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TNF superfamily member 13, APRIL, inhibits allergic lung inflammation

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Summary

The T cell functions of APRIL (TNFSF13) remain largely undefined. We previously showed that APRIL suppressed Th2 cytokine production in cultured CD4⁺ T cells and Th2 antibody responses. Here we show that APRIL suppresses allergic lung inflammation, which is associated with diminished expression of the transcription factor c-maf. Mice deficient in the *April* gene (*April*^{−/−} mice) had significantly aggravated lung inflammation compared to WT mice in the ovalbumin-induced allergic lung inflammation model. Likewise, blockade of APRIL in WT mice by the APRIL-receptor fusion protein, TACI-Ig, enhanced lung inflammation. Transfer of APRIL-sufficient, ovalbumin-specific, TCR-transgenic CD4⁺ T (OT-II) cells to *April*^{−/−} mice restored the suppressive effect of APRIL on lung inflammation. Mechanistically, the expression of the Th2 cytokine transcription factor c-maf, but not GATA-3, was markedly enhanced in *April*^{−/−} CD4⁺ T cells at the RNA and protein level and under non-polarizing (ThN) and Th2-polarizing conditions. Since c-maf transactivates the IL-4 gene, the increased c-maf expression in *April*^{−/−} mice readily explains increased Th2 cytokine production. Independent of its effect on IL-4, APRIL suppressed IL-13 expression. APRIL thus may regulate lung inflammation in a dual way, by acting on c-maf expression and by directly controlling IL-13 production.

Keywords

asthma; Th2 transcription; IL-13; IL-4; immune response

Introduction

A proliferation inducing ligand, APRIL or TNFSF13, is a TNF superfamily member. APRIL and B-cell activation factor, BAFF or TNFSF13B, share two receptors, the transmembrane activator and calcium modulator and cyclophilin ligand interactor, TACI or TNFRSF13B, and B cell maturation antigen, BCMA or TNFRSF17 [1]. APRIL in addition binds heparan sulfate proteoglycans, HSPGs [2, 3], while BAFF specifically binds to the BAFF receptor, BAFF-R or TNFRSF13C [1]. Both APRIL and BAFF are expressed in monocytes, macrophages, dendritic cells, neutrophils, T cells, B cells, osteoclasts, tumor cells, as well as airway and intestinal epithelial cells [1, 4–7]. TACI, BCMA, BAFF-R, and HSPGs are expressed on B cells, while BAFF-R and HSPGs are also found on T cells [1–3]. Though controversial, increasing evidence indicates that TACI is expressed also on T cells [6, 8, 9].

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Little is known about the T cell functions of APRIL. Existing data are controversial about the effects of APRIL on T cell activation, proliferation, survival and expansion [1, 3, 4, 6, 9–11]. We previously showed that APRIL increased the incidence of collagen-induced arthritis (CIA) and the production of IL-17 and IgG2a autoantibody, and that APRIL suppressed Th2 cytokine production by CD4⁺ T cells *in vitro* and Th2 antibody responses *in vivo* [12].

A common Th2 response-mediated disorder is allergic lung inflammation, an underlying cause for asthma. IL-13, IL-4, IL-5 and IL-9 produced by Th2 T cells are among the drivers of allergic lung inflammation [13]. GATA-3 and c-maf are the two most important transcription factors in the control of Th2 cytokine production. GATA-3 transactivates the IL-5 and IL-13 genes and facilitates the conversion of the IL-4, IL-5, IL-13 locus to an open conformation [14]. C-maf is critical for IL-4 production. It directly transactivates the IL-4 gene and promotes Th2 cell differentiation mainly by an IL-4-dependent mechanism [15, 16].

As reported here, we investigated the role of APRIL in allergic lung inflammation and the mechanism by which APRIL inhibits Th2 cytokine production.

