1   **A genome-wide screen in macrophages identifies new regulators of IFNγ-inducible MHCII**
2   **that contribute to T cell activation**
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Abstract

Cytokine-mediated activation of host immunity is central to the control of pathogens. A key cytokine in protective immunity is interferon-gamma (IFNγ), which is a potent activator of antimicrobial and immunomodulatory effectors within the host. A major role of IFNγ is to induce major histocompatibility complex class II molecules (MHCII) on the surface of cells, which is required for CD4$^+$ T cell activation. Despite its central role in host immunity, the complex and dynamic regulation of IFNγ-induced MHCII is not well understood. Here, we integrated functional genomics and transcriptomics to comprehensively define the genetic control of IFNγ-mediated MHCII surface expression in macrophages. Using a genome-wide CRISPR-Cas9 library we identified genes that control MHCII surface expression, many of which have yet to be associated with MHCII. Mechanistic studies uncovered two parallel pathways of IFNγ-mediated MHCII control that require the multifunctional glycogen synthase kinase 3 beta (GSK3β) or the mediator complex subunit MED16. Both pathways are necessary for IFNγ-mediated induction of the MHCII transactivator CIITA, MHCII expression, and CD4$^+$ T cell activation. Using transcriptomic analysis, we defined the regulons controlled by GSK3β and MED16 in the presence and absence of IFNγ and identified unique networks of the IFNγ-mediated transcriptional landscape that are controlled by each gene. Our analysis suggests GSK3β and MED16 control distinct aspects of the IFNγ-response and are critical for macrophages to respond appropriately to IFNγ. Our results define previously unappreciated regulation of MHCII expression that is required to control CD4$^+$ T cell responses by macrophages. These discoveries will aid in our basic understanding of macrophage-mediated immunity and will shed light on mechanisms of failed adaptive responses pervasive in infectious disease, autoimmunity, and cancer.

Introduction

Activation of the host response to infection requires the coordinated interaction between antigen presenting cells (APCs) and T cells (1-3). For CD4$^+$ T cells, the binding of the T cell receptor (TCR) to the peptide-loaded major histocompatibility complex class II (MHCII) on the surface of APCs is necessary for both CD4$^+$ T cell activation and their continued effector function in peripheral tissues (3-5). Dysregulation of MHCII control leads to a variety of conditions including the development autoimmunity and increased susceptibility to pathogens and cancers (6-10). While MHCII is constitutively expressed on dendritic cells and B cells, the production of the cytokine IFNγ promotes MHCII expression broadly in other cellular populations including macrophages (11-14). The induction of MHCII in these tissues activates a feedforward loop wherein IFNγ-producing CD4$^+$ T cells induce myeloid MHCII expression, which in turn amplifies CD4$^+$ T cell responses (14-16). Thus, IFNγ-mediated MHCII expression is essential for protective immunity.

The IFNγ-dependent control of MHCII is complex (5, 12, 17-19). Binding of IFNγ to its receptor induces cytoskeletal and membrane rearrangement that results in the activation of Janus kinases 1 and 2 (JAK1 and JAK2) and STAT1-dependent transcription (20, 21). STAT1 induces IRF1, which then drives the expression of the MHCII master regulator, CIITA (22, 23). The activation of CIITA opens the chromatin environment surrounding the MHCII locus and recruits transcription factors, including CREB1 and RFX5 (5, 24). MHCII is also regulated post-translationally to control the trafficking, peptide loading, and stability of MHCII on the surface of cells (25-27). While recent evidence points to additional regulatory mechanisms of IFNγ-mediated MHCII expression, including the response to oxidative stress, these have not been investigated directly in macrophages (17).

In non-inflammatory conditions, macrophages express low levels of MHCII that is uniquely dependent on NFAT5 (15). While basal MHCII expression on macrophages plays a

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85   role in graft rejection, it is insufficient to control intracellular bacterial pathogens, which require

86   IFNγ-activation to propagate protective CD4$^+$ T cell responses (28-30). Many pathogens

87   including *Mycobacterium tuberculosis* and *Chlamydia trachomatis* inhibit IFNγ-mediated MHCII

88   induction to evade CD4$^+$ T cell-mediated control and drive pathogen persistence (31-33).

89   Overcoming these pathogen immune evasion tactics is essential to develop new treatments or

90   immunization strategies that provide long-term protection (28). Without a full understanding of

91   the global mechanisms controlling IFNγ-mediated MHCII regulation in macrophages, it has

92   proven difficult to dissect the mechanisms related to MHCII expression that cause disease or

93   lead to infection susceptibility.

94   Here we globally defined the regulatory networks that control IFNγ-mediated MHCII

95   surface expression on macrophages. Using CRISPR-Cas9 to perform a forward genetic screen,

96   we identified the major components of the IFNγ-regulatory pathway in addition to many genes

97   with no previously known role in MHCII regulation. Follow-up studies identified two critical

98   regulators of IFNγ-dependent CIITA expression in macrophages, MED16 and GSK3β. Loss of

99   either MED16 or GSK3β resulted in significantly reduced MHCII expression on macrophages,

100   unique changes in the IFNγ-transcriptional landscape, and prevented the effective activation of

101   CD4$^+$ T cells. These results show that IFNγ-mediated MHCII expression in macrophages is

102   finely tuned through parallel regulatory networks that interact to drive efficient CD4$^+$ T cell

103   responses.

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105

106   **Results**

107

108   **Optimization of CRISPR-Cas9 editing in macrophages to identify regulators of IFNγ-**

109   **inducible MHCII.**

110   To better understand the regulation of IFNγ-inducible MHCII we optimized gene-editing

111   in immortalized bone marrow-derived macrophages (iBMDMs) from C57BL6/J mice. iBMDMs

112   were transduced with Cas9-expessing lentivirus and Cas9-mediated editing was evaluated by

113   targeting the surface protein CD11b with two distinct single guide RNAs (sgRNA). When we

114   compared CD11b surface expression to a non-targeting control (NTC) sgRNA by flow

115   cytometry, we observed less than 50% of cells targeted with either of the CD11b sgRNA were

116   successfully edited (Figure S1A). We hypothesized that the polyclonal Cas9-iBMDM cells

117   variably expressed Cas9 leading to inefficient editing. To address this, we isolated a clonal

118   population of Cas9-iBMDMs using limiting dilution plating. Using the same CD11b sgRNAs in a

119   clonal population (clone L3) we found 85-99% of cells were deficient in CD11b expression by

120   flow cytometry compared to NTC (Figure S1B). Successful editing was verified by genotyping

121   the CD11b locus for indels at the sgRNA targeting sequence using Tracking of Indels by

122   Decomposition (TIDE) analysis (34). Therefore, clone L3 Cas9$^+$ iBMDMs proved to be a robust

123   tool for gene editing in murine macrophages.

124   To test the suitability of these cells to dissect IFNγ-mediated MHCII induction we next

125   targeted Rfx5, a known regulator of MHCII expression, with two independent sgRNAs (35). We

126   stimulated Rfx5 targeted and NTC cells with IFNγ for 18 hours and quantified the surface

127   expression of MHCII by flow cytometry (Fig 1A and 1B). In cells expressing the non-targeting

128   sgRNA, IFNγ stimulation resulted in a 20-fold increase in MHCII. In contrast, cells transduced

129   with either of two independent sgRNAs targeting Rfx5 failed to induce the surface expression of

5

130   MHCII following IFNγ stimulation. Thus, L3 cells are responsive to IFNγ and can be effectively

131   used to interrogate IFNγ-mediated MHCII expression in macrophages.

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133   **Forward genetic screen identifies known and novel regulators of MHCII surface**

134   **expression in macrophages**

135   To define the genetic networks required for IFNγ-mediated MHCII expression, we made

136   a genome-wide library of mutant macrophages with sgRNAs from the Brie library to generate

137   null alleles in all protein-coding genes (36). After verifying coverage and minimal skew in the

138   initial library, we conducted a forward genetic screen to identify regulators of IFNγ-dependent

139   MHCII expression (Figure 1C and Table S1). The loss-of-function library was stimulated with

140   IFNγ and 24 hours later, we selected MHCII^{high} and MHCII^{low} expressing cells by fluorescence

141   activated cells sorting (FACS). Following genomic DNA extraction, sgRNA abundances for each

142   sorted bin were determined by deep sequencing.

143   As our knockout library relied on the formation of Cas9-induced indels and was exclusive

144   to protein-coding genes, we focused our analysis on genes expressed in macrophages under

145   the conditions of interest, which we determined empirically in the isogenic cell line by RNA-seq

146   (Table S2). We assumed that sgRNAs targeting non-transcribed genes are neutral in their effect

147   on IFNγ-induced MHCII expression, which afforded us ~32,000 internal negative control

148   sgRNAs (37). To test for statistical enrichment of sgRNAs and genes, we used the modified

149   robust rank algorithm (α-RRA) employed by Model-based Analysis of Genome-wide

150   CRISPR/Cas9 Knockout (MAGeCK), which first ranks sgRNAs by effect and then filters low

151   ranking sgRNAs to improve gene significance testing (38). We tuned the sgRNA threshold

152   parameter to optimize the number of significant hits without compromising the calculated q-

153   values of known positive controls that are expected to be required for IFNγ-mediated MHCII

154   expression. Further, by removing irrelevant sgRNAs that targeted genes not transcribed in our

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155   conditions, we removed potential false positives and improved the positive predictive value of

156   the screen (Figure S2A and S2B).

157   Guide-level analysis confirmed the ability to detect positive control sgRNAs which had

158   robust enrichment in the MHCII^{low} population (Fig S2C). Using the previously determined

159   parameters, we tested for significantly enriched genes that regulated MHCII surface levels. As

160   expected, sgRNAs targeting known components of the IFNγ-receptor signal transduction

161   pathway, such as Ifngr1, Ifngr2, Jak1 and Stat1, as well as regulators and components of

162   IFNγ−mediated MHCII expression, such as Ciita, Rfx5, and Rfxank were all significantly

163   enriched (Figure 1D) (5, 22). These results validated our approach to identify functional

164   regulators of IFNγ-mediated MHCII expression.

165   Stringent analysis revealed a significant enrichment of genes with no known involvement

166   in interferon responses and antigen presentation. To identify functional pathways that are

167   associated with these genes, we performed KEGG pathway analysis on the positive regulators

168   of IFNγ-induced MHCII that met the FDR cutoff (FigS2D) (39-41). However, gene membership

169   for the ten most enriched KEGG pathways was largely dominated by known regulators of IFNγ

170   signaling. To circumvent this redundancy and identify novel pathways enriched from our

171   candidate gene list, the gene list was truncated to remove the 11 known IFNγ signaling

172   regulators. Upon reanalysis, several novel pathways emerged, including mTOR signaling

173   (Figure S2E). Thus, our genetic screen uncovered previously undescribed pathways that are

174   critical to control IFNγ-mediated MHCII surface expression in macrophages.

175   The results of the genome-wide CRISPR screen highlight the sensitivity and specificity

176   of our approach and analysis pipeline. To gain new insights into IFNγ-mediated MHCII

177   regulation, we next validated a subset of candidates that were not previously associated with

178   the IFNγ-signaling pathway. Using two independent sgRNAs for each of 15 candidate genes, we

179   generated loss-of-function macrophages in the L3 clone (Figure 1E and S2F). MHCII surface

180 expression was quantified by flow cytometry for each cell line in the presence and absence of

181 IFNγ activation. For all 15 candidates, we observed deficient MHCII induction following IFNγ

182 stimulation with at least one sgRNA. For 9 of15 candidate genes, we observed a significant

183 reduction in MHCII surface expression with both gene-specific sgRNAs These results show that

184 our screen not only identified known regulators of IFNγ-mediated MHCII induction, but also

185 uncovered new regulatory networks required for MHCII expression on macrophages.

186 We were interested in better understanding the IFNγ-mediated transcriptional activation

187 of MHCII to determine if a subset of candidates reveal new regulatory mechanisms of MHCII-

188 expression. Based on the screen and validation results, we examined the known functions of

189 the candidates that were confirmed with two sgRNAs, and identified MED16 and Glycogen

190 synthase kinase 3β (GSK3β) for follow-up study. MED16 is a subunit of the mediator complex

191 that regulates transcription initiation while GSK3β is a multifunctional kinase that controls

192 signaling pathways known to regulate transcription (42, 43). Thus, we hypothesized that MED16

193 and GSK3β would be required for effective IFNγ-mediated transcriptional control of MHCII.

