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BCL6 Controls Th9 Cell Development by Repressing *Il9* Transcription

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Abstract

The transcriptional repressor B-cell lymphoma 6 (BCL6) is required for the development of T helper (Th) follicular cells and it has been shown to suppress Th2 cell differentiation. We demonstrate that BCL6 is a key regulator of Th9 cell development. BCL6 expression is transiently downregulated in polarized Th9 cells and forced expression of BCL6 in Th9 cells impairs Th9 cell differentiation. In contrast, BCL6 knockdown up-regulated interleukin (IL)-9 production in Th9 cells. The function of BCL6 in Th9 cells is under the control of IL-2/Janus kinase 3 (JAK3)/signal transducer and activator of transcription 5 (STAT5) signaling pathway. Using chromatin immunoprecipitation (ChIP), we show that in Th9 cells, BCL6 and STAT5 bind to adjacent motifs in the *Il9* promoter. Furthermore, we found that STAT5 binding was associated with the abundance of a permissive histone mark at the *Il9* promoter, while under conditions where BCL6 binding was predominant a repressive histone mark was prevalent. The effects of STAT5 and BCL6 on IL-9 transcription were further demonstrated using an IL-9-luciferase reporter assay where BCL6 repressed STAT5-mediated *Il9* transactivation. In experimental autoimmune encephalomyelitis (EAE), forced expression of BCL6 in myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$-specific Th9 cells resulted in decreased IL-9 production and induction of IFNγ causing an exacerbation of the clinical disease. Our findings demonstrate a novel role of BCL6 in the regulation of Th9 cell development and their encephalitogenicity.

INTRODUCTION

Following antigen stimulation, naïve $CD4^+$ T cells differentiate into one of several functional classes of effector cells. In addition to the classical Th1 and Th2 lineages, Th17 cells have been described and extensively characterized. Recently, a new subset of IL-9-producing Th cells induced *in vitro* by IL-4 and transforming growth factor-β1 has been identified (1, 2). Traditionally associated with the Th2 response, IL-9 is a pleiotropic cytokine that impacts inflammation by exerting broad effects on a variety of cell types such

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as $CD4^+$ T cells, mast cells and epithelial cells. Recent reports by our group and others demonstrated that IL-9 exerts pro- or anti-inflammatory properties depending on the inflammatory milieu by regulating Th17 and regulatory $CD4^+FoxP3^+$ T cells (Tregs) expansion and survival (3–6). Moreover, adoptive transfer of Th9 cells has shown divergent functions from other transferred subsets in models of tumor immunity, autoimmune encephalomyelitis, and allergic airway disease (7–9).

Networks of cytokines and transcription factors are critical for determining $CD4^+$ T cell fates and effector cytokine production. Indeed, each subset utilizes a master regulatory transcription factor and a particular signal transducer and activator of transcription (10). The relationships are as follows: Th2, GATA-binding protein 3 (GATA-3)/STAT5; Th1, T-box transcription factor expressed in T cells (T-bet)/STAT4; Th17, retinoid orphan receptor γt (RORγt)/STAT3; inducible Treg, forkhead box protein 3 (Foxp3)/STAT5. Recent studies suggest that T follicular helper cells may also fit the paradigm with the factors being B-cell lymphoma 6 (Bcl-6)/STAT3. Interestingly, in many instances, the STAT involved also plays a role in the induction of the master transcriptional regulator (reviewed in (11)). The *Il9* locus is responsive to multiple factors that bind and induce a conserved non-coding sequence (CNS) in reporter assays including IRF4, PU.1, NF-κB, and Smad/Notch complexes (3, 12–14). Recently, transcription factors of the STAT family, STAT5 and STAT6, were shown to be critical for Th9 cell development (15, 16). The *Bcl6* gene, originally identified as an oncogene for B cell lymphoma, encodes a transcriptional repressor protein that regulates T cell differentiation by repressing Th1 and Th2 cell development (17–19). BCL6 knockout (KO) mice exhibit significant growth retardation and invariably die by ten weeks of age (20, 21). BCL6KO mice have multiple immunological defects, including lack of germinal center formation and spontaneous development of severe Th2-type inflammatory disease, particularly affecting the heart and lungs (20, 21). The DNA motifs recognized by BCL6 are highly homologous to the core consensus binding sequence TTC-NNN-GAA (where N is any nucleotide) of STAT5 (20, 22), a positive regulator of Th9 cell development (16), which suggests that BCL6 may play a role in the transcriptional regulation of the *Il9* locus and Th9 cell development.

In the present study, we analyzed the role of BCL6 in the regulation of Th9 cell development and encephalitogenicity. We demonstrate that BCL6 controls Th9 cell differentiation by direct binding and regulation of the *Il9* locus. Furthermore, BCL6 function in Th9 cells is regulated by the IL-2/JAK3/STAT5 signaling pathway.

MATERIALS AND METHODS

Mice and Reagents

C57BL/6 and Rag2−/− mice were purchased from the Jackson Laboratories and $MOG_{35–55}$ T cell receptor transgenic mice (2D2) were previously described (23). Mice were housed in the pathogen-free animal facility at Harvard Medical School, New Research Building, in accordance with the guidelines of the Committee of Animal Research at the Harvard Medical School and the National Institutes of Health animal research guidelines as set forth in the Guide for the Care and Use of Laboratory Animals. The following fluorescent conjugated primary mAb were purchased from BD Biosciences (San Jose, CA): anti-mouse

IL-9-PE, IL-17-APC, and anti-mouse CD4-Pacific Blue. JAK3 inhibitor VI (420126) and STAT5 inhibitor (573108) were obtained from Calbiochem (San Diego, CA). 7AAD stain for dead cell exclusion was obtained from BD Biosciences.

