BCL-6 regulates chemokine gene transcription in macrophages

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The transcriptional repressor protein BCL-6, implicated in the pathogenesis of B cell lymphoma, regulates lymphocyte differentiation and inflammation. We investigated the mechanism for the T helper cell subset 2 (T_H2)-type inflammation that occurs in BCL-6$^{-/-}$ mice. Using chimeric mice we found that the T_H2-type inflammation is dependent upon nonlymphoid cells. We identified three chemokines, MCP-1, MCP-3 and MRP-1, which are negatively regulated by BCL-6 in macrophages. Promoter analysis revealed that BCL-6 is a potent repressor of MCP-1 transcription. Our results provide a mechanism for the regulation of T_H2-type inflammation by BCL-6 and link T_H2 differentiation to innate immunity.

The gene encoding BCL-6 (*BCL6*) is rearranged by chromosomal translocations in about 30% of diffuse large cell lymphoma cases, as well as in a significant percentage of other B cell lymphomas[1–4]. *BCL6* encodes a zinc-finger–containing transcriptional repressor protein[5–9]. However, the regulatory pathways that BCL-6 affects are not well understood in part because the target genes that BCL-6 regulates have not been well characterized.

BCL-6–deficient (BCL-6$^{-/-}$) mice have shown that BCL-6 is essential for the formation of germinal centers and for a normal antibody response[10,11]. BCL-6$^{-/-}$ mice have also revealed, unexpectedly, that BCL-6 is major negative regulator of T helper cell subset 2 (T_H2) differentiation and T_H2-type inflammation. BCL-6$^{-/-}$ mice have a high rate of severe T_H2-type inflammatory disease and frequently die at an early age. Approximately 80% of BCL-6$^{-/-}$ mice develop myocarditis or inflammation of other organs such as spleen, gut, liver and skin, which is characterized by infiltration of monocytes, eosinophils, and T_H2 cells[10,11]. Abnormal numbers of IgE-positive B cells and intraepithelial mast cells are also seen in BCL-6$^{-/-}$ mice[10]. Immunization of BCL-6$^{-/-}$ mice with a protein antigen in adjuvant leads to the induction of a T_H2-type inflammation, suggesting that the hyper-T_H2 response can be antigen driven[12].

In this study we found a critical role for nonlymphoid cells in the development of the eosinophilic infiltrates and abnormal production of

IgE that occurs in BCL-6$^{-/-}$ mice. Macrophages grown from BCL-6$^{-/-}$ mice have increased expression of specific chemokines compared to wild-type cells. Chemokines are small secreted factors that are critical regulators of leukocyte recruitment, activation and inflammatory processes[13,14]. Although various functions of chemokines have been well studied, considerably less is known about the transcriptional regulation of chemokine expression. BCL-6 represents an example of a transcription factor that specifically represses chemokine transcription,
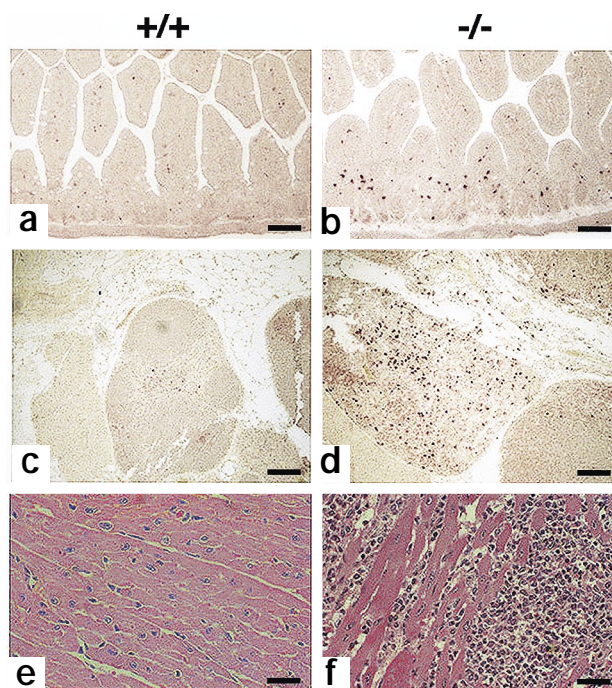
Figure 1. Inflammatory wild-type disease in BCL-6$^{-/-}$ and chimeric BCL-6$^{-/-}$ mice. The figure shows representative sections of wild-type (left column) and inflammation-affected chimeric mice (right column). Histochemical chloroacetate esterase–labeled (CAE$^+$) intraepithelial mast cells (red) were identified in (**a**) BCL-6$^{-/-}$ mice and (**b**) all three types of chimeric mice, but not in wild-type controls. In lymph nodes, an increased amount of IgE$^+$ cells (brown immunostain) were not detected in (**c**) wild-type controls or the three types of chimeras but were detected in (**d**) the BCL-6$^{-/-}$ mice. In myocardium, significant eosinophilic and mononuclear infiltration was not observed from (**e**) wild-type or chimeric mice but was observed from (**f**) BCL-6$^{-/-}$ mice. Scale bars: 0.2 mm for **a**, **b**, **c**, **d** and 0.05 mm for **e**, **f**.

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Table 1. Germinal center formation and inflammatory response in BCL-6⁻/⁻ embryonic stem cell–derived chimeras

Complementation		BCL-6 status in cells			GC formation	Inflammatory disease		
Host	Embryonic stem cells	T	B	Nonlymphoid		Hyper-IgE	Eosinophilic infiltration	Intraepithelial mast cells in gut
BCL-6$^{+/+}$	–	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	60/60	1/22[a]	0/60	1/60[a]
BCL-6$^{-/-}$	–	BCL-6$^{-/-}$	BCL-6$^{-/-}$	BCL-6$^{-/-}$	0/64	12/21	40/64	45/59
RAG-1$^{-/-}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	3/3	0/3	0/3	0/3
RAG-1$^{-/-}$	BCL-6$^{-/-}$	BCL-6$^{-/-}$	BCL-6$^{-/-}$	Chimeric	0/6	0/6	0/6	6/6
IgM$^{-/-}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	6/6	0/6	0/6	1/6[a]
IgM$^{-/-}$	BCL-6$^{-/-}$	Chimeric	BCL-6$^{-/-}$	Chimeric	0/5	0/5	1/5	3/5
TCRβδ$^{-/-}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	BCL-6$^{+/+}$	2/2	0/2	0/2	0/2
TCRβδ$^{-/-}$	BCL-6$^{-/-}$	BCL-6$^{-/-}$	chimeric	chimeric	3/6[b]	0/6	0/6	5/6
BCL-6 function required in:					B cells	non-B, non-T	non-B, non-T	B or T

[a]Occasionally, low levels of IgE$^+$ cells and intraepithelial CAE$^+$ mast cells are detected above the average background values for wild-type animals. [b]Low chimerism might have resulted in insufficient number of T cells in the three chimeras that failed to develop germinal centers. See Fig. 1 for examples of the three major aspects of the inflammatory disease. The experimental procedure is outlined in the Methods section.

and chemokine genes represent important transcriptional targets for BCL-6. Thus, BCL-6 is now identified as a regulator of chemokine expression and innate immune responses.