194

195 **MED16 is uniquely required for IFNγ-mediated CIITA expression.**

196 We first examined the role of MED16 in controlling IFNγ-mediated MHCII expression.

197 MED16 was the sixth ranked candidate from our screen results, with robust enrichment of all 4

198 sgRNAs in the MHCII^{low} population (Fig2A). Our validation results confirmed that MED16 was

199 indeed an essential positive regulator of MHCII expression (Fig1E). As part of the mediator

200 complex, MED16 bridges the transcription factor binding and the chromatin remodeling that are

201 required for transcriptional activation (44). These changes then recruit and activate RNA

202 polymerase II to initiate transcription. While the core mediator complex function is required for

203 many RNA polymerase II dependent transcripts, distinct sub-units of the mediator complex can

204 also play unique roles in gene regulation (42, 44). To examine if MED16 was uniquely required

205   for IFNγ-dependent MHCII expression, we probed our genetic screen data for all mediator

206   complex subunits. None of the other 27 mediator complex subunits in our library showed any

207   significant changes in MHCII expression (Figure 2B). To test the specific requirement of

208   MED16, we generated knockout macrophages in Med16 (Med16 KO) and using two

209   independent sgRNAs targeted three additional mediator complex subunits, Med1, Med12 and

210   Med17 (Figure S3 and materials and methods). We treated with IFNγ and quantified the surface

211   levels of MHCII by flow cytometry. In support of the screen results, Med1, Med12 and Med17

212   showed similar MHCII upregulation compared to NTC cells, while Med16 targeted cells

213   demonstrated defects in MHCII surface expression (Figure 2C and Figure 2D). These results

214   suggest that there is specificity to the requirement for MED16-dependent control of IFNγ-

215   induced CIITA that is unique among the mediator complex subunits.

216   To understand the mechanisms of how MED16 regulates MHCII-induction, we assessed

217   the transcriptional induction of MHCII in Med16 KO cells. In macrophages, the IFNγ-mediated

218   transcriptional induction of MHCII subunits requires the transcriptional activation of CIITA that

219   then, in complex with other factors like RFX5, initiates transcription at the MHCII locus (14, 17).

220   To determine whether MED16 controls the transcriptional induction of MHCII, we stimulated

221   NTC, Med16 KO and Rfx5 targeted cells with IFNγ for 18 hours and isolated RNA. Using qRT-

222   PCR, we observed that loss of RFX5 did not impact the induction of CIITA, but had a profound

223   defect in the expression of H2-Aa compared to NTC cells (Fig2E). Loss of MED16 significantly

224   inhibited the induction of both CIITA and H2-Aa. These data suggest that MED16 controls the

225   induction of MHCII through upstream regulation of CIITA.

226

227   **Loss of GSK3β prevents the IFNγ-dependent induction of CIITA.**

228   We next examined the mechanisms of GSK3β control of IFNγ-mediated MHCII

229   expression in more detail. GSK3β is involved in many cellular pathways including mTor and Wnt

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230   (43, 45, 46). While GSK3β was previously suggested to repress collagen production via CIITA,

231   no role in regulating IFNγ-mediated MHCII expression has previously been described (47). In

232   addition to its high ranking in the screen and the strong effects of multiple sgRNAs (Figure 3A),

233   pathway analysis uncovered a significant enrichment of genes within the mTor pathway, which

234   controls GSK3β function, suggesting this signaling network is critical for MHCII expression

235   (Figure S2E) (46). Our validation studies further showed that GSK3β is required for the effective

236   induction of IFNγ-dependent MHCII (Figure 1E). To begin to understand the mechanisms

237   controlling GSK3β-dependent regulation of MHCII expression we generated Gsk3β knockout

238   cells (Gsk3β KO) and verified that the loss of Gsk3β strongly inhibited IFNγ-mediated MHCII

239   surface expression (Figure 3B and Figure S3B).

240   To confirm the genetic evidence using an orthogonal method, GSK3β function was

241   inhibited chemically using the well-characterized small molecule CHIR99021 (48, 49). NTC

242   macrophages treated with either CHIR99021 or DMSO were stimulated cells with IFNγ and

243   MHCII expression was analyzed by flow cytometry. Consistent with the genetic experiments,

244   inhibition of GSK3β activity reduced the induction of surface MHCII, and was similar to a genetic

245   loss of Gsk3β alone (Figure 3C and 3D). The GSK3β chemical inhibitor facilitated additional

246   experiments in other cells that were not possible with Med16 KO cells. Thus, we repeated this

247   experiment in primary bone marrow-derived macrophages from HoxB8 conditionally

248   immortalized progenitor cells and observed identical results (Figure 3E) (50). Therefore, GSK3β

249   activity is required for the effective induction of IFNγ-mediated MHCII in immortalized and

250   primary murine macrophages.

251   We next examined if the IFNγ-mediated transcriptional induction of CIITA or H2-Aa were

252   reduced in Gsk3β KO cells. Loss of GSK3β significantly inhibited the expression of both CIITA

253   and H2-Aa after IFNγ-treatment compared to NTC controls. These data suggest that GSK3β,

254 similar to MED16, is an upstream regulator of IFNγ-mediated MHCII induction that controls the

255 expression of CIITA following IFNγ-activation.

256 These qRT-PCR studies (Figure 3F and 3G) suggested that GSK3β was required for the

257 transcriptional activation of CIITA. We hypothesized that GSK3β inhibition with CHIR99021

258 would block MHCII expression only if the inhibitor was present shortly after IFNγ stimulation. To

259 test this hypothesis, iBMDMs were stimulated with IFNγ then treated with DMSO for the length

260 of the experiment or with CHIR99021, 2, 6, 12, and 18 hours post-stimulation. When MHCII was

261 quantified by flow cytometry we saw a reduction in MHCII expression when CHIR99021 was

262 added two or six hours after IFNγ (Figure 3H). CHIR99021 addition at later time points resulted

263 in similar MHCII expression compared to DMSO treated cells. When the expression of H2-Aa

264 mRNA was quantified from a parallel experiment, a significant reduction in mRNA expression

265 was only observed in macrophages that were treated with CHIR99021 two hours following IFNγ-

266 activation (Figure 3I). Thus, GSK3β activity is required early after IFNγ stimulation to activate the

267 transcription of MHCII.

268 We were interested in understanding the pathways GSK3β regulates that contribute to

269 CIITA induction. One previous study in Raw264.7 cells found a requirement for GSK3β to

270 activate STAT3 following IFNγ stimulation (51). While STAT1, and not STAT3, is thought to

271 control the majority of CIITA induction, a minor role for STAT3 remained possible. To test the

272 contribution of STAT1 and STAT3 to IFNγ-induced MHCII, we targeted both Stat1 and Stat3

273 with two independent sgRNAs to generate loss-of-function macrophages. As expected, when

274 these cells were stimulated with IFNγ, Stat1 prevented the increase of MHCII surface

275 expression. In contrast, neither sgRNA targeting Stat3 showed any difference in MHCII

276 expression compared to non-targeting control (Figure S3C). Thus, while GSK3β may regulate

277 STAT3 dependent pathways following IFNγ, a loss in STAT3 functionality does not explain the

278 contribution of GSK3β to IFNγ-mediated MHCII induction. Together these results suggest that

279   GSK3β, similarly to MED16, controls IFNγ-mediated MHCII expression upstream of the

280   transcriptional induction of CIITA.

281

GSK3α controls IFNγ-induced MHCII expression in the absence of GSK3β

283   Throughout the experiments above we observed that cells treated with CHIR99021

284   inhibited MHCII even more robustly than the Gsk3β KO cells. CHIR99021 not only inhibits

285   GSK3β but also the paralog GSk3α (52). This led us to consider the role of GSK3α in IFNγ-

286   mediated MHCII expression. While we did not observe enrichment for GSK3α in the screen

287   (Figure 1D and Table S1), we could not exclude the possibility that GSK3α can play a

288   secondary function during IFNγ-activation. Given the increased inhibition of MHCII with

289   CHIR99021, we hypothesized that GSK3α can partially compensate for total loss of GSK3β,

290   resulting in some remaining IFNγ-induced MHCII expression. To test this hypothesis, we treated

291   NTC and Gsk3β KO macrophages with CHIR99021 or DMSO and quantified MHCII surface

292   expression. Consistent with our previous results, we found robust inhibition of MHCII expression

293   on NTC macrophages treated with CHIR99021 (Figure 4A and 4B). In support of a minor role

294   for GSK3α, CHIR99021 treatment of Gsk3β KO macrophages further reduced surface MHCII

295   expression after IFNγ-stimulation.

296   To exclude the possibility of CHIR99021 off-target effects we next targeted Gsk3α

297   genetically. To enable positive selection of a second sgRNA, we engineered vectors in the

298   sgOpti background with distinct resistance markers for bacterial and mammalian selection that

299   facilitated multiplexed sgRNA cloning (see materials and methods) (53). These vectors could be

300   used to improve knockout efficiency when targeting a gene with multiple sgRNAs or target

301   multiple genes simultaneously (Figure S4A). We targeted Gsk3α with two unique sgRNAs in

302   either NTC or Gsk3β KO macrophages and stimulated the cells with IFNγ. Cells targeting Gsk3α

303   alone upregulated MHCII expression similarly to NTC control cells (Figure 4C and 4D). In

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304   contrast, targeting Gsk3α in Gsk3β KO macrophages led to a further reduction of MHCII surface

305   expression, similar to what was observed with CHIR99021 treatment. This same trend was

306   observed when we examined CIITA mRNA expression after IFNγ-activation (Figure 4E).

307   Therefore, blocking both GSK3α/β function results in a more severe reduction of IFNγ-

308   stimulated MHCII expression in macrophages than GSK3β alone. Taken together, we conclude

309   that GSK3α regulates IFNγ-mediated CIITA induction only in the absence of the GSK3β.

310

311   **GSK3β and MED16 function through distinct mechanisms to control IFNγ-mediated CIITA**

312   **expression.**

313   Since the loss of either MED16 or GSK3β reduced IFNγ-mediated CIITA transcription, it

314   remained possible that these two genes control MHCII expression through the same regulatory

315   pathway. While Med16 KO macrophages are greatly reduced in IFNγ-mediated MHCII induction,

316   there remains a small yet reproducible increase in MHCII surface expression. We determined if

317   this effect on MHCII expression after IFNγ-activation required GSK3 activity by treating with

318   CHIR99021. While DMSO-treated Med16 KO cells showed a reproducible 2-3 fold increase in

319   MHCII expression after IFNγ stimulation, CHIR99021 treated Med16 KO cells showed no

320   change whatsoever (Figure 5A and 5B). These results led us to hypothesize that MED16 and

321   GSK3β control IFNγ-mediated CIITA induction and MHCII expression through independent

322   mechanisms.

323   To test this hypothesis, we compared the transcriptional profiles of Med16 KO and

324   Gsk3β KO cells to NTC cells by performing RNAseq on cells that were left untreated or were

325   stimulated with IFNγ (See materials and methods). Principal component analysis of these 6

326   transcriptomes revealed distinct effects of IFNγ-stimulation ("condition"; PC1) and genotype

327   (PC2) gene expression (Figure 5C). Both Med16 and Gsk3β knockout macrophages had

328   distinct transcriptional signatures in the absence of cytokine stimulation, which were further

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329   differentiated with IFNγ-stimulation. The PCA analysis suggested that MED16 and GSK3β

330   control distinct transcriptional networks in macrophages following IFNγ-activation.

