

1 **Integrative profiling of early host chromatin accessibility responses in human**  
2 **neutrophils with sensitive pathogen detection.**

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## **Abstract**

Sepsis is a leading cause of death globally where neutrophils respond to pathogens via tightly regulated antimicrobial effectors. Combining early neutrophilic responses and pathogen detection may reveal insights for disease recognition. We performed ATAC-seq of human neutrophils challenged with six toll-like receptor ligands and two organisms; and RNA-seq after *Escherichia coli* (EC) exposure for 1 and 4 hours along with ATAC-seq. ATAC-seq of neutrophils retains more pathogenic DNA reads than standard library preparation methods. Only a fraction of differential chromatin regions overlap between challenges. Shared signatures exist for ligands but rest are unique in position, function, and challenge. Epigenomic changes are plastic, only ~500 are shared by EC challenges over time, resulting in varied differential genes and associated processes. We also identify three classes of chromatin mediated gene regulation based on their relative locations. These and transcription factor footprinting reveal timely and challenge specific mechanisms of transcriptional regulation in neutrophils.

## 60 Introduction

61  
62 Sepsis, a life-threatening sequela of bloodstream infections due to dysregulated host  
63 response, is the leading cause of death related to infections worldwide with rising  
64 incidences. Most common bloodstream infection causing bacteria are *Staphylococcus*  
65 *aureus* (SA) and *Escherichia coli* (EC) with frequencies of 20.5% and 16% respectively in  
66 culture positive patients<sup>1</sup>. Time is of the essence in sepsis, as every hour delay in appropriate  
67 antibiotic therapy decreases survival by 7.6%<sup>2</sup>. Understanding early host-pathogen interplay  
68 in sepsis can offer invaluable clinical insights critical to saving lives. Neutrophils are the  
69 first responders to infection and have been extensively studied for their role in infection and  
70 inflammatory processes, particularly sepsis<sup>3</sup>. Neutrophils recognize pathogen associated  
71 molecular patterns (PAMPs) via toll like receptors (TLRs)<sup>4</sup> and danger associated  
72 molecular patterns (DAMPs) via receptors such as RAGE for HMGB1<sup>5</sup>. PAMPs are  
73 derived from the cell walls of live or dead pathogenic organisms (exogenous signals),  
74 whereas DAMPs are derived from the host (endogenous signals) and each are specifically  
75 recognized by different TLRs<sup>6</sup>. Both result in inflammatory responses involved in sepsis.  
76 Neutrophils responding to PAMPs and DAMPs are capable of unleashing immediate,  
77 antimicrobial effector functions, including neutrophil extracellular trap (NET) production,  
78 phagocytosis, superoxide production and release of cytokines for further recruitment of  
79 other neutrophils and macrophages in a tightly regulated manner<sup>7,8</sup>. Moreover, studies have  
80 described that the neutrophil life span may be extended from 5-8 hours in the periphery to  
81 days upon interaction with both PAMPs and DAMPs<sup>9,10</sup>. These responses are tightly  
82 regulated to avoid collateral damage like increased vascular permeability and hypotensive shock  
83 resulting from release of heparin binding proteins by neutrophil activation via adherence to  
84 endothelial cells<sup>11</sup> and lung injury and poor patient outcome because of a cytokine storm  
85 resulting from hyperresponsiveness and dysregulation of apoptosis in lung neutrophils<sup>12</sup>.

86  
87 Despite possessing tightly regulated yet diverse functions, neutrophils have been regarded  
88 as a terminally differentiated cell type with limited ability to produce transcripts or proteins.  
89 The inference from this assumption is that the chromatin structure of a neutrophil is  
90 dynamically limited. Specifically in comparison to monocytes, neutrophils were shown to  
91 have much lower gene expression and largely repressed chromatin<sup>13</sup>. However, despite the  
92 fact that neutrophils have reduced transcriptional activity overall, they possess a much  
93 more dynamic range of transcripts and CpG patterns when compared to other cell types<sup>14</sup>.  
94 Neutrophils also show heterogeneity in their methylation patterns between individuals<sup>15</sup> and  
95 undergo active chromatin remodeling, methylation/acetylation patterns associated with  
96 gene transcription and cytokine production<sup>16-19</sup>. Neutrophils employ an inhibitor program  
97 to safeguard their epigenome from unregulated activation thereby protecting the host<sup>20</sup>.  
98 Epigenetic signatures have also been shown to play a role in the cellular function of septic  
99 patients<sup>21</sup>. Specifically, bacteria can affect the chromatin structure of host immune cells via  
100 histone modifications, DNA methylation, restructuring of CTCF loops, and non-coding  
101 RNA<sup>19,22,23</sup>. Such chromatin changes allow for repositioning of inflammatory genes into a  
102 transcriptionally active state, recruitment of cohesion near the enhancer regions, and result  
103 in swift transcriptional response in the presence of EC<sup>19</sup>. Even though chromatin remodeling

104 is shown in neutrophils in response to external stimuli, the exact regulation of transcription  
105 by changes in chromatin accessibility is not well understood.

106  
107 Since the epigenome reacts before gene expression, we are interested in profiling chromatin  
108 responses in neutrophils to infections for early disease recognition. In the current study, we  
109 first explore the relevant chromatin elements involved in TLR-mediated responses to  
110 1hour exposures from various pathogen ligands or whole SA and EC on a genome-wide  
111 scale using ATAC-seq to elucidate differences in the host response. We also assay the  
112 temporal fluctuation between 1 and 4 hours post EC exposure in the neutrophil epigenome  
113 and resulting transcriptome to better understand the processes and pathways involved in  
114 immune response. Neutrophilic chromatin accessibility patterns may reveal what pathogen  
115 an individual has encountered and/or how they are responding to the infection. Our analyses  
116 reveal chromatin accessibility, enriched motifs, and functional signatures unique to each  
117 challenge. We also observe time specific chromatin accessibility changes resulting in  
118 transcriptional changes at two time points. Based on the chromatin accessibility patterns, we  
119 classify three categories of transcriptional regulation in neutrophils. Additionally, since  
120 prokaryotes lack chromatin, employing ATAC-seq results in increased pathogen to host  
121 ratio of DNA, enhancing rare microbial reads. The coupling of host response profiles with  
122 microbial reads in a single assay may offer diagnostic advantages while gaining  
123 unprecedented insights into early host-pathogen dynamics and neutrophil biology.

124

## 125 **Results**

126

### 127 **Neutrophils are activated in response to ligand and whole organism challenges**

128 Purified neutrophils from 4 female healthy volunteers were challenged with an array of  
129 toll-like receptor (TLR) agonists for one hour, namely lipotechoic acid (LTA) (TLR2),  
130 lipopolysaccharide (LPS) (TLR4), flagellin (FLAG) (TLR5), resiquimod (R848) (TLR7/8);  
131 as well as  $\beta$ -glucan peptide (BGP) that signals via the dectin-1 receptor<sup>24</sup> for fungal  
132 infection and the DAMP high mobility group box 1 (HMGB1), a cytokine released in sterile  
133 inflammation (such as early traumatic events, thought to signal through RAGE and TLR4)<sup>5,25</sup>  
134 (Fig. 1b). Neutrophil activation was confirmed by IL8 and TNF $\alpha$  qRT-PCR (Fig. 1c). Since  
135 it is important that the ATAC-seq is performed on intact nuclei, sytox green assay was  
136 performed to estimate the extracellular DNA as an indication of NETs. No NETs were  
137 observed in response to any stimuli at the time of ATAC-seq (Fig. 1d).

138

### 139 **Pathogen DNA from challenges is enriched in ATAC-seq**

140 Neutrophils possess largely closed chromatin, and prokaryotes lack chromatin. ATAC-seq  
141 on neutrophils yields several features that increase sensitivity for pathogen reads. First,  
142 negative isolation of neutrophils reduces the number of human cells and potentially captures any  
143 circulating or phagocytized pathogens. Second, by surveying only open chromatin, ATAC-seq  
144 increases the pathogen to host ratio of DNA in the sample compared to traditional library  
145 preparation methods. To demonstrate this, neutrophils were challenged with SA in  
146 incremental colony forming units (CFU) per mL for 1 hour. These were then parallelly  
147 subjected to genome-wide sequencing using a standard SPRI library preparation and ATAC-  
148 seq. As suspected, the relative abundance of SA reads obtained by ATAC-seq was higher  
149 than the SPRI method, for all concentrations (Fig. 1e). In fact, the relative abundance retained

150 by ATAC-seq at  $10^3$  CFU/mL is comparable to the  $10^5$  CFU/mL SPRI preparation method; a  
151 marked improvement in sensitivity. Contaminant signals are present given that the  
152 neutrophils were only challenged with SA, these are likely short, low complexity reads that  
153 that do not map specifically. Despite the contamination, ATAC-seq samples display 3 times  
154 more reads for the pathogen compared to SPRI.

### 155 156 **Differential accessibility of chromatin in the genome is challenge and time specific**

157 Neutrophils have limited accessible chromatin; however, insert size distributions and  
158 enrichment at transcription start sites (TSS) were consistent across samples (Supplemental  
159 Figs. 2a and 2b). Strong correlation of genome wide peak counts across technical replicates  
160 (from  $r^2 = 0.70 - 0.95$ ) were observed for any given ligand stimulation (Supplementary Fig.  
161 3) while lower correlation was observed between donors, suggesting donor specific  
162 heterogeneity.

