swelling of paws 2-3 times per week. Mouse paws were scored as described in Table 4.1. The maximal arthritic score per paw was 4, and the maximal arthritic score per mouse was 16. Arthritis was considered positive when two consecutive positive evaluations were obtained (Campbell et al. 1997). Mouse paw images were photographed.

Table 4.1. Visual scoring system for evaluating arthritis severity (Yamanishi 2002; Nurieva 2003; Lee 2005)

<table>
<thead>
<tr>
<th>Severity score</th>
<th>Gross pathology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Erythema and mild swelling confined to the mid-foot (tarsals) or ankle joint</td>
</tr>
<tr>
<td>2</td>
<td>Erythema and mild swelling extending from the ankle to the mid-foot</td>
</tr>
<tr>
<td>3</td>
<td>Erythema and moderate swelling extending from the ankle to the metatarsal joints</td>
</tr>
<tr>
<td>4</td>
<td>Erythema and severe swelling encompass the ankle, foot, and digits/joint deformity or ankylosis</td>
</tr>
</tbody>
</table>

4.17.2. Histological examination of arthritis

At the end of the experiments, mouse paws were fixed in 10% buffered neutral formalin, and decalcified with 10% formic acid. Samples were submitted to the Histopathology Core of the Sylvester Cancer Center at the University of Miami, Miller School of Medicine, where specimens were embedded, sectioned, and stained with H&E. Slides were examined under the microscope. Histological images were photographed.
4.17.3. Serum autoantibody production assay

Blood samples were collected from the orbital sinus of mice on day 34. Serum mouse CII-specific antibody levels were determined with ELISA (see section 4.18).

4.17.4. Cytokine production assay

Inguinal LN cells, isolated one week after a single chick CII/CFA immunization were cultured at $2 \times 10^6$ /ml (200µl/well) in 96-well round-bottom plates in the presence or absence of denatured chick CII (100µg/ml) or anti-CD3 (2C11, 2.5µg/ml) for 4 days. Cytokine production in the supernatant was determined by ELISA (see section 4.18).

4.18. ELISA for cytokine and antibody production (Xiao et al. 2008)

ELISA antibody pairs and standards for IgA, IgG1, IgG2a, IgG2b, IgG3, IgM and IgE, as well as IFN-γ, IL-4, IL-17, TGF-β and IL-6 were purchased from BD Pharmingen, and IL-5, IL-13, IL-10 and IL-23 from eBioscience. Serum total Ig levels, serum CII or OVA specific antibody isotype levels and cytokine production in culture supernatants were determined by sandwich ELISA, according to the manufacturer’s instructions. In the detection of serum CII or OVA specific antibody isotypes, 96-well plates were coated with 2µg/ml of mouse CII or 100µg/ml of OVA in PBS, and biotin-conjugated anti-mouse Ig isotype antibodies were used for detection. TNF-α and IL-1β ELISA was performed by using mouse TNF-α ELISA Ready-SET-Go (eBioscience) and Mouse IL-1β/IL-1F2 Immunoassay (R&D), respectively.
4.19. Statistical analysis

Unpaired t test, paired t test, and two-way ANOVA followed by Bonferroni post-tests were performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). P < 0.05 was considered as significant. Data are mean ± SEM.
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