331   Transcriptional analysis confirmed a critical role of GSK3β and MED16 in regulating

332   IFNγ-dependent CIITA and MHCII expression in macrophages compared to NTC controls

333   (Figure 5D and 5E). However, the extent to which MED16 or GSK3β controlled the overall

334   response of macrophages to IFNγ remained unclear. To directly assess how MED16 and

335   GSK3β regulate the general response to IFNγ, we queried IFNγ-regulated genes from our

336   dataset that are annotated as part of the cellular response to IFNγ stimulation

337   (GeneOntology:0071346). Hierarchical clustering found that, of the 20 most induced IFNγ-

338   regulated transcripts, the expression of 8 were unaffected by loss of either GSK3β and MED16

339   (Figure 5F, Cluster 2). Importantly, these genes included a major regulator of the IFNγ

340   response, IRF1, as well as canonical STAT1-target genes (GBP2, GBP3, GBP5, GBP6 and

341   GBP7). This suggests that neither GSK3β nor MED16 are global regulators of the IFNγ

342   response in macrophages, but rather are likely to exert their effect on particular genes at the

343   level of transcription or further downstream. In contrast, only two genes, out the top 20 IFNγ-

344   regulated genes, were similarly reduced in both Med16 KO and Gsk3β KO cells (Cluster 4), one

345   of which was H2-Ab1. This shows that while GSK3β and MED16 both regulate IFNγ-mediated

346   MHCII expression, they otherwise control distinct aspects of the IFNγ-mediated response in

347   macrophages. The remaining clusters from this analysis showed specific changes in either

348   Med16 KO or Gsk3β KO cells. Clusters 1 and 3 showed a subset of genes that were more

349   robustly induced in Gsk3β KO cells compared to NTC and Med16 KO cells. These genes

350   included NOS2, IL12RB1 and chemokines CCL2, CCL3, CCL4, and CCL7. In contrast, Cluster

351   5 showed a subset of genes that were reduced only in macrophages lacking MED16, including

352   IRF8 and STAT1; as these effects were modest, and did not reach statistical significance, they

353   may be suggestive of an incomplete positive feedforward in which MED16 plays a role. Further

354   stringent differential gene expression analysis (FDR<0.05, absolute LFC>1) of the IFNγ-

355   stimulated transcriptomes identified 69 and 90 significantly different genes for MED16 and

356   GSK3β respectively. Of these differentially expressed genes (DEGs), eight non-MHCII genes

357   were shared between MED16 and GSK3β, including five genes that are involved in controlling

358   the extracellular matrix (MMP8, MMP12, TNN, and CLEC12a). Taken together these results

359   suggest that while MED16 and GSK3β both regulate IFNγ-mediated CIITA and MHCII

360   expression in macrophages, they otherwise control distinct regulatory networks in response to

361   IFNγ.

362   We next used the transcriptional dataset to understand what aspects of IFNγ-mediated

363   signaling MED16 and GSK3β specifically control. To resolve the transcriptional landscape of

364   Med16 KO macrophages and to understand the specific effect that MED16 loss has on the host

365   response to IFNγ, we analyzed the DEGs for upstream regulators whose effects would explain

366   the observed gene expression signature. The analysis correctly predicted a relative inhibition on

367   IFNγ signaling compared to NTC due to the muted induction of CIITA, H2-Ab1 and CD74. This

368   analysis also identified signatures of IL-10, STAT3, and PPARγ activation that included SOCS3

369   induction and PTGS2 downregulation (Figure 5G and Figure S5A and S5B). As the DEG

370   analysis relied on a stringent threshold that filtered the great majority of the transcriptome from

371   analysis, we sought to incorporate a more comprehensive analysis capable of capturing genes

372   with more modest effects based on pathway enrichment. To this end, we performed gene set

373   enrichment analysis (GSEA) using a ranked gene list derived from the differential gene

374   expression analysis (54). Of the ~10,000 gene sets tested, 11 sets were enriched for NTC +

375   IFNγ and 76 for MED16 + IFNγ (FDR<0.1). To reduce pathway redundancy and infer biological

376   relevance from the gene sets, we consolidated the signal into pathway networks (Figure S5C),

377   and observed a significant enrichment for genes involved in xenobiotic and steroid metabolism,

378   including many cytochrome p450 family members and glutathione transferases. We also

379   observed an elevated type I interferon transcriptional response in Med16 KO cells stimulated

380   with IFNγ that included components of IFNα/β signal transduction (IFNAR2), transcription

381   factors (STAT2, IRF7) and antiviral mediators (OAS2, IFITM1, IFITM2, IFITM3, IFITM6) (Figure

382   5H and 5I). Thus, MED16 is a critical regulator of the overall interferon response in

383   macrophages.

384   We next examined the regulatory networks that were specifically controlled by GSK3β.

385   As observed by the initial PCA (Fig5C), the transcriptional landscape of GSK3β deficient

386   macrophages was altered in unstimulated cells. We hypothesized that these widespread

387   differences may alter cellular physiology and explain, in part, the varied responsiveness of

388   Gsk3β KO cells to IFNγ. DEG analysis of unstimulated macrophages identified 284 differentially

389   expressed genes due to GSK3β loss. Functional enrichment by STRING identified 3 major

390   clusters that included dysregulation of chemokines, cell surface receptors, growth factor

391   signaling, and cellular differentiation (FigS5D). We next examined the response of Gsk3β KO

392   macrophages following IFNγ stimulation. GSEA identified a strong enrichment for chemotaxis

393   and extracellular matrix remodeling pathways including several integrin subunits and matrix

394   metalloproteinase members. These results suggest that GSK3β is an important regulator of both

395   macrophage homeostasis and the response to IFNγ. Altogether the global transcriptional

396   profiling suggests that while MED16 and GSK3β are both critical regulators of IFNγ-mediated

397   MHCII expression, they each control distinct aspects of the macrophage response to IFNγ.

398

399   **Loss of MED16 or GSK3 inhibits macrophage-mediated CD4$^+$ T cell activation.**

400   While the data to this point suggested that MED16 and GSK3β control the IFNγ-

401   mediated induction of MHCII, in addition to distinct aspects of the IFNγ-response, it remained

402   unclear how loss of GSK3β or MED16 in macrophages altered the activation of CD4$^+$ T cells. To

403   test this, we optimized an *ex vivo* T cell activation assay with macrophages and TCR-transgenic

404   CD4$^+$ T cells (NR1 cells) that are specific for the *Chlamydia trachomatis* antigen Cta1 (55).

405   Resting NR1 cells were added to non-targeting control macrophages that were untreated, IFNγ

406   stimulated, Cta1 peptide-pulsed, or IFNγ-stimulated and Cta1 peptide-pulsed. Five hours later,

407   we harvested T cells and used intracellular cytokine staining to identify IFNγ producing cells by

408   flow cytometry. Only macrophages that were treated with IFNγ and pulsed with Cta1 peptide

409   were capable of stimulating NR1 cells to produce IFNγ (Figure 6A-6C). Additionally, when Rfx5

410   deficient macrophages were pulsed with peptide in the presence and absence of IFNγ, we

411   observed limited IFNγ production by NR1 cells in both conditions suggesting this approach is

412   peptide-specific and sensitive to macrophage MHCII surface expression.

413   We next determined the effectiveness of macrophages lacking GSK3 components to

414   activate CD4$^+$ T cells. Macrophages deficient in GSK3α, GSK3β or GSK3α/β along with NTC

415   and RFX5 controls were left untreated or stimulated with IFNγ for 16 hours, then all cells were

416   pulsed with Cta1 peptide. Resting NR1 cells were then added and the production of IFNγ by

417   NR1 cells from each condition was quantified by flow cytometry five hours later. In agreement

418   with our findings on MHCII expression, loss of GSK3α did not inhibit the production of IFNγ by

419   NR1 cells (Figure 6D-6F). In contrast, Gsk3β KO cells reduced the number of IFNγ$^+$ NR1 cells

420   over two-fold and reduced the mean fluorescence intensity of IFNγ production over 4-fold.

421   Furthermore, macrophages deficient in GSK3α and GSK3β were almost entirely blocked in their

422   ability to activate IFNγ production by NR1 cells. Thus, macrophages deficient in GSK3 function

423   are unable to serve as effective antigen presenting cells to CD4$^+$ T cells.

424   The *ex vivo* T cell assay was next used to test the effectiveness of Med16 KO

425   macrophages as APCs. NR1 cells stimulated on IFNγ activated Med16 KO macrophages were

426   reduced in the number of IFNγ$^+$ T cells by 10-fold and the fluorescence intensity of IFNγ by 100-

427   fold compared to NTC (Figure 6G-GI). Similar to what we observed with MHCII expression,

17

428   there was a small yet reproducible induction of IFNγ^+ NR1 cells incubated with IFNγ-activated

429   Med16 KO macrophages. We hypothesized that inhibition of GSK3 and MED16 simultaneously

430   would eliminate all NR1 activation on macrophages. Treatment of Med16 KO macrophages with

431   CHIR99021 prior to IFNγ-stimulation and T cell co-incubation, eliminated the remaining IFNγ

432   production by NR1 cells seen in the DMSO treated Med16 KO condition. Altogether these

433   results show that GSK3β and MED16 are critical regulators of IFNγ mediated antigen

434   presentation in macrophages and their loss prevents the effective activation of CD4$^+$ T cells.

435

436

Discussion

437

438   IFNγ-mediated MHCII is required for the effective host response against infections. Here,

439   we used a genome-wide CRISPR library in macrophages to globally examine mechanisms of

440   IFNγ-inducible MHCII expression. The screen correctly identified major regulators of IFNγ-

441   signaling, highlighting the specificity and robustness of the approach. In addition to known

442   regulators, our analysis identified many new positive regulators of MHCII surface expression.

443   While we validated only a subset of these candidates, the high rate of validation suggests many

444   new regulatory mechanisms of IFNγ-inducible MHCII expression in macrophages. While the

445   major pathways identified from the candidates in CRISPR screen were related to IFNγ-signaling,

446   we also identified an important role for other pathways including the mTOR signaling cascade.

447   Within the top 100 candidates of the screen several genes related to metabolism and lysosome

448   function including LAMTOR2 and LAMTOR4 were found. Given the known effects of IFNγ in

449   modulating host metabolism, these results suggest that the metabolic changes following IFNγ-

450   activation of macrophages is critical for key macrophage functions including the surface

451   expression of MHCII (56). In addition, we found the small lysosome associated GTPase Arl8a is

452   an important regulator of IFNγ-mediated MHCII surface expression. Interestingly, a paralog,

453   Arl8b, was previously described as a regulator of MHCII and CD1d, by controlling lysosomal

454   function (57, 58). Future studies will need to dissect the metabolism specific mechanisms that

455   macrophages use to control the IFNγ response, including the regulation of MHCII.

456   In this study, we focused our follow up efforts from validated candidates on genes that

457   might control MHCII transcriptional regulation. We identified MED16 and GSK3β as strong

458   regulators of IFNγ-mediated CIITA induction. Using global transcriptomics we found that loss of

459   either MED16 or GSK3β in macrophages inhibited subsets of IFNγ-mediated genes including

460   MHCII. Importantly, the evidence here strongly supports a model where MED16 and GSK3β

461   control IFNγ-mediated MHCII expression through distinct mechanisms (Figure 7). Our results

19

462   uncover previously unknown regulatory control of CIITA-mediated expression that is biologically

463   important to activate CD4$^+$ T cells.

464   MED16 is a subunit of the mediator complex that is critical to recruit RNA polymerase II

465   to the transcriptional start site (42). While the mediator complex can contain over 20 unique

466   subunits and globally regulate gene expression, individual mediator subunits control distinct

467   transcriptional networks by interacting with specific transcription factors (42, 44). Our data

468   shows that MED16 is uniquely required among the mediator complex for IFNγ-mediated MHCII

469   expression. How MED16 controls CIITA expression remains an open question. One recent

470   study showed that MED16 controls NRF2 related signaling networks that respond to oxidative

471   stress (59). A major finding of our MED16 transcriptional analysis was the identification of

472   several metabolic pathways involved in oxidative stress and xenobiotics. Given the previous

473   work that described how oxidative stress and the NRF2 regulator Keap1 regulated IFNγ-

474   mediated MHCII expression in human melanoma cells, NRF2 regulation and redox

475   dysregulation could explain a possible mechanism for MED16 control of MHCII (17). Intriguingly,

476   the effect of MED16 loss was negligible on many STAT1 and IRF1 targets, and, in fact, resulted

477   in a type I interferon gene signature. Whether this signature is causative of or secondary to the

478   dysregulated response to type II interferons remains unknown.

479   Previous studies showed that CDK8, a kinase that can associate with the mediator

480   complex, controls a subset of IFNγ-dependent gene transcription (60). However, our results

481   strongly support a model where MED16 acts independently of CDK8. Not only was CDK8 not

482   identified in the initial CRISPR screen, but our transcriptional profiling showed that the major

483   IFNγ-dependent genes controlled by CDK8, TAP1 and IRF1, remain unchanged in Med16 KO

484   macrophages. Thus, understanding what transcription factors MED16 interacts with in the future

485   will be needed to fully determine the mechanisms of MED16-dependent transcription and its

486   control over CIITA and IFNγ-mediated gene expression.

487   While we hypothesize that MED16 directly controls CIITA transcription, GSK3 likely

488   regulates MHCII through signaling networks upstream of transcription initiation. GSK3α and

489   GSK3β are multifunctional kinases that regulate diverse cellular functions including

490   inflammatory and developmental cascades (43). Our studies found that loss of GSK3β but not

491   GSK3α blocked efficient IFNγ-mediated MHCII expression. However, ours results suggest that

492   even though GSK3α is not a primary regulator of IFNγ-mediated MHCII expression, it can

493   partially compensate for the loss of GSK3β. Thus, GSK3α and GSK3β are partially redundant in

494   their control of IFNγ-mediated MHCII expression highlighting the interlinked regulation of MHCII.

495   This finding supports using genetic interactions studies in the future to fully understand the IFNγ-

496   mediated regulatory networks in macrophages.