Results and Discussion

April^{-/-} mice have significantly aggravated lung inflammation

We previously showed that, *in vitro*, CD4⁺ T cells from APRIL^{-/-} mice exhibited enhanced IL-4, IL-5, IL-10 and IL-13 production under Th neutral, non-polarizing (ThN) conditions, and augmented IL-13 production under Th2 conditions (Supporting Information Fig. 1 A–D and F–I) [12]. *In vivo*, upon ovalbumin immunization with aluminum potassium sulfate (alum) as adjuvant, *April*^{-/-} mice responded with an up-regulated antigen-specific IgG1 (Th2) response [12]. Since Th2 cytokines, especially IL-13, are essential for the development of allergic lung inflammation, we hypothesized that APRIL inhibits allergic lung inflammation. To test this we induced allergic lung inflammation in *April*^{-/-} and WT mice using the classical ovalbumin model (Fig. 1A). *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. 1B–C), by augmented inflammatory cell infiltration in the lung detected by H&E staining (Fig. 1D–E), by enhanced mucus secretion detected by Periodic acid-Schiff (PAS) staining (Fig. 1F–G), by elevated serum ovalbumin-specific IgE levels (Fig. 1H), by enhanced Th2 cytokine (IL-13, IL-5) and increased inflammatory cytokine (IL-6) production by restimulated bronchial LN cells (Fig. 1I). IL-4 and INF-γ production by bronchial LN cells was undetectable.

TACI-Ig treatment blocking APRIL in WT mice also enhances allergic lung inflammation

APRIL binds to TACI, one of its cognate receptors. We generated TACI-Ig to block APRIL in WT mice and analyzed its effect on allergic lung inflammation. APRIL blocking TACI-Ig activity was assessed *in vitro* by its ability to block APRIL-induced IgA production in mouse IgD⁺ B cells (data not shown). During induction of allergic lung inflammation, from day -1 to +2 relative to aerosol, WT mice were given TACI-Ig or control mouse IgG (mIgG) daily (Fig. 1J). We found that TACI-Ig treatment enhanced lung inflammation, as indicated by elevated total cell numbers and eosinophil numbers and percentage in the BALF (Fig. 1K–L), by exacerbated inflammatory cell infiltration and mucus secretion in the lung (Fig. 1M–P), by enhanced serum ovalbumin-specific IgE levels (Fig. 1Q), and by augmented cytokine (IL-13, IL-5 and IL-6) production by restimulated bronchial LN cells (Fig. 1R). TACI-Ig can also bind BAFF with comparable affinity [1], and BAFF was also reported to be a negative regulator of Th2 cytokine production and allergic lung inflammation [8, 17]. Therefore, the effect of TACI-Ig treatment on lung inflammation may

reflect the effects of both APRIL and BAFF blockade. Accordingly, we observed that TACI-Ig treatment had more profound effect on lung inflammation than *April* knockout based on larger eosinophil numbers and higher eosinophil percentage in the BALF from TACI-Ig treated mice (compare Fig. 1B and K). The examination of representative lung slides stained with H&E and PAS was not sensitive enough to compare the difference between the effects of TACI-Ig treatment and *April* gene deletion on lung inflammation (compare Fig. 1E and N, G and P). The effects of TACI-Ig treatment and genetic deletion of *April* on serum ovalbumin-specific IgE levels were comparable (Fig. 1H and Q). It has been shown that BAFF does not inhibit serum ovalbumin-specific IgE levels although it does suppress allergic lung inflammation [17]. Thus the diminished serum ovalbumin-specific IgE levels reflect the blockade of APRIL by TACI-Ig. TACI-Ig was not added to the *in vitro* assay of Th2 cytokine production by restimulated bronchial LN cells. The presence of APRIL in this assay explains the difference in cytokine production of restimulated bronchial LN cells from *April*^{-/-} and TACI-Ig treated WT mice (compare Fig. 1I and R). To further validate the role of APRIL, a BAFF only inhibitor will be tested in the future.

