

Generation of *IL10* and *TGFB1* coexpressed mice displaying resistance to ovalbumin-induced asthma

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Abstract Asthma is a common chronic inflammatory disease in the airways with wide prevalence, and it is thought to be caused by the combinational factors in environment and genetics. A large body of studies has suggested that cell immunity played a vital role in regulating the airway hyperreactivity (AHR) and inflammation. Therefore, we here developed a mouse model of asthma by microinjecting the pronucleus with a vector spontaneously coding human *IL10* and *TGFB1* gene to explore the possible interaction between these two potent molecules during asthma progression. From the total 35 newborn mice, we successfully obtained 3 founders expressing exogenous genes. In the transgenic mice, we observed profoundly enhanced expression of *IL10* and *TGFB1*. In the condition of ovalbumin challenge, transgenic mice displayed a 1.9-fold higher MCh50 score than wild-type counterparts, indicating reminiscent AHR. Meanwhile, a three-fold decrease of cell counts in bronchoalveolar lavage fluid (BALF) was recorded as well. These results suggested that *IL10* and *TGFB1* cooperatively protected the respiratory system in response to antigenic stimulus. To interrogate the respective

behaviors of the two genes, we quantified the expression of downstream genes in *IL10* signaling or *TGFB1* signaling. We observed that the examined genes in *IL10* signaling were significantly repressed, especially *IL5*, which showed 5.4-fold decreased expression. Most genes were not altered in *TGFB1* signaling, and the production of endogenous *TGFB1* was significantly inhibited. These evidences collectively proved that the activation of *IL10* and *TGFB1* protected the host from antigen-induced asthma, possibly through *IL10* signaling. This study shed some light on the modulations of *IL10* and *TGFB1*, and related networks to asthma progression.

Keywords Asthma · *IL10* · *TGFB1* · Mouse model

Introduction

Asthma is a non-communicable disorder of the airway characterized by airway hyperresponsiveness and airflow limitation, affecting more than 200 million people (Patella 2015). Increasing evidence of factors provoking asthma has been reported (Al-Khateeb and Al Khateeb 2015; Zhang et al. 2015), but the details of the underlying mechanisms remain unknown. In the aspect of genetic predisposition, substantial advances have been achieved in the last decade. Data indicated that *TGFβ* signaling and several interleukin pathways were implicated in the

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pathogenesis of asthma (Makinde et al. 2007; Olin and Wechsler 2014). These modulators elicit some clinical potentials to improve asthma care (Olin and Wechsler 2014).

The transforming growth factor β (TGF β), a superfamily of small-secreted homodimeric proteins involved in pro-inflammatory responses and the remodeling of several organs and tissues, comprises of three isoforms, TGF β 1, TGF β 2 and TGF β 3 (Hinck 2012). Among the family members, TGF β 1 was extensively studied. In asthma patients, TGF β 1 was described to be significantly higher (Kokturk et al. 2003). However, increased airway reactivity and inflammation was observed in a murine model of impaired TGF β signaling (Schramm et al. 2003). These conflicting findings indicated that TGF β functioned distinctly in the pathogenesis of asthma, ascribing to the complexity of the signaling (Duvernelle et al. 2003; Al-Alawi et al. 2014).

Interleukins (ILs) are a large group of cytokines involved in immune responses, inflammatory reactions, and hematopoiesis (Sims et al. 1988). Regarding asthma, different interleukins exerted distinct effects. Blocking IL4, IL5, and IL13 showed remission in both eosinophilic and noneosinophilic asthma (Dunn and Wechsler 2015). By contrast, IL10 and IL12 were proven to prevent AHR, reduce infiltration of eosinophils and neutrophils, and suppress other interleukins, such as IL4 and IL5 (Hsu et al. 2010). In a study of virus-induced asthma, IL10 expression was found to be up-regulated (Grissell et al. 2005). Collective clues suggested that IL10 appeared to be a robust upstream modulator of AHR and asthma, perhaps via interacting with other cytokines.

To explore the role of the aforementioned pathways in asthma, we microinjected a vector containing a cassette encoding human *TGFB1* and *IL10* gene linked by a 2A peptide into the mouse pronucleus, and prepared transgenic mice. Transgenic mice showed inhibited AHR and reduced BALF counting when exposed to OVA, indicating that spontaneous expression of *hIL10* and *hTGFB1* conferred protective effects in mice. Furthermore, we discovered that the IL10 signaling was activated, while TGFB1 signaling not changed. This differential activation of the two pathways could be switched by the behavior of IL5, which was a crucial mediator in the both pathways.

