Cutting Edge: STAT6 Serves as a Positive and Negative Regulator of Gene Expression in IL-4-Stimulated B Lymphocytes[1]

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STAT6 plays an important role in IL-4-mediated B cell activation and differentiation. To identify primary and secondary target genes of STAT6, gene expression profiles of IL-4-stimulated B cells from STAT6$^{+/+}$ vs STAT6$^{-/-}$ mice were compared. Statistical analysis revealed that 106 distinct probe sets including 70 known genes were differentially expressed between the 2 genotypes. These genes include transcription factors, kinases, and other enzymes, cell surface receptors, and Ig H chains. Surprisingly, although 31 genes were expressed at higher levels in STAT6$^{+/+}$ B cells, 39 genes were expressed at higher abundance in STAT6$^{-/-}$ B cells. This result implies both positive and negative regulatory functions of STAT6 in IL-4-mediated gene expression. Furthermore, IL-4 induces expression of the transcription factor Krox20, which is required for maximal IL-4-induced transcription. ***The Journal of Immunology,*** **2002, 168: 996–1000.**

The cytokine IL-4 is a multifunctional cytokine produced by a subset of activated T cells, mast cells, and basophils. It is crucial for the development of type I allergic reactions and for immune protection against helminthic parasites. These responses are in part mediated through the effects of IL-4 on B cells (for a review, see Ref. 1). Binding of IL-4 to its receptor triggers activation of Janus kinase (JAK)[2] 1 and JAK3 tyrosine kinases, which phosphorylate tyrosine residues in the cytoplasmic domain of the IL-4Rα. These residues then serve as docking sites for several proteins containing phosphotyrosine binding or Src homology 2 domains including signal transducer and activator of transcription family member STAT6. STAT6 is tyrosine phosphorylated by JAK kinases, which induces its homodimerization and subsequent translocation into the nucleus, where it can regulate gene transcription.

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Received for publication November 12, 2001. Accepted for publication December 10, 2001.

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[2] Abbreviations used in this paper: JAK, Janus kinase; BCL-6, B cell lymphoma 6; WT, wild-type; DN, dominant negative; GFP, green fluorescent protein.

IL-4-induced proliferation and survival have been shown to be at least partially independent of STAT6 (2–4), whereas IL-4-induced differentiation appears to be largely dependent on STAT6. Previous work has shown that B cells from STAT6-deficient mice fail to produce significant amounts of IgG1 or IgE after infection with the nematode *Nippostrongylus brasiliensis* (4). Furthermore, on stimulation with IL-4, STAT6-deficient B cells fail to up-regulate FcϵRII/CD23 or MHC class II (2–4), which play important roles in Ag capture and presentation, respectively. In consequence, STAT6-deficient mice show impaired immune responses to infection with nematodes and diminished allergic responses in murine models of asthma (4, 5).

STAT6-binding elements were identified in the Iϵ promoter (6), the CD23 promoter (7), and other enhancer elements. STAT6 activation alone, however, is not sufficient to *trans*-activate these genes in primary cells but in addition requires de novo protein synthesis which is efficiently blocked by cycloheximide (6, 8). This finding implies that IL-4 likely induces the expression of a cascade of essential secondary transcription factors and proteins. To identify factors that are regulated in a STAT6-dependent manner in IL-4-stimulated B cells, a combination of gene targeting and gene expression profiling was used.

Materials and Methods

Antibodies, mice, and cell culture

Anti-CD24-PE and anti-B220-FITC were from BD PharMingen (San Diego, CA); anti-caspase-6 was from Cell Signaling Technology; anti-B cell lymphoma 6 (BCL-6) and anti-STAT6 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-extracellular signal-related kinase 1/2 was from Promega (Madison, WI). STAT6$^{-/-}$ mice on the BALB/c background were a gift from Dr. M. Grusby (Boston, MA) (2). Naive splenic B cells were isolated as described previously (9). B cells were >95% B220 positive as confirmed by flow cytometry (data not shown). The murine B cell line M12.4.1 has been described previously (6).

Affymetrix GeneChips analysis

Freshly isolated naive (CD43^{-}B220^{+}) B cells pooled from each two to four BALB/c STAT6$^{+/+}$ or STAT6 $^{-/-}$ mice were stimulated with LPS (1.5 μg/ml) and murine recombinant IL-4 (1:25, generously provided by W. Paul, National Institutes of Health, Bethesda, MD). After 24 h, total RNA was isolated, processed, and hybridized with Affymetrix U74A GeneChips. Chips were scanned and analyzed using a target intensity normalization of 2500 for all chips as suggested by the manufacturer.