Results

Non-B, non-T cells needed for T$_H$2-type inflammation

Because BCL-6 is expressed at various levels in multiple cell types, including B cells, T cells and the myeloid lineage[15–18], we carried out a series of genetic complementation experiments to identify the responsible cell types for each of the major phenotypes observed in the BCL-6$^{-/-}$ mice. We obtained chimeric mice by injecting BCL-6$^{-/-}$ embryonic stem cells into the blastocysts of either RAG-1$^{-/-}$ (ref. 19), TCRβδ$^{-/-}$ (ref. 20) or IgM$^{-/-}$ (ref. 21) mice. RAG-1$^{-/-}$ mice are genetically incapable of producing mature T cells or B cells, TCRβδ$^{-/-}$ mice cannot produce any mature T cells, and IgM$^{-/-}$ mice cannot produce any mature B cells. Chimeric animals made with these mutant blastocysts have both BCL-6$^{+/+}$ and BCL-6$^{-/-}$ cells in all cell types except in the targeted lineages where all the cells are BCL-6$^{-/-}$.

These mice were analyzed for their ability to form germinal centers after immunization with sheep red blood cells. In addition, they were also examined for signs of the hyper T$_H$2 inflammatory disease using three major criteria: hyper-IgE production, eosinophilic infiltration in various tissues and presence of intraepithelial mast cells (**Fig. 1**, **Table 1**). First, germinal centers failed to develop in the RAG-1$^{-/-}$ and IgM$^{-/-}$ chimeras but formed normally in the TCRβδ$^{-/-}$ chimeras. This data confirmed the observation that germinal center formation is dependent upon BCL-6 function in B cells but not T cells[22]. Second, although intraepithelial mast cells can be detected in all three types of chimeras, lack of BCL-6 function in either B, or T, or both lineages did not result in increased IgE production or eosinophilic infiltration of tissues. These results indicate that the full scale T$_H$2-type inflammatory disease is not a lymphoid-autonomous phenotype, and therefore must be initiated by cells of a non-B, non-T lineage.
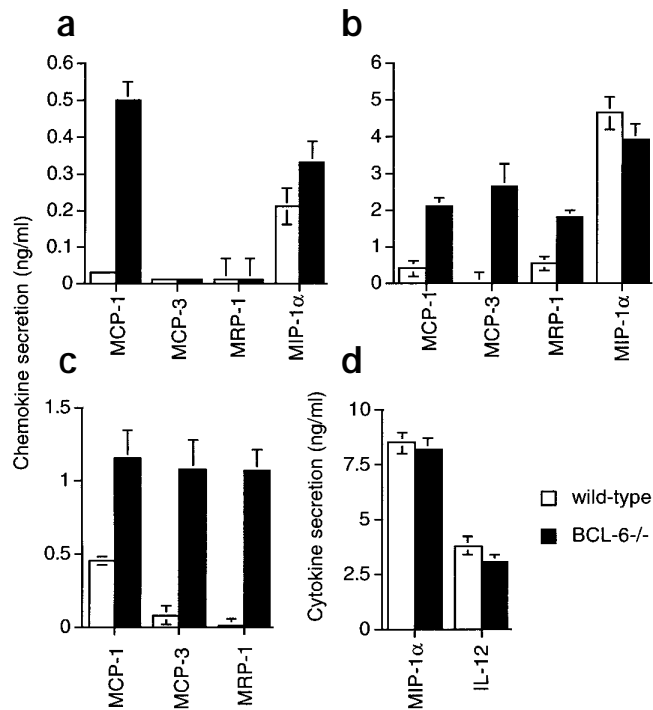
Increased chemokine production by macrophages

As macrophages are a major class of nonlymphoid cells that can regulate inflammatory processes, and as BCL-6 is expressed in monocytic cells[18], we analyzed the role of BCL-6 in macrophage gene expression.

Very pure populations of macrophages can be grown from bone marrow precursors by stimulation with recombinant mouse M-CSF. This type of generation of macrophages *in vitro* minimizes the influence of other BCL-6$^{-/-}$ cells on the development of the cells. We used bone marrow isolated from BCL-6$^{-/-}$TCRα$^{-/-}$ double mutant mice, as in theory these progenitor cells should not be perturbed by inflammatory T$_H$2 cells. BCL-6$^{-/-}$ TCRα$^{-/-}$ double mutant mice are unable to produce αβ T cells[20] and do not develop the T$_H$2-type inflammation characteristic of BCL-6$^{-/-}$ mice (A.L. Dent, unpublished data). (Macrophages from TCRα$^{-/-}$ and TCRα$^{-/-}$ BCL-6$^{-/-}$ mice are referred to henceforth as wild-type and BCL-6$^{-/-}$, respectively.)

Because of the critical importance of chemokines in inflammatory processes[13,14], we analyzed M-CSF–cultured BCL-6$^{-/-}$ macrophages for abnormalities in chemokine production. First, chemokine gene expression was
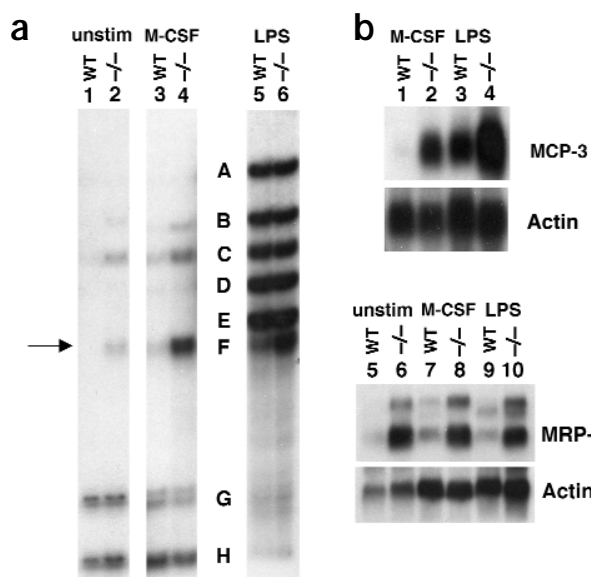
Figure 2. Chemokine gene expression in wild-type (WT) and BCL-6⁻/⁻ knockout (–/–) macrophages. (**a**) Ribonuclease protection assay. RNA was prepared from macrophages either left in medium (unstim.) or stimulated with M-CSF (10 ng/ml) or LPS (2 μg/ml) for 5 h. L32 and GAPDH are control mRNAs to show similar amounts of input RNA. Lanes 1–4 were exposed to film for 20 h, lanes 5 and 6 were exposed to film 2 h. (A, RANTES; B, MIP-1β; C, MIP-1α; D, MIP-2; E, IP-10; F, MCP-1; G, L32; H, GAPDH.) The arrow indicates increased MCP-1. (**b**) Northern analysis. RNA was prepared from macrophages stimulated as in part a above. Each lane contains 7 μg total RNA. RNA input is shown by actin probing of the respective blots.

