NFκB attenuates IL-5 production and upregulates T-box transcription factors in Th2-like T cells

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Abstract IL-5 plays important roles in eosinophil differentiation, expansion, and recruitment. The regulation of IL-5 seems critical for the treatment of eosinophil-mediated allergic reactions. However, the precise mechanisms for IL-5 regulation remain unknown. In this study, we investigated how IL-5 production is regulated. The transduction of GATA-3 into a murine T cell hybridoma resulted in acquiring the ability to produce IL-5 in response to an antigenic stimulus like Th2 cells. This production was dependent on the cAMP-PKA pathway, but not on p38 activation. Transduction of NIK largely impaired IL-5 production. RelA and RelB similarly impaired IL-5 production. RelA decreased not only IL-5 protein amount but mRNA. RelA also inhibited *Il5*-luciferase reporter activity. The transduction of GATA-3 decreased the expression of *Tbx21* and *Eomes*, but

the additional transduction of RelA abrogated the decreased expression of GATA-3-induced *Tbx21* and *Eomes*. Furthermore, the transduction of T-bet or Eomes into the GATA-3-transduced T cell hybridoma impaired IL-5 production. These results suggested that strong enhancement of the NFκB pathway downregulates IL-5 production and upregulates T-box protein expression to shift an immune response from Th2 to inflammatory Th1.

Keywords IL-5 · GATA-3 · NFκB · Rel · T-bet · Eomes

Introduction

Helper T cells (Th) are classified into at least 3 types: T helper 1 (Th1), Th2, and Th17. Th1 cells produce IFN-γ and evoke immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5, and IL-13 and participate in immunity against parasites and allergic responses. Th17 cells produce IL-17A, IL-17F, and IL-22 and induce immunity against extracellular pathogens and certain autoimmune diseases. Th differentiation and functions are tightly controlled by specific transcription factors. Th1 differentiation is favored by T-bet (Szabo et al. 2000), Th2 is enhanced by GATA-3 (Zheng and Flavell 1997), and Th17 is controlled by RORγ (Ivanov et al. 2006).

IL-5 was initially identified as a mouse B cell growth and differentiation factor, and subsequent

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studies revealed that IL-5 plays important roles in the differentiation, function, and recruitment of eosinophils (Rothenberg and Hogan 2006; Kouro and Takatsu 2009; Takatsu et al. 2009). IL-5 is produced by Th2 cells, Tc2 cells, mast cells, eosinophils, NK cells, NKT cells, and natural helper cells/innate lymphoid cells (Mosmann and Coffman 1989; Tominaga et al. 1988; Desreumaux et al. 1992; Warren et al. 1995; Sakuishi et al. 2007; Moro et al. 2010; Ikutani et al. 2012; Kuraoka et al. 2004). The expression of IL-5 is induced by bt_2cAMP , a chemical analogue of cAMP (Snijderwint et al. 1993; Lee et al. 1993). Activation of adenylate cyclase by cAMP leads to protein kinase A (PKA) activation, resulting in IL-5 production in a murine T cell line, EL4, and some other types of cells (Lee et al. 1993). Physiological stimuli such as TCR stimulation induce IL-5 expression in Th2 cells. Recently, IL-33, the IL-1 family cytokine was shown to robustly induce IL-5 (Schmitz et al. 2005). p38 upregulates IL-5 production in a murine Th2 clone (Chen et al. 2000) and in human T cells (Mori et al. 1999). cAMP elevates the p38 level and p38 phosphorylates GATA-3 (Chen et al. 2000). A large amount of IL-5 production seems to be restricted to highly differentiated cells, such as repeatedly induced Th2 cells (Islam et al. 2011). IL-5 production is regulated by GATA-3 (Zhang et al. 1997), a transcription factor that is induced by the IL-4-Stat6 signaling pathway (Ouyang et al. 1998). GATA-3 regulates not only the generation of T cells but also Th2 differentiation (Zheng and Flavell 1997). GATA-3 participates in the remodeling of chromatin at Th2 cytokine gene loci (Ansel et al. 2006; Wilson et al. 2009), which may induce IL-4 expression. IL-5 expression is directly transactivated at the *Il5* promoter region by GATA-3, whereas IL-4 expression does not necessarily require GATA-3 (Zhang et al. 1997; Zhang et al. 1998; Ranganath et al. 1998; Lee et al. 2000). On the other hand, mechanisms for the downregulation of IL-5 have not been completely elucidated. Only T-box transcription factor has been reported; Eomes downregulates IL-5 expression in memory Th2 cells (Endo et al. 2011).

