

1 **Co-activation of NF- κ B and MYC renders cancer cells addicted to IL6**
2 **for survival and phenotypic stability**

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28 **Highlights**

- 29 • NF- κ B and MYC co-activation originates (pre)plasmablast-like cancer
30 • NF- κ B/MYC⁺ renders cancer cells addicted to IL6 for survival and phenotypic stability
31 • NF- κ B/MYC⁺ cancers are alike a fraction of human plasmablastic lymphoma
32 • t(8;14)[MYC- IGH] multiple myeloma is linked to a NF- κ B/MYC co-activation signature
33

34 **Summary**

35 NF- κ B and MYC are found co-deregulated in human B and plasma-cell cancers. In physiology, NF- κ B is
36 necessary for terminal B-to-plasma cell differentiation, whereas MYC repression is required. It is thus
37 unclear if NF- κ B/MYC co-deregulation is developmentally compatible in carcinogenesis and/or impacts
38 cancer cell differentiation state, possibly uncovering unique sensitivities. Using a mouse system to trace
39 cell lineage and oncogene activation we found that NF- κ B/MYC co-deregulation originated cancers with a
40 plasmablast-like phenotype, alike human plasmablastic-lymphoma and was linked to t(8;14)[MYC-
41 IGH] multiple myeloma. Notably, in contrast to NF- κ B or MYC activation alone, co-deregulation rendered
42 cells addicted to IL6 for survival and phenotypic stability. We propose that conflicting oncogene-driven
43 differentiation pressures can be accommodated at a cost in poorly-differentiated cancers.

44
45 **Significance**

46 Our studies improve the understanding of cancer pathogenesis by demonstrating that co-deregulation of
47 NF- κ B and MYC synergize in forming a cancer with a poorly-differentiated state. The cancers in the mouse
48 system share features with human Plasmablastic lymphoma that has a dismal prognosis and no standard of
49 care, and with t(8;14)[MYC-IGH] Multiple myeloma, which is in overall resistant to standard therapy.
50 Notably, we found that NF- κ B and MYC co-deregulation uniquely render cells sensitive to IL6 deprivation,
51 providing a road-map for patient selection. Because of the similarity of the cancers arising in the compound
52 mutant mouse model with that of human Plasmablastic lymphoma and t(8;14)[MYC-IGH] Multiple
53 myeloma, this model could serve in preclinical testing to investigate novel therapies for these hard-to-treat
54 diseases.

55
56 **Keywords**

57 NF- κ B, MYC, IL6, B-cell, plasma-cell, plasmablast, B-cell terminal differentiation, Diffuse large B-cell
58 lymphoma, plasmablastic lymphoma, multiple myeloma, phenotypic stability

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68 **Introduction**

69 Diffuse-large-B-cell-lymphoma (DLBCL) and Multiple myeloma (MM) are the most frequent
70 hematological malignancies overall, each comprising multiple disease entities with different genetic
71 profiles and response to treatment (Kumar et al., 2017; Young et al., 2019). There is no clear interconnection
72 between these two diseases and reports of co-occurrence are extremely rare. However, DLBCL and MM
73 share the same normal cell counterpart albeit at different stages of differentiation, i.e. a mature B-cell and
74 a terminally-differentiated B-cell (Plasma-cell), respectively.

75
76 B-to-plasma cell differentiation is a multi-stage process that involves an intricate network of factors. It
77 initiates through the downregulation of *PAX5* in an activated B-cell, a transcription factor critical for B-cell
78 identity, allowing the expression of factors such as *XBPI* and *JCHAIN* (Nutt et al., 2015). Subsequent
79 upregulation of *BLIMP1* and *IRF4* expression, at least in part downstream of the NF- κ B pathway, is key
80 for the reinforcement of the plasma-cell program and full terminal B-cell differentiation characterized by
81 cell cycle arrest and substantial Ig secretion (Grumont and Gerondakis, 2000; Heise et al., 2014; Klein et
82 al., 2006; Morgan et al., 2009; Nutt et al., 2015; Saito et al., 2007).

83
84 Notably, genetic alterations leading to the activation of the NF- κ B pathway are found in ~40% of DLBCLs
85 corresponding primarily to the so-called activated B-cell subset (ABC-DLBCL) and in about 20% of MM
86 patients (Annunziata et al., 2007; Compagno et al., 2009; Davis et al., 2010; Keats et al., 2007; Lenz et al.,
87 2008a). However, in more than 80% of cases, MM cancer cells constitutively engage the NF- κ B pathway
88 through stimuli received from the cancer microenvironment (Demchenko and Kuehl, 2010; Hideshima et
89 al., 2005; Staudt, 2010). NF- κ B signaling plays a crucial role in the survival of mature B-cells and Plasma-
90 cells in physiology and pathology and is critical for both ABC-DLBCL and MM cell lines (Staudt, 2010;
91 Tornatore et al., 2014).

92
93 The knowledge that NF- κ B directly induces the expression of genes essential in B-to-plasma cell
94 differentiation and that ABC-DLBCL cancer cells despite being mature B-cells display features of
95 plasmacytic differentiation suggest that a block of B-to-plasma cell is required in the pathogenesis of ABC-
96 DLBCL. Consistently, the activity of *BLIMP1*, key for B-to-plasma cell differentiation, is lost exclusively
97 in DLBCL of the ABC subtype through *BLIMP1* genetic aberrations (~30% of cases), and indirectly by
98 deregulated *BCL6* expression from chromosomal translocations (~26% of cases) (Mandelbaum et al.,
99 2010; Pasqualucci et al., 2011; Tam et al., 2006; Zhang et al., 2015). We and others previously demonstrated
100 using mouse models that disruption of *Blimp1* precluded B-to-Plasma cell differentiation and synergized

101 with NF- κ B activation for the development of lymphomas resembling ABC-DLBCL (Calado et al., 2010;
102 Mandelbaum et al., 2010; Zhang et al., 2015).

103
104 The expression of the proto-oncogene MYC is deregulated through diverse mechanisms in most cancers,
105 including ABC-DLBCL and MM (Anderson, 2011; Janz, 2006; Shaffer et al., 2006). In fact, around 70%
106 of ABC-DLBCLs and at least 40% of MMs display MYC positivity at the protein level (Hu et al., 2013;
107 Szabo et al., 2016; Xiao et al., 2014). Amongst other properties, MYC has a crucial role in regulating cell
108 cycle entry of mammalian cells in both physiology and pathology and is critical in both ABC-DLBCL and
109 MM cell lines (Holien et al., 2012; Lenz et al., 2008b; Shaffer et al., 2008).

110 The survival properties of the NF- κ B pathway, and MYC's role in cell cycle, favor the hypothesis that these
111 factors are synergistic in carcinogenesis. However, it may be important in this hypothesis to account for
112 specific oncogene-driven differentiation pressures. In fact, and in contrast to the key role of NF- κ B in
113 Plasma-cell differentiation it has been shown that MYC opposes this process and that MYC expression is
114 repressed by BLIMP1 for terminal B-cell differentiation to ensue (Lin et al., 2000; Lin et al., 1997; Shaffer
115 et al., 2002). It is thus unclear whether co-deregulation of NF- κ B and MYC is developmentally compatible
116 for carcinogenesis, and whether a potential synergy impacts the differentiation state of cancer cells, possibly
117 uncovering unique sensitivities.

118
119 In this work we used a system in the mouse to trace cell lineage and oncogene activation and found that co-
120 deregulation of NF- κ B and MYC synergize to form cancer with a poorly-differentiated Plasma-cell state.
121 The mouse cancers resembled a fraction of human Plasmablastic lymphoma, a rare disease with a dismal
122 prognosis, and were linked at the gene expression level with MM carrying t(8;14)[MYC-IGH] that are in
123 overall resistant to standard therapy (Montes-Moreno et al., 2010; Valera et al., 2010). In contrast to
124 activation of either NF- κ B or MYC alone, co-deregulation rendered cells sensitive to IL6 deprivation, and
125 in the absence of IL6 the synergy between NF- κ B and MYC in the formation of a cancer with a poorly-
126 differentiated Plasma-cell state was lost. This work evidences that poorly-differentiated cancer cells can
127 accommodate conflicting oncogene-driven differentiation pressures at the cost of a critical dependency.

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135 **Results**

136 **Experimental design**

137 To investigate the oncogenic activity of constitutively active NF- κ B signaling and MYC over-expression,
138 alone or in combination, we used the *CD19^{creERT2}* transgene that targets Cre expression in B cells
139 encompassing all stages of development except terminally differentiated B-cells (Plasma-cells) in a
140 temporally regulated manner through tamoxifen administration (**Fig. 1**)(Yasuda et al., 2013). To induce
141 activation of the NF- κ B canonical pathway and/or MYC over-expression we generated compound mutant
142 mice carrying the *CD19^{creERT2}* allele together with a *ROSA26* allele *IKK2ca^{stopFL}* and/or a *ROSA26* allele
143 containing a *MYC* cDNA driven by a CAG promoter (**Fig. 1A**; *MYC^{stopFL}*; (Calado et al., 2012; Sasaki et
144 al., 2006)). Activation of the *IKK2ca^{stopFL}* allele by Cre-mediated recombination can be traced by expression
145 of GFP, whereas activation of the *MYC^{stopFL}* allele is marked by expression of a signaling deficient truncated
146 version of human CD2 (**Fig. 1A**). Mice carrying the *CD19^{creERT2}* transgene in combination with a *ROSA26*
147 reporter allele containing a cDNA encoding YFP preceded by a *loxP* flanked STOP cassette (*YFP^{stopFL}*;
148 (Srinivas et al., 2001)) were used as controls (**Fig. 1B**).

149

150 ***CD19cre^{ERT2}* allows the study of oncogenic mutations alone and in combination in a single mouse**

151 In a *bona fide* model system of cancer the introduction of oncogenic mutations are tissue specific,
152 temporally controlled, to trace the fate of cells in which mutations were introduced, and restricted to a small
153 number of cells, mimicking the sporadic nature of oncogenic events. The ability to test the outcome of
154 multiple oncogenes alone and in combination in the same mouse at the same time would be ideal to
155 investigate synergy, dysergy or neutrality. We first immunized *CD19^{creERT2} IKK2ca^{stopFL} MYC^{stopFL}*
156 (hereafter termed *C-IM*) and control *CD19^{creERT2} YFP^{stopFL}* (hereafter termed *C-YFP*) mice and injected
157 tamoxifen at days 6, 7, 8, and 9 after immunization (**Fig. 1C**). Analysis at day 10 after the 1st tamoxifen
158 injection (i.e. at day 15 after immunization) revealed that control mice had a small fraction of YFP⁺ (~2%)
159 in the spleen (**Fig. 1D**). *C-IM* mice on other hand had three Cre-recombined populations: GFP⁺hCD2^{neg}
160 cells (~1%) representing cells with constitutive NF- κ B activation alone, GFP^{neg}hCD2⁺ cells (~1%)
161 representing cells where only MYC activation occurred, and GFP⁺hCD2⁺ (~0.5%) representing cells
162 carrying both mutations (**Fig. 1D**). The Cre-recombined populations in *C-YFP* and *C-IM* mice were
163 overwhelmingly B-cells (**Fig. 1D**). We concluded that the *CD19^{creERT2}* system displays ideal properties to
164 investigate the function of oncogenic events in carcinogenesis.

165

166 **NF- κ B signaling and MYC over-expression synergize for hyperplasia of a Plasma-cell like population**

167 To determine the impact of enforced NF- κ B activation and MYC over-expression on B-cell fate, we
168 established cohorts of control *C-YFP* mice, of experimental *C-IM* mice and mice carrying the *CD19^{creERT2}*

169 allele in combination with either the *IKK2ca^{stopFL}* allele (hereafter termed *C-IKK2*) or *MYC^{stopFL}* (hereafter
170 termed *C-MYC*) in accord with the experimental design in (**Fig. 1C**). We first examined the blood of mice
171 for reporter positive cells at 55 days after the 1st tamoxifen injection and multiple timepoints thereafter to
172 trace their persistence or disappearance. The fractions of YFP⁺ cells in *C-YFP* and of MYC single
173 expressing cells in *C-MYC* and *C-IM* decayed over the time of analysis, whereas the cellular population
174 with enforced NF-κB activation in *C-IKK2* and *C-IM* remained constant (**Fig. S1A-C**). In contrast, the
175 fraction of cells in which NF-κB and MYC were co-deregulated increased over the time of analysis (**Fig.**
176 **S1A and B**). These data suggest that co-deregulation of NF-κB and MYC promote cellular expansion.
177 We next analyzed the spleens of mice at day 55 and 95 after tamoxifen injection and characterized
178 phenotypically the reporter positive populations using flow-cytometry. Similarly to the analysis at day 10
179 (**Fig. S1D**) we found at these time-points a YFP⁺ population in *C-YFP* mice and three distinct reporter
180 positive populations: GFP^{neg}hCD2⁺ i.e. MYC⁺, GFP⁺hCD2^{neg} i.e. IKK2ca⁺, and GFP⁺hCD2⁺ i.e.
181 IKK2ca⁺MYC⁺ (**Fig. 1E and F**). In contrast to the analysis at day 10, the IKK2ca⁺MYC⁺ population was at
182 day 55 of analysis no longer homogenous with two distinct subpopulations emerging at day 95 (**Fig. 1E**
183 and **F**). Further analysis using B-cell and Plasma-cell markers revealed the appearance of Plasma-cell like
184 cells (CD19^{low}CD138⁺), being particularly noticeable within IKK2ca⁺MYC⁺ cells (~10% at day 55; ~50%
185 at day 95) compared to the other reporter positive cell populations (**Fig. 1E and F**). This data showed that
186 expression of MYC from the *MYC^{stopFL}* allele did not impair the loss of the B-cell phenotype and acquisition
187 of Plasma-cell like markers (Lin et al., 2000; Lin et al., 1997). Further, the data demonstrated that NF-κB
188 signaling and MYC over-expression synergized for hyperplasia of a Plasma-cell like population.