In vitro T Cell Differentiation, Retroviral Cell Transduction and Cytokine Assay

Naïve $CD4^+$ T cells were purified from C57BL/6 WT mice using anti-CD4 beads (Miltenyi, Auburn, CA) and flow sorted into naïve $CD4^+CD62L^{hi}$ T cells by flow cytometry on a FACSAria T cell sorter (BD Biosciences). $CD4^+$ T cells were stimulated with plate-bound anti-CD3 (4 μg/ml) (145-2C11; BD Biosciences, San Diego, CA) and soluble anti-CD28 (2 μg/ml; BD Biosciences) for 4 days in a serum-free culture medium (X-VIVO-20; Lonza, Hopkinton, MA) supplemented with 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, non-essential amino acids, L-glutamine and 100 μU/ml penicillin/100 μg/ml streptomycin in the presence of recombinant cytokines. Polarization of Th9 cells was in the presence of human TGF-β1 (3 ng/ml) plus mouse IL-4 (10 ng/ml). Cells were supplemented with recombinant IL-2 (40 ng/ml) where indicated. Rat anti-mouse IL-2 antibody (BD Biosciences) was used for blocking of IL-2 activity *in vitro*. All recombinant proteins were from R&D Systems. JAK3 (250 μM) and STAT5 (50 μM) inhibitors were added along with the polarization medium at the start of differentiation. For intracellular flow cytometry staining, cells were restimulated with 12-O-tetradecanoylphorbol-13-acetate (20 ng/ml; Sigma, St. Louis, MO), ionomycin (300 ng/ml; Sigma), and 2 mM monensin (GolgiStop, BD Biosciences) for 4 h at 37°C. Cells were washed, stained for surface markers, fixed and permeabilized, and fluochrome-conjugated antibodies were added. For the measurement of cytokines released in the culture supernatants, cell culture supernatants were collected on day 4 after differentiation and the secreted cytokines were determined by fluorescent bead-based Luminex technology (Luminex, Austin, TX) for the indicated cytokines, in accordance with the manufacturers' instructions. For intracellular cytokine staining of infiltrated T cells, mice were sacrificed and perfused with PBS. Spinal cord tissues were digested with collagenase IV (Sigma-Aldrich) for 30 min at 37°C, resuspended in 30% Percoll, and loaded onto 70% Percoll. After centrifuge at 1300 × *g* for 20 min, the CNS inflammatory cells were retrieved from the 30/70% Percoll interface. Lymphocytes were prepared for intracellular flow cytometry staining as described above using anti-IL-9-PE and anti-IFNγ-APC conjugated antibodies.

For forced expression of BCL6, naïve $CD4^+$ T cells were transduced with a MSCV-based retroviral vector (24). Briefly, the RV particles were produced by co-transfecting the plasmids encoding for the ecotropic envelope gag-pol with the retroviral vector with Effectene (QIAGEN) into the Phoenix packaging cells. RV supernatant was harvested 48hr after transfection and centrifuged prior to use. $CD4^+CD62L^+$ T cells isolated by magnetic-activated cell sorting (MACS) from WT mice were activated with anti-CD3 and anti-CD28 for 24 hr, retroviral supernatant was added to the cells together with polybrene (8 μg/ml), and cells were incubated at 37°C. After incubation, live cells were sorted with the Dead Cell Removal Kit (Miltenyi) and cells were expanded for an additional 4 days under Th9 cell polarization condition.

Expression Analysis by Real-Time PCR

RNA was purified using Stratagene RNA kit and transferred directly into the RT reagent using the Applied Biosystems Taqman reverse transcriptase reagents. Samples were subjected to real-time PCR analysis on PRISM 7000 Sequencer Detection System (Applied Biosystems, Foster City, CA) under standard conditions. Genes analyzed were detected using commercially available assays (Applied Biosystems). Relative mRNA abundance was normalized against GAPDH.

Western blot

Cells were lysed in RIPA buffer (Thermo Scientific) with a protease inhibitor (Roche Diagnostics) and a phosphatase inhibitor mixture (Sigma-Aldrich); 20 μg total protein was loaded into each well of a SDS-PAGE gel for separation by electrophoresis and then transferred on nitrocellulose membrane. The resulting blots were blocked for 1 h with TBS-Tween 20 containing 5% bovine serum albumin and then probed for 3 hrs at room temperature with primary Abs directed against: phospho-STAT5 (Tyr694), phospho-JAK3 (Tyr980/981), STAT5, JAK3, or BCL6 (Cell Signaling Technology). β-actin mouse mAb (Sigma) was used as the loading control. Blots were then washed five times and probed for 1 h with the appropriate HRP-conjugated secondary Ab. Membranes were developed with Immobilon Western Chemiluminescent HRP substrate (Millipore).

RNA interference

To knock down the expression of *Stat5a* and *Bcl6*, $CD4^+$ T cells were purified and nucleoporated with small interfering RNA (siRNA) specific for *Stat5a* or *Bcl6* (Santa Cruz Biotechnology). Scrambled siRNA was used as control. Cells were transfected using an Amaxa nucleoporator system. Briefly, 5–10 × 10^6 $CD4^+$ T cells were resuspended in 100 μl Nucleofector solution and transfected with 100 nM siRNA using Amaxa Nucleofector (Lonza, Basel, Switzerland). After transfection, the cells were incubated for 6 h at 37°C followed by polarization under Th9 conditions.

Passive EAE Model

For the generation of myelin-specific Th9 cells, $CD4^+CD62L^+$ cells were prepared from spleens of $MOG_{35–55}$ T cell receptor transgenic mice (2D2) using MACS beads. $CD4^+$ cells were transduced with MSCV-BCL6-GFP or control empty vector MSCV-GFP followed by stimulation with $MOG_{35–55}$ peptide (20 μg/ml) in the presence of IL-4 (20 ng/ml) and TGF-β1 (3 ng/ml) for 3 days in the presence of splenic $CD11c^+$ dendritic cells isolated from 2D2 mice. One million cells were transferred into Rag2−/− recipient mice via i.p. injection followed by immunization with the emulsion made of 50 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK; New England Peptide) and complete Freund's adjuvant (CFA). Each animal also received i.p. injections of 69 ng pertussis toxin (PT) on days 1 and 3 after transfer. The EAE clinical score was determined as follows: 0, no disease; 0.5, partial tail paralysis; 1, complete tail paralysis; 2, partial hind limb paralysis; 3, complete hind limb paralysis; 4, complete hind limb and partial front limb paralysis; and 5, moribund or dead animals.

ChIP and qPCR

$CD4^+$ T cells were purified by MACS sorting and were polarized to Th9 phenotype. ChIP was performed according to the protocol described in (25) with the following modifications: Chromatin was sheared using Micrococcal Nuclease (New England Biolabs) and Protein A Magnetic Beads (New England Biolabs) were used. Cell lysates were used for immunoprecipitation with anti-STAT5, anti-BCL6, anti-mono-methyl-Histone H3 lysine 4 (H3K4me1), and anti-pan-methyl-H3K9 (all from Cell Signaling Technology) and were compared to control IgG. One region of the *Il9* promoter containing putative STAT5 and BCL6 binding sites as well as a region of the *Bcl6* promoter containing putative STAT5 binding sites was amplified by SYBR Green qPCR (Applied Biosystems) and quantified in duplicate with the percentage of input method. The following primers were used: *Il9* Promoter: Site 1 Fwd: ACTGAGTTCCAGACTCCCGT, Rev: GCCCAGCACAGAACTGAAGA; Site 2 Fwd: GGATCCTCAAGGCCAATGCT, Rev: ACACCTCTGAGAAGTCGCTC; Site 3 Fwd: ACAGAAGTGTGCTGTCTGGT; Rev: CCCCTTGAGCCACTGGATAC. *Bcl6* Promoter: Site 1 Fwd: CTGCGGAGCAATGGTAAAGC, Rev: ATAATCACCTGGTGTCCGGC; Site 2 Fwd: CGAGGAGCCGAGTTTATGGG, Rev: GAGAGTGCGCTTTGCTTTCC; Site 3 Fwd: CGAATGACAGTCCCGACGAT, Rev: GCTTGGGATGCTCCTGTTGT.

