## Interleukin-1 receptor associated kinase 1/4 and bromodomain and extra-terminal inhibitions converge on NF-κB blockade and display synergistic antitumoral activity in activated B-cell subset of diffuse large B-cell lymphoma with *MYD88* L265P mutation

The outcome of patients with diffuse large B-cell lymphoma (DLBCL) is very heterogeneous and is most likely dictated by their cell of origin (COO), defining two main molecular subtypes, i.e., germinal center B-cell (GCB) and activated B-cell (ABC).[1] Upon treatment with multi-agent chemotherapy (cyclophosphamide, doxorubicin, vincristine and prednisone) combined with the monoclonal anti-CD20 antibody rituximab (R-CHOP), almost a third of the patients, corresponding mainly to the ABC subtype of the disease, does not achieve complete remission (CR) or relapses shortly after CR.[2] However, the COO does not fully account for the different outcomes. Massive sequencing analyses recently uncovered molecular subtypes of DLBCL with distinct outcomes. In this regard, Chapuy *et al*. have described five different molecular subtypes with distinct pathogenic mechanisms and prognosis, independently of the COO. Interestingly, the C5 cluster (mostly ABC subtypes), enriched in *MYD88* L265P and *CD79B* mutations, maintained a shorter survival compared to the other ABC cluster.[3]

ABC-DLBCL tumors rely almost exclusively on constitutive nuclear transcription factor κB (NF-κB) signaling for their survival, a phenomenon that has been linked to a variety of genetic alterations that aberrantly activate the B-cell receptor (BCR) and the Toll-like receptor (TLR) signaling pathways.[1] Within the TLR axis, mutations in the gene codifying for the adaptor protein myeloid differentiation primary response gene 88 (*MYD88*) enhance interleukin-1 receptor-associated kinase 1 and 4 (IRAK1 and IRAK1) activity, providing sustained activation of NF-κB through most of the TLR. The p.L265P mutation, characterized by a change from leucine (CTC) to proline (CCG) in the MYD88 Toll/interleukin (IL)-1 receptor domain, recruits MYD88 to the cytoplasmic tail of TLR to form an active complex. Beside NF-κB, this complex promotes Janus kinase-signal transducer and activator of transcription 3 (JAK-STAT3) signaling through a pathway involving interleukin (IL)-6 and IL-10 secretion.[4]

Preclinical data have indicated that *MYD88*-mutant ABC-DLBCL cells were sensitive to pharmacological blockade of IRAK4 kinase activity, being IRAK4-compromised cells especially responsive to the Bruton's tyrosine kinase (BTK) inhibitor ibrutinib or the BCL-2 antagonist venetoclax, as almost all ABC-DLBCL display BCL2 amplification/overexpression.[5,6] Considering that both IRAK1 and IRAK4 are required for ABC-DLBCL cell survival,[4] we investigated the effect of a 24-72 hour treatment with a selective and orally bioavailable IRAK1/4 inhibitor (IRAKi, Merck),[7] in three well-characterized *MYD88*-mutated cell lines, OCI-LY3, OCI-LY10, HBL-1, using proliferation as a read out. Three germinal center B-cell (GCB)-DLBCL cell lines (SUDHL-4, SUDHL-8 and OCI-LY8) with wild-type MYD88 *($MYD88^{wt}$)* were analyzed in the same settings, as a control. We observed a partial and transitory response to IRAKi in ABC-DLBCL cells only, when using the compound at the physiological dose of 50 μM (Figure 1A). Treatment-related cytotoxicity decreased from 25.5% at 24 hours to 19% at 72 hours, respectively, despite an efficient blockade of IRAK1 and IRAK4 phosphorylation at Thr209 and Thr345 residues, in the three *MYD88*-mutated cell lines (Figure 1B). Interestingly, the destabilization of the anti-apoptotic protein and key mediator of IRAKi activity, MCL-1,[8] was not sufficient to confer a significant cytotoxicity to the compound (Figure 1B). A gene expression profiling (GEP) analysis in the three *MYD88*-mutated cell lines exposed for 6 hours to the inhibitor, further showed that IRAK1/4 blockade significantly altered the expression of the top NF-κB gene signatures associated to B-cell lymphoma,[9] namely *NFKB_ALL_OCI_LY10* and *NFKB_BOTH OCILY3ANDLY10*, with normalized enrichment score (NES) values reaching 1.8, while in contrast a third gene set, *NFKB_OCILY10_ONLY*, was slightly upregulated (NES: -1.20), according to GSEA analysis (Figure 1C; *Online Supplementary Table S1*). In agreement, the transcription of several NF-κB-regulated genes known to promote ABC-DLBCL pathogenesis, including *IL6, IL10, IRF4* and *CCL3*, were either unaffected or even increased after treatment with IRAKi (Figure 1D, *Online Supplementary Table S1*). Consistently, in an OCI-LY3 mouse xenograft model the compound failed to elicit a significant tumor growth inhibition (*Online Supplementary Figure S1A*).