Materials and methods

Design and construction the expression vector

An expression vector containing the human *IL10* (Accession number: NM_000572.2) and *TGFB1* (Accession number: NM_000660.5) cDNA was designed as follows. Total RNA was extracted from in vitro cultured human fibroblast cells by using the TRIzol reagent (Life technologies, USA) and was then reverse-transcribed into cDNA following the instruction of a PrimeScript 1st cDNA Synthesis Kit (Takara, Japan). Subsequently the cDNA encoding *hIL10* or *hTGFB1* was cloned under the following conditions: denaturation at 95 °C for 5 min, 32 cycles of annealing at 60 °C for 30 s and extension at 72 °C for 1 min followed by a final extension at 72 °C for 10 min, using primers in Table 1. Be worthy of note, a fragment encoding 2A peptide derived from porcine teschovirus-1 (Kim et al. 2011), was chemically synthesized and incorporated in the 5' of the *hTGFB1* reverse primer. Therefore, a fragment containing 2A-*hTGFB1* would be amplified.

The *hIL10* fragment was inserted into the pcDNA-3.1 (+) plasmid by the *EcoRI* and *NheI* digestion. The 2A-*hTGFB1* fragment was integrated into the downstream of *hIL10* by *NheI* and *XhoI* digestion. Hence, the destine vector was generated, designated as pCMV-*hIL10*-2A-*hTGFB1*, depicted as Fig. 1. The CMV-*hIL10*-2A-*hTGFB1* vector was cut for linearization by *AhdI* and purified by the Endo-free plasmid mini kit (OMEGA, USA). The linearized vector was stored in −20 °C for microinjection.

Generation of transgenic mouse by pronuclear microinjection

Six to eight week-old C57 female mice were super-ovulated by injection of 10 IU PMSG (pregnant mare serum gonadotropin) and 10 IU hCG (human chorionic gonadotropin) (Ningbo second hormone factory, Ningbo) 48 h later and then mated with male mice. Zygotes were collected in which male pronucleus was microinjected with 5 ng/ul of the CMV-*hIL10*-2A-*hTGFB1* vector. The injection dosage was empirically determined, based on the criteria of more than 50 % embryos alive post-injection. After injection, embryos were surgically transferred into the uterus of pseudo-

Table 1 Primers for gene cloning, transgenesis identification and qPCR

Name	Sequence (5'–3')	T _m (°C)	Product length (bp)
<i>For molecular clone</i>			
hIL10-F1	CGAgaattcATGCACAGCTCAGCACTGCTCTGTT	60	552
hIL10-R1	CCGgctagcTCAGTTTCGTATCTTCATTGTCA		
hTGFB1-F1	CCGgctagc <u>GGAAGCGGAGCTACTAACTTCAGCC</u> <u>TGCTGAAGCAGGCTGGAGACGTGGAGGAGAA</u> <u>CCCTGGACCTATGCCGCCCTCCGGGCT</u>	60	1257
hTGFB1-R1	CAGctcgagTCAGCTGCACTTGCAGGAGC		
<i>For transgenesis identification and qPCR</i>			
hIL10-F2	GACATCAGGGTGGCGACTCT	60	152
hIL10-R2	GGGGGTTGAGGTATCAGAGGT		
hTGFB1-F2	CCTGCAAGACTATCGACATGGA	60	383
hTGFB1-R2	CCTCAGCAGACGCAGCTCTG		
IL5-F	TGGGGGTACTGTGGAAATGC	60	162
IL5-R	CCTTCCATTGCCCACTCTGT		
IL6-F	TCTTGGGACTGATGCTGGTG	58	155
IL6-R	CATTTCCACGATTTCACAGA		
mTGFB1-F	CGCGAAGACTTCACCCCAAAG	57	242
mTGFB1-R	GTAAAAATCGAGATGAGCGCT		
COL1A1-F	AACTTTGCTTCCCAGATGTCC	59	333
COL1A1-R	GCCTCGGTGTCCCTTCATT		
Fn1-F	GCCAACTCCGTCACCCTC	58	244
Fn1-R	GCAGGACCTCCACACGGTA		
PDGFA-F	CCAGGACGGTCATTTACGAG	59	274
PDGFA-R	CGATGGTCTGGGTTCAGGTT		
LAMA1-F	ATCAACAACACGGCGGTCA	60	217
LAMA1-R	GGCCCTCAGCTCTTGTGCT		
GAPDH-F	AACTTTGGCATTGTGGAAGG	60	223
GAPDH-R	ACACATTGGGGGTAGGAACA		

