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1 **CSF1R and BTK inhibitions as novel strategies to disrupt**
2 **the dialogue between mantle cell lymphoma and macrophages**

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4 Antonin Papin^{1,2,7}, Benoit Tessoulin^{1,3,7,‡}, Céline Bellanger^{1,2,7,‡},
5 Anne Moreau^{4,7}, Yannick Le Bris^{1,5,7}, Hervé Maisonneuve^{6,7}, Philippe Moreau^{1,3,7},
6 Cyrille Touzeau^{1,3,7}, Martine Amiot^{1,2,7}, Catherine Pellat-Deceunynck^{1,2,7},
7 Steven Le Gouill^{1,3,7,‡} and David Chiron^{1,2, 7,‡,*}

8
9 ¹CRCINA, INSERM, CNRS, Université d'Angers, Université de Nantes, Nantes, France.

10 ²GDR3697 Micronit, CNRS

11 ³Service d'Hématologie Clinique, Unité d'Investigation Clinique, CHU, Nantes, France.

12 ⁴Service d'Anatomie Pathologique, CHU, Nantes, France.

13 ⁵Service d'Hématologie Biologique, CHU, Nantes, France

14 ⁶Centre Hospitalier de la Roche sur Yon, Roche sur Yon, France.

15 ⁷L'Héma-NexT, i-Site NexT, Nantes, France

16 [‡]Equal contribution

17
18 * Corresponding author:

19 Tel: +33 228080297

20 Address: 8 quai Moncousu, 44007 Nantes, France

21 E-mail: david.chiron@univ-nantes.fr

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28 **Disclosure**

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32
33
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35 **Abstract**

36 The microenvironment strongly influences mantle cell lymphoma (MCL) survival, proliferation
37 and chemoresistance. However, little is known regarding the molecular characterization of
38 lymphoma niches. Here, we focused on the interplay between MCL cells and associated
39 monocytes/macrophages. Using circulating MCL cells (n=58), we showed that, through the
40 secretion of CSF1 and, to a lesser extent, IL-10, MCL polarized monocytes into specific
41 CD163+ M2-like macrophages (M ϕ MCL). In turn, M ϕ MCL favored lymphoma survival and
42 proliferation *ex vivo*. We next demonstrated that BTK inhibition abrogated CSF1 and IL-10
43 production in MCL cells leading to the inhibition of macrophage polarization and
44 consequently resulting in the suppression of microenvironment-dependent MCL expansion.
45 *In vivo*, we showed that CSF1 and IL-10 plasma concentrations were higher in MCL patients
46 than in healthy donors, and that monocytes from MCL patients overexpressed CD163.
47 Further analyses of serial samples from ibrutinib-treated patients (n=8) highlighted a rapid
48 decrease of CSF1, IL-10 and CD163 in responsive patients. Finally, we showed that
49 targeting the CSF1R abrogated M ϕ MCL-dependent MCL survival, irrespective of their
50 sensitivity to ibrutinib. These data reinforced the role of the microenvironment in lymphoma
51 and suggested that macrophages are a potential target for developing novel therapeutic
52 strategies in MCL.

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63 **Introduction**

64 Mantle cell lymphoma (MCL) is a rare and incurable B-cell malignancy, representing 3-10%
65 of non-Hodgkin lymphomas (NHLs)(1,2). MCL cells are naive CD19⁺ IgM⁺ B-cells
66 characterized by the expression of the B1-cell marker CD5 and the absence of CD23(3).
67 Conventional MCL cells initially accumulate in the lymph nodes and disseminate early on into
68 the peripheral blood or the bone marrow(4). In addition to the translocation t(11;14)(q13;q32),
69 leading to the overexpression of Cyclin D1, conventional MCL cells are characterized by the
70 overexpression of the oncogene SOX11. A SOX11-negative leukemic non-nodal MCL
71 subtype is now well characterized and displays a limited number of genomic alterations,
72 indolent clinical course, spleen involvement and a high percentage of circulating tumoral
73 cells(5). Both subtypes can evolve into more aggressive forms (blastoid/pleomorphic)
74 characterized by increased genomic instability and a high proliferation index(5). Several
75 studies have described the nature of MCL genomic secondary alterations, such as frequent
76 *ATM* or *TP53* mutations as well as recurrent copy number abnormalities and the deletion of
77 *CDKN2A* or *TP53*, those being associated with a bad prognosis(6–8).

78 In addition to intrinsic tumoral abnormalities, the major role of the immune and stromal
79 microenvironments in the expansion and chemoresistance of B-cell lymphomas is now
80 widely accepted(9,10). MCL, one of the most aggressive B-cell lymphomas, does not escape
81 this logic, and several studies have recently confirmed the role of the microenvironment in
82 the survival, proliferation and chemoresistance of this NHL(11–16). Nevertheless, the
83 composition of the MCL microenvironment and the resulting interactions that occur in the
84 tumor niches remain largely unknown.

85 Among accessory cells, tumor-associated macrophages are known to play a critical role in
86 solid tumor progression(17,18) and have also been described in several B-cell
87 malignancies(19–22). Previous studies suggested the presence of macrophages in MCL
88 lymph nodes(23,24) but their phenotype and the molecular dialogue that occurs between
89 MCL cells and associated macrophages remain unknown.

90 In the present work, we have studied the dynamic interactions between MCL and its myeloid
91 microenvironment. Using primary co-culture models *ex vivo*, we have demonstrated that
92 primary MCL cells polarize monocytes into specific associated macrophages (M Φ MCL),
93 which support MCL growth and survival. Furthermore, we identified mechanism-based
94 targeted strategies that disrupt the dialogue between MCL and M Φ MCL *ex vivo* and *in vivo*.

