

Bcl-6 and NF- κ B cistromes mediate opposing regulation of the innate immune response

Grant D. Barish, Ruth T. Yu, Malith Karunasiri, et al.

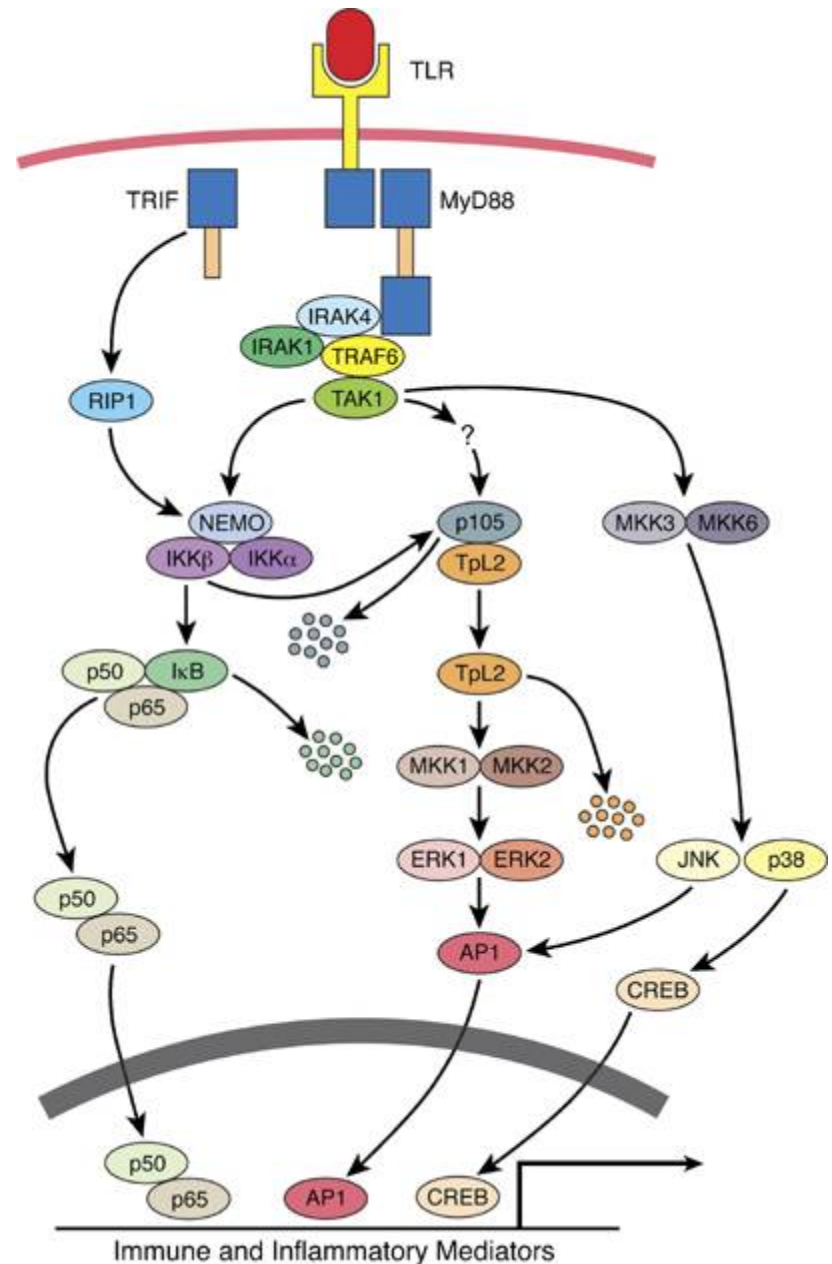
INTRODUCTION

Toll-like receptors (TLRs) are the most important receptor family of the innate immune system.

They are pattern recognition receptors that can be activated by several different pathogen associated molecular patterns as well as by some endogenous ligands

Different combinations of TLRs are expressed by a variety of cell types, particularly in the immune system, but also by epithelial and endothelial cells

Following interaction with their ligands they induce the transcription of a wide set of genes involved in inflammation (pro-inflammatory cytokines, cytokine receptors, adhesion molecules) through a complex signalling pathway that ultimately leads to activation of NFkB and AP-1.



TLRs play two fundamental roles in the immune response:

- they initiate the inflammatory process at the site of infection

AND

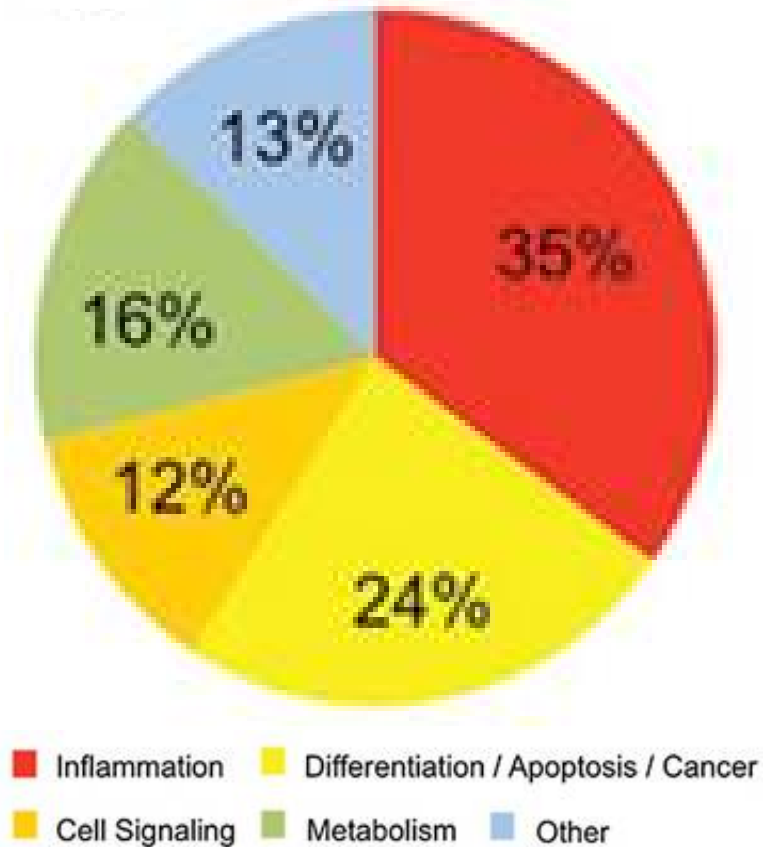
- by activating the local dendritic cells they link the innate to the adaptive immune response

As they trigger a potent, self amplifying inflammatory cascade and as they can also respond to endogenous ligands, their activation must be tightly regulated.