Figure 3. Cytokine secretion by stimulated wild-type and BCL-6^{-/-} macrophages. (**a**) M-CSF and (**b**) IL-6 were used at 10 ng/ml. (**c**,**d**) LPS was used at 2 μg/ml. Supernatants were collected after a 20-h stimulation with the indicated stimuli for measurement by enzyme-linked immunosorbent assays (ELISA). Bars indicate the s.e., data shown is representative of three separate experiments performed with different preparations of macrophages.

MCP-1, tenfold more MCP-3 and 100-fold more MRP-1 than the LPS-stimulated wild-type cells. IL-12 and MIP-1α production following LPS-stimulation was similar between BCL-6^{-/-} and wild-type cells (**Fig. 3**). M-CSF stimulation did not lead to significant secretion of MRP-1 from the macrophages, although BCL-6^{-/-} macrophages expressed similar levels of MRP-1 RNA after both M-CSF and LPS stimulation. This may perhaps be explained by the fact that some cytokines require a post-transcriptional signal for their secretion from macrophages[23], although this phenomenon has not been previously described for chemokines. IL-6 is known to be a potent inducer of MCP-1 expression[24] and therefore we also analyzed chemokine secretion following IL-6 stimulation of the macrophages. IL-6 stimulation of BCL-6^{-/-} macrophages led to markedly amplified secretion of MCP-1, MRP-1 and MCP-3 compared to wild-type, whereas MIP-1α levels after IL-6 stimulation were similar in BCL-6^{-/-} cells and wild-type cells. Thus, three chemokine genes are strongly and specifically up-regulated in BCL-6^{-/-} macrophages: MCP-1, MRP-1 and MCP-3.

Increased expression of chemokines in BCL-6^{-/-} mice

We then tested chemokine expression in spleen cells taken directly from BCL-6^{-/-} mice, with and without stimulation of T cells with antibodies to CD3. CD3 stimulation of both wild-type and BCL-6^{-/-} spleen cells induced similar high levels of MIP-1α compared to unstimulated spleen cells (**Fig. 4**). Wild-type spleen cells did not produce detectable levels of MCP-1, MCP-3 and MRP-1, with or without CD3 stimulation (**Fig. 4**). In contrast, both unstimulated and anti-CD3–stimulated BCL-6^{-/-} spleen cells produced similar high levels of MCP-1, MCP-3 and MRP-1 (**Fig. 4**). We next tested whether the abnormally high MCP-1, MCP-3 and MRP-1 expression in BCL-6^{-/-} spleen cells derived from myeloid cells such as macrophages. Spleen cells from BCL-6^{-/-} mice were depleted of myeloid cells by fluorescence activated cell sorting (FACS), by using fluorescent-labeled antibodies to Mac-1 (CD11b). Depletion of Mac-1^{+} cells from unstimulated BCL-6^{-/-} spleen populations eliminated almost all MCP-1, MCP-3 and MRP-1 production (**Fig. 4**). The high constitutive levels of these three chemokines in BCL-6^{-/-} spleen cannot be explained simply by an increase in Mac-1^{+}

analyzed using a multiprobe ribonuclease protection assay (RPA). RNA was prepared from macrophages either maintained in culture medium without M-CSF, restimulated with M-CSF or stimulated with lipopolysaccharide (LPS) for 5 h. We observed that stimulation of both wild-type and BCL-6^{-/-} macrophages by LPS led to the very strong induction of multiple chemokine genes (**Fig. 2a**). These chemokines were induced to similar levels in both wild-type and BCL-6^{-/-} cells, indicating that these two macrophage populations responded to LPS almost identically for most chemokines analyzed. However, a large increase in MCP-1 expression in BCL-6^{-/-} compared to wild-type macrophages was noticeable after M-CSF and LPS stimulation, as well as in the unstimulated cells.

Because there are over 20 different chemokine genes in the mouse, it is possible that BCL-6 regulates chemokine genes other than the nine represented on the RPA panel. We therefore obtained cDNAs for chemokines not on the RPA panel and used these to screen northern blots containing RNA from wild-type and BCL-6^{-/-} macrophages cultured in medium alone or stimulated with either M-CSF or LPS (**Fig. 2b**). Seven of the chemokine cDNAs tested were not expressed by either wild-type or BCL-6^{-/-} macrophages: Exodus-1 (also called MIP-3α or LARC), Exodus-2 (SLC or 6Ckine), Exodus-3 (ELC or MIP-3β), MDC (ABCD-1), MCP-2, MCP-5 and SDF-1α (data not shown). An eighth chemokine, MRP-2 (MIP-1γ) was expressed relatively equally by wild-type and BCL-6^{-/-} macrophages (data not shown). However, two chemokines tested by northern blot were expressed at substantially higher levels by BCL-6^{-/-} macrophages when stimulated with M-CSF or LPS: MRP-1 (C10) and MCP-3 (MARC) (**Fig. 2b**). MRP-1 was expressed at similar levels under all conditions tested, whereas MCP-3 displayed a pattern of induction by LPS similar to what was seen with MCP-1.
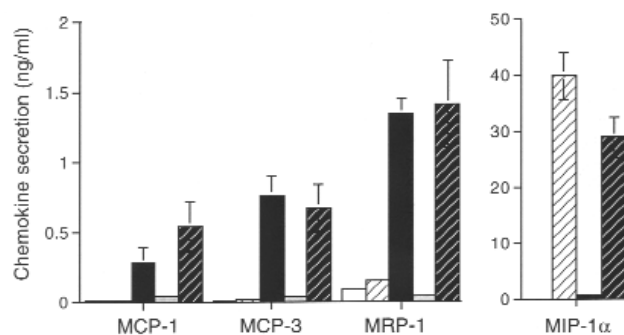
Next we used specific ELISAs to measure chemokine secretion following stimulation of the macrophage populations (**Fig. 3**). The ELISA results were compatible with the mRNA analysis by showing that LPS-stimulated BCL-6^{-/-} macrophages secreted almost threefold more

Figure 4. Constitutive expression of chemokines by Mac-1^{+} cells from BCL-6^{-/-} spleen. Supernatants were taken from wild-type and BCL-6^{-/-} spleen cells that were plated in untreated or anti-CD3–coated tissue culture wells for 48 h. Bars indicate the s.e. of averages from stimulation of three or four individual mice. (Open bars, wild-type; open hatched bars, wild-type + anti-CD3; filled bars, BCL-6^{-/-}; shaded bars, BCL-6^{-/-} with Mac-1 depletion; filled hatched bars, BCL-6^{-/-} + anti-CD3.)