In this study, we examined the regulation mechanism of IL-5 production. We show that *Gata3* transduction into a T cell hybridoma led to IL-5 production in a cAMP-PKA pathway-dependent manner. We also show that high levels of Rel proteins reduce the *Gata3*-induced IL-5 production and induce T-box protein in Th2-like T cells.

Materials and methods

Mice

OT-II mice transgenic for chicken ovalbumin 323–339 (cOVA323-339)-specific I-A^b-restricted T cell receptor (Barnden et al. 1998) were provided by Dr William R. Heath (The Walter and Eliza Hall Institute of Medical Research, Parkville VIC, Australia) through Dr. Nakayama (Chiba University, Chiba, Japan). Mice were housed under specific-pathogen free conditions in Laboratory Animal Research Center, Dokkyo Medical University (Mibu, Japan) and were handled according to the Guidelines for the Care and Use of Laboratory Animal Research Center, Dokkyo Medical University (protocol #0663).

Cells and cell culture

DO11.10 T cell hybridoma (Haskins et al. 1983) was maintained in RPMI 1640 (Wako, Osaka, Japan) containing 5 % FCS (Equitech-Bio, Kerrville, TX, USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). Cells were stimulated with immobilized anti-CD3 mAb (10 µg/ml; 145-2C11 (BioLegend, San Diego, CA, USA)) or the combination of 0.01–1 mM bt_2cAMP (Sigma-Aldrich), 10 ng/ml PMA (Sigma-Aldrich), and 0.5 µM ionomycin (Merck-Millipore, Billerica, MA, USA). For some experiments, 10 µM PKA inhibitor H-89 (Sigma-Aldrich) or 10 µM p38 inhibitor SB203580 (Merck-Millipore) was added. OT-II splenocytes were stimulated with 1 µM cOVA323-339 supplemented with 10 ng/ml rIL-2 (PeproTech, Rocky Hill, NJ, USA).

Gene transduction

Murine *Gata3*, *Tbx21*, *Eomes*, *Rel*, *Rela* and *Relb*, and the wild-type and loss-of-function forms of human NIK (*MAP3K14*) were obtained by RT-PCR. IκBαM, a suppressor mutant of human IκBα (*NFKAIA*) in pMX-IG (Inami et al. 2004) was a gift from Dr. T. Nakayama, Chiba University. Full-length cDNA was inserted into pMXs, pMXs-IRES-EGFP (pMXs-IG, kindly provided by Dr. T. Kitamura, University of Tokyo) (Nosaka et al. 1999), or pMXs-IRES-human nerve growth factor receptor (hNGFR, kindly provided by Dr. T. Nakayama, Chiba University).

(Shinnakasu et al. 2008). Human NFATc1 cDNA (Northrop et al. 1994) was inserted into pEGFP-C1, and the EGFP-hNFATc1 region was inserted into pMXs. PLAT-E cells (kindly provided by Dr. T. Kitamura (Morita et al. 2000)) were transfected with retroviral vector (kindly provided by Dr. T. Kitamura (Nosaka et al. 1999)) using FuGENE HD (Roche (Basel, Switzerland) or Promega (Fitchburg, WI, USA)) and the supernatant at day 2 was mixed with the cell suspension in the presence of 8 µg/ml polybrene (Sigma-Aldrich). OT-II CD4⁺ cells stimulated for 1 day were transduced using PLAT-E supernatant on RetroNectin (Takara, Otsu, Japan)-coated wells by incubation for over night. Cells were washed and expanded in the presence of 10 ng/ml rhIL-2 for 2–4 days.

