

ORIGINAL ARTICLE

Bcl6 in pulmonary epithelium coordinately controls the expression of the CC-type chemokine genes and attenuates allergic airway inflammation

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Summary

Background There is synteny in the CC-type chemokine gene clusters between humans (CCL2/MCP-1, CCL7/MCP-3, CCL11/eotaxin, CCL8/MCP-2, CCL13/MCP-4, and CCL1/I-309) and mice (CCL2, CCL7, CCL11, CCL12/MCP-5, CCL8, and CCL1).

Objective As many putative Bcl6/STAT-binding sequences are observed in the clusters, we examined the roles of a transcriptional repressor Bcl6 and the regional histone modification in the expression of these chemokine genes in pulmonary epithelium.

Methods We generated transgenic (Tg) mice carrying the *Bcl6* or the dominant-negative (*DN*)-*Bcl6* gene under the control of the surfactant protein C (SPC) promoter that induces the exogenous gene expression in the distal lung epithelium. For *in vitro* studies, A549, alveolar type II-like epithelial cell line transfected with the *SPC-DN-Bcl6* gene were stimulated with IL-4 + TNF- α , and Bcl6 or STAT6 binding to and histone modification of the cluster in the transfectants were analysed by chromatin immunoprecipitation assays. Tg mice sensitized with ovalbumin (OVA) were challenged with OVA inhalation. The amounts of mRNAs in each sample were analysed by quantitative RT-PCR.

Results The amount of Bcl6 bound to the cluster decreased in A549 cells stimulated with IL-4 and TNF- α , whereas STAT6 binding increased in association with regional histone H3-K9/14 acetylation and H3-K4 methylation. The expression of all chemokine genes in the gene cluster was augmented in activated A549 cells transfected with the *DN-Bcl6* gene. We also induced allergic airway inflammation in Tg mice. Expression of the chemokine genes and infiltrated cell numbers in the lungs of these Tg mice with allergic airway inflammation were inversely correlated with the amount of Bcl6 in the lungs.

Conclusion and Clinical Relevance Expression of the pulmonary epithelium-derived CC-type chemokine genes in the cluster is orchestrated by the conserved machinery related to Bcl6. Thus, Bcl6 in pulmonary epithelium may be a critical regulator for pathogenesis of various pulmonary inflammatory diseases.

Keywords allergy, Bcl6, chemokines, transcription factors, transgenic/knockout mice

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Introduction

Histone acetylation is generally associated with transcriptional activity, whereas histone deacetylation leads to gene silencing. The balance between histone acetylation induced by histone acetyltransferases and histone deacetylation induced by the histone deacetylases serves as a key epigenetic mechanism for transcriptional factor-dependent gene expression. Histone modifications such as acetylation have a major influence on chromatin structure

by affecting the local environment, facilitating the binding of transcriptional factors to expression of their target gene [1]. The transcriptional repressor Bcl6 is well conserved between humans and mice at the amino acid level [2, 3]. The NH₂-terminal half of the protein contains the BTB/POZ repressor domains [4] and the Krüppel-type zinc finger motifs in the COOH-terminal region bind to specific DNA sequences [5]. The BTB/POZ domain can recruit the histone deacetylase complex to silencer regions to repress gene expression [6]. This gene is expressed in various

tissues including the lungs, and Bcl6-deficient (Bcl6-KO) mice displayed T helper type 2 inflammation, including eosinophilia in multiple organs, especially the lungs [7, 8].

There are about 40 kinds of chemokines at present, and these proteins have been divided into subfamilies (CXC, CC, C, and CX3C). As chemokines play important roles in inflammatory diseases, chemokines derived from pulmonary epithelium contribute to the migration and accumulation of diverse cells in lungs [9]. Thus, pulmonary epithelium of Bcl6-KO mice may produce excess amounts of chemokines that are involved in the development of T_H2-type inflammation in Bcl6-KO mice. However, the roles of Bcl6 in the regulation of chemokine expression from pulmonary epithelium have never been reported. Recently, we have found synteny of the CC-type chemokine genes in the clusters between humans and mice. It has been reported that the production of CCL2 and CCL7 in the cluster is augmented in macrophages of Bcl6-KO mice and CCL2 is known as a target gene for Bcl6 [10]. However, the regulatory mechanism of others in the cluster by Bcl6 is unclear. As we found multiple Bcl6/STAT-binding sequences outside of the CCL2 gene in the clusters, Bcl6 may coordinately regulate the expression of these chemokine genes in the clusters of both humans and mice. Here, we prove that Bcl6 controls pulmonary inflammation through coordinated negative regulation of the CC-type chemokine genes in the cluster of pulmonary epithelial cells.

Materials and methods

Mice

Bcl6-KO mice were backcrossed to the C57BL/6J strain more than 10 generations [11]. SPC-FL-Bcl6 Tg and SPC-DN-Bcl6 Tg mice were generated using the conventional methods. Briefly, the *mBcl6* cDNA (2.8 kb) and its dominant-negative cDNA (*DN-Bcl6*) (0.8 kb) were subcloned into pcDNA3 to generate *pcDNA3-FL-Bcl6* and *pcDNA3-DN-Bcl6*, respectively [12, 13]. The *FL-Bcl6* cDNA or *DN-Bcl6* cDNA was inserted into the *Bam*HI/*Bam*HI site between the 3.7 kb human surfactant protein C (SPC) promoter and upstream from SV40 intron and polyadenylation signal sequences in a plasmid [14, 15] (a generous gift from Gary Hoyle, Tulane University Health Science Center). The 6.6 kb *SPC-FL-Bcl6* and the 5.1 kb *SPC-DN-Bcl6* constructs were released by digestion with BssHII and purified by agarose gel electrophoresis. A 3 ng/mL solution of the linear construct was micro-injected into pronuclei of fertilized (C57BL/6×SJL) F2 eggs. Mice that developed from injected eggs were genotyped by a PCR reaction for the SV40 intron. Each transgenic (Tg) mouse was backcrossed onto the C57BL/6J strain more than 10 generations. Mice were maintained under specific pathogen-free conditions in the animal centre of the Graduate School of Medicine, Chiba Uni-

versity (Chiba, Japan). The care of all animals used in the present study was in accordance with Chiba University Animal Care guidelines.

Cell culture, gene transfection, and stimulation of stable transfectants

A549, human alveolar type II-like epithelial cell line, obtained from American Type Culture Collection (ATCC), was cultured in DMEM (Sigma, St. Louis, MO, USA) containing 10% FCS (Sigma), penicillin G, and streptomycin sulphate at 37 °C in 5% CO₂. The co-transfection protocol was as follows: A549 cells were plated in six-well culture dishes and incubated in a medium without antibiotics following overnight growth. The next day, FuGENE 6 Reagent (Roche Diagnostics, Indianapolis, IN, USA) was diluted in OptiMEM (Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) (9 µL FuGENE 6 Reagent+200 µL OptiMEM for one condition). The diluted FuGENE 6 transfection reagent was incubated for 5 min at room temperature. Next, 209 µL of this mixture was added to 3 µg plasmid DNA [*SPC-DN-Bcl6* construct or *SPC* construct (empty control)] and 0.3 µg *pcDNA3* vector plasmid (containing the Neomycin resistance gene). After the incubation period, the FUGENE-6/plasmid DNA mixture was added to A549 cells and incubated at 37 °C in 5% CO₂. Twenty-four hours after co-transfection, stable transfectants were selected in Geneticin (G418; Wako, Osaka, Japan) at a concentration of 1200 µg/mL. Thereafter, the selection medium was replaced every 3 days. After 2 weeks of selection in G418, clones of resistant cells were isolated and allowed to proliferate in a medium containing G418 at 600 µg/mL. The integration of co-transfected plasmid DNA was confirmed by PCR analysis and Northern blot analysis. Stable transfectant cells were stimulated with IL-4 (10 ng/mL; PeproTec Inc.)+TNF-α (50 ng/mL; PeproTec Inc., Rocky Hill, NJ, USA) for 16 h or with IL-1β (10 ng/mL; PeproTec Inc.) for 6 h as described elsewhere [16, 17].