497   Because GSK3 regulates a range of pathways, careful work will be needed to determine

498   which GSK3 regulated networks are responsible for controlling CIITA expression. One major

499   function of GSK3 is to modulate the activation of the Wnt signaling cascade (43). Inhibition or

500   loss of GSK3 results in the constitutive stabilization of Beta-Catenin and TCF expression. If the

501   constitutive activation of Beta-catenin and Wnt signaling prevents effective CIITA expression

502   remains to be determined. Interestingly, another Wnt signaling pathway member FZD4 was

503   identified in our screen as required for MHCII expression in our screen, supporting a possible

504   role for Wnt in IFNγ-induced MHCII regulation. It is tempting to speculate that Wnt signaling

505   balances IFNγ-induced activation, resulting in distinct MHCII upregulation between cells with

506   different Wnt activation states. While there is data supporting interactions between Wnt

507   pathways and Type I IFN during viral infections, this has not been explored yet in the context of

508   IFNγ (61, 62).

509   Previous studies suggested that GSK3 controls IFNγ mediated STAT3 activation, LPS-

510   mediated nitric oxide production, and IRF1 transcriptional activity but our results in

511   macrophages clearly show these do not explain the requirement for GSK3-dependent MHCII

21

512 expression (51, 63, 64). In contrast, we found no role for STAT3 in IFNγ-mediate MHCII

513 expression and significantly higher expression of inducible Nitric Oxide Synthase in Gsk3β KO

514 macrophages. In addition, we observed a significant increase in a number of chemokines that

515 are critical to mobilizing cells to the site of infections. These results show that GSK3 is a central

516 regulator of the balanced host response during infection, and that targeting GSK3 function is

517 likely to make the host susceptible to disease. In line with this prediction, GSK3 was recently

518 found to be co-opted by the *Salmonella enterica* serovar Typhimurium effector SteE to skew

519 infected macrophage polarization and allow infection to persist (65, 66). Our results suggest

520 another possible effect of targeting GSK3 may be the inefficient upregulation of MHCII on

521 Salmonella-infected macrophages in response to IFNγ. While it is known that *Salmonella* and

522 other pathogens including *M. tuberculosis* and *C. trachomatis*, modulate the expression of

523 MHCII, the precise mechanisms underlying many of these virulence tactics remains unclear (27,

524 28). Our screening results provide a framework to test the contribution of each candidate MHCII

525 regulator during infection with pathogens that target MHCII. These directed experiments would

526 allow the rapid identification of possible host-pathogen interactions. It will be important to

527 determine if augmenting specific MHCII pathways identified by our screen overcomes pathogen-

528 mediated inhibition and induces robust MHCII expression to better activate CD4$^+$ T cells and

529 protect against disease.

530   Beyond infections, our dataset provides an opportunity to examine the importance of

531 newly identified MHCII regulators in other diseases such as tumor progression and

532 autoimmunity. Of course, MHCII is not the only surface marker that is targeted by pathogens

533 and malignancy. Other important molecules including MHCI, CD40 and PD-L1 are induced by

534 IFNγ stimulation and are targeted in different disease states (67-70). Employing our screening

535 pipeline for a range of surface markers will identify regulatory pathways that are shared and

536 unique at high resolution and provide insights into targeting these pathways therapeutically.

537   Taken together, the tools and methods developed here identified new regulators of IFNγ-

538   inducible MHCII that will illuminate the underlying biology of the host immune response.

539

540

541 **METHODS**

542

543 *Mice*

544 C57BL/6J (stock no. 000664) were purchased from The Jackson Laboratory. NR1 mice were a

545 gift of Dr. Michael Starnbach (55). Mice were housed under specific pathogen-free conditions

546 and in accordance with the Michigan State University Institutional Animal Care and Use

547 Committee guidelines. All animals used for experiments were 6–12 weeks of age.

548

549 *Cell culture*

550 Macrophage cell lines were maintained in Dulbecco's Modified Eagle Medium (DMEM; Hyclone)

551 supplemented with 5% fetal bovine serum (Seradigm). Cells were kept in 5% CO2 at 37C. For

552 HoxB8- conditionally immortalized macrophages, bone marrow from C57BL6/J mice was

553 transduced with retrovirus containing estradiol-inducible HoxB8 then maintained in media

554 containing 10% GM-CSF conditioned supernatants, 10% FBS and 10uM Beta-Estradiol as

555 previously described (50). To generate BMDMs cells were washed 3x in PBS to remove

556 estradiol then plated in 20% L929 condition supernatants and 10% FBS. 8-10 days later cells

557 were plated for experiments as described in the figure legends.

558

559 *CRISPR Screen and Analysis*

560 The mouse BRIE knockout CRISPR pooled library was a gift of David Root and John Doench

561 (Addgene #73633) (36). Using the BRIE library, 4 sgRNAs targeting every coding gene in mice

562 in addition to 1000 non-targeting controls (78,637 sgRNAs total) were packaged into lentivirus

563 using HEK293T cells and transduced in L3 cells at a low multiplicity of infection (MOI <0.3) and

564 selected with puromycin two days after transduction. Sequencing of the input library showed

565 high coverage and distribution of the library (FigS1). We next treated the library with IFNγ

566 (10ng/ml) and 24 hours later the cells were fixed and fluorescence activated cell sorting (FACS)

567   was used to isolate the MHCII^{high} and MHCII^{low} bins. Bin size was guided by the observed

568   phenotypes of positive control sgRNAs, such as RFX5, which were tested individually and to

569   ensure sufficient coverage (>25x unselected library) in the sorted populations. Genomic DNA

570   was isolated from sorted populations from two biological replicate experiments using Qiagen

571   DNeasy kits. Amplification of sgRNAs by PCR was performed as previously described using

572   Illumina compatible primers from IDT (36), and amplicons were sequenced on an Illumina

573   NextSeq500.

574   Sequence reads were first trimmed to remove any adapter sequence and to adjust for p5

575   primer stagger. We used bowtie 2 via MAGeCK to map reads to the sgRNA library index without

576   allowing for any mismatch. Subsequent sgRNA counts were median normalized to control

577   sgRNAs in MAGeCK to account for variable sequencing depth. Control sgRNAs were defined

578   as non-targeting controls as well as genes not-transcribed in our macrophage cell line as

579   determined empirically by RNA-seq (Table S2). To test for sgRNA and gene enrichment, we

580   used the 'test' command in MAGeCK to compare the distribution of sgRNAs in the MHCII^{high} and

581   MHCII^{low} bins. Notably, we included the input libraries in the count analysis in order to use the

582   distribution of sgRNAs in the unselected library for the variance estimation in MAGeCK.

583

584   *sgRNA cloning*

585   sgOpti was a gift from Eric Lander & David Sabatini (Addgene plasmid #85681) (53). Individual

586   sgRNAs were cloned as previously described (71). Briefly, annealed oligos containing the

587   sgRNA targeting sequence were phosphorylated and cloned into a dephosphorylated and

588   BsmBI (New England Biolabs) digested SgOpti (Addgene#85681) which contains a modified

589   sgRNA scaffold for improved sgRNA-Cas9 complexing. A detailed cloning protocol is available

590   in supplementary methods. To facilitate rapid and efficient generation of sgRNA plasmids with

591   different selectable markers, we further modified the SgOpti vector such that the mammalian

592   selectable marker was linked with a distinct bacterial selection. Subsequent generation of

25

593   SgOpti-Blasticidin-Zeocin (BZ), SgOpti-Hygromycin-Kanamycin (HK), and SgOpti-G418-

594   Hygromycin (GH) allowed for pooled cloning in which a given sgRNA was ligated into a mixture

595   of BsmBI-digested plasmids. Successful transformants for each of the plasmids were selected

596   by plating on ampicillin (SgOpti), zeocin (BZ), kanamycin (HK), or hygromycin (GH) in parallel.

597   In effect, this reduced the cloning burden 4x and provided flexibility with selectable markers to

598   generate near-complete editing in polyclonal cells and/or make double knockouts.

599

600   *Flow cytometry*

601   Cells were harvested at the indicated times post-IFNγ stimulation by scrapping to ensure intact

602   surface proteins. Cells were pelleted and washed with PBS before staining for MHCII. MHCII

603   expression was analyzed on the BD LSRII cytometer or a BioRad S3E cell sorter. All flow

604   cytometry analysis was done in FlowJo V9 or V10 (TreeStar)

605

606   *Chemical inhibitors*

607   CHIR99021 (Sigma) was resuspended in DMSO at 10 mM stock concentration. DMSO was

608   added at the same concentration to the inhibitors as a control. Cells were maintained in 5%

609   CO_2. Cells were stimulated with 6.25ng/ml of IFNγ (Biolegend) for the indicated times in each

610   figure legend before analysis.

611

612   *Isolation of Knockout cells*

613   Cells transduced with either MED16 or GSK3β sgRNAs were stimulated with IFNγ then stained

614   for MHCII 24 hours later. Cells expressing low MHCII were then sorted using a BioRad S3e cell

615   sorter and plated for expansion. Gene knockouts were confirmed by amplifying the genomic

616   regions encoding either MED16 or GSK3β from each cell population in addition to NTC cells

617   using PCR. PCR products were purified by PCR-cleanup Kit (Qiagen) and sent for Sanger

618   Sequencing (Genewiz). The resultant ABI files were used for TIDE analysis to assess the

619   frequency and size of indels in each population compared to control cells.

620

621   *RNA isolation*

622   Macrophages were homogenized in 500uL of TRIzol reagent (Life Technologies) and incubated

623   for 5 minutes at room temperature. 100uL of chloroform was added to the homogenate,

624   vortexed, and centrifuged at 12,000 x g for 20 minutes at 4C to separate nucleic acids. The

625   clear, RNA containing layer was removed and combined with 500uL of ethanol. This mixture

626   was placed into a collection tube and protocols provided by the Zymo Research Direct-zol RNA

627   extraction kit were followed. Quantity and purity of the RNA was checked using a NanoDrop and

628   diluted to 5ng/uL in nuclease-free water.

629

630   *RNA-sequencing Analysis*

631   To generate RNA for sequencing, macrophages were seeded in 6-well dishes at a density of 1

632   million cells/well. Cells were stimulated for 18 hours with IFNγ (Peprotech) at a final

633   concentration of 6.25 ng/mL, after which RNA was isolated as described above. RNA quality

634   was assessed by qRT-PCR as described above and by TapeStation (Aligent); the median RIN

635   value was 9.5 with a ranger of 8.6 to 9.9. A standard library preparation protocol was followed to

636   prepare sequencing libraries on poly-A tailed mRNA using the NEBNext® Ultra™ RNA Library

637   Prep Kit for Illumina®. In total, 18 libraries were prepared for dual index paired-end sequencing

638   on a HiSeq 2500 using a high-output kit (Illumina) at an average sequencing depth of 38.6e6

639   reads per library with > 93% of bases exceeding a quality score of 30. FastQC (v0.11.5) was

640   used to assess the quality of raw data. Cutadapt (v2.9) was used to remove TruSeq adapter

641   sequences with the parameters --cores=15 -m 1 -a

642   AGATCGGAAGAGCACACGTCTGAACTCCAGTCA -A

643   AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT. A transcriptome was prepared with the

644   rsem (v1.3.0) command rsem-prepare-reference using bowtie2 (v2.3.5.1) and the gtf and

645   primary Mus musculus genome assembly from ENSEMBL release 99. Trimmed sequencing

646   reads were aligned and counts quantified using rsem-calculate-expression with standard

647   bowtie2 parameters; fragment size and alignment quality for each sequencing library was

648   assessed by estimating the read start position distribution (RSPD) via --estimate-rspd. Gene

649   counts as determined by rsem were used as input for differential expression analysis in DESeq2

650   according to standard protocols. Briefly, counts were imported using tximport (v1.16.0) and

651   differential expression was performed with non-targeting control ("NTC") and unstimulated

652   ("Condition A") as reference levels for contrasts. For visualization via PCA, a variance stabilizing

653   transformation was performed in DESeq2. Pathway enrichment utilized R packages gage and

654   fgsea or Ingenuity Pathway Analysis (Qiagen). Gene-set enrichment analysis (GSEA) was

655   performed utilized gene rank lists as calculated from defined comparisons in DeSeq2 and was

656   inclusive of gene sets comprised of 10-500 genes that were compiled and made available by

657   the Bader lab (72). Pathway visualization and network construction was performed in

658   CytoScape 3.8 using the apps STRING and EnrichmentMap. Pathway significance thresholds

659   were set at an FDR of 0.1 unless specified otherwise.