In fact some of them, particularly TLR 4 have been associated to the hyperinflammatory response that causes septic shock as well as to chronic inflammatory processes.

Bcl 6 is a transcriptional repressor known for its role in B-cell proliferation, but also implicated in modulation of macrophage inflammatory response

This work will investigate the role of Bcl 6 in regulating and modulating TLR 4-induced transcriptional response in macrophages at a chromatin level



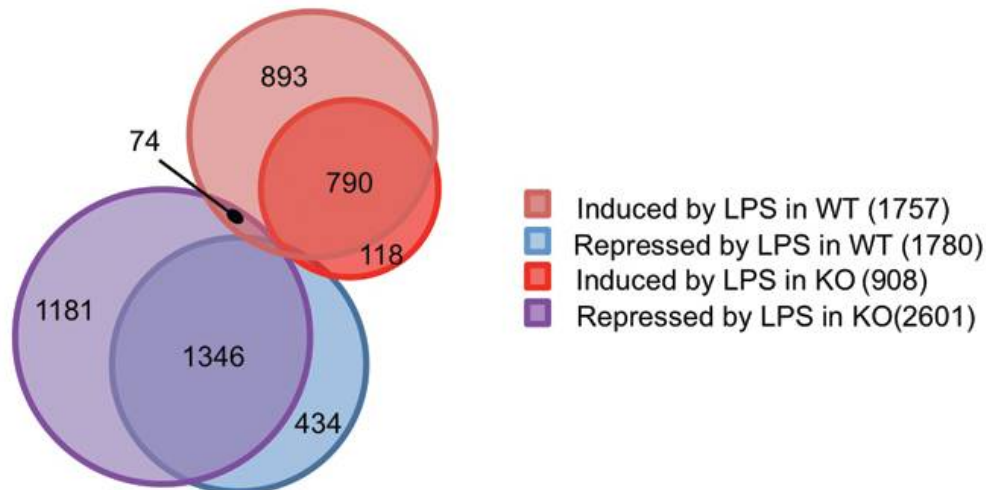
Firstly Bcl6 regulated gene network was identified by comparing genome wide expression analysis (by microarray) in WT to Bcl 6 ^{-/-} unstimulated BMDMs

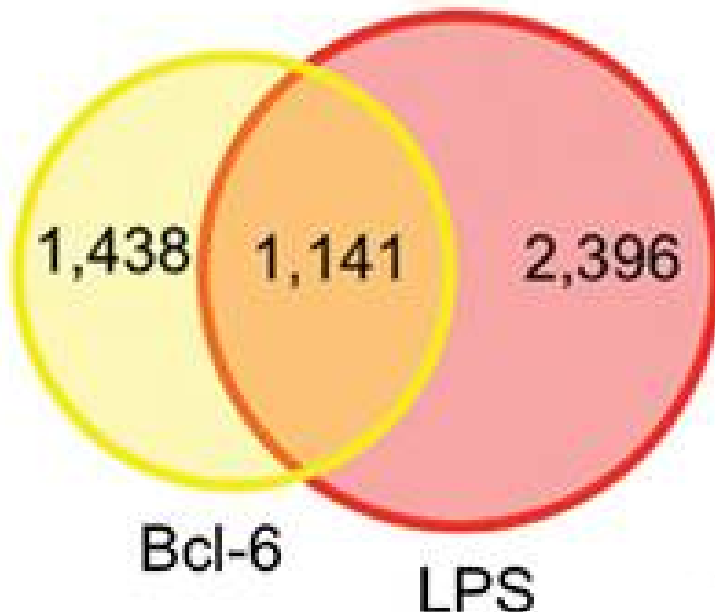
More than 2500 genes showed altered expression in Bcl 6 ^{-/-} cells

35% of these genes were found to be involved in inflammation
24% in differentiation, apoptosis and cancer
16 % in metabolism
12 % in cell signaling
and the remaining 13 % in other functions

Subsequently whole genome microarray analysis was repeated on LPS-stimulated cells.

Exposure to LPS resulted in a change in the level of expression for more than 3500 genes in both WT and Bcl 6 ^{-/-} BMDMs