163  
164 Differential accessibility of chromatin in neutrophils is readily apparent when comparing the  
165 accessible chromatin landscape in response to the challenges with that of unstimulated  
166 neutrophils using Diffbind. For each challenge, filtering of the differential region calls with  
167  $p < 0.05$  and  $\text{abs}(\log\text{FC}) \geq 1$  resulted in a total of 28,812 differential regions for the LTA  
168 challenge; 35,453 for with LPS; 34,038 with FLAG; 33,604 for R848; 29,198 for BGP;  
169 32,916 with HMGB1; 10,201 with SA; 31,197 with the EC-1h (EC1h) exposure; and 22,511  
170 with EC-4h (EC4h) exposure. Despite the differences in the numbers of differential regions in each  
171 challenge, their genomic distribution identified using ChIPseeker (Fig. 2a) is similar. More than  
172 80% of the differential regions in each challenge were found in either distal intergenic or  
173 intronic regions.

174  
175 Landscape of differential chromatin accessibility is challenge dependent. This uniqueness is  
176 down to the gene level where associated differential regions between challenges are  
177 disparate (Fig. 2b). A map of the differential regions around the TCF7L2 gene portrays this  
178 uniqueness clearly. Overlap analyses of differential regions across the entire genome  
179 between challenges showed that the vast majority of these regions did not overlap and were  
180 challenge specific (Figs. 2c and 2d). A total of 19,710; 21,597; 21,978; 22,712; 17,791;  
181 20,703 regions were unique to the tested ligands BGP; FLAG; HMGB1; LPS; LTA; and  
182 R848 respectively. Similar patterns were observed with the whole organism challenges as  
183 well. In the SA; EC1h; and EC4h challenges, there were 9,417; 28,818; and 20,766 unique  
184 regions respectively. There are no overlapping differential regions across all of the  
185 challenges. 6 out of the 9 challenges tested were the most to have any overlapping  
186 differential regions. A total of 115 regions were shared by a combination of 6 challenges,  
187 603 were common between 5 challenges, 2121 between 4 challenges, 6429 between 3  
188 challenges, and 23,149 regions were common between a combination of two challenges.  
189 Interestingly, out of the 115 differential regions common to 6 challenges, 108 are common  
190 across all the ligand challenges (Fig. 2c). There is only 1 common differential region  
191 between the whole organism challenges. However, comparing the signature between the  
192 whole organism challenge and their corresponding ligands, a few commonalities exist (Fig.  
193 2d). There are 72 common differential regions between the SA and LTA challenges, 548  
194 common between the two EC time points, EC1h and LPS have 356 in common, and EC4h  
195 and LPS have 188 in common. Surprisingly, however, only 6 differential regions are

196 common to the two EC time points and LPS. On average, ~64% of the differential regions  
197 from the merged peaksets are unique to the ligand challenges while with the whole organism  
198 challenges, ~92.3% of the regions are unique to the challenge.

199  
200 Unique chromatin accessibility signatures in response to challenges are not limited to  
201 specific positions of differential chromatin accessibility but correspond to differences in the  
202 regulated functional pathways as well. Differential regions were assigned to genes by using  
203 a combination of prediction tools and surveying overlaps with known regulatory regions.  
204 This was done rather than assigning regions to genes immediately downstream so as to  
205 account for distal regulation as well. Prediction methods included T-gene<sup>26</sup> and GREAT<sup>27</sup>.  
206 Overlap analyses were performed against the HACER database as well as the predicted  
207 regulatory regions identified in primary human cancers<sup>28</sup>. Applying this combinatorial  
208 method, a minimum, ~91.6% of the differential regions were associated with genes while on  
209 average ~95.6% of the differential regions were successfully associated with genes across  
210 all challenges. In addition, on average, 78.3% of these overlapped previously identified  
211 regions with histone marks from the IHEC. GOterm and Pathway enrichment analyses with  
212 the assigned genes and the number of differential regions associated with each of them  
213 revealed dissimilar functional enrichment signatures between challenges (Fig. 3a). Signaling  
214 by receptor tyrosine kinases is the only common pathway enriched amongst all 9 challenges.  
215 Metabolism of carbohydrates, NOTCH1 signaling pathways, and deactivation of beta-  
216 catenin transactivating complex are unique to the whole organism challenges. The EC  
217 challenge over time resulted in similar pathway enrichments of the differentially accessible  
218 chromatin regions. Pathways like TLR4 cascade, neutrophil degranulation, response to  
219 infectious diseases, and Fc gamma receptor dependent phagocytosis are enriched. Overall,  
220 other than for a few overlapping enriched pathways, each challenge elicited varied  
221 functional responses upon stimulating the neutrophils.

222  
223 Similar to patterns with position and function, there are unique enriched motifs specific to  
224 each challenge. Importantly however, despite large proportions of differential regions being  
225 unique to each challenge, these still contain common enriched motifs shared across  
226 challenges. For example, even though there are no common differential regions across all 9  
227 challenges, 24 enriched motifs are common to them (Fig. 3b). Additionally, comparing  
228 enriched motifs in all the differential regions across the challenges, there are motifs unique  
229 to each. There were 5 unique motifs in the BGP challenge; 6 in the FLAG challenge; 5 in  
230 HMGB1; 5 in LPS; 6 in LTA; 7 in R848; 2 in SA; 14 in EC1h; and 5 in EC4h  
231 (Supplemental table 1). Hence, it is important to combine both specific positions as well as  
232 the enriched motifs to identify signatures unique to each challenge.

233  
234 **Transcriptional plasticity of neutrophils in response to EC challenges**  
235 RNA-seq analysis of EC challenged neutrophils at two time points – 1 hour (EC1h) and 4 hours  
236 (EC4h) using edgeR revealed a temporal pattern in gene expression suggesting plasticity in  
237 neutrophil transcription. Although most genes remain unchanged in expression in the presence of  
238 EC, there are differences in the number of differentially expressed genes and the magnitude of  
239 change between the two time points (Fig. 4a). There are 66 up and 56 down regulated genes at 1  
240 hour of exposure while there are 2554 up and 2656 down regulated genes at 4 hours. There are  
241 only 93 genes up regulated and 10 genes down regulated common to both time points.

242 Interestingly, there are a few genes that are either up regulated at 1 hour and down regulated at 4  
243 hours or vice versa. In this category, 7 genes were up at 1 hour and down at 4 and 10 were down  
244 at 1 hour and up regulated at 4 hours.

245  
246 Plasticity is also reflected in the biological processes enriched at the two time points (Fig. 4b).  
247 GOterm enrichment of genes grouped in above mentioned categories portrays a transcriptional  
248 landscape in neutrophils that are responsive to external stimuli. For example, immunoglobulin  
249 production is transiently up regulated at 1 hour, suggesting immediate response to the challenge.  
250 Transport and metabolism are predominant in the down regulated genes at 1 hour. Similarly, at 4  
251 hours of EC challenge, processes involved in neutrophil activation and degranulation, response  
252 to molecules of bacterial origin, intrinsic apoptotic signaling pathway, and processes utilizing  
253 autophagy mechanisms are enriched in the up regulated genes. More diverse processes are  
254 enriched in the down regulated genes at 4 hours and interestingly, many overlaps with those  
255 enriched in the up regulated genes at 4 hours. As expected, however, many processes involved in  
256 immune response are enriched in the genes that are up regulated at both time points. These  
257 include neutrophil migration, cellular response to lipopolysaccharides, positive regulation of  
258 inflammatory response, chemokine mediated signaling, NF-kappaB signaling, and regulation of  
259 DNA binding transcription factor activity. Surprisingly there are no enriched processes in the  
260 continuously down regulated genes. Interestingly, SRP-dependent co-translational protein  
261 targeting to membrane was down regulated at 1 hour and up regulated at 4 hours. On the  
262 contrary, Fc-epsilon and Fc-gamma receptor signaling pathways, regulation of complement  
263 activation, and regulation of protein processing are all up regulated at 1 hour and down regulated  
264 at 4 hours.

### 266 **Transcriptional plasticity of neutrophils is a result of complex accessible chromatin** 267 **crosstalk**

268 Combination of ATAC-seq and RNA-seq revealed a complex combination of differentially  
269 open and closed chromatin regions affecting gene expression changes. For each category of  
270 gene expression, a count of the whether the associated peaks are differentially closed or  
271 open at each time shows that there is no specific pattern (Fig. 4c). That is, genes being up  
272 regulated at 1 hour are not all a result of just differential chromatin regions either opening or  
273 closing. For genes that are differentially expressed only at 1 hour, there likely are chromatin  
274 accessibility changes occurring at 4 hours that are maintaining gene expression level with  
275 the control. This is readily apparent when looking at the distribution of the openness or  
276 closedness of associated differential regions (Fig. 4c). This phenomenon is clear when  
277 surveying the associated differential regions for each gene (Fig. 4d). For example, out of the  
278 7 genes that are up regulated at 1 hour and down regulated at 4 hours, differential chromatin  
279 regions were found to be associated only with 1 gene, PTK7. For this gene, 9 associated  
280 differential chromatin regions either open or close in a combinatorial manner to affect the  
281 gene expression. These 9 associated regions include 6 regions that are differentially closed  
282 at 1 hour, 1 that is differentially open at 1 hour and 2 that are open at 4 hours. Interestingly  
283 however, there is no direct correlation between gene expression and the number of  
284 associated differential regions (Spearman correlation – EC1h: 0.0321 EC4h: 0.0289). In  
285 general, there are more chromatin accessibility changes earlier while transcriptional changes  
286 largely occur at the later time point.