The restriction endonuclease sites are in lowercase; the 2A sequence is underlined

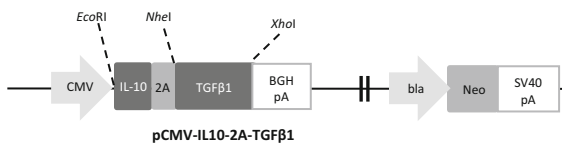


Fig. 1 Schematic representation of the vector for *IL10* and *TGFB1* integration. *CMV* cytomegalovirus promoter, *BGH pA* bovine growth hormone polyadenylation signal, *bla* an ampicillin resistance promoter, *Neo* a neomycin selection marker, *SV40 pA* simian virus 40 polyadenylation signal

pregnant female mice. Mice were housed in a specific pathogen-free facility with ad libitum access to food and water.

Identification of transgenic mouse

The toe clips of newborn mice were lysed and DNA was extracted by the phenol–chloroform method. PCR was performed by using primer hIL10-F2/R2 and hTGFB1-F2/R2 listed in Table 1. PCR parameters were: denaturing at 95 °C for 5 min, 32 cycles of annealing at 60 °C for 30 s and extension at 72 °C for 1 min followed by a final extension at 72 °C for 10 min. Afterwards, the PCR products were subjected to electrophoresis on a 2.0 % agarose gel. The PCR products were additionally, cloned into the pMD18-T vector (Takara, China) for Sanger sequencing in order to confirm the transgenesis.

Transcription analysis by qRT-PCR

Total RNA was isolated from the lung tissue by TRIzol reagent according to manufacturer's instructions (Life technologies, USA). Subsequently, RNA was reversely transcribed into cDNA using a first-strand cDNA synthesis kit (Takara, Japan). In the quantification of mRNA expression, 1 μ l of cDNA was used on an ABI 7500 real-time machine (ABI, CA, USA) using SYBR Green reagent (Takara, Japan) and primer pairs listed in Table 1. GAPDH was employed as internal control and used for normalization of Ct values.

Translation analysis by western blot

The lung tissue was lysed by the RIPA Lysis Buffer (Beyotime, China) supplemented with PMSF as protease inhibitor and the lysis solution was denatured at 100 °C for 10 min. 50 μ g of protein was loaded to a polyacrylamide gel and the gel was run at a voltage of 120 V for about 90 min. Then a transferring module was prepared and proteins in the gel were transferred onto a PVDF membrane at a current of 350 mA for 40 min in ice. Successively, the membrane was incubated with 1:1000 diluted primary antibodies: antibody SAB1409249 for IL10, SAB2108510 for TGFB1 and A5441 for β -actin as internal control (Sigma, USA). 1 h later, the membrane were soaked in 1:4000 diluted HRP-conjugated goat anti-mouse IgG or HRP-conjugated goat anti-rabbit IgG for 30 min. Finally, the membrane was incubated with the ECL reagent (Thermo Scientific, USA) for 1 min, and exposed to a X-ray film in the dark room.

Sensitization and measurement of airway reactivity

Sensitization of mice as well as the measurement of airway reactivity were performed in a head-out body plethysmograph as previously described (Schramm et al. 2003). The methacholine (MCh) dose resulting in a 50 % decrease of the tidal mid-expiratory flow (EF50), a marker for airway resistance, was measured. In brief, animals were immunized with 100 μ g of OVA-alum or PBS-alum on days 0 and 14 i. p. On days 25–27, mice were aerosol challenged with 1 % OVA in PBS for 20 min. AHR was measured on day 28 in untreated (PBS-alum) and sensitized (OVA-alum)

wild-type (WT), hIL10-hTGFB1-transgenic (Tg) mice. For this experiment, animals were divided into four groups: matched WT mice untreated (WT, control), WT mice sensitized (WT, OVA), Tg mice untreated (Tg, control), Tg mice sensitized (Tg, OVA).

Collection and counting of BALF

After AHR measurement, BALF was collected as previously described (Schramm et al. 2003). Briefly, the lung and airways were flushed 3 times with 1 ml PBS. Cells were automatically counted with a CASY system according to the manufacturer's instructions.