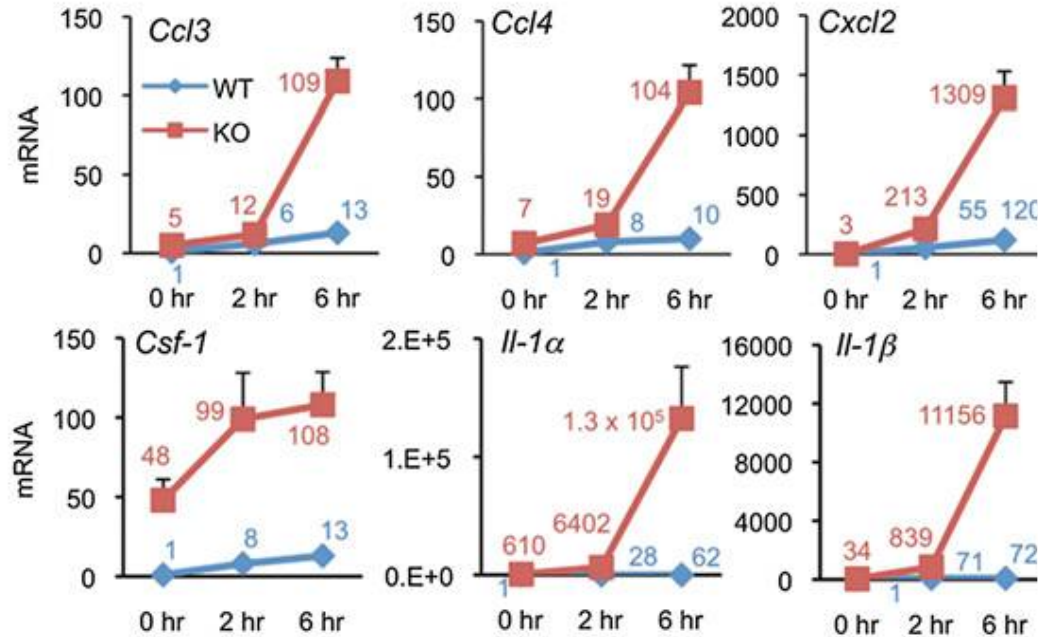




LPS induced transcriptome was then compared to Bcl 6 regulated gene network and 1141 genes were found to be co-regulated.

In 60 % of these genes co-regulation was such as loss of Bcl 6 had the same effect of LPS stimulation

The expression of some inflammation-related genes was also analysed by q RT PCR at different time points following LPS stimulation



When Wt were compared to Bcl -/- cells many of these genes showed a striking difference in basal expression (particularly IL-1 alpha) and consistently loss of Bcl 6 resulted in a hypersensitive response to LPS.

All together these data suggest that BCL 6 may prevent an hyperinflammatory response to LPS induced TLR 4 activation and it may also help to control basal transcription in TLR 4 gene network.

Surprisingly Bcl 6 can be found among TLR 4 regulated genes. Its transcription is transiently induced within 2 hours following LPS exposure, very likely with the function of attenuating the inflammatory response induced by TLR 4 itself

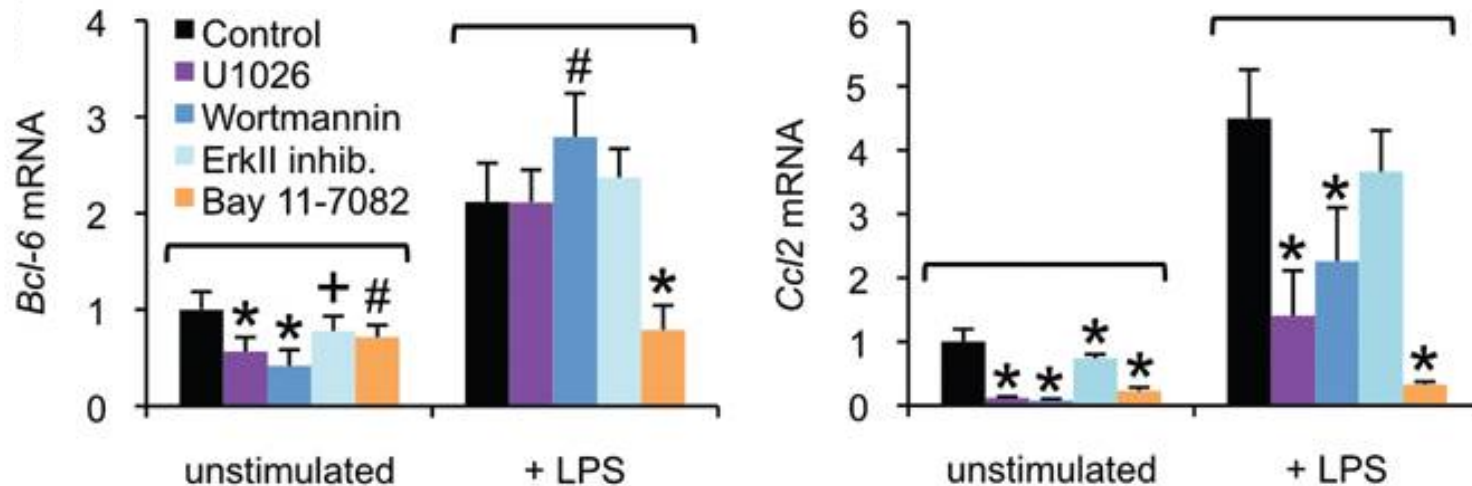
In order to identify which signalling pathway mediates response to LPS, BMDMs were treated with different TLR 4 inhibitors:

UO 126 (Mek1 and Mek2 inhibitor)

Erk II inhibitor (Erk 1/2inhibitor)

Wortmannin (PI3K inhibitor)

Bay 11-7082 (Nf-kB inhibitor)



Following LPS stimulation only Bay 11-7082 appeared to strongly inhibited TLR 4 transcriptional response by downregulating both Ccl2 and Bcl 6 mRNA levels

ChIP-seq Condition	Bcl-6 unstim.	Bcl-6 LPS	p65 LPS	Bcl-6 / p65 all
Cell signaling	23%	9%	18%	7%
Cytoskeleton / Cell adhesion	19%	22%	19%	18%
Differentiation / Apoptosis / Cancer	18%	13%	15%	20%
Inflammation	28%	48%	23%	47%
Metabolism	7%	--	9%	8%
Other	5%	8%	16%	--

To better understand the chromatin based mechanism of Bcl 6 regulation , Bcl 6 cistrome was determined by ChiPSeq performed in Wt BMDMs.