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118 **Methods**

119 *Culture and co-culture of primary cells*

120 Primary MCL cells were obtained after informed consent from patients according to protocols
121 approved by local institutional review boards (REFRACT-LYMA cohort; ethical approval
122 GNEGS-2015-09-13(25)) and in accordance with the Declaration of Helsinki. Patients'
123 characteristics are summarized in the supplemental Table S1. Briefly samples from 58
124 patients (69% male; median age, 70 years) were used in this study, 61% at diagnosis and
125 representing the different subtypes of the disease (37 conventional, 10 blastoid/pleomorphic,
126 9 leukemic non-nodal). Peripheral blood (PB) MCL cells were isolated after Ficoll-Hypaque
127 separation and stored in liquid nitrogen. PB MCL cells (median of circulating cells, 50%) were
128 separated from other mononuclear cells using anti-human CD19-conjugated magnetic beads
129 (Miltenyi, Paris, France) with purity > 90%. For autologous cocultures, monocytes from
130 patient were isolated using anti CD14-conjugated magnetic beads (Miltenyi, Paris, France).
131 For allogeneic coculture experiments, PB primary monocytes from healthy donors were
132 obtained by elutriation (CIC Biotherapy 0503, Nantes, France). For IL-10, CSF1 plasma
133 concentrations and CD163 expression on monocytes, PB was obtained from age-matched
134 (>60 years) healthy donors. Samples used for *in vitro* co-cultures or molecular
135 characterisations were listed in Table S1.

136 For *in vitro* generation of classically activated M1 and alternatively activated M2
137 macrophages, monocytes were differentiated with CSF2 (GM-CSF, 20 ng/ml, 5 days) or
138 CSF1 (M-CSF, 50 ng/ml, 5 days) before activation with IFN γ (10 ng/ml, 2 days) or IL-10 (25
139 ng/ml, 2 days), respectively (26,27).

140 CD19⁺ primary MCL cells were cultured at 10⁶ cells/ml alone or with monocytes or *in vitro*
141 pre-differentiated macrophages at 2.10⁵ cells/ml (5:1 ratio). Transwell assays were realized
142 with a 0.4 μ m pore polycarbonate membrane and 6.5 mm inserts (Corning, NY, USA). After
143 co-cultures, MCL cells were separated from macrophages by removal of non-adherent cells
144 and identified using B-cell markers by flow cytometry (CD19, CD20). Adherent macrophages

145 were detached using PBS-EDTA 0,02% (15 minutes at 4°C). All cells were maintained in
146 RPMI-1640 medium (Gibco) supplemented with 10% fetal calf serum and 2mM glutamine.

147

148 *Mantle cell lymphoma cell lines*

149 JeKo-1, MINO, REC-1, MAVER-1 and GRANTA-519 were purchased from DSMZ
150 (Braunschweig, Germany) and Z138 from ATCC (Manassas, USA). UPN1 and SP53 were
151 kindly provided by Dr. V. Ribrag (Institut Gustave Roussy Villejuif, France) and Pr. S. Chen-
152 Kiang (Cornell University, NY) respectively. NTS-3 and NTS-4 has been generated in our
153 laboratory (CRCINA)(12). Cell lines are routinely identified using a flow cytometry-based
154 barcode as previously described(28) as well as MHC class I sequencing and are tested for
155 mycoplasma contamination. Values for MCL cell lines are the mean of at least 3 independent
156 experiments.

157

158 *Bioinformatics analysis*

159 For mRNA relative expression level, CD19+ peripheral blood B cells from healthy donors
160 (Normal B Cell, NBC, n = 15), MCL cells (n = 183) and myeloid cells (Monocytes, n = 6;
161 M ϕ MCL, n = 4; M1, n = 3; M2, n = 5) datasets were collected from the GEO database
162 (GSE50006, GSE19243, GSE35426, GSE16455, GSE36000, GSE21452, GSE70910,
163 GSE76803, GSE28490, GSE124931, GSE95405, GSE20484). In order to overcome data
164 normalization biases, only Affimetrix Human Genome U133 Plus 2.0 series with raw data
165 were retained. Briefly, raw .CEL files were downloaded and processed in R-3.5.1 using the
166 affy package, optical noise/background correction was performed by MAS5.0 or gcrma with
167 standard options, expression batches were finally normalized by quantiles using the limma
168 package(29,30). Principal Component Analysis was performed by FactoMineR and
169 factoextra packages. A hierarchical ascendant clustering was performed using Euclidean
170 distances and Ward.D2 method. Heatmap and radarchart were carried out with the
171 ComplexHeatmap and fmsb package, respectively.

172 For deconvolution analysis of tumor bulk gene expression data (LN MCL, n=161, GSE16455,
173 GSE93291, GSE16024, GSE36000, GSE70910) we used the Cibersort program (31).
174 CD19+ sorted MCL cells from 4 LN (GSE70910) were used as an internal control for the
175 deconvolution analysis.

176

177 *Other Methods.*

178 Cell cycle and viability assays as well as real-time quantitative reverse transcription
179 polymerase chain reaction (qRT-PCR, control gene *RPL37A*) and Immunoblot protocols
180 have been previously described(15). Antibodies and reagents are detailed in supplemental
181 Table S2. Statistical analyses were performed using two-sided Mann Whitney, Wilcoxon
182 matched-pairs signed-rank or t-tests as stated in the figure legends. Analyses were
183 performed using GraphPad Prism and R statistical softwares and all tests were considered
184 statistically significant at $p < .05$.

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200 **Results**

201 **Monocyte-derived macrophages support primary MCL cell survival and proliferation.**

202 Using a deconvolution algorithm for tumor bulk gene expression data(31), we first observed
203 that MCL lymph nodes (LN) were characterized by macrophage infiltration (median, 12%,
204 n=161, Figure S1A). CD68+ macrophages were also highlighted in MCL LN by IHC (n=10),
205 arguing for a potential dialogue between MCL and its myeloid microenvironment *in vivo*
206 (Figure S1B).

