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BCL6 Controls Th9 Cell Development by Repressing Il9 Transcription

Ribal Bassil,*1 William Orent,*1 Marta Olah,* Ahmed T. Kurdi,* Michael Frangieh,* Thomas Buttrick,* Samia J. Khoury,*7 and Wassim Elyaman*

The transcriptional repressor B cell lymphoma 6 (BCL6) is required for the development of 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 upregulated IL-9 production in Th9 cells. The function of BCL6 in Th9 cells is under the control of IL-2/JAK3/STAT5 signaling pathway. Using chromatin immunoprecipitation, 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, whereas under conditions in which 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 in which BCL6 repressed STAT5-mediated Il9 transactivation. In experimental autoimmune encephalomyelitis, forced expression of BCL6 in myelin oligodendrocyte glycoprotein 35–55-specific Th9 cells resulted in decreased IL-9 production and induction of IFN-γ, 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. The Journal of Immunology, 2014, 193: 000–000.

The transcriptional repressor B cell lymphoma 6 (BCL6) is required for the development of 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 upregulated IL-9 production in Th9 cells. The function of BCL6 in Th9 cells is under the control of IL-2/JAK3/STAT5 signaling pathway. Using chromatin immunoprecipitation, 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, whereas under conditions in which 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 in which BCL6 repressed STAT5-mediated Il9 transactivation. In experimental autoimmune encephalomyelitis, forced expression of BCL6 in myelin oligodendrocyte glycoprotein 35–55-specific Th9 cells resulted in decreased IL-9 production and induction of IFN-γ, 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.

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 STAT (10). The relationships are as follows: Th2, GATA-binding protein 3/STAT5; Th1, T-box transcription factor expressed in T cells (T-bet)/STAT4; Th17, retinooid orphan receptor γ/STAT3; inducible Treg, forkhead box protein 3 (Foxp3)/STAT5. Recent studies suggest that Th follicular 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 noncoding sequence in reporter assays, including IRF4, PU.1, NF-kB, 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 mice exhibit significant growth retardation and invariably die by 10 wk of age (20, 21). BCL6 knockout 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 (in which N is any nucleotide) of the Il9 locus and Th9 cell development.

In the current 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.
C57BL/6 and Rag2−/− mice were purchased from Jackson Laboratory, and myelin oligodendrocyte glycoprotein (MOG)35–55 TCR 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-ALX (BD Biosciences). After centrifuge at 1300 × g for 30 min at 37˚C, resuspended in 30% Percoll, and loaded onto 70% Percoll. Briefly, the retrovirus particles were produced by cotransfecting the plasmid pCMV-IREs-2BCL6 (420126) and STAT5 inhibitor (573108) were obtained from Calbiochem (San Diego, CA). The 7-aminoactinomycin D stain for dead cell exclusion was obtained from BD Biosciences.

In vitro T cell differentiation, retroviral cell transduction, and cytokine assay

Naive CD4+ T cells were purified from C57BL/6 wild-type (WT) mice using anti-CD44 beads (Miltenyi Biotec, Auburn, CA) and flow sorted into naive CD4+CD62Lhigh T cells by flow cytometry on a FACS Aria T cell sorter (BD Biosciences). CD4+ T cells were stimulated with plate-bound anti-CD3 (4 µg/ml; 145-2C11; BD Biosciences) and soluble anti-CD28 (2 µg/ml; BD Biosciences) for 4 d in a serum-free culture medium (X-VIVO-20; Lonza, Hopkinton, MA) supplemented with 50 µM 2-ME, 1 mM sodium pyruvate, nonessential amino acids, 1-glutamine, and 100 U/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 rIL-2 (40 ng/ml), where indicated. Rat anti-mouse IL-2 Ab (BD Biosciences) was used for blocking of IL-2 activity in vitro. All recombinant proteins were from R&D Systems. JK3 (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 resuspended with 12-O-tetradecanoylphorbol-13-acetate (20 ng/ml; Sigma-Aldrich, St. Louis, MO), ionomycin (300 ng/ml; Sigma-Aldrich), and 2 mM monensin (GolgiStop; BD Biosciences) for 4 h at 37˚C. Cells were washed, stained for surface markers, and permeabilized, and fluorochrome-conjugated Abs 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. After sacrifice, the CNS was harvested, and the relative mRNA abundance was normalized against GAPDH. Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (Thermo Scientific) with 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 probed for 1 h with TBS-Tween 20 containing 5% BSA and then probed for 3 h at room temperature with primary Abs directed against the following: phospho-STAT5 (Tyr613), phospho-JAK3 (Tyr505), STAT5, JAK3, or BCL6 (Cell Signaling Technology). β-actin mouse mAb (Sigma-Aldrich) 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 nucleoporation system. Briefly, 5 × 106 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 experimental autoimmune encephalomyelitis model