Data analysis

Data obtained from U74A GeneChips version 1 were masked by Microarray Suite software resulting in a data set of 10,043 probe sets. Average differences from the different data sets were grouped into STAT6$^{+/+}$ vs STAT6$^{-/-}$ or STAT6$^{-/-}$ LPS vs STAT6$^{-/-}$ LPS plus IL-4, respectively.

0022-1767/02/$02.00

Average differences of <5 were set to 5. Statistical significance was calculated using Cyber-T software as described previously (10). A gene was considered differentially expressed between the compared groups if: 1) p was <0.000099 (1/10,043); 2) the absolute fold difference between the mean average differences from each group was ≥2; and 3) the mean average difference change was ≥500. This ensures a false positive error rate of <1 of 10,043 genes.

Northern blots, Western blots, and flow cytometry

Total RNA was isolated from B cells using the RNEasy Kit (Qiagen, Valencia, CA), and equal amounts of RNA were used to generate cRNA as described previously (11). Northern blotting of these RNA was performed as described (12). Western blotting was conducted as previously described (13). For flow cytometry, cells were sequentially incubated with Fc block and primary fluorochrome-conjugated Ab, washed, and fixed as suggested (BD PharMingen).

Retroviral transduction

A dominant negative (DN) version of Krox20 (Krox20DN) containing *S379R* and *D380Y* point mutations was generated from wild-type (WT) Krox20 (Krox20WT) by site-directed mutagenesis (QuickChange; Stratagene, La Jolla, CA) followed by sequencing. The full length cDNAs of Krox20WT, Krox20DN, or STAT6 were subcloned into pMIG upstream of an internal ribosome entry site and green fluorescent protein (GFP). Generation of ecotropic retroviruses and infection of M12 cells were performed as described (14). After infection, cells were rested for 48 h before additional experiments.

Results and Discussion

Positive and negative modulation of gene expression by STAT6

To identify genes the expressions of which are modulated by STAT6, LPS-stimulated B cells were cultured with IL-4 for 24 h, after which total RNA was isolated and processed to obtain gene expression profiles. The time point of 24 h was chosen to detect a maximum of both early and late genes that are altered in response to IL-4. The mitogen LPS is known to induce activation of NF-κB, which, together with STAT6, is required for Ig H chain class switching to IgE (15). Statistical analysis of the expression profiles identified 114 differentially expressed probe sets at the level of statistical significance. These probe sets represented 106 distinct cDNAs, 70 of which were known genes (Fig. 1) and 36 of which were expressed sequence tags (Table I). Differentially expressed genes were grouped into transcription factors/transcription-related proteins, kinases/phosphatases, other enzymes, cytokines/cell surface receptors, Ig H chains, and miscellaneous. A number of previously described IL-4-inducible genes were identified as STAT6 dependent by this approach, including the transcription factor *e4bp4/nfil3* (Fig. 1, Nos. 1 and 54) (16), as well as the known STAT6 target genes *CD23* and *il4rα* (Fig. 1, Nos. 38 and 50). It should also be noted that STAT6 expression in $STAT6^{-/-}$ B cells was detectable (Fig. 1, No. 7), because only the Src homology 2 domain of STAT6 was targeted by homologous recombination in the $STAT6^{-/-}$ mice, leaving the 5′ portion of the gene intact (2). Thus, a partial STAT6 mRNA is still expressed in $STAT6^{-/-}$ mice and detected by GeneChip, which, however, is not translated into functional STAT6 protein (Fig. 3*d*). In STAT6-deficient B cells, 31 of 70 genes were expressed at lower levels, but surprisingly, expression of 39 genes was detected at higher levels than in $STAT6^{+/+}$ B cells. This finding implies that STAT6 not only induces gene expression but may also mediate repression of genes in a direct or indirect manner.