Luciferase Reporter Assay

Reporter vector coding for the Firefly Luciferase under the control of the *Il9* promoter encompassing nucleotides −1310 to +32 bp was cloned into the promoterless pGL3 Basic luciferase reporter gene vector (Promega). Murine stem cell virus vector expressing wild-type mouse *Stat5a* (MSCV) was previously described (26). Plasmid encoding mouse *Bcl6* (mBCL6/pCMV-SPORT6.1) was purchased from Open Biosystems. Reporter assays were carried out as described previously (3). Briefly, 293T cells were transfected with 0.2 μg of the reporter vector coding for the Firefly Luciferase under the control of the *Il9* promoter and with 0.2 μg of the STAT5A or BCL6 encoding plasmids. Cells were cultured for 24 hrs before harvesting and the relative *Il9* promoter activity was measured using Promega kit in accordance with the manufacturer's instructions.

Statistical Analysis

The Mann-Whitney test was used for clinical disease analysis. Data are expressed as mean ± SEM and were compared using the Unpaired Student's *t* test for experiments with two groups by Prism software v5. Data were considered statistically significant at $P < 0.05$.

RESULTS

BCL6 Is a Negative Regulator of IL-9 Expression in Th9 Cells

The transcriptional repressor BCL6 has emerged as a multifunctional regulator of lymphocyte differentiation and immune responses. Here, we investigated the possibility whether BCL6 is involved in murine Th9 cell development. We measured BCL6 protein expression in Th9 cells polarized *in vitro* with IL-4 and TGF-β1 and found it decreased as early as 8 hours following differentiation as shown by Western blot. BCL6 downregulation

was transient suggesting an intrinsic mechanism for the control of BCL6 expression in Th9 cells (Figure 1A). To understand the functional relevance of the regulation of BCL6 expression in Th9 cells, we asked whether BCL6 forced expression influences Th9 cell development *in vitro*. To address this, we transduced naïve $CD4^+CD4^{low}CD62L^{hi}$ T cells with MSCV-BCL6-GFP retrovirus (BCL6-RV-GFP), cultured the cells under Th9 conditions for 4 days, and measured intracellular cytokine expression using flow cytomtery. We found that the frequency of IL-9-positive cells was altered by ~60% after BCL6 (GFP^+) overexpression compared to BCL6-negative (GFP^-) Th9 cells suggesting that the transcription factor BCL6 is a negative regulator of Th9 cells (Figure 1B). To ascertain the involvement of BCL6 in Th9 cell generation, we used BCL6-specific siRNA to knockdown BCL6 gene expression in Th9 cells. Naïve $CD4^+$ T cells were nucleoporated with BCL6-specific siRNA and subsequently polarized to the Th9 lineage and harvested 4 days after transfection. Consistent with the data obtained with BCL6 over-expression, BCL6 knockdown resulted in an up-regulation of IL-9 expression at both the protein and gene levels as measured by bead-based Luminex assay and Taqman PCR, respectively (Figure 1C, D). Moreover, to address the functional role of increased BCL6 level at later stage of Th9 differentiation, we treated Th9 cells with BCL6-specific siRNA or scrambled siRNA on day 3 after polarization. Cells were kept for another 3 days under Th9 conditions followed by analysis for IL-9 production by Luminex. We found that BCL6 knockdown did not alter IL-9 production suggesting that BCL6 is primarily involved in the early development of Th9 cells (Figure 1E).

Activation of IL-2/JAK3/STAT5 Axis Inversely Correlates With BCL6 Down-regulation in Th9 Cells

IL-2 is a major growth factor for activated T cells and plays an important role in murine and human Th9 cell expansion and differentiation *in vitro* (27, 28). Additionally, activation of STAT5 is an important component of IL-2 signaling and has been recently proposed as a positive regulator of Th9 cells (16, 29). To measure the IL-2 signaling pathway downstream components in Th cells, we first extracted gene expression profiling from RNA-sequencing (RNA-Seq) data deposited on Gene Expression Omnibus (GEO) (30). Analysis of IL-2 signaling in murine Th1, Th2, Th9, Th17 and inducible $CD4^+FoxP3^+$ regulatory T cells (iTregs) differentiated *in vitro* according to standard protocols shows that the expression of Il2ra, Stat5a and Stat5b is up-regulated in Th9 cells (Supplementary Figure 1A). Using Taqman PCR to verify the transcriptome sequencing results in different Th subsets, we found that mRNA levels of *Il2ra*, *Stat5a* and *Stat5b* are indeed elevated in Th9 cells compared to other cell phenotypes (Supplementary Figure 1B). Moreover, temporal analysis of IL-2 expression in polarized Th9 cells demonstrates that IL-2 is transiently induced in Th9 cells and this expression inversely correlates with a transient decrease in BCL6 mRNA expression in these cells (Figure 2A).

Given that IL-2 is required for Th9 cells polarized *in vitro* and is abundantly produced by these cells as measured by Luminex bead-based assay (Supplementary Figure 1C), and since IL-2/STAT5 pathway has been shown to regulate BCL6 signals in follicular T helper cells (31, 32), we sought to analyze the activity of the key transducers of IL-2 signaling, STAT5 and its upstream kinase JAK3, in polarized Th9 cells. Using bead-based Luminex assay for

phosphorylated STAT5αβ, we compared the activity of STAT5 in different T helper subsets differentiated *in vitro* for several time points between 0–120 minutes according to standard protocols in the absence of exogenous IL-2. We found that STAT5αβ activation is predominantly induced in Th9 cells as early as 15 minutes after polarization (Figure 2B). Furthermore, we confirmed that STAT5 signaling is activated in Th9 cells, by measuring STAT5αβ phosphorylation and its upstream protein kinase, JAK3. Thus, naïve $CD4^+$ T cells were stimulated under Th9 conditions in the presence or absence of recombinant IL-2 (0–60 minutes), and cells were lysed and used for immunoblot analysis using antibodies specific for phosphorylation-dependent and independent JAK3 and STAT5. We found that the phosphorylation of both JAK3 and STAT5 was induced in Th9 cells and this was sustained in the presence of added recombinant IL-2 (Figure 2C). Quantification of phospho-JAK3 and STAT5 by measurements of the optical density of the corresponding bands is shown (Supplementary Figure 1D). Altogether, these data suggest the JAK3/STAT5 signaling cascade is activated in Th9 cells and is further enhanced by IL-2 signaling.