Statistical analysis

All the data were presented as mean $\pm$ SD. Two-tailed *T* test was performed to evaluate the statistical significance between two means of equal variance. One asterisk (*) indicates statistical difference complying with the criteria $P < 0.05$, and two asterisks (**) indicate $P < 0.01$.

Results

Generation and characterization of transgenic mice

To produce mice over-expressing *hIL10* and *hTGFB1* genes, a vector harboring the cDNA regions of the two genes was injected into 452 male pronucleus, and then 430 living zygotes were surgically transferred into the uterus of 17 pseudo-pregnant female mice, of which 12 were eventually pregnant. After 21 days of gestation, a total of 35 mice were delivered. Newborn pups were genotyped by the primer pairs hIL10-F2/R2 and hTGFB1-F2/R2 (Table 1), respectively located in *hIL10* and *hTGFB1* coding region. Results showed that 4 mice (#12, #13, #15 and #24) carried the both genes, and 1 was male and 3 were female (Fig. 2a).

To confirm the expression of exogenous genes, qPCR and western blotting were performed. As depicted in Fig. 2b, we detected both *IL10* and *TGFB1* mRNA in 3 mice (#12, #13 and #24), but expression was undetectable in #15. In the protein level, three individuals originating from the transgenic founders expressed increased IL10 and TGFB1 proteins compared with matched wide-type counterparts (Fig. 2c).

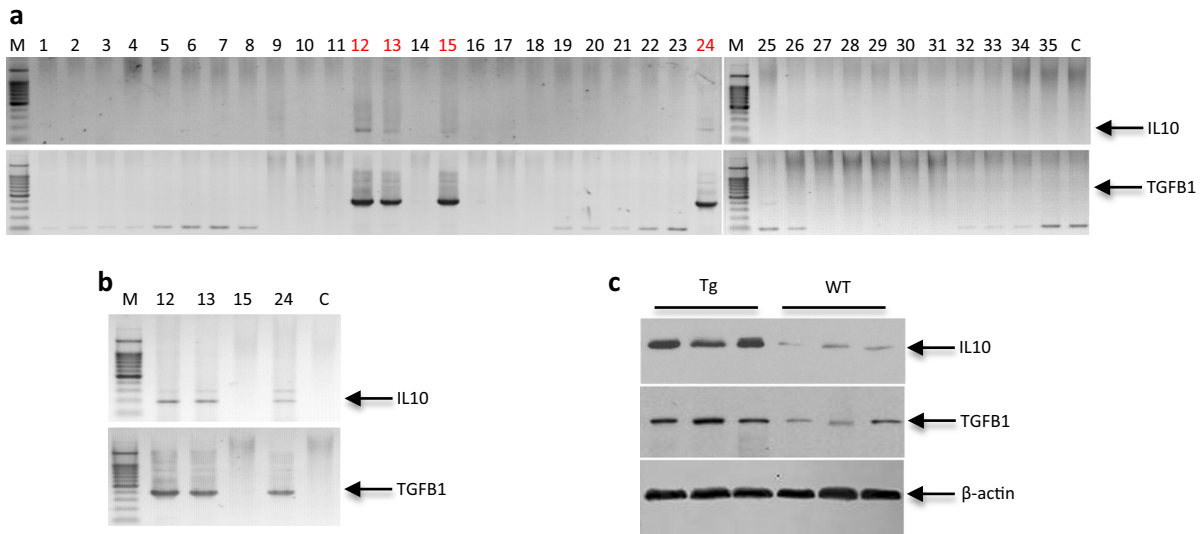


Fig. 2 Identification of transgenic mice. **a** Amplification of exogenous genes from all newborn mice according to their DNA sequence. **b** Identification of RNA expression among the mice harboring exogenous DNA. IDs of transgenic mice are in red. M: 200 bp DNA ladder. **c** Protein characterization of three F2

individuals derived from the transgenic founders and matched wild-type controls. Protein expression was analyzed by western blot method, and β -actin was loaded as endogenous reference. (Color figure online)

Importantly, we observed a protein band at 19 kDa for IL10, 28 kDa for TGFB1. No fused protein was detected, inferring 2A was efficiently cleaved. These results indicated that we generated genetically modified mice with an efficiency of 3/35, in which IL10 and TGFB1 were effectively and separately produced.