189 **NF-κB or MYC expression leads to B cell lymphoma while co-deregulation to Plasma-cell like cancers**

190 To assess a role of constitutive NF-κB signaling and MYC over-expression in carcinogenesis we aged
191 control *C-YFP*, and experimental mice (*C-IKK2*, *C-MYC*, *C-IM*). *C-IM* displayed a dramatically reduced
192 life span due to cancer occurrence (~190 days after tamoxifen injection, p<0.0001) compared to all other
193 genotypes (**Fig. 2A**). *C-IKK2* and *C-MYC* mice also succumbed to cancer to a variable degree, albeit at a
194 much later time-point (~500 days after tamoxifen injection; **Fig. 2A**). *C-MYC* and *C-IKK2* mice presented
195 splenomegaly and accumulation of reporter positive cells in the spleen and lymph-nodes (**Fig. 2B**). In both
196 cases, the cancer cells expressed the B-cell marker CD19 indicating B-cell lymphoma development (**Fig.**
197 **2C**). Macroscopic examination of cancer-bearing *C-IM* mice showed splenomegaly and hepatomegaly
198 (**Fig. 2B**, not shown). Cancer-bearing *C-IM* mice displayed clonal accumulation of IKK2ca⁺MYC⁺ cells in
199 the spleen, liver and bone marrow, with cells carrying single mutations being largely absent (**Fig. 2D** and
200 **E and S2A**, not shown). Cancer cells in *C-IM* expressed the Plasma-cell marker CD138 (**Fig. 2D**), and
201 histological examination of *C-IM* spleens showed compared to control mice a diffuse cell pattern with loss
202

203 of follicular structure and B220 and Pax5 expression, but positivity for Irf4 and the proliferative marker
204 Ki67 (**Fig. 2F**). Further suggesting a Plasma-cell like cancer, *C-IM* had an aberrant accumulation of IgM
205 paraprotein (**Fig. S2B** and C). These data highlighted a unique synergy between NF- κ B signaling and MYC
206 over-expression for the formation of Plasma-cell like cancers.

207
208 **Cancers with NF- κ B and MYC co-deregulation display a phenotype alike that of (pre)plasmablast**

209 B-to-Plasma cell differentiation is a multi-stage process that involves an intricate network of factors (**Fig.**
210 **3A**, (Nutt et al., 2015)). To characterize the stage of B-to-Plasma cell differentiation of NF- κ B⁺MYC⁺
211 cancer cells we performed gene expression profiling (GEP) by RNA sequencing of FACS-sorted
212 GFP⁺hCD2⁺ cancer cells. We next compared the GEP of cancer cells with that of discrete B-cell and
213 Plasma-cell populations (Shi et al., 2015). In agreement with the phenotypical characterization (**Fig. 2**),
214 NF- κ B⁺MYC⁺ cancer cells clustered with normal Plasma-cell populations in the loss of the expression of
215 genes associated with the B-cell phenotype, including *Pax5*, *Ms4a1* (CD20), and *CD19* (**Fig. 3B**, and C).
216 However, when analyzing genes which expression is increased in a Plasma-cell, the NF- κ B⁺MYC⁺ cancer
217 cells displayed an intermediate B-to-Plasma cell GEP, clustering on their own (**Fig. 3B**). Such an B-to-
218 Plasma cell GEP state was highlighted by intermediate expression of *Blimp1*, *Irf4*, and *Xbp1* that are critical
219 for B-to-Plasma cell differentiation and of other Plasma-cell expressed genes such as *CD138* (*Sdc1*) and
220 *Jchain* (**Fig. 3B-D**). Gene signatures have previously been generated for Plasmablasts and Plasma-cells
221 (Shi et al., 2015). Using these signatures we performed gene set enrichment analysis (GSEA) and found
222 that the GEP of *C-IM* cancer cells was enriched for genes associated with Plasmablasts whereas the GEP
223 of normal Plasma-cells was enriched for genes present in the Plasma-cell signature (**Fig. 3E**). These data
224 suggested that enforced NF- κ B activation and MYC over-expression synergized in the development of a
225 cancer at a poorly-differentiated plasma-cell stage alike that of (pre)plasmablast.

226
227 **NF- κ B and MYC co-deregulation confers proliferative and survival advantage to (pre)Plasmablasts**

228 To better understand the contribution of NF- κ B and MYC co-deregulation we used a classical
229 (pre)Plasmablast (B220^{low}CD138⁺) differentiation assay *in vitro* in which B-cells are cultured in the
230 presence of LPS (Andersson et al., 1972). For this purpose, we crossed the *IKK2ca^{stopFL}*, *MYC^{stopFL}*, and
231 control *YFP^{stopFL}* alleles with *CD19^{cre}* that constitutively targets Cre expression in B-cells (Rickert et al.,
232 1997). In agreement with the *in vivo* data, we found a profound synergy in the accumulation of
233 B220^{low}CD138⁺ cells in cultures derived from *CD19^{cre} IKK2ca^{stopFL} MYC^{stopFL}* B-cells compared to those
234 where NF- κ B or MYC deregulation occurred alone (**Fig. 4A** and **B**). Analysis of the fraction of
235 B220^{low}CD138⁺ cells per division revealed an increased proliferative capacity upon NF- κ B and MYC co-

236 deregulation compared to all other genotypes (**Fig. 4C**). This increased proliferative capacity was
237 accompanied by reduced apoptosis as measured by cleaved caspase 3 (**Fig. 4D**). These data showed that
238 co-deregulation of NF- κ B and MYC provided an advantage in proliferative capacity and survival of
239 (pre)Plasmablasts.

240

241 **NF- κ B and MYC co-deregulation render cells addicted to IL6 for survival**

242 To uncover dependencies of cancer cells with NF- κ B and MYC co-deregulation we compared the GEP of
243 the *C-IM* cancer cells with that of normal Plasma-cells. Compared to plasma-cells, the GEP of *C-IM* cancer
244 cells was depleted for genes associated with the gene signature “Hallmarks_Apoptosis” (**Fig. 5A**) and
245 pathway analysis revealed the enrichment of the “IL6 JAK/STAT” and “IL-6 signaling in MM” signatures
246 in the GEP of *C-IM* cancer cells, reflected in part by the reduced expression of the STAT3 pathway inhibitor
247 *Socs3*, and increased expression of *Il6st*, that encodes the IL6 co-receptor gp130 (**Fig. 5B-C**). The IL6
248 JAK/STAT pathway was previously shown to play a key role in the survival of MM cancer cells *ex vivo*
249 and *in vivo* (Klein et al., 1990a). IL6 ligation to IL6ra and gp130 leads to STAT3 phosphorylation
250 (pSTAT3), homodimerization and nuclear translocation where it activates the transcription of multiple
251 genes including the anti-apoptotic factors *BclxL* and *Mcl1* (Gaudette et al., 2014; Jourdan et al., 2003;
252 Peperzak et al., 2013). Consistent with previous work demonstrating that IL6 is induced by NF- κ B
253 activation (Libermann and Baltimore, 1990), we found increased IL6 expression in the *in vitro* cultures
254 derived from B-cells of mice carrying the *IKK2ca^{stopFL}* allele alone or in combination with *MYC^{stopFL}* (**Fig.**
255 **S3A**). Notably, IL6 production was highly enriched in the (pre)Plasmablast population (~85% of cells)
256 compared to the activated B-cell population (~15% of cells; **Fig. S3A**). To investigate the survival
257 dependency on IL6 of (pre)Plasmablasts with NF- κ B and MYC single or double de-regulation, we cultured
258 B-cells with LPS in the absence or presence of an anti-IL6 neutralizing antibody (**Fig. 5D**). Suggestive of
259 increased dependency and/or selection, the (pre)Plasmablasts with NF- κ B and MYC co-deregulation
260 showed the highest levels of pSTAT3 amongst all genotypes (**Fig. 5E**). Treatment of the cell cultures with
261 anti-IL6 was effective in reducing the levels of STAT3 phosphorylation in all genotypes (**Fig. 5E**). The
262 proliferative capacity of the (pre)Plasmablasts was unaltered by the anti-IL6 neutralizing antibody(**Fig.**
263 **S3B**). However, we found a significant increase in the fraction of (pre)Plasmablasts marked for apoptosis
264 in the condition where NF- κ B and MYC were co-deregulated and a trend when MYC was deregulated
265 alone (**Fig. 5F**). We next looked at the expression of anti-apoptotic proteins known to be downstream of
266 IL6 signaling. With exception of the (pre)Plasmablasts carrying NF- κ B activation alone, IL6 neutralization
267 led to significant reduction in BclxL protein levels (**Fig. 5G**), whereas this was the case for Mcl1 protein
268 levels in (pre)Plasmablasts carrying NF- κ B/MYC co-deregulation and deregulation of MYC alone (**Fig.**

269 **5H)**. These data showed that (pre)Plasmablasts with NF- κ B and MYC co-deregulation and with
270 deregulation of MYC alone are particularly sensitive to IL6 deprivation *in vitro*. Additional analysis by
271 GEP demonstrated that such sensitivity to IL6 deprivation is (pre)Plasmablast specific, given the unaltered
272 GEP profile of B cells with NF- κ B/MYC co-deregulation upon IL6 neutralization (**Fig. 5I** and **J**). To
273 investigate whether IL6 neutralization delayed cancer occurrence in *C-IM* mice, we aged cohort of mice
274 and performed a single course of IL6 neutralization. Injection of anti-IL6 antibody significantly increased
275 the length of cancer free survival of *C-IM* mice (**Fig. 5K**) suggesting a dependency of IKK2ca⁺MYC⁺
276 cancer cells on IL6 also *in vivo*.

277

278 **IL6 is critical for NF- κ B⁺MYC⁺ (pre)plasmablast-like cancer phenotypic stability**

279 We next wanted to determine whether IL6 dependency upon NF- κ B and MYC co-deregulation was
280 associated with cellular transformation at a poorly-differentiated Plasma-cell state. For that we generated
281 compound mutant *C-IM* mice lacking IL6 (hereafter termed *C-IM-IL6KO*). Analysis of mice at 100 days
282 after tamoxifen injection revealed a trend for a reduced fraction of cells with NF- κ B and MYC co-
283 deregulation, whereas cells with NF- κ B or MYC deregulation alone showed either a trend for increased
284 fraction of cells or no difference, respectively (**Fig. 6A** and **B**). When characterizing the fraction of
285 CD19^{low}CD138⁺ cells (Plasma-cell like) within each reporter positive population the impact of IL6
286 deprivation was unique to cells carrying NF- κ B and MYC co-deregulation both in spleen (**Fig. 6C**) and
287 bone marrow (**Fig. S4A**). Also only in the Plasma-cell like cells within the IKK2ca⁺MYC⁺ population we
288 observed a significant increase in the fraction of cleaved caspase 3 positive cells (**Fig. 6D**). In contrast, IL6
289 deprivation had little impact on the cell cycle status of CD19^{low}CD138⁺ cells within the IKK2ca⁺MYC⁺
290 population, whereas a slight increase in cells at the S/G2M phase was observed in the MYC activation alone
291 condition (**Fig. 6E**). These data showed that NF- κ B and MYC co-deregulation uniquely rendered cells
292 sensitive to IL6 deprivation *in vivo*. These data also suggest that the apparent dependency of
293 (pre)Plasmablasts with MYC deregulation alone on IL6 *in vitro* (**Fig. 5F** and **H**) could be a consequence of
294 LPS induced NF- κ B activation.

295 To investigate if IL6 had a role in the synergy between NF- κ B and MYC in cell transformation we generated
296 cancer cohorts of *C-IM* mice and *C-IM-IL6KO* mice following the previously described protocol of study
297 (**Fig. 1B**). Compared to *C-IM* mice, *C-IM-IL6KO* had significantly prolonged cancer latency, with a median
298 survival of 265 day for *C-IM-IL6KO* compared to 187 for *C-IM* (**Fig. 6F**). Notably, *C-IM-IL6KO* cancers
299 had a significant increased fraction of cleaved caspase 3 positive cells compared to *C-IM* cancers and were
300 enriched in their gene GEP for the gene signature “Hallmark_Apoptosis” (**Fig. 6G** and **H**). To better
301 characterize the state of B-to-Plasma cell differentiation of *C-IM-IL6KO* cancer cells we performed GEP

302 by RNA sequencing of FACS-sorted GFP⁺hCD2⁺ cancer cells and compared their GEP with that of discrete
303 B-cell and Plasma-cell populations (Shi et al., 2015). Two out of 6 *C-IM-IL6KO* cancers analyzed by
304 RNAseq clustered with the B-cell populations in the expression of genes associated with the B-cell
305 phenotype, whereas 4/6 *C-IM-IL6KO* cancers clustered together with the *C-IM* cancers and the Plasma-cell
306 populations in the loss of the expression of genes associated with the B-cell phenotype (**Fig. 6I**).
307 Histological analysis revealed that roughly 15% (3/21) of *C-IM-IL6KO* cancers presented a B-cell like
308 phenotype (**Fig. S4B**). When analyzing genes which expression is increased in Plasma-cells the four *C-IM-*
309 *IL6KO* that for the B-cell signature clustered with *C-IM* no longer grouped together with these cancers (**Fig.**
310 **6I**). Whereas *C-IM-IL6KO* cancers clustered with the Plasma-cell populations (**Fig. 6I**), the *C-IM* cancers,
311 as before (**Fig. 3B**) had an intermediate B-to-Plasma cell GEP, clustering on their own (**Fig. 6I**). Compared
312 to *C-IM* cancers the *C-IM-IL6KO* B-cell like cancers had in accordance to their phenotype reduced
313 expression of *Blimp1*, *Irf4*, and *Xbp1*, whereas expression of *Pax5* was elevated (**Fig. 6K and L**). The *C-*
314 *IM-IL6KO* Plasma-cell like cancers were depleted in genes characteristically expressed by Plasmablasts
315 compared to *C-IM* cancers (**Fig. 6M**), and had increased expression of *Blimp1* (**Fig. 6N**). These data suggest
316 that compared to the *C-IM* cancers, the *C-IM-IL6KO* Plasma-cell like cancers were more alike a well-
317 differentiated Plasma-cell. In summary, IL6 was critical in the synergy between NF-κB and MYC
318 deregulation for survival and transformation of a cell with a poorly-differentiated Plasma-cell phenotype.
319 Phenotypic stability

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321 **In the context of NF-κB activation MYC interferes with the expression of Plasma-cell factors**
322 Previous work in cell lines suggested that repression of *MYC* by BLIMP1 is necessary for B cell terminal
323 differentiation (Lin et al., 2000; Lin et al., 1997). In this work we showed that MYC over-expression driven
324 from the *MYC*^{stop^{FL}} allele does not interfere with the loss of the B-cell phenotype (**Fig. 3 and 4**). To better
325 understand how MYC overexpression may interfere with the state of Plasma-cell differentiation we
326 performed LPS stimulation of B-cells *in vitro* that either carrying NF-κB deregulation alone or together
327 with MYC. We next purified reporter positive B220^{low}CD138⁺ cells from these cultures and performed GEP
328 using RNA-seq. Comparison of these GEP with previously derived Plasmablast and Plasma-cell signatures
329 revealed that the GEP of cells with NF-κB and MYC co-deregulation was enriched for genes present in the
330 Plasmablast signature whereas the GEP of cells with NF-κB deregulation alone enriched for genes present
331 in the Plasma-cell signature (**Fig. 7A**). In agreement with these data cells with NF-κB deregulation alone
332 expressed higher levels of genes key for the Plasma-cell differentiation process, namely *Blimp1*, *Irf4*, and
333 *Xbp1* compared to cells with NF-κB and MYC co-deregulation (**Fig. 7B**). The level of the B-cell identity
334 gene *Pax5* were identical between genotypes (**Fig. 7B**). These data indicates that MYC overexpression did

335 not impact the loss of the B-cell phenotype, but it interfered with the reinforcement of the Plasma-cell
336 program curtailing full Plasma-cell differentiation.