For the generation of myelin-specific Th9 cells, CD4+CD62Lhigh cells were prepared from spleens of MOG35–55 TCR transgenic mice (2D2) using MACS beads. CD4+ cells were transduced with MSCV-BCL6-GFP or control vector MSCV-GFP followed by stimulation with MOG35–55 peptide (20 µg/ml) in the presence of IL-4 (20 ng/ml) and TGF-β1 (3 ng/ml) for 3 d in the presence of splenic CD11c+ dendritic cells isolated from 2D2 mice. One million cells were transduced into Rag2−/− recipient mice via i.p. injection, followed by immunization with the emulsion made of 50 µg MOG35–55 (MEGVYKRSFSRVHLYRNKG; New England Peptide) and CFA. Each animal also received i.p. injections of 69 µg pertussis toxin on days 1 and 3 after transfer. The experimental autoimmune encephalomyelitis (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.

Chromatin immunoprecipitation and quantitative PCR

CD4+ T cells were purified by MACS sorting and were polarized to Th9 phenotype. Chromatin immunoprecipitation (ChIP) was performed according to the protocol described previously (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-monomethyl histone 3(lysine 4) (H3K4me1), and anti–pan-methyl-H3K9 (all from Cell Signaling Technology) and were compared with 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 qualitative PCR (Applied Biosystems) and quantified in duplicate with the percentage of input method. The following primers were used: Il9 promoter: site 1 forward, 5′-ACCTAGTTCGACAGGTTTCCT-3′, and reverse, 5′-GCCACAGACAGGCTGCCTT-3′; site 2, forward, 5′-GGGACTCCAGGCAAAGCCTG-3′, and reverse, 5′-ACCATCTGGAAGTGTGCTGTC-3′; site 3, forward, 5′-ACGAACTTGGTGCCCTGCT-3′, and reverse, 5′-ACCCCTTTGAGGCCCCAGCG-3′; site 4, forward, 5′-CTGGCGGAGCTATCGTCCTG-3′, and reverse, 5′-AGTATCTCTCCACGCTGCTG-3′; and reverse, 5′-CTGGTGATTGGCTTGTCCTG-3′. 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). MSCV expressing WT mouse Stat5a 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 reporter vector coding for the pRL-TK luciferase under the control of the Il9 promoter and with 0.2 µg STAT5A or BCL6 encoding plasmids. Cells were cultured for 24 h before harvesting, and the relative Il9 promoter activity was measured using Promega kit in accordance with the manufacturer’s instructions.

Materials and Methods

Mice and reagents

BCL6 IS A NEGATIVE REGULATOR OF Th9 CELLS

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Statistical analysis
The Mann–Whitney U test was used for clinical disease analysis. Data are expressed as mean ± SEM and were compared using the unpaired Student 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. In this study, we investigated the possibility as to 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 h following differentiation, as shown by Western blot. BCL6 downregulation was transient, suggesting an intrinsic mechanism for the control of BCL6 expression in Th9 cells (Fig. 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 naive CD4⁺CD4₄lowCD62Lhigh T cells with MSCV-BCL6-GFP retrovirus (BCL6-RV-GFP), cultured the cells under Th9 conditions for 4 d, and measured intracellular cytokine expression using flow cytometry. We found that the frequency of IL-9⁺–positive cells was altered by ~60% after BCL6 (GFP⁺) overexpression compared with BCL6-negative (GFP⁻) Th9 cells, suggesting that the transcription factor BCL6 is a negative regulator of Th9 cells (Fig. 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, subsequently polarized to the Th9 lineage, and harvested 4 d after transfection. Consistent with the data obtained with BCL6 overexpression, BCL6 knockdown resulted in an upregulation of IL-9 expression at both the protein and gene levels, as measured by bead-based Luminex assay and TaqMan PCR, respectively (Fig. 1C, 1D). 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 d 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 (Fig. 1E).