We then considered the possibility to enhance IRAKi activity in *MYD88* L265P ABC-DLBCL by combining the compound with the BET bromodomain inhibitor CPI203 (kindly provided by Constellation Pharmaceuticals), as this BRD4 antagonist has been shown to effectively suppress a NF-κB gene signature that includes *IL6, IL10* and *IRF4*, in ABC-DLBCL.[10] After exposing the same ABC-DLBCL cell lines as above to a 50 μM dose of IRAKi, followed by a 24-hour treatment with 0.5 μM CPI203, a new GEP analysis was performed. As shown in Figure 2A, IRAKi-CPI203 combination induced a significant downregulation of NF-κB-related genes when compared to IRAKi single agent, with NES comprised between 1.46 and 1.99. Of note, the combination therapy allowed to a significant disruption of *NFKB_OCILY10_ONLY* gene signature with a NES of 1.89. Among the genes included in the *NFKB_ALL_OCILY3_LY10* gene set, a selected list of nineteen factors underwent a ≥2-fold increase in their rank metric score between this analysis and the previous one (*Online Supplementary Table S2*), suggesting that their improved modulation may be associated with the combinational effect of IRAKi and CPI203. From this list, we identified only four genes (*LTA, MARCKS, CD44* and *HEATR1*) that were not included in the core component of the NF-κB target genes affected by either IRAKi or CPI203 as single agents, but which underwent a significant downregulation upon treatment with the drug combination. Among these genes, we were unable to detect significant levels of *LTA* and *HEATR1* transcripts in the three ABC-DLBCL cell lines (*data not shown*). In contrast, upon exposure of the three *MYD88*-mutated cell lines to the IRAKi we observed a 1.2- to 2-fold transcriptional increase of *MARCKS* and *CD44*, together with *IL6* and *IL10* used here as hallmarks of NF-kB activation. These genes were all reduced down to 0.5-fold in cells treated with the drug combination (Figure 2B). Accordingly, IRAKi-CPI203 treatment led to the accumulation of the intracellular inhibitor of NF-κB, IκB, and to the consequent reduction in CD44 and MARCKS protein levels, while IRAKi and CPI203 single agents slightly affected the expression of these factors (Figure 2C). As expected, in two out of the three cell lines, CPI203-based treatments led to the decrease in MYC protein and mRNA, used here as hallmarks of BRD4 inhibition (Figures 2B and C). Also confirming a previous report linking bromodomain inhibitor therapy with IRAK1 downregulation in B-cell lymphoma,[11] IRAK1-pThr209 levels underwent a slight downregulation after CPI203 treatment and this effect was remarkably potentiated upon addition of

IRAKi to the cell cultures (Figure 2C). In line with the increased blockade of NF-κB signaling, the addition of CPI203 synergistically improved IRAKi cytostatic effect in the three cell lines, as attested by an 86% blockade in cell proliferation, significantly higher than the 19% activity achieved by IRAKi alone (combination index [CI]: 0.52, Figure 2D). Importantly, the co-operation between the IRAKi and CPI203 involved a remarkable downregulation of MCL-1 (Figure 2C), which was accompanied by a 36% increase in the relative apoptosis rate when compared with IRAKi and CPI203 used separately (Figure 2F).