Figure 5. Analysis of chemokine RNA expression by heart tissue. Lanes 1–3 show RPA analysis, lanes 4–7 show RT-PCR for the indicated genes. Lanes 1, 4 and 5, RNA from inflamed BCL-6^{−/−} hearts. Lanes 2 and 6, RNA from a BCL-6^{−/−} TCRα^{−/−} heart. Lanes 3 and 7, RNA from a wild-type heart. Similar chemokine expression was observed with two other BCL-6^{−/−} hearts (not shown).
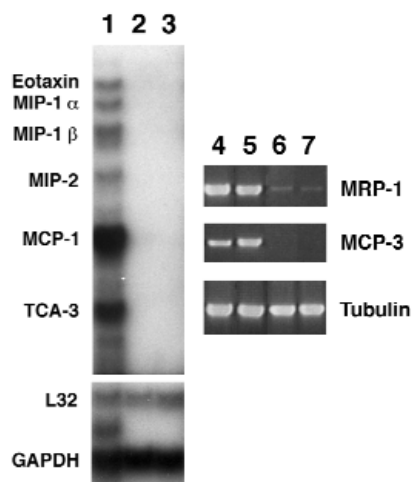
cells because BCL-6^{−/−} spleen contains on average, only twofold more Mac-1^{+} cells than wild-type spleen. Furthermore, increased secretion of MCP-1 was also observed in spleen cells from BCL-6^{−/−}TCRα^{−/−} double mutant mice (data not shown), indicating that the constitutive chemokine secretion from Mac-1^{+} spleen cells was not simply due to ongoing T$_H$2-type inflammatory disease. Thus, myeloid cells from BCL-6^{−/−} mice secrete abnormally high levels of the chemokines MCP-1, MCP-3 and MRP-1 and provide a potential mechanism for the inflammatory disease that develops in BCL-6^{−/−} mice. Significantly, BCL-6 does not appear to regulate chemokine expression in T cells (**Fig. 4**), showing that increased chemokine production is a unique feature of BCL-6^{−/−} myeloid cells.

To determine whether the same chemokines up-regulated in BCL-6^{−/−} macrophages were also expressed in inflamed heart tissue from BCL-6^{−/−} mice, RNA was prepared from obviously inflamed hearts from BCL-6^{−/−} mice, as well as hearts from TCRα^{−/−}BCL-6^{−/−} mice and wild-type mice. Chemokines were examined by RPA and reverse transcription polymerase chain reaction (RT-PCR) analysis (**Fig. 5**). The inflamed heart tissue from BCL-6^{−/−} mice expressed multiple chemokines, with MCP-1 transcripts most prominently expressed. MRP-1 and MCP-3 transcripts were also expressed at high levels in the inflamed hearts compared to the control hearts as assayed by RT-PCR. Heart tissue from TCRα^{−/−} BCL-6^{−/−} mice and wild-type mice had no detectable chemokine expression measured by RPA and only trace MRP-1 detected by RT-PCR (**Fig. 5**). The background level of MRP-1 was similar between the wild-type and the TCRα^{−/−} BCL-6^{−/−} hearts, and thus BCL-6 does not appear to regulate basal levels of chemokine expression in the heart. These data indicate that the chemokine expression in the inflamed heart was due to the inflammatory process. Overall, the pattern of chemokine expression in the inflamed heart is reminiscent to that seen with M-CSF–stimulated BCL-6^{−/−} macrophages (**Fig. 2a**), with the exception of TCA-3 and eotaxin expression. TCA-3 mRNA may be due to infiltrating T$_H$2 cells because we have found that BCL-6[−/−] T$_H$2 cells produce TCA-3 (A.L. Dent, unpublished data), similar to wild-type T$_H$2 cells[25]. T$_H$2 cells do not appear to produce eotaxin[25] (and A.L. Dent, unpublished data), however T$_H$2 cells produce IL-13, which has been shown to induce eotaxin expression in lung epithelium[26]. By extension, eotaxin expression in the inflamed heart may be due to induction by T$_H$2-derived IL-13.

## Chemokine genes as transcriptional targets of BCL-6

The results shown above suggested that BCL-6 is a transcriptional repressor of chemokine gene expression in macrophages. The idea that BCL-6 plays a functional role in macrophages is supported by a previous study that show high levels of BCL-6 mRNA in myeloid cells[18]. BCL-6 protein can be detected in M-CSF–cultured macrophages by both Western blot and gel shift assays with DNA probes, as well as in freshly isolated myeloid cells by intracellular staining (data not shown). We therefore wondered whether BCL-6 could directly repress chemokine gene transcription in macrophages. Sequence analysis of the mouse MCP-1 promoter revealed three potential high affinity BCL-6 binding sites[7,11]: at −150 bp, −1550 bp and −2760 bp from the start of transcription. As the −150 bp site is closest to the start of transcription, and is also 100% conserved between the mouse and human MCP-1 promoters (data not shown), we focused on this proximal site. We tested the ability of an oligonucleotide probe MCP-WT spanning the −150 bp region of the MCP-1 promoter to compete for BCL-6 binding in a gel shift assay using a radiolabeled high affinity BCL-6 binding site (**Fig. 6**). As a source of BCL-6, we used nuclear extracts from a mouse B cell lymphoma line that expresses high levels of BCL-6, K46 (A.L. Dent, unpublished data). The radiolabeled probe for the gel shift assay was a sequence from the promoter of the human chemokine IL-8 that contains a good match to the BCL-6 consensus site (**Fig. 6**). The MCP-WT probe was able to compete for BCL-6 binding only slightly less effectively than the IL-8 probe itself. Both the IL-8 and MCP-WT probes had a higher affinity for BCL-6 than a previously characterized[11] site in the CD23b promoter (**Fig. 6**). A probe corresponding to the MCP-WT sequence except for mutations of two bases in the BCL-6 consensus, MCP-MU, was ineffective at competing for binding to BCL-6 (**Fig. 6**).