337

338 ***C-IM* cancers are alike a fraction of plasmablastic lymphoma**

339 We next investigated if the cancers occurring in the *C-IM* mice resembled human disease. We excluded
340 human-ABC-DLBCLs given that these lymphomas retain the B-cell phenotype in the vast majority of
341 cells (Alizadeh et al., 2000). PBL on other hand is a human cancer that carries a Plasmablast-like phenotype
342 and in which B-cell markers have been lost, despite being classified under DLBCL (Montes-Moreno et al.,
343 2010; Swerdlow, 2008; Valera et al., 2010). Notably, at least 70% of PBL cases carry Ig/MYC
344 translocations or *MYC* locus gain, and a recent PBL patient derived cell line was shown to be IL6 dependent
345 (Mine et al., 2017; Tadesse-Heath et al., 2010; Valera et al., 2010). The *C-IM* cancers were generated
346 through *MYC* overexpression, displayed a critical dependency on IL6 and had a poorly-differentiated
347 Plasma-cell state similar to that of a (pre)Plasmablast. However, for the development of cancers in the
348 mouse model system we also enforced the activation of the NF- κ B pathway and currently it is unknown if
349 PBL displays constitutive NF- κ B signaling. We therefore assembled a cohort of 34 PBL cases and
350 performed immunohistochemistry to determine *MYC* positivity, activation of the NF- κ B canonical and
351 alternative pathway through determination of nuclear p50 and p52, respectively, and activation of the
352 STAT3 pathway using an anti-pSTAT3 antibody (Compagno et al., 2009). All of PBL cases analyzed were
353 strongly positive for *MYC*, whereas ~15% (5/33) were positive for nuclear p50, ~25% (8/33) were positive
354 for nuclear p52, and ~15% (6/33) were positive for both, suggesting that ~55% (19/33) of PBL cases in the
355 study cohort display activation of the NF- κ B pathway (**Fig 7C**). We also found that ~44% (15/34) of PBL
356 cases in the study cohort displayed phosphorylated STAT3 (**Fig 7C**). In summary, ~28% (9/32) of PBL
357 cases in the assembled cohort displayed co-deregulation of NF- κ B, *MYC* and STAT3 phosphorylation (**Fig**
358 **7C**), indicating that the *C-IM* cancers are alike a fraction of PBL cancers.

359

360 **NF- κ B and *MYC* co-deregulation identifies multiple myeloma patients with poor prognosis**

361 We investigated whether oncogene associated gene signatures could also be predictive of patient outcome.
362 Signatures associated with NF- κ B or STAT3 activation were *per se* not predictive of patient outcome (**Fig.**
363 **S5A and B**). In contrast, we found that signatures associated with *MYC* activity were highly predictive of
364 patient outcome, in agreement to what was previously found at the protein level by others ((Moller et al.,
365 2018); **Fig. S5C**). The lack of predictive value of both NF- κ B or STAT3 gene signatures was puzzling. We
366 therefore performed GEP of B220^{low}CD138⁺ cells derived *in vitro* from B-cells without oncogene activation
367 and with NF- κ B and *MYC* deregulation alone or together. We next generated signatures of upregulated

368 genes unique to each genotype i.e. specific to a condition where only enforced NF- κ B occurred
369 (IKK2.muTex), where only MYC de-regulation was induced (Myc.muTex), and for the condition where
370 both NF- κ B and MYC were enforced simultaneously (Myc.IKK2.muTex; **Fig S5D**). In contrast to the NF-
371 κ B and STAT3 gene signatures, all three muTex signatures were highly predictive of overall survival (OS)
372 and progression free survival (PFS) (**Fig. 7D** and **S5E** and **F**). Notably, each muTex gene signature
373 specifically enriched into discrete MM subsets according to their hallmark translocations (**Fig. 7E** and **S5E**
374 and **F**). MM cases carrying either t(14;16)[IGH-MAF] or t(4;14)[FGFR3/WHSC1-IGH] positively
375 correlated in their GEP with the IKK2.muTex signature whereas a negative correlation with this signature
376 was observed for MM cases carrying t(8;16)[MYC-IGH] (**Fig. S5E**). The Myc.muTex gene signature was
377 found to be significantly enriched in MM cases carrying t(6;14)[CCND3-IGH] (**Fig. S5F**), and the
378 Myc.IKK2.muTex gene signature was significantly enriched in MM cases carrying t(8;16)[MYC-IGH]
379 (**Fig. 7E**). These data suggest that gene signatures generated from phenotypically relevant cells with
380 enforced NF- κ B and MYC single and co-deregulation identify MM patients with poor prognosis and were
381 linked to discrete genomic alterations providing a road-map for patient selection in specific therapeutic
382 settings. The observation that a signature unique to NF- κ B and MYC co-deregulation associates with MM
383 carrying t(8;16)[MYC-IGH] indicates a role for the NF- κ B and IL6 signaling pathway in these hard to treat
384 MM patient subset.

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402 Discussion

403 In this work we studied oncogenic events that in physiology impose conflicting differentiation pressures.
404 Specifically, we investigated carcinogenesis upon activation of NF- κ B and MYC in the context of B-to-
405 Plasma cell differentiation. We found that NF- κ B and MYC co-deregulation synergized for the
406 development of a cancer with a phenotype of a poorly-differentiated Plasma-cell, resembling a
407 (pre)Plasmablast. Notably, in contrast to single NF- κ B or MYC activation, co-deregulation rendered cells
408 sensitive to IL6 deprivation, and IL6 was critical for the NF- κ B/MYC synergy in forming a cancer with a
409 poorly-differentiated Plasma-cell phenotype. We propose that poorly-differentiated cancer cells can
410 accommodate at a cost conflicting oncogene-driven differentiation pressures.

411
412 The current work provides cues for the order and timing of mutation acquisition in the pathogenesis of
413 mature B-cells. The finding that B-cell specific co-deregulation of NF- κ B and MYC originates cancers
414 devoid of B-cell markers supports that a block in B-to-Plasma cell differentiation, such as loss of BLIMP1
415 activity, is required for the pathogenesis of ABC-DLBCL. Given that cancer cells in ABC-DLBCL retain
416 the B-cell phenotype, the cancers the current mouse model are not alike ABC-DLBCL. Instead, the NF-
417 κ B/MYC mouse cancers seem alike a fraction of human PBL and are linked with t(8;14)[MYC-IGH] MM
418 at the gene expression level. We found that ~55% of PBLs display NF- κ B and MYC activation, and others
419 have shown that a Plasmablastic morphology in MM is linked to high MYC expression and dismal survival
420 prognosis (Lorsbach et al., 2011; Moller et al., 2018).

421
422 Surprisingly, *BLIMP1* genetic aberrations are also found in a fraction of PBL and MM (Chapman et al.,
423 2011; Montes-Moreno et al., 2017). Their impact on BLIMP1 activity, timing of occurrence, disease
424 formation and/or progression requires investigation. However, it is tempting to speculate that partial loss
425 of BLIMP1 activity could alter the transcriptional landscape of cancer cells impacting the differentiation
426 state and expression of cell cycle genes such as *MYC* (Montes-Moreno et al., 2017; Shaffer et al., 2008).
427 Interestingly, *BLIMP1* genetic aberrations associate with poor prognosis in MM (Solimando et al., 2019).

428
429 Seminal work by Potter and colleagues described the development of Plasma-cell like cancers in mice upon
430 pristane injection (Anderson and Potter, 1969). Additional studies revealed that the occurrence of such
431 cancers involves chronic inflammation, IL6 signaling and the formation of *Myc* chromosomal translocations
432 (Cheung et al., 2004; Dechow et al., 2014; Hilbert et al., 1995; Potter et al., 1985; Potter and Wiener, 1992;
433 Rutsch et al., 2010; Suematsu et al., 1992). Other work showed that activation of MYC in B-cells to mostly
434 originates cancers with a B-cell phenotype, although some mouse models displayed also Plasma-cell like
435 cancers in a fraction of cases (Chesi et al., 2008; Harris et al., 1988; Kovalchuk et al., 2000; Park et al.,

436 2005). It is accepted that NF- κ B induces and is induced by inflammatory signals being considered the key
437 link between inflammation and carcinogenesis (Taniguchi and Karin, 2018). However, despite this
438 knowledge and that NF- κ B favors B-to-Plasma cell differentiation, direct evidence of the participation of
439 this pathway for the development of Plasma-cell like cancers was missing. Therefore, the current work
440 provides a rationale for Plasma-cell like carcinogenesis in previous mouse models. The resemblance of the
441 NF- κ B/MYC mouse cancers with a fraction of PBLs and t(8;14)[MYC-IGH] MM indicates a role for the
442 NF- κ B pathway in the pathogenesis and/or progression of the cancers.

443
444 Our work and that of others suggests that knowledge on the differentiation state of cancer cells with respect
445 to the normal cell counterpart allows a better definition of risk and of therapeutic opportunities (Paiva et
446 al., 2017; Tarte et al., 2003). Human PBL and t(8;14)[MYC-IGH] MM patients have very poor prognosis
447 and limited treatment options. The similarity of the NF- κ B/MYC mouse cancers with these diseases may
448 allow their use to identify therapeutic opportunities and strategies. We showed that a single course of anti-
449 IL6 treatment was beneficial in delaying cancer progression. Supporting a possible role for IL6 in PBL, is
450 the observation that this cytokine was required for the growth of a patient derived cell line (Mine et al.,
451 2017). In MM, Inhibition of IL6 activity is of long-standing interest (Matthes et al., 2016). Initial proof of
452 principle studies were highly promising, however, the results from randomized trials were unconvincing
453 (Chari et al., 2013; Klein et al., 1991; Kurzrock et al., 2013; San-Miguel et al., 2014; Voorhees et al., 2013).
454 It is possible that the results of randomized trials reflect existing MM cancer cell heterogeneity and the co-
455 occurrence of IL6 dependent and independent clones (San-Miguel et al., 2014). This hypothesis is supported
456 by work demonstrating that IL6 is not crucial in physiology for the survival of all Plasma-cells (Cassese et
457 al., 2003). We found that B220^{low}CD138⁺ cells carrying single MYC or NF- κ B deregulation were IL6
458 independent *in vivo*. However, the failure of randomized trials and success of proof of principle studies
459 could be at least in part due to patient selection (Klein et al., 1991; Klein et al., 1990b). Given this
460 knowledge and the low toxicity of anti-IL6 treatment, although not curative, IL6/IL6R blocking may still
461 be beneficial for a fraction of relapsed/refractory MM patients, particularly those displaying
462 (pre)Plamablastic features and/or carrying t(8;14)[MYC-IGH].

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470 **Figure Legends**

471 **Figure 1. NF- κ B signaling and MYC over-expression synergize for hyperplasia of a Plasma-cell like**
472 **population.** See also Fig S1.

473 **(A-B)** Scheme illustrating the genetic systems used in the study. Triangles represent loxP sites. R26p and
474 CAGp represent Rosa26 promoter and CAG promoter. **(B)** Schematic representation of the protocol of the
475 study. Black arrow: immunization time-point with sheep red blood cells (SRBC); red arrow: day counting
476 after the first tamoxifen administration; Blue arrow: tamoxifen administration time-point; yellow arrow:
477 analysis time-point. **(D-F)** Representative flow cytometric analysis of Cre-mediated recombination in *C-*
478 *YFP* and *C-IM* mice at day 10 (D), day 55 (E) and day 100 (F) after the first tamoxifen administration.
479 Histograms represent CD19 expression within the recombined populations and bottom panels illustrate B-
480 cell (CD19⁺) and Plasma-cell like (CD19^{low}CD138⁺) populations within the individual reporter positive
481 populations: GFP^{neg}hCD2⁺ i.e. MYC⁺, GFP⁺hCD2^{neg} i.e. IKK2ca⁺, and GFP⁺hCD2⁺ i.e. IKK2ca⁺MYC⁺.

482

483 **Figure 2. NF- κ B or MYC expression leads to B cell lymphoma while co-deregulation to Plasma-cell**
484 **like cancers.** See also Fig S2.

485 **(A)** Cancer free survival curve for control *C-YFP* mice, and experimental *C-IKK2*, *C-MYC*, and *C-IM* mice.

486 **(B)** Representative images of spleen and mesenteric lymph nodes from aged mice of the indicated
487 genotypes. **(C)** Representative flow cytometric analysis of cancers in spleen of *C-MYC* and *C-IKK2* mice.