Flow cytometry

Fluorochrome-conjugated mAbs were purchased from BioLegend or eBioscience (San Diego, CA, USA) unless otherwise described. hNGFR was stained by biotinylated anti-hNGFR mAb (C40-1457, BD Biosciences, Franklin Lakes, NJ, USA) followed by streptavidin-PE or -APC (BioLegend). For intracellular cytokine staining, cells were fixed with 4 % paraformaldehyde (WAKO, Osaka, Japan), permeabilized with 0.1 % saponin, stained with anti-IL-5-PE, anti-IL-4-PE, anti-IL-13-PE, or anti-IFN-γ-PE or -APC. Cells were analyzed on a FACSCalibur (BD Biosciences) using the FlowJo software (TreeStar). In some experiments, cells were sorted on a FACSARIA (BD Biosciences).

ELISA

IL-5 concentration in culture supernatant of DO11.10 T cell hybridoma was determined by sandwich ELISA using specific mAbs (TRFK5 (capture antibody) and TRFK4 (antibody for detection), BioLegend).

RT-Quantitative-PCR (RT-qPCR)

Total RNA was prepared with ISOGEN (Nippon Gene, Tokyo, Japan) and converted to cDNA using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qPCR was performed then using Thunderbird SYBR qPCR mix (Toyobo, Japan, Tokyo) on an ABI5700 (Invitrogen). Specific primers

are listed in a table in the Supplementary Methods section. Data were normalized to *Actb* and *Hprt*.

Luciferase assay

DO11.10 T cell hybridoma were electroporated with 20 µg of 3.6 kb IL5 promoter-inserted pGL4 and 0.5 µg of phRL-TK (thymidine kinase promoter-driven Renilla luciferase expression vector for internal control) (Promega, Tokyo, Japan) at 250 V, 975 µF on a Gene Pulser II (BioRad, Hercules, CA, USA). Cells were left unstimulated or stimulated with 10 ng/ml PMA (Sigma-Aldrich), 0.5 µg/ml ionomycin (Sigma-Aldrich) and 1 mM bt₂cAMP (Sigma-Aldrich) for 6 h, and harvested. Luciferase activities were measured by Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

Results

GATA-3 induces IL-5 production in T cell hybridoma

Because GATA-3 is known as a master regulator of Th2 cytokines, we initially tried to investigate IL-4 production mechanisms by the transduction of *Gata3*. Retroviral transduction of *Gata3* bicistronically expressing *egfp* into DO11.10 T cell hybridoma, however, did not lead to IL-4 production (data not shown). Interestingly, it did lead to IL-5 production in response to PMA/ionomycin/bt₂cAMP stimulus (Fig. 1a), which supports the findings of previous reports (Zhang et al. 1998; Ranganath et al. 1998; Lee et al. 2000). *Gata3* transduction into 2B4 T cell hybridoma or OT-II CD4⁺ T cells similarly led to IL-5 production to a lesser extent (Supplementary Figure S1a). On the other hand, retroviral transduction of *Tbx21*, a master regulator of Th1 cytokines, into DO11.10 T cell hybridoma resulted in IFN-γ production (Supplementary Figure S2). *Gata3*- and *Tbx21*-transduced DO11.10 cells seemed similar to Th2-like and Th1-like cells, respectively, according to the cytokines produced. In order to investigate what molecule regulates IL-5 production, DO11.10 was transduced with pMXs-Gata3, without any marker such as EGFP, and then cloned and selected for IL-5 production.