Next we tested whether BCL-6 could repress transcription from the MCP-1 promoter. NIH3T3 cells, which do not express BCL-6, were transiently transfected with a reporter plasmid containing 1.2 kb of the MCP-1 promoter driving the luciferase gene and an expression plasmid for BCL-6. A β-galactosidase reporter construct driven by the cytomegalovirus promoter (CMV–β-gal) was used as an internal control for transfection efficiency and nonspecific repression. In this assay, BCL-6 was able to repress the MCP-1 promoter by an average of 96% (**Fig. 7a**). In contrast, the CMV–β-gal reporter was not

Figure 6. Gel shift analysis of BCL-6 binding sites. All lanes contain K46 nuclear extract and radiolabeled IL-8 probe. Lanes 1 and 11, no addition. Lane 2, supershift with anti–BCL-6. Lanes 3–10, competition reactions with the indicated unlabeled probes. Lanes 3, 5, 7 and 9 contain tenfold molar excess and lanes 4, 6, 8 and 10 contain 100-fold molar excess over the labeled probe. Top-strand probe sequences are indicated below. BSS 5.2 and BSS 29.1 are high affinity BCL-6 sites identified by binding site selection[7] and shown for comparison. The IL-8 sequence is found at position −450 bp in the human IL-8 promoter sequence. The MCP-WT sequence is found at position −150 bp in mouse MCP-1 promoter. MCP-MU is identical to MCP-WT except for two base changes indicated in lower case letters. The CD23 site has been previously published[11].

a

b
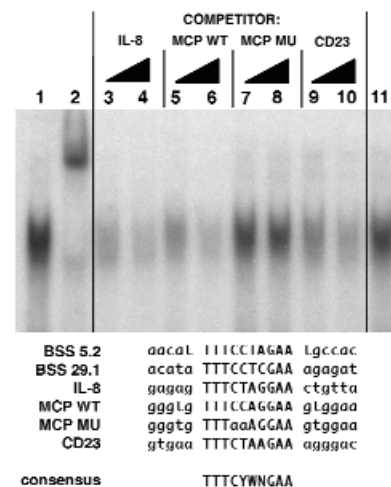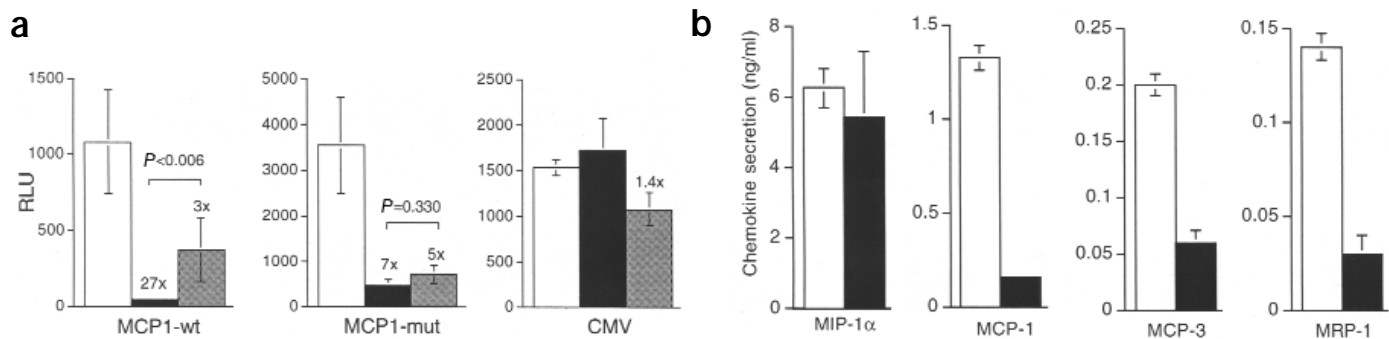
Figure 7. Repression of chemokine expression by BCL-6. (**a**) NIH3T3 cells were transfected with expression and reporter constructs as described in the text and in the Methods section. Relative light units (RLU) were obtained for the indicated reporter plasmids. Expression plasmids: open bars, pCGN; filled bars, pCGN-BCL6; shaded bars, pCGN-BCL6Δ. Luciferase values from MCP-1–wild-type (MCP1-wt) and MCP-1–mutant (MCP1-mut) plasmids were normalized to cotransfected CMV–β-gal levels. The MCP-1–mutant plasmid consistently produced higher luciferase levels than the MCP-1–wild-type plasmid. The CMV–β-gal levels are not directly comparable to the MCP-1 luciferase values due to the differences in the reporter systems. Student's t-tests were used to compute the P values indicated. (**b**) Macrophages from BCL-6$^{-/-}$ mice were infected with either GFP-control (open bars) or GFP–BCL-6 (filled bars) retroviruses and GFP$^+$ cells were isolated by cell sorting. GFP$^+$ cells were stimulated with 10 μg/ml LPS, and supernatants collected after 20 h. Bars indicate s.e.

repressed by BCL-6 (**Fig. 7a**), which is consistent with a lack of consensus BCL-6 binding sites in the CMV promoter. To further assess the specificity of repression of the MCP-1 promoter by BCL-6, we mutated the –150 bp BCL-6 site to a nonbinding sequence (equivalent to MCP-MU in **Fig. 6**). A luciferase reporter construct with the mutated MCP-1 promoter showed an average 86% repression by BCL-6, indicating that either part of the repression was independent of DNA binding by BCL-6, or that there were cryptic BCL-6 sites in the mutated MCP-1 promoter that could mediate repression. To distinguish between these two possibilities, we tested the ability of a truncated form of BCL-6 (BCL-6Δ), which cannot bind DNA, to repress the MCP-1 promoter. The truncated BCL-6 repressed the wild-type MCP-1 promoter only 66% compared to 96% with full length BCL-6, a highly statistically significant difference, whereas there was no significant difference between repression of the mutant MCP-1 promoter by truncated BCL-6 and full-length BCL-6 (**Fig. 7a**). Thus, BCL-6 potently represses the MCP-1 promoter by a combination of direct and indirect mechanisms. The mechanism for indirect repression by BCL-6 is not known, but one possibility is that BCL-6 can interact with other transcription factors that bind to the MCP-1 promoter and thus recruit in corepressor proteins and histone deacetylases[27]. We have also obtained direct and indirect repression of the MCP-1 promoter by BCL-6 in the macrophage cell line RAW 264.7. However, the overall degree of repression by BCL-6 in RAW264.7 cells was significantly lower, most likely because these cells already express significant levels of BCL-6 (data not shown).