207 To understand the potential role of this interplay, we first set up an *ex vivo* co-culture of
208 peripheral blood primary MCL cells (PB MCL cells) and monocytes isolated from healthy
209 donors. After 7 days of co-culture, we observed that monocytes differentiated into adherent
210 macrophages (Figure S1C), which we called MCL-associated-macrophages (M Φ MCL)
211 throughout this study. PB MCL cells poorly survived when cultured alone. In contrast, the
212 presence of monocytes greatly improved their survival after 7 days *ex vivo* (median Annexin-
213 V^{neg}; alone, 6.5%; co-culture, 85.9%; n= 17; p<0.001; Figure 1A). The pro-survival advantage
214 of the co-culture, observed as early as 48h, was maintained for several weeks and after
215 several months of culture, the t(11;14) EBV^{neg} MCL cell line NTS4 (from sample 2b)
216 remained dependent on M Φ MCL for survival (data not shown). Using culture inserts to avoid
217 contact between the two cell types, we determined that the pro-survival impact of monocytes
218 was partly dependent on soluble factors (median survival alone, 5%; co-culture, 32.9%;
219 p<0.001; Figure 1A). Indeed, whereas direct contact with monocytes induced a 13-fold
220 increase of PB MCL cell survival, soluble factors supported a 6.5-fold survival increase
221 (Figure 1A).

222 We have previously shown that in contrast to lymph node MCL cells, circulating MCL cells
223 rarely proliferate (Figure S2A)(12). Here, we have demonstrated that monocyte co-culture
224 supported the proliferation of primary MCL cells in 8/16 samples tested (median BrdU⁺ cells
225 in co-culture, 14.75%; Figure 1B, S2B), confirming the microenvironment-dependent
226 expansion of MCL cells. Of note, monocyte-dependent proliferation was significantly lower in
227 leukemic non-nodal (light grey bars, n=5) compared to conventional (dark grey bars, n=7) or

228 aggressive (black bars; n=4) MCL subtypes ($p < 0.05$, Figure 1B). This monocyte-dependent
229 increase in proliferation was confirmed at the molecular level by the induction of *MKI67*
230 expression and the inhibition of the tumor suppressor Rb (ratio phospho (p)Rb/Rb) (Figure
231 S2C-D). As observed for survival, cell cycle induction was, at least, partly dependent on
232 soluble factors (Figure S2E). Of note, autologous monocytes/MCL cocultures displayed
233 similar results (n=4, autologous vs. allogeneic coculture $p > 0.35$, Figure 1C).

234

235 **M ϕ MCL are M2-like macrophages.**

236 In order to better characterize the interplay between MCL and its myeloid microenvironment,
237 co-cultures of PB MCL cells in contact with *in vitro* pre-differentiated, classically activated
238 (M1) or alternatively activated (M2) macrophages were set up. Even though M1
239 macrophages display anti-tumoral activities in several models, both M1 and M2
240 macrophages provided a similar pro-survival benefit to PB MCL cells (Figure S3A). As
241 observed for monocytes, this pro-tumoral effect was, at least, partly due to soluble factors
242 (Figure S3B), suggesting that both M1 and M2 macrophages secrete MCL pro-survival
243 factors. Of note, M2 macrophages induced significantly more proliferation in PB MCL cells
244 compared to M1 macrophages (median with M1 = 3%, with M2 = 9%, $p < 0.01$; Figure S3C).

245 To define the precise nature of MCL-associated-macrophages (M ϕ MCL) we analyzed their
246 transcriptome along with the one of undifferentiated monocytes, M1 and M2 macrophages.
247 To compare the different groups, we performed a Principal Component Analysis (PCA), and
248 observed that M ϕ MCL segregated with M2 macrophages (Figure S4A). Accordingly,
249 hierarchical clustering based on 17256 genes highlighted greater similarities of M ϕ MCL with
250 alternatively activated M2 macrophages (Figure 2A).

251 Previously defined genes signatures allowed the robust prediction of monocytes, M1-like or
252 M2-like phenotypes (Figure S4B)(31). It is noteworthy that *ex vivo* generated M ϕ MCL and
253 macrophages infiltrated in MCL lymph nodes *in vivo* displayed a similar CSF1-differentiated
254 M2-like macrophage signature (Figure 2B). In line with an M2-like profile, M ϕ MCL were
255 characterized by the expression of the M2-like marker CD163, even though at a lower level

256 than *in vitro* pre-differentiated M2 macrophages (Figure 2C, S4C). M ϕ MCL arising from
257 allogeneic or autologous CD14⁺ monocytes displayed a similar phenotype (Figure S4C) and
258 the presence of CD163⁺ cells was confirmed in MCL LN by IHC or cytometry (Figure S4D-E).
259 Given the key role of soluble factors in M ϕ MCL/MCL interplay (Figure 1), we analyzed the
260 expression profile of genes coding for cytokines (n=24) and chemokines (n=28) in M ϕ MCL
261 (Figure S4F). Even though hierarchical clustering based on these 52 genes highlighted
262 greater similarities with M2 macrophages (Figure S4G), PCA demonstrated that M ϕ MCL
263 formed a specific subtype of macrophages, producing both M2 and M1 associated factors
264 (Figure S4H). Among them, the expression of known pro-tumoral cytokines in MCL such as
265 IGF1, IL10 and IL6 (32–34) were confirmed in additional M ϕ MCL samples (n = 5, Figure 2C).

266

267 **MCL cells secrete the M2 polarizing factors IL-10 and CSF1.**

268 To determine how primary MCL cells polarized monocytes into specific CD163⁺ M2-like
269 M ϕ MCL, the expression of known macrophage-polarizing factors(35) was analyzed. We first
270 interrogated publicly available gene expression datasets and observed that *CSF1* and *IL10*
271 transcripts, in contrast to *CSF2*, *IL4*, *IL34* or *IL13* (*data not shown*), were significantly
272 overexpressed in MCL samples *in vivo*, when compared to normal B cells (NBC)(Figure 3A).
273 Of note, *CSF1* but not *IL10* expression was significantly higher in MCL LN compared to MCL
274 PB (Figure S5A). To confirm that these soluble factors were indeed produced by MCL cells,
275 we assessed their expression in MCL cell lines (n=9) and purified PB MCL cells (n=20) by
276 RT-qPCR (Figure 3B-C). Whereas only 3/9 cell lines (JeKo, Mino, Granta) co-expressed
277 *CSF1* and *IL10*, transcripts for both factors were detected in 19 out of 20 primary MCL
278 samples. *CSF1*, but not *IL10* mRNA, was significantly overexpressed in aggressive
279 (blastoid/pleomorphic) MCL subtypes and correlated with proliferation in co-culture *ex vivo*
280 (BrdU⁺ cells in co-culture) and in tissues *in vivo* (*MKI67*) (Figure S5B-D). IL-10 (7/10) and
281 CSF1 (7/10) expressions were then confirmed at the protein level in the MCL/M ϕ MCL co-
282 culture supernatant (D7), with all samples tested secreting detectable amounts of at least
283 one of both the factors (median CSF1, 4.35 pg/mL; median IL-10, 5.67 pg/mL)(Figure 3D).