Fig. 1 *Gata3* transduction leads to IL-5 production in T cell hybridoma. **a** DO11.10 T cell hybridoma was retrovirally transduced with control pMXs-IG or pMXs-*Gata3*-IG (*Gata3*), and IL-5 production in response to PMA/ionomycin/bt₂cAMP was measured by intracellular staining. **b–d** *Gata3*-transduced DO11.10 T cell hybridoma was treated with various stimuli (**b**), with fixed doses of PMA + ionomycin and various concentrations of bt₂cAMP (**c**), or with PMA + ionomycin + bt₂cAMP

in the absence or presence of H89 or SB203580 (**d**), and IL-5 production was measured by intracellular staining. **d** *Gata3*-transduced DO11.10 T cell hybridoma was stimulated as in (**d**), except without the addition of inhibitors, and IL-5, IL-4, and IL-13 were measured. Numbers in figures indicate mean fluorescence intensity (MFI). Data are representative of 2 (**c–e**) or 3 (**a**, **b**) independent experiments

PMA and, ionomycin alone did not induce IL-5 production (Fig. 1b and data not shown, respectively), but bt₂cAMP alone led to IL-5 production from the *Gata3*-transduced DO11.10 hybridoma. PMA upregulated and PMA plus ionomycin maximized the bt₂cAMP-induced production of IL-5. The production of IL-5 induced by bt₂cAMP was dose dependent, and it reached the maximum when 0.3 mM bt₂cAMP was added (Fig. 1c). Previous reports demonstrated that

IL-5 production is regulated by the cAMP-PKA pathway (Lee et al. 1993; Klein-Hessling et al. 2003) or induced by p38 activation (Mori et al. 1999; Chen et al. 2000). As expected, the addition of H89, a PKA inhibitor, impaired IL-5 production, but the addition of SB203580, a p38 inhibitor, did not affect the production (Fig. 1d). These data confirmed that bt₂cAMP-induced production of IL-5 by DO11.10 was mediated by PKA. The production of Th2-type cytokines, IL-4,

IL-5, and IL-13, are relatively linked. The production of IL-13 was similarly detected, but that of IL-4 was not detected (Fig. 1e).

NF κ B pathway downregulates IL-5 production in T cell hybridoma

In order to identify the key modulator(s) for IL-5 production, signaling molecules were tested by retroviral transductions. According to the transcription factor-binding search (<http://www.cbrc.jp/research/db/TFSEARCH.html>), many AP-1 recognition sites are present in the *Il5* promoter. Thus, the MAPK

pathway was first examined. Transduction of wild-type or a gain-of-function *Xenopus* MEK (*map2k1*) did not alter IL-5 production (Supplementary Figure S3a). GATA3 and NFAT are both implicated in IL-5 gene transcription (Rao et al. 1997; Zhang et al. 1997; Zhang et al. 1999), and an NFAT-binding site exists in the *Il5* promoter region (Mordvinov and Sanderson 2001). Thus, EGFP-fused human NFATc1 was retrovirally transduced to *Gata3*-transduced DO11.10 and the IL-5 production was examined. However, IL-5 production in response to bt_2cAMP and the combination with PMA was comparable to the EGFP control transduction (Supplementary Figure S3b). The

Fig. 2 NF κ B activation impairs IL-5 production. **a** *Gata3*-transduced DO11.10 T cell hybridoma was retrovirally transduced with pMXs-IG (control) or pMXs-human NIK-IG (*MAP3K14*), and IL-5 production in response to bt_2cAMP , bt_2cAMP + PMA, bt_2cAMP + ionomycin, or bt_2cAMP + PMA + ionomycin + bt_2cAMP was measured by intracellular staining. EGFP⁺ cells were gated (1st column) and analyzed for IL-5 production (2nd to 5th columns). **b** *Gata3*-transduced DO11.10 T cell hybridoma was retrovirally transduced with pMXs-IG (control), pMXs-c-Rel-IG (*Rel*), pMXs-RelA-IG (*Rela*), or pMXs-RelB-IG (*Relb*), and IL-5 production in response to anti-CD3 mAb or PMA + ionomycin + bt_2cAMP was measured by intracellular staining. EGFP⁺ cells were gated