6655 Bcl 6 interaction sites were identified in unstimulated cells, mostly located in intergenic or intronic regions.







Each peak was then associated to the gene with the closest transcription start site allowing identification of 4354 genes, whose function was classified as inflammatory in 28% of cases

Motif analysis of Bcl 6 binding peaks revealed canonical Bcl6 binding sites in 74% of cases

Pu1 binding sites was identified as the second most frequent motif, suggesting that Bcl 6 may maintain quiescence in macrophages in part by inhibiting Pu 1 enhancer activity

This overlapping was confirmed by performing ChIPSeq with an anti Pu 1 antibody

Interestingly NFkB binding site was also found to be highly enriched.

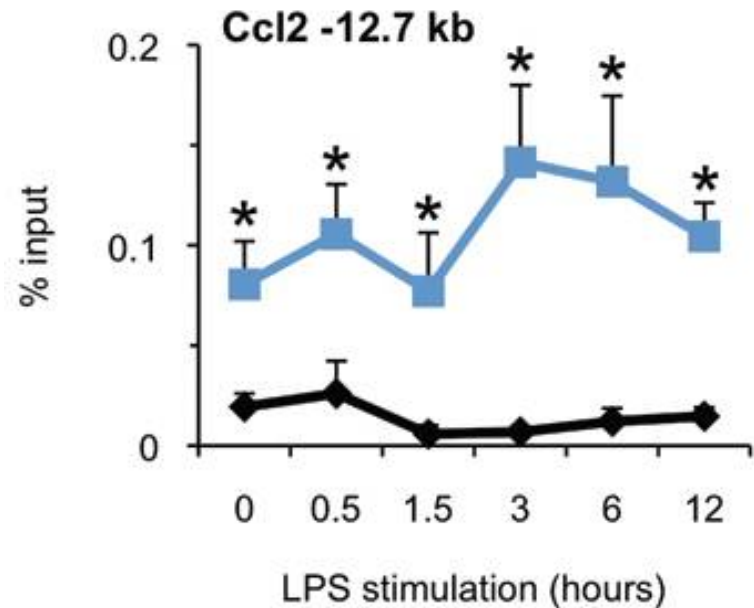
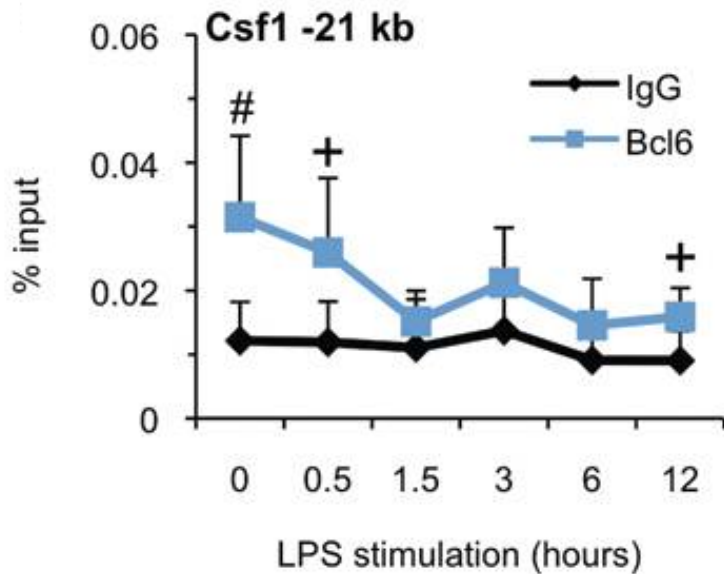
<u>Motif</u>	<u>p-value</u>	<u>Binding site</u>
	5e ⁻²²³	Bcl-6
	3e ⁻¹⁸⁴	
	1e ⁻⁷³⁷	Pu.1
	2e ⁻¹⁵⁸	
	6e ⁻⁸³	NF-κB
	3e ⁻¹⁶	

ChIP-seq Condition	Bcl-6 unstim.	Bcl-6 LPS	p65 LPS
Peak count	6,655	1,842	31,070
Promoter	5%	4%	10%
Exon	3%	3%	5%
Intron	42%	37%	39%
Intergenic	50%	56%	48%
p300 or Pu.1 overlap	44%	51%	64%
p300 overlap	28%	24%	28%
Pu.1 overlap	40%	50%	59%
Bcl-6 combined overlap (unstim. + LPS)	100%	100%	8%
Bcl-6 unstim. overlap	100%	11%	7%
Bcl-6 LPS overlap	39%	100%	3%
NF- κ B p65 LPS overlap	32%	45%	100%

To explore Bcl 6 role in the inflammatory response Bcl 6 ChIPSeq was repeated on LPS stimulated cells.

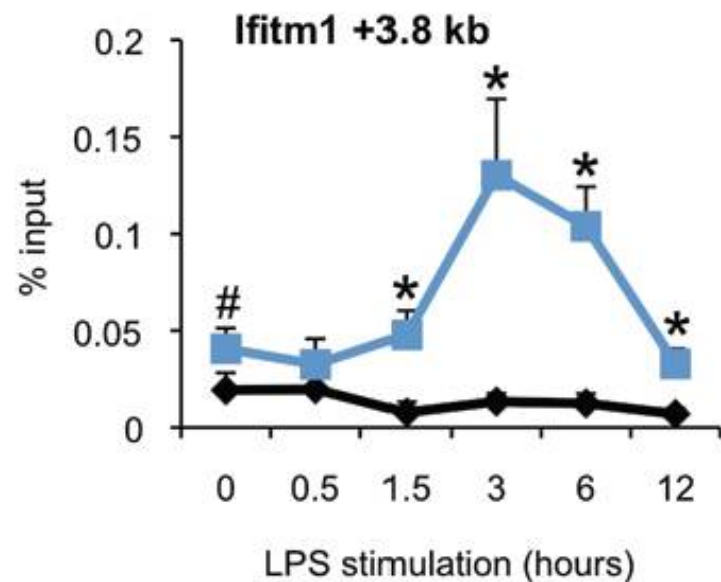
Treatment with LPS produced a marked change in Bcl 6 distribution