284 To understand the role of the CSF1 and IL-10 in the initiation of MCL/monocyte interplay, we
285 cultured monocytes with previously generated MCL/monocyte co-culture supernatants (7
286 days) in the presence of inhibitors of the CSF1R (GW2580 (36–38), blocking antibodies) or
287 IL10R (blocking antibodies). We demonstrated that inhibition of the CSF1R significantly
288 reduced monocyte survival (median viability reduction of 80% with GW2580, of 44% with
289 anti-CSF1R, n= 6, Figure 3E). Inhibition of CSF1R with GW2580 significantly reduced the
290 M2-like marker CD163 on remaining viable monocytes (median CD163 reduction of 45%, n=
291 5) (Figure 3E). Significant inhibition was also observed with anti-CSF1R monoclonal
292 antibodies (median reduction of 28%, n= 5). Inhibition of IL-10R resulted in a slight, but not
293 significant, reduction of the CD163 level on M ϕ MCL (median reduction of 16%, n= 5, Figure
294 3E). These data showed that MCL-secreted CSF1, but not IL-10, was essential for initiating
295 the dialogue between MCL and monocytes.

296 In addition to inhibiting monocytes survival and polarization, CSF1R neutralization resulted in
297 the inhibition of monocyte-dependent survival in all primary MCL samples tested (Figure 3F)
298 (median monocyte-dependent survival reduction by GW2580 of 91%). Besides, we observed
299 that CSF1R inhibition also resulted in an inhibition of primary MCL cells viability as well as a
300 downregulation of macrophages CD163 expression in coculture experiments with pre-
301 differentiated M ϕ MCL and M2, but not M1, macrophages (Figure S6A).

302 In accordance with the absence of CSF1R expression on MCL cells (data not shown),
303 GW2580 did not display any direct cytotoxicity on primary MCL cells (Figure S6B), confirming
304 that the loss of viability was the consequence of the inhibition of MCL/M ϕ MCL interplay. In
305 addition, CSF1R inhibition with other inhibitors such as BLZ945 (37), the clinically available
306 PLX3397 or anti-CSF1R mAb confirmed the results obtained with GW2580 (Figure S6C).

307 Taken together, the results showed that MCL cells secrete the M2-polarizing factors IL-10
308 and CSF1, the latter being essential for the initiation of the pro-tumoral dialogue between
309 malignant B-cells and associated macrophages.

310

311 **Ibrutinib disrupts the dialogue with M ϕ MCL by inhibiting MCL-specific CSF1 and IL-10**
312 **secretion.**

313 Previous studies reported modulations of the MCL secretome, including IL-10, upon BTK
314 inhibition with ibrutinib, both *in vitro*(39) and *in vivo*(40). To study the modulation of IL-10 and
315 CSF1 upon ibrutinib treatment, Mino and Granta cell lines, which produce both factors, were
316 first used (Figure 3B). As previously reported(41), it was confirmed that, in contrast to Mino
317 cells, Granta was resistant to both cytotoxic and cytostatic ibrutinib effects *in vitro* (0.5 μ M;
318 data not shown). Both *CSF1* and *IL10* mRNA were dramatically reduced after 72h of
319 ibrutinib treatment (0.5 μ M) in the ibrutinib-sensitive Mino cells whereas no modulation was
320 observed in the ibrutinib-resistant Granta cells (Figure 4A). These modulations were
321 confirmed at the protein level using ELISA (Figure 4B). Of note, we observed an induction of
322 the CSF1 expression in cell lines with a low baseline level (UPN1, JeKo) after BCR activation
323 upon anti-IgM stimulation, confirming that CSF1 is a direct target of the BCR signaling
324 network (Figure S7A).

325 In accordance with production of M2-polarizing factors, the co-culture of both cell lines
326 induced CD163 expression on PB primary monocytes from healthy donors (Figure 4C). As
327 expected, ibrutinib inhibited the M2-like polarization exclusively with the ibrutinib-sensitive
328 Mino cells (mean reduction of 43%, n=3, Figure 4C). Taken together, the results suggested
329 that ibrutinib treatment counteracted CD163+ M ϕ MCL polarization through inhibition of MCL-
330 specific IL-10 and CSF1 secretion.

331 In addition to impairing M ϕ MCL polarization, ibrutinib treatment resulted in the inhibition of
332 M ϕ MCL-dependent pro-survival and proliferative effects in 8 out of 14 and 4 out of 4
333 samples, respectively ($p < 0.01$) (Figure 4D-E). Even though BCR signaling was constitutively
334 activated, independently of monocyte coculture (Figure S7B), ibrutinib did not induce any
335 cytotoxicity in primary MCL cells cultured alone *ex vivo* (Figure S7C). Besides, ibrutinib did
336 not induce any cytotoxicity in monocytes/macrophages alone (data not shown). Thus, given
337 that BCR inhibition abrogated MCL survival in coculture, it appears that ibrutinib acted mainly
338 through the disruption of the interplay between tumoral cells and M ϕ MCL.

339 **Early *in vivo* CD163 modulation on PB monocytes is observed upon ibrutinib**
340 **treatment of MCL patients.**

341 In accordance with the ability of primary MCL cells to produce CSF1 and IL-10, a significantly
342 higher level of these M2-polarizing factors was detected in the plasma of MCL patients
343 compared to age-matched healthy donors (HD) (median HD IL-10, 0pg/mL; CSF1, 0pg/mL;
344 n= 9; median MCL IL-10, 1.2pg/mL; CSF1, 5.6pg/mL; n=28, Figure 5A). Likewise, CD163
345 was overexpressed at the surface of CD14+ PB monocytes in several MCL samples
346 compared to healthy donors (median CD163 MFIr HD = 4.3, n=8; MCL= 7.5, n=32, Figure
347 5A), which was consistent with the CD163-inducing properties of CSF1 and IL-10. There was
348 no significant correlation between the level of IL-10, CSF1 nor CD163 and the status of the
349 disease (Diagnosis/Relapse).