To investigate whether IL-2 is involved in the down-regulation of BCL6 in polarized Th9 cells, we measured BCL6 protein expression in Th9 cells differentiated *in vitro* for several days in the presence of recombinant IL-2 or anti-IL-2 neutralizing antibody. We found that while BCL6 protein levels were transiently decreased during Th9 cell differentiation, supplementation with recombinant IL-2 induced sustained suppression of BCL6 protein expression (Figure 2D). Interestingly, when Th9 cells were differentiated in the presence of anti-IL-2 neutralizing antibody, BCL6 expression was maintained throughout the polarization suggesting that IL-2 signaling is a negative regulator of BCL6 in Th9 cells (Figure 2E). Quantification of BCL6 levels by densitometry is shown (Supplementary Figure 1D).

IL-2/JAK3/STAT5 Inhibition Up-regulates BCL6 Expression and Suppresses Th9 Cell Differentiation

Recent reports demonstrated that STAT5 signaling promotes Th9 cell differentiation and that STAT5 and BCL6 are cross-regulated in Th1 cells (33, 34). Given that JAK3 is an upstream positive regulator of STAT5 (35), we hypothesized that BCL6 may be regulated by STAT5 in Th9 cells. Thus, we analyzed the effects of the inhibition of IL-2/JAK3/STAT5 signaling on the outcome of Th9 cell differentiation and BCL6 expression. $CD4^+$ T cells were differentiated under Th9 conditions in the presence or absence of JAK3 (250 μM) or STAT5 (100 μM) specific inhibitors for 4 days. We found that addition of either inhibitor up-regulated BCL6 mRNA expression as measured by Taqman PCR (Figure 3A). Moreover, JAK3 and STAT5 inhibitors suppressed the production of IL-9 as measured by intracellular cytokine staining as well as by Luminex bead-based assay (Figure 3B, C).

Next, we used STAT5A-specific siRNA to inhibit STAT5 signaling at the gene level. Thus, naïve $CD4^+$ T cells were nucleoporated with STAT5A-specific siRNA or with scrambled siRNA and subsequently polarized to the Th9 lineage and harvested 4 days after transfection. Silencing of STAT5A specifically inhibited IL-9 production but did not affect the production of others cytokines, including IL-5, IL-17 and IFNγ (Figure 3D). Quantitative RT-PCR confirmed that STAT5A siRNA reduced the expression of *Il9* and

Stat5a mRNA (Figure 3E). To check whether the reduction in IL-9 in STAT5A siRNA-treated cells was due to differential cell survival, we performed 7AAD flow staining of nucleoporated cells, which revealed no changes between control and STAT5A siRNA-treated cells (**data not shown**).

Further analysis of STAT5A-specific siRNA showed that the treatment was associated with an increase in *Bcl6 mRNA* expression (Figure 3E), in agreement with the elevated *Bcl6* expression in Th9 cells treated with IL-2/JAK3/STAT5 inhibitors (Figure 3A) and the decrease in BCL6 protein expression in Th9 cells exposed to IL-2 (Figure 2C). These findings suggest that BCL6, a transcriptional repressor that has been shown to modulate STAT signaling in B cells (36, 37) plays a role in the regulation of Th9 cell differentiation under the control of STAT5 signaling.

Opposite Roles of BCL6 and STAT5 in Binding and Regulating the *Il9* Promoter in Th9 Cells

Because IL-2 enhances the differentiation of Th9 cells and exerts opposite effects on STAT5 and BCL6 expression, we hypothesized that these transcription factors may have opposing roles in the regulation of the *Il9* promoter in Th9 cells. To investigate this, we searched the *Il9* promoter for potential binding sites for STAT5 and BCL6 using Biobase database. We identified three overlapping putative binding sites for STAT5 and BCL6 upstream of the transcription start site (TSS) (Figure 4A). To determine the *Il9* promoter occupancies by STAT5 and BCL6, binding motifs were used to design chromatin immunoprecipitation (ChIP) experiments. Primer sets flanking the STAT5 and BCL6 binding at three sites in the *Il9* promoter were designed to amplify the immunoprecipitated ChIP DNA by qPCR (Figure 4A). $CD4^{+}CD62L^{hi}$ T cells were differentiated under Th9 cell polarizing conditions for 1–4 days and then analyzed by ChIP-PCR. We found that STAT5 binds significantly to sites 1, 2 and 3 in the *Il9* promoter in Th9 cells 2 days after cell polarization (Figure 4B and Supplementary Figure 2). We next investigated whether STAT5 binding to the *Il9* promoter in Th9 cells is modulated by IL-2 signaling. We observed that IL-2 blockade using IL-2 neutralizing antibody abolished STAT5 binding to the *Il9* promoter. Similarly, pharmacological inhibition of both JAK3 and STAT5 abolished STAT5 recruitment to the *Il9* promoter in Th9 cells which correlates with the suppression of IL-9 expression (Figure 4B and Supplementary Figure 2). We confirmed the specificity of STAT5 binding by amplifying a region of the *Il9* promoter that does not contain STAT5 binding sites (**data not shown**).

Given that STAT5 and BCL6 have opposite effects on IL-9 expression in Th9 cells, we investigated whether the two transcription factors compete for binding to the same binding motifs in the *Il9* promoter. Indeed, we found that BCL6 binding to the *Il9* promoter inversely correlates with STAT5 binding to site 2 and to a lesser extent to sites 1 and 3. Strikingly, this process was under the control of IL-2 signaling since inhibition of either IL-2, JAK3 or STAT5 suppressed STAT5 binding while it facilitated BCL6 recruitment to the same motif, particularly to site 2 and to a lesser extent to sites 1 and 3 in the *Il9* promoter (Figure 4C and supplementary Figure 2). We next studied whether chromatin modifications at the STAT5 and BCL6 binding motifs within the *Il9* promoter could explain the regulation

of IL-9 by IL-2 signaling. Analysis of histone 3 (H3) methylation in Th9 cells revealed increased permissive H3 lysine 4 monomethylation (H3K4me1) modifications at site 2 in the *Il9* promoter with decreased repressive H3 lysine 9 pan-methylation (H3K9-pan-me) chromatin modifications on day 2 after *in vitro* polarization. Interestingly, IL-2 neutralization increased the H3K9-pan repressive mark while it has no significant influence on the H3K4me1 permissive mark (Figure 4D). Previously, STAT5 has been shown to bind the *Bcl6* promoter and down-regulate its transcription in B-lymphoma cells and other hematopoietic cell lines (38). To investigate a similar outcome in Th9 cells, we analyzed STAT5 recruitment to the *Bcl6* promoter in polarized Th9 cells exposed to IL-2/JAK3/STAT5 blocking agents. Interestingly, we found that under basal conditions, STAT5 binds significantly to the *Bcl6* promoter and this process was abolished when cells were exposed to anti-IL-2 blocking antibody or to JAK3 and STAT5 inhibitors (Figure 4E), which correlates with the identified role of STAT5 as a positive regulator of Th9 cell development.