1842 interaction sites were identified, but overall the Bcl 6 quiescence cistrome was reduced from 6655 to 726 specific sites (- 90%), while 1116 additional sites were induced



To better focalise on Bcl 6 cistrome dynamic during LPS induced response some inflammation-related genes were analysed by ChIP PCR at different time points following LPS stimulation

LPS signal resulted in loss of BCL 6 binding for CSF1 and in gain of BCL 6 binding for Ifitm1, while Ccl2 retained Bcl 6 -chromatin interaction



Three main data emerge from this first part of the experimental work:

- Bcl 6 has an essential role in maintaining macrophage quiescence and in modulating TLR induced inflammatory response
- its cistrome is highly dynamic and dramatically change in response to LPS stimulation
- its binding sites frequently colocalise with enhancer sequences(as Pu 1 and p300 binding sites) as well as with NFkB binding sites

- NF-κB cistrome analysis using p65 as binding protein
- A low number of enriched regions (less than 200) can be found in quiescent macrophages due to cytoplasmic sequestration
- After LPS stimulation the peaks increase to more than 31.000

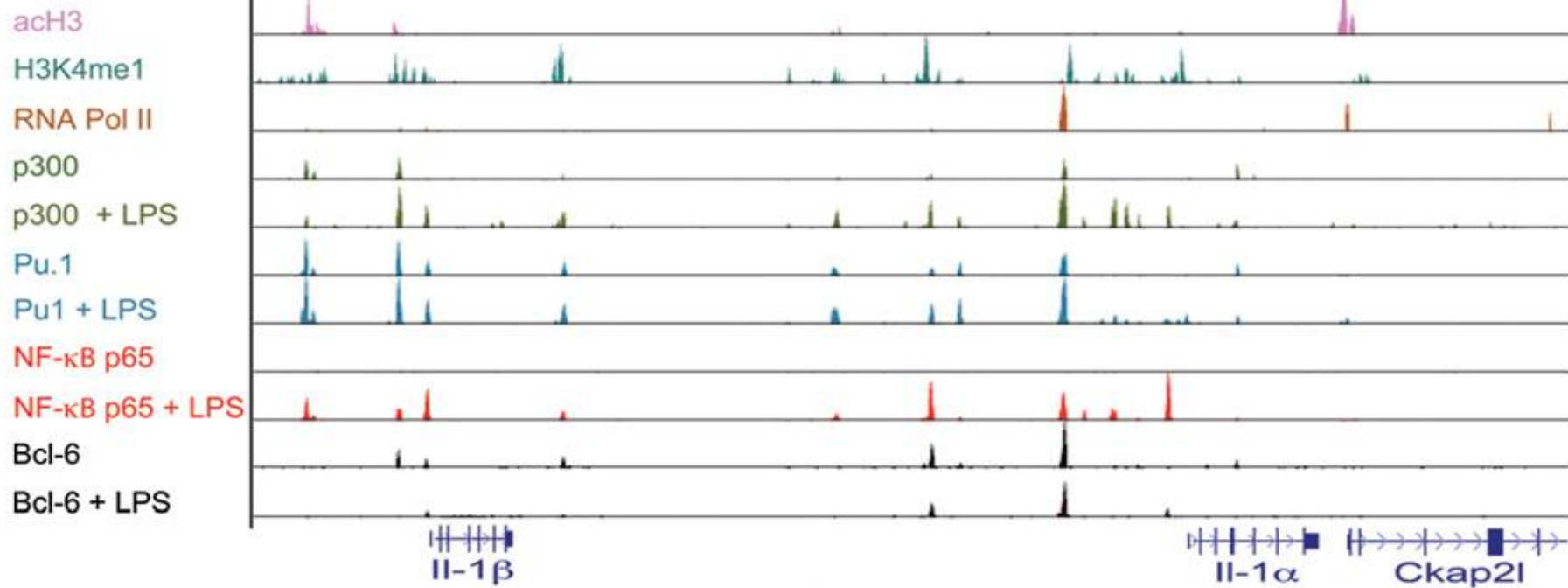
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A