660

661   *Quantitative real time PCR*

662   PCR amplification of the RNA was completed using the One-step Syber Green RT-PCR Kit

663   (Qiagen). 25ng of total RNA was added to a master mix reaction of the provided RT Mix, Syber

664   green, gene specific primers (5uM of forward and reverse primer), and nuclease-free water. For

665   each biological replicate (triplicate), reactions were conducted in technical duplicates in 96-well

666   plates. PCR product was monitored using the QuantStudio3 (ThermoFisher). The number of

667   cycles needed to reach the threshold of detection (Ct) was determined for all reactions. Relative

668   gene expression was determined using the 2^-ddCT method. The mean CT of each

669   experimental sample in triplicate was determined. The average mean of glyceraldehyde 3-

670 phosphate dehydrogenase (GAPDH) was subtracted from the experimental sample mean CT

671 for each gene of interest (dCT). The average dCT of the untreated control group was used as a

672 calibrator and subtracted from the dCT of each experimental sample (ddCT). 2^-ddCT shows

673 the fold change in gene expression of the gene of interest normalized to GAPDH and relative to

674 to untreated control (calibrator).

675

676 *T cell activation assays*

677 CD4$^+$ T cells were harvested from the lymph nodes and spleens of naive NR1 mice and

678 enriched with a mouse naïve CD4 negative isolation kit (BioLegend) following the

679 manufacturer's protocol. CD4$^+$ T cells were cultured in media consisting of RPMI 1640

680 (Invitrogen), 10% FCS, l-glutamine, HEPES, 50 μM 2-ME, 50 U/ml penicillin, and 50 mg/ml

681 streptomycin. NR1 cells were activated by coculture with mitomycin-treated splenocytes pulsed

682 with 5 μM Cta1$_{133-152}$ peptide at a stimulator/T cell ratio of 4:1. Th1 polarization was achieved by

683 supplying cultures with 10 ng/ml IL-12 (PeproTech, Rocky Hill, NJ) and 10 μg/ml anti–IL-4

684 (Biolegend) One week after initial activation resting NR1 cells were co-incubated with untreated

685 or IFNγ-treated macrophages of different genotypes, that were or were not pulsed with Cta1

686 peptide. Six hours following co-incubation NR1 cells were harvested and stained for intracellular

687 IFNγ (BioLegend) using an intracellular cytokine staining kit (BioLegend) as done previously.

688 Analyzed T cells were identified as live, CD90.1$^+$ CD4$^+$ cells.

689

690 *Statistical Analysis and Figures*

691 Statistical analysis was done using Prism Version 7 (GraphPad) as indicated in the figure

692 legends. Data are presented, unless otherwise indicated, as the mean $^+$/- the standard

693 deviation. Figures were created in Prism V7 or were created with BioRender.com

694

704   **Figure Legends**

705

706   **Figure 1. Genome-wide CRISPR Cas9 Screen Identifies regulators of IFNγ–dependent**

707   **MHCII expression.**

708

709   **(A)** Cas9+ iBMDMs (Clone L3) expressing the indicated sgRNAs were left untreated or treated

710   with IFNγ (6.25ng/ml) for 24 hours. Surface MHCII was quantified by flow cytometry. Shown is a

711   representative histogram of MHCII surface staining and **(B)** the quantification of the mean

712   fluorescence intensity (MFI) in the presence and absence of IFNγ stimulation from 3 biological

713   replicates. **** p<.0001 by one-way ANOVA with tukey correction for multiple hypotheses.

714   These data are representative of three independent experiments. **(C)** A schematic

715   representation of the CRISPR-Cas9 screen conducted to identify regulators of IFNγ-inducible

716   MHCII surface expression on macrophages. A genome-wide CRISPR Cas9 library was

717   generated in L3 cells using sgRNAs from the Brie library (4 sgRNAs per gene). The library was

718   treated with IFNγ and MHCII^{hi} and MHCII^{low} populations were isolated by FACS. The

719   representation of sgRNAs in each population in addition to input library were sequenced. **(D)**

720   Shown is score for each gene in the CRISPR-Cas9 library that passed filtering metrics as

721   determined by the alpha-robust rank algorithm (a-RRA) in MAGeCK from two independent

722   screen replicates. **(E)** The L3 clone was transduced with the indicated sgRNAs for candidates (2

723   per candidate gene) in the top 100 candidates from the CRISPR-Cas9 screen. All cells were left

724   untreated or treated with 10ng/ul of IFNγ for 24 hours then were analyzed by flow cytometry.

725   The fold-increase in MFI was calculated for triplicate samples for each cell line (MFI IFNγ+/MFI

726   IFNγ-). The results are representative of at least two independent experiments. Candidates that

727   were significant for two sgRNAs (Red) or one sgRNA (Blue) by one-way ANOVA compared to

31

728   the mean of NTC1 and NTC2 using Dunnets multiple comparison test. Non-significant results

729   are shown in Grey bars.

730

731

732   **Figure 2. The mediator complex sub-unit MED16 is uniquely required for IFNγ-mediated**

733   **MHCII surface expression.**

734

735   **(A)** Shown is the normalized mean read counts from FACS sorted MHCII^{low} and MHCII^{hi}

736   populations for the four sgRNAs targeting MED16 within the genome-wide CRISPR-Cas9

737   library. **(B)** The mean of the log fold change (normalized counts in MHCII^{hi}/normalized counts in

738   MHCII^{low}) for each mediator complex subunit that passed quality control metrics described in

739   materials and methods. The bar colors indicate the number of sgRNAs out of four possible that

740   pass the alpha cutoff using the MAGeCK analysis pipeline as described in material and

741   methods. **(C)** Med16 KO cells or L3 cells targeted with the indicated sgRNA were left untreated

742   or were treated with 6.25 ng/ml of IFNγ for 18 hours. Cells were then analyzed for surface

743   MHCII expression by flow cytometry. Shown are representative comparing the MHCII surface

744   expression of indicated mediator complex subunit (Black solid line) treated with IFNγ overlayed

745   with NTC (Grey dashed line) treated with IFNγ. **(D)** Quantification of the MFI of surface MHCII

746   from the experiment in (C) from three biological replicates. These results are representative of

747   two independent experiments. **(E)** NTC L3 cells, RFX5 sg#1 cells, and Med16 KO cells were left

748   untreated or were treated with 6.25 ng/ml of IFNγ. 18 hours later cells RNA was isolated and

749   qRT-PCR was used to determine the relative expression of CIITA and **(H)** H2aa compared to

750   GAPDH controls from three biological replicates. The results are representative of three

751   independent experiments. ***p<.001 as determined one-way ANOVA compared to NTC cells

752   with a dunnets test.

753

754

Figure 3. Inhibition of GSK3β results in decreased IFNγ-mediated CIITA and MHCII expression.

(A) Shown is the normalized mean read counts from FACS sorted MHCII^{lo} and MHCII^{hi} populations for the four sgRNAs targeting Gsk3β within the genome-wide CRISPR-Cas9 library. **(B)** NTC L3 cells and Gsk3β KO cells were treated with 6.25 ng/ml of IFNγ. 18 hours later cells were stained for surface MHCII and analyzed by flow cytometry. Shown is a representative flow cytometry plot overlaying Gsk3β KO (blue line) with NTC (grey line). The results are representative of 5 independent experiments. **(C)** NTC L3 cells or Gsk3β KO were treated with DMSO or 10μM CHIR99021 as indicated then left untreated or stimulated with IFNγ for 18 hours. MHCII surface expression was then quantified by flow cytometry. A representative flow cytometry plot of DMSO treated IFNγ treated NTC cells is overlaid with either NTC CHIR99021 treated cells **(Left)** or Gsk3β KO DMSO treated cells **(Right)**. **(D)** The mean fluorescence intensity was quantified from three biological replicates. These results are representative of three independent experiments. **(E)** Bone marrow derived macrophages from conditionally immortalized HoxB8 progenitor cells from C57BL6/J mice were treated with DMSO or 10 μM CHIR99021 and left untreated or stimulated with IFNγ for 18 hours. The MHCII surface levels were then quantified by flow cytometry. Shown is the mean fluorescence intensity from 3 biological replicates in each condition. **(F)** NTC L3 cells, Rfx5 sg#1 cells, and Gsk3β KO cells were left untreated or were treated with 6.25ng/ml of IFNγ. 18 hours later cells RNA was isolated and qRT-PCR was used to determine the relative expression of CIITA and **(G)** H2-Aa compared to GAPDH controls from three biological replicates. The results are representative of

33

777   three independent experiments. **(H)** Immortalized bone marrow macrophages were treated with

778   IFNγ. Control cells were treated with DMSO and for the remaining cells CHIR999021 was added

779   at the indicated times following IFNγ treatment. 24 hours after IFNγ stimulation the levels of

780   surface MHCII were quantified by flow cytometry. Shown is the MFI for biological triplicate

781   samples. **(I)** In parallel to **(H)**, 24 hours after IFNγ stimulation RNA was isolated and the relative

782   expression of H2-Aa was quantified relative to GAPDH from biological triplicate samples. The

783   data are representative of three independent experiments. ***p<.001 **p<.01 *p<.05 by one-

784   way ANOVA with a Tukey Correction test.

785

786

787   **Figure 4. GSK3α controls IFNγ-mediate MHCII expression only in the absence of GSK3β.**

788

789   **(A)** NTC L3 cells or Gsk3β KO were treated with DMSO or 10uM CHIR99021 then left untreated

790   or stimulated with IFNγ for 18 hours. MHCII surface expression was then quantified by Flow

791   cytometry. A representative flow cytometry plot of DMSO treated IFNγ treated NTC cells

792   (Dashed line) is overlaid with either NTC **(Left)** or Gsk3β KO **(Right)** treated with CHIR99021.

793   The mean log fold change in MFI (IFNγ-treated/untreated) was quantified from three biological

794   replicates. These results are representative of three independent experiments. **(C)** L3 cells or

795   Gsk3β KO transduced with the indicated sgRNAs were treated with IFNγ and 18 hours later the

796   surface levels of MHCII were quantified by flow cytometry. A representative histogram of NTC

797   cells (dotted line) is overlaid with cells targeted with the indicated sgRNA (solid line) after 18

798   hours of IFNγ treatment. In **(D)** the mean fluorescence intensity of surface MHCII was quantified

799   from 3 biological replicates from this experiment. **(E)** L3 cells or Gsk3β KO transduced with the

800   indicated sgRNAs were treated with IFNγ and 18 hours later RNA was isolated and the

34

801   expression of CIITA was quantified relative to GAPDH using qRT-PCR. Results are from three

802   independent wells and are representative of two independent experiments. ***p<.001, **p<.01,

803   *p<.05 by one-way ANOVA with a Tukey correction test.

804

805   **Figure 5. Transcriptomic analysis reveals distinct regulatory mechanisms of IFNγ**

806   **signaling mediated by MED16 and GSK3β.**

807

808   **(A)** Med16 KO cells were treated with DMSO or CHIR99021 then left untreated or stimulated

809   with IFNγ overnight. The following day MHC II cell surface expression was determined by flow

810   cytometry. Shown is a representative histogram with the indicated treatment in untreated

811   (Grey/Black line) or IFNγ-treated (Colored line) cells. **(B)** The quantification of the MFI of MHCII

812   from four biological replicates. ***p<.001 by one-way ANOVA with Tukey correction. **(C)** The

813   Global transcriptomes of NTC, Gsk3β KO and Med16KO was determined in the presence and

814   absence of IFNγ-stimulation for 18 hours by RNA sequencing. Shown is the principal

815   component analysis of the transcriptomes from three biological replicates for each condition. **(D)**

816   Dotplot showing the normalized read counts for CIITA and **(E)** H2-Aa **(F)** Shown is a heatmap

817   showing the relative expression (log normalized, row-scaled) of the most varied 20 genes

818   involved in the cellular response to type II interferon (Gene Ontology GO:0071346). **(G)** Shown

819   is a Dotplot visualizing the normalized counts of the type I IFN signature Socs3 from all RNAseq

820   conditions. Clustering was used to **(H)** Significant gene sets from Med16 KO cells that were

821   uniquely regulated from the RNAseq dataset were analyzed by gene set enrichment analysis

822   (GSEA) then subjected to Leading Edge analysis, which identified a significant enrichment of

823   the cellular responses to type I interferons (normalized enrichment score 2.81, FDR<0.01). **(I)**

824   Shown is a heatmap demonstrating the relative expression of the type I interferon signature

825   identified in IFNγ-stimualted Med16 KO macrophages from the RNAseq analysis. (J) Shown is a

826   heatmap demonstrating the relative expression of unique differentially expressed genes from

827   the Gsk3β KO in the presence (Top) and absence (Bottom) of IFNγ-stimulation. (K) These

828   differentially expressed genes were used in GSEA to identify Leading Edge networks that are

829   specific to Gsk3β KO cells. (Top) Shown is the leading-edge analysis of the UPAR pathway that

830   was identified from IFNγ-stimulated Gsk3β KO cells. (Bottom) Shown is the leading-edge

831   analysis of the Granulocyte chemotaxis pathway that was identified as differentially regulated in

832   resting Gsk3β KO cells.