***April*-sufficient CD4⁺ T cells restore the suppressive effect of APRIL in *April*^{-/-} mice**

Elevated Th2 cytokine production was observed when purified CD4⁺ cells from *April*^{-/-} mice were stimulated with anti-CD3 antibody *in vitro* [12], suggesting that the suppressive signal of APRIL is intrinsic to CD4⁺ T cells. It is known that APRIL is expressed in T cells [4–7, 9, 10, 18]. Consistent with a previous study [4], we detected *April* mRNA expression in Th2-polarized CD4⁺ T cells (Fig. 2B). APRIL is a secreted soluble ligand [19] which renders it capable to function in autocrine and/or paracrine ways. The transfer of antigen-specific WT CD4⁺ T cells to *April*^{-/-} mice may be able to restore the suppressive effect of APRIL on lung inflammation and abolish the difference in severity of allergic lung inflammation between *April*^{-/-} and WT mice. To test this hypothesis, purified APRIL sufficient, ovalbumin-specific, TCR transgenic CD4⁺ T (OT-II) cells, 1×10⁶ cells/mouse, were adoptively transferred i.v. to *April*^{-/-} and WT mice, two days before ovalbumin/alum priming. *April*-sufficient OT-II cell transfer abolished the difference between *April*^{-/-} and WT mice in the severity of lung inflammation following aerosol challenge as indicated by comparable total cell numbers and eosinophil numbers and percentage in BALF, comparable lung inflammatory cell infiltration, comparable mucus secretion, comparable serum ovalbumin-specific IgE levels and comparable Th2 cytokine production (Fig. 2C–E and data not shown). OT-II (CD4⁺GFP⁺) cell expansion was monitored in the recipients following immunization to make sure that the cell transfer was successful. In peripheral blood, OT-II cell frequency in blood increased to day 5 and then decreased. On day 15, OT-II cell frequency was at a very low level in blood, but at a high level in the BALF, bronchial LN and lungs (Fig. 2F). No difference in OT-II cell frequency was detected between *April*^{-/-} and WT mice (Fig. 2F). Our data suggest that the effect of APRIL is CD4⁺ T cell autonomous in terms of APRIL source and does not require APRIL production by other cells. It is possible that other cells producing APRIL may also contribute to suppression of lung inflammation in WT mice.

C-maf expression is enhanced in CD4⁺ T cells from *April*^{-/-} mice

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 CD4⁺ T cells from *April*^{-/-} and WT mice. We found that c-maf expression was significantly enhanced in *April*^{-/-} CD4⁺ T cells at both the RNA and protein level and under both ThN and Th2 conditions. GATA-3 expression was unchanged (Fig. 3A–C). Since IL-4 can induce c-maf expression [20], augmented c-maf expression in *April*^{-/-} CD4⁺ T cells could have been caused secondarily by the increased IL-4 production. However, 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.

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 [15, 16]. Therefore, the increased c-maf expression in cultured *Apri*^{-/-} CD4⁺ T cells readily explains our previous observation (Supporting Information Fig. 1 A–D and F–I) [12] that, under ThN conditions, IL-4 expression was up-regulated and upon secondary stimulation followed by an increase of the expression of other Th2 cytokines (IL-5, IL-10 and IL-13) in *Apri*^{-/-} CD4⁺ T cells. Although c-maf levels were upregulated in *Apri*^{-/-} T cells, the addition of IL-4 under Th2-polarizing conditions abolished the difference between *Apri*^{-/-} and WT CD4⁺ T cells in Th2 cytokine production upon secondary stimulation. The exception was IL-13, which remained increased in *Apri*^{-/-} CD4⁺ T cells even under Th2 conditions, suggesting that APRIL is required for suppressing IL-13 production in an IL-4 independent, IL-13 specific pathway. The increased c-maf expression in *Apri*^{-/-} cells also explains our previous observation (Supporting Information Fig. 1E and J) [12] that IL-17 production was enhanced in *Apri*^{-/-} CD4⁺ T cells under Th2 conditions upon secondary stimulation, since it was found recently that c-maf promotes IL-17 production [21]. Furthermore, up-regulated c-maf expression was observed in human asthmatic airways after allergen challenge and spontaneously (unchallenged) increased eosinophil numbers were found in the BALF of c-maf transgenic mice [22]. These findings directly support our conclusion that the increased c-maf expression in *Apri*^{-/-} CD4⁺ T cells results in augmented allergic lung inflammation in *Apri*^{-/-} 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. IL-4 independent IL-13 expression could also be postulated by the previous observation that IL-13 levels, unlike other Th2 cytokines, were not reduced in c-maf^{-/-} T cells without exogenous IL-4 [16]. In addition, IL-13 is not always coexpressed with other Th2 cytokines in a single T helper cell [23]. Taken together, these data raise the intriguing possibility that subsets of IL-13-producing Th2 cells exist whose formation is IL-4 independent [16] but may involve APRIL-mediated signaling.

APRIL has been shown to bind to mouse T cells by interacting with proteoglycans [2]. However, proteoglycans may just function to provide a platform for ligand multimerization and cross-linking to facilitate APRIL binding to its receptors [2, 3, 24]. Whether TACI is expressed on T cells is controversial, but increasing evidence indicates the expression [6, 8, 9]. Using PE Rat anti-Mouse CD267 (TACI) clone 8F10 from BD Pharmingen, the same clone as used in a recent study which showed the expression [9], we did not detect TACI expression on CD4⁺ T cells. Detailed future studies are required to clarify this conflicting issue and which receptor is used by APRIL to suppress c-maf expression.