To test whether BCL-6 could repress expression of MCP-1, MCP-3 and MRP-1 in macrophages, we used a retrovirus system to introduce functional BCL-6 into BCL-6$^{-/-}$ macrophages. Phoenix ecotrophic retrovirus packaging cell lines were transfected with either the empty retrovirus construct or a BCL-6–encoding retrovirus construct. The cDNA for BCL-6 was cloned behind an internal ribosomal entry site (IRES) that allowed coexpression of green fluorescence protein (GFP) and BCL-6. GFP-control and GFP–BCL-6 retroviruses were used to infect BCL-6$^{-/-}$ macrophages. Two days after infection, GFP-positive cells were isolated by FACS. The GFP-positive cells were replated and restimulated with LPS. After 24 h, chemokine expression in the cell supernatants was measured by ELISA. Infection of BCL-6$^{-/-}$ macrophages with a GFP–BCL-6 retrovirus reproducibly suppressed MCP-1, MCP-3 and MRP-1 expression by 70–85% compared to cells

infected with the GFP-control retrovirus (**Fig. 7b**). The BCL-6 retrovirus did not repress MIP-1α expression after LPS stimulation. Thus, ectopic expression of BCL-6 in BCL-6$^{-/-}$ macrophages leads to a specific decrease in MCP-1, MCP-3 and MRP-1 expression. These results are consistent with the idea that BCL-6 can directly and specifically repress the transcription of a subset of chemokine genes in macrophages.

Discussion

This study has given us more insights into how BCL-6 functions to regulate inflammation and T$_H$2 differentiation. Although previous studies have shown that BCL-6 has a critical function in regulating T$_H$2-type inflammation[10,11], the cell types and genes affected by loss of BCL-6 were unclear. Here we provide evidence that the T$_H$2-type inflammation in BCL-6$^{-/-}$ mice requires a nonlymphoid cell population and that BCL-6 regulates chemokine gene expression in macrophages. Because chemokines have well established roles in cell migration and inflammatory processes, it is likely that deregulated chemokine expression in macrophages of BCL-6$^{-/-}$ mice underlies part of the severe T$_H$2 inflammatory phenotype of these mice. Our results therefore show that BCL-6 has a critical role in maintaining normal levels of chemokine gene expression in macrophages under different stimulation conditions.

An important aspect of the increased chemokine secretion by BCL-6$^{-/-}$ macrophages is that it may contribute to the abnormal T$_H$2 differentiation seen in BCL-6$^{-/-}$ mice[10–12]. In particular, MCP-1 has been implicated in the differentiation of T$_H$2 cells because MCP1$^{-/-}$ mice have defective T$_H$2 responses[28]. This suggests that MCP-1 may promote T$_H$2 differentiation, as has been implicated in other studies[29–31]. However, in tests using recombinant MCP-1, MRP-1 and MCP-3 to stimulate highly purified naïve CD4 T cells from either wild-type or BCL-6$^{-/-}$ mice, we have not observed significant enhancement of differentiation into the T$_H$2 lineage (A.L. Dent, unpublished data). Nonetheless, it is likely that overexpression of MCP-1, MRP-1 and MCP-3 *in vivo* can promote the development of T$_H$2 responses in an indirect manner, such as modulating the ability of antigen presenting cells (APCs) to activate T cells. One possible mechanism for this indirect influence of chemokines is the inhibitory effect of MCP-1 on IL-12 production from activated monocytes and macrophages[32]. Decreased production of IL-12 would restrict T$_H$1 differentiation and favor T$_H$2 differentiation. Another possibility is that chemokines such

as MCP-1, MRP-1 and MCP-3 recruit T cells to special microenvironments where T_H2 differentiation is favored.

How nonlymphoid cells promote the T_H2-type inflammation in BCL-6$^{-/-}$ mice is not entirely clear. The most likely explanation is that the nonlymphoid cell population consists of "professional" APCs, such as macrophages, that can influence inflammation as well as T cell differentiation. Indeed, APCs are known to strongly influence T_H2 differentiation[33,34]. Additionally, we have recently identified specific activation conditions where T_H2 differentiation of BCL-6$^{-/-}$ T cells is greatly amplified over wild-type T cells, indicating that BCL-6$^{-/-}$ T cells have an intrinsic defect favoring differentiation into T_H2 cells (L.M. Toney and A.L. Dent, unpublished data). The increased intraepithelial mast cells, which are an indication of an abnormal T_H2 response, are present in all BCL-6$^{-/-}$ chimeric mice and are consistent with a defect specific to BCL-6$^{-/-}$ T cells. Taken together, the data indicate that the T_H2-type inflammation in BCL-6$^{-/-}$ mice is due to defects in both BCL-6$^{-/-}$ T cells and BCL-6$^{-/-}$ APCs. We therefore propose a model in which BCL-6$^{-/-}$ macrophages, by overexpressing chemokines, recruit abnormal numbers of T cells to sites of inflammation. The BCL-6$^{-/-}$ T cells recruited by these chemokines undergo accelerated differentiation into T_H2 effector cells in response to signals from BCL-6$^{-/-}$ APCs, and the subsequent production of IL-4, IL-5 and IL-13 can promote eosinophilic infiltration and IgE formation by B cells. The initial stimulus for the T_H2-type inflammatory response is not clear, as the inflammation is not associated with any infectious agent[10]. Thus, the severe and often lethal inflammation of BCL-6$^{-/-}$ mice may be due to abnormal responses to endogenous bacteria, foreign substances or loss of tolerance to self-antigens.

Before the work presented here, the transcriptional targets of BCL-6 were largely unknown. To date, the only extensively characterized target gene for BCL-6 is the IgE sterile promoter[35]. Although the increase in IgE in BCL-6$^{-/-}$ mice may be partly due to loss of repression of the IgE sterile promoter by BCL-6, the increased IgE also requires a nonlymphoid component as shown by our results. Because there is no precedent for APCs (or other nonlymphoid cells) directly inducing IgE production by B cells, the increased IgE is probably indirectly promoted by BCL-6$^{-/-}$ APCs via increased T_H2 differentiation and subsequent production of IL-4 and IL-13.