350 We next evaluated IL-10 and CSF1 plasma concentrations as well as CD163 expression on
351 CD14+ PB monocytes in 8 patients treated by anti-CD20 monoclonal antibody
352 (Obinutuzumab) in combination with ibrutinib (protocol detailed in Figure S8A). PB samples
353 were collected before (D0) and after 8 days of treatment (D8). IL-10 or CSF1 concentration
354 was decreased at D8 in 8/8 and 2/3 evaluable plasma samples, respectively (IL10, n=8,
355 p<0.01) (Figure 5B). In addition, CD163 expression on CD14+ PB monocytes was decreased
356 in 7 out of 8 patients at D8 (median CD163+ reduction of 58%, n=7, p<0.05, Figure 5B). In
357 contrast to ibrutinib (Figure 4A), the anti-CD20 mAb Obinutuzumab did not directly modulate
358 *IL10* or *CSF1 in vitro* (Figure S8B).

359 The follow up of patients treated with anti-CD20 mAb and ibrutinib for several cycles (Pt# A1,
360 A2, A5, A9) revealed that CD163 inhibition was durable and associated with a clinical
361 response (>2 years) (Figure S8C). In fact, whereas the three patients characterized by a
362 dramatic reduction in CD163 at D8 (Pt# A1, A2, A5) achieved a durable complete response,
363 Pt# A9, who displayed an increase in CD163 expression and a limited decrease of IL-10
364 plasma concentration, progressed under treatment. Taken together, our retrospective
365 analysis suggested that CD163 modulation upon ibrutinib treatment is associated with a

366 clinical response. These results warrant further investigation with a larger cohort of ibrutinib-
367 treated patients.

368

369 **CSF1R inhibition as an alternative for disrupting MCL/M ϕ MCL dialogue in ibrutinib-**
370 **resistant patients**

371 Ibrutinib did not efficiently counteract M ϕ MCL-dependent MCL survival in 6 out of 14 samples
372 (Figure 4D), including two patients who previously demonstrated ibrutinib resistance *in vivo*
373 (Pt#2 and #12). To assess whether alternative strategies targeting the MCL/M ϕ MCL dialogue
374 could bypass ibrutinib resistance, the CSF1R inhibitor GW2580 was tested in both ibrutinib-
375 sensitive (Pt#7, 11, 15, 16) and resistant (Pt#2, 5, 18, 19) primary cells. We showed that
376 GW2580 reduced MCL cell viability in all samples tested, irrespective of their sensitivity to
377 ibrutinib (Figure 6A), suggesting that targeting the CSF1/CSF1R axis could be of major
378 interest in ibrutinib-resistant patients. To assess whether the association of BTK and CSF1R
379 inhibitors could also be beneficial for ibrutinib-sensitive patients, the efficacy of
380 ibrutinib/GW2580 combination was tested at lower concentrations (125 nM). We showed
381 additive (Pt#11 and #47) or supra-additive (Pt#7 and #15) effects of the combination in
382 ibrutinib-sensitive samples (n= 4, p<0.05, Figure 6B, left) but not in ibrutinib-resistant
383 samples (n= 4, Figure 6B, right).

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394 **Discussion**

395 Several studies have highlighted the central role of the microenvironment in the
396 expansion and chemoresistance of B-cell lymphomas, including MCL(11–16,42). The few
397 works that have examined the role of macrophages in MCL suggested a protumoral
398 function(23,24), especially through the induction of a VEGF-dependent lymphangiogenesis
399 (24). However little was known about the molecular interplay between MCL cells and
400 associated monocytes/macrophages. Here we showed that, through secretion of IL-10 and
401 CSF1, MCL polarizes monocytes into M2-like macrophages, which in turn favor tumor
402 survival and proliferation.

403 IL-10 production by MCL cells has been previously reported *in vitro* and *in vivo*(39,40).
404 In contrast, this is, to our knowledge, the first study reporting a CSF1 paracrine loop in MCL.
405 CSF1 and IL-10 are involved in monocyte polarization into alternatively activated M2-like
406 tumor-associated macrophages (TAM)(43). In addition, CSF1 and its receptor CSF1R are
407 central to myeloid cell biology and promote migration, survival, and proliferation of
408 monocytes(44). It is noteworthy that both *ex vivo* generated M ϕ MCL and *in vivo* MCL-
409 infiltrated macrophages displayed a M2-like signature close to macrophages differentiated by
410 the CSF1 (Figure 2B, S4B), highlighting the relevance of our *ex vivo* coculture model and
411 reinforcing the key role of the CSF1 in MCL/monocyte interplay.

412 CSF1 production has been reported in several solid tumor models and has been
413 associated with a poor prognosis(45,46). Regarding B-cell malignancies, *CSF1* transcript
414 overexpression correlated with chronic lymphocytic leukemia (CLL) progression, however,
415 CSF1 protein was not detected in the plasma of CLL patients(47). In our study, and using the
416 same technology (ELISA), we detected a significant amount of CSF1 protein in 17 out of 28
417 MCL plasmas studied, highlighting a high production in MCL (Figure 5). In addition, we
418 showed that *CSF1* was more expressed in the most aggressive forms of MCL and was
419 associated with primary MCL cells proliferation (BrdU+) *ex vivo*. This was reinforced by a
420 positive correlation between *CSF1* and *MKI67* (proliferation index) expression *in vivo* (GEP

421 analysis, n=183) and suggested an association between MCL/ M ϕ MCL interplay and tumor
422 aggressiveness (Figure S5).