As mentioned above, APRIL is expressed in Th2 polarized CD4⁺ T cells and binds to T cells. To confirm the observation that APRIL inhibits c-maf expression, we conducted assays to analyze c-maf expression with TACI-Ig treatment to block APRIL in the WT CD4⁺ T cell culture and APRIL treatment to restore APRIL function in the *Apri*^{-/-} CD4⁺ T cell culture. As mentioned above, the functions of APRIL and TACI-Ig were assessed by inducing and blocking IgA production in mouse IgD⁺ B cells, respectively. However, TACI-Ig did not upregulate c-maf expression in WT CD4⁺ cells and APRIL did not suppress c-maf expression in *Apri*^{-/-} CD4⁺ cells (Fig. 3D). In contrast, the increased severity of lung inflammation observed in *Apri*^{-/-} mice was mimicked in WT mice by TACI-Ig treatment, as described above. Taken together, these data suggest that the suppressive signal delivered by APRIL already exists in CD4⁺ T cells before cell isolation and can only be induced or

blocked *in vivo* but not *in vitro*. One possibility is that additional costimuli participate in the regulation of c-maf expression by APRIL *in vivo*.

Concluding Remarks

Our data suggest that APRIL inhibits allergic lung inflammation by suppressing c-maf expression and directly reducing IL-13 production in CD4⁺ T cells. 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 side effects.

Materials and methods

Mice

April^{-/-} mice [12] were backcrossed onto C57BL/6 background for over 14 generations. The allergic lung inflammation animal protocol was approved by the University of Miami Institutional Animal Care and Use Committee (IACUC).

Induction of allergic lung inflammation, TACI-Ig treatment and OT-II cell transfer

Mice were sensitized on day 0 and boosted on day 5 by i.p. injection of ovalbumin coprecipitated with alum. On day 12, mice were aerosol challenged with ovalbumin. Lung inflammation was evaluated on day 15 (Fig. 1A). More details were provided previously [13]. For some experiments, TACI-Ig or control mIgG (Sigma-Aldrich, St. Louis, MO) was administered at 100μg/mouse by i.p. injection daily from day 11 to 14 (Fig. 1J). OT-II cells were purified to 93–97% purity by using the mouse CD4 (L3T4) microbeads (Miltenyi Biotec, Auburn, CA) or the mouse CD4 selection kit, EasySep (StemCell Technologies, Seattle, WA). OT-II cells were transferred at 1×10⁶ cells/mouse by tail vein injection, two days before the first ovalbumin/alum injection (Fig. 2A).

Lung inflammation assessment

BAFF cells on slides stained with Wright-Giemsa were counted (> 200 cells/slide) to determine counts for macrophages, eosinophils and lymphocytes. For lung histology, five H&E stained and six PAS stained lung sections of each mouse were scored in a blinded fashion using the scoring systems described previously [13]. Serum ovalbumin-specific IgE production and cytokine production by ovalbumin (100μg/ml) restimulated bronchial LN cells were determined by sandwich ELISA as in our previous study [12].

Generation of TACI-Ig

TACI-Ig was constructed by fusing the extracellular domain of mouse TACI cDNA (Met 1 - Thr 129, 387bp) [25] to an Ig κ chain signal peptide (92 bp) at the 5' end and to the mouse IgG1-Fc sequence (680bp) at the 3' end. TACI-Ig was purified from the culture supernatant of transfected 3T3 cells.

Cell culture, RT-PCR and Western blot to determine expression of GATA-3 and c-maf

CD4⁺ cells purified by using the mouse CD4 selection kit, EasySep (StemCell Technologies, Seattle, WA) (>99% purity) were cultured under ThN and Th2 conditions for four days, and re-stimulated for two days with only anti-CD3. More details were provided previously [12]. For some experiments, during the primary and secondary stimulation, mouse TACI-Ig or IgG control at 30μg/ml was added to the WT CD4⁺ T cell culture, and mouse APRIL (PeproTech, Rocky Hill, NJ) at 1μg/ml or PBS control was added to the *April*^{-/-} CD4⁺ T cell culture.

Quantitative TaqMan real-time RT-PCR were performed using gene expression assays and the 7300 Real-Time PCR system from Applied Biosystems, Foster City, CA. Relative expression or fold change was calculated using the ΔC_t method. Western blotting was carried out under reducing conditions using mouse anti-mouse/human GATA3 (BD Pharmingen, San Diego, CA) and rabbit polyclonal to mouse c-maf (Abcam, Cambridge, MA).