Activation of IL-2/JAK3/STAT5 axis inversely correlates with BCL6 downregulation 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 (30). Analysis of IL-2 signaling in murine Th1, Th2, Th9, Th17, and inducible CD4⁺Foxp3⁺ Tregs differentiated in vitro according to standard protocols shows that the expression of Il2ra, Stat5α, and Stat5β is upregulated in Th9 cells (Supplemental Fig. 1A). Using TaqMan PCR to verify the transcriptome-sequencing results in different Th subsets, we found that mRNA levels of Il2ra, Stat5α, and Stat5β are indeed elevated in Th9 cells compared with other cell phenotypes (Supplemental Fig. 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 (Fig. 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 (Supplemental Fig. 1C), and because IL-2/STAT5 pathway has been shown to regulate BCL6 signals in follicular Th 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 Th subsets differentiated in vitro for several time points between 0 and 120 min 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 min after polarization (Fig. 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 rIL-2 (0–60 min), and cells were lysed and used for immunoblot analysis using Abs 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 rIL-2 (Fig. 2C). Quantification of phospho-JAK3 and STAT5 by measurements of the OD of the corresponding bands is shown (Supplemental Fig. 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 downregulation of BCL6 in polarized Th9 cells, we measured BCL6 protein expression in Th9 cells differentiated in vitro for several days in the presence of rIL-2 or anti–IL-2 neutralizing Ab. We found that, whereas BCL6 protein levels were transiently decreased during Th9 cell differentiation, supplementation with rIL-2 induced sustained suppression of BCL6 protein expression (Fig. 2D). Interestingly, when Th9 cells were differentiated in the presence of anti–IL-2 neutralizing Ab, BCL6 expression was maintained throughout the polarization, suggesting that IL-2 signaling is a negative regulator of BCL6 in Th9 cells (Fig. 2E). Quantification of BCL6 levels by densitometry is shown (Supplemental Fig. 1D).

IL-2/JAK3/STAT5 inhibition upregulates 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 d. We found that addition of either inhibitor upregulated BCL6 mRNA expression as measured by TaqMan PCR (Fig. 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 (Fig. 3B, 3C).

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 d after transfection. Silencing of STAT5A specifically inhibited IL-9 production but did not affect the production of other cytokines, including IL-5, IL-17, and IFN-γ (Fig. 3D). Quantitative RT-PCR confirmed that STAT5A siRNA reduced the expression of Il9 and...
BCL6 mRNA (Fig. 3E). To check whether the reduction in IL-9 in STAT5A siRNA-treated cells was due to differential cell survival, we performed 7-aminoactinomycin D flow staining of nucleoparticleted 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 (Fig. 3E), in agreement with the elevated Bcl6 expression in Th9 cells treated with IL-2/JAK3/STAT5 inhibitors (Fig. 3A) and the decrease in Bcl6 protein expression in Th9 cells exposed to IL-2 (Fig. 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) (Fig. 4A). To determine the Il9 promoter occupancies by STAT5 and BCL6, binding motifs were used to design 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 quantitative PCR (Fig. 4A). CD4^+CD62L^high T cells were differentiated under Th9 cell-polarizing conditions for 1–4 d 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 d after cell polarization (Fig. 4B, Supplemental Fig. 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 Ab 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 (Fig. 4B, Supplemental Fig. 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 because inhibition of either IL-2, JAK3, or STAT5 suppressed STAT5 binding, whereas it facilitated BCL6 recruitment to the same motif, particularly to site 2.