833

834   **Figure 6. IFNγ-stimulated macrophages require MED16 or GSK3 to activate CD4$^+$ T cells.**

835

836   **(A)** Macrophages were left untreated, treated with 10ng/ml IFNγ overnight, 5μM peptide for 1

837   hour or both IFNγ and peptide as indicated. TCR-transgenic NR1 CD4$^+$ T cells specific for the

838   peptide Cta1 from *Chlamydia trachomatis* were then added to L3 macrophages of the indicated

839   genotypes at a 1:1 ratio. 4 hours after the addition of T cells, NR1 cells were harvested and the

840   number of IFNγ-producing CD4$^+$ T cells was quantified by intracellular staining and flow

841   cytometry. Shown is a representative flow cytometry plot gated on live/CD4$^+$ cells. Gates for

842   IFNγ^+ T cells were determined using an isotype control antibody. **(B)** The percent of live CD4$^+$ T

843   cells producing IFNγ and **(C)** the MFI of IFNγ production by live CD4$^+$ T cells was quantified

844   from triplicate samples. These results are representative of three independent experiments. **(D)**

845   L3 cells targeted with the indicated sgRNAs were left untreated or treated overnight with IFNγ

846   then pulsed with Cta1 peptide for 1 hour. NR1 cells were then added at a 1:1 ratio and 4 hours

847   later NR1 cells were harvested and the number of IFNγ-producing CD4$^+$ T cells was quantified

848   by intracellular staining and flow cytometry. Shown is a representative flow cytometry plot gated

849   on live/CD4$^+$ cells. Gates for IFNγ^+ T cells were determined using an isotype control antibody.

850 **(E)** The percent of live CD4$^+$ T cells producing IFNγ and **(F)** the MFI of IFNγ production by live

851 CD4$^+$ T cells was quantified from triplicate samples. These results are representative of three

852 independent experiments. **(G)** NTC L3 cells or Med16 KO cells were left untreated or treated

853 overnight with DMSO, IFNγ and DMSO or IFNγ and CHIR999021 then pulsed with Cta1 peptide

854 for 1 hour. NR1 cells were then added at a 1:1 ratio and 4 hours after the addition of T cells,

855 NR1 cells were harvested and the number of IFNγ-producing CD4$^+$ T cells was quantified by

856 intracellular staining and flow cytometry. Shown is a representative flow cytometry plot gated on

857 live/CD4$^+$ cells. Gates for IFNγ$^+$ T cells were determined using an isotype control antibody. **(H)**

858 The percent of live CD4$^+$ T cells producing IFNγ and **(I)** the MFI of IFNγ production by live CD4$^+$

859 T cells was quantified from triplicate samples. These results are representative of three

860 independent experiments.

861

862 **Figure 7. Model of GSK3β– and Med16-mediated control of IFNγ-activated MHCII**

863 **expression.** Shown is a model of how GSK3β and MED16 regulate IFNγ-mediated MHCII

864 expression. In the absence of IFNγ **(Left)** GSK3β controls the transcription of many macrophage

865 genes related to inflammation such as CCLs. In contrast, Med16 KO cells shows minimal

866 transcriptional changes in resting macrophages. Additionally, IFNγ-mediated gene expression is

867 low. Following the activation of macrophages with IFNγ **(Right)**, STAT1 becomes

868 phosphorylated and translocates to the nucleus to drive gene transcription. The IFNγ-mediated

869 induction of IRF1 does not require either GSK3β or MED16. While GSK3β continues to

870 negatively regulate inflammatory genes like CCLs it also positively regulates the transcriptional

871 activation of CIITA following IFNγ-activation. Through a parallel but distinct mechanism, IFNγ-

872 mediated induction of CIITA also requires MED16 function. The expression of CIITA then

873   recruits other transcription factors such as RFX5 to the MHCII locus where it induces the

874   expression of MHCII, which allows for the activation of CD4$^+$ T cells.

875

876   **Figure S1. Optimization of CRISPR-Cas9 editing in iBMDMs.** Immortalized C57BL6/J

877   macrophages were transduced with lentivirus expressing Cas9 then selected using Hygromycin.

878   Polyclonal transductants were then transduced with a second lentivirus encoding two different

879   sgRNAs targeting CD11b or a non-targeting control then selected with puromycin. **(A)**

880   Transductants were then stained for surface CD11b one-week later and analyzed by flow

881   cytometry. Shown is a representative histogram of CD11b from the polyclonal Cas9 line. **(B)**

882   Single cell clones were isolated from the polyclonal Cas9 line by limiting dilution. One

883   clone, clone L3 was transduced with two different sgRNAs targeting CD11b or a non-targeting

884   control then selected with puromycin. Transductants were then stained for surface CD11b one-

885   week later and analyzed by flow cytometry. Shown is a representative histogram of CD11b from

886   the L3 Cas9 clone.

887

888   **Figure S2. Adaptations to the MAGeCK analysis pipeline identifies high confidence**

889   **regulators of IFNγ-mediated MHCII expression following a Genome-wide CRISPR Cas9**

890   **screen.**

891

892   **(A)** At the selected alpha cutoff of 0.025, the number of significant genes by FDR level and the

893   number of false positives (gray bar within black bar) when using all guides; proportion of

894   significant genes that were false positives annotated above each bar (left half). This analysis

895   was repeated using only transcribed genes as determined by RNAseq analysis (Table S2). **(B)**

896   Genes that passed quality filtering and were expressed within L3 cells at the RNA level as

897   determined by transcriptomics were ranked by the FDR as determined in MAGeCK. Highlighted

898   in Red are the top hits many of which are within the canonical IFNγ-signaling and MHCII

899   expression pathway. Vertical dotted lines indicate genes that are below a calculated FDR of .2

900   and include the follow-up candidates Med16 and GSK3β. **(C)** The distribution of sgRNAs based

901   on the Log2-Fold Change as determined by MAGeCK from three groups of sgRNA targets;

902   Positive controls (Known IFNγ/MHCII pathway), Test Guides (all targeting guides in BRIE library)

903   and non-targeting controls (~1000 included in BRIE library) are shown. Positive control sgRNAs

904   are left shifted compared to negative controls indicating an enrichment in the MHCII^{low}

905   population of these sgRNAs. **(D)** Significant genes from the genome-wide screen were used to

906   identify enriched pathways from the KEGG pathway database. Shown are the top 10 enriched

907   pathways from the screen results indicating a significant enrichment of IFNγ-related pathways

908   ranked by FDR and the size of the circle indicates the Normalized Enrichment Score. **(E)** To

909   identify new pathways unrelated to IFNγ signaling KEGG pathway enrichment was repeated

910   with the top 11 genes related to IFNγ removed from the query list. Shown are the top 10

911   pathways that were identified by KEGG ranked by FDR and the size of the circle indicates the

912   Normalized Enrichment Score. **(F)** The L3 clone was transduced with the indicated sgRNAs for

913   candidates in the top 100 candidates from the CRISPR-Cas9 screen. All cells were treated with

914   10ng/ul of IFNγ for 24 hours then were analyzed by flow cytometry. Shown are representative

915   flow cytometry plots from the data quantified in Figure 1E. The results are representative of at

916   least two independent experiments. *p<.05 **p<.01 ***p<.001 by one-way ANOVA compared to

917   the mean of NTC1 and NTC2 using Dunnets multiple comparison test.

918

919   **Figure S3. Confirmation of KO lines using TIDE analysis.** Genomic DNA was isolated

920   from NTC, Med16 KO and Gsk3β KO cells and the PCR was used to amplify the region

921   encoding either Med16 or GSK3β. TIDE analysis was used to quantify the editing efficiency of

922   the indels in each cell line using trace plots following Sanger sequencing. Shown is the TIDE

923   analysis profile indicating the percent editing efficiency for (A) Med16 KO and (B) Gsk3β KO

924   cells compared to NTC control cells. (C) L3 cells and cells transduced with sgRNAs targeting

925   either Stat1 or Stat3 were left untreated or were stimulated with IFNγ for 18 hours. The surface

926   levels of MHCII were then quantified by flow cytometry and the mean fluorescence intensity

927   was determined from triplicate samples. These results are representative of two independent

928   experiments. ***p<.001 by one-way ANOVA compared using Dunnets multiple comparison test

929   compared to L3 controls.

930

931   **Figure S4. Development of a multi-vector sgRNA system to rapidly edit one gene or**

932   **simultaneously edit multiple genes. (A)** Shown is a schematic of the sgOpti derivatives that

933   were generated. sgOpti_V1 was previously published and contains an ampicillin bacterial

934   selection marker and a puromycin mammalian selction marker. sgOpti_V2 and sgOpti_V3 were

935   generated by subcloning distinct bacterial and mammalian selections markers to deliver multiple

936   sgRNAs to cells expressing sgOpti_V1. sgOpti_V2 contains a Kanamycin bacterial selection

937   marker and a Hygromycin B mammalian selection maker while sgOpti_V3 contains a zeocin

938   bacterial selection marker and a Blasticidin mammalian selection marker. **(B)** Cells transduced

939   with the indicated sgRNAs or a clonal IFNγR KO were left untreated or treated with IFNγ for 24

940   hours and analyzed by flow cytometry for the surface expression of the IFNγ-inducible marker

941   CD271. Shown is the percent of cells that induced CD271 compared to untreated cells for each

942   cell line.

943

944   **Figure S5. Transcriptomic analysis of MED16 and GSK3β reveals mechanisms of IFNγ-**

945   **mediated control. (A)** RNAseq analysis of NTC, Gsk3β KO and Med16 KO cells was

946   completed as described in the materials and methods. Shown are representative scatter plots of

947   normalized absolute read counts for genes that were highly variable among the conditions from

948   the heatmap in Figure 5F. **(B)** Differential gene expression analysis from MED16 KO cells

949   following IFNγ treatment was used to identify dysregulated pathways using gene set enrichment

950   analysis (GSEA). Shown are visual representations of the pathway networks identified using

951   EnrichmentMap and CytoScape. We found a strong downregulation (Blue) of genes involved in

952   antigen processing and presentation and an upregulation (Red) in genes related to Xenobiootic

953   metabolism, glutathione activity, and serine hydrolase and matrix metalloprotease activity. **(C)**

954   GSEA of differentially expressed genes in the Med16 KO after IFNγ-stimulation identified a type

955   I IFN signature. Shown is a pathway map generated by ingenuity pathway analysis highlighting

956   the genes that are downregulated (Blue) or upregulated (Orange) in the Type I IFN pathway.

957   The darkness of the color indicates the magnitude of the differential expression. **(D)** Differential

958   expression analysis of the Gsk3β KO in untreated conditions were used in GSEA. Shown is a

959   visual representation of the dysregulated genes placed into pathway networks using CytoScape.

960   Genes that are upregulated are shown in Red and downregulated genes are shown in Blue. The

961   darkness of the shading indicates the magnitude of the change as determined in the RNAseq

962   analysis.