50 kbp

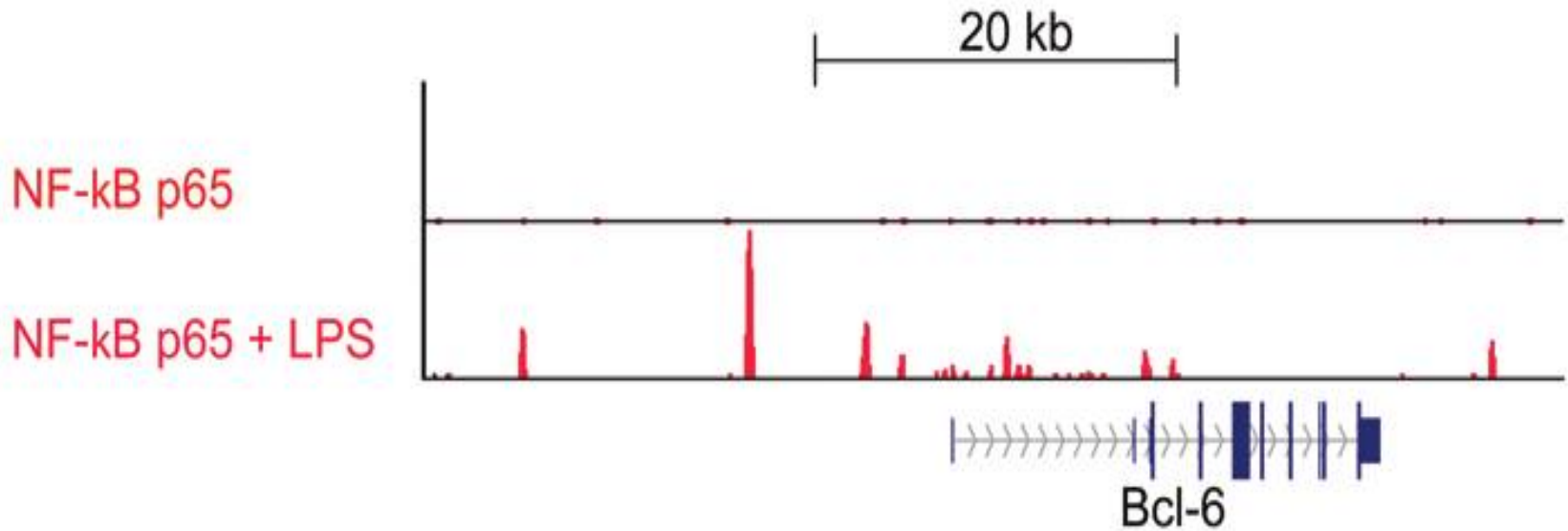


C

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The genes regulated by the NF- κ B cistrome have a wide range of functions

C



- NF-kB regulates transcription of Bcl-6 gene
- ChIP-seq for p65 in LPS-stimulated macrophages shows peaks near (within 50 kb) the gene for Bcl-6 suggesting a NF-kB-mediated regulation
- Thus Bcl-6 is both a constitutive and inducible repressor

E

<u>Motif</u>	<u>p-value</u>	<u>Binding site</u>
	6e ⁻²³⁴	Bcl-6
	2e ⁻¹⁰³	
	2e ⁻⁹⁰	
	2e ⁻¹⁸⁰	Pu.1
	6e ⁻¹⁷²	
	9e ⁻⁶¹	NF-κB

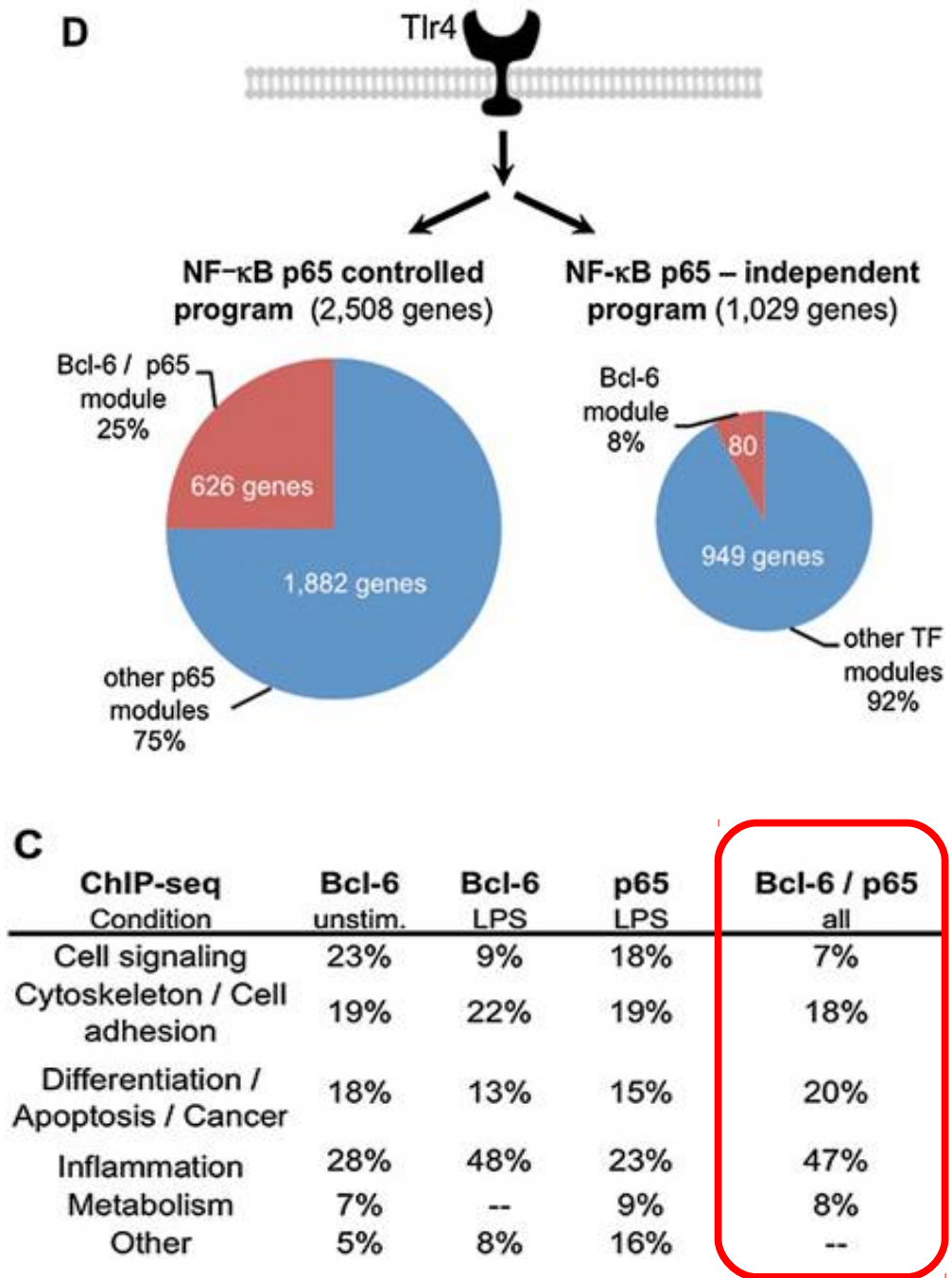
- Some genes of the NF-κB cistrome are coregulated by Bcl-6
- DNA fragments obtained by ChIP-seq for NF-κB show Bcl-6 and Pu.1 consensus sequences, too