In order to further validate the activity of the drug combination, primary lymph node biopsies from DLBCL patients with either *MYD88*$^{wt}$ or *MYD88* L265P were co-cultured in the presence of a feeding stromal monolayer as previously reported,[12] and treated with the different drugs as above. While IRAKi-CPI203 was almost inactive in *MYD88*$^{wt}$ cells, the combination induced a 16% augmentation in relative apoptotic cell death in the *MYD88* L265P primary co-culture (Figure 3A, left panel), which was accompanied by a 12% decrease in the fraction of cells with high contents of *IL6* mRNA, a percentage superior to what observed upon treatment with each drug alone (Figure 3B, right panel).

Among the above mentioned genes, CD44 expression and IL-6 serum levels have been described as prognostic markers in DLBCL.[13,14] In order to investigate the role of these two factors in the response of ABC-DLBCL cell lines to IRAKi-based treatment, HBL-1 and OCI-LY3 cells were stimulated with 0.5 μM of the CD44 ligand, hyaluronic acid (HA), or exposed to a 5 μg/mL dose of the IL-6 blocking antibody tocilizumab, prior to a 72-hour treatment with the drugs. In the case of HA, cells were exposed to IRAKi (50 μM) +/- CPI203 (0.5 μM), while effect of tocilizumab pretreatment was evaluated in IRAKi-treated cells. Cell response was determined by fluorescence microscopy recounting of cells with high contents in F-actin and by MTT assay, respectively. As shown on Figure 3B, both IRAKi and CPI203 were able to block actin polymerization by 50.4% and 54.5%, respectively, while the drug combination achieved a total 77.9% decrease in cells with high contents in F-actin following stimulation of