(1st column) and analyzed for IL-5 production (2nd and 3rd columns). **c** OT-II CD4⁺ T cells were retrovirally transduced with *Gata3* (bicistronically with hNGFR) together with control (pMXs-IG) or *Rela*. hNGFR⁺ (*GATA-3*⁺) and IL-5 production in response to PMA + ionomycin + bt_2cAMP was measured by intracellular staining. EGFP^{hi} cells were gated and analyzed. Histograms show IL-5 production with isotype control staining (left). Bars show mean fluorescence intensity (MFI) + SD of triplicate wells with a statistical difference (Student's *t* test) (right). Numbers indicate MFI (**a**, **b**) and percentages of IL-5 positive cells (**c**). Data are representative of at least 2 independent experiments

transcription factor-binding search also showed NF κ B binding sites near the *Il5* transcription initiation site. Human NIK was retrovirally transduced to *Gata3*-transduced DO11.10 bicistronically with EGFP. The transduction efficiency of NIK was relatively low, but EGFP⁺ cells showed drastically decreased IL-5 production (Fig. 2a). On the other hand, loss-of-function human NIK slightly decreased IL-5 production (Supplementary Figure S4). A gain-of-function human I κ B α (constitutively attenuating the NF κ B pathway) transduction slightly decreased IL-5 production. These results imply that attenuation of the NF κ B pathway enhances, but a complete shutdown of the NF κ B pathway impairs, IL-5 production.

Next, the Rel family molecules were tested. Murine c-Rel (*Rel*), RelA (*Rela*), or RelB (*Relb*) was transduced to *Gata3*-transduced DO11.10. Although the transduction level of *Rel* and the suppressive effects were relatively low, all Rel-related molecules tested showed impaired IL-5 production against both anti-CD3 and PMA/ionomycin/bt₂cAMP stimuli (Fig. 2b). When 2B4 used, the inhibition of IL-5 production by *Rela* or *Relb* was similarly observed, although IL-5 production levels were lower (Supplementary Figure S5). We also observed that double transduction of *Gata3* and *Rela* substantially lowered the IL-5 production from OT-II CD4⁺ T cells (Fig. 2c).

RelA transcriptionally inhibits IL-5 expression

Next, we examined whether the inhibition caused by *Rela* transduction happened at a transcriptional level. Control (pMXs-IG) or *Rela* (*Rela*) was transduced to

Gata3-transduced DO11.10, sorted for EGFP⁺ and expanded. The cells were stimulated with PMA + ionomycin + bt₂cAMP and IL-5 protein amount in the culture supernatant was measured by ELISA. The IL-5 amount produced by *Rela*-transduced cells was drastically lower compared to control transduction. (Fig. 3a). Then, the cells were stimulated to examine *Il5* transcript by qPCR. Similarly, the *Il5* transcript level was severely impaired in *Rela*-transduced cells compared to pMXs-IG transduced cells (control) (Fig. 3b). Furthermore, *Il5* transcription was studied using a 3.6 kb *Il5* promoter linked to luciferase. The cells were transfected with the reporter plasmid and stimulated. As expected luciferase activity of *Rela*-transduced cells was lower compared to the control transduction (Fig. 3c). These results demonstrate that RelA inhibits IL-5 production at a transcriptional level.

RelA upregulates T-box protein expression

Because the NF κ B pathway downregulated IL-5 but upregulated IFN- γ production, we hypothesized that GATA-3 downregulates Rel molecules. However, all *Rel*, *Rela*, and *Relb* levels were comparable or rather enhanced in *Gata3*-transduced DO11.10 and Th2 cells as compared to non-transduced DO11.10 and Th1 cells, respectively (Supplementary Figure S6). This finding suggested that Rel indirectly regulates IL-5 expression. It is well known that T-bet and GATA-3 reciprocally regulate the level of the other one (Usui et al. 2003, 2006). GATA-3 also suppressed Eomes expression (Yagi et al. 2010). Thus, T-box protein