Chromatin immunoprecipitation assay

Putative Bcl6-binding sites (5'-TTC[T/C][T/A][G/A]GAA-3') as the γ-activated site (GAS) motif were analysed using Match public version (BIOBASE Biological Databases GmbH). In this study, the GAS motif was also considered to be putative STAT-binding sites [18]. Many putative Bcl6-binding sequences, all of which are completely identical or highly homologous to the consensus sequence, were detected in the clusters of human and mouse genes (Fig 1 and Table S1). To analyse chromatin associated with Bcl6 and STAT6, and histone modification in the gene cluster of human, a chromatin immunoprecipitation (ChIP) assay was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) and then conducted according to the modified manufacturer's recommendations as described

[12]. The following antibodies (Abs) were used: two kinds of Bcl6-specific rabbit polyclonal Abs (Cell signaling technology, Danvers, MA, USA), anti-STAT6-specific rabbit polyclonal Abs (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-acetylated histone H3-K9/14 Abs (UpState Biotechnology), anti-mono/di/trimethylated histone H3-K4 Abs (UpState Biotechnology). Normal rabbit IgG Abs (Santa Cruz Biotechnology) was used as a control. After precipitation, the supernatant from the 'no-Abs' sample was analysed as an unfractionated input chromatin.

Immunoprecipitated chromatin DNA samples were analysed by PCR using primers (Table S2) designed to amplify all the putative binding sites and C1000 Thermal Cycler (Bio Rad, Benicia, CA, USA). After an initial 7-min incubation at 95 °C, 27 cycles of PCR were performed using the following conditions: denaturation at 95 °C for 1 min, annealing at 56 °C for 1 min, and polymerization at 72 °C for 1 min. PCR products were analysed by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining.

Allergic airway inflammation and cytokine production by splenocytes

Mice were sensitized intraperitoneally with ovalbumin (OVA) (Sigma-Aldrich, St Louis, MO, USA) (10 µg) adsorbed on an aluminium hydroxide Gel (Alum) (Sigma-Aldrich) (4 mg) on days 0 and 5. The sensitized mice were challenged with 1% OVA inhalation for 30 min on day 14 or in some experiments on days 14 and 15. Bronchoalveolar lavage (BAL) was performed on day 16 (supplemental methods). Seventeen days after the last sensitization, splenocytes were cultured in the presence or absence of OVA (500 µg/mL). Supernatants were harvested 72 h later and stored at -80 °C.

Bronchoalveolar lavage

After mice were euthanized with pentobarbital sodium (Nembutal, Dai-Nippon Pharmaceuticals, Osaka, Japan), BAL was immediately performed with six serial injections of phosphate-buffered saline (PBS) (0.5 mL) and aspiration of the fluid. BAL fluid was centrifuged and the supernatant was stored at -80 °C. Each cell pellet was resuspended in PBS for cell counting. After cytopinning, preparations were stained with Diff-quick (Sysmex International Reagents, Kobe Japan) for the analysis of differential cell counts.

Preparation of total RNA and cDNA from bronchoalveolar lavage cells, lungs, and spleen

After BAL was performed, BAL cells, right four lobes of the lungs, and the spleen were stored at -80 °C for a few days. The lungs and spleen were thawed at 0 °C in TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and total

RNA was extracted using a modified guanidine-isothionate-phenol-chloroform method as recommended by the manufacturer's instructions. Five µg total RNA were immediately reverse-transcribed using a Superscript III Reverse Transcriptase (Life Technologies) and oligo(dT) (Amersham Pharmacia Biotech, Piscataway, NJ, USA) as recommended by the manufacturer's instructions.

Real-time quantitative reverse transcription-polymerase chain reaction

A real-time PCR was carried out using an ABI/PRISM 7000 sequence detection system and analysed using ABI PRISM 7000 SDS v1.0 software (Applied Biosystems, Carlsbad, CA, USA). PCR conditions were as follows: 4 µL of cDNA were mixed with 12.5 µL of Power SYBR Green PCR Master Mix (Applied Biosystems) and 1.5 µL of primer mix (15 pmol) and adjusted to 25 µL. The thermal cycler conditions were followed as per the manufacturer's instructions (Applied Biosystems). Standard curves for the value of mRNA were generated using cDNA from A549 cells or murine splenocytes. The amplification of standard cDNA and sample cDNA was carried out in 96-well reaction plates. Each plate always contained the same standard. The cycling conditions included a hot start at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Product accumulation was monitored on the basis of SYBR green fluorescence. mRNA levels were expressed as the absolute number of copies normalized against β -actin mRNA. The primers used were as follows: mouse β -actin: 5'-CCAGCCTTCCTTCTTGGGTAT-3' (forward), 5'-TGGCATAGAGGTCTTTACGGATGT-3' (reverse); human β -actin: 5'-GGATGCAGAAGGAGATCACTG-3' (forward), 5'-CGATCCACACGGAGTACTTG-3' (reverse); *FL-Bcl6*: 5'-CCGGCTCAATAATCTCGTGAA-3' (forward), 5'-GGTGCGTAGAGTGGTGAGTGA-3' (reverse); *DN-Bcl6*: 5'-CCCTGTGAAATCTGTGGCACTC-3' (forward), 5'-ACACGCGGTATTGCACCTTG-3' (reverse); human *CCL2*: 5'-CAATCAATGCC CAGTCAC (forward), 5'-GATTCTTGGGTGTGGAGTG (reverse). We used primers for *CCL2* (mouse), *CCL7* (mouse and human), *CCL11* (mouse and human), *CCL8* (mouse and human), *CCL1* (mouse and human), *CCL13* (human), *CCL12* (mouse), and *CCL17* (human and mouse) from TAKARA products. A primer pair of *FL-Bcl6* was used for the sequence of the 5' side of Bcl6 outside of the *DN-Bcl6* region to distinguish *FL-Bcl6* from *DN-Bcl6*. A primer pair of *DN-Bcl6* could also detect *FL-Bcl6*. *CCL17* mRNA was analysed as a negative control because we could identify no Bcl6 consensus-binding sequence within 10 kb of flanking regions of the *CCL17* gene.

Enzyme-linked immunosorbent assay for chemokines and cytokines

The concentration of CCL2 and CCL11 in BAL fluids was measured using the Quantikine Mouse JE/MCP1

Immunoassay kit (R&D, Minneapolis, MN, USA), and the amounts of IL-4 and IFN- γ in splenocyte culture supernatants were measured using the OptEIA ELISA sets (BD PharMingen, San Diego, CA, USA) according to the manufacturer's instructions.

Histological examination

After BAL, the left lobes of the lungs were extracted from mice, washed with PBS, and fixed with 4% formaldehyde in sodium phosphate buffer for more than 2 days at room temperature. After fixation, the lungs were embedded in paraffin and haematoxylin-eosin staining was performed.