287

288 Based on the *in silico* linked chromatin accessibility changes and the transcriptional  
289 expression, the regulatory mechanisms were classified into three proposed categories – 1.  
290 Differential regions only in the promoter (1h: 84; 4h: 86); 2. Differential regions only in  
291 distal sites while the promoter region is primed for expression (1h: 19,450; 4h: 23,206) ; and  
292 3. Differential regions in the promoter as well as distal sites (1h: 2245; 4h: 1015). Although  
293 these three mechanisms are prevalent, there are differentially expressed genes that do not  
294 have any associated differential chromatin regions. In the first category, Differential regions  
295 were present only in the TSS  $\pm$  2.5 kb regions (Fig. 5a). An example for this category is the  
296 FAM66C gene which is affiliated with the long non-coding RNA (lncRNA) class. This de  
297 novo differential opening in the promoter region results in  $\sim$ 3 logFC increase at 4 hours in  
298 the presence of EC. In the second category, regions in the promoter are open, however, there  
299 is no difference between the EC challenge in comparison to control. In this category, gene  
300 expression is fine-tuned by distal regulatory regions. The BBS2 gene, a member of the  
301 Bardet-Biedl syndrome gene family and forms a part of the BBSome multiprotein complex,  
302 is down regulated at 4 hours (-1.4 logFC) while there exists an associated differentially open  
303 region present at 1 hour. This region being open facilitates the maintenance of gene  
304 expression at 1 hour (Fig. 5b). The third category is a combination of differential chromatin  
305 regions in the promoter region and in associated distal sites. An example is the HMGCS1  
306 gene that encodes a protein with protein homodimerization, and isomerase activity is up  
307 regulated  $\sim$ 3 logFC only at 4 hours (Fig. 6a). Interestingly, the accessible chromatin  
308 signature associated is complex. A differential chromatin region is fixed in the promoter at  
309 both time points and yet differential gene expression occurs only at one. This is likely a  
310 result of the associated distal regions – two that are differentially open at 1 hour and one  
311 each of differentially open and closed regions at 4 hours. These distal interactions are further  
312 supported by the Hi-C predicted interacting regions<sup>19</sup>. These distal regions fall within  
313 226,569 Hi-C predicted interactions across the genome. A complex interplay of interacting  
314 chromatin regions facilitates expression of the associated gene. These interactions facilitate  
315 the activation or repression of binding motifs to fine tuning the regulation. Transcription  
316 factor footprinting of enriched motifs in open distal differential regions associated with the  
317 HMGCS1 gene revealed a temporal pattern of binding (Fig. 6B). The Sp2 binding motif  
318 remains unbound at both time points while the ETS1 motif is only bound at 1 hour and the  
319 n-Myc motif is bound only at 4 hours. Similar patterns of binding are observed in motifs  
320 unique to each time point at these differential regions as well. A combination of varied  
321 differentially accessible chromatin regions and transcription factor binding motifs provide  
322 an intricate means of transcriptional regulation in neutrophils.

323

## 324 Discussion

325

326 In this study we explore the chromatin accessibility signatures affecting immune response in  
327 neutrophils challenged with external infecting stimuli using ATAC-seq. ATAC-seq on  
328 neutrophils provides a unique advantage, interrogating the host response biology, while  
329 simultaneously gathering information about pathogens with higher detection sensitivity.  
330 Identifying the pathogen responsible for sepsis is an area of intense research. Additionally,  
331 ATAC-seq can evaluate the accessibility changes in intergenic and enhancer regions<sup>29</sup>,  
332 showing cell type specific enhancer usages. Even though next generation sequencing has  
333 provided technological advances, gathering useful host and microbial information



334 simultaneously is challenging. Only a fraction of the reads generated from human  
335 samples correspond to pathogens and, even if successfully retained in the sea of human  
336 DNA, contig assembly is very difficult, so classification must be able to proceed accurately  
337 with short reads from noisy data<sup>30,31</sup>. There is a large body of work dedicated to defining  
338 positivity via setting thresholds (adjusted based on pathogen load and misclassification due  
339 to incomplete reads), species identification to differentiate pathogen from contaminant, and  
340 subtraction from negative controls<sup>30,31</sup>. Our assay design improves sensitivity by capitalizing  
341 on negative isolation of neutrophils, reducing the amount of background human DNA as  
342 well as cell free microbial DNA in the final sequencing sample and then only sequencing  
343 open chromatin from the eukaryote and prokaryote. It performs drastically better than  
344 standardized diagnostic library preparation methods (Fig. 1e). This assay requires  
345 a small sample volume making it ideal towards clinical adoption in sepsis diagnosis.

346  
347 Neutrophils are known to form subpopulations at the site of inflammation in response to the  
348 stimulus<sup>32</sup> but it was unclear whether epigenetic changes are challenge specific. We find  
349 challenge specific genome wide chromatin changes (Figs. 2b, 2c, and 2d) despite similar  
350 genomic distributions (Fig. 2a). These unique chromatin changes reflect in common and  
351 unique enriched pathways as well specific transcription factor binding motifs unique to each  
352 challenge (Fig. 3). Only signaling by receptor tyrosine kinases is common to all challenges.  
353 This supports the challenge specific response nature since the only common pathway is  
354 sensing external cues. Although there are only about ~500 differential regions common to  
355 the two EC challenge time points, their associated enriched pathways are identical (Fig. 3a).  
356 Interestingly however, there aren't many shared pathways between EC and the  
357 corresponding ligand, LPS portraying the differences between single ligand and whole  
358 organism stimulation. Apart from the receptor tyrosine kinase pathway, VEGFA signaling  
359 pathways is shared which is involved in neutrophil recruitment<sup>33</sup>. These chromatin  
360 accessibility signatures support the earlier discovered stimulus specific gene expression  
361 changes in response to LPS and EC<sup>34</sup>. The unique chromatin accessibility signatures also  
362 expose unique transcription factor binding motifs specific to challenges (Fig. 3b and  
363 supplemental table 1).

364  
365 Early events in immune cells involved in sepsis can and should be captured in their  
366 epigenome, as this is the first step in the cellular response to a cell's environment. These  
367 chromatin accessibility events could be a source for new diagnostic tools and even novel  
368 molecular targets for new therapies. At one hour, under many stimuli in this first responder  
369 cell type, we find challenge specific genome wide changes in chromatin  
370 accessibility (Figs. 2b, 2c, and 2d). This phenomenon is also readily apparent when  
371 comparing the differential chromatin accessibility at the two time points of the challenges  
372 (31,197 at 1h vs 22,511 at 4h). Measurements of gene and protein expression capture  
373 events much later than epigenomic changes and hence may be less informative. For  
374 example, it has been shown that enhancer profiling was better at determining cell identity  
375 than mRNA<sup>35</sup>. This delayed transcription vs epigenetics is supported by our data. While  
376 more chromatin accessibility changes are observed at 1h, more differential expression of  
377 genes occurred at 4h and in a time specific manner (Figs. 4b, 4c and 4d). Hence, we propose  
378 that a combination of unique differentially accessible chromatin regions as well as motif  
379 signatures we have identified may be more illuminating of the neutrophil's pathogen

380 exposure. These exposure-specific chromatin accessibility changes are rapidly induced and,  
381 while many maybe transient, may leave a longer-lasting “mark” on the epigenome,  
382 potentially spawning a new forensic and diagnostic modality, an advantage over current  
383 tools, as the epigenome is the earliest detectable signal.

384  
385 Plasticity in neutrophils has been widely accepted recently<sup>36,37</sup> but the mechanisms driving  
386 this have yet to be successfully delineated. Current focus on the role of epigenetics has  
387 vastly expanded the understanding<sup>38</sup>, but much is still unknown. A study of unchallenged  
388 neutrophils from healthy volunteers identified over 2000 genes with a significant  
389 epigenetic component explaining their expression<sup>39</sup> and the role of epigenetics in sepsis  
390 induced immunosuppression in various immune cells has been identified<sup>36</sup>. Subsequently,  
391 significant chromatin restructuring was observed in response to a three hour EC infection<sup>19</sup>.  
392 While these chromatin restructures facilitate the opening of the inflammatory response  
393 armament, the exact accessible chromatin interactions are still unknown. With the EC  
394 infection time series in our study, we find that both transcription and chromatin accessibility  
395 are plastic in neutrophils. This is readily apparent in the low overlap in the differential  
396 regions between the two time points. This can be attributed to the changes in chromatin  
397 structure and the need for opening/closing of transcription factor binding sites giving rise to  
398 transient gene expression. Based on the differential regions and gene associations, we  
399 classified three broad categories of accessible chromatin regulation that occur within earlier  
400 identified CTCF anchored loops<sup>19</sup>. The categories include – differential regions only in the  
401 promoter, differential regions only in the distal enhancer regions, and finally genes regulated  
402 by a combination of both. Of these categories, genes with the differential regions only  
403 within the promoter were the fewest (Fig. 5a) and showed new H3K27ac modifications  
404 within the promoter. Genes with primed promoter regions being regulated only by distal  
405 enhancer regions were the highest (Fig. 5b) and showed histone marks in the promoter,  
406 suggesting promoter activation, under both challenged and unstimulated states further  
407 supporting our classification. The third category is a combination of both differential regions  
408 in promoters as well as distal regions and the HMGCS1 gene, implicated in the regulation of  
409 inflammation<sup>40</sup>, is an example of how this results in gene expression (Fig. 6a). Although  
410 similar mechanisms of epigenetic transcriptional regulation are known in macrophages<sup>41,42</sup>  
411 and the additive or competitive roles of multiple distal enhancers for gene are known<sup>43,44</sup>,  
412 this is the first evidence for such regulation in neutrophils. We also see time specific binding  
413 of transcription factors (Fig. 6B) within the HMGCS1 associated distal regions, possibly  
414 involved in cooperative activation or repression similar to other systems<sup>45–48</sup> or by inhibiting  
415 the binding of different transcription factors<sup>20</sup> to result in the observed gene expression.  
416 Overall, we show that neutrophils undergo plastic transcriptional expression under intricate  
417 accessible chromatin regulation that is unique to the stimulus faced (Fig. 7) and that this  
418 methodology can potentially be used in combination with these signatures as a putative  
419 diagnostic tool.