The up-regulated chemokine genes in this study also increase our knowledge of BCL-6 target genes. Specifically, MCP-1 appears to be a *bona fide* target gene for BCL-6 because BCL-6 strongly represses the MCP-1 promoter and retrovirus-introduced BCL-6 can inhibit the overexpression of MCP-1 by BCL-6$^{-/-}$ macrophages. It is likely that MCP-3 and MRP-1 are also target genes for BCL-6, because overexpression of these genes in BCL-6$^{-/-}$ macrophages can be repressed by introduction of a functional *BCL6*. The role of BCL-6 in transcription of MRP-1 and MCP-3 will require further molecular characterization of the promoters for these genes. Although the murine MCP-3 promoter sequence has not been characterized, we have found that the sequence of the human MCP-3 promoter contains several consensus BCL-6 sites (A.L. Dent, unpublished data). Undoubtedly the chemokine genes shown here are only part of the overall array of genes directly regulated by BCL-6. Much more work is required to understand the range of genes that are critically regulated by BCL-6, and how these genes relate to the development of T_H2 cells, germinal center B cells and B cell lymphoma.

Methods

Mice. Animals were maintained under specific-pathogen free conditions in animal facilities certified by the American Association of Laboratory Animal Care. Chimeric mice were pro-

duced by blastocyst complementation as previously described[10]. RAG-1$^{-/-}$, IgM$^{-/-}$, TCRβδ$^{-/-}$ and TCRα$^{-/-}$ mice were obtained from Jackson Laboratories (Bar Harbor, ME). BCL-6$^{-/-}$ mice were maintained on a mixed C57BL/6-129 background and were genotyped by PCR as previously described[12]. Mice were genotyped for TCRα mutations using the following oligonucleotides in a three primer PCR reaction: 5′–TCTTCTAACATCAGTCCTCTTG GC–3′; 5′–TTGGCTCAAGAGAAGACAGGAAGG–3′; 5′–GAGGCCACTTGTGTAGCG CCAAGT-3′.

Histology. For analysis of inflammatory disease in BCL-6$^{-/-}$ mice and BCL-6$^{-/-}$ chimeric mice, tissue processing and immunohistochemistry was performed essentially as previously described[10]. A polyclonal sheep antibody to mouse IgE (ICN Biochemical Research, Costa Mesa, CA) was used at 1:5000 dilution on 1mM EDTA-antigen retrieved sections, counterstained with a biotin-conjugated rabbit secondary antibody to goat or sheep followed by Avidin-HRP (Dako, Carpinteria, CA). Specificity of the antiserum was assayed on STAT6 and CD40 knockout mice (not shown). CAE staining for intraepithelial mast cells was performed as described previously[36]. Intraepithelial mast cells are typically seen only in mice that have been exposed to intraluminal pathogens. However, our mouse facility is free of intraluminal pathogens, and thus intraepithelial mast cells appear to be a unique marker of the BCL-6$^{-/-}$ phenotype.

Macrophage cultures and stimulations. Bone marrow was flushed from the femurs of TCRα$^{-/-}$ and BCL-6$^{-/-}$ TCRα$^{-/-}$ mutant mice using needles and syringes filled with PBS buffer. Bone marrow cells were plated in sterile plastic bacterial petri dishes (Falcon catalog no. 1029) at 2×10^5 per ml in complete DMEM medium supplemented with 10 ng/ml of murine rM-CSF (R&D Systems, Minneapolis) and 20% horse serum (Gibco-Brl, Rockville, MD). Complete DMEM (Gibco) contained 10% fetal calf serum (Hyclone, Logan, UT), 2 mM glutamine (Gibco), 10 mM MEM nonessential amino acids (Gibco), 100 U/ml penicillin + 100 μg/ml streptomycin (Gibco), 10 mM HEPES buffer (Gibco) and 55 μM 2-mercaptoethanol (Gibco). 10 ml (2×10^6) bone marrow cells were plated per 10-cm dish and cultured for 9 days at 37 °C + 5% CO$_2$. At the end of the culture period, macrophages were scraped off the dish using a plastic spatula, washed with PBS buffer, resuspended in complete DMEM and counted. 10^6 macrophages were plated in 2 ml in wells of a six-well tissue culture plate unless otherwise noted. The cells adhered overnight and then fresh media was added plus any stimulating factors the next day. For RPA, the macrophages were stimulated for 5 h before RNA preparation. For measuring cytokines by ELISA, supernatants were collected after 16–20 h. Wild-type and BCL-6$^{-/-}$ macrophages were always cultured and stimulated in parallel. LPS (LPS *E. coli* 055:B5; Sigma, St. Louis, MO) was used at 2–10 μg/ml for macrophage stimulations. Recombinant mouse IL-6 was obtained from Gibco.

Cytokine assays. The MCK-5 multiprobe RPA specific for chemokine genes was purchased and used according to the manufacturer's protocol (Pharmingen). RNA was prepared from macrophage cultures or mouse hearts using Trizol (Gibco). Murine MCP-1 was measured by ELISA using reagents purchased from Pharmingen. Recombinant mouse MCP-1 standard was obtained from R&D Systems. Murine MCP-3 (MARC), MRP-1 (C10), and MIP-1α were measuring using ELISA kits (R&D Systems). Murine IL-12 p70 was measured by ELISA using antibodies purchased from Genzyme (Cambridge, MA), and standards purchased from R&D Systems. ELISA assays were run with standards and samples in duplicate or triplicate. ELISA results were analyzed with a BioRad plate reader (Hercules, CA) and software. Northern blots were prepared and probed according to standard protocols[37] using RNA from stimulated macrophages. RT-PCR was performed using an Enhanced Avian Reverse Transcriptase PCR Kit (Sigma). The primers used were β-tubulin: 5′–ATGTTGCTCTCAGCCTCGGTGAAC–3′ and 5′–TGTCCATGAAGGAGGTGGAT GAG–3′, MRP1: 5′–ATTCTAAACCAAAAGACATCATGG–3′ and 5′–TTCAGGGT AAATGTAAATTACTAGAT–3′, MCP-3: 5′–GGGCCAAGAAGCAAAATTCCTCA–3′ and 5′–ATATGAAACTTCAGTAGTCATACATT–3′. MRP-1 and MRP-2 cDNA probes were a gift from B. Youn (Indiana University School of Medicine). Exodus-1, Exodus-2, and Exodus-3 cDNAs were gifts of R. Hromas (Indiana University School of Medicine). The remaining chemokine cDNA probes were purchased as IMAGE clones from Genome Systems Inc. (St. Louis, MO): MCP-2 (no. 620243, Genbank no. AA178071), MCP-3 (no. 1181707, Genbank no. AA711435), MCP-5 (no. 59927, Genbank no. AI3266125), MDC (no. 619257, Genbank no. AA175762) and SDF-1 (no. 126091, Genbank no. AA8555531).