488 Left panels of each genotype show the frequency of reporter positive cells in the spleen; right panels of
489 each genotype show the expression of CD19 within reporter positive cells (red), and total splenocytes from
490 a control C57BL6 mouse (grey).

(D) Representative flow cytometric analysis of cancers in spleen and bone
491 marrow of *C-IM* mice. Left panels on each tissue show the frequency of double reporter positive cells; right
492 panels on each tissue show the expression of the plasma cell marker CD138 within the double reporter
493 positive population (red), and within the reporter negative population (grey).

(E) Analysis of clonality by
494 RNA sequencing. The fraction of reads mapped to each individual IgH-V gene out of all the reads mapped
495 to IgH-V genes is shown. Top panel: control plasma cells (Shi et al., 2015), bottom panel: GFP⁺hCD2⁺ *C-*

496 *IM* cancer cells (FACS purified). **(F)** Representative histological and immunohistochemical analysis of a
497 *C-YFP* control spleen (top panels) and of a cancer in the spleen of *C-IM* mice (bottom panels) for H&E,
498 B220, Pax5, Irf4, and Ki67.

499

500 **Figure 3. Cancers with NF- κ B and MYC co-deregulation display a poorly-differentiated Plasma-cell**
501 **state.**

502

503 **(A)** Schematic representation of the Plasma-cell differentiation process highlighting discrete populations
and the associated expression of B-cell and Plasma-cell factors throughout the process. **(B)** Transcriptional

504

504 analysis of *C-IM* cancers compared to discrete B-cell and Plasma-cell populations by RNAseq. GCB:
505 Germinal Center B-cells, B1: B1 B-cells, FOB: Follicular B-cells, MZB: Marginal Zone B-cells, SPLPC:
506 Spleen Plasma-cells, BMPC: Bone Marrow Plasma-cells, SPLPB: Spleen Plasmablasts (Shi et al., 2015).
507 Seven *C-IM* cancers are depicted. B-cell signature: expression profile of the top 50 downregulated genes
508 in BMPCs compared with FOBs, Plasma-cell signature: expression profile of the top 50 upregulated genes
509 in BMPCs compared to FOBs, in addition to 4 genes of particular immunological interest (*Slc3a2*, *Prdm1*,
510 *Ly6c1*, *Cd28*). Log2 FPKM expression values of genes are shown in the heatmaps, color-coded according
511 to the legend. (C) RNAseq expression data for genes involved in B-cell identity and function (*Pax5*, *Ms4a1*,
512 *CD19*) and factors related to Plasma-cell differentiation (*Prdm1*, *Irf4*, *Xpb1*, *Sdc1*, *Jchain*), in the
513 populations mentioned in (B). TPM, transcripts per million. (D) Quantitative RT-PCR for plasma cell
514 differentiation factors in seven *C-IM* cancers and spleen CD19^{low}CD138⁺ plasma cells from C57BL6 mice.
515 Data was normalized to a house-keeping gene (*Hprt1*) and then to the expression on naïve B cells (2e-
516 $\Delta\Delta Ct$).

517

518 **Figure 4. NF- κ B and MYC co-deregulation confers proliferative and survival advantage to**
519 **(pre)Plasmablasts.**

520 (A) Representative flow cytometric analysis of the proliferation profiles illustrating the frequency of
521 (pre)plasmablasts (CD138⁺) on day 3 of LPS stimulation of B-cells from the indicated genotypes *in vitro*.
522 Cell Trace Violet (CTV) dye dilution was used to assess proliferation. (B) Cell numbers of B220^{low}CD138⁺
523 (pre)plasmablasts recovered from *in vitro* cultures at the indicated time-points. Black dashed lines represent
524 control mice (control), yellow solid lines CD19^{cre} IKK2ca^{stopFL} mice (IKK2ca), blue solid lines CD19^{cre}
525 MYC^{stopFL} (MYC), and red solid lines CD19^{cre} IKK2ca^{stopFL} MYC^{stopFL} (IKK2caMYC). (C) Distribution of
526 B220^{low}CD138⁺ (pre)plasmablasts within each cell division as assessed by CTV dilution at day 4 of *in vitro*
527 cultures. Mice genotypes are represented as in (B). (D) Frequency of cleaved caspase-3⁺ within
528 CD19^{low}CD138⁺ in spleen of mice assessed by *ex vivo* intracellular flow cytometric analysis. Mice of the
529 indicated genotypes were analyzed between 16 and 29 weeks of age.

530

531 **Figure 5. NF- κ B and MYC co-deregulation render cells addicted to IL6 for survival.** See also Fig S3.

532 (A) Enrichment for genes in the signature “Hallmark_Apoptosis” using GSEA and the GEP of spleen
533 CD19^{low}CD138⁺ Plasma-cells from C57BL6 mice and *C-IM* cancer cells. (B) Pathway analysis enrichment
534 (Metacore) of the GEP of *C-IM* cancer cells compared to spleen CD19^{low}CD138⁺ Plasma-cells. The top 5
535 enriched pathways are shown. (C) RNAseq expression data for genes related to the IL-6 signaling pathway,
536 *Socs3* and *Il6st*. GCB: Germinal Center B-cells, B1: B1 B-cells, FOB: Follicular B-cells, MZB: Marginal
537 Zone B-cells, SPLPC: Spleen Plasma-cells, BMPC: Bone Marrow Plasma-cells, SPLPB: Spleen

538 Plasmablasts (Shi et al., 2015), *C-IM*: GFP⁺hCD2⁺ *C-IM* cancer cells (FACS purified). **(D)** Representative
539 flow cytometric analysis of the proliferation profiles illustrating the frequency of (pre)plasmablasts
540 (CD138⁺) on day 2, 3, and 4 of LPS stimulation of B-cells from CD19cre IKK2ca^{stopFL} MYC^{stopFL}
541 (IKK2caMYC) *in vitro*, in the presence of either control antibody (control Ig) or anti-IL6 neutralizing (anti-
542 IL6). Cell Trace Violet (CTV) dye dilution was used to assess proliferation. **(E)** MFI of pSTAT3 in
543 B220^{low}CD138⁺ (pre)plasmablasts at day 2 of *in vitro* culture of B-cells from the indicated genotypes. Black
544 circles represent control mice (WT), yellow circles CD19cre IKK2ca^{stopFL} mice (IKK2), blue circles
545 CD19cre MYC^{stopFL} (MYC), and red circles CD19cre IKK2ca^{stopFL} MYC^{stopFL} (IKK2MYC). White
546 background: control Ig, dashed background: anti-IL6. **(F)** Fold change (anti-IL6/control Ig) of the fraction
547 of cleaved caspase 3⁺ cells within B220^{low}CD138⁺ (pre)plasmablasts at day 2 of *in vitro* culture of B-cells
548 from the indicated genotypes as in (E). **(G)** MFI of BCL-xL in B220^{low}CD138⁺ (pre)plasmablasts at day 2
549 of *in vitro* culture of B-cells from the indicated genotypes as in (E). White background: control Ig, dashed
550 background: anti-IL6. **(H)** MFI of MCL-1 in B220^{low}CD138⁺ (pre)plasmablasts at day 2 of *in vitro* culture
551 of B-cells from the indicated genotypes as in (E). White background: control Ig, dashed background: anti-
552 IL6. **(I)** Principal component (PC) analysis plots of RNA-seq analysis of activated B cells (B220⁺) and
553 B220^{low}CD138⁺ (pre)plasmablasts at day 3 of *in vitro* culture of B-cells from CD19cre IKK2ca^{stopFL}
554 MYC^{stopFL} (IKK2.MYC). **(J)** Enrichment for genes in the signature “Hallmark_Apoptosis” using GSEA
555 and the GEP of B220^{low}CD138⁺ (pre)plasmablasts at day 3 of *in vitro* culture of B-cells from CD19cre
556 IKK2ca^{stopFL} MYC^{stopFL} in the presence of control Ig (IKK2.MYC control Ig) or anti-IL6 antibody
557 (IKK2.MYC anti IL6). **(K)** Cancer free survival curve for *C-IM* mice treated with control Ig (solid red line)
558 or anti-IL6 antibody (dashed red line).

559
560 **Figure 6. IL6 is critical for the formation of NF- κ B⁺MYC⁺ cancers at a poorly-differentiated Plasma-**
561 **cell state.** See also Fig S4.

562 **(A)** Representative flow cytometric analysis of Cre-mediated recombination in spleen of *C-IM* and *C-*
563 *IM-IL6KO* mice at day 100 after the first tamoxifen administration (protocol of study as in Fig. 1C). **(B)**
564 Frequency in spleen of reporter positive populations: GFP^{neg}hCD2⁺ i.e. MYC⁺, GFP⁺hCD2^{neg} i.e. IKK2ca⁺,
565 and GFP⁺hCD2⁺ i.e. IKK2ca⁺MYC⁺. **(C)** Frequency in spleen of CD19^{low}CD138⁺ cells within each reporter
566 positive populations as in (B) and within reporter negative cells. **(D)** Frequency in spleen of cleaved caspase
567 3⁺ CD19^{low}CD138⁺ cells within each reporter positive populations as in (B) and within reporter negative
568 cells. **(E)** Frequency in spleen of CD19^{low}CD138⁺ cells at the S/G2M phase of the cell cycle within each
569 reporter positive populations as in (B) and within reporter negative cells. **(F)** Cancer free survival curve for
570 *C-IM* and *C-IM-IL6KO* mice. **(G)** Frequency of cleaved caspase-3⁺ within CD19^{low}CD138⁺ cancer cells of
571 spleen of *C-IM* and *C-IM-IL6KO*. **(H)** Enrichment for genes in the signature “Hallmark_Apoptosis” using

572 GSEA in the GEP of *C-IM-IL6KO* and *C-IM* cancer cells (GFP⁺hCD2⁺ FACS sorted). **(I)** Transcriptional
573 analysis of *C-IM* and *C-IM-IL6KO* cancer cells compared to discrete B-cell and Plasma-cell populations by
574 RNAseq. GCB: Germinal Center B-cells, B1: B1 B-cells, FOB: Follicular B-cells, MZB: Marginal Zone
575 B-cells, SPLPC: Spleen Plasma-cells, BMPC: Bone Marrow Plasma-cells, SPLPB: Spleen Plasmablasts
576 (Shi et al., 2015). Seven *C-IM* and six *C-IM-IL6KO* cancers are depicted. B-cell signature: expression
577 profile of the top 50 downregulated genes in BMPCs compared with FoBs, Plasma-cell signature:
578 expression profile of the top 50 upregulated genes in BMPCs compared to FOBs, in addition to 4 genes of
579 particular immunological interest (*Slc3a2*, *Prdm1*, *Ly6c1*, *Cd28*). Log₂ FPKM expression values of genes
580 are shown in the heatmaps, color-coded according to the legend. **(J)** Analysis of clonality by RNA
581 sequencing. The fraction of reads mapped to each individual IgH-V gene out of all the reads mapped to
582 IgH-V genes is shown (Shi et al., 2015). Top panel: a GFP⁺hCD2⁺ *C-IM-IL6KO* B-cell cancer, bottom
583 panel: a GFP⁺hCD2⁺ *C-IM-IL6KO* Plasma-cell cancer. **(K)** Enrichment for genes in the signature
584 “Bcell_signature_Shi” using GSEA of the GEP of *C-IM* and *C-IM-IL6KO* (B-cell cancers) cancer cells. **(L)**
585 RNAseq expression data for factors related to Plasma-cell differentiation (*Prdm1*, *Irf4*, *Xpb1*) and B-cell
586 identity (*Pax5*) in cancer cells of *C-IM* and *C-IM-IL6KO* (B-cell cancers). **(M)** Enrichment for genes in the
587 signature “Plasmablast_signature_Shi” using GSEA of the GEP of *C-IM* and *C-IM-IL6KO* (Plasma-cell
588 cancers) cancer cells. **(N)** RNAseq expression data for factors related to Plasma-cell differentiation (*Prdm1*,
589 *Irf4*, *Xpb1*) and B-cell identity (*Pax5*) in cancer cells of *C-IM* and *C-IM-IL6KO* (Plasma-cell cancers).

590

591 **Figure 7. NF-κB/MYC⁺ cancers are alike a fraction of PBL and linked to t(8;14)[MYC- IGH] MM at**
592 **the gene expression level.** See also Fig S5.

593 **(A)** Enrichment for genes in the signature “Plasmablast_signature_Shi” (left) and “PC_signature_Shi”
594 (right) using GSEA of the GEP of B220^{low}CD138⁺ (pre)Plasmablasts at day 3 of *in vitro* culture of B-cells
595 from CD19cre IKK2ca^{stopFL} and CD19cre IKK2ca^{stopFL} MYC^{stopFL} mice. **(B)** RNAseq expression data for
596 factors related to Plasma-cell differentiation (*Prdm1*, *Irf4*, *Xpb1*) and B-cell identity (*Pax5*) in
597 B220^{low}CD138⁺ (pre)Plasmablasts at day 3 of *in vitro* culture of B-cells from CD19cre IKK2ca^{stopFL} and
598 CD19cre IKK2ca^{stopFL} MYC^{stopFL} mice. **(C)** Left, representative immunohistochemical analysis of human
599 biopsies of tonsil and PBL for MYC, nuclear NF-κB p50, nuclear NF-κB p52, and phospho STAT3
600 (pSTAT3). A cutoff of >30% positive cells was used to determine positivity. Right, cumulative data of the
601 immunohistochemical analysis. **(D)** Correlation between the gene enrichment of the “Myc.IKK2.muTex”
602 signature with MM patient overall survival (left) and progression free survival (right; MMRF dataset). **(E)**
603 Association between the “Myc.IKK2.muTex” signature and MM chromosomal abnormalities.