420

## 421 **Methods**

422

### 423 *Study participants*

424 Four healthy volunteer females 30-40 years old were recruited and informed consent  
425 obtained (Stanford University IRB-37618).

426

427 *Negative selection isolation and activation of neutrophils*

428 *FACs and Flow cytometric analysis*

429 Neutrophils were isolated using a negative selection method that allowed for seamless  
430 dovetailing with the ATAC-seq method (Fig. 1a, Supplemental Fig. 1). Neutrophils were  
431 isolated using the Stem Cell EasySep Direct Human Neutrophil Isolation Kit as per the  
432 manufacturer's protocol and resuspended in PBS with 1% FBS and 2mM EDTA.  $8.0 \times 10^5$   
433 cells per condition were fixed in 1% PFA for 10 min at room temperature and subsequently  
434 stained with primary antibodies: mouse anti-human PEcy7-CD16 (BD #557744), mouse  
435 anti-human PerCPcy5.5-CD66b (Biolegend # 305107), and mouse anti-human V500-CD45  
436 (BD # 560779) as well as the corresponding IgG controls. Flow cytometric and statistical  
437 analysis were performed using FlowJo V. 10.0.8.

438

439 *Toll Like Receptor (TLR) samples*

440 Neutrophils isolated from 4 healthy volunteers were plated at 50,000 cells per well and  
441 stimulated in duplicate with the following ligands for 1 hour: lipoteichoic acid (LTA)  
442 100ng/mL (Invivogen), LPS (Sigma) 100ng/mL, Flagellin (FLAG) 300ng/mL, resiquimod  
443 (R848) (Invivogen), 10uM, CpG Class C ODN 2395 5uM,  $\beta$ -glucan peptide (BGP)  
444 100ug/mL (Invivogen), high mobility group box 1 (HMGB1) (R & D) 1ug/mL<sup>4,5,24,25</sup> (Fig.  
445 1b).

446

447 *Live Organism Challenge samples*

448 Blood from 2 healthy volunteers was spiked with a specific CFU/mL of either EC ATCC  
449 25922 or SA ATCC 29312. The Stem Cell EasySep Direct Human Neutrophil Isolation Kit  
450 was applied as per the manufacturer's protocol to 2mL of blood after 1 hour of SA  
451 treatment and 1 and 4 hours of EC treatment. After isolation, cells were counted, divided  
452 into 50,000 cell samples (50uL) in duplicate (Fig. 1b).

453

454 *Quantitative RT-PCR of IL8 and TNF $\alpha$*

455 Total RNA was isolated from  $8.0 \times 10^5$  cells prepared as above with the RNeasy kit  
456 (QIAGEN). RNA was DNase treated using the TURBO DNA-free DNase treatment  
457 (Ambion). One step qRT-PCR was performed in the Rotor Gene Q using the Rotor Gene  
458 SYBR Green RT-PCR kit.  $\Delta\Delta C_t$  was calculated using GAPDH. Primer sets are as follows:  
459 *IL8 F* 5'CAGTTTTGCCAAGGAGTGCT, *IL8 R* 5'ACTTCTCCACAACCCTCTGC, *TNF F*  
460 5'GCTGCACTTTGGAGTGATCG, *TNF R* 5'ATGAGGTACAGGCCCTCTGA, *GAPDH F*  
461 5'TGCACCACCAACTGCTTAGC, *GAPDH R* 5'GGCATGGACTGTGGTCATGA

462

463 *Sytox Assays for Neutrophil Extracellular Traps*

464 Neutrophils were isolated as above using either the TLR sample or live organism sample  
465 preparation as appropriate. Cells were plated at  $2.0 \times 10^5$  per well in triplicate. A positive  
466 control was created by stimulating cells with 25nM PMA (Sigma). 5mM Sytox green (Life  
467 Technologies) was used to detect the presence of NETs<sup>49</sup>. Fluorescence intensity was  
468 measured using the Tecan Infinite M200 Pro.

469

470 *ATAC-seq and RNA-seq library prep and sequencing*

471 All treated and untreated control cells from 4 donors and 6 ligands as well as 2 donors and 2  
472 whole organism challenges were collected as described above and ATAC-seq was performed  
473 as described<sup>29</sup>. Excess primers libraries were removed using the AMPpure bead kit. In  
474 parallel with ATAC-seq, genome-wide sequencing using a standard SPRI<sup>50</sup> library  
475 preparation was performed on the SA challenged neutrophils with AMPure XP from  
476 Beckman Coulter.

477 RNA was extracted from isolated neutrophils after the 1 and 4 hour EC challenges as well  
478 untreated controls using the miRNeasy Micro kit from Qiagen and libraries were generated  
479 using KAPA PolyA enrichment mRNA library prep. All libraries were sequenced at the  
480 Stanford Functional Genomics Facility on the Illumina HiSeq.

481

## 482 *ATAC-seq analysis*

### 483 *Data processing and peak calling*

484 The datasets generated for this study are available under BioProject - xxxxxxxx. Fastq files  
485 were analyzed from raw data all the way to peak calls using the PEPATAC pipeline  
486 (<http://code.databio.org/PEPATAC/>) against the hg19 build of the human genome. Briefly,  
487 reads were trimmed of adapters using Trimmomatic<sup>51</sup> and aligned to hg19 using Bowtie2<sup>52</sup>  
488 with the `-very-sensitive -X 2000` parameters. Duplicates were removed using PICARD tools  
489 (<http://broadinstitute.github.io/picard/>). Reads with MAPQ <10 were filtered out using  
490 Samtools<sup>53</sup>. Reads mapping to the mitochondria or chromosome Y were removed and not  
491 considered. Technical replicates were merged using Samtools yielding one sample per  
492 donor per stimulation. Peaks were called using MACS2<sup>54</sup> with the `-q 0.3 --shift 0 --nomodel`  
493 parameters. Given the closed nature of neutrophil chromatin and our interest only in  
494 differential regions, we chose to relax the FDR cutoff to produce sufficient regions for  
495 further differential studies. Correlation between replicates was generated and a single peakset  
496 was generated across replicates for each challenge.

497

### 498 *Microbial identification*

499 Reads generated from both SPRI as well as ATAC-seq were preprocessed by trimming with  
500 Trimmomatic. Using Kraken<sup>55</sup>, human reads were removed from the samples, and relative  
501 abundances for pathogens were determined as counts per million reads. Replicates were  
502 averaged together and log2 transformed for an abundance value.

503

### 504 *Differential analysis*

505 Differentially accessible regions were identified from the merged peaksets using the  
506 DiffBind R package<sup>56</sup>. A p-value of 0.05 and  $abs(\log FC) \geq 1$  were set as the  
507 threshold. Consensus bed files were generated with Diffbind with a threshold of 0.66  
508 overlap. Overlapping/ common regions between peak sets were determined using the  
509 DiffBind tool and visualized with the UpSet package<sup>57</sup> in R.

510

### 511 *Assigning genes associated with differential regions*

512 Differential regions in each sample were associated with genes following multiple  
513 approaches – 1. T-gene<sup>26</sup> from the MEME suite was used to predict regulatory links between  
514 the differential region and the genes. Only associations with p-value < 0.05 and correlation  
515  $\geq 0.4$  were included. 2. Using GREAT<sup>27</sup> and implementing the basal plus extension

516 algorithm and defining a 2.5Kb region each for proximal upstream and downstream  
517 respectively, and a distal region up to 500Kb. 3. Surveying overlap of differential regions  
518 with previously reported association links<sup>28,58</sup> using Bedtools<sup>59</sup>. Additional support for these  
519 associations was derived by incorporating predicted Hi-C interactions from EC stimulation  
520 of neutrophils for 3 hours<sup>19</sup>. A functional pathway enrichment analysis was performed using  
521 the ChIPseeker<sup>60</sup> package on R with the custom developed table with the differential regions  
522 and their associated genes. This uses a hypergeometric model to assess the enrichment of  
523 genes associated with a pathway. A Benjamin-Hochberg adjusted p-value of 0.01 was used  
524 as a cutoff.

525

### 526 *Annotating differential regions*

527 Genomic distribution of differential regions and enrichment around transcription start sites  
528 were estimated using ChIPseeker. A custom background of histone marks was collected  
529 from the International Human Epigenome Consortium (<http://ihec-epigenomes.org>). We  
530 selected for mature neutrophil samples and women of Northern European ancestry. Sample  
531 files in bigbed format were converted to bed format using the UCSCtools package. Motif  
532 analysis was performed using HOMER<sup>61</sup> and known motifs with a cutoff of  $p < 0.01$  were  
533 selected. Each differential region was annotated with known overlapping histone marks and  
534 a list of motifs. Presence-absence heatmaps of the enriched motifs in each sample were  
535 plotted using heatmap.2 from within the gplots package in R. Footprinting of transcription  
536 factors was enriched in regions of interest was performed using the FootprintPipeline  
537 (<https://github.com/aslihankarabacak/FootprintPipeline>).