IL-4 induced STAT6-dependent expression of a selection of genes identified by GeneChip analysis, including *krox20*, homeodomain-interacting protein kinase 2 (*hipk2*), p300/CREB-binding protein cointegrating protein *pcip*, and *caspase-6*, was confirmed by Northern blot (Fig. 2). The differential expression of *hipk2* was selective, because *hipk1* and *hipk3* were not differentially expressed byGeneChip (data not shown). Also, expression of

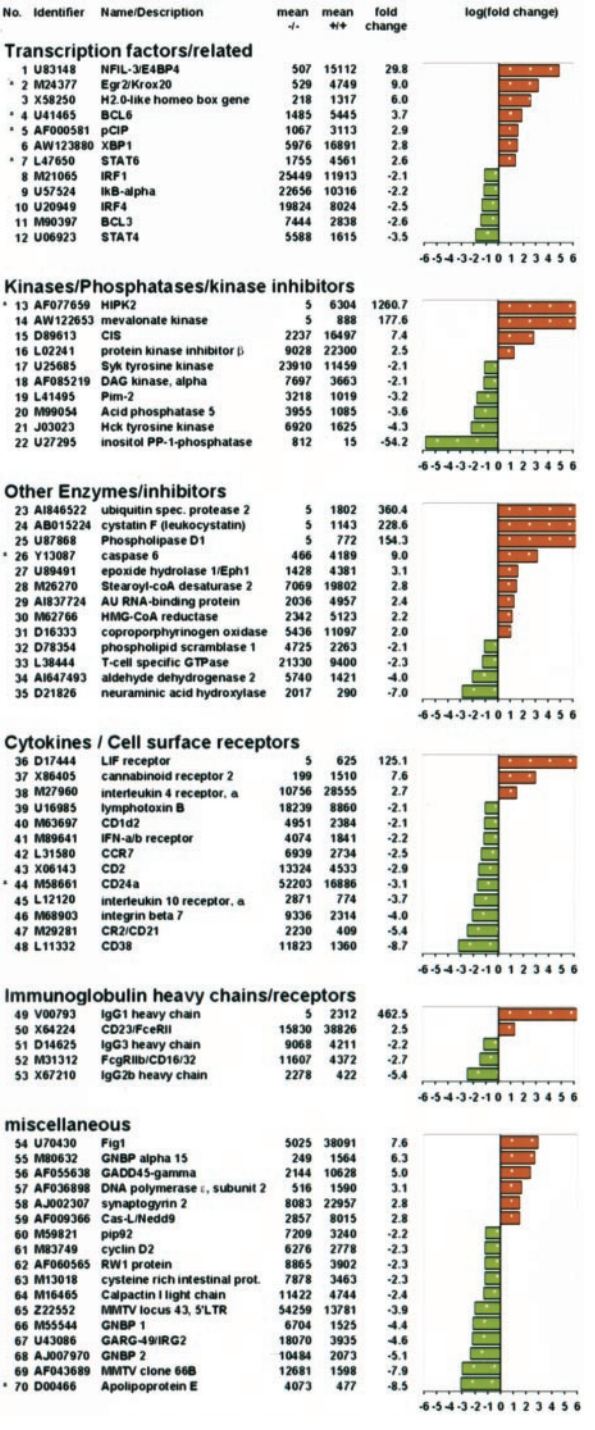

FIGURE 1. Genes differentially expressed between LPS plus IL-4-stimulated B cells from $STAT6^{+/+}$ vs $STAT6^{-/-}$ mice. Data represent genes differentially expressed after statistical analysis of four independent gene expression profiles from $STAT6^{+/+}$ and five profiles from $STAT6^{-/-}$ B cells cultured with LPS plus IL-4 for 24 h. Red bars, Expression in $STAT6^{+/+}$ > expression in $STAT6^{-/-}$. Green bars, Expression in $STAT6^{-/-}$ > expression in $STAT6^{+/+}$, $p < 0.000099$. Fold difference is graphed on a logarithmic scale (base 2); i.e., a 32-fold change equals log 5. *, further data provided in the text. IRF-1, IFN-regulatory factor 1; DAG kinase, 1,2-diacylglycerol kinase; HMG-CoA, hydroxymethylglutaryl-CoA; MMTV, mouse mammary tumor virus; HIPK2, homeodomain-interacting protein kinase 2; spec., specific; prot., protein; CIS, cytokine-inducible SH2 protein; Hck, hematopoietic cell kinase; GNBP, guanylate-binding protein; XBP, X-box-binding protein; GADD45, growth arrest and DNA damage-inducible protein 45; GARG, glucocorticoid-attenuated response gene 49.