Statistical analysis

Values are expressed as mean + SD. Differences between two groups were examined for statistical significance using StatView5.0 (the Students *t*-test). Data were considered to be significantly different when the *P*-value was <0.05.

Results

Synteny of the CC-type chemokine genes in the clusters between humans and mice, and their coordinative expression in lungs

Many CC-type chemokine genes are clustered on the chromosome 17q11.2 of humans and on the 11q21.2 of mice. These clusters include genes encoding *CCL2*, *CCL7*, *CCL11*, *CCL8*, *CCL13*, and *CCL1* of humans and *CCL2*, *CCL7*, *CCL11*, *CCL12*, *CCL8*, and *CCL1* of mice (Fig. 1). As we identified a multiple region of consensus Bcl6-binding sequences in the clusters of human and mouse genes, respectively, we investigated the levels of these chemokine mRNAs in the lungs from naive Bcl6-KO and wild-type (WT) mice by qRT-PCR. Bcl6-KO mice showed higher

amounts of these chemokine mRNAs while *CCL12* mRNA was not detected in both mice (Fig. 2). Expression of the *CCL17* gene, which is located outside of the cluster (chromosome 8 in mice), was also augmented in Bcl6-KO mice as compared with WT mice. However, Bcl6-binding sequences were not detected around and within the *CCL17* gene, suggesting that increment of the CC-type chemokine expressions in the lungs of Bcl6-KO mice is caused by the secondary effect of Th2-type inflammation.

The relation between Bcl6-binding and histone modification in the CC-type chemokine gene cluster of A549 cells

We investigated Bcl6 binding to these putative sequences in the cluster of A549 cells by ChIP assays. Bcl6 binding was observed in these sites throughout the cluster of A549 cells at a resting state (Fig. 3). No overt PCR products were detected in a control ChIP assay using normal rabbit IgG Abs. The major Bcl6-binding sites were in the region downstream from *CCL11* to upstream from *CCL13*. When A549 cells were stimulated with IL-4 and TNF- α , which is known to induce the expression of *CCL11*, *CCL13*, and *CCL17* mRNA [16, 17, 19], Bcl6 binding to these sites throughout the cluster decreased 16 h after stimulation.

We attempted to examine the relation between Bcl6 binding and histone modification in the cluster of A549 cells by overexpression of the *Bcl6* gene. However, we could not generate any stable transfectants with the *SPC-FL-Bcl6* gene because of the apoptosis induction by Bcl6 overexpression [20]. Thus, as an alternative, we attempted to reduce Bcl6 function in A549 cells by overexpression of the *DN-Bcl6* gene. The *SPC-DN-Bcl6* gene encodes the DNA-binding domain but not the repressor domain of Bcl6, and thus its product (DN-Bcl6) competitively inhibits binding of Bcl6 to the target sites [12, 13]. A549 cells were transfected with the *SPC-DN-Bcl6* or *SPC-control* gene and two clones (DN1 and DN2 or CR1 and CR2, respectively) of each stable transfectant were established. These transfectants were stimulated with IL-4 and TNF- α . The amounts of acetylated histone H3-K9/14 and methylated histone H3-K4 in the cluster of those activated transfectants were examined 16 h after stimulation by a ChIP assay. The amounts in both DN1 and CR1 cells after activation were more than those in resting DN1 and CR1 cells (Figs 4a and b). The acetylated histones in DN1 cells were 1.5–2-fold more than those in CR1 cells regardless of the activation (Figs 4a and c). The methylated histones in DN1 cells were 2–4-fold more than those in CR1 cells after activation, while there was no significant difference in the methylated histones between DN1 and CR1 cells at a resting state (Figs 4b and c). In particular, it is of interest that acetylation of histone H3-K9/14 and methylation of histone H3-K4 in the region of downstream from *CCL11* to upstream from *CCL13* were augmented in DN1 cells as

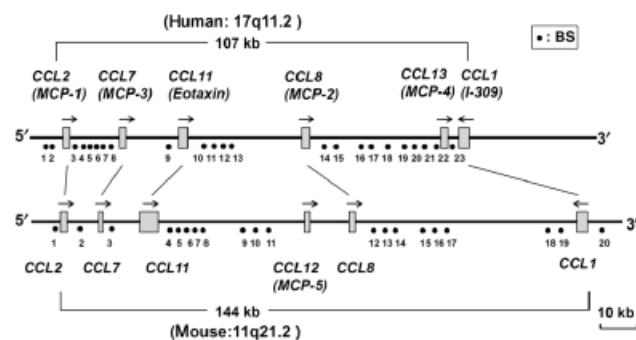


Fig. 1. Chemokine genes and putative Bcl6-binding sites (BS) in the cluster. There is synteny of the CC-type chemokine genes in the clusters between human and mouse genes. The positions of putative BS are indicated as closed circles, with those of humans and mice numbered consecutively from 1 to 23 and from 1 to 20, respectively. Numbering of binding sites begins at the 5' flanking region of the *CCL2*.

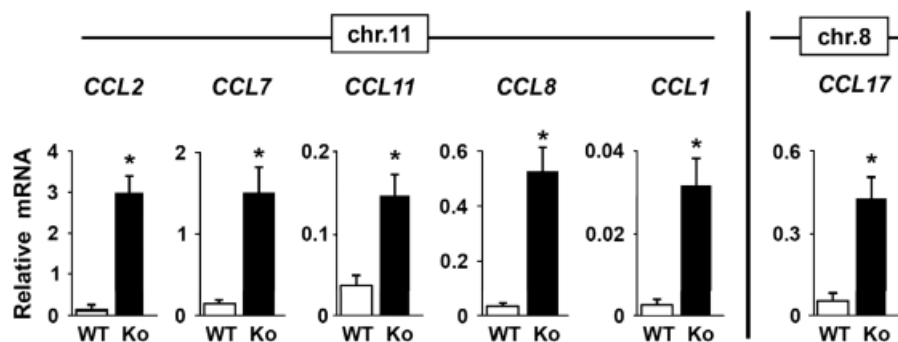


Fig. 2. Expression of the chemokine (*CCL2*, *CCL7*, *CCL11*, *CCL8*, *CCL13*, *CCL1*) genes in the gene cluster on chromosome (chr.) 11 and that of the *CCL17* gene on chr. 8 in lungs of Bcl6-KO mice. The amounts of mRNAs in the lungs from wild-type (WT) (open bar, $n = 4$) and Bcl6-KO mice (closed bars, $n = 4$) were determined by qRT-PCR. The vertical axis indicates the normalized relative intensity of PCR products. Data are presented as the mean $\pm$ SD from two independent experiments. * $P < 0.05$.