604

605

606 **Supplemental Figure Legends.**

607 **Supplemental Figure 1. Dynamics of reporter positive subpopulations over-time.**

608 (A) Representative flow cytometric analysis of Cre-mediated recombination in the blood of *C-YFP*, *C-*
609 *MYC*, *C-IKK2*, and *C-IM* at days 55, 95, 135, 175 after the first tamoxifen administration. (B) Fraction of
610 cells within the individual reporter positive populations: YFP⁺ in *C-YFP* mice and GFP^{neg}hCD2⁺ i.e. MYC⁺,
611 GFP⁺hCD2^{neg} i.e. IKK2ca⁺, and GFP⁺hCD2⁺ i.e. IKK2ca⁺MYC⁺ in *C-IM* mice. Frequencies were
612 normalized to day 55 after the first tamoxifen administration. (C) Left, fraction of GFP^{neg}hCD2⁺ i.e. MYC⁺
613 in *C-MYC* mice. Right, fraction of GFP⁺hCD2^{neg} i.e. IKK2ca⁺ in *C-IKK2* mice. Frequencies were
614 normalized to day 55 after the first tamoxifen administration.

615

616 **Supplemental Figure 2. Characterization of *C-IM* cancer properties.**

617 (A) Southern blot analysis of cancer clonality using a JH4 probe. Dashed red line represents germline IgH
618 configuration. Red arrow denotes clonal IgH. (B) ELISA of total serum IgM, IgG1 and IgG2b concentration
619 in aged *C-YFP* and *C-IM* mice. (C) Serum protein electrophoresis of representative samples from aged *C-*
620 *YFP* and *C-IM*. The position of albumin and of various globulin components of the serum is indicated.

621

622 **Supplemental Figure 3. (pre)Plasmablasts are a source of IL6**

623 (A) Representative flow cytometric analysis of intracellular staining for IL6 in *in vitro* day3 LPS stimulated
624 B-cells from the indicated genotypes. Top panels: of activated B cells (B220⁺), Bottom panels:
625 B220^{low}CD138⁺ (pre)Plasmablasts. (B) Distribution of B220^{low}CD138⁺ (pre)plasmablasts within each cell
626 division as assessed by CTV dilution at day 4 of *in vitro* cultures in the presence of control Ig or anti-IL6
627 neutralizing antibody. Control mice (WT), CD19cre MYC^{stopFL} (MYC), CD19cre IKK2ca^{stopFL} mice
628 (IKK2ca), CD19cre IKK2ca^{stopFL} MYC^{stopFL} (IKK2MYC).

629

630 **Supplemental Figure 4. Analysis of *C-IM-IL6KO* mice.**

631 (A) Left, frequency in bone marrow of *C-IM* and *C-IM-IL6KO* mice at day 100 after first tamoxifen
632 injection of reporter positive populations: GFP^{neg}hCD2⁺ i.e. MYC⁺, GFP⁺hCD2^{neg} i.e. IKK2ca⁺, and
633 GFP⁺hCD2⁺ i.e. IKK2ca⁺MYC⁺. Right, frequency in bone marrow of *C-IM* and *C-IM-IL6KO* mice at day
634 100 after first tamoxifen injection of CD19^{low}CD138⁺ cells within each reporter positive populations and
635 within reporter negative cells. (B) Representative histological and immunohistochemical analysis of a B-
636 cell cancer in the spleen of a *C-IM-IL6KO* mice (top panels) and of a Plasma-cell cancer in the spleen of *C-*
637 *IM-IL6KO* mice (bottom panels) for H&E, B220, Pax5, Irf4, and Ki67.

638

639 **Supplemental Figure 5. Gene signature enrichment in MM.**

640 **(A)** Correlation between the enrichment of a “NF-κB signature” (PASQUALUCCI) and the “NF-κB
641 signature” (STAUDT) with MM patient overall survival (MMRF dataset). **(B)** Correlation between the
642 enrichment of a “STAT3 signature” (STAUDT) with MM patient overall survival (MMRF dataset). **(C)**
643 Correlation between the enrichment of multiple MYC signatures with MM patient overall survival (MMRF
644 dataset). **(D)** Venn diagram showing the overlap in genes upregulated in the GEP of B220^{low}CD138⁺ cells
645 derived *in vitro* from B-cells of CD19cre IKK2ca^{stopFL} mice (IKK2), CD19cre MYC^{stopFL} (MYC), CD19cre
646 IKK2ca^{stopFL} MYC^{stopFL} (IKK2MYC) mice. **(E)** Correlation between the enrichment of the “IKK2.muTex”
647 signature with MM patient overall survival (left) and progression free survival (right; MMRF dataset).
648 Association between the “IKK2.muTex” signature and MM chromosomal abnormalities. **(F)** Correlation
649 between the enrichment of the “Myc.muTex” signature with MM patient overall survival (left) and
650 progression free survival (right; MMRF dataset). Association between the “Myc.muTex” signature and
651 MM chromosomal abnormalities.

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673 **Materials and methods**

674 **Mouse models and tumor cohorts:** CD19^{creERT2}, CD19^{cre}, IKK2^{ca^{stop}FL}, MYC^{stopFL}, IL6^{KO/KO}, and
675 YFP^{stopFL} alleles have been previously described. For T cell-dependent immunization, 8- to 12-
676 week old mice were injected intravenously with 10⁹ defibrinated Sheep Red Blood Cells (SRBCs)
677 (TCS Bioscience) in PBS. Mice were administered tamoxifen by oral gavage, dissolved in
678 sunflower seed oil (both from Sigma). For full details on the experimental protocol, please see
679 Figure 1E. Mouse cohorts were monitored twice a week for tumor development and euthanized if
680 signs of tumor development occurred. Experiments were conducted using age-matched animals.
681 Sex/gender was randomly distributed. All mice were on the C57BL/6 background. Mice were bred
682 at The Francis Crick Institute under specific pathogen-free conditions. All animal experiments
683 were carried out in accordance with national and institutional guidelines for animal care and were
684 approved by The Francis Crick Institute Biological Resources Facility Strategic Oversight
685 Committee (incorporating the Animal Welfare and Ethical Review Body) and by the Home Office,
686 UK.

687
688 **In vivo treatment with anti-IL6 neutralizing antibody:** To study the impact of IL6 on cancer
689 progression, C-IM mice were treated with InVivoMab anti-mouse IL-6 (bioXcell), or a control
690 antibody (InVivoMab rat IgG1 isotype control, anti-horseradish peroxidase; bioXcell).
691 Administration of the antibodies was performed intraperitoneally three times per week for three
692 weeks, starting on day 150 after tamoxifen administration. Mice were given 200ug of antibody per
693 injection, diluted in InVivoPure dilution buffer, as per manufacturer's instructions.

694
695 **In vitro stimulations:** B cells were isolated from splenocyte suspensions using CD43 microbeads
696 (Miltenyi Biotec), and plated at a density of 1x10⁶/mL in B-cell media (DMEM high glucose with
697 Glutamax (Gibco), supplemented with Fetal Bovine Serum (GE Healthcare), non-essential
698 aminoacids (Gibco), HEPES buffer (Gibco), sodium pyruvate (Gibco), Penicillin-Streptomycin
699 (Gibco), and 2-Mercaptoethanol (Sigma-Aldrich)). B cells were stimulated with
700 Lipopolysaccharide (LPS; Sigma-Aldrich) at a concentration of 10ug/mL. Where indicated, LPS
701 stimulation was performed in the presence of blocking anti-IL-6 antibody or control IgG
702 (Biolegend), at a concentration of 1ug/mL.

703

704 **Histology and Immunohistochemistry:** Mouse spleens were fixed with 10% neutral buffered
705 formalin (NBF) (Fischer Scientific) and embedded in paraffin. Sections were stained with
706 hematoxylin and eosin (H&E) (Sigma). Antigen retrieval was performed with citrate buffer pH6,
707 23 minutes in the microwave. Ki67 staining was performed using the Ventana Discovery Ultra,
708 CC1 for 48 minutes. All slides were counterstained with Harris Haematoxylin (Fisher Scientific),
709 in Tissue Tek Prisma staining machine. Images were acquired with Zeiss Axio Scan.Z1 Slide
710 Scanner and visualized with ZEN lite Blue Edition (Zeiss). For human Plasmablastic samples,
711 tumor biopsies were fixed with 10% NBF and embedded in paraffin. Antigen retrieval (heat-
712 induced epitope retrieval) was performed with citrate buffer using a pressure cooker and a
713 commercial unmasking solution (Vector labs). The detection system used was from Biogenex
714 (Super Sensitive Polymer HRP IHC Detection System). Images were acquired with the
715 Panoramic 250 Flash Scanner, uploaded into an online server (Casecenter) and visualized using
716 Panoramic Viewer (all 3DHistec).

717
718 **Flow cytometry:** Single-cell suspensions of spleen and bone marrow were prepared in FACS
719 buffer (2% FBS, 2 mM EDTA), in PBS (Gibco) and were treated with ACK Lysing Buffer (Gibco)
720 for erythrocyte lysis. Single-cell suspensions were stained with antibodies, see Key Resources
721 Table for details. Dead cells were excluded using Zombie NIR™ Fixable Viability Kit
722 (BioLegend). For the assessment of cell division, cells were stained with CellTrace™ Violet
723 (Invitrogen) as per manufacturer's specifications, prior to in vitro stimulation. For the detection of
724 cleaved caspase-3, MCL1 and BCL-xL, samples were fixed for 20 min on ice after surface marker
725 and viability dye staining, followed by intracellular staining using BD Cytotfix/Cytoperm staining
726 kit (BD Biosciences) as per manufacturer's specifications. For the detection of STAT3 and
727 phospho STAT3, samples were fixed for 15 min at 37°C after surface marker and viability dye
728 staining using Fixation Buffer (Biolegend), followed by permeabilization for 1h at -20°C with
729 True-Phos™ Perm Buffer (Biolegend) as per manufacturer's specifications. For the detection of
730 IL-6, cells were cultured in the presence of brefeldin A (Sigma) for 4h, followed by fixation for
731 15 min at room temperature in 4% paraformaldehyde (Thermo Fisher). Samples were acquired on
732 an LSR-Fortessa (BD Biosciences) with FACS-Diva software (BD Biosciences) and data were
733 analyzed with FlowJo software (v10.3, Tree Star).

734

735 **Gene expression analysis:** For gene expression profiling of C-IM and C-IM-IL6KO tumors,
736 reporter positive cells (GFP+ hCD2+) were FACS sorted using a FACS Aria III or a FACS Aria
737 Fusion (BD Biosciences). For gene expression profiling of in vitro derived activated B cells and
738 plasmablasts, cells were FACS sorted at day 3 of LPS stimulation according to the expression of
739 CD19 and CD138 markers (activated B cells – CD19⁺ CD138^{neg}; plasmablasts – CD19^{low}
740 CD138⁺). RNA was extracted using AllPrep DNA/RNA Mini and Micro Kits (Qiagen) as per
741 manufacturer’s specifications. RNA sequencing was performed at The Francis Crick Institute
742 Advanced Sequencing Unit. RNA sequencing was carried out on the Illumina HiSeq 2500 and
743 4000 platforms and typically generated around 25 million 101bp strand-specific paired-end reads
744 per sample. Adapter trimming was performed with cutadapt (version 1.9.1) (Martin M, 2011) with
745 parameters “--minimum-length=25 --quality-cutoff=20 -a AGATCGGAAGAGC –A
746 AGATCGGAAGAGC”. The RSEM package (version 1.3.0) (Li and Dewey, 2011) in conjunction
747 with the STAR alignment algorithm (version 2.5.2a) (Dobin et al., 2013) was used for the mapping
748 and subsequent gene-level counting of the sequenced reads with respect to mm10 Ensembl genes
749 downloaded from the UCSC Table Browser (Karolchik et al., 2004) on 19th February 2016. The
750 parameters used were “--star-output-genome-bam –paired-end --forward-prob 0”. Differential
751 expression analysis was performed with the DESeq2 package (version 1.12.3) (Love et al., 2014)
752 within the R programming environment (version 3.3.1) (REF). An adjusted p-value of ≤ 0.05 was
753 used as the significance threshold for the identification of differentially expressed genes. The
754 Subjunc aligner from the Subread package (version 1.5.1) (Liao et al., 2013) was used for the
755 quantification of Ighv transcripts as described in Shi et al., 2015. The parameters used were “--
756 allJunctions -I 16 -u”. Gene set enrichment analysis (GSEA) (version 2.2.3) (Subramanian et al.,
757 2005) pre-ranked analysis was performed using the Wald statistic with respect to custom signatures
758 obtained from the literature. All parameters were kept as default except for enrichment statistic
759 (classic), min size (5) and max size (50 000). Gene set enrichment analysis for differentially
760 expressed genes was performed by Gene Ontology Pathway and Biological processes using
761 GeneGo MetaCore (<https://portal.genego.com/>).

762

763 **Quantitative real time PCR:** Total RNA was reverse transcribed using the SuperScript® III First-
764 Strand Synthesis System with Oligo(dT)₂₀ (Invitrogen). For qRT-PCR analysis, the Power SYBR
765 Green Master Mix was used, followed by quantification with the StepOnePlus System (Applied

766 Biosystems). Samples were assayed in duplicate, and messenger abundance was normalized to that
767 of HPRT1. Primers sequences used for Prdm1, Xbp1, Sdc1 and Irf4 amplification can be found in
768 the table attached.

769
770 **Statistical analyses:** Data were analyzed using unpaired two-tailed Mann-Whitney test for two-
771 way comparisons. Statistical significance for multiple comparisons was calculated using the False
772 Discovery Rate approach by using the Two-Stage Step-Up method of Benjamini, Krieger and
773 Yekutieli. P-value of 0.05 or less was considered significant. Prism (v8, GraphPad) was used for
774 statistical analysis. Data in text and figures are represented as box plots with floating bars
775 representing min and max values, with median value represented as a line.

776
777 **Analysis of tumor clonality:** Southern blotting of EcoRI-digested genomic DNA from C-IM
778 tumors using a JH probe spanning the JH4 exon and part of the downstream intronic sequence.

779
780 **Serum protein electrophoresis:** Serum from C-IM tumor mice and C-EYFP aged mice was
781 diluted 1:2 in barbital buffer and analyzed on a Hydragel K20 system (Sebia) according to
782 manufacturer's instruction.