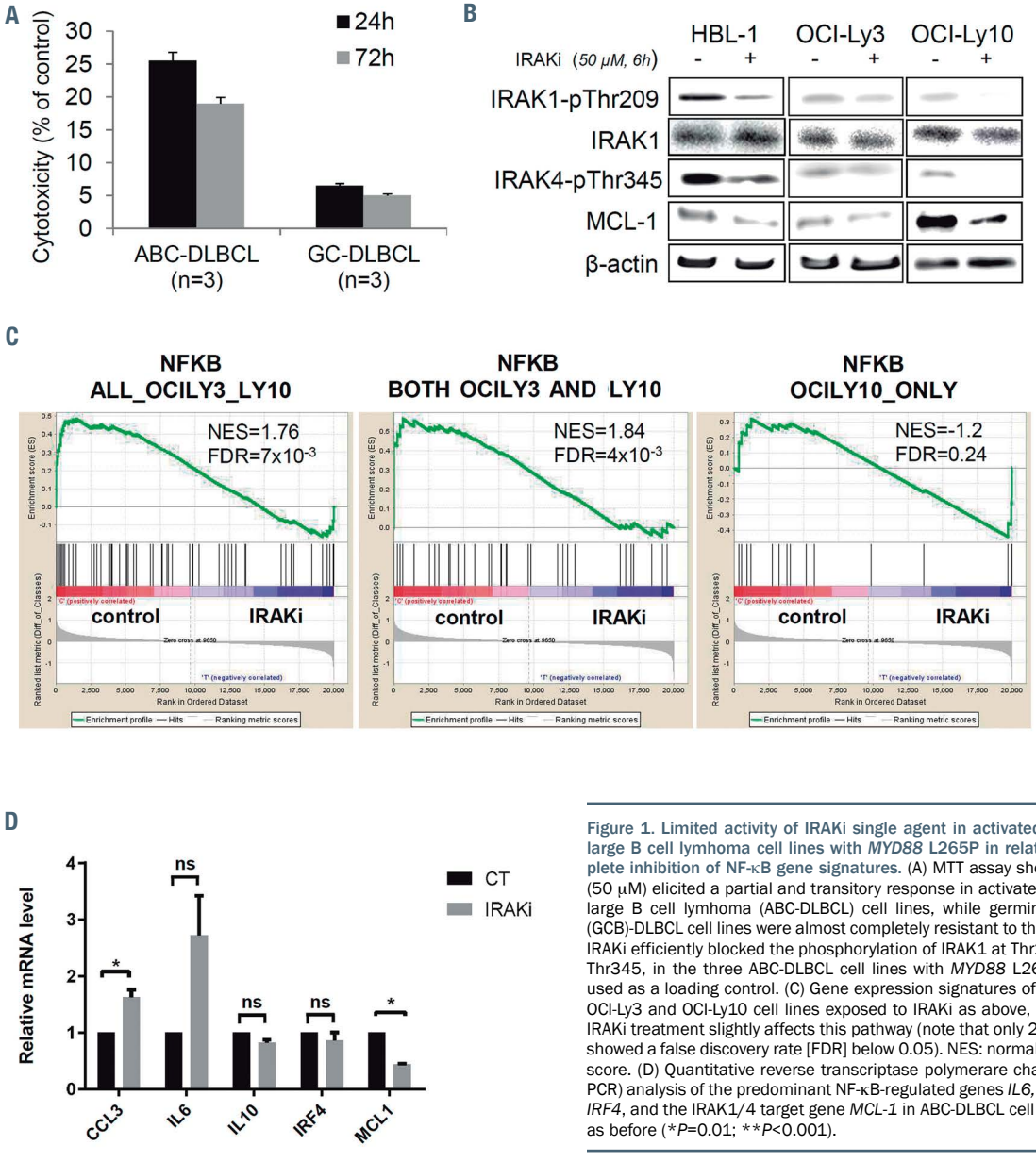


**Figure 1. Limited activity of IRAKi single agent in activated B-cell - diffuse large B cell lymhoma cell lines with *MYD88* L265P in relation with incomplete inhibition of NF-κB gene signatures.** (A) MTT assay showing that IRAKi (50 μM) elicited a partial and transitory response in activated B-cell - diffuse large B cell lymhoma (ABC-DLBCL) cell lines, while germinal center B-cell (GCB)-DLBCL cell lines were almost completely resistant to the compound. (B) IRAKi efficiently blocked the phosphorylation of IRAK1 at Thr29 and IRAK4 at Thr345, in the three ABC-DLBCL cell lines with *MYD88* L265P. β-actin was used as a loading control. (C) Gene expression signatures of NF-κB in HBL-1, OCI-Ly3 and OCI-Ly10 cell lines exposed to IRAKi as above, highlighting that IRAKi treatment slightly affects this pathway (note that only 2 out 3 gene sets showed a false discovery rate [FDR] below 0.05). NES: normalized enrichment score. (D) Quantitative reverse transcriptase polymerare chain reaction (RQ-PCR) analysis of the predominant NF-κB-regulated genes *IL6, IL10, CCL3*, and *IRF4*, and the IRAK1/4 target gene *MCL-1* in ABC-DLBCL cell treated by IRAKi as before (*$P$=0.01; **$P$<0.001).