Increased resistance to albumin-induced asthma in transgenic mouse

Albumin is a kind of antigen that can cause asthma (Daubeuf and Frossard 2014). To assess the cooperative role of IL10 and TGFB1 in allergic asthma, we determined the AHR representing asthma severity in condition of antigen challenging. Antigen caused a significant increase of AHR (MCh50, WT-OVA: 26.3 ± 2.1 mg/ml vs WT-PBS: 54.2 ± 4.3 mg/ml, $P = 0.005$; Fig. 3a). In contrast, over-expression of IL10 and TGFB1 protected mice from Ag-dependent AHR (MCh50, WT-OVA: 26.3 ± 2.1 mg/ml vs Tg-OVA: 47.7 ± 6.4 mg/ml, $P = 0.014$; Fig. 3a). Accordingly, we also observed transgenesis-related remission characterized by BALF counting: IL-10 and TGFB1 dramatically prevented the antigen caused increase of BALF number (BALF, WT-OVA: $12.63 \pm 1.65 \times 10^5$ /ml vs Tg-OVA: $4.82 \pm 1.26 \times 10^5$ /ml, $P = 0.000$;

Fig. 3b). Here we drew a conclusion that transgenes elicited beneficial effect on the respiratory system in the context of antigen stimulation.

Activation of IL10 signaling not TGFB1 signaling in transgenic mouse

As both IL10 and TGFB1 elicited dual roles in asthma progression (Duvernelle et al. 2003; Al-Alawi et al. 2014; Hsu et al. 2010; Grissell et al. 2005), we were interested in the contribution of each molecule to the disease. Herein, we tested the relative activity of signaling triggered by IL10 or TGFB1 by measuring the expression of several key members in the two pathways.

Generally, IL10 functions as an anti-inflammatory cytokine by inhibiting the release of $\text{TNF-}\alpha$, IL1, IL6 and IL12 (Bogdan et al. 1991). In an in vivo study, IL10 was found to suppress the expression of *IL5* and prevented AHR (Hsu et al. 2010). Compared with the WT counterparts, the expression of all the tested pro-inflammatory cytokines was decreased when exposed to OVA in mice over-expressing *IL10*. Especially, *IL5* showed the maximum suppression, with a 5.4-fold decrease. These data indicated that the over-

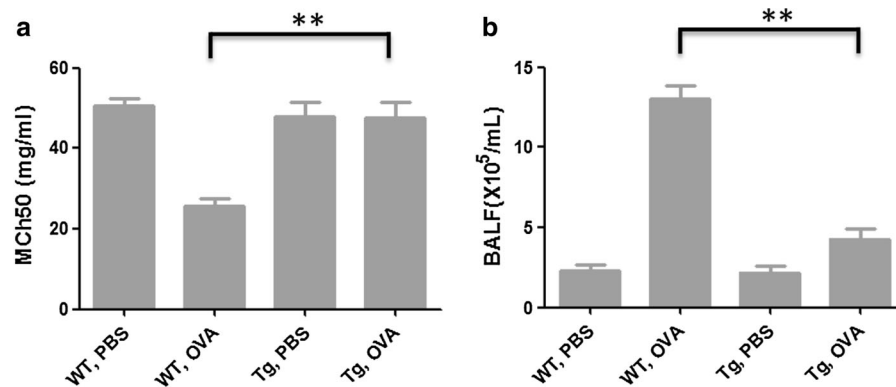


Fig. 3 Analysis of AHR (a) and BALF (b) of transgenic mice and wild-type mice challenged with OVA. The group ‘WT, PBS’ consists of 6 mice, and the other groups each consists of 3

mice. Data is displayed as mean $\pm$ standard deviation; * $P < 0.05$; ** $P < 0.01$

expression of *IL10* effectively suppressed the transcription of the downstream genes.

During the airway remodeling in condition of allergic asthma, *TGFB1* was previously demonstrated that it simulated IL6, mediated proliferation of collagen, fibronectin, platelet-derived growth factor and laminin (Dekkers et al. 2010). Hence, we measured the transcription alterations of *COL1A1*, *Fnl*, *PDGFA* and *LAMA1*. Besides, the expression of *TRAIL* (TNF-related apoptosis-inducing ligand) was also investigated, which was reported to reciprocally activate *TGFB1*. Results showed that the transcription of these genes in the Tg mice was slightly changed than that in WT counterparts, including *COL1A1*, *Fnl* and *LAMA1* gene. In contrast to *IL10*, data here suggested that *TGFB1* did not adequately provoke the transcription of the pathway.