RT-PCR to determine APRIL expression

SYBR Green real-time RT-PCR reagents (SuperArray Bioscience Corporation, Frederick, MD) and the 7300 Real-Time PCR system (Applied Biosystems) were used to determine APRIL expression in Th2 polarized CD4⁺ T cells from the above cell culture after restimulation. β -actin was used as the endogenous control.

Statistical analysis

Paired or unpaired t test was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA). $P < 0.05$ was considered as significant. Data are mean $\pm$ SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

alum	aluminum potassium sulfate
APRIL	a proliferation inducing ligand
BAFF	B-cell activation factor of the TNF family
BAFF-R	BAFF receptor
BALF	bronchoalveolar lavage fluid
BCMA	B cell maturation antigen
HSPGs	heparan sulfate proteoglycans
mIgG	mouse IgG
PAS	Periodic acid-Schiff
TACI	transmembrane activator and calcium modulator and cyclophilin ligand interactor
ThN: Th neutral	non-polarizing conditions

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Figure 1.

April^{-/-} mice and TACI-Ig treated WT mice have significantly aggravated allergic lung inflammation. (A–I) *April*^{-/-} (–/–) and WT (+/+) mice. (J–R) TACI-Ig and mIgG treated WT mice. (A and J) Scheme for induction of allergic lung inflammation (A) and TACI-Ig treatment in the allergic lung inflammation model (J). (B and K) Inflammatory cell number and percentage in the BALF. (C and L) BALF cells on slides stained with Giemsa-Wright. The eosinophils are stained blue in nuclei and red in cytoplasm, indicated by arrows (original 40x). (D, E, M and N) Histological analysis of lung – H&E staining. Lung inflammation is indicated by increased perivascular infiltration of eosinophils and lymphocytes (original 40x). (F, G, O and P) Histological analysis of lung – PAS staining. Mucus produced by goblet cells is stained purple, indicated by arrows (original 40x). (H and Q) Ovalbumin-specific IgE antibody titers detected by ELISA. The serum titration shown is within the linear range. For B–H, *p<0.05, **p<0.01; unpaired t test; n=8–9. Data from 5 independent experiments (n=1–2 each) were combined. For K–Q, *p<0.05, **p<0.01;

paired t test; n=4. Data from 2 independent experiments (n=2 each) were combined. (I and R) Cytokine production by ovalbumin restimulated bronchial LN cells determined by ELISA. For I, *p<0.05; unpaired t test; n=6 (IL-13); n=4 (IL-5). Cells from 2 mice were pooled in the culture, if necessary. Data from 4–5 independent experiments were combined. For R, *p<0.05, **p<0.01; paired t test; n=4. Data from 2 independent experiments (n=2 each) were combined.

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Figure 2.

The transfer of *April*-sufficient OT-II cells abolishes the difference of allergic lung inflammation between *April*^{-/-} and WT mice. (A) Scheme for OT-II cell transfer in the allergic lung inflammation model. (B) APRIL expression in Th2-polarized CD4⁺ T cells determined by SYBR Green real-time RT-PCR. β -actin was used as the endogenous control and average Ct was shown. n=2. (C) Inflammatory cell number and percentage in the BALF. (D) Ovalbumin-specific IgE antibody titers detected by ELISA. The serum titration shown is within the linear range. (E) Cytokine production by ovalbumin restimulated bronchial LN cells determined by ELISA. (F) OT-II (CD4⁺GFP⁺) cell expansion in peripheral blood,

bronchial LNs, BALF, and lungs detected by FACS. n=14–15 (C–D); n=10–15 (E–F). Data from 6 independent experiments were combined. $-/-$ = *ApriI*^{-/-}; $+/+$ = WT.

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Figure 3.

C-maf, not GATA-3, expression is enhanced in cultured *April*^{-/-} CD4⁺ T cells. (A) C-maf and GATA-3 expression detected by quantitative TaqMan real-time RT-PCR. Relative expression was calculated relative to the unstimulated WT samples, normalized to β -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 ($n = 1-3$ each) were combined. (B) C-maf and GATA-3 expression detected by western blots. (C) Quantification of western Blot bands. Relative intensity was calculated relative to β -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 ($n = 2-3$ each) were combined. (D) C-maf expression detected by quantitative

TaqMan real-time RT-PCR after TACI-Ig (mIgG as a control) or APRIL (PBS as a control) treatment. 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 (n=1 each) were combined. $-/-$ = *April*^{-/-}; $+/+$ = WT.

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