423 Even though transcriptome analysis showed that MCL associated-macrophages
424 (M ϕ MCL) shared more similarities with M2 macrophages (Figure 2), M ϕ MCL expressed both
425 M1 and M2-associated soluble factors (Figure S4F-H). This reflected the phenotypic and
426 functional plasticity of macrophages polarization and suggested that additional factors to IL-
427 10 and CSF-1 might be involved in M ϕ MCL polarization. Besides soluble factors, cellular
428 contacts might also play a role in MCL/M ϕ MCL interplay. We observed that M ϕ MCL
429 displayed a specific immune checkpoints profile and expressed both PD1 and PDL1 (Figure
430 S4F), suggesting that immune checkpoint inhibitors could be of interest as another
431 therapeutic avenue in MCL.

432 Given the central role of the myeloid microenvironment in numerous solid and
433 hematological malignancies, several targeted strategies are under investigation. Among
434 them, the CSF2-dependent re-education of M2-like TAM into classically activated anti-
435 tumoral M1 macrophages has been proposed in several models such as glioblastoma or
436 multiple myeloma(48,49). However, based on our *ex vivo* data, M1 macrophages could also
437 provide pro-survival signal in MCL. The depletion of tumor-associated macrophages using
438 targeted therapies has therefore appeared to be more attractive in MCL. Accordingly, we
439 demonstrated that targeting the CSF1R using the small molecule GW2580 efficiently
440 counteracted M ϕ MCL protumoral effects *ex vivo* (Figures 3,6). GW2580 is an orally
441 bioavailable and selective CSF1R kinase inhibitor whose efficacy and selectivity have been
442 previously demonstrated in different models (36–38,50,51). In addition, potential off target
443 effects have been ruled out using other well described selective small molecules such as
444 PLX3397 or BLZ945 as well as anti-CSF1R mAb. Success of such a strategy has been
445 recently confirmed *in vivo* using clodrolip or anti-CSF1R mAb in a CLL mouse model(22). In
446 humans, several anti-CSF1R mAb (LY3022855, Emactuzumab, AMG820) and CSF1R
447 inhibitors (PLX3397, Pexidartinib) are currently being evaluated in phase I/II clinical studies
448 that will soon document the efficacy of these targeted therapies (36).

449 In the present study, we demonstrated that ibrutinib also directly counteracted the
450 MCL/M ϕ MCL dialogue through the modulation of the malignant B-cells secretome. We
451 showed that BTK inhibition resulted in a dramatic decrease of CSF1 and IL-10 *ex vivo* and *in*
452 *vivo*. Indeed, whereas higher levels of CSF1 and IL-10 were detected in the plasma of MCL
453 compared to HD, ibrutinib treatment rapidly decreased concentrations of both cytokines.
454 Furthermore, CD163 was overexpressed at the surface of circulating monocytes in MCL
455 compared to HD, which was consistent with the CD163-inducing properties of CSF1 and IL-
456 10. Of interest, the longitudinal follow up of 4 patients treated with ibrutinib highlighted a
457 downregulation of CD163 on PB monocytes in 3 responsive patients *in vivo*. In contrast, only
458 limited modulation was observed in the resistant patient (Pt #A9). Taken together, our results
459 are in favor on monitoring of CSF1, IL-10 and CD163 for the follow up of patients' response
460 to ibrutinib-based treatment. Even though a larger cohort of MCL patients treated with
461 ibrutinib is now necessary to confirm the strength of this soluble and cellular signature, it is
462 noteworthy that modulation of PB cytokines have also been associated to *in vivo* ibrutinib
463 response in other B-cell malignancies(52).

464 Single-agent ibrutinib displayed unprecedented clinical efficacy in MCL and is now
465 approved for use in several B-cell malignancies. Nevertheless, several mechanisms of
466 resistance such as mutation acquisition(53), compensatory pathway activation i.e., NF κ B(41)
467 or kinome adaptive reprogramming(42) have been described and retrospective studies
468 revealed poor outcomes for ibrutinib relapsed/refractory MCL patients(54). Here we showed
469 that targeting the CSF1R could be an alternative for disrupting the MCL/M ϕ MCL pro-tumoral
470 dialogue, especially for ibrutinib-refractory patients for who poor therapeutic alternatives are
471 available. In addition, we observed (supra)additive cytotoxicity when BTK and CSF1R
472 inhibitors were combined at low doses *ex vivo*, suggesting that this strategy could also be
473 beneficial for ibrutinib-sensitive patients.

474 In conclusion, by modeling the dialogue between MCL cells and their protective
475 immune niches, we uncovered a novel rational combination that could overcome drug
476 resistance. Our data reinforces the central role of the microenvironment in MCL and shows

477 that monocytes/macrophages are a potential target for developing novel therapeutic
478 strategies in MCL.

479

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485

486 **Authorship contribution**

487 AP designed and performed experiments, analyzed data and wrote the article

488 BT participated in bioinformatics analysis

489 CB performed experiments and participated in bioinformatics analysis

490 AM provided biopsy samples and analyzed data

491 YLB provided samples

492 HM provided samples

493 PM participated in the design of the study

494 CT provided samples

495 MA participated in the design of the study and reviewed the article

496 CPD participated in the design of the study, in the data analysis and in the writing of the
497 article

498 SLG participated in the design of the study, in the data analysis and in the writing of the
499 article

500 DC designed and performed experiments, analyzed data and wrote the article

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691 **Figure Legends**

692 **Figure 1. Allogeneic and autologous monocytes support MCL cell survival and**
693 **promote cell proliferation.** (A) The percentage of PB MCL live cells was assessed by
694 Annexin-V staining after 7 days of culture alone (-) or with allogeneic monocytes, either in
695 contact (n = 17, left panel) or separated by transwell inserts (n = 13, right panel). Wilcoxon
696 matched pairs sign rank test. *** p < .001. Red lines represent medians. (B) Cell cycle
697 analysis (BrdU/PI) of PB MCL cells (n = 16) after 7 days of co-cultures with monocytes
698 according to their molecular subtypes (light grey: leukemic non-nodal (n = 5), dark grey:
699 conventional (n = 7), black: aggressive (n = 4)). Mann Whitney test. * p < .05. (C) Percentage
700 of live cells (AnnexinV staining, n = 4, left panel) and cell cycle analysis (BrdU/PI, n = 5, right
701 panel) of PB MCL cells cultured alone or in contact with autologous or allogeneic monocytes
702 for 7 days. Paired t-test. * p < .05 ** p < .01.