Discussion

Transgenic technology is a valuable and feasible approach to explore the function of interested genes, or to clarify the potential mechanisms of human diseases. Murine disease model by means of gene manipulation was extensively used in genetics. In the present study, we physically injected 430 fertilized eggs with 5 ng/ μ l of the vector coding human *IL10* and *TGFB1* gene, produced 35 newborn mice, and 11.4 % of the pups were successfully integrated with the exogenous genes into the genome. However, in the transcriptional and translational level, 1 individual failed to express the mRNA or protein (Fig. 2b). The

reduced or completely abolished transgene expression was likely to be caused by “position effect”, because the transgene was randomly inserted into a transcription-inactive region of the genome or been regulated by some silencing elements (Palmiter et al. 1982; Overbeek et al. 1986). Notably, we inserted a 2A peptide between the genes to simultaneously over-express them in equal stoichiometry. In the generation of animal disease model, the simultaneous expression of multiple genes using a single construct is sometimes of importance (Daniels et al. 2014). To this end, internal ribosomal entry sites (IRESs) were usually inserted between genes (Szymczak and Vignali 2005). However, IRESs are somewhat large in size and the translation efficiency is much lower for the genes located after the IRES (Kim et al. 2011). To overcome the limitations, 2A peptide, a “self-cleaving” small peptide was developed (Ryan et al. 1991). Asthma is a complicated disease in which many molecules are involved, hence we simultaneously over-expressed two related genes to singularize the phenotype by connecting exogenous molecules with a 2A peptide. The two proteins were successfully separated into two dependent parts after translation with high efficiency, as we detected cleaved proteins and no fused protein was observed (Fig. 2c). In addition, 2A peptide confers the equal proteins in stoichiometry of the two linked genes, which is ideal for the interaction survey of interested genes. As a result, transgenic mice showed increased resistance to antigen-induced airway disability and relieved inflammation in the lungs.

Regarding asthma, interleukins and *TGFB* are able to control inflammation and AHR in different manners