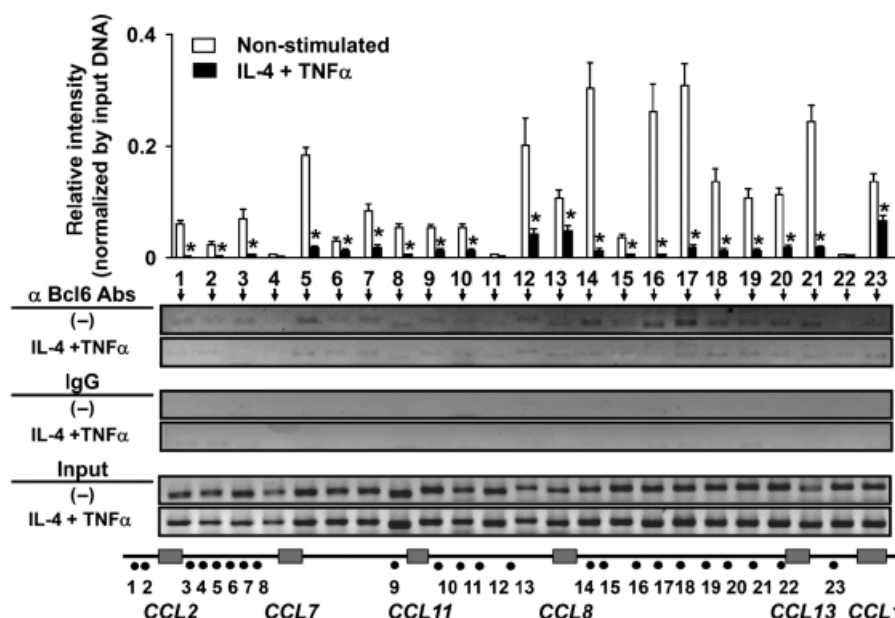


Fig. 3. Bcl6 binding in the cluster of A549 cells. Bcl6 binding was analysed in the cluster of A549 cells before (open bar) and 16 h after (closed bar) stimulation with IL-4 and TNF- α by a ChIP assay. Numbers on the horizontal axis indicate putative Bcl6-binding sites in the cluster shown in the expedient map for the human chemokine gene cluster. The vertical axis indicates the relative band intensities of PCR products (lower panel) normalized by input DNA bands (lower panel) measured by a densitometer. Data are presented as the mean $\pm$ SD from three samples. * $P < 0.05$.

compared with CR1 cells after activation (Fig. 4c), as the region was shown to have extreme binding of endogenous Bcl6 in resting A549 cells. These results suggest that Bcl6 binds to the specific sites throughout the CC-type chemokine gene cluster and inhibits histone acetylation in chromatin of the cluster in pulmonary epithelium, leading to repression of expression of the chemokine genes.

Effect of DN-Bcl6 on expression of the CC-type chemokine genes in the cluster of A549 cells

As Bcl6-binding sequences resemble the sequence motif bound by STAT factors [7], STAT6 binding to these putative Bcl6-binding sites was examined in the transfectants stimu-

lated with IL-4 and TNF- α by ChIP assay. STAT6 binding was not detected on the clusters of both DN1 and CR1 cells before stimulation (data not shown). STAT6 binding was detected on the clusters of both DN1 and CR1 cells after stimulation with IL-4 and TNF- α and the amount in activated DN1 cells was more than that in activated CR1 cells (Fig. 5a). These amounts of STAT6 binding are roughly correlated with the amounts of acetylated and methylated histones in the cluster. These results suggest that STAT6 competitively binds to the Bcl6-binding sites in the cluster of A549 cells activated with IL-4 and TNF- α and induces regional histone modification.

The expression of these chemokine genes (*CCL2*, *CCL7*, *CCL11*, *CCL8*, *CCL13*, and *CCL1*) in the cluster was

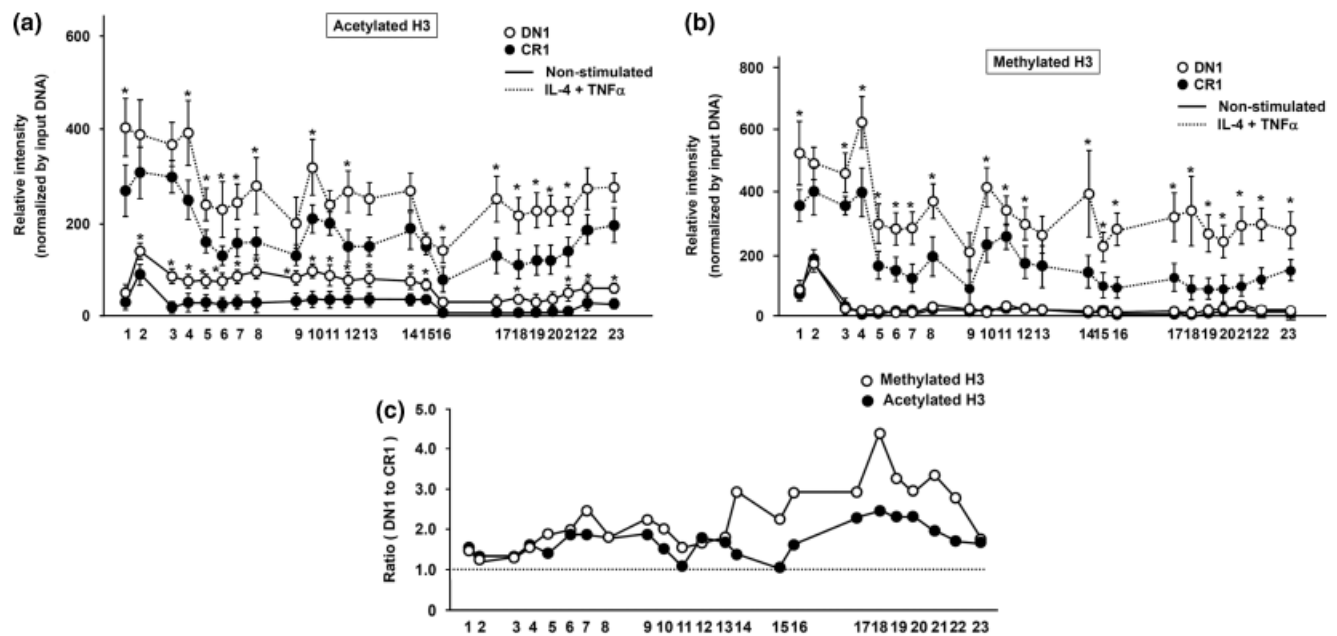


Fig. 4. Effect of DN-Bcl6 on histone modification in the cluster of A549 cells. DN1 and CR1 cells were stimulated with IL-4 and TNF- α . (a and b) Histone modification was analysed in the cluster of DN1 (open circle) and CR1 (closed circle) cells before (solid lines) and 18 h after (broken lines) stimulation with IL-4 and TNF- α by a ChIP assay. Numbers on the horizontal axis indicate putative Bcl6-binding sites in the cluster. ChIP assay for acetylated histone H3-K9/14 (a) and for methylated histone H3-K4 (b). The vertical axis indicates the relative intensity of PCR products normalized by input DNA. Data are presented as the mean $\pm$ SD from three samples. * $P < 0.05$ (c). Ratios of acetylated histone H3-K9/14 (closed circle) and methylated histone H3-K4 (open circle) in DN1 cells to those in CR1 cells after stimulation.

examined in the transfectants stimulated with IL-4 and TNF- α for 16 h or with IL-1 β for 6 h by qRT-PCR. The expression was induced in those cells after stimulation, and the amounts of these mRNAs in activated DN1 and DN2 cells were more than those in activated CR1 and CR2 cells (Fig. 5b). In contrast, the amounts of *CCL17* mRNA in the DN1 and DN2 cells were similar to those in the CR1 and CR2 cells. These results strongly suggest that Bcl6 coordinately regulates the expression of the CC-type chemokine genes in the cluster by inhibiting the regional histone acetylation. We also investigated the effect of Bcl6 down-regulation by siRNA on the chemokine gene expression in A549 cells. The results were similar to those in DN1 and DN2 cells after stimulation (data not shown).