963

964   **Table S1. CRISPR Screen Analysis**

965

966   **Table S2. RNAseq Analysis**

967

968   **Table S3. Oligonucleotides used in the study**

969

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1181

Figure 1
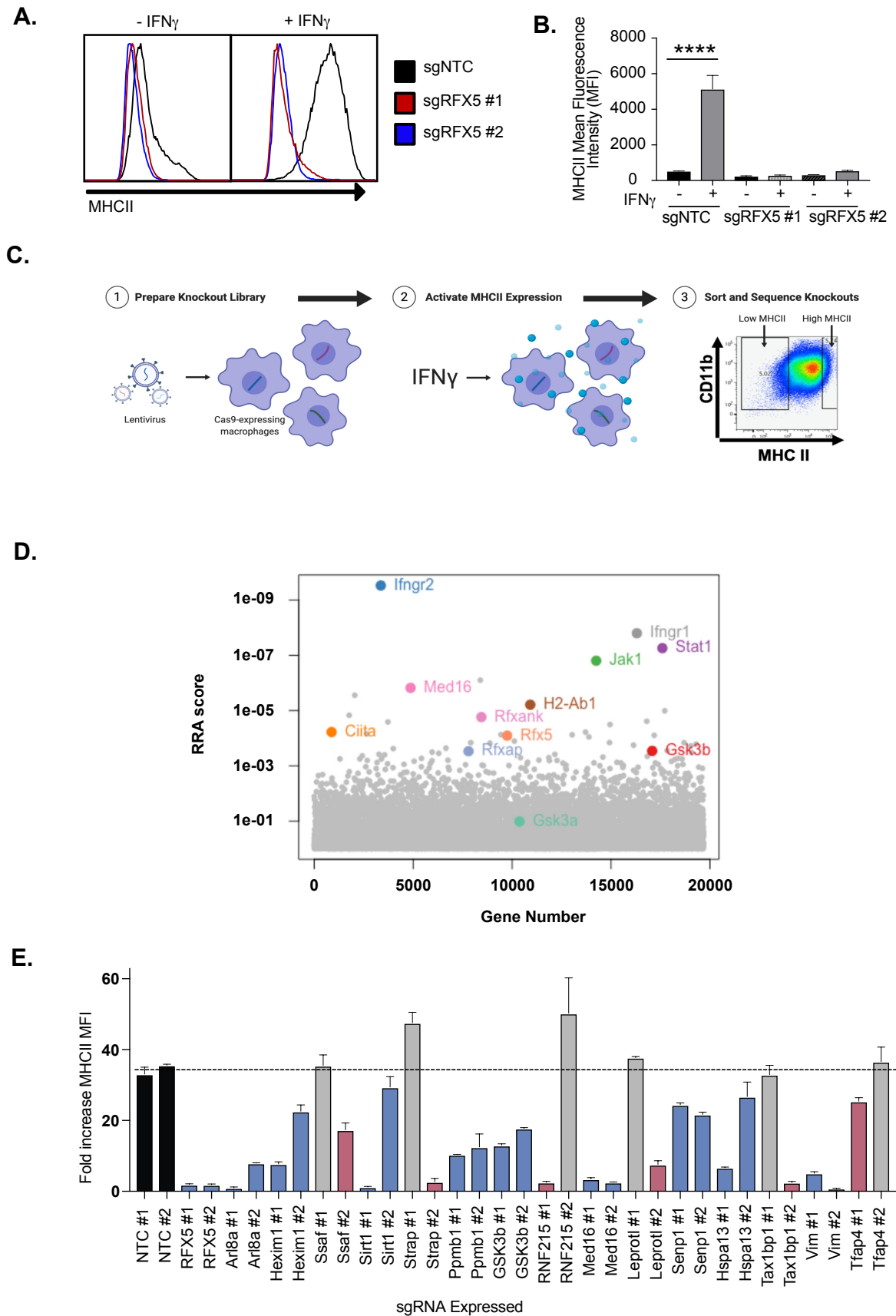
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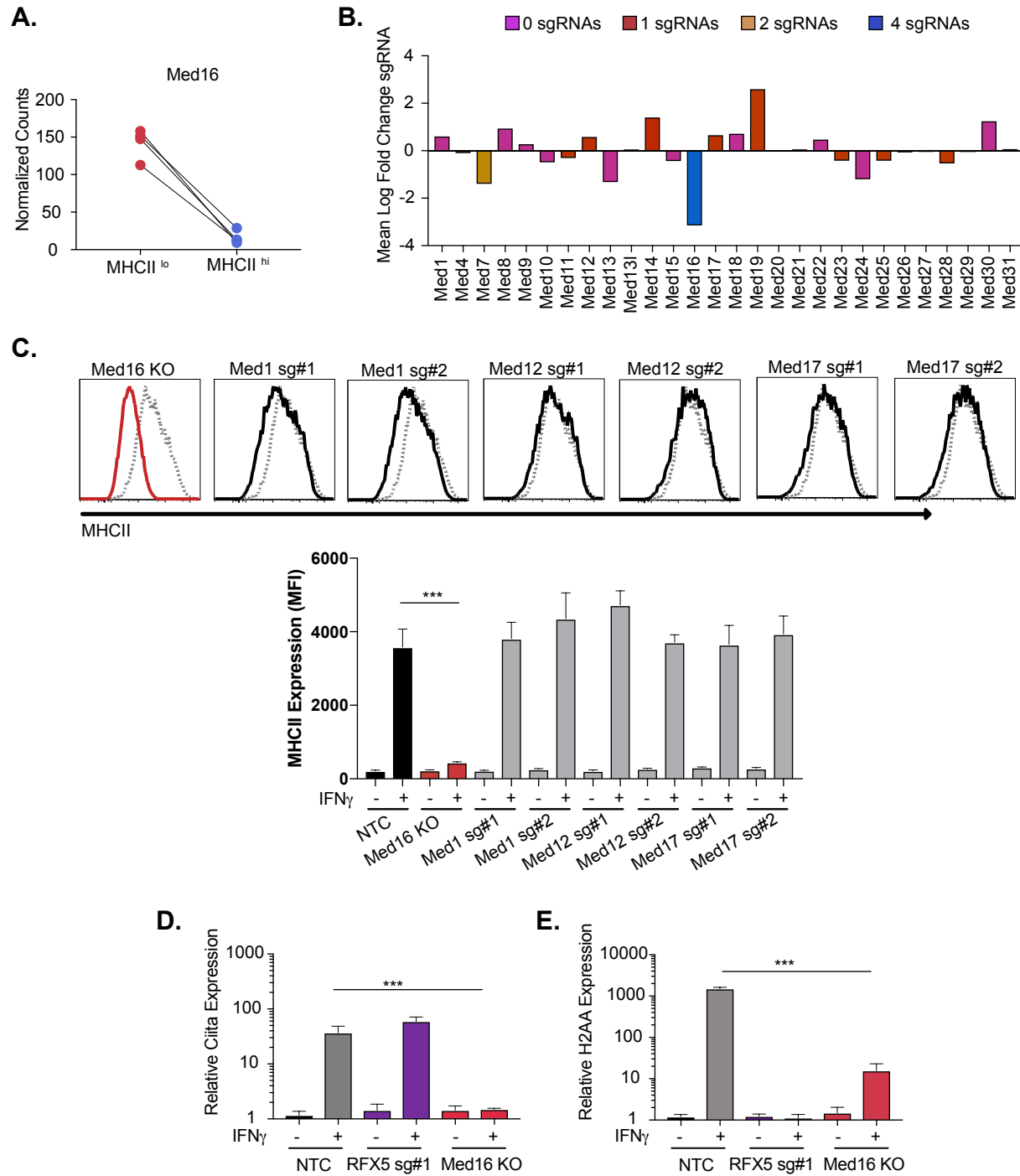
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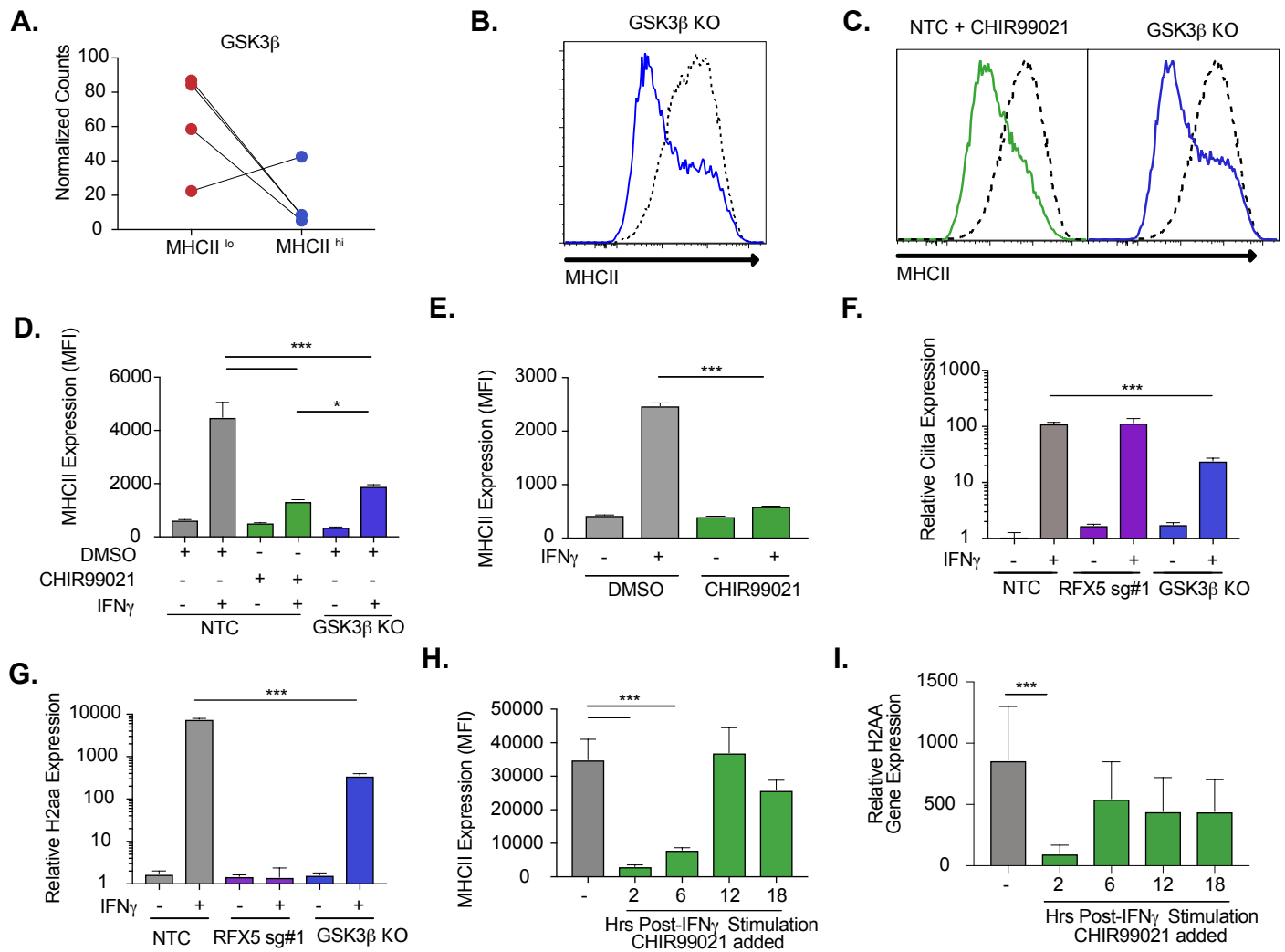
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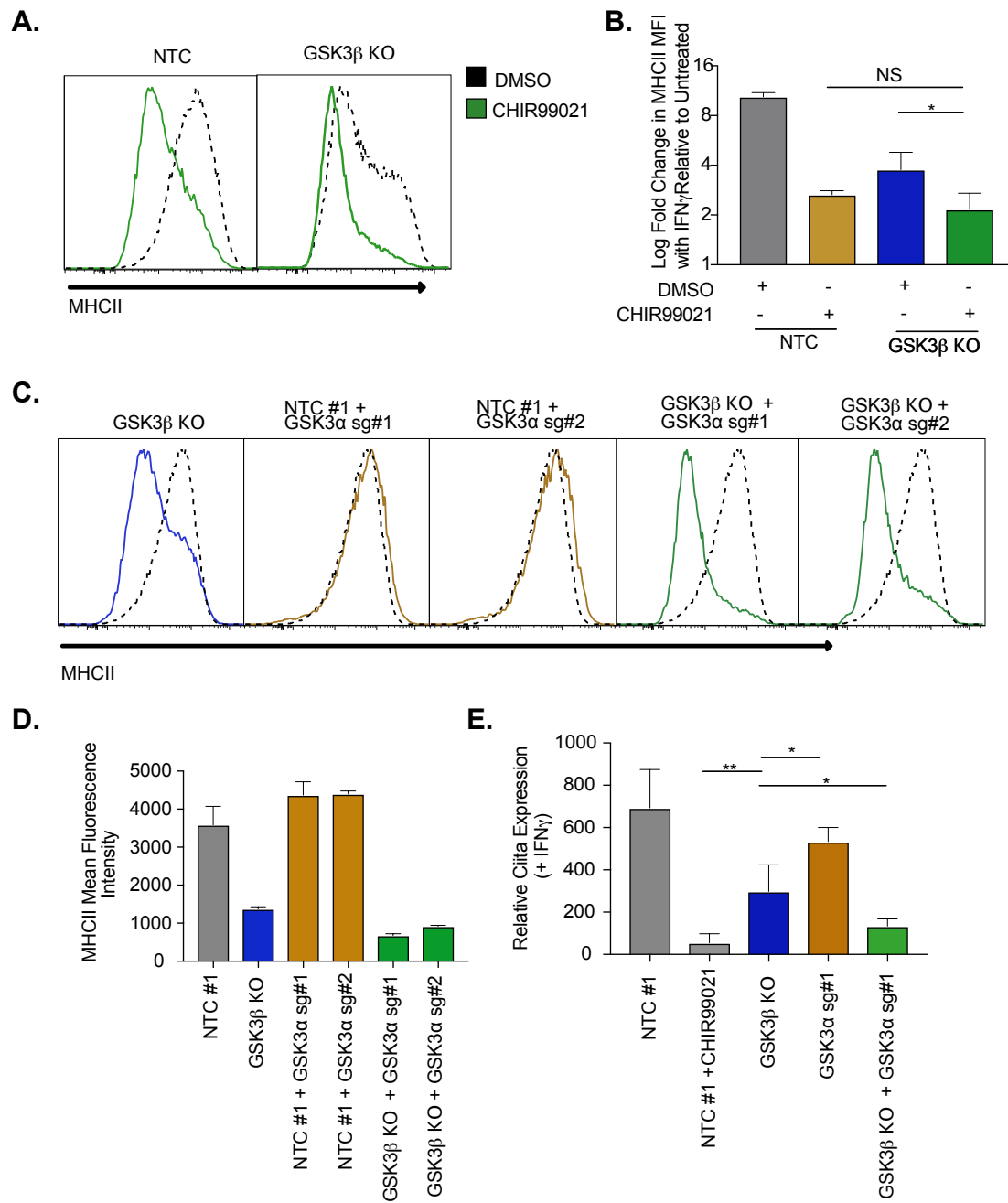