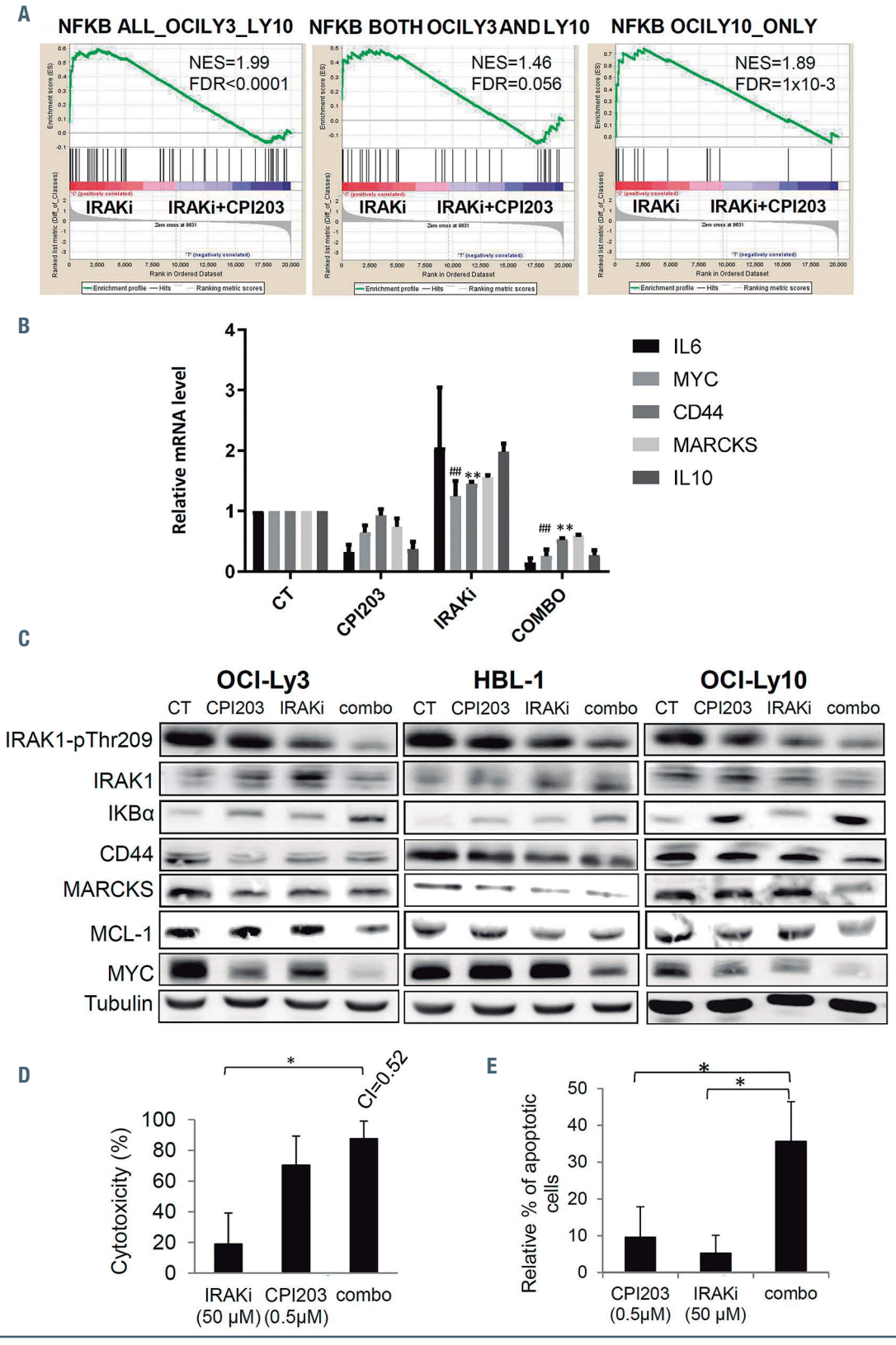


**Figure 2. The BETi CPI203 synergizes with IRAKi in activated B-cell - diffuse large B cell lymphoma mediated by the inhibition of NF-κB downstream pathways.** (A) Enrichment plots from gene set enrichment analysis (GSEA) analysis comparing IRAKi single agent *vs.* IRAKi-CPI203 combo in the 3 cell lines treated for 6 hours (Affymetrix HG-U219; GSEA), showing a significant improvement of NF-κB signature decrease by the addition of CPI203 to IRAKi. (B) Quantitative reverse transcriptase polymerare chain reaction (RQ-PRC) analysis of NF-κB downstream genes in the three cell lines exposed to IRAKi, CPI203 or CPI203-IRAKi combo as before. (*$P$=0.01; **$P$<0.001). (C) CPI203-IRAKi combination led to intracellular accumulation of IκB, and subsequent downregulation of IRAK1, MYC, CD44, MARCKS and MCL-1 proteins in activated B-cell - diffuse large B cell lymphoma (ABC-DLBCL) cells with *MYD88*$^{L265P}$. (D) OCI-Ly3, OCI-Ly10, HBL-1 cells were exposed for 24 hours to 0.1-0.5 μM CPI203 and/or 50-500 μM IRAKi. Cytotoxicity was evaluated by MTT assay and combination index (CI) was determined using the Calcusyn software. Shown are the cytotoxicity and the mean CI value calculated for cell treatment with 0.5 μM CPI203 and 50 μM IRAKi. (E) The drug combination led to a synergistic antitumoral effect *in vitro* in these 3 cell lines, inducing a median 36% increase in apoptosis rate when compared to single agent treatments (*$P$<0.04).