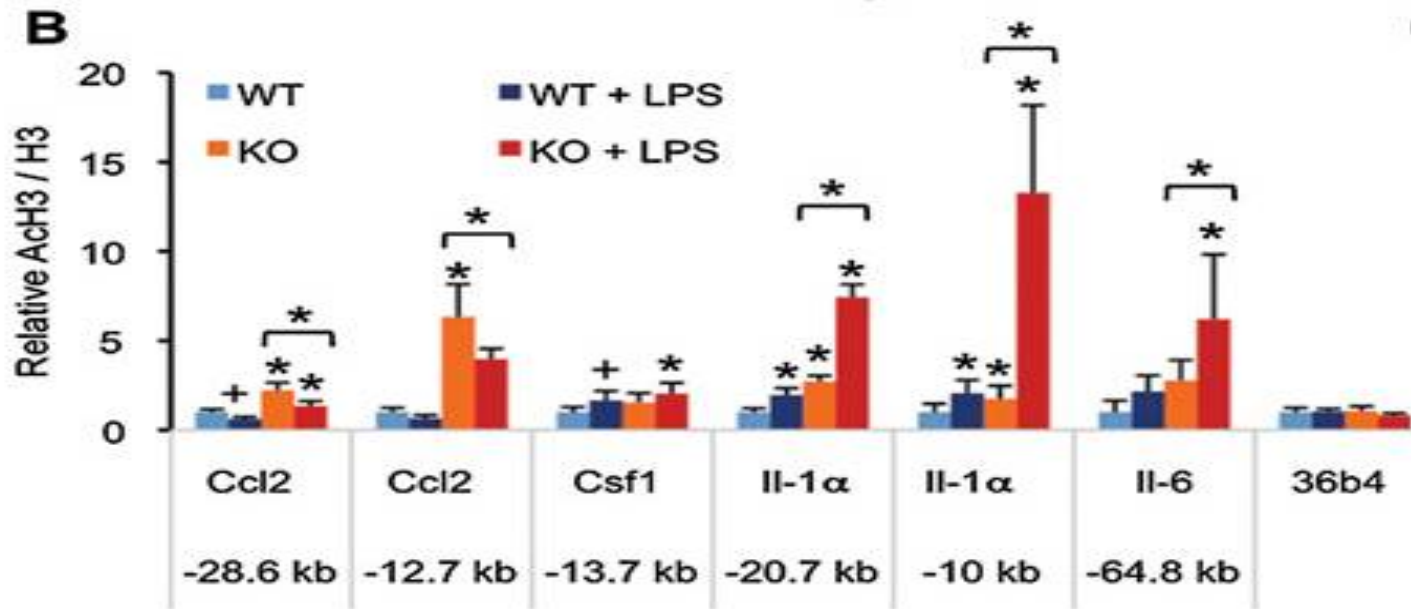
- NF- κ B and Bcl-6 sites are adjacent
- Those sites represent 32% and 45% of Bcl-6 cistrome of quiescent or stimulated macrophages respectively
- Thousands of sites for NF- κ B and Bcl-6 colocalize within nucleosomal distance (200 bp)
- This suggests a possible regulation mediated by cistrome competition and binding site masking

A

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- NF- κ B has a key role in Tlr4 signaling
- Of all the genes regulated by NF- κ B, 25% are coregulated by Bcl-6
- Most (47%) of those genes have an inflammatory role