Table I. *Expressed sequence tags differentially expressed between LPS plus IL-4-stimulated B cells from STAT6$^{+/+}$ vs STAT6$^{-/-}$ mice*[a]

No.	Identifier	Name/Description	Mean −/−	Mean +/+	Fold Change
1	AA041969	mj06h03.r1 *Mus musculus* cDNA, 5′ end	5	3,883	776.53
2	AI553553	vw40g01.x1 *M. musculus* cDNA, 3′ end	5	2,848	569.67
3	AA289585	*M. musculus* clone L5 uniform group of 2-cell-stage gene *fa*	5	1,577	315.43
4	AI845798	UI-M-AQ1-aeb-h-07-0-UI.s1 *M. musculus* cDNA, 3′ end	5	636	127.21
5	AW061234	UI-M-BH1-amj-b-11-0-UI.s2 *M. musculus* cDNA, 3′ end	91	1,831	20.07
6	AW123907	UI-M-BH2.1-app-h-01-0-UI.s1 *M. musculus* cDNA, 3′ end	1,469	14,586	9.93
7	AI845798	UI-M-AQ1-aeb-h-07-0-UI.s1 *M. musculus* cDNA, 3′ end	4,693	21,406	4.56
8	AW047875	UI-M-BH1-als-g-03-0-UI.s1 *M. musculus* cDNA, 3′ end	2,019	9,131	4.52
9	AI843476	UI-M-AQ1-aec-g-07-0-UI.s1 *M. musculus* cDNA, 3′ end	8,941	38,225	4.28
10	AI153421	uc53a11.r1 *M. musculus* cDNA, 5′ end	6,995	24,128	3.45
11	AI648925	uk32h09.x1 *M. musculus* cDNA, 3′ end	513	1,769	3.45
12	AI854008	UI-M-BH0-aiv-h-12-0-UI.s1 *M. musculus* cDNA, 3′ end	1,636	5,426	3.32
13	AA693125	vr57c08.s1 *M. musculus* cDNA, 5′ end	3,638	11,581	3.18
14	C85523	C85523 *M. musculus* cDNA, 3′ end	6,435	19,708	3.06
15	AW120511	UI-M-BH1-ana-a-04-0-UI.s2 *M. musculus* cDNA, 3′ end	1,200	3,247	2.71
16	AA880275	vv99h02.r1 *M. musculus* cDNA, 5′ end	2,841	7,388	2.60
17	AI852574	UI-M-BH0-aiu-a-02-0-UI.s1 *M. musculus* cDNA, 3′ end	2,973	7,644	2.57
18	AA710132	vt45a05.r1 *M. musculus* cDNA, 5′ end	4,174	10,655	2.55
19	AI844736	UI-M-AL1-ahq-c-10-0-UI.s1 *M. musculus* cDNA, 3′ end	1,574	4,007	2.55
20	AA673486	vp49f04.r1 *M. musculus* cDNA, 3′ end	1,351	3,385	2.51
21	AI837100	UI-M-AK0-adc-d-02-0-UI.s1 *M. musculus* cDNA, 3′ end	16,512	40,472	2.45
22	AI849432	UI-M-AJ1-ahc-f-04-0-UI.s1 *M. musculus* cDNA, 3′ end	4,987	11,723	2.35
23	AA881294	vx11c02.r1 *M. musculus* cDNA, 5′ end	14,506	33,199	2.29
24	AB030505	AB030505 *M. musculus* cDNA, 5′ end	5,021	11,234	2.24
25	AW125713	UI-M-BH2.2-aql-c-12-0-UI.s1 *M. musculus* cDNA, 3′ end	3,311	7,390	2.23
26	AW120725	UI-M-BH2.3-any-a-12-0-UI.s1 *M. musculus* cDNA, 3′ end	10,167	22,287	2.19
27	AW122731	UI-M-BH2.2-aot-e-08-0-UI.s1 *M. musculus* cDNA, 3′ end	1,605	3,374	2.10
28	AA261092	mz65g04.r1 *M. musculus* cDNA, 5′ end	30,103	14,327	−2.10
29	AI120844	ub73a05.r1 *M. musculus* cDNA, 5′ end	19,710	8,393	−2.35
30	AI851230	UI-M-BH0-ajx-h-10-0-UI.s1 *M. musculus* cDNA, 3′ end	6,481	2,746	−2.36
31	AI842065	UI-M-AN1-afg-a-10-0-UI.s1 *M. musculus* cDNA, 3′ end	2,859	1,147	−2.49
32	AA816121	vp44a01.r1 *M. musculus* cDNA, 5′ end	11,744	4,651	−2.53
33	AI117236	ub84h01.r1 *M. musculus* cDNA, 5′ end	3,510	1,323	−2.65
34	AI662509	mt33c08.x1 *M. musculus* cDNA, 3′ end	6,518	1,708	−3.82
35	AI647612	uk41h01.x1 *M. musculus* cDNA, 3′ end	3,853	652	−5.91
36	AI461767	ub78g10.x1 *M. musculus* cDNA, 3′ end	729	5	−145.75