To analyze the functional relevance of the binding of STAT5 and BCL6 to their target sequences in the *Il9* locus, we investigated the ability of STAT5 and BCL6 to regulate the activity of the *Il9* promoter in reporter assays. We used the reporter construct pGL3-Il9 containing the firefly luciferase gene under the control of the *Il9* promoter. First, we found that co-transfection of the pGL3-Il9 luciferase reporter construct with a plasmid encoding constitutively active mutant of STAT5 ($STAT5a^{S711F}$) (26) in 293T cells resulted in a significant increase in *Il9* transcription (Figure 4F). In agreement with the function of BCL6 as a negative regulator of Th9 cell development, we found that BCL6 decreased *Il9* promoter activity significantly in a luciferase reporter assay. Strikingly, co-transfection of BCL6 with STAT5 resulted in a very significant suppression of STAT5 positive activity ($p < 0.0001$) suggesting that BCL6 is a dominant negative regulator of the *Il9* transcription in Th9 cells (Figure 4F). STAT5A overexpression in 293T cells is comparable in the presence or absence of exogenous BCL6 (Supplementary Figure 3).

BCL6 Overexpression in Myelin-Specific Th9 Cells Regulates their Encephalitogenicity

Previous studies demonstrated that adoptive transfer of myelin-reactive Th9 cells induces experimental autoimmune encephalomyelitis (EAE) (7). To investigate whether BCL6-mediated IL-9 repression modulates EAE, we carried out adoptive transfer experiments with $MOG_{35–55}$-pulsed Th9 cells following BCL6 overexpression. To generate myelin-specific Th9 cells overexpressing BCL6, $MOG_{35–55}$-TCR transgenic $CD4^+CD62L^{hi}$ naive cells were sorted from 2D2 mice and were transduced with MSCV-BCL6-GFP retrovirus or with control vector and subsequently polarized into Th9 cells for three days in the presence of $MOG_{35–55}$ (20 μg/ml) peptide and splenic dendritic cells. Analysis of the cytokine profile of BCL6-GFP-positive 2D2 $CD4^+$ T cells demonstrate a decrease in IL-4 and IL-9 expression associated with an increase in IFNγ expression compared to control cells as shown by Taqman PCR (Figure 5A). No changes were detected in IL-17 expression (**data not shown)**. To analyze the effects of BCL6 on the encephalitogenicity of Th9 2D2 cells, cells were transferred into Rag2−/− lymphopenic mice followed by reactivation with $MOG_{35–55}$/CFA immunization and mice were monitored for clinical disease development. We found that EAE was markedly enhanced in recipients of Th9 MSCV-BCL6 compared with Th9 MSCV-RV control mice (Mean maximal score for Th9 MSCV-BCL6-treated mice 3 ± 0.3

compared with Th9 MSCV-RV-treated mice 1.4 ± 0.2; $p < 0.001$ by Mann-Whitney test) (Figure 5B). To test whether the cytokine profile of transferred cells is maintained in recipient mice, spinal cord tissues were collected ~10 days post-transfer, 2D2 cells were identified using the surface markers Vα3.2/Vβ11 and cells were analyzed for cytokine expression confirming that BCL6 forced expression converts Th9 cells into Th1-like phenotype as shown by an increase in IFNγ and suppression of IL-9 production (Figure 5C).

DISCUSSION

Previous reports uncovered a key role of BCL6 as a transcriptional regulator in various cell types including B cells, T lymphocyte subsets within and outside of the germinal centers, including $CD4^+$ follicular helper T cells (39), T regulatory (40) and T helper cells and in the control of T-cell-dependent inflammation and autoimmunity (17, 20). The role of BCL6 in the regulation of T helper cells including Th1, Th2 and Th17 cells has been reported by several groups (17, 18, 21, 41–43). Here we provide evidence demonstrating that BCL6 is a repressor of Th9 cell development through direct regulation of the *Il9* promoter. We report that BCL6 expression is reduced early in polarized Th9 cells under IL-4 plus TGF-β1 conditions in an IL-2-dependent manner. At the transcription level, BCL6 binds and induces *Il9* repression, and this process is in competition with the STAT5 signaling, a positive regulator of Th9 cell differentiation. In autoimmunity, silencing of BCL6 in myelin-reactive Th9 cells increases IL-9 and IL-4 expression and decreases IFNγ production leading to a reduction of their encephalitogenicity upon adoptive transfer in lymphopenic hosts.

IL-2, a growth factor linked to human autoimmune diseases (44), is a critical element for the development of Th1 (45) and Th2 (46) cells while inhibiting Th17 (47) and T follicular helper cells (48). The alpha-subunit of the IL-2 receptor (IL-2Rα) plays a pivotal role in the regulation of T cell function. Levels of a soluble form of IL-2Rα (sIL-2Rα) lacking the transmembrane and cytoplasmic domains were shown to be increased in several autoimmune diseases including multiple sclerosis (49). In addition, IL-2Rα genetic variants correlate with the levels of soluble IL-2Rα in subjects with type 1 diabetes and MS (50, 51). Addition of IL-2 to murine and human Th9 cells enhances their differentiation (27, 28, 52). However, the molecular mechanisms of IL-2-mediated amplification of Th9 cell differentiation are still unclear. IL-2 is produced mainly by activated T cells after engagement of the T-cell receptor and the CD28 co-stimulatory molecule (53). Our data demonstrate that in addition to Th1 and Th2 cells, IL-2 is massively produced early after Th9 cell polarization suggesting a role of IL-2 signaling in the regulation of Th9 cell development. Three subunits constitute the IL-2 receptor (IL-2R): the α chain (IL-2Rα, also known as CD25), the β chain (IL-2Rβ, also known as CD122, shared by the IL-15R), and the common cytokine receptor γ chain (γc, also known as CD132, shared by IL-4R, IL-7R, IL-9R, IL-15R and IL-21R). The IL-2/IL-2R complex mediates downstream signaling through IL-2Rβ and γc by inducing the association and phosphorylation of the tyrosine kinases Janus kinase 1 (JAK1) and JAK3 (54, 55), leading to the activation of STAT5 pathway (56). Here we provide novel evidence characterizing a regulatory network where STAT5 functions as an upstream regulator of the expression and activity of BCL6 and promoting the development of Th9 cells. These findings are in agreement with a recent

report describing involvement of STAT5 signaling in Th9 cell polarization in animal models of allergic airway inflammation (16, 29).

The molecular dissection of BCL6-mediated Th9 cell differentiation inhibition indicates that BCL6 directly represses the *Il9* locus. We have previously reported that Th9 cell differentiation is defective in GATA3-deficient mice suggesting that GATA3 is required for IL-9 transcription (1). Although our present report provides direct evidence demonstrating that BCL6 directly regulates the *Il9* promoter, it remains possible that GATA3, a known target of BCL6 (17), may provide an additional mechanism linking BCL6 to the regulation of Th9 cell differentiation.