Gel shift assays. DNA sequences used for gel shift assays are shown in **Fig. 5**. The oligonucleotidess shown and complementary oligonucleotides were custom synthesized (Gibco), and annealed to each other to create double stranded probes used in the gel shift assays. 100 ng of IL-8 probe was labeled to high specific activity with [^{32}P]γ–ATP (6000 Ci/mmol, Amersham, Arlington Heights, IL) and T4 polynucleotide kinase (Roche, Indianapolis, IN). Radiolabeled oligonucleotide corresponding to 10^5 cpm was used in each gel shift reaction. The gel shift reactions were performed in EMSA buffer: 4% glycerol, 1 mM EDTA, 5 mM DTT, 10 mM Tris at pH 7.5. Each reaction also contained 1 mg/ml of bovine serum albumin and 2 μg polydI-dC (Sigma). 5 μg nuclear extract was used per reaction. Rabbit anti-BCL-6 (N-3, Santa Cruz Biotech, Santa Cruz, CA) was used at 1 μg per reaction. Nuclear extracts were prepared as described[11,38]. Nuclear extracts were incubated in the gel shift reaction for 20 min and then loaded on a pre-run 4% native polyacrylamide gel with 0.5X TBE running buffer. K46 mouse B lymphoma cells were obtained from L.M. Staudt (NCI, Bethesda).

Luciferase assays. The CMV–β-gal reporter (pCMV–β-gal) was a gift from M. Harrington (Indiana University School of Medicine). A plasmid containing the mouse MCP-1 promoter driving luciferase (pMCP-Luc) was constructed from pGL3-basic (Promega, Madison, WI) and a PCR-generated MCP-1 promoter fragment. A proofreading competent Taq reagent (AccuTaq, Sigma) was used to ensure high fidelity PCR. 1.2 kb of the MCP-1 promoter (GenBank Accession no. U12470) was obtained by PCR of C57BL/6 mouse DNA with the following primers: 5′–TCATGTATATGGCTTTCCAGGTCT–3′ (sense strand, distal to transcription start) and 5′–TGCACTTCTGGCTGCTCTGAG GCA–3′ (antisense strand, proximal to transcription start). The PCR product terminates 28-bp downstream of the MCP-1 TATA site. XmaI and XhoI sites were added to the MCP-1 promoter by a second round of PCR with the primers: 5′–ATATCACCCGGGT CATGTATATGGCTTTCCAGGTCT–3′ and 5′–TAGATTCTCGAGTGCACTTCTGGCT GCTCTGAGGCA–3′, and XmaI and XhoI were used to clone the MCP-1 promoter fragment into pGL3-basic. For the construction of the MCP-1 mutant promoter, the BCL-6 site was mutated by a two step PCR–based strategy using the above flanking primers and the following complementary primers to mutate the BCL-6 site: 5′–TCTCTCTTCCACTTC C**TTT**AAACA–3′ and 5′–TGTTT**AA**AGGAAGTGGAAGAGAGA–3′ (mutated bases are in bold). The mutant promoter was cloned into pGL3 via XhoI and XmaI. The wild-type and mutant MCP-1 promoter sequences were verified by sequencing. For transfection assays, 0.9 µg of luciferase reporter plasmid, 0.1 µg of pCMV–β-gal along with 1.0 µg of pCGN, pCGN-BCL-6 or pCGN-BCL6Δ expression plasmids[7,11] were added to NIH3T3 cells in one well of a six-well dish using the Fugene reagent (Roche). Cells were lysed and analyzed for luciferase activity in a luminometer using Luciferase Assay System reagents (Promega Biotech, Madison, WI) 48 h after transfection. The expression plasmid pCGN-BCL-6Δ was constructed by digesting the BCL-6 cDNA with EcoRI, deleting the 3′ region and cloning the remaining cDNA into pCGN. The BCL-6Δ is missing four of six zinc fingers and does not bind to a consensus BCL-6 probe in a gel shift assay (A.L. Dent, unpublished data). β-galactosidase activity was also measured in a luminometer using Galacto Light Plus reagents (Tropix, Bedford, MA). Luciferase levels were normalized by using β-galactosidase activity as a measure of transfection efficiency.

Retrovirus infections. Bone marrow cells were cultured with M-CSF to obtain macrophages as described above. On day four of culture, supernatants containing ecotropic retrovirus were added to the cells along with 5 µg/ml polybrene (Sigma). 48 h after infection, macrophages were scraped off and sorted for GFP expression using FACS. Cell sorting was performed at the Indiana University Cancer Center flow cytometry laboratory. Typically 5–10% of the infected macrophages were GFP-positive. GFP-positive cells were cultured in wells of a 96-well plate at 10^5 cells/ml along with 10 µg/ml LPS. GFP-control and GFP–BCL-6 retroviruses were grown by transfecting Phoenix ecotrophic retrovirus packaging cells with MIEG3 and MIEG3–BCL-6 retrovirus constructs. Phoenix packaging cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) with the permission of G. Nolan (Stanford University). The pMIEG3 plasmid was obtained from D.A. Williams (Indiana University School of Medicine). pMIEG3 is a murine stem cell virus (MSCV) retroviral construct in which the gene encoding GFP is driven by the MSCV long terminal repeat. pMIEG3 contains an IRES that allows coexpression of the GFP gene and a second gene. The human BCL-6 cDNA with an optimized translation initiation codon (obtained from L.M. Staudt, NCI, NIH) was cloned into pMIEG3 via NotI and XhoI restriction sites. Phoenix cells were transfected on 10-cm dishes with 20 µg pMIEG3 or pMIEG3-BCL-6 using the Calcium Phosphate technique. Retrovirus containing supernatant was collected 48 h after transfection.

Spleen cell stimulations. Dissociated spleen cells from wild-type and mutant mice, after red blood cells lysis (RBC lysis reagent, Sigma), were stimulated on wells coated with 10 µg/ml anti-CD3 (NA/LE 145-2C11, Pharmingen). 5×10^6 cells were stimulated in 1 ml complete DMEM in one well of a 24-well plate for 48 h, at which time supernatants were collected. Mac-1+ cells were depleted by staining of the spleen cells with phycoerythrin-conjugated Mac-1 (M1/70, Pharmingen) antibody, followed by FACS in which gates were set for Mac-1–negative cells. Cells obtained from FACS were stimulated identically to unseparated cells. Wild-type spleen cells contain 8.5±2.2% Mac-1+ cells and BCL-6−/− spleen contains 16.2%±1.5% Mac-1+ cells (average±s.e.). Gr-1+ granulocytes comprise about 40% of the Mac-1+ population in both types of mice as measured by flow cytometry using FITC-labeled Gr-1 antibody (Pharmingen).

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