[a] Data represent genes differentially expressed after statistical analysis of four independent gene expression profiles from STAT6$^{+/+}$ and 5 profiles from STAT6$^{-/-}$ B cells cultured with LPS plus IL-4 for 24 h, $p < 0.00009$. Mean +/+: expression level, STAT6$^{+/+}$. Mean −/−: expression level, STAT6$^{-/-}$. Fold difference: positive values = STAT6$^{+/+}$ > STAT6$^{-/-}$; negative values = STAT6$^{-/-}$ > STAT6$^{+/+}$.

caspase-6 mRNA was selective because other caspases, including caspases 1–3, 7–9, 11, 12, and 14, were not differentially expressed (data not shown).

Interestingly, IL-4 induced STAT6-dependent repression of *apoe* (Fig. 2*f*; compare Fig. 1, No. 70). Furthermore, on LPS plus IL-4 stimulation, CD24 expression was repressed in STAT6$^{+/+}$ B cells, whereas CD24 levels remained largely unchanged on LPS or LPS plus IL-4 stimulation in STAT6$^{-/-}$ B cells (Fig. 3*a*; compare Fig. 1, No. 44). Down-regulation of CD24 by IL-4 was also observed in M12.4.1 B cell lines (data not shown).

Importantly, the GeneChip analysis together with Western blot analysis identified the transcriptional repressor *bcl-6* as an IL-4-inducible, STAT6-dependent gene (Fig. 1, No. 4, and Fig. 3*b*). Furthermore, activation with IL-4 or LPS plus IL-4 for 24 h resulted in a moderate ~2-fold, nevertheless highly reproducible, up-regulation of caspase-6 protein levels in STAT6$^{+/+}$, but not in STAT6$^{-/-}$ B cells (Fig. 3*c*). STAT6-dependent induction of caspase-6 protein levels correlated with differential caspase-6 enzyme activity in a delayed manner after prolonged stimulation of at least 72 h (data not shown).

Taken together, these experiments identify several novel IL-4 modulated STAT6-dependent genes. Surprisingly, there is both STAT6-dependent induction and repression of gene expression in response to IL-4 stimulation. Interestingly, in STAT6-deficient B cells, only eight genes were differentially expressed in LPS vs LPS plus IL-4-stimulated samples (data not shown). None of these eight genes was among those described in Fig. 1. This result therefore suggests that expression of genes was repressed in a STAT6-dependent manner and not selectively up-regulated by IL-4 only in STAT6-deficient B cells.

Previous work has shown that STAT6 can interfere with NF-κB activity by competition for overlapping STAT6 and NF-κB DNA-binding elements (17). Because LPS mediates NF-κB activation, this mechanism may provide an explanation for down-regulation of gene expression by STAT6 in LPS plus IL-4-stimulated B cells. Another potential mechanism for STAT6-mediated negative regulation of gene expression involves the IL-4 and STAT6-mediated induction of transcriptional repressors. BCL-6-deficient mice exhibit pathologically enhanced Th2-type inflammatory responses, suggesting that BCL-6 is a physiologic repressor of IL-4-mediated effects, including regulation of Iε transcription and class switching to IgE (18, 19). Some of the genes with mRNA levels that were less abundant in STAT6$^{+/+}$ B cells than in STAT6$^{-/-}$ B cells, including the BCL-6 target gene *cyclin D2* (20), might therefore be targets of BCL-6-mediated repression. It is striking that a number of genes that were less abundantly expressed in STAT6$^{+/+}$ B cells (*iκb-α*, *irf4*, *bcl3*, *stat4*, *dag kinase α*, *hck*, *il-10Rα*, *ifn-αβ receptor*, *fcgrIIb*, *cyclin d2*, *gnbp 1* and *2*) are normally down-regulated in germinal center B cells (21). Interestingly, BCL-6 is required for the formation of germinal centers (18, 19). Our data therefore suggest that STAT6 may influence B cell differentiation via transcriptional repressors.