The BCL6 consensus-binding site resembles the interferon-gamma activated sequence motif recognized by the STAT family of transcription factors, raising speculation that BCL6 may repress some cytokine response genes (57). Our data demonstrate that STAT5 not only binds and activates the *Il9* promoter transactivation but also competes with BCL6 binding activity to the *Il9* promoter in an IL-2-dependent manner. These findings support previous studies demonstrating that STAT5 and BCL6 bind to distinct but overlapping sequence motifs, supporting the proposal that STAT factors and BCL6 directly compete for DNA binding (20, 58). BCL6 repression could thus involve direct competition for STAT5 binding, as well as epigenetic modifications induced by BCL6-associated factors. Indeed, we observed an increase in the repressive H3K9 pan-methylation mark surrounding BCL6 binding site in Th9 cells cultured with anti-IL-2 neutralizing antibody. It should be noted that the described interplay between STAT5 and BCL6 is likely modulated for the long-term because STAT5 was found to act as a transcriptional repressor on the BCL6 gene itself (38). We also found that in Th9 cells, STAT5 is recruited to the BCL6 promoter and this was regulated by IL-2 signaling.

T helper cells exhibit various degrees of plasticity, namely, the capacity to change their phenotype when exposed to a new inflammatory milieu or upon regulation of transcription factors. An increasing body of evidence demonstrates that the regulation of Th9 cell development is a complex process requiring multiple cis-regulatory elements and specific trans-activating transcription factors including PU.1, IRF4, and RBPJ that are in common with other $CD4^+$ T cell lineages particularly Th2 cells (12, 14, 59, 60). Our identification of BCL6 transcriptional program in Th9 cell differentiation provides additional evidence of the plasticity of Th9 cells where BCL6 forced expression resulted in a down-regulation of Th9/Th1 ratio as shown by a decrease in IL-4/IL-9 expression and an increase in IFNγ production leading to EAE exacerbation following adoptive transfer. It is worth noting that other transcription factors such as STAT1 have been reported to modulate Th9 cell plasticity such that genetic deletion of STAT1 in these cells increased the Th9/Th1 ratio while IL-27 suppressed IL-9 production and up-regulated IFNγ expression in Th9 cells in a STAT1-dependent manner (61).

In conclusion, our work indicates that IL-2/BCL6 pathway orchestrates *Il9* transcription and modulates the pathogenicity of Th9 cells in EAE mice. Further studies are therefore warranted to determine the effects of IL-9 on the plasticity of T helper cells in the context of autoimmune responses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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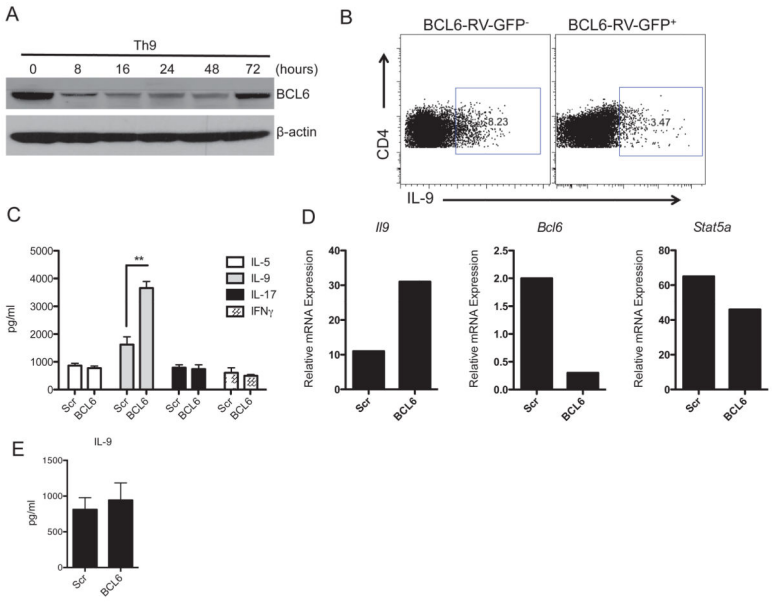

Figure 1. BCL6 Is a Negative Regulator of Th9 Cells

(**A**) BCL6 is transiently downregulated in polarized Th9 cells. Immunoblot of BCL6 in Th9 cells polarized for 0–72 hours in the absence of exogenous recombinant IL-2. β-actin was used as internal control for protein loading. (**B**) BCL6 forced expression reduced IL-9 expression. Naïve $CD4^+$ T cells were prepared from the spleens of WT mice and cultures were transduced with BCL6-RV-GFP encoding for BCL6-GFP. Cells were differentiated under Th9 cell conditions for 4 days followed by intracellular cytokine staining and BCL6-GFP-positive cells were gated on the basis of the GFP expression. (**C**) BCL6 knockdown in Th9 cells up-regulates IL-9 production. Naïve $CD4^+$ T cells were transfected with siRNA specific for BCL6 or with scrambled (Scr) siRNA using Amaxa followed by polarization under Th9 cell conditions for 4 days. Cytokine analysis of IL-5, IL-9, IL-17 and IFNγ expression in the supernatants was assessed by Luminex. (**D**) A representative gene expression of *Il9*, *Bcl6* and *Stat5a* in Th9 cells that were transfected with siRNA specific for BCL6 is shown. Transfection with scrambled siRNA was used as a control. (**E**) IL-9 production in Th9 cells. Th9 cells were differentiated for 3 days followed by transfection with siRNA specific for BCL6 or with scrambled siRNA. IL-9 release in the culture supernatants was measured by Luminex. Data are representative of three experiments with similar results. $^{**}p < 0.005$.

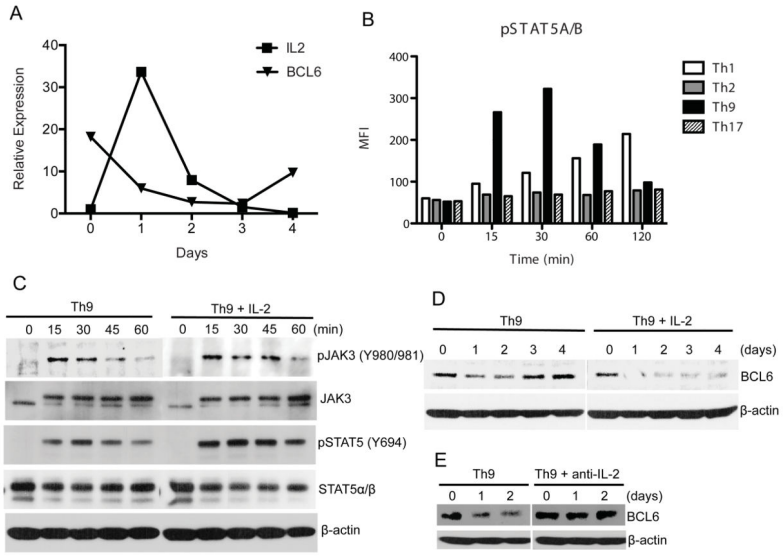

Figure 2. Activation of IL-2/JAK3/STAT5 Axis Inversely Correlates With BCL6 Down-regulation in Th9 Cells