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-I9 containing the firefly luciferase gene under the control of the Il9 promoter in 293T cells (Fig. 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, cotransfection of pGL3-I9 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 (Fig. 4F). STAT5A overexpression in 293T cells is comparable in the presence or absence of exogenous rIL-2, and BCL6 expression was assessed on days 0, 1, and 2. β-actin expression was assessed on days 0, 1, and 2.

**BCL6 overexpression in myelin-specific Th9 cells regulates their encephalitogenicity**

Previous studies demonstrated that adoptive transfer of myelin-reactive Th9 cells induces EAE (7). To investigate whether BCL6-mediated IL-9 repression modulates EAE, we carried out adoptive transfer experiments with MOG35-55-pulsed Th9 cells following BCL6 overexpression. To generate myelin-specific Th9 cells overexpressing BCL6, MOG35-55-TCR transgenic CD4+
CD62L<sup>high</sup> 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 3 d in the presence of MOG<sub>35-55</sub> (20 μg/ml) peptide and splenic dendritic cells. Analysis of the cytokine profile of BCL6-GFP–positive 2D2 CD4<sup>+</sup> T cells demonstrates a decrease in IL-4 and IL-9 expression compared with control cells as shown by TaqMan PCR (Fig. 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<sup>−/−</sup> lymphopenic mice, followed by reactivation with MOG<sub>35-55</sub>/CFA immunization, and mice were monitored for clinical disease development. We found that EAE was markedly enhanced in recipients of Th9 MSCV-BCL6–treated cells compared with Th9 MSCV-RV–treated 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 U test) (Fig. 5B). To test whether the cytokine profile of transferred cells is maintained in recipient mice, spinal cord tissues were collected ~10 d posttransfer, 2D2 cells were identified using the surface markers V<sub>α</sub>3.2/V<sub>β</sub>11, 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 IFN-γ and suppression of IL-9 production (Fig. 5C).

**Discussion**

Previous reports uncovered a key role of BCL6 as a transcriptional regulator in various cell types, including B cells and T lymphocyte subsets within and outside the germinal centers, including CD4<sup>+</sup> follicular Th cells (39), T regulatory (40), and Th cells, and in the control of T cell–dependent inflammation and autoimmunity (17, 20). The role of BCL6 in the regulation of Th cells, including Th1, Th2, and Th17 cells, has been reported by several groups (17, 18, 21, 41–43). In this study, 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 IFN-γ 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 Th follicular cells (48). The α-subunit of the IL-2R (IL-2Rα) plays a pivotal role in the regulation of T cell function. Levels of a soluble form of IL-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 multiple sclerosis (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 TCR and the CD28 costimulatory 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-2R, as follows: the α-chain (IL-2Rα), also known as CD25, the β-chain (IL-2Rβ, also known as CD122, shared by the IL-15R), and
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 and 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. Naive CD4+ T cells from WT mice were polarized under Th9 cell conditions for 2 d in the presence of anti–IL-2 neutralizing Ab, 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 inflammatory protein are anti-STAT5, anti-BCL6, and control IgG. Total input DNA before inflammatory protein 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 d. (E) ChIP analysis of STAT5 binding to the Bcl6 promoter in Th9 cells differentiated in vitro for 2 d. (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 lysed 24 h later, and luminescence was measured. Representative of three independent experiments. *p < 0.01, **p < 0.005, ***p < 0.0001.
the common cytokine receptor γ-chain (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 common cytokine receptor γ-chain by inducing the association and phosphorylation of the tyrosine kinases JAK1 and JAK3 (54, 55), leading to the activation of STAT5 pathway (56). In this study, we provide novel evidence characterizing a regulatory network in which 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 IFN-γ-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 Ab. It should be noted that the described interplay between STAT5 and BCL6 is most 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.

Th 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, which 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 in which 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 IFN-γ 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 as shown by genetic deletion of STAT1 in these cells increased the Th9/Th1 ratio, whereas IL-27 suppressed IL-9 production and upregulated IFN-γ 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 Th cells in the context of autoimmune responses.

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Disclosures

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References