Function of Bcl6 in pulmonary epithelium on antigen-induced airway inflammation

In order to examine the function of Bcl6 in pulmonary epithelium on the expression of the CC-type chemokine genes in the cluster, we generated Tg mice carrying the *FL-Bcl6* or the *DN-Bcl6* gene under the control of the SPC promoter, *SPC-FL-Bcl6* or *SPC-DN-Bcl6*. We confirmed that the expression and functions of the exogenous genes were restricted to the lungs, especially pulmonary epithelium, of *SPC-FL-Bcl6* Tg and *SPC-DN-Bcl6* Tg mice (Fig. S1). Although A549 cells were induced to apoptosis by excess amounts of the *FL-Bcl6* transgene, we found no

apparent histological difference in alveolar structure and integrity in distal lung tissue among these two types of Tg and WT mice before immunization (data not shown).

Expression of the CC-type chemokine genes was analysed in the lungs of immunized Tg mice 48 h after re-challenge with OVA by qRT-PCR. The amounts of these chemokine (*CCL2*, *CCL7*, *CCL11*, *CCL8*, *CCL13*, and *CCL1*) mRNAs increased significantly in the lungs of *SPC-DN-Bcl6* Tg mice compared with those in WT mice (Fig. 6a). The amounts of chemokine mRNAs examined in the lungs of *SPC-FL-Bcl6* Tg mice were less than those in WT mice, although there was no statistically significant difference in gene expression, except for *CCL11* mRNA (Fig. 6b). Furthermore, we confirmed more amounts of *CCL2* and *CCL11* proteins in BAL fluids from *SPC-DN-Bcl6* Tg mice than those in WT mice 48 h after the re-challenge (Fig. 6c). On the other hand, the level of *CCL17* mRNA in the lungs of *SPC-DN-Bcl6* Tg and *SPC-FL-Bcl6* Tg mice showed a tendency towards an increase and a decrease as compared with WT mice, respectively, although there was no significant difference in the expression among these mice (Figs 6a and b). The cell population in BAL fluids from these Tg mice was analysed 48 h after antigen re-challenge. The total cell numbers in BAL fluids from *SPC-DN-Bcl6* Tg and *SPC-FL-Bcl6* Tg mice were more and less than those in WT mice, respectively (Fig. 6d). The numbers of eosinophils in BAL fluids from *SPC-DN-Bcl6* Tg mice and those of eosinophils, macrophages, and lymphocytes in

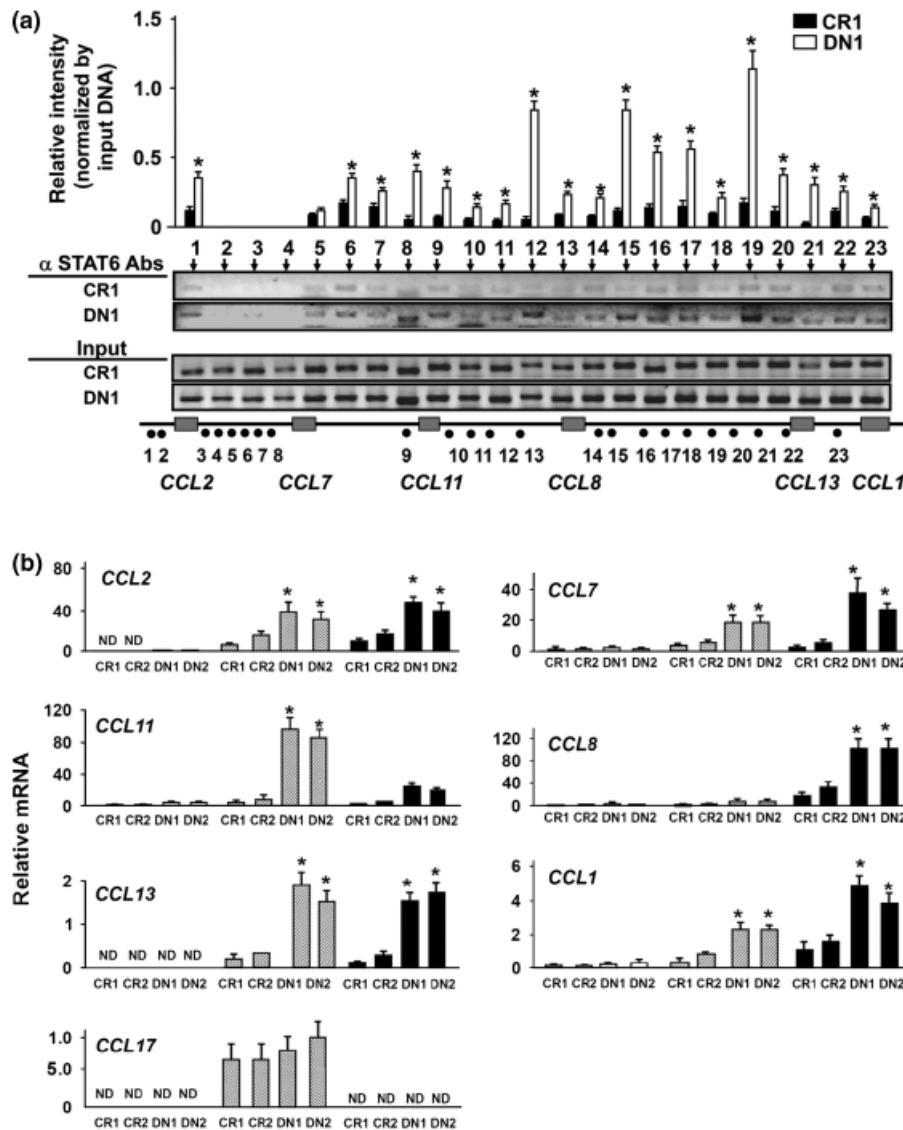


Fig. 5. Effect of DN-Bcl6 on expression of the CC-type chemokine genes in the cluster of A549 cells. DN1, DN2, CR1, and CR2 cells were stimulated with IL-4 and TNF- α . (a) STAT6 binding was detected in the clusters of DN1 (open bar) and CR1 (closed bar) cells 16 h after stimulation by a ChIP assay. Numbers on the horizontal axis indicate putative Bcl6-binding sites in the cluster. The vertical axis indicates the relative band intensities of PCR products (lower panel) normalized by input DNA bands (lower panel) measured by a densitometer. Data are presented as the mean $\pm$ SD from three samples. * P < 0.05. (b) The amounts of chemokine mRNAs were analysed in those cells without stimulation (open bar), 16 h after stimulation with IL-4 and TNF- α (hatched bar), or 4 h after stimulation with IL-1 β (closed bars) by qRT-PCR. Data are presented as the mean $\pm$ SD from three samples. * P < 0.05.

those from SPC-FL-Bcl6 Tg mice were significantly affected. SPC-FL-Bcl6-Tg mice showed pulmonary infiltrates, despite their reduction of inflammatory cells in BAL fluid, similar to those of WT mice 48 h after re-challenge with OVA (data not shown), whereas the pulmonary infiltrates in SPC-DN-Bcl6-Tg mice were greater than those in WT mice (Fig. 6e). Thus, it is strongly suggested that Bcl6 in pulmonary epithelium controls the migration and accumulation of diverse cells in airway inflammation by inhibiting the production of the CC-type chemokines.