538

### 539 *RNA-seq analysis*

#### 540 *Data processing and differential expression analysis*

541 Quality of the paired-end reads generated for each replicate was performed using FastQC<sup>62</sup>  
542 and trimmed with Trim Galore (<https://github.com/FelixKrueger/TrimGalore>). Resulting reads  
543 were aligned to hg19 using HISAT2<sup>63</sup> with the --rna-strandness RF parameter. The  
544 generated SAM files were sorted and then converted to BAM using Samtools. Counts were  
545 generated using the R package Rsubread<sup>64</sup> in a strand specific manner. Differential gene  
546 expression analysis was performed using edgeR<sup>65</sup> and genes with FDR corrected p-value  $<$   
547  $0.05$  and  $\logFC \geq 1$  or  $\logFC \leq -1$  were selected. GOterm enrichment analysis was  
548 performed and comparisons between time points were made using the compareCluster  
549 function from the cluterProfiler R package<sup>66</sup> which uses a hypergeometric model to assess  
550 the enrichment of genes associated with a pathway. A Benjamin-Hochberg adjusted p-value  
551 of 0.01 was used as a cutoff.

552

### 553 **Data Availability**

554

555 The datasets generated for this study are available under BioProject - xxxxxxxx.

556

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565

### 566 **Author Contributions**

567 S.T., S.Y., and H.Y.C. designed the project. S.T. performed the ATAC-seq and N.A. and  
568 X.Y. performed the RNA-seq. N.R., S.T., U.L. and S.C. performed all bioinformatics  
569 analysis and generated the figures with critical feedback from S.Y. and H.Y.C. N.R. wrote  
570 the manuscript with feedback/input from all other authors.

571

### 572 **Competing Interests**

573 H.Y.C. is a co-founder of Accent Therapeutics, Boundless Bio, and an advisor to  
574 10x Genomics, Arsenal Biosciences, and Spring Discovery. No other authors have  
575 competing interests to report.

576

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721 287.

722  
723

## 724 **Figure legends**

725

726 **Fig. 1. Neutrophil activation in response to challenges.** **a.** Schematic of neutrophil  
727 isolation, stimulation, and ATAC-seq. Blood is collected from healthy volunteers in EDTA  
728 tubes and unwanted cells are removed using a magnetic bead selection. Tn5 transposase  
729 (green ovals) carrying an adaptor payload (red and blue) complementary to next generation  
730 flow cells and inserts randomly into regions of open chromatin. Unstimulated and stimulated  
731 neutrophils are the sequenced using Illumina technology. **b.** Table of tested challenges  
732 including 6 ligands, 2 whole organisms, and 1 time series. **c.** Healthy donor neutrophils  
733 produce IL8 or TNF in response to ligands or live organism challenge (ligand donors n= 4,  
734 live organism donors n= 2, mean and SE are represented). **d.** Healthy volunteer neutrophils  
735 do not produce NETs via sytox green assay in response to pathogen ligands at 1 hour or  
736 immediately following live organism challenge supporting this time point for ATAC-seq.  
737 (PMA is a positive control) (ligand donors n=4, live organism donors n=2). **e.** ATAC-seq is  
738 more sensitive to SA reads than traditional SPRI library preparation. Whole blood was  
739 spiked at increasing concentrations with live SA and prepared for sequencing using  
740 traditional DNA extraction and library preparation (SPRI method) compared to neutrophil  
741 isolation and ATAC-seq method. (n = 2) (WB: whole blood only, no organisms). Relative  
742 abundance plots illustrate that reads align to SA (red) as well as other bacteria, however the  
743 species that would be contamination are still low in relative abundance.

744

745 **Fig. 2. Comparison of locations of differential regions across challenges.** **a.** Whole  
746 genome distribution of differential regions within promoters, UTRs, exons, introns,  
747 downstream regions, and distal regions as determined using ChIPseeker. Very similar  
748 distribution patterns across sample, more than 80% of differential regions are distal. **b.**  
749 Depiction of the unique signatures across the challenges. Differential regions around the  
750 TCF7L2 gene. From top, all transcripts generated by the TCF7L2 gene, the TCF7L2 gene,  
751 differential regions associated at EC4h, EC1h, SA, LPS, LTA, R848, HMGB1, FLAG, and  
752 BGP respectively. **c.** Upset plot of overlapping differential regions across ligand challenges.  
753 Consensus set of regions were generated using Diffbind and the presence absence was  
754 visualized using Upset on R. Majority of the differential regions are unique to challenges. **d.**  
755 Upset plot of overlapping regions between the whole organism challenges and their  
756 corresponding ligands. Minimal overlap between whole organisms and ligands.

757  
758 **Fig. 3. Comparison of functional profiles of differential regions.** **a.** Reactome pathway  
759 enrichment analysis of the genes associated with the differential regions in each challenge.  
760 Gene assignments to each peak were carried out as described in the methods. Enrichment  
761 analysis performed using ChIPseeker and compared using the comparecluster function from  
762 clusterprofiler. **b.** Presence-absence heatmap of enriched motifs in the differential regions  
763 from each challenge. Enriched motifs were determined using HOMER and filtering for a p-  
764 value of  $p < 0.01$ . Although a lot of enriched motifs are shared, there are motifs unique to  
765 each challenge.

766  
767 **Fig. 4. Paired ATAC-seq and RNA-seq of neutrophils challenged with EC at 1 and 4**  
768 **hours.** **a.** Comparison of expression of genes at the two time points. logFC calculated using  
769 edgeR for each gene at the two time points were plotted as points if the p value is less than  
770 0.05. Points are colored based on the gene expression patterns at the two time points. down:  
771 genes down-regulated at both time points; down1h and down4h: genes down-regulated only  
772 at 1 hour and 4 hour time points respectively; downup: genes down-regulated at 1 hour and  
773 up regulated at 4 hours; NC: genes that are not differentially expressed at either time point;  
774 up: genes up-regulated at both time points; up1h and up4h: genes up-regulated only at 1  
775 hour and 4 hour time points respectively; and updown: genes that are up-regulated at 1 hour  
776 and down-regulated at 4 hours. **b.** GOterm enrichment analysis of the genes categorized  
777 based on their pattern over time. Grouped genes analyzed using the enrichGO feature within  
778 clusterprofiler. **c.** Distribution of open and closed differential regions at each time point with  
779 respect to the gene expression patterns. Combination of open and closed regions at each  
780 time point and each gene expression pattern except for genes that are up regulated at 1 hour  
781 and down regulated at 4 hours. **d.** Counts of open and closed differential regions associated  
782 with each gene at each time point. Typically, more associated differential regions for each  
783 gene at 1 hour than at 4 hours. Additionally, combination of multiple open and closed  
784 differential regions associated with most genes.

785  
786 **Fig. 5. Mechanisms of control of transcription by accessible chromatin in neutrophils.**  
787 **a.** Differential regions only in the promoter. Promoter is defined as the regions  $\pm 2.5$ Kb  
788 around the TSS for each gene. Example for this category is the FAM66C gene. **b.**  
789 Differential regions only in distal regions, promoter is primed for expression. Example for  
790 this category is the BBS2 gene. In A and B, from the top, RNA-seq coverage at 1 hour for

791 EC and control; RNA-seq coverage at 4 hours; ATAC-seq coverage at 1 hour; ATAC-seq  
792 coverage at 4 hours; genes in the region from hg19; open (green) differential regions  
793 associated with the gene at 1 hour and open or closed differential regions not associated with  
794 the gene of interest (gray); differential regions associated at 4 hours; location on the  
795 chromosome; H3K27ac histone marks in the presence of EC; and absence of EC lifted over  
796 from earlier study<sup>19</sup>; and Hi-C interactions in the presence of EC lifted over from earlier  
797 study<sup>19</sup>.

798  
799 **Fig. 6. Intricate combinatorial chromatin accessibility regulation of transcription in**  
800 **neutrophils. a.** Third mechanism of gene regulation in neutrophils involves differential  
801 regions in both the promoter as well as distal sites. An example in this category is the  
802 HMGCS1 gene. The differential region in the promoter is fixed and unique to an EC  
803 infection. Tracks are similar to those in Fig. 5. Differentially closed regions are shown in red  
804 **b.** Transcription factor footprinting of enriched motifs identified in the distal associated  
805 differential regions. Footprinting using the ATAC-seq reads was performed using the  
806 FootprintingPipeline. Time dependent binding of transcription factors affecting gene  
807 expression.

808  
809 **Fig. 7. Model for intricate accessible chromatin regulation of gene expression in**  
810 **neutrophils.** A schematic depicting the three mechanisms of regulation identified in our  
811 analyses. Upon exposure to stimuli, the closed chromatin in neutrophils opens up in stimulus  
812 specific patterns. Accessible chromatin regulation of genes occurs in one of three  
813 mechanisms where – 1. Differential regions occur only in the promoter region; 2.  
814 Differential regions occur only at distal sites; and 3. Differential regions occur at promoter  
815 and distal sites.