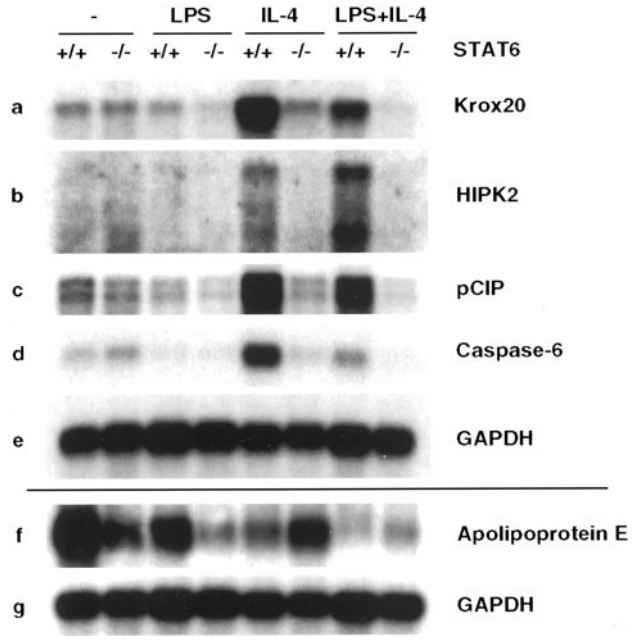

FIGURE 2. IL-4 both induces and inhibits gene expression in a STAT6-dependent manner in B cells. B cells from STAT6$^{+/+}$ or STAT6$^{-/-}$ mice were cultured with LPS, IL-4, or LPS plus IL-4 or left unstimulated for 24 h. Expression of Krox20 (*a*), homeodomain-interacting protein kinase 2 (HIPK2, *b*), pCIP (*c*), caspase-6 (*d*) or apolipoprotein E (*f*) was detected by Northern blot by sequential hybridization with the respective probes. Blots were then reprobed with GAPDH to demonstrate equal loading (*e* plus *g*).

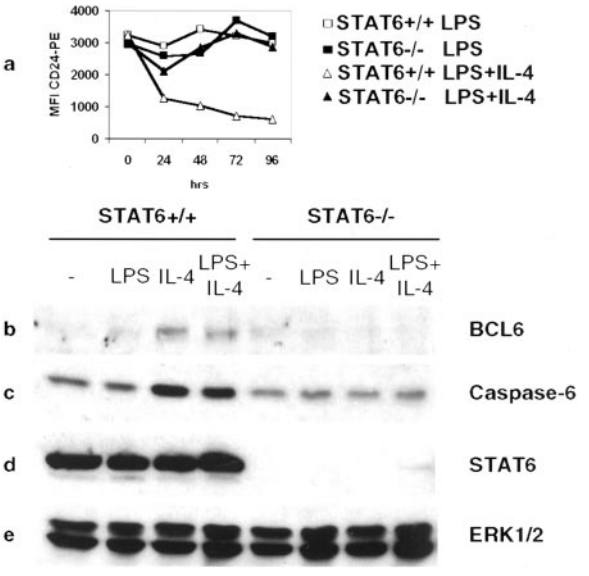

FIGURE 3. Differential protein expression in B cells from STAT6$^{+/+}$ and STAT6$^{-/-}$ B cells. *a*, B cells from STAT6$^{+/+}$ or STAT6$^{-/-}$ mice were stimulated with LPS or LPS plus IL-4 for the indicated time. Surface expression of CD24/HSA was detected by flow cytometry. MFI, Median fluorescence intensity. B cells from STAT6$^{+/+}$ or STAT6$^{-/-}$ mice were stimulated with LPS, IL-4, or LPS plus IL-4 or left unstimulated for 24 h. Expression of BCL6 (*b*), caspase-6 (*c*), or STAT6 (*d*) was detected by Western blot. Reprobing of the membrane with extracellular signal-related kinase 1/2 (ERK1/2) antiserum demonstrates equal loading (*e*).