703

704 **Figure 2. MCL cells polarize monocytes into specific M ϕ MCL with M2-like features.** (A)
705 An ascendant hierarchical clustering was constructed with ward.D2 method of Euclidian
706 distance. M2 were generated from human monocytes cultured with CSF1/IL-10 (see
707 methods), M2' with CSF1 alone (GSE20484) and M1 with LPS/IFN γ (GSE95405) (B)
708 Radarchart representation of the ciphersort signature for M ϕ MCL and MCL-infiltrated
709 macrophages in lymph nodes. (C, left panel) CD163 Mean Fluorescence Intensity ratio
710 assessed by flow cytometry for M1 (n = 6), M2 (n = 6) and M ϕ MCL (n = 9) macrophages.
711 Mann Whitney test. ** p < .01 *** p < .001. (right panel) *IGF1*, *IL10* and *IL6* induction
712 measured by qRT-PCR (relative to undifferentiated human monocytes) for M1 (n = 3), M2 (n
713 = 3) and M ϕ MCL (n = 5) macrophages. t- test. * p < .05 ** p < .01.

714

715 **Figure 3. MCL cells express the M2-polarising factors CSF1 and IL-10.** (A) Expression of
716 *CSF1* and *IL10* in MCL cells (n = 183) compared to normal B cells (NBC, n = 15) according
717 to GEP public databases (see Methods). Mann-Whitney test. n.s, not significant. **** p
718 < .0001 ** p < .01. (B) qRT-PCR analysis of *CSF1* and *IL10* gene expression in 9 MCL cell

719 lines (realized in triplicate) and (C) twenty CD19⁺ MCL samples purified from PB. Expression
720 was normalized to Granta cell line. (D) Concentration of CSF1 and IL-10 proteins evaluated
721 by ELISA in the supernatant of M ϕ MCL/MCL co-culture (7 days, n = 10). (E) Percentage of
722 lived CD14 cells (left panel) and CD163 MFI modulation (on CD14 live cells) (right panel)
723 after 3 days of culture with previously generated MCL/M ϕ MCL coculture supernatants (n = 5)
724 in the presence of CSF1R inhibitors (GW2580 1 μ M, anti-CSF1R antibodies 5 μ g/mL), or of
725 anti-IL-10R antibodies (5 μ g/mL). Paired t-test *p < .05 **p < .01 ***p < .001 (F) Percentage
726 of primary MCL live cells in coculture with monocytes with or without GW2580 treatment
727 (1 μ M) (n = 8). Cell death was assessed by Annexin-V staining. Wilcoxon matched pairs sign
728 rank test. **p < .01. Red lines represent medians.

729

730 **Figure 4. Ibrutinib counteracts MCL/M ϕ MCL dialogue through inhibition of CSF1.** (A)
731 qRT-PCR analysis of *CSF1* and *IL10* expression in ibrutinib-sensitive Mino cells and
732 ibrutinib-resistant Granta cells with or without ibrutinib treatment (72h; 0.5 μ M). Gene
733 expression has been normalized to the non-treated control condition of each cell line
734 (realized in triplicate) (B) Concentration of CSF1 and IL-10 proteins evaluated by ELISA in
735 the supernatant of Mino and Granta cells with or without ibrutinib treatment (72h; 0.5 μ M;
736 triplicate). (C) (upper panel) Gating strategy to evaluate the CD163 expression on CD14⁺
737 after 3 days of co-culture between monocytes and Mino or Granta cells with or without
738 ibrutinib treatment (72h; 0.5 μ M) (lower panel) CD163 MFI modulation on CD14⁺ cells
739 representing 3 independent experiments. (D) Percentage of primary MCL live cells in
740 coculture with monocytes (Annexin-V staining) with or without ibrutinib treatment (72h;
741 0.5 μ M; n = 14). Wilcoxon matched pairs sign rank test. *** p < .01. (E) Cell cycle analysis
742 (BrdU/PI) of primary MCL cells after 5 days of co-cultures with monocytes with or without
743 ibrutinib treatment (72h; 0.5 μ M; n = 4). Paired t- test. *** p < .001.

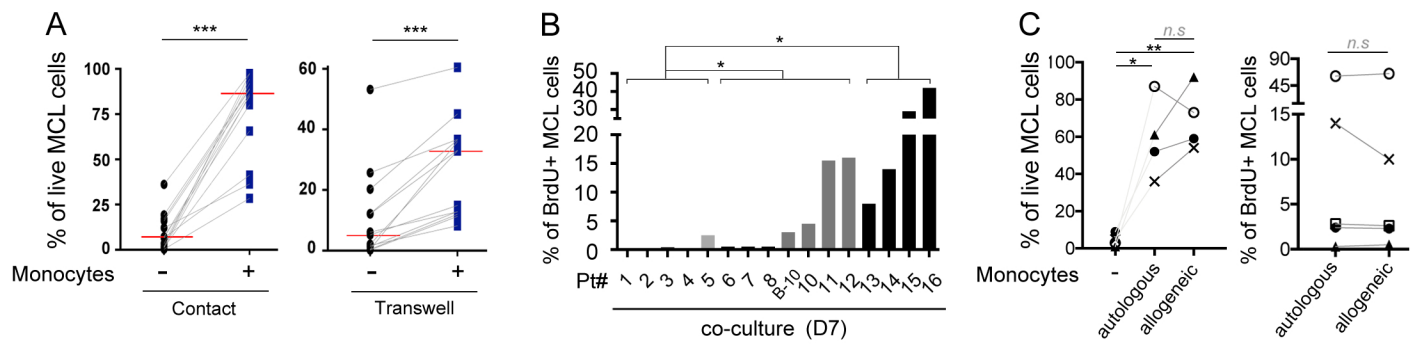
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745 **Figure 5. CD163 modulation on circulating monocytes *in vivo* might be an early marker**
746 **of ibrutinib response.** (A) Plasma concentration of CSF1 and IL-10 proteins in MCL