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Figure 1

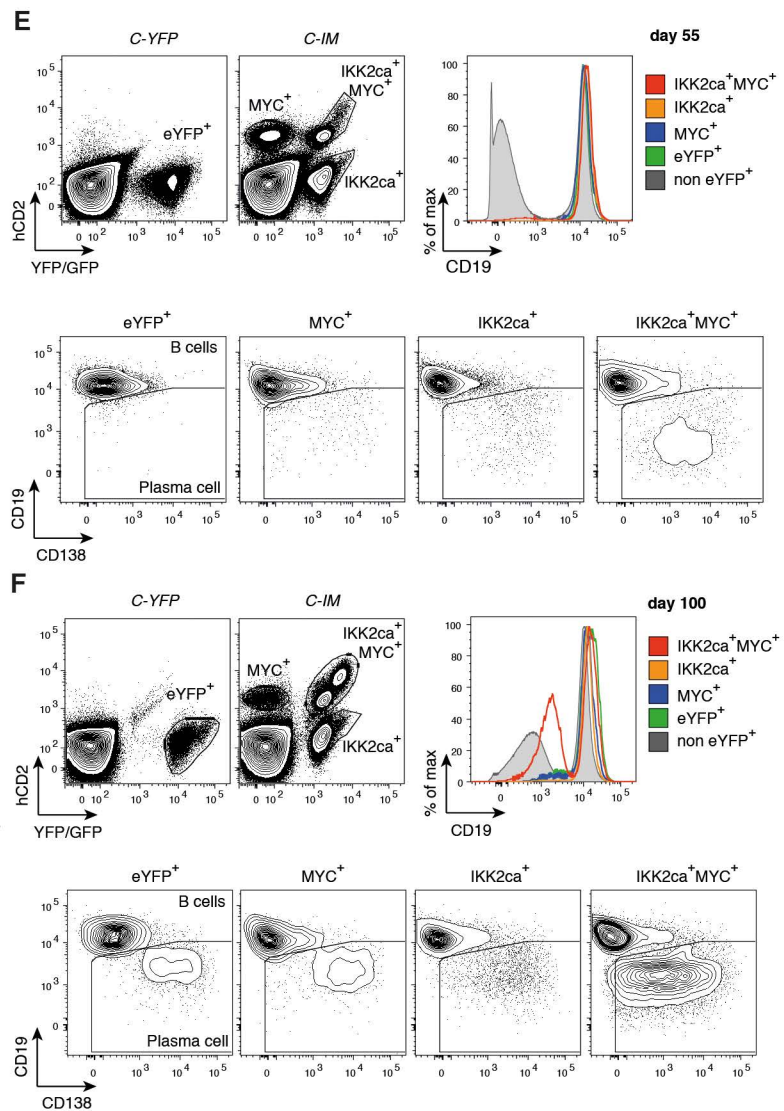
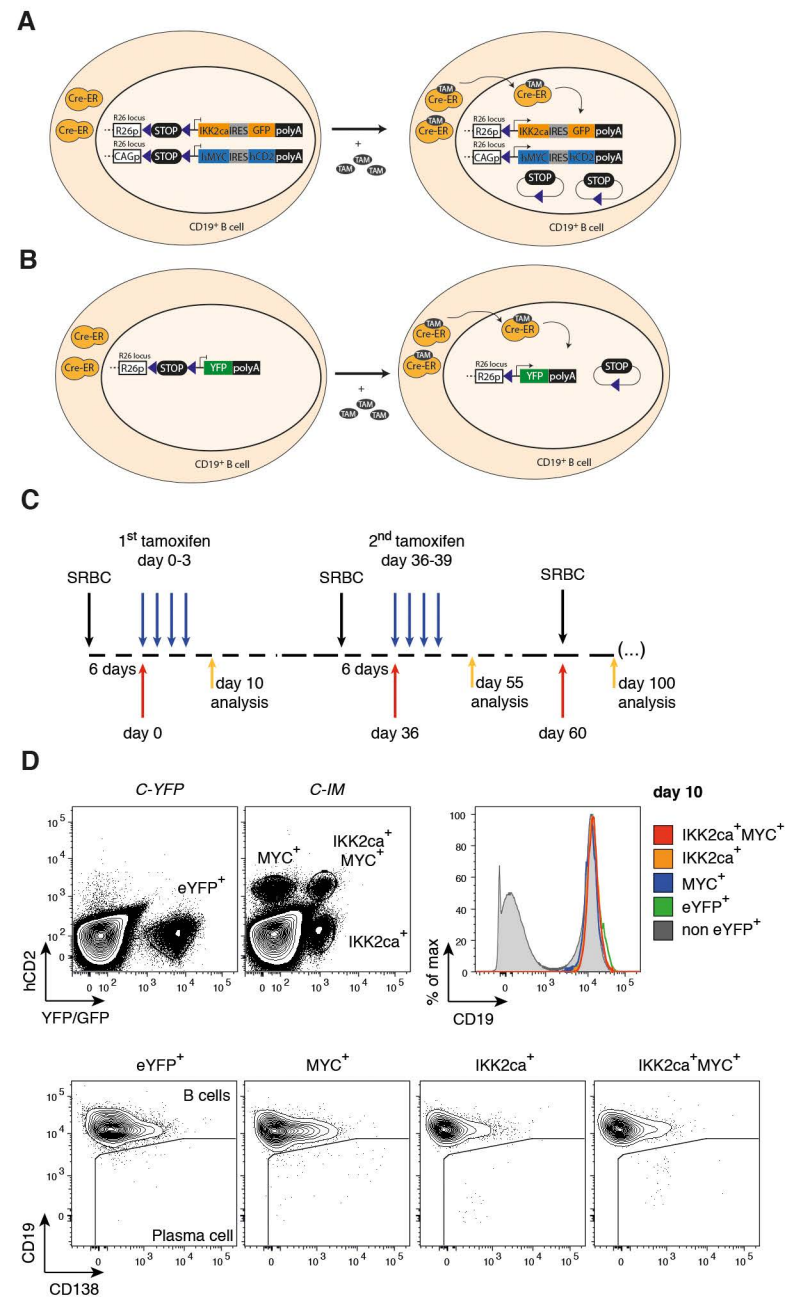


Figure 2

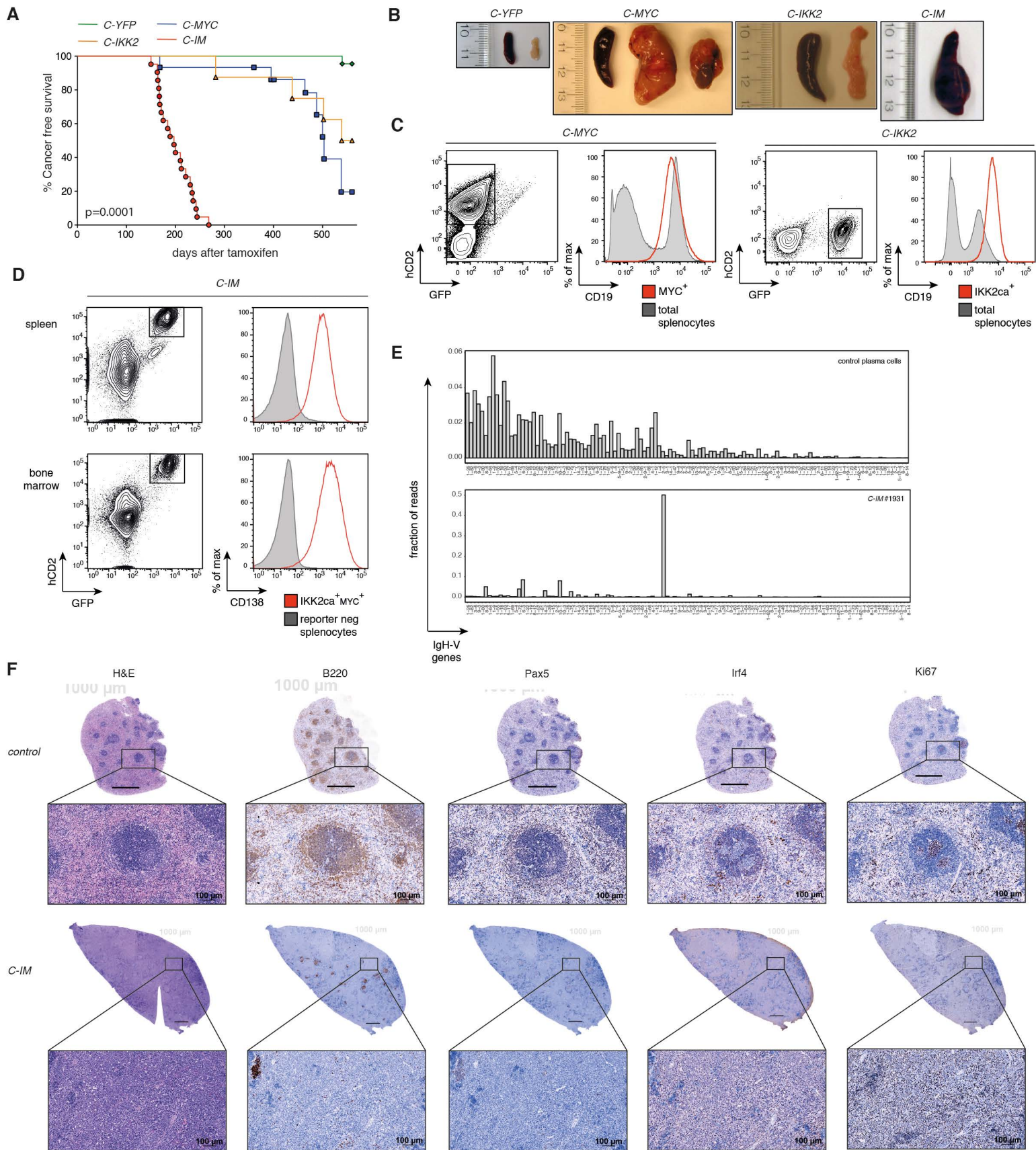
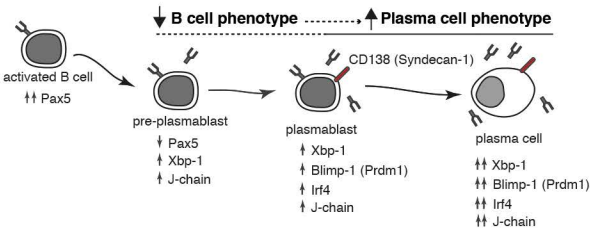
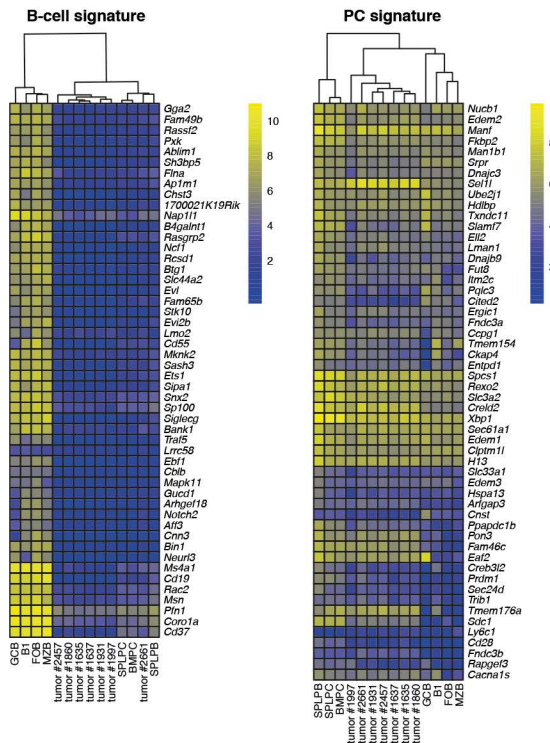


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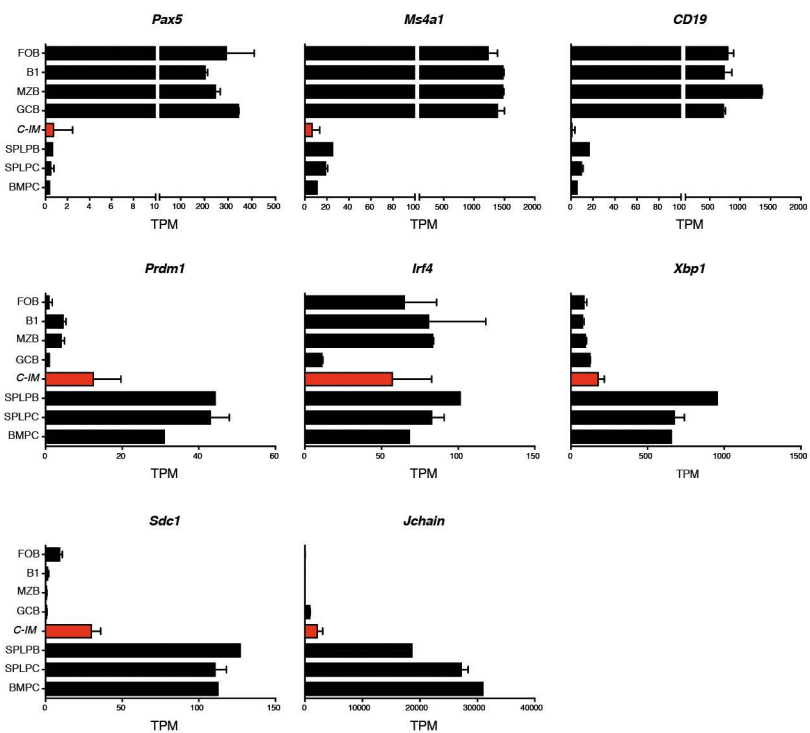
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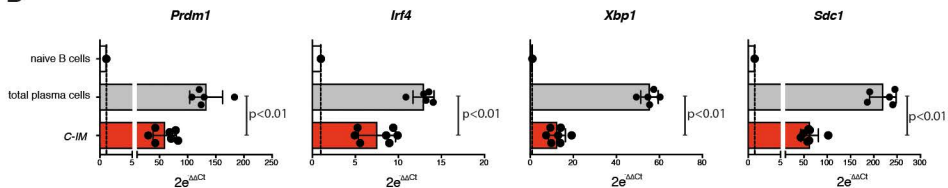
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C



