Bcl-6 and NF-κB 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 1/2 inhibitor)
- Wortmannin (PI3K inhibitor)
- Bay 11-7082 (Nf-κB 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.
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.
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
The genes regulated by the NF-kB cistrome have a wide range of functions.

<table>
<thead>
<tr>
<th>ChIP-seq Condition</th>
<th>Bcl-6 unstim.</th>
<th>Bcl-6 LPS</th>
<th>p65 LPS</th>
<th>Bcl-6 / p65 all</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell signaling</td>
<td>23%</td>
<td>9%</td>
<td>18%</td>
<td>7%</td>
</tr>
<tr>
<td>Cytoskeleton / Cell adhesion</td>
<td>19%</td>
<td>22%</td>
<td>19%</td>
<td>18%</td>
</tr>
<tr>
<td>Differentiation / Apoptosis / Cancer</td>
<td>18%</td>
<td>13%</td>
<td>15%</td>
<td>20%</td>
</tr>
<tr>
<td>Inflammation</td>
<td>28%</td>
<td>48%</td>
<td>23%</td>
<td>47%</td>
</tr>
<tr>
<td>Metabolism</td>
<td>7%</td>
<td>--</td>
<td>9%</td>
<td>8%</td>
</tr>
<tr>
<td>Other</td>
<td>5%</td>
<td>8%</td>
<td>16%</td>
<td>--</td>
</tr>
</tbody>
</table>
• 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
Some genes of the NF-kB cistrome are coregulated by Bcl-6

DNA fragments obtained by ChIP-seq for NF-kB show Bcl-6 and Pu.1 consensus sequences, too
- NF-κB 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-κB and Bcl-6 colocalize within nucleosomal distance (200 bp)
- This suggests a possible regulation mediated by cistrome competition and binding site masking

<table>
<thead>
<tr>
<th></th>
<th>ChIP-seq Condition</th>
<th>Bcl-6 unstim.</th>
<th>Bcl-6 LPS</th>
<th>p65 LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak count</td>
<td>6,655</td>
<td>1,842</td>
<td>31,070</td>
<td></td>
</tr>
<tr>
<td>Promoter</td>
<td>5%</td>
<td>4%</td>
<td>10%</td>
<td></td>
</tr>
<tr>
<td>Exon</td>
<td>3%</td>
<td>3%</td>
<td>5%</td>
<td></td>
</tr>
<tr>
<td>Intronic</td>
<td>42%</td>
<td>37%</td>
<td>39%</td>
<td></td>
</tr>
<tr>
<td>Intergenic</td>
<td>50%</td>
<td>56%</td>
<td>48%</td>
<td></td>
</tr>
<tr>
<td>p300 or Pu.1 overlap</td>
<td>44%</td>
<td>51%</td>
<td>64%</td>
<td></td>
</tr>
<tr>
<td>p300 overlap</td>
<td>28%</td>
<td>24%</td>
<td>28%</td>
<td></td>
</tr>
<tr>
<td>Pu.1 overlap</td>
<td>40%</td>
<td>50%</td>
<td>59%</td>
<td></td>
</tr>
<tr>
<td>Bcl-6 combined overlap</td>
<td>100%</td>
<td>100%</td>
<td>8%</td>
<td></td>
</tr>
<tr>
<td>(unstim. + LPS)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bcl-6 unstim. overlap</td>
<td>100%</td>
<td>11%</td>
<td>7%</td>
<td></td>
</tr>
<tr>
<td>Bcl-6 LPS overlap</td>
<td>39%</td>
<td>100%</td>
<td>3%</td>
<td></td>
</tr>
<tr>
<td><strong>NF-κB p65 LPS overlap</strong></td>
<td><strong>32%</strong></td>
<td><strong>45%</strong></td>
<td><strong>100%</strong></td>
<td></td>
</tr>
</tbody>
</table>
• NF-kB has a key role in Tlr4 signaling
• Of all the genes regulated by NF-kB, 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 hyperacetilation patterns on inflammatory genes.

LPS exposure cause the histone acetyltransferase p300 to bind near inflammatory genes.

Thus Bcl-6 acts as an inhibitor of the Tlr4-mediated acetylation blocking p300.
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.
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.