Discussion

In this study, we have identified synteny of the CC-type chemokine genes in the clusters between humans and mice. It has been reported that the expression of *CCL2* and *CCL7* in the cluster is augmented in macrophages of Bcl6-KO mice [10]. In addition to these two chemokines, we demonstrated that the expression of all CC-type chemokine genes in the cluster was augmented in the lungs of Bcl6-KO mice. We have identified many putative Bcl6-binding sequences throughout the cluster regions, and

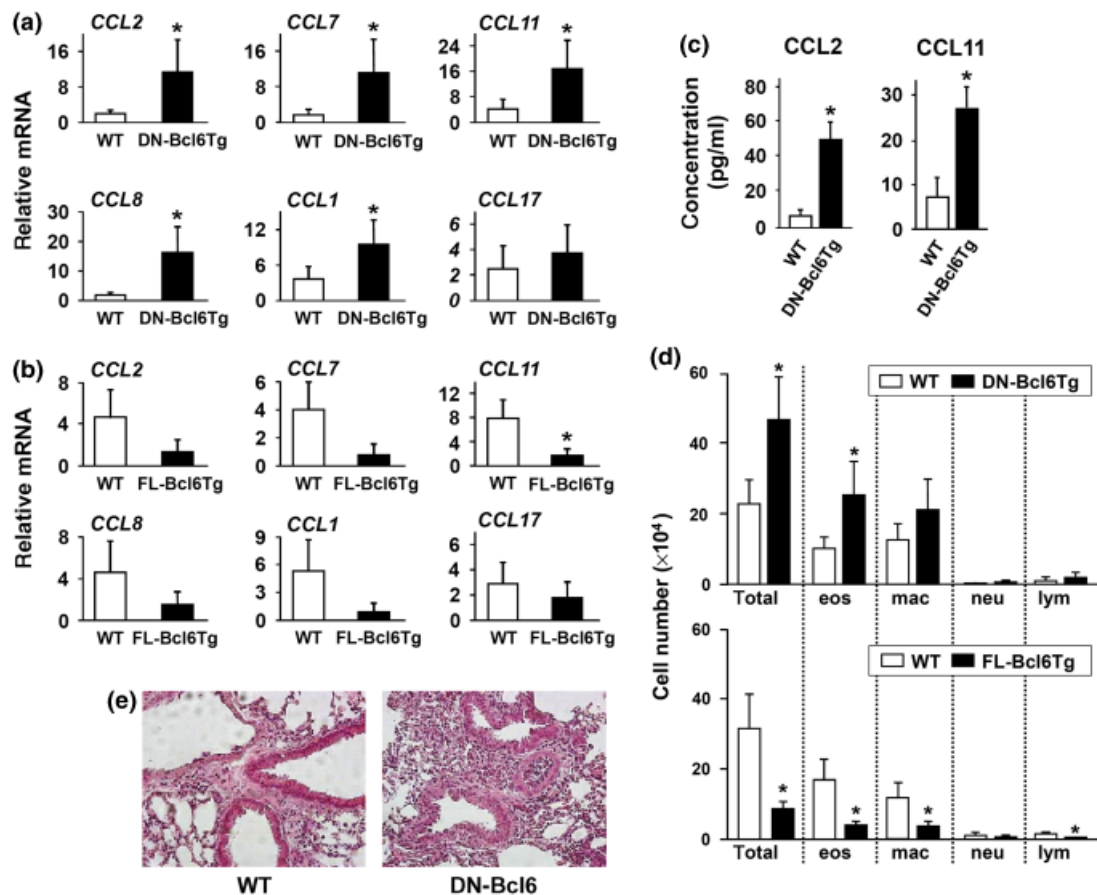


Fig. 6. Effect of Bcl6 in pulmonary epithelium on the antigen-induced airway inflammation. Transgenic (Tg) and wild-type (WT) mice were immunized with ovalbumin (OVA) and analysed 48 h after re-challenged with OVA on day 14 after immunization. (a and b) Relative amounts of chemokine mRNAs in the lungs of SPC-DN-Bcl6 Tg (closed bar, $n = 12$) mice and WT littermates (open bar, $n = 12$) (a), and SPC-FL-Bcl6 Tg (closed bar, $n = 9$) mice, and WT littermates (open bar, $n = 9$) (b). The amount of mRNA was analysed by qRT-PCR. (c) The concentration of CCL2 and CCL11 in BAL fluids from the SPC-DN-Bcl6 Tg and WT mice. The amount of protein was analysed by ELISA. (d) Cell numbers of total, eosinophils (eos), macrophages (mac), neutrophils (neu), and lymphocytes (lym) in BAL fluids from the Tg mice and WT littermates. All data are presented as the mean $\pm$ SD from three independent experiments. * $P < 0.05$. (e) Representative pathological findings for lungs from SPC-DN-Bcl6-Tg and WT littermates 48 h after re-challenge with OVA (haematoxylin–eosin staining, magnification = $\times 400$) are shown.

Bcl6 binds to the sequences to deacetylate the regional histones in A549 cells at a resting state. The chromatin structure with histone H3-K9/14 deacetylation throughout the cluster may not be able to accept other important transcriptional activators to the promoter and regulatory regions of those chemokine genes. However, expression of the *CCL17* gene, which locates on the distinct chromosomes (16q13 in humans and 8qC5 in mice), was not affected by Bcl6. Thus, expression of the CC-type chemokine genes in the clusters on the chromosome 17q11.2 of humans and on the chromosome 11q21.2 of mice is orchestrated by the conserved machinery related to Bcl6. The level of histone H3-K9/14 acetylation in the cluster region of DN1 cells at a resting state was more than that in CR1 cells. DN-Bcl6 cannot induce regional histone deacetylation but competitively inhibits Bcl6 binding to the cluster region in DN1 cells. As chromatin modification

such as histone acetylation is believed to facilitate the accessibility of the DNA for the transcriptional machinery [21], these results suggest that Bcl6 is required for maintaining the chromatin structure of the cluster region to the closed condition with the histone deacetylation to inhibit binding of other transcriptional activators. Therefore, this interactive binding of Bcl6 in the cluster of A549 cells at a resting state is involved in tuning for chromatin remodelling in the chemokine gene cluster.

Histone H3-K9/14 acetylation plays an important role in transcriptional activation [22, 23]. STAT factors, which also bind to Bcl6-binding sequences [7], are known to recruit histone acetyltransferases including p300 [24]. STAT6 was considered to bind to the gene cluster of A549 cells stimulated with IL-4 and TNF- α , because IL-4 signalling can potentially activate STAT6 in various cell types [25]. Although exogenous DN-Bcl6 can compete the

binding of STAT6 to the Bcl6-binding sites of DN1 cells, STAT6 binding and histone H3-K9/14 acetylation were augmented in the gene cluster of activated DN1 cells. Therefore, STAT6 binding can be consequently reinforced to overcome the binding of DN-Bcl6 to the gene cluster following the initiation signal for the gene activation [26]. These results also suggest a possibility for the involvement of other transcriptional activators such as NF- κ B, which is activated by TNF- α signalling [27, 28]. In addition, almost all of these chemokine genes in the cluster were activated in A549 cells stimulated with IL-1 β , and the amounts of these chemokine mRNAs in DN1 and DN2 cells were more than those in CR1 and CR2 cells. As IL-1 β signalling activates NF- κ B [29], NF- κ B may be suitable for another transcriptional activator involved in the chemokine gene regulation in the A549 cells. We analysed NF- κ B binding to some putative NF- κ B-binding regions in the cluster by a ChIP assay. However, modest bindings of this molecule were observed in each region of CR1 cells after stimulation with IL-1 β (data not shown). Furthermore, the binding was not reinforced in DN1 cells (data not shown). These results suggest that activator molecules other than NF- κ B and STAT factors are involved in expression of the chemokine genes in the cluster.