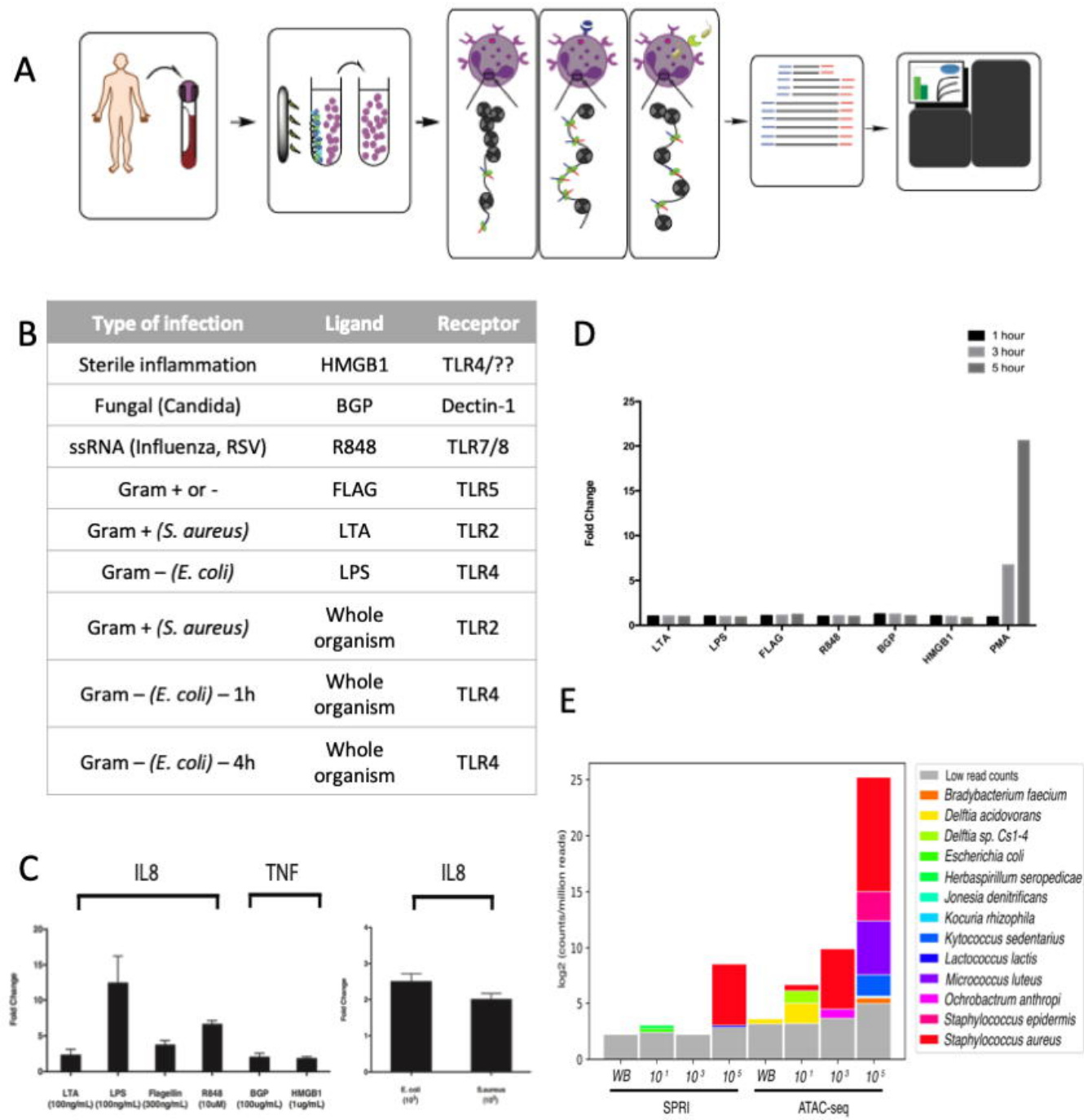
816  
817 **Supplemental Fig. 1.** Flow cytometry with gating strategy depicted confirms 98.1% purity of  
818 CD66b/CD16 double positive neutrophils.

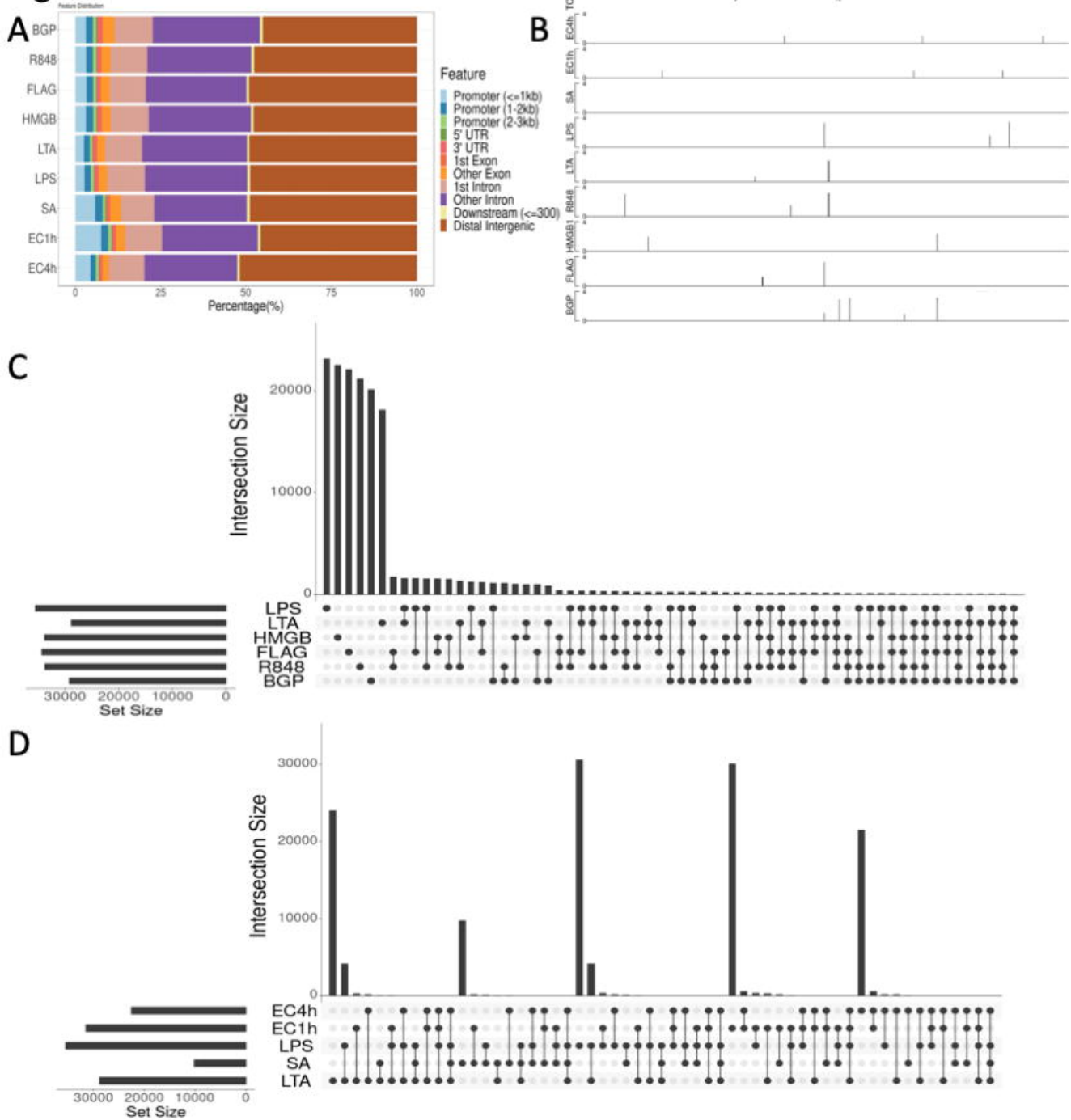
819  
820 **Supplemental Fig. 2. a.** Representative QC plots demonstrating library prep results in expected  
821 insert size distribution and **b.** reads are enriched around transcription start sites (TSS).

822  
823 **Supplemental Fig. 3.** Quality control for DiffBind method of identification of differentially  
824 accessible regions of chromatin. Correlation heat maps and principal component analysis (PCA)  
825 of differentially accessible chromatin. We found that for any given challenge across donors,  
826 stimulated samples cluster together, control samples cluster together, and the stimulated and  
827 control cluster away from each other, suggesting high quality data and accessible chromatin  
828 region identification that allows for analysis of four healthy donor data.

829  
830 **Supplemental Table 1.** List of known motifs identified using Homer that are unique to each  
831 challenge.