Fig. 3 Rel transcriptionally suppresses IL-5 expression. *Gata3*-transduced DO11.10 T cell hybridoma was retrovirally transduced with pMXs-IG (control) or pMXs-*Rela*-IG (*Rela*), and IL-5 production in response to PMA + ionomycin + bt₂cAMP was measured by ELISA (a), *Il5* transcript by RT-qPCR (b), and

Il5 promoter activity by luciferase assay (c). *Il5* transcript was normalized by *Hprt*. *Il5* promoter activity was normalized by *Renilla* luciferase activity. Bars show Mean + SD of triplicate samples. * $p < 0.05$ (Student's *t* test). Data are representative of 2 independent experiments

Fig. 4 Rel transduction restores T-box molecule expression, which in turn decrease IL-5 expression. **a** *Gata3* transduction decreased T-box molecule expression. *Tbx21* (left) and *Eomes* (right) mRNA levels in *Gata3*-transduced DO11.10 T cell hybridoma were measured by RT-qPCR. **b** *Gata3*-transduced DO11.10 T cell hybridoma was retrovirally transduced with pMXs-IG (control) or pMXs-*Rela*-IG (*Rela*), sorted for EGFP⁺, and *Tbx21* and *Eomes* mRNA levels were measured by RT-qPCR. **c** *Gata3*-transduced DO11.10 T cell hybridoma was retrovirally transduced with pMXs-IG (control), pMXs-*Tbx21*-IG (*Tbx21*), or pMXs-*Eomes*-IG (*Eomes*), and IL-5 and

IFN- γ production were measured by intracellular staining as described in Fig. 1. EGFP⁺ cells were gated (1st column) and analyzed for IL-5 and IFN- γ production (2nd and 3rd column, respectively). Numbers in histograms indicate MFI. **d** OT-II CD4⁺ T cells were retrovirally transduced with *Gata3* (bicistronically with hNGFR) together with Empty (pMXs-IG) or *Rela* and IFN- γ production in response to PMA + ionomycin + bt₂cAMP was measured by intracellular staining. hNGFR⁺ (GATA-3⁺) and EGFP⁺ cells were gated and analyzed. Numbers indicate percentages of IFN- γ ⁺ cells. Data are representative of 2 independent experiments

expression in *Gata3*-transduced DO11.10 was examined. As expected, *Tbx21* and *Eomes* levels were severely decreased in *Gata3*-transduced DO11.10 (Fig. 4a). Then, we investigated whether Rel increases T-box protein expression. Transduction of *Rela* considerably enhanced *Tbx21* and *Eomes* expression (Fig. 4b). To test whether these T-box proteins impair IL-5 production, *Tbx21* or *Eomes* were transduced into *Gata3*-transduced DO11.10 cells. Transduction of

Tbx21 moderately impaired the IL-5 production, whereas the production was severely impaired by *Eomes* (Fig. 4c). At the same time, *Tbx21* and *Eomes* induced IFN- γ production. However, knockdown of *Eomes* or *Tbx21* did not alter the decreased IL-5 production (Supplementary Figure S7). These results suggest that Rel does not simply induce T-box family protein to directly inhibit IL-5 production. We also examined IFN- γ production in GATA-3-expressing

cells. *Rela* transduction resulted in enhanced IFN- γ production in GATA-3-expressing OT-II CD4⁺ T cells (Fig. 4d). These results suggested that strong NF κ B signals favors Th1 responses in T cells.