Histone H3-K4 methylation is also considered to be a key epigenetic modification for gene expression [30]. Transcriptionally active euchromatin preferentially contains methylated histones at H3-K4, H3-K36, and H3-K79. Particularly, methylation at H3-K4 correlates well with an active or a permissive state of transcription, suggesting that this histone methylation is a hallmark of actively transcribed chromatin. Interestingly, DN1 cells showed enhancement of histone H3-K4 methylation as well as that of histone H3-K9/14 acetylation in the cluster as compared with those in CR1 cells after stimulation. These results suggest that Bcl6 indirectly interferes with histone H3-K4 methylation by inducing regional histone deacetylation. Thus, histone methylation related to active transcription is implicated in Bcl6-mediated chromatin remodelling. However, a great deal of uncertainty remains about the machinery for histone methylation.

To elucidate a role of Bcl6 in the regulation of the chemokine genes in the gene cluster of alveolar epithelium, we established SPC-DN-Bcl6- and -FL-Bcl6 Tg mice and utilized a model for allergic airway inflammation. The human SPC gene promoter fragment has been previously demonstrated to direct expression of transgenes to distal lung epithelium, including type II alveolar epithelial cells and Clara cells of the terminal bronchioles in mice [31, 32]. In our experiments, we also confirmed the SPC promoter activity by detecting excess amounts of each transgene mRNA. Indeed, any excess amounts of the transgene mRNAs were not detected in organs examined other than lungs by qRT-PCR (Fig S1). Furthermore, production of the cytokines by splenocytes mainly T cells

from Tg mice was similar to that from WT mice (data not shown). Therefore, the exogenous *FL-Bcl6* and *DN-Bcl6* genes are expressed selectively in the lungs, especially in pulmonary epithelium.

The expression profile of the chemokine genes in the lungs of those Tg mice correlated with an extent of pulmonary infiltrates, in particular eosinophils and macrophages. Some of the chemokines were considered to be produced by lung macrophages. However, the large amounts of chemokine mRNAs in the lungs of DN-Bcl6 Tg mice might be attributed to intrapulmonary accumulation of macrophages rather than changes in their ability of gene expression because this study suggested that expression of the chemokine genes in lung macrophages of the Tg mice was not significantly different from that in WT mice. A critical role of CCL2 and CCL11 has been reported in eosinophilic pulmonary inflammation in the murine model of asthma [33]. Other chemokines in the cluster, CCL7 (human and mouse), CCL8 (human and mouse), CCL12 (mouse), and CCL13 (human), also have potent chemotactic functions for various inflammatory cells including eosinophils [9, 34, 35]. On the other hand, CCL1 (human and mouse) is a chemoattractant for macrophages. Thus, chemokines derived from pulmonary epithelium may sufficiently contribute to the pathogenesis of pulmonary inflammatory diseases such as asthma, although chemokines are produced by many cell types including macrophages, eosinophils, and pulmonary fibroblasts. Furthermore, pulmonary epithelium is strictly implicated in the development of not only bronchial asthma but also other diseases such as chronic obstructive pulmonary diseases and interstitial pneumonia/pulmonary fibrosis [36]. Thus, Bcl6 in pulmonary epithelium may control these pulmonary diseases by orchestrating the expression of the CC-type chemokine genes in the cluster.

In summary, Bcl6 plays an important role in coordinate expression of the CC-type chemokine genes in the cluster as a transcriptional repressor. As airway inflammation is potentially mediated by chemokines produced by pulmonary epithelium, Bcl6 in the epithelium may be a critical regulator for the pathogenesis of various pulmonary inflammatory diseases.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Specific expression of the exogenous genes in pulmonary epithelium of SPC-FL-Bcl6 Tg and SPC-DN-Bcl6 Tg mice.

Table S1. Putative Bcl6-binding sequences in the chemokine gene clusters.

Table S2. Primers for ChIP assay to assess Bcl6-binding to the chemokine gene cluster of human.

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Online Supplement to

Bcl6 in pulmonary epithelium coordinately controls expression of the CC-type chemokine genes and attenuates allergic airway inflammation

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Supplemental information

Table 1: Putative Bcl6-binding sequences in the chemokine gene clusters.

All of sequences **bolded** are completely identical or highly homologous to the consensus sequence and were detected in the clusters in human (A) and mouse (B), respectively.

(A) Human.

Human chemokine genes and intergene (bp)	GeneBank accession number	# of binding site	Position relative to CCL2 trascription starting site (+1)	Putative binding sequence
		1		CTTCTTGGAAG
		2	-137	CTTCCTGGAAA
CCL2 (1,927)	NM 002982			
13,017		3	2,455	TTTCTAGGAAA
		4	4,097	GTTCCAGGAAA
		5	6,076	GTTCCAGGAAG
		6	6,560	CTTCTATGAAT
		7	9,983	TTTCTAGGAAT
		8	14,442	ATTCTAAGAAT
CCL7 (2,017)	NM 006273			
13,430		9	18,207	ATTCTTGGAAG
CCL11 (2,513)	NM 002986			
30,877		10	34,811	CTTCCAGGAAC
		11	35,179	ATTCCAGGAAG
		12	37,387	CTTCTTGGAAG
		13	40,930	CTTCCATGAAA
CCL8 (2,356)	NM 005623			
35,049		14	73,771	TTTCCATGAAA
		15	75,296	ATTCTATGAAT
		16	84,764	TTTCCAGGAAT
		17	90,603	TTTCTAAGAAA
		18	93,865	GTTCTAAGAAC
		19	100,258	ATTCCAGGAAG
		20	100,473	CTTCCTGGAAA
		21	101,778	CTTCTATGAAA
		22	101,060	TTTCCTAGAAA
CCL13 (2,159)	NM 005408			
2,248		23	102,567	CTTCCTTGAAT
CCL1	NM 002981			

(B) Mouse.

Murine chemokine genes and intergenes (bp)	GeneBank accession number	# of binding site	Position relative to CCL2 trascription starting site (+1)	Putative binding sequence
		1	-151	CTTCCTGGAAA
<i>CCL2</i> (1,883)	NM 011333			
8,258		2	6,247	CTCCTGGAAA
<i>CCL7</i> (1,818)	NM 013654			
1,021		3	15,450	ATTCTAAGAG
<i>CCL11</i> (5,138)	NM 011330			
38,889		4	30,853	GTTCTAGGAT
		5	36,420	CTTCTAGGAAG
		6	38,843	TTTCCTGGAAC
		7	39,237	GTTCCAGGAGA
		8	42,196	CTTCCTGGAAG
		9	43,486	CTTCCTGGAAG
		10	44,640	CTTCCTGGAAA
<i>CCL12</i> (1,556)	NM 011331			
1,178				
<i>CCL8</i> (1,615)	NM 021443			
59,857		11	74,569	GTTCTCAGAAA
		12	75,238	GTTCCCAGAAA
		13	77,406	GTTCTCAGAAA
		14	78,046	GTTCTGGAAC
		15	78,249	GTTCTGGAAG
		16	78,768	CTTCCAAGAAT
		17	82,699	GTTCTTTGAAG
		18	83,082	ATTCTTTGAAT
		19	105,780	ATTCTGGAAG
		20	114,584	CTTCCTGGAAC
<i>CCL1</i>	NM 011329			

Table 2: Primers for ChIP assay to assess Bcl6-binding to the chemokine gene cluster of human.