Krox20 modulates LPS plus IL-4-induced CD23 expression in B cells

To test the function of the IL-4-induced transcription factor Krox20 in B cell differentiation, M12.4.1 B cells were stably transduced with STAT6, Krox20WT, or Krox20DN by retroviral infection using a bicistronic vector also encoding GFP. The point mutations in Krox20DN that were used here have been shown to cause dominant congenital neuropathies in humans and alterations of gene transcription in mouse Schwann cells by competing with WT Krox20 (22).

Transduced cells were stimulated with LPS, IL-4, or LPS plus IL-4 for 48 h (Fig. 4), and levels of CD23 expression in GFP$^+$ cells were assessed by flow cytometry. Overexpression of STAT6 or Krox20WT resulted in an only moderate increase in CD23 expression after stimulation with LPS plus IL-4 ($n = 4$, $p = 0.41$ or $p = 0.12$, respectively; Fig. 4). This suggests that neither STAT6 nor Krox20 limited CD23 expression under these conditions. Strikingly, expression of Krox20DN resulted in a highly significant inhibition (between 55 and 81%; $n = 4$, $p < 0.01$) of LPS plus IL-4-induced CD23 expression (Fig. 4). The effect of Krox20DN was specific to activation by IL-4, because this mutant did not significantly alter CD23 expression in cells that were only stimulated with LPS (Fig. 4). A potential Krox20-binding element is present at position −266 of the CD23 promoter, which is ~120 bp upstream of the STAT6-binding element (23). Taken together, these data provide strong evidence for an important role of Krox20 in IL-4-mediated CD23 expression in B cells.

This result supports the idea that IL-4-mediated differentiation of B cells requires not only direct activation of genes by STAT6 alone but also secondary transcriptional regulators, including Krox20, which are induced only on IL-4 stimulation by STAT6. These transcription factors therefore may regulate transcription of IL-4-modulated genes in a cascade-like manner. Other transcriptional regulators that mediate IL-4-modulated STAT6-dependent gene expression may include NF-IL3, IFN-regulatory factors 1 and 4, STAT-4, BCL3, XBP-1, and BCL-6 (Fig. 1). A similar cascade

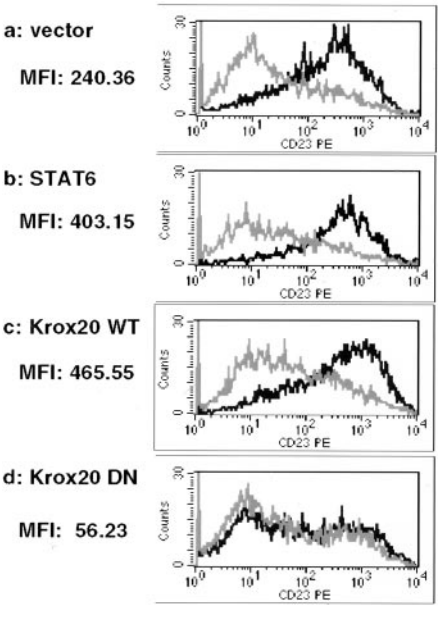

FIGURE 4. Krox20 plays an essential role for IL-4-induced CD23/FcεRII expression. M12.4.1 B cells were infected with retroviruses encoding either GFP alone (*a*, vector) or GFP plus STAT6 (*b*), Krox20WT (*c*), or Krox20DN (*d*). Transduced cells were stimulated with LPS (gray line), or LPS plus IL-4 (black line) for 48 h, and CD23 expression in GFP$^+$ cells was assessed by flow cytometry. One representative of four independent experiments is shown.

of transcriptional regulation has previously been identified on IFN-γ-induced expression of MHC II, which requires STAT1-mediated induction of the MHC II *trans* activator CIITA (24).

In summary, our data provide novel insights into the mechanism of action of STAT6 in IL-4-stimulated B cells. The identified genes and their products therefore represent novel targets for the development of therapeutic drugs for the treatment of allergic disease states.

Acknowledgments

We thank V. Miljkovic for excellent technical services; Dr. M. Grusby, Dr. X. Jiang, Dr. Y. Kim, Dr. J. Milbrandt, Dr. W. Paul, and Dr. M. Rosenfeld for valuable gifts; and Dr. K. Calame, Dr. C. Schindler, J. Losman, and J. Mostecki for critical reading of the manuscript.

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