Discussion

We demonstrated that Rel molecules downregulate IL-5 production by upregulating T-box protein expression. Previous reports have demonstrated that Rel supports the Th1 response. c-Rel-deficient mice have slight symptoms of myelin oligodendrocyte glycoprotein (MOG)-induced experimental autoimmune encephalomyelitis, and MOG-immunized T cells result in normal IL-4 production but impaired IFN- γ production (Hilliard et al. 2002). c-Rel-deficient T cells also showed impaired IFN- γ production against *Toxoplasma* antigen (Mason et al. 2004). RelB-deficient CD4⁺ T cells have defects in Th1 differentiation (Corn et al. 2005). RelA-deficiency has a mild effect on Th1 differentiation, but RelA-deficient mice are susceptible to *Leishmania major* infection (Mise-Omata et al. 2009). A previous report has suggested that RelA regulates T-bet function through heterodimerization of these proteins (Hwang et al. 2005a). On the other hand, NF κ B1 (p50/p105) is reciprocally important for IL-5 production. p50-deficient T cells show impaired IL-5 production (Barnes and Karin 1997; Yang et al. 1998; Das et al. 2001). This impaired production is caused by the fact that p50 deficiency impairs GATA-3 expression (Das et al. 2001). NF κ B family proteins dimerize to exert their function. Rel proteins have a transactivation domain, whereas p50 and p52 do not. Constituents of the dimers could be important irrespective of whether the outcome is activation or inhibition of gene expression.

From our results, it seems that Rel does not directly regulate IL-5 production, but indirectly modulates IL-5 expression by upregulating T-box proteins. T-bet is induced under Th1-inducing conditions (Szabo et al. 2000). Eomes is induced by Runx3 (Yagi et al. 2010). The functions of T-box proteins and GATA-3 are reciprocal (Usui et al. 2003; Usui et al. 2006). T-bet downregulates GATA-3 expression (Usui et al. 2006) as well as the binding of GATA-3 to DNA (Szabo et al. 2000; Hwang et al. 2005b). Similarly, Eomes regulates GATA-3 binding to the *Il5* promoter by direct binding through the T-box region (Endo et al. 2011). It is

possible that Rel protein-induced T-box proteins inhibit GATA-3 binding to DNA. Putative NF κ B binding sites are present at approximately −7.2, −6.5, −6.4, −5.2, −2.8, −1.7, and 0.5 kb and −9.7, −8.4, −6.8, −6.6, and −0.1 kb in the *Tbx21* and *Eomes* promoters, respectively. Thus, Rel protein may regulate *Tbx21* and *Eomes*. However, knockdown either of *Eomes* or *Tbx21* did not restore IL-5 production. Either of them might be enough to suppress IL-5 production. Also, the mechanisms by which Rel induces T-bet and Eomes need to be elucidated.

Our data do not exclude the possibility that IL-5 induction does not require the Rel family molecules. Transduction of the loss-of-function NIK lowered the IL-5 production (Supplementary Figure S4), suggesting that diminished NF κ B activation hampers the IL-5 expression. p50 plays an essential role in IL-5 production (Barnes and Karin 1997; Yang et al. 1998; Das et al. 2001). Diminished activation of NF κ B activation including p50 may decrease IL-5 production. Our data suggested that neither strong NFAT- nor strong MAPK- pathway activation is required for IL-5 production. However, our data also do not exclude the possibility that NFAT and MAPK proteins are not required for IL-5 production. NFAT-binding sites are present in the *Il5* promoter region, and previous data demonstrate that NFAT plays important roles in IL-5 production (De Boer et al. 1999). Certain amounts of these proteins would be sufficient for IL-5 expression.

Our data demonstrate that Rel molecules downregulate IL-5 production. This seems to conflict with a previous report showing that activation of the NF κ B pathway enhances IL-5 production (Inami et al. 2004). This enhancement was mediated by a locus controlled by histone hyperacetylation of the *Il5* locus. In the report, NF κ B molecule(s) involved in this enhancement were not identified. There is the possibility that this can be attributed to the presence of p50 and/or p52. Our data suggest that Rel effects on the production of IL-5 are indirect. It is still possible that the NF κ B pathway controls accessibility to the gene locus during functional development of T cells and modulates expression after functional maturation.

The regulation of IL-5 is thought to be important for eosinophilic allergic responses. Thus far, the specific regulation of IL-5 is only mediated by mAb. Although the precise mechanisms of IL-5 production have to be clarified, our data shed light on new targets for IL-5 regulation.

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