(**A**) Opposite regulation of IL-2 and BCL6 in polarized Th9 cells. Naïve $CD4^+$ T cells were cultured under Th9 conditions for 4 days in the absence of exogenous recombinant IL-2 and BCL6 and IL-2 mRNA expression was assessed by Taqman PCR. (**B**) Increased STAT5 activation in Th9 cells. Polarized T helper and inducible Tregs were differentiated *in vitro* for 0–120 minutes (min) and phospho-STAT5 (pSTAT5) was assessed using bead-based Luminex assay. (**C**) Representative immunoblot of phospho-dependent and -independent JAK3 and STAT5 in naïve $CD4^+CD62L^{hi}$ polarized under Th9 conditions for the indicated time points (0–60 min) with recombinant IL-4 plus TGF-β1 in the presence or absence of exogenous recombinant IL-2. Phospho-epitope of each protein kinase is indicated. β-actin was used as internal control for protein loading. (**D**) Recombinant IL-2 inhibited BCL6 expression in Th9 cells. Naïve $CD4^+$ T cells were polarized under Th9 cell conditions in the presence or absence of exogenous recombinant IL-2 and BCL6 expression was assessed for up to 4 days. β-actin expression is shown. (**E**) Anti-IL-2 neutralizing antibody rescued the decrease in BCL6 expression in Th9 cells. Naïve $CD4^+$ T cells were polarized under Th9 cell conditions in the presence or absence of anti-IL-2 neutralizing antibody and BCL6 expression was assessed on days 0, 1 and 2 days. β-actin expression is shown.

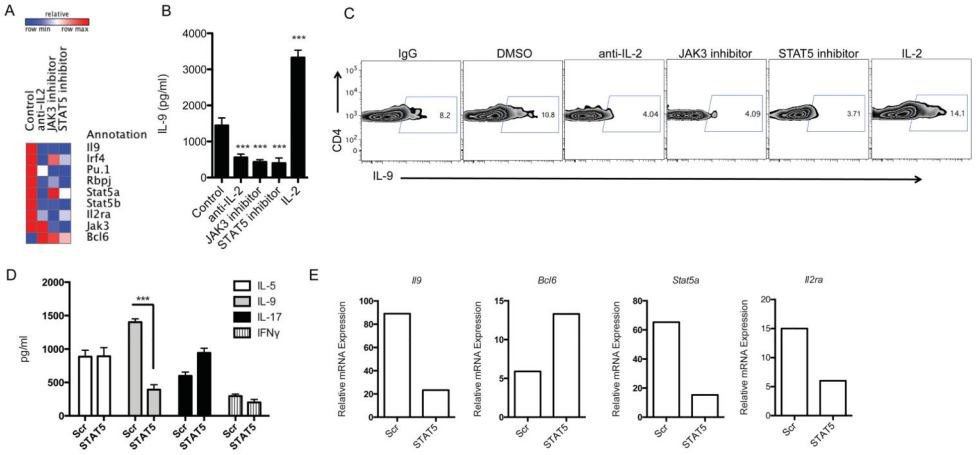

Figure 3. Opposite Role of STAT5A and BCL6 in the Regulation of Th9 Cell Development

(A, B, C) Inhibition of IL-2/JAK3/STAT5 signaling decreases IL-9 production in Th9 cells. (**A**) Heatmap of gene regulated in Th9 cells treated with anti-IL-2 neutralizing antibody, and JAK3 and STAT5 inhibitors. Naive $CD4^+$ T cells from WT mice were differentiated into Th9 cells in the presence or absence of the indicated treatment and gene expression was measured by Taqman PCR. (**B, C**) IL-9 protein expression by bead-based Luminex assay (**B**) as well as by intracellular flow cytometry (**C**) in treated Th9 cells is shown. (**D, E**) STAT5 knockdown reduces IL-9 and up-regulated BCL6 expression in Th9 cells. Naïve $CD4^+$ T cells transfected using Amaxa with siRNA specific for STAT5A or scrambled (Scr) siRNA followed for polarization under Th9 cell conditions for 4 days. (**D**) Cytokine analysis of IL-5, IL-9, IL-17 and IFNγ expression in the supernatants was assessed by Luminex. (**E**) A representative gene expression of *Il9*, *Bcl6*, *Stat5a* and *Il2ra* in Th9 cells that were transfected with siRNA specific for STAT5A, or scrambled siRNA and measured by Taqman PCR. Data are representative of three experiments with similar results. ***$p < 0.0001$.

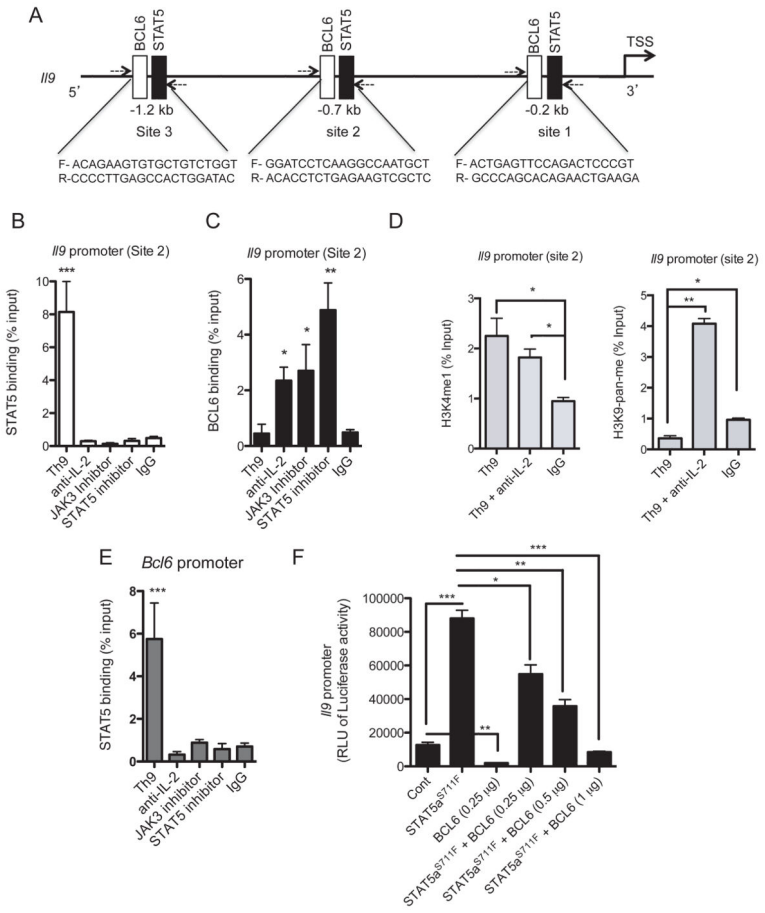

Figure 4. IL-2-Mediated Control of STAT5 and BCL6 Differential Binding to the *Il9* Promoter