D



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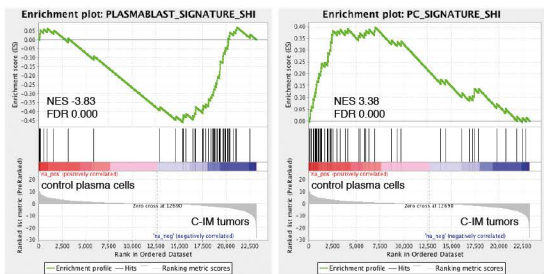
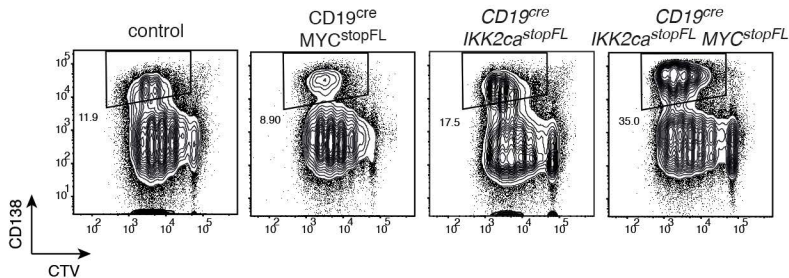
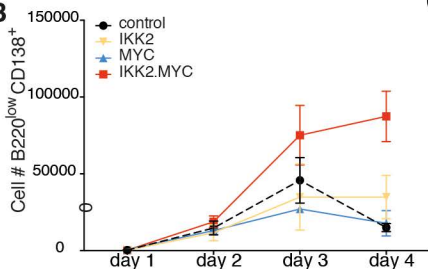


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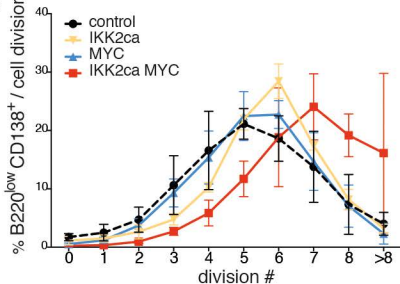
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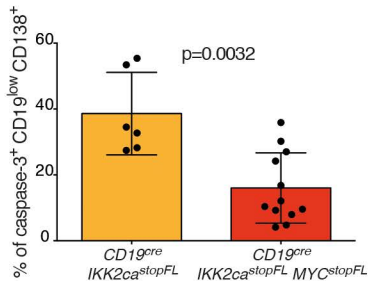


Figure 5

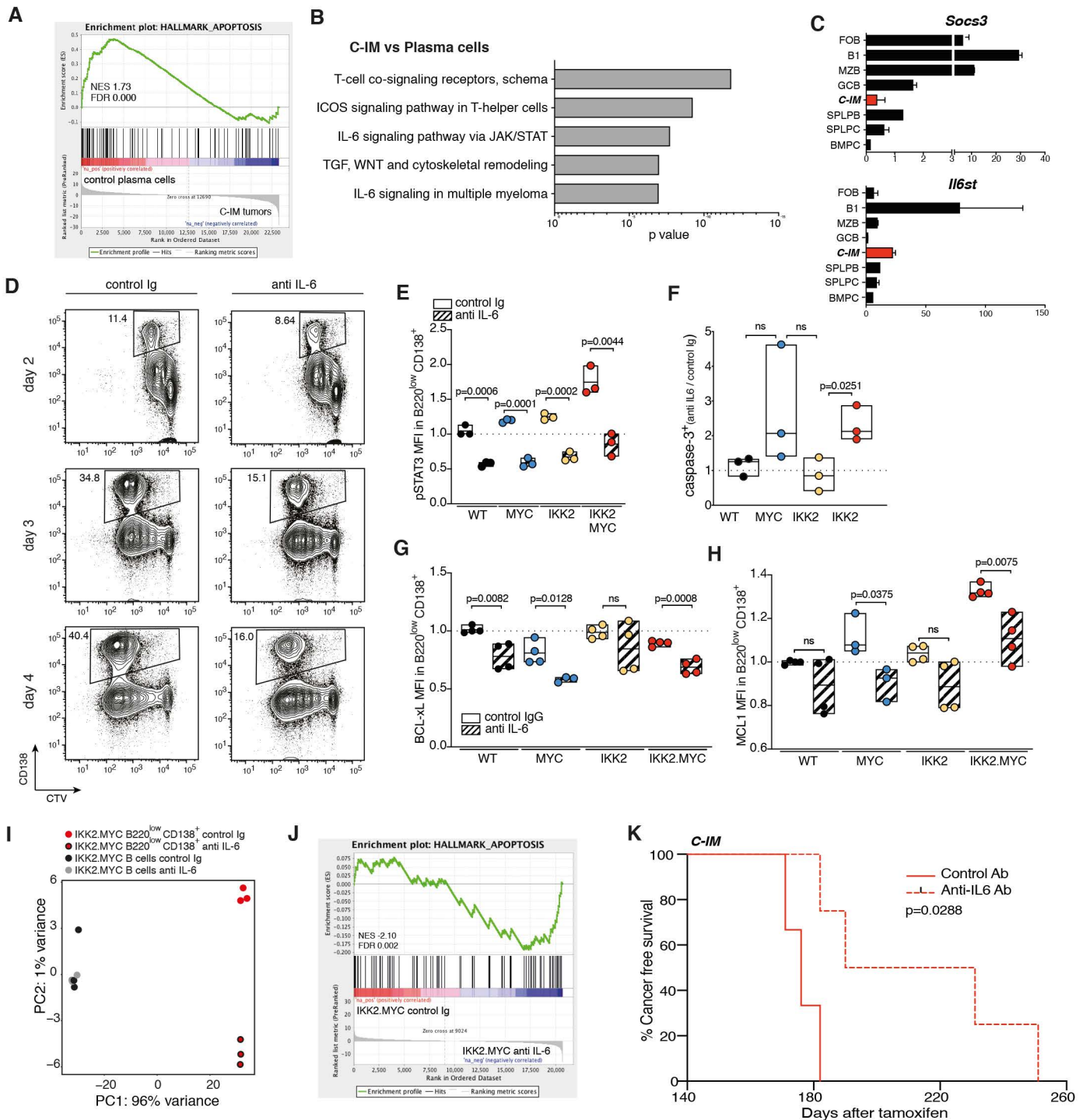


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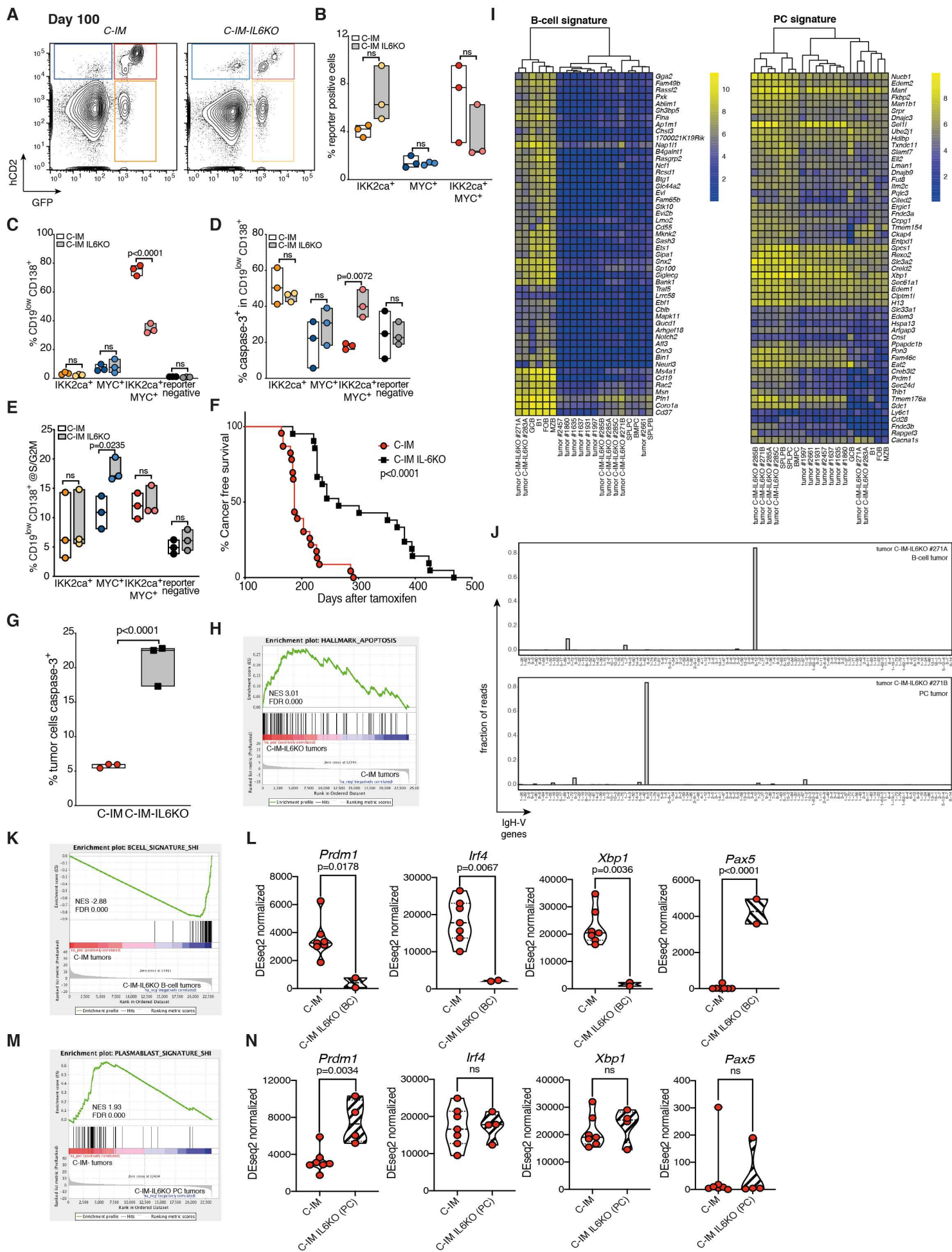
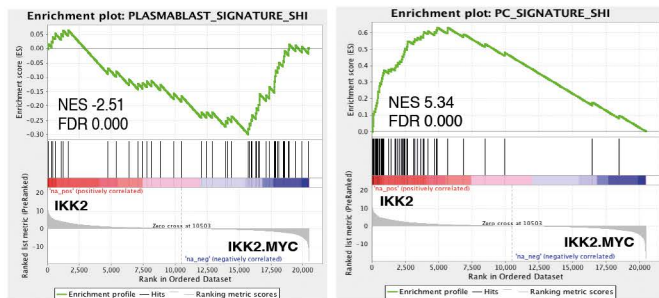
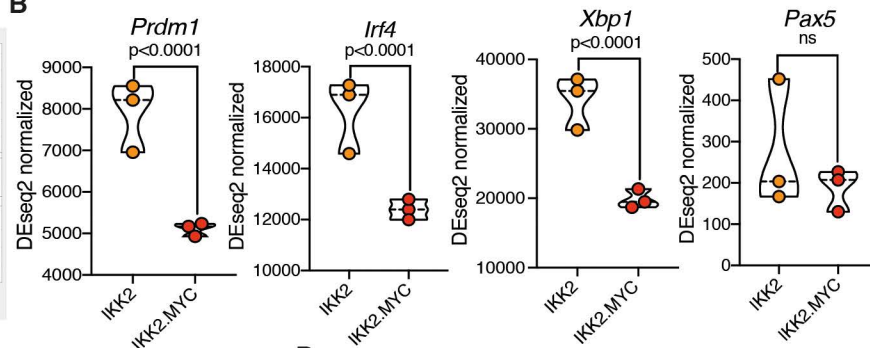