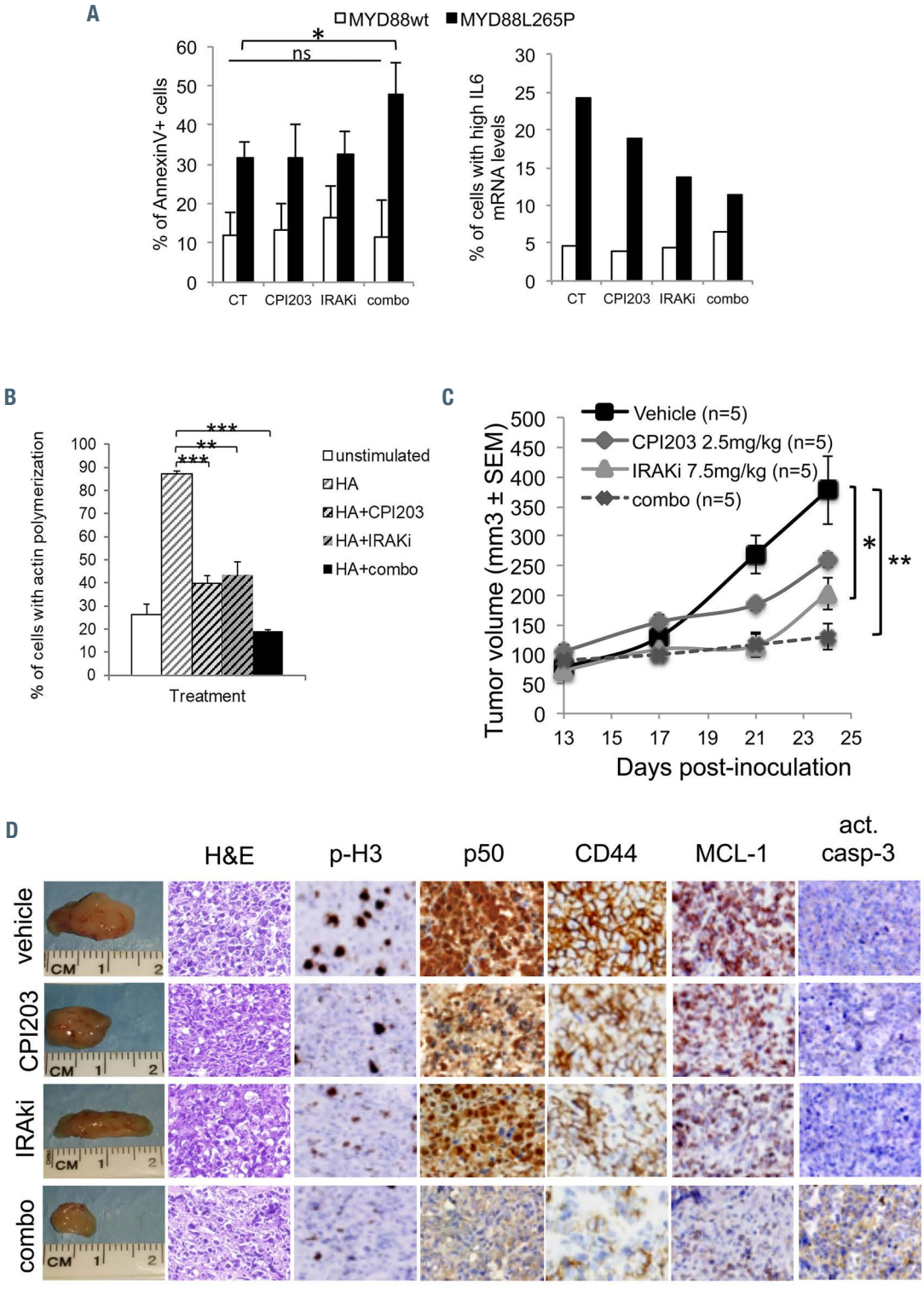


**Figure 3. IRAKi and CPI203 combination is active in activated B-cell - diffuse B-cell lymphoma primary cultures and impairs tumor growth *in vivo*.** (A) Left panel: antitumoral activity of CPI203 (0.5 μM) and/or IRAKi (50 μM) was evaluated after a 24-hour culture of primary lymph node biopsies from activated B-cell - diffuse B-cell lymphoma patients with either *MYD88*$^{wt}$ or *MYD88* L265P by cytofluorimetric quantification of AnnexinV$^{+}$ cells. Cell of origin (COO) and *MYD88* mutational status of the patients were determined by allele-specific polymerase chain reaction and gene expression analysis, as previously.[15] *Right panel*: DLBCL cultures treated as above were labeled with an IL-6 Hu-Cyanine 5 SmartFlare RNA detection probe (Merck Millipore), and percentage of viable cells with high contents in *IL6* mRNA was determined by flow cytometry, as previously.[16] (B) HBL-1 y OCI-LY3 cell lines were preincubated for 24 hours with 0.5 μM CPI203 and/or 50 μM IRAKi, followed by a 24-hour stimulation with 0.5 μM HA, labeling with 50 μM Phalloidin-TRITC (Sigma-Aldrich) and recounting of red fluorescent cells on a Nikon H5505 microscope by means of a 20X/1.30 NA oil objective (Nikon) with the use of Isis Imaging System v5.3 software (MetaSystems GmbH) (***$P$<0.001). (C) NOD/SCID IL2Rγ-null (NSG) mice were inoculated subcutaneously with $10^7$ OCI-LY3 cells and after 13 days, tumor-bearing animals (n=5 mice per group) received intraperitoneal (i.p.) injection of 2.5 mg/kg CPI203 (BID) and/or i.p. administration of 7.5 mg/kg IRAKi (BID), or an equal volume of vehicle, for 11 days, in a five/two (on/off) schedule. Tumor volumes were measured each 2-3 days with external calipers. (D) Immunohistological analysis of consecutive tumor sections from representative animals reveals a notable decrease in mitotic index and in the NF-κB-regulated CD44, as well as a strong downregulation of MCL-1 and induction of apoptosis in IRAKi-CPI203 combo group.

CD44 by HA. In contrast, the anti-IL-6 antibody failed to sensitize ABC-DLBCL cells to IRAKi-based treatment (*Online Supplementary Figure S1B*). Thus, these results suggest a significant activity of the drug combination towards CD44 downstream signaling, while IL-6 expression may not be directly involved in the effect of these agents.