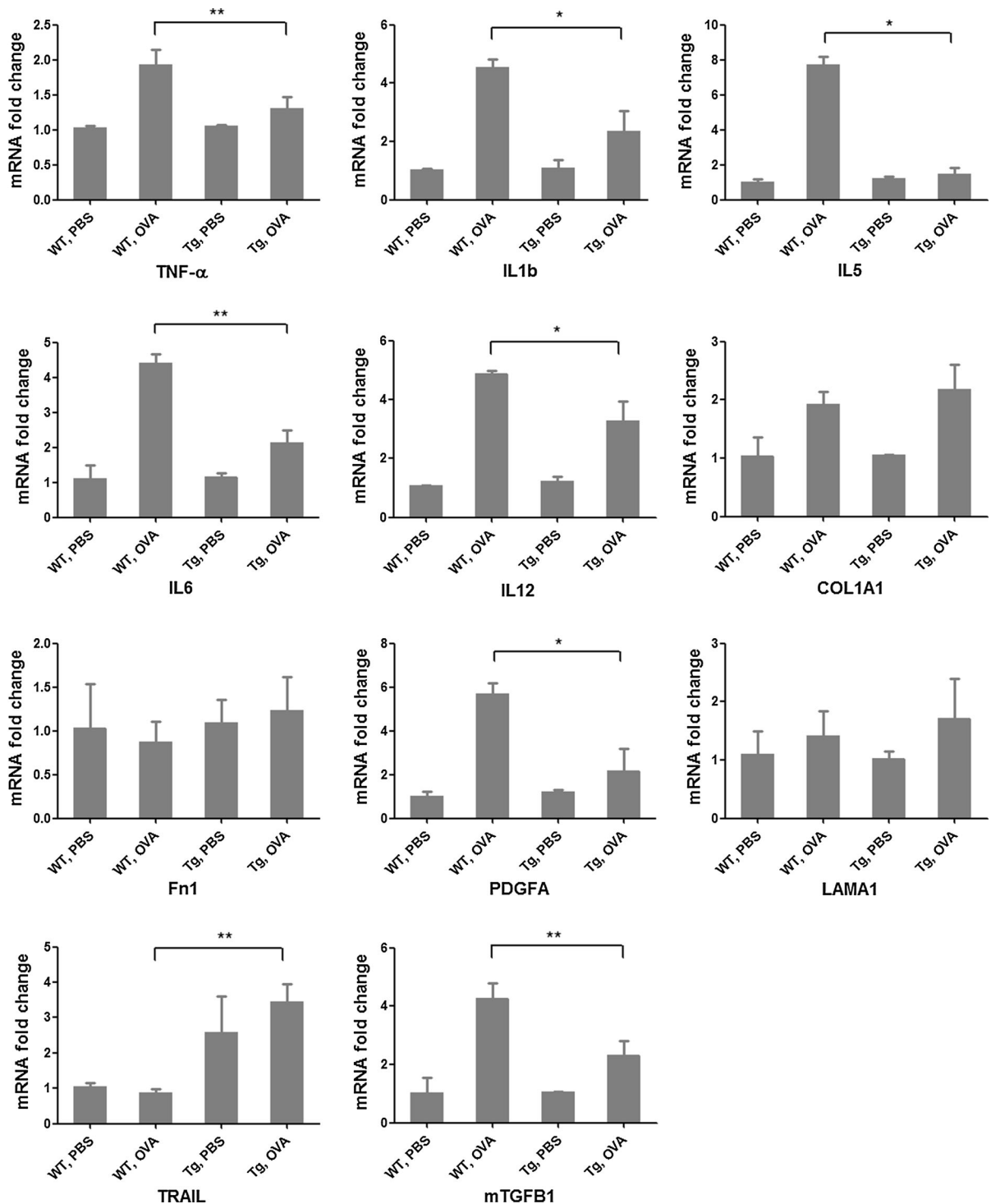


Fig. 4 Profiling of downstream genes in IL10 pathway or TGFβ1 pathway. QRT-PCR was performed to quantify the gene expression in the lungs of mice. The group 'WT, PBS' consists

of 6 mice, and the other groups each consists of 3 mice. *Gapdh* was chosen as endogenous reference. Data is displayed as mean $\pm$ standard deviation; * $P < 0.05$; ** $P < 0.01$

and pathways (Al-Alawi et al. 2014; Bentley et al. 1996). IL10 functions as an anti-inflammatory cytokine in various immune cells by inhibiting the release of pro-inflammatory cytokines, such as TNF- α , IL1, IL6 and IL12 (Moore et al. 2001). In the cellular actions, IL10 suppresses the ability of antigen-presenting cells to inactivate T cells and to attenuate eosinophilic airway inflammation (Berkman et al. 1995). By contrast, TGFB exhibits bi-directional regulation to asthma pathogenesis (Duvernelle et al. 2003; Al-Alawi et al. 2014). Notably, TGFB also simulates some interleukins, such as IL6, and is positively modulated by IL5 (Michaeloudes et al. 2011). Therefore, there are some crosstalk between the two transgenes, partly bridged by IL5 and IL6. In the current study, the mouse over-expressing *IL10* and *TGFB1* showed suppressed production of TNF- α , IL1, IL5, IL6, IL12, representing the activation of IL10 triggered pathways. However, most of the TGFB1 mediated factors were unchanged in mRNA transcription, such as *COL1A1*, *Fn1* and *LAMA1* (Dekkers et al. 2010). The produce of biologically inactive transgene protein, or the intervention of other molecules, could explain the unchanged TGFB1 signaling. It was difficult to test the biological activity of exogenous TGFB1 in vivo. Here we observed that IL10 elicited significant repression on *IL5*, *IL6* and other molecules, which was more robust than that simulated by TGFB1. As IL5 induces TGFB1 expression, we speculated that the repression of IL5 expression caused the decreased endogenous mouse TGFB1 (mTGFB1) production, resulting in the inability of TGFB1. Actually, the *mTGFB1* was overtly decreased (Fig. 4). Of course, more direct evidence was required to support the speculation.

In summary, we successfully generated mouse model of allergic asthma by spontaneous insertion human *IL10* and *TGFB1* gene into the mouse genome, reaching a integrating efficiency of 11.4 %. Transgenic mouse were feathered in the relieved AHR and reduced BALF counting in the lung. In the molecular clues, downstream genes of IL10 were significantly repressed, while the TGFB1 signaling was not altered. This could be caused, at least partly by the interaction between the two pathways, in which IL5 played pivotal roles. This study demonstrated that IL10 elicited protective effect to allergic asthma through inhibiting its downstream factors, especially IL5. IL5 plays a central role in the activation, proliferation and

survival of eosinophils (Gleich 2000), which modulates the pathobiology of asthma (Nair 2013).

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