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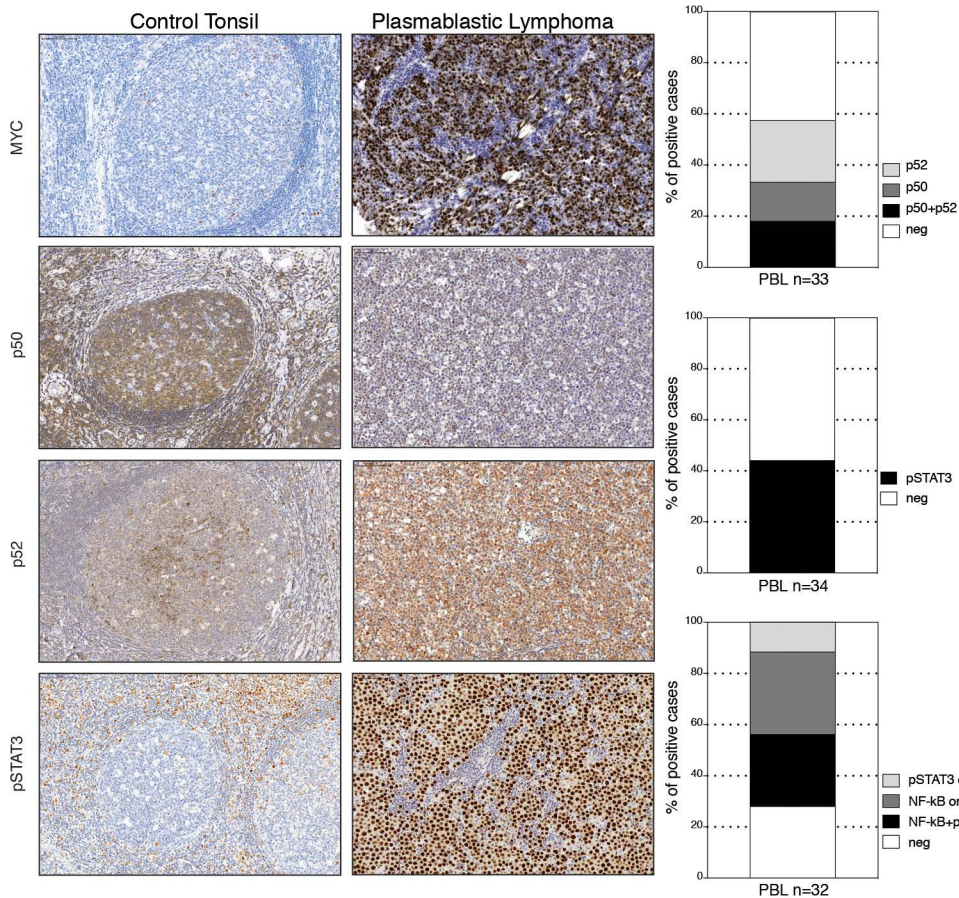
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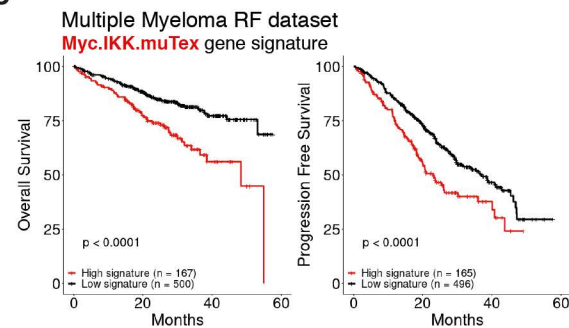
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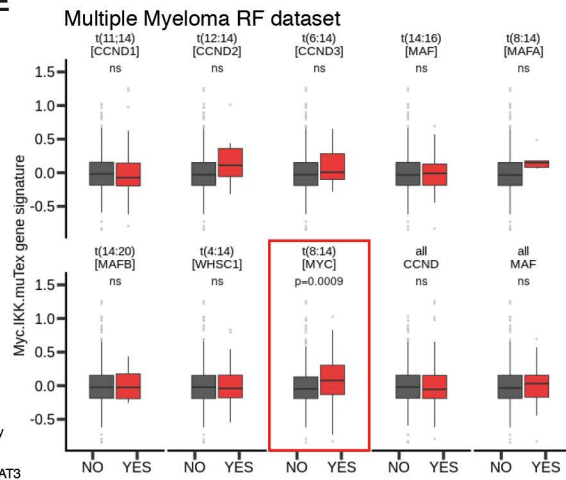
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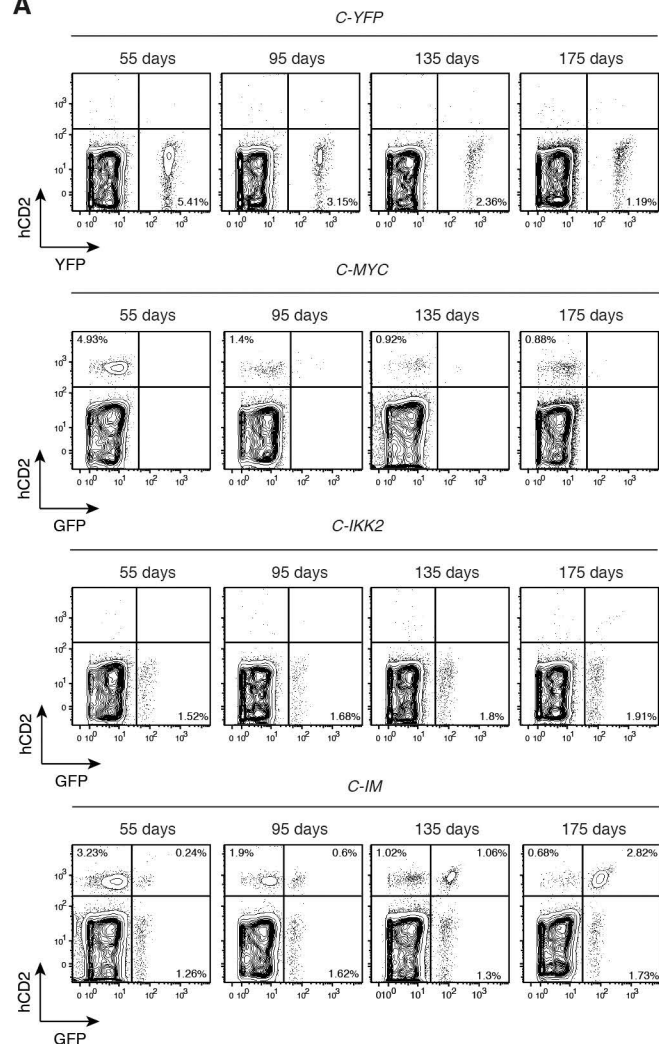


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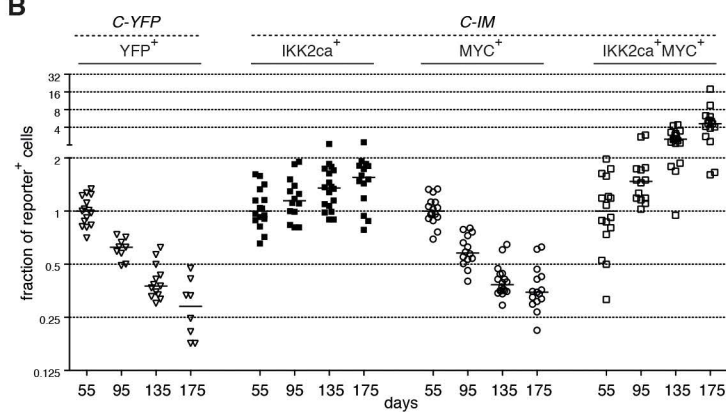


Supplemental Figure 1

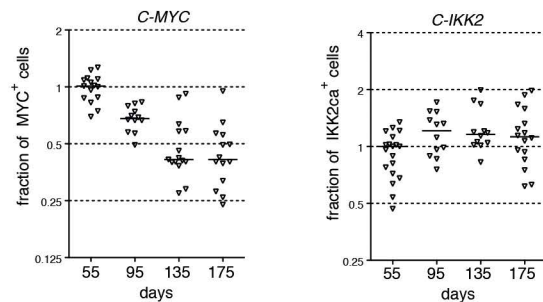
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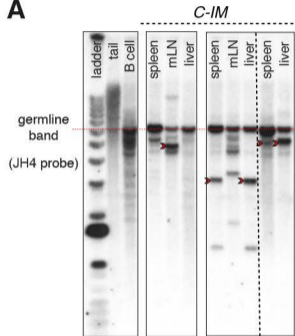


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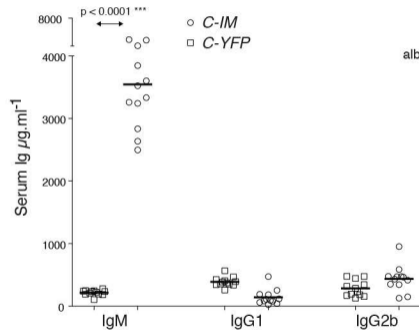


Supplemental Figure 2

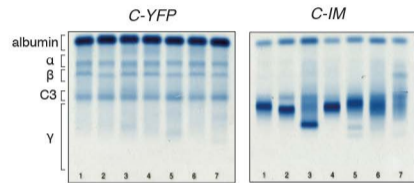
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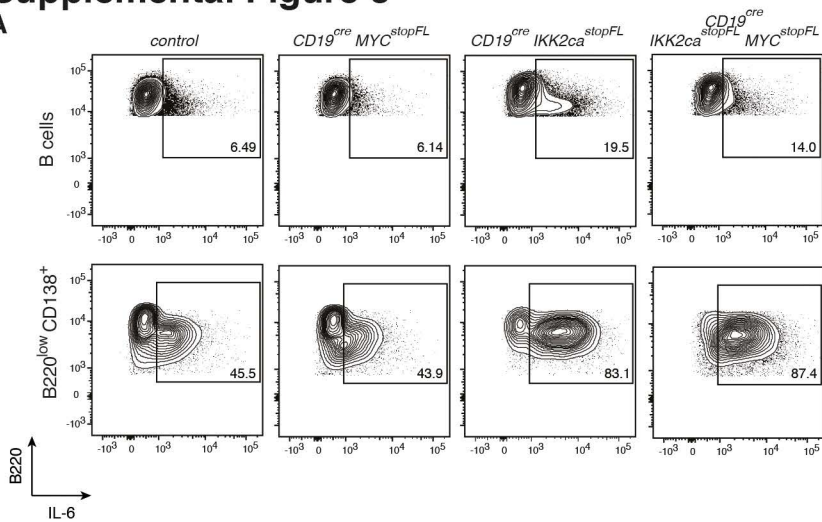
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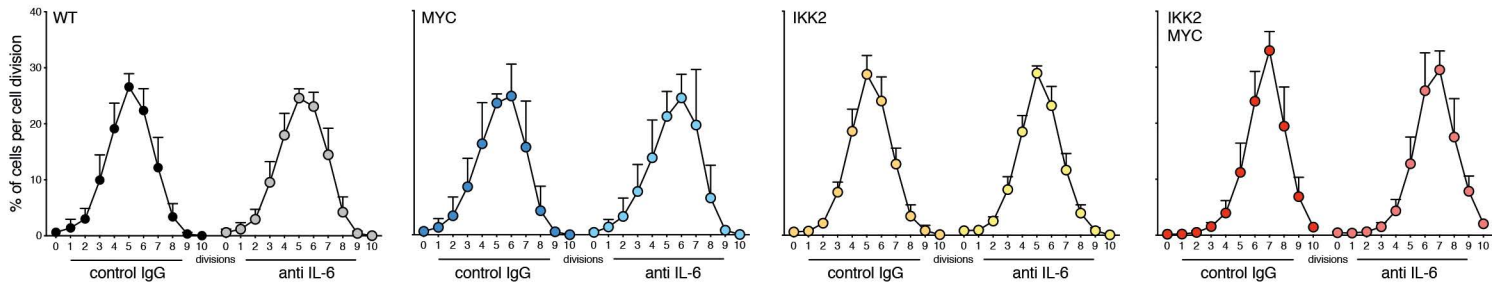
Barbosa RR et al.

Supplemental Figure 3

A

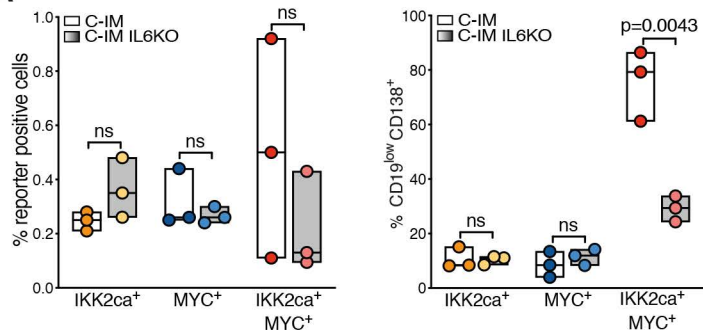


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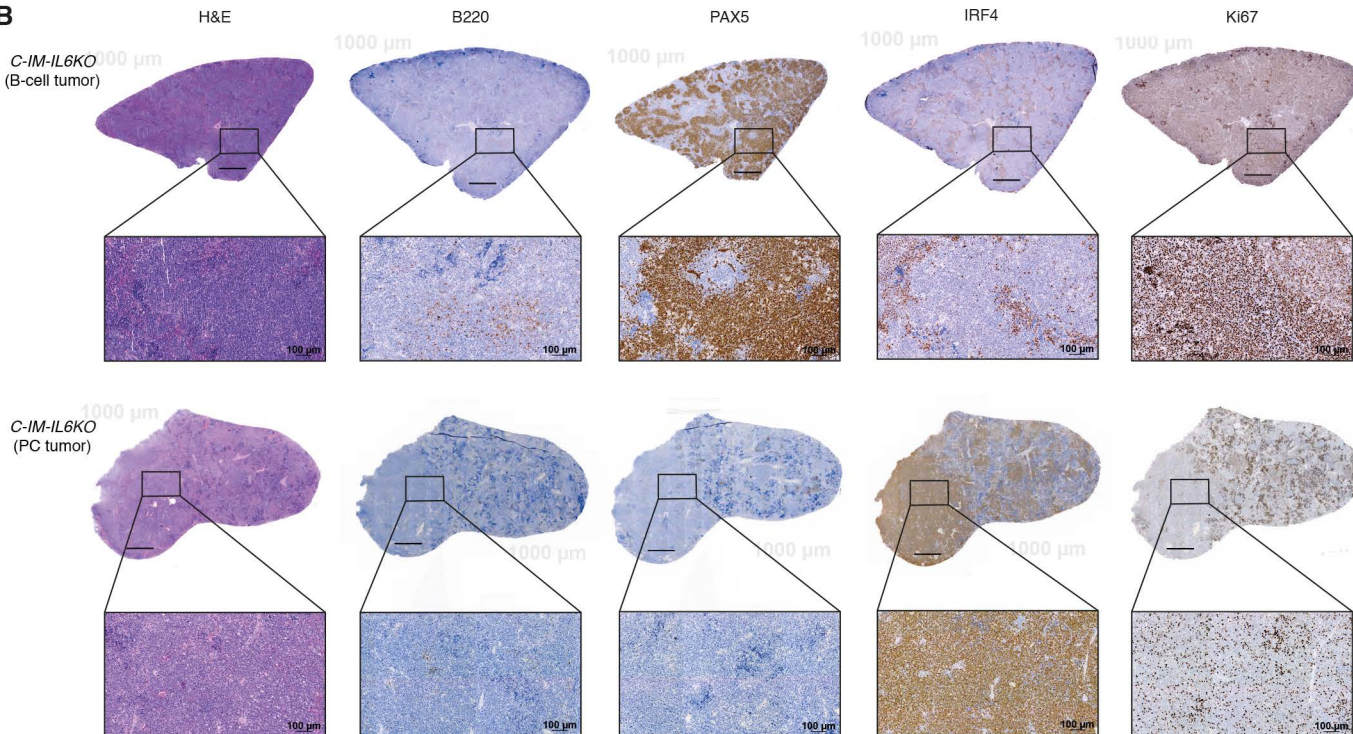


Supplemental Figure 4

A



B



Supplemental Figure 5