(**A**) Three predicted binding sites for BCL6 (Open box) and STAT5 (filled boxes) in the *Il9* promoter. PCR primer sets for each site are shown. (**B, C**) ChIP analysis of BCL6 and STAT5 binding to the *Il9* promoter. Regulation of BCL6 and STAT5 binding to site 2 within the *Il9* promoter. Naïve $CD4^+$ T cells from WT mice were polarized under Th9 cell conditions for 2 days in the presence of anti-IL-2 neutralizing antibody, JAK3 or STAT5 inhibitor. ChIP-SYBR Green PCR was performed to determine (**B**) STAT5 and (**C**) BCL6 binding to the *Il9* promoter. Abs used for IP are anti-STAT5, anti-BCL6 and control IgG. Total input DNA before IP was used for normalization of data. (**D**) ChIP analysis of H3K4 (left panel) and H3K9 (right panel) modifications normalized to input controls at the BCL6 and STAT5 binding site 2 within the *Il9* promoter in Th9 cells differentiated in vitro for 2 days. (**E**) ChIP analysis of STAT5 binding to the *Bcl6* promoter in Th9 cells differentiated in vitro for 2 days. The graphs represent quantitative PCR analysis of the ratio of enriched *Il9* and *Bcl6* promoters with transcription factor binding sites to the input DNA. Data represent mean ± SE of a representative experiment each performed in triplicate. **(F)** HEK 293T cells were transfected with active mutant of STAT5 ($STAT5a^{S711F}$), BCL6, or combination of $STAT5a^{S711F}$ and BCL6 together with a constant amount of *Il9* promoter-luciferase vector. Cells were lyzed 24 hrs later and luminescence was measured. Representative of three independent experiments. $*p < 0.01$, $**p < 0.005$; $***p < 0.0001$.

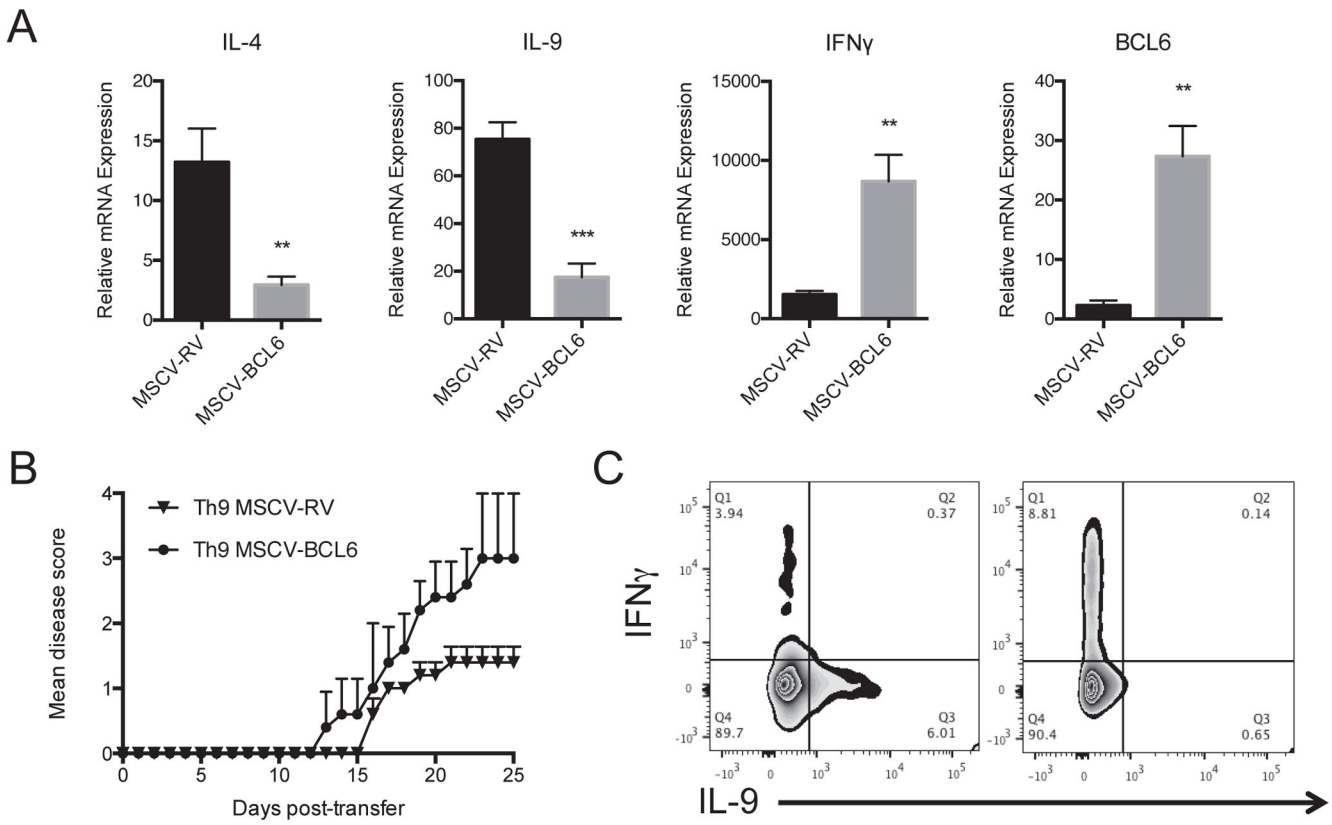

Figure 5. BCL6 Regulates Th9 Cell Pathogenicity in EAE mice

(**A**) A representative gene expression of IL-4, IL-9, IL-17A, IFNγ and BCL6 in Th9 overexpressing BCL6 and control cells. $MOG_{35–55}$-specific $CD4^+$ T cells were isolated from 2D2 mice and were transduced with MSCV-BCL6-GFP retrovirus virus (RV) or with control empty vector (MSCV-RV) followed by *in vitro* polarization under Th9 conditions for 4 days in the presence of $MOG_{35–55}$ peptide (20 μg/ml) and irradiated antigen-presenting cells. Cells were lyzed and analyzed for gene expression by Taqman. (**B**) A representative EAE experiment showing clinical scores (means ± s.e.m.) in mice after adoptive transfer of $MOG_{35–55}$-stimulated 2D2 polarized under Th9 cell conditions with or without BCL6 overexpression (10 mice/group). Rag2−/− mice received MSCV-BCL6-transduced Th9 cells or control cells followed by immunization with $MOG_{35–55}$ peptide (100 μg/ml). (**C**) Intracellular staining of transferred 2D2 cells. Infiltrated cells were prepared from the spinal cords of recipient mice (3 mice/group) followed by flow cytometry intracellular staining of IFNγ and IL-9. Data are representative of two experiments with similar results. $^{**}p < 0.01$; $^{***}p < 0.001$.