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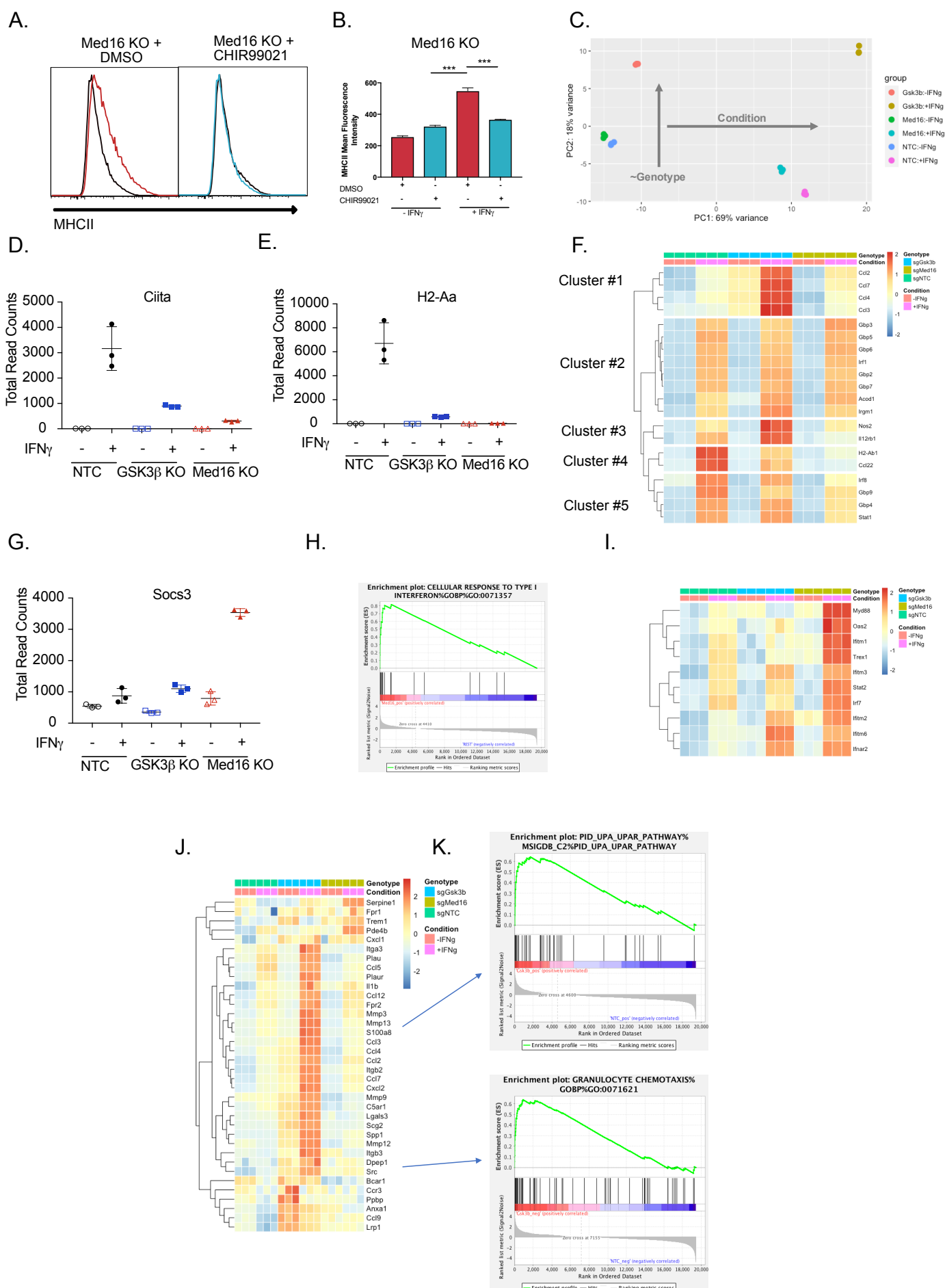
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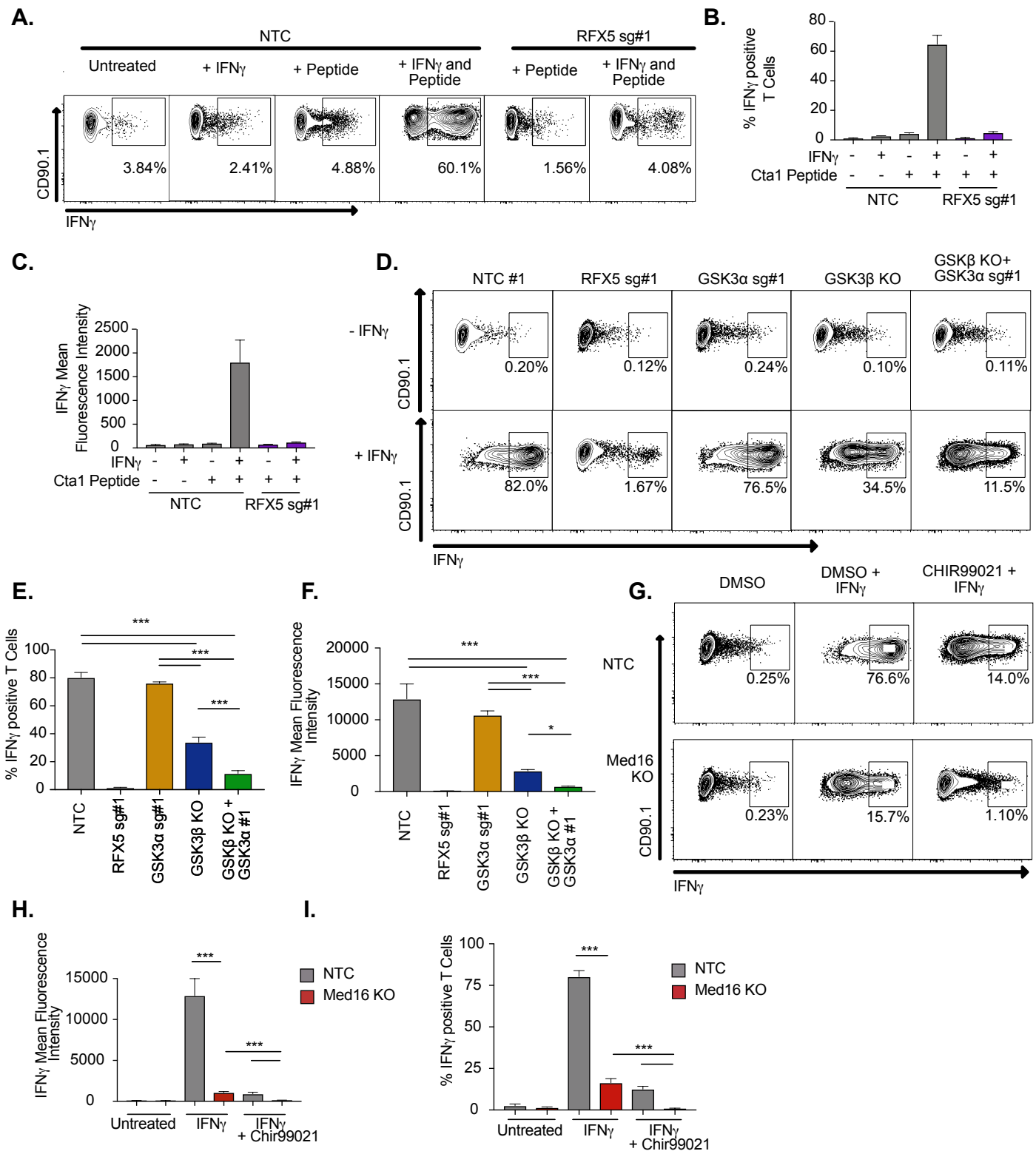
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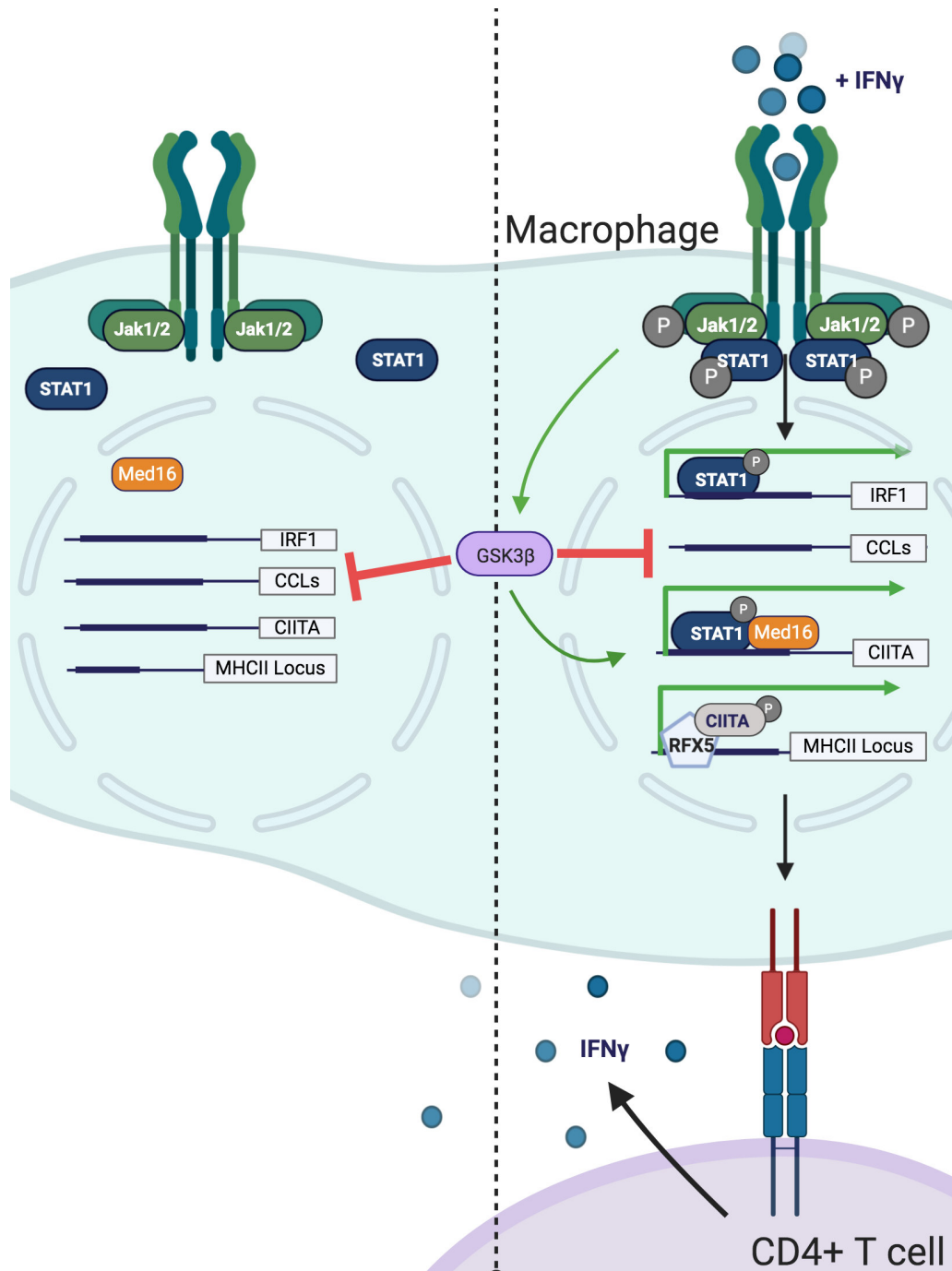
Figure S1

Figure S2

Figure S3.

A.

Med16-1 FWD

total eff. = 98.8 %

$R^2 = 0.99$
p < 0.001
p ≥ 0.001

B.

GSK3b

total eff. = 97.6 %

$R^2 = 0.98$
p < 0.001
p ≥ 0.001

C.

Figure S4

A.

Bacterial Selection

Mammalian Selection

sgOpti_V1 - Amp/Puro   sgOpti_V2 - Kan/Hyg   sgOpti_V3 - Zeo/Blast

B.

Figure S5