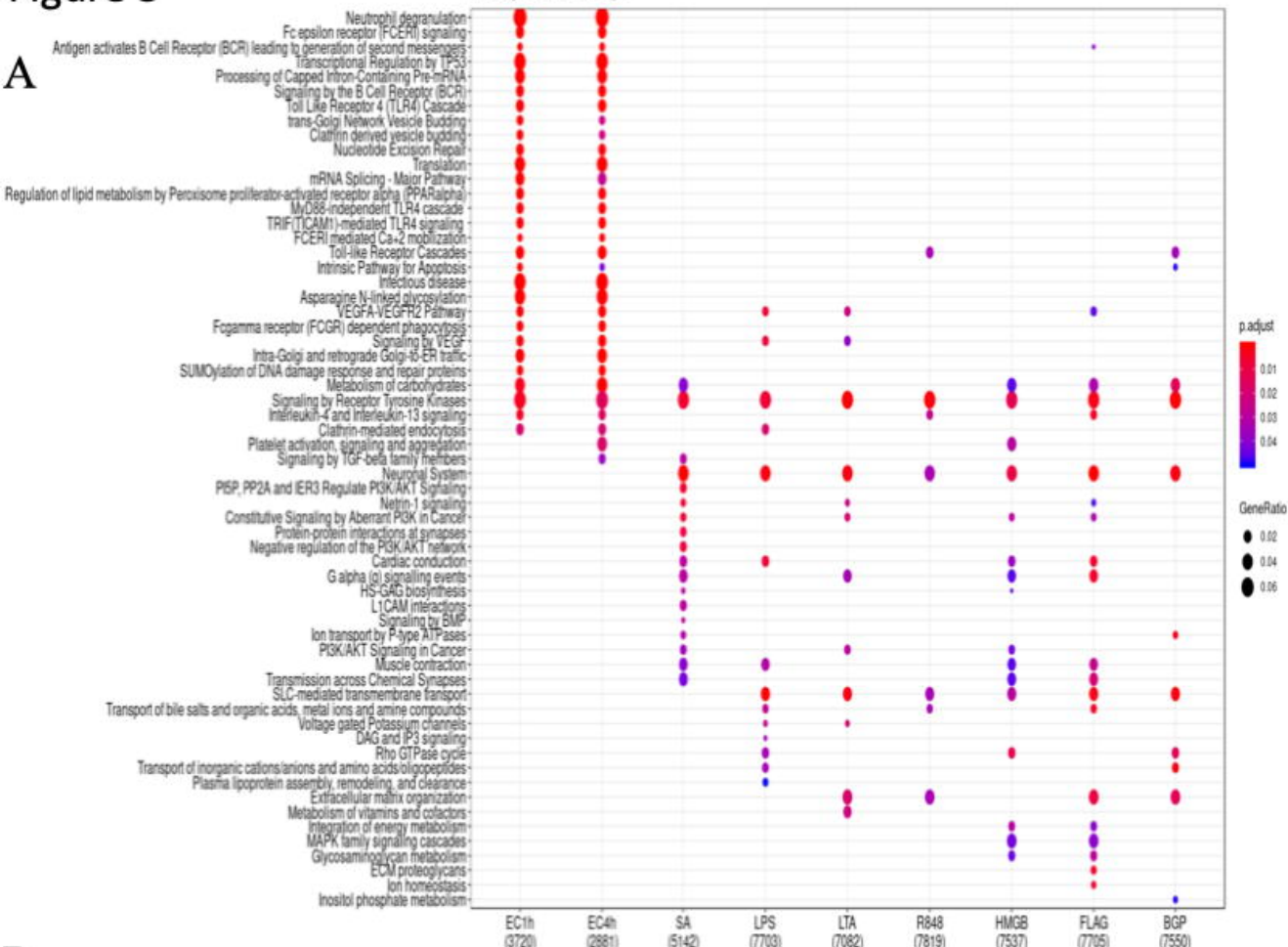
# Figure 1



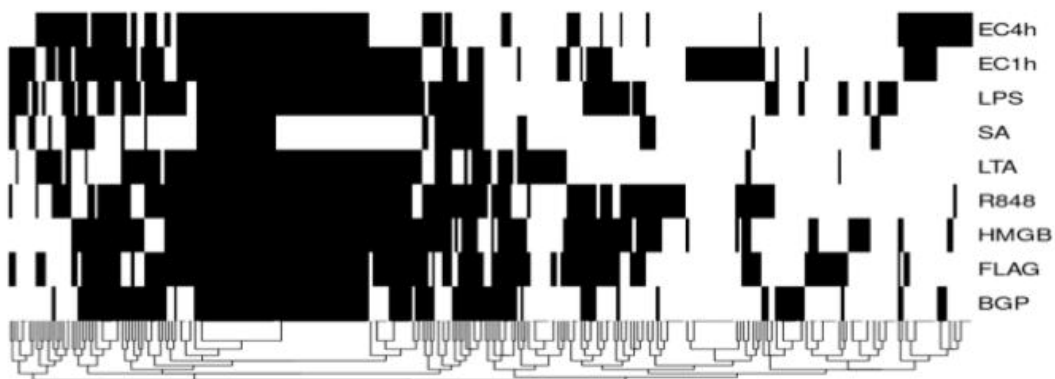
**Figure 2**

# Figure 3

A

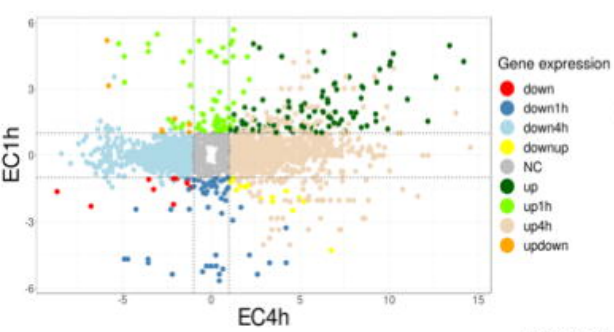


B

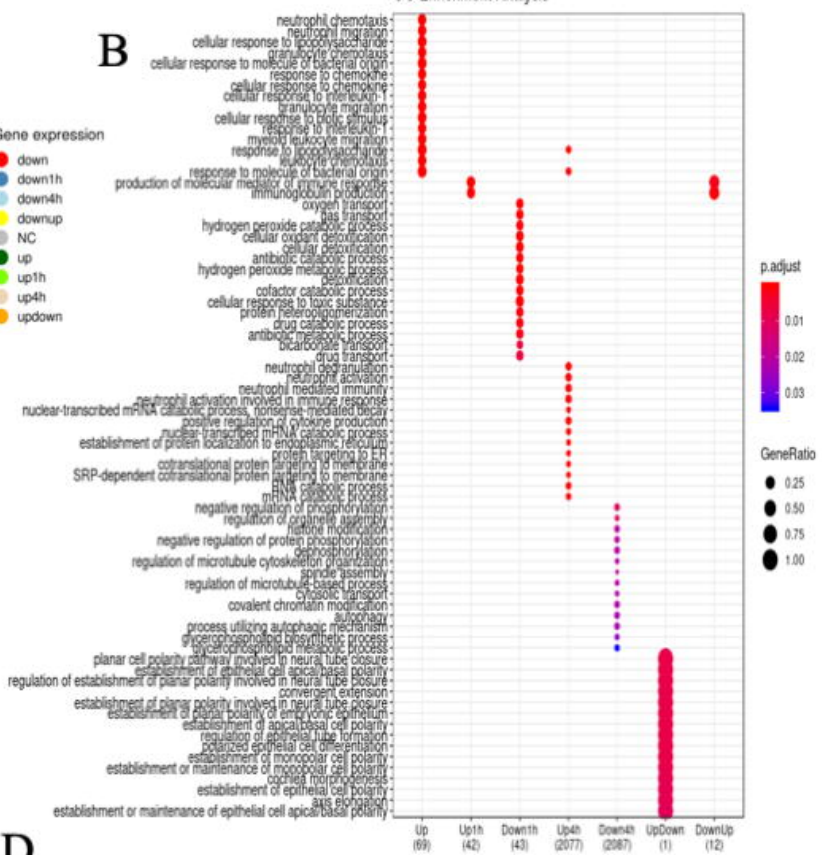


# Figure 4

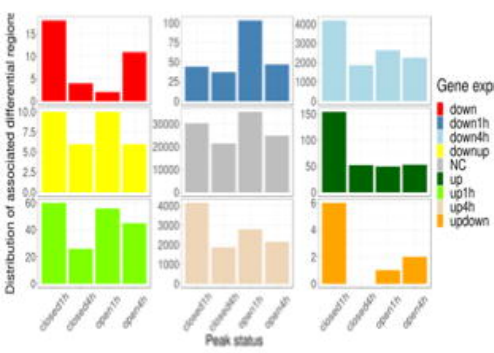