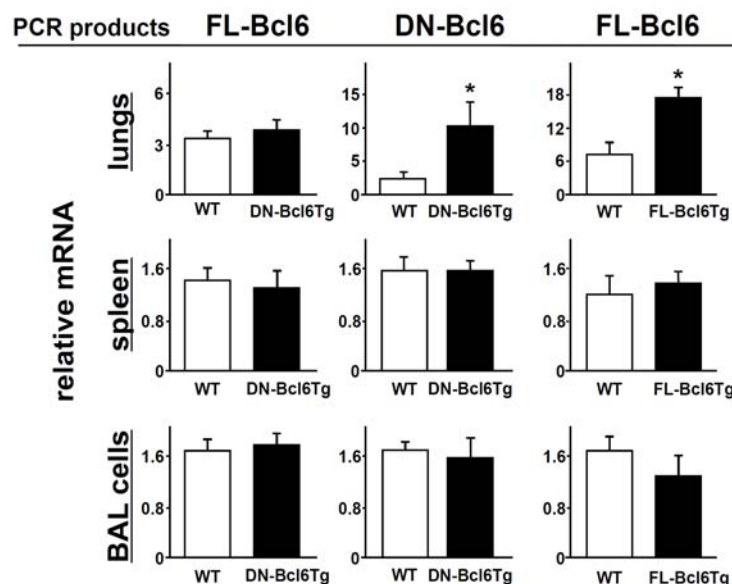
Primers (# of Bcl6-binding site)	Sequences
1	(F) 5'- GGGAAATTGAATGCGGTCCACCAA-3' (R) 5'- TCCTCTGCTCATGCCTCAATCCAT-3'
2	(F) 5'- CCCATTGCTCATTGGTCTCAGC-3' (R) 5'- TGGCTGCTGTCTCTGCCTCTTATT-3'
3	(F) 5'- ACCTGGAGTAATACAGACACTGCC-3' (R) 5'- GCCCGAATTCGCATCTGGTTCATT-3'
4	(F) 5'- TGAAACTGAACACAGGGCATTGGC-3' (R) 5'- TGGTGCTTCTGTAACAAACACCTAC-3'
5	(F) 5'- GCCAAGGAGAAGAAATGGACAAGC-3' (R) 5'- TCTCATTGGACAAGGAAGACTGGG-3'
6	(F) 5'- ACTGCATACTCATTAAACAATACCTGCTC-3' (R) 5'- TGACACATGCCTTAACATAAATGAACC-3'
7	(F) 5'- AGAAGAGCTTGAGGAGTATTGGT-3' (R) 5'- TGAAAGAGGGAACATTACAATTGATGC-3'
8	(F) 5'- CAGACACTGAATGAAGGCCTTGTG-3' (R) 5'- AGGACACGAGTCCACAAAGAGACA-3'
9	(F) 5'- AACCACGTCAGACAAACACCTCTG-3' (R) 5'- TTGGGAATGCTGGAAAGACCTCAG-3'
10	(F) 5'- GTTCCTGGCCTGGAGAACAATTCA-3' (R) 5'- ACTCTCTCATCCTGGGCCTCATT-3'
11	(F) 5'- GACAAAGAACAAGCTGGGAGTCCA-3' (R) 5'- TGGTGACTCCTTGACAGTAGCCAT-3'
12	(F) 5'- AGGAAGTGCAGCCCATGAGATGAT-3' (R) 5'- GGGAGAAGAGAGAGAATTCCTTGGCA-3'
13	(F) 5'- TAGAGCAGTTTCCAGTAGGGACCA -3' (R) 5'- TTGGGATAAGGCCAGGAAATCTGC-3'
14	(F) 5'- CCCTCTAATCTGTTCCCTTGGT-3' (R) 5'- TGGCATGCACACGAATTGGAAGAC-3'
15	(F) 5'- CCTTGGTCAGTCTTGCAAAGGGAT-3' (R) 5'- GGTTGAACCTCGAATCAGGAAACA-3'
16	(F) 5'- ACTGTGAATCCATCTGGTCTGGGT-3' (R) 5'- CCCTCAAAGACTATTATGAACACTTCACGC-3'
17	(F) 5'- CCATGTGACATTAGTGTGAGTGGA-3' (R) 5'- ACTGTGCCTTAGGAGACTGGAAGT-3'
18	(F) 5'- AGAGTGGTCCTCAGACAAGCCTTA -3' (R) 5'- GGGTCAGTTAAGCATGGGTTC AAG -3'
19	(F) 5'- ACAGAACCAGCTTGGGCTTCATC -3' (R) 5'- TGGTTGAACATGTCCCTTTCCAC -3'
20	(F) 5'- GGCCAAACACCTCACTTCTTTGCT-3' (R) 5'- CAGATTAAGTCAGATTCTGATGACAGGG-3'
21	(F) 5'- AAAGTTGAACTGCCTTGGTGAT-3' (R) 5'- GTGCTGTGCTAAGGACTCTGTTCA-3'
22	(F) 5'- TTATGGCTTCCCATGGTGAATGGCTG-3' (R) 5'- AGCTTGGAACCTCAGAGGACCTT-3'
23	(F) 5'- TGAGGGAAACACAGCATGAGACCT-3' (R) 5'- TGATTGAGATGCCCAACCCAGCTTA-3'

(F) Forward primer, (R) Reverse primer

Figure 1: Specific expression of the exogenous genes in pulmonary epithelium of SPC-FL-Bcl6 Tg and SPC-DN-Bcl6 Tg mice.

In order to examine function of Bcl6 in pulmonary epithelium on expression of the CC-type chemokine genes in the cluster, we generated Tg mice carrying the *FL-Bcl6* or *DN-Bcl6* gene under the control of SPC promoter, *SPC-FL-Bcl6* or *SPC-DN-Bcl6*. We confirmed that expression and functions of the exogenous genes are restricted to lungs, especially pulmonary epithelium, of SPC-FL-Bcl6 Tg and SPC-DN-Bcl6 Tg mice.

The exogenous *FL-Bcl6* and *DN-Bcl6* mRNAs were detected by qRT-PCR on the RNA encoding the repressor domain and the DNA binding domain of Bcl6, respectively. Although the amount of *FL-Bcl6* mRNA in lungs of naïve SPC-DN-Bcl6 Tg mice was similar to that of WT mice, the amount of *DN-Bcl6* mRNA in the lungs of SPC-DN-Bcl6 Tg mice was more than that in WT mice (*upper*). In contrast, amounts of *FL-Bcl6* mRNAs in lungs of naïve SPC-FL-Bcl6 Tg mice were significantly more than those in WT mice. In contrast, amounts of *FL-Bcl6* and *DN-Bcl6* mRNAs in the whole spleen tissues (*middle*), BAL cells including mainly macrophages (*lower*) and primary pulmonary fibroblasts (data not shown) of SPC-FL-Bcl6 Tg and SPC-DN-Bcl6 Tg mice were similar to those in WT mice.



SPC-DN-Bcl6 Tg (closed bar, $n = 6$) mice and WT littermates (open bar, $n = 6$). SPC-FL-Bcl6 Tg (closed bar, $n = 6$) mice and WT littermates (open bar, $n = 6$). Data are presented as the mean $\pm$ SD from three independent experiments. * $P < 0.05$.