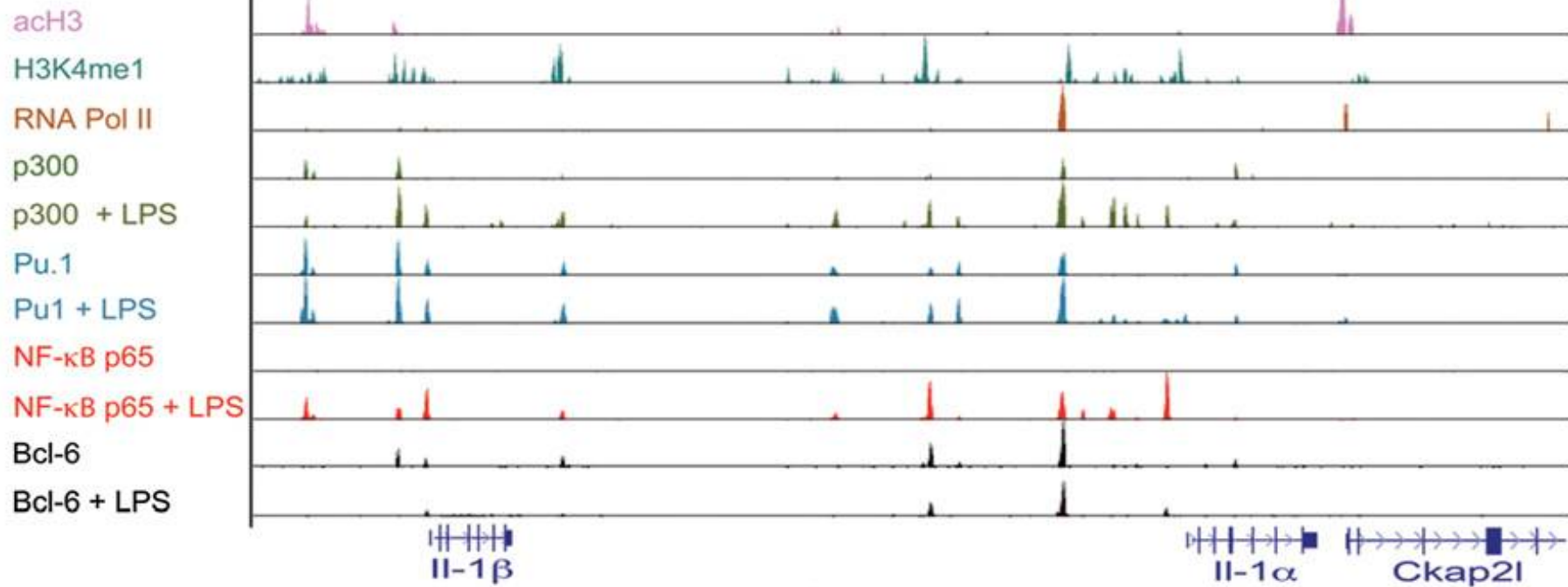




- Cistromic differences based on stimulation mat suggest a chromatin remodeling
- Bcl-6 presence and stimulation with LPS don't cause variation in H3K4 monometilation (a flag for enhancers)
- However Bcl-6 $-/-$ cells show hyperacetylation patterns on inflammatory genes
- LPS exposure cause the hystone acetyltransferase p300 to bind near inflammatory genes
- Thus Bcl-6 acts as an inhibitor of the Tlr4-mediated acetylation blocking p300

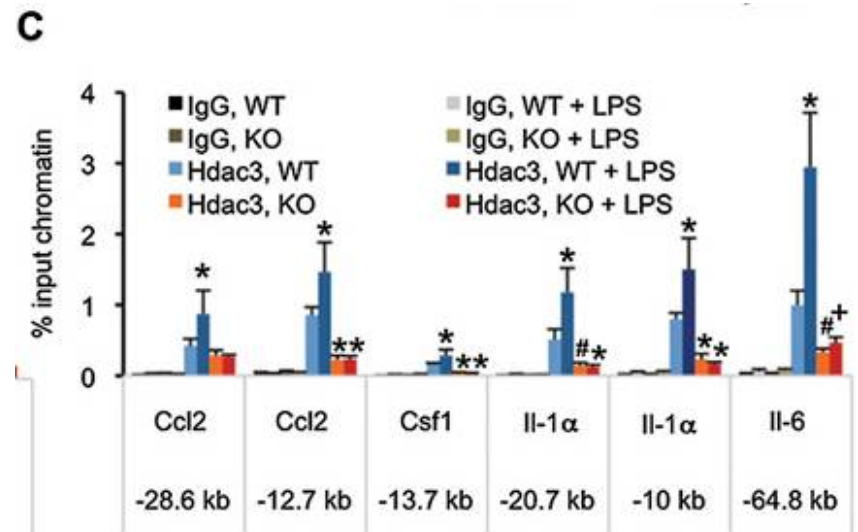
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50 kbp



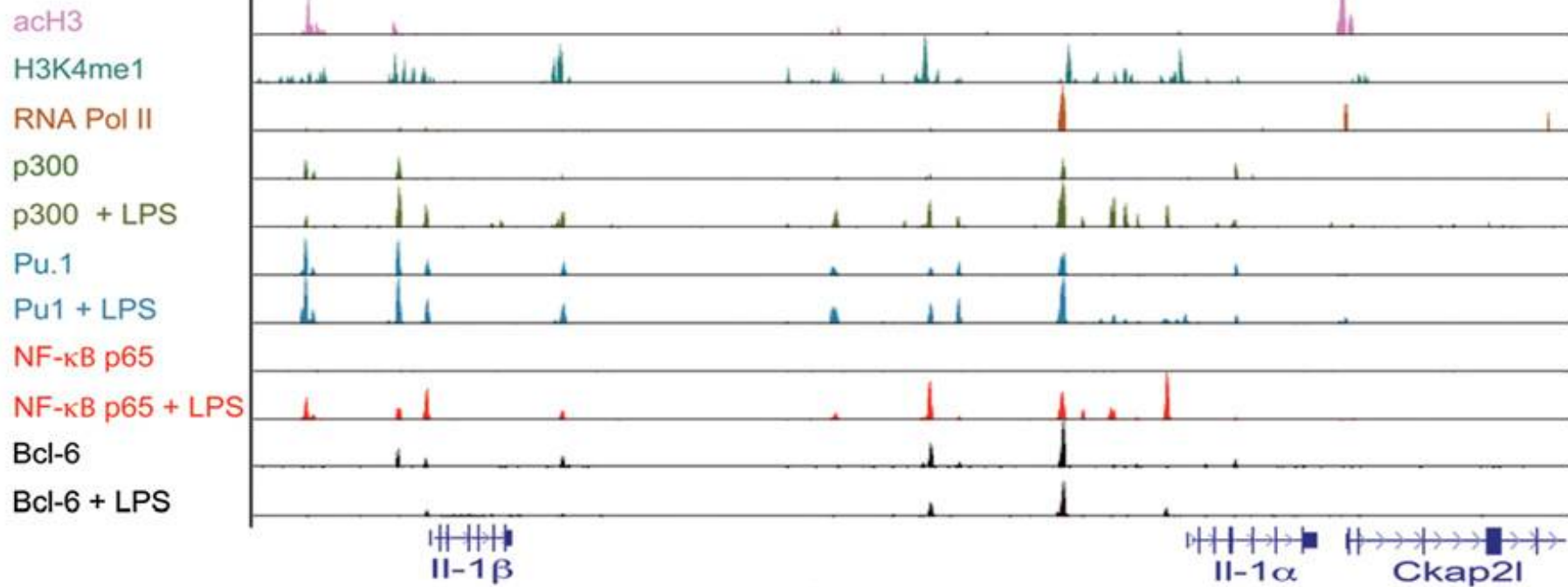
- LPS stimulation is shown to cause the binding of the Hystone deacetylase 3 near the same set of inflammatory genes suggesting a system to avoid overexpression of the genes

Bcl-6 $-/-$ lack this feature



A

50 kbp



Conclusions

- Bcl-6 has a key role in maintaining macrophages into quiescence and controlling the level of the inflammation
- This is possible with a cistrome-based antagonism