## A



## B



## C



## D

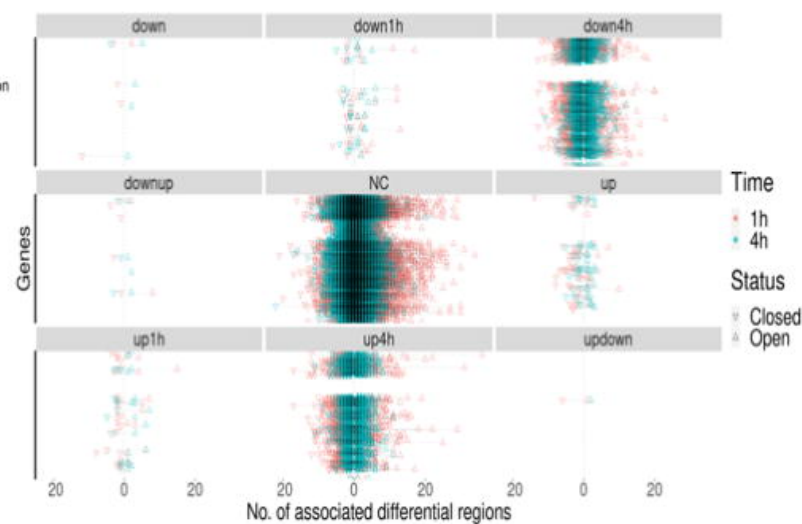
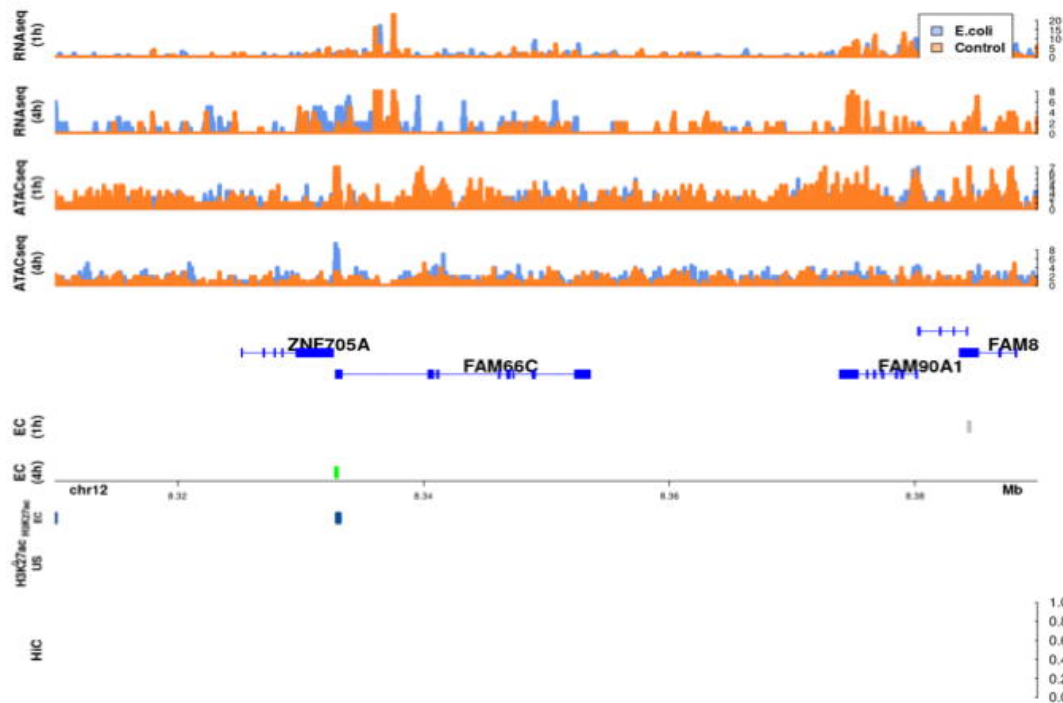
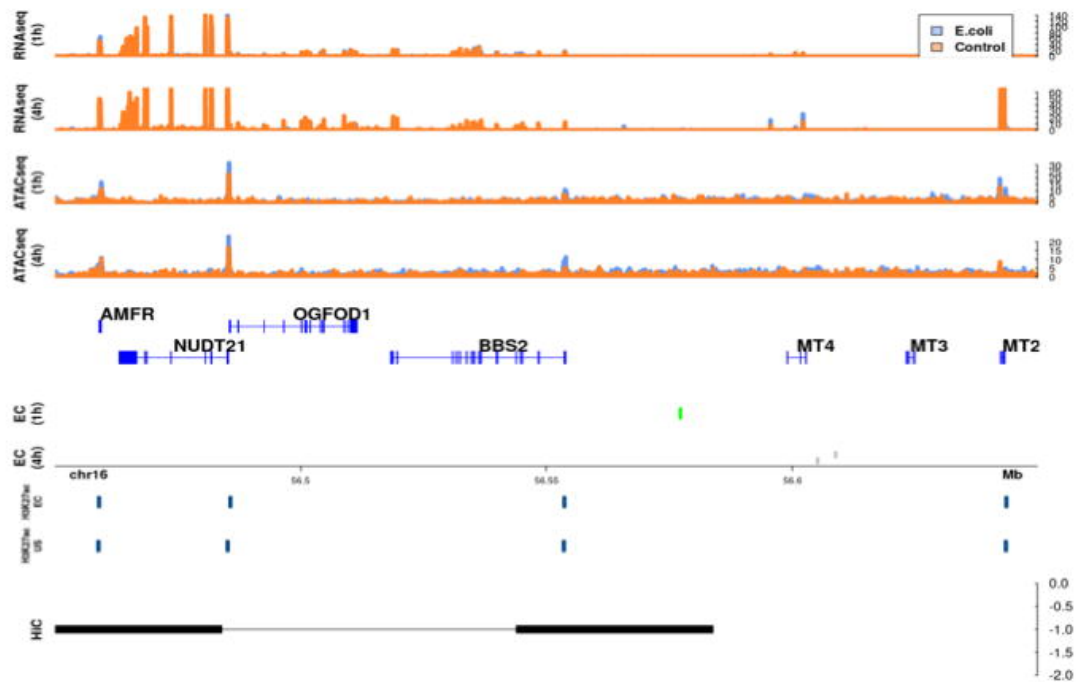


Figure 5

A



B





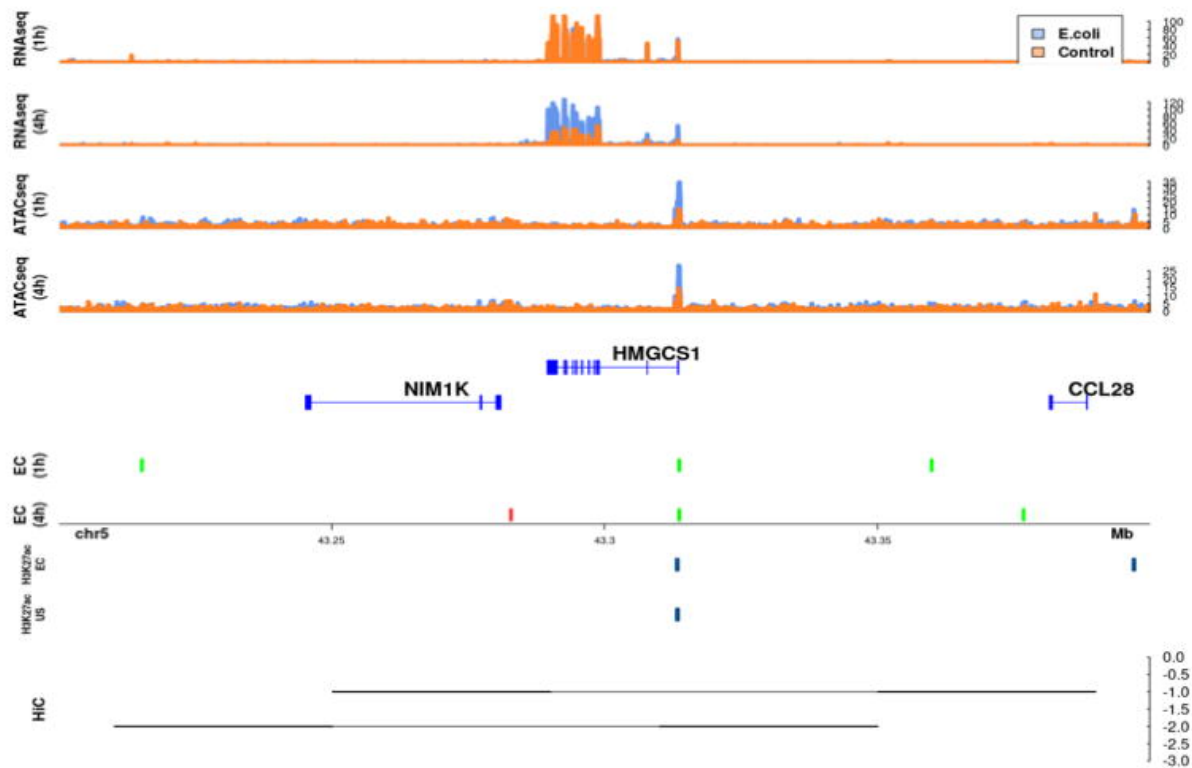
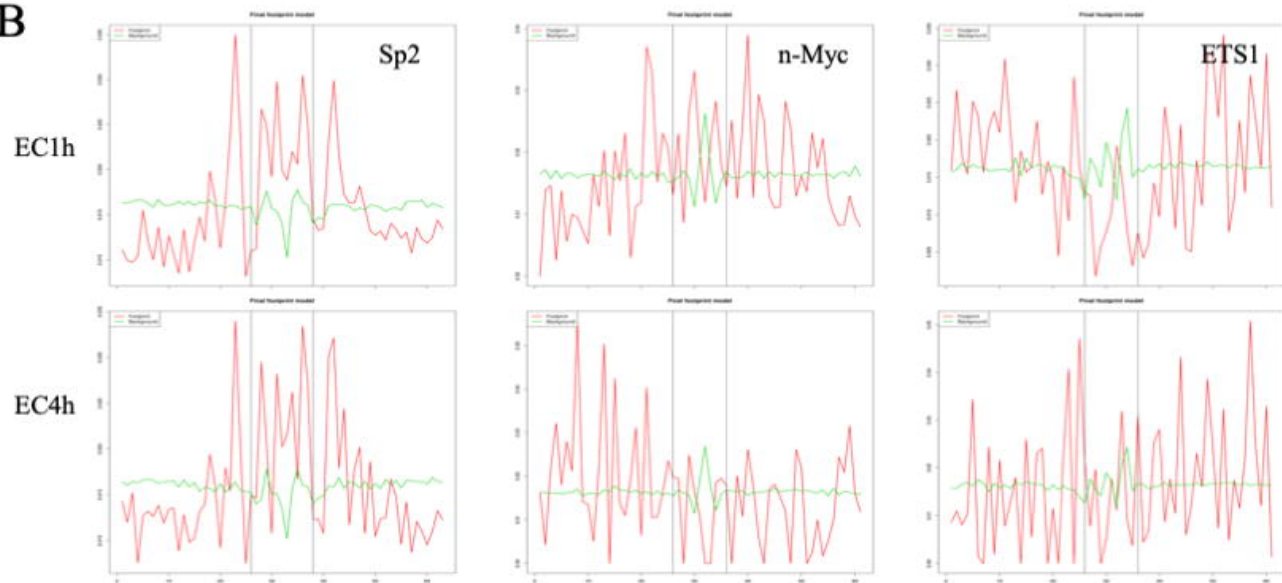
**Figure 6****A****B**

Figure 7