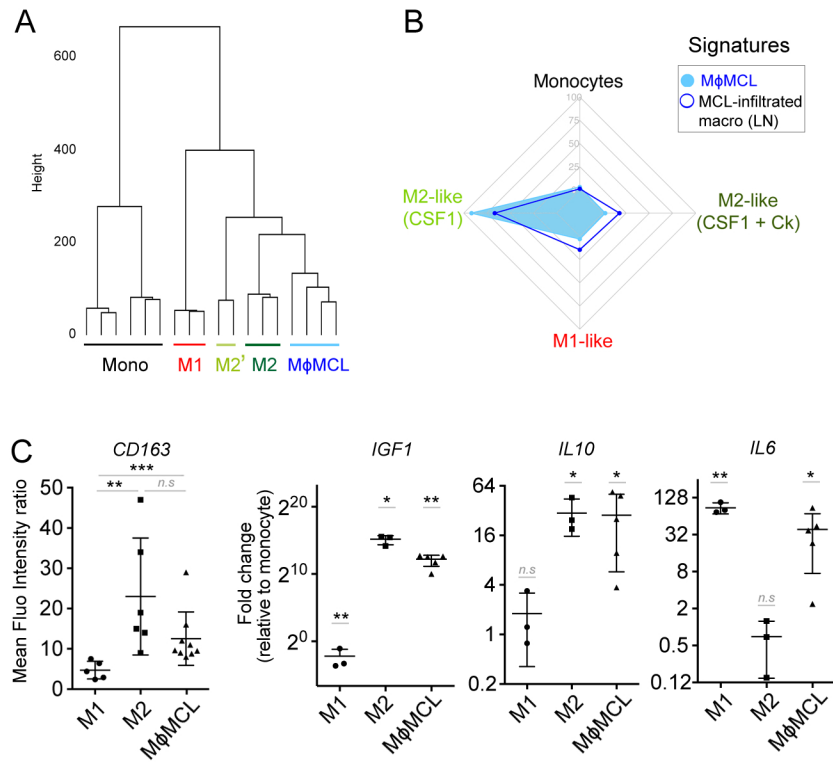
747 patients (n = 28) and age-matched healthy donors (HD, n = 9)) by ELISA. (lower panel)
748 Mean fluorescence intensity ratio (MFI_r) of CD163 on circulating monocytes (CD14⁺ cells) in
749 MCL patients (n = 32) compare to age-matched HD (n = 8). Mann-Whitney test. * p < .05 ** p
750 < .01 ***P < .001. (B) Plasma concentration of CSF1 and IL-10 and CD163 MFI_r modulation
751 on monocytes (CD14⁺) of patient treated with anti-CD20 and ibrutinib (Clinical trial:
752 NTC02558816). Biological parameters have been evaluated before treatment with ibrutinib
753 and obinutuzumab (D0, black bars) and after 8 days of treatment (D8, grey bars). Variation of
754 CD163 expression is normalized to D0 (% of D0).

755

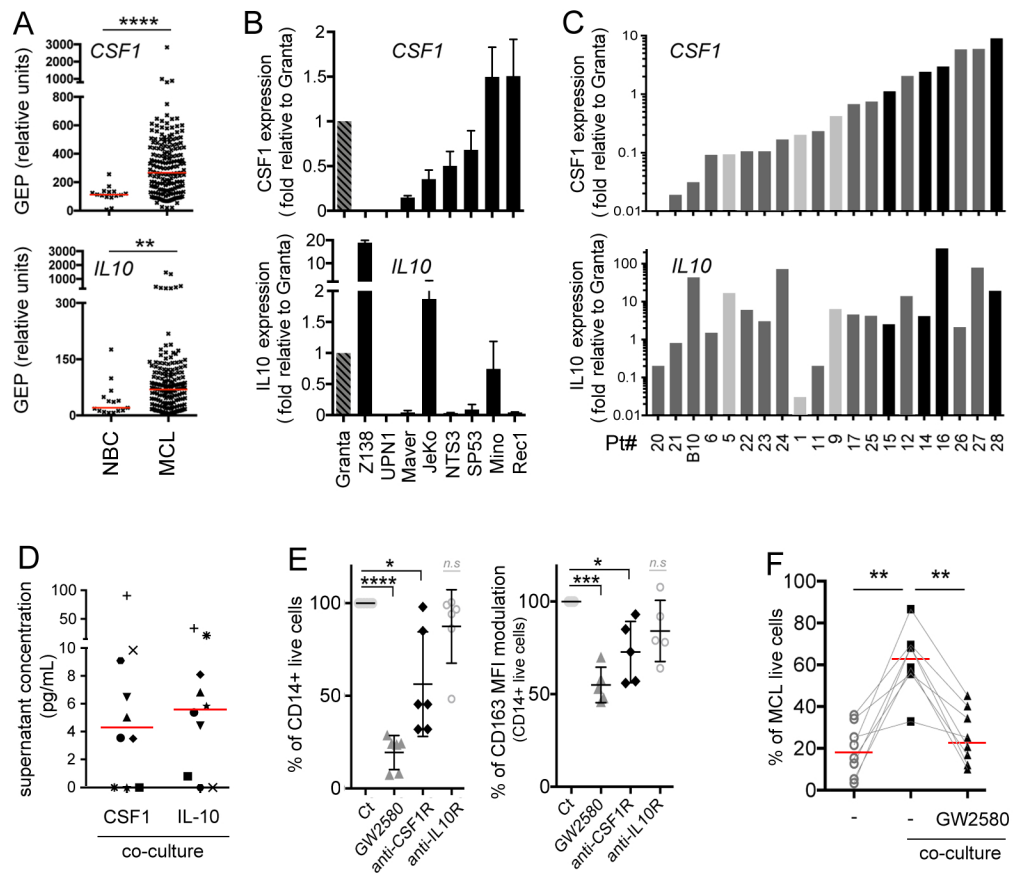