Finally, in order to assess the efficacy of the drug combination *in vivo*, NSG mice were subcutaneously injected with OCI-LY3 cells, and tumor-bearing animals received daily doses of either IRAKi (5 mg/kg, intraperitoneal [i.p.], BID), CPI203 (2.5 mg/kg, i.p., BID), the combination of both agents, or the equivalent volume of vehicle, for 11 days. Figure 3C shows that CPI203 and IRAKi single agents induced a 31.5% and 46.3% tumor growth inhibition (TGI), respectively, while the combination of both drugs significantly improved this effect with a 65.6% TGI, when compared to vehicle-receiving animals (*$P$=0.011; **$P$=0.007). No significant toxicity was observed in any of the treatment arms. Histological analysis of the corresponding tumors revealed an improved reduction of mitotic index together with an accumulation of apoptotic cells by the combination therapy, as assessed by phosphohistone H3 and activated-caspase-3 staining (Figure 3D). In agreement with the *in vitro* results, an enhanced reduction in the levels of CD44 and MCL-1, and an improved downregulation of nuclear p50 used as a read of NF-κB activity, was observed in the combination group when compared with the other arms (Figure 3D).

Collectively, our results suggest that IRAK1/4 inhibition is modestly effective in *in vitro* and *in vivo* models of ABC-DLBCL with *MYD88* L265P, achieving only a partial inhibition of NF-κB signaling. We confirm that BET inhibition is an efficient strategy to counteract NF-κB over-activation in these models, offering synergistic anti-tumoral and pro-apoptotic activities with IRAK inhibition, mediated by the downregulation of the NF-κB-regulated factors, CD44 and MCL-1, and the consequent blockade of cell motility and triggering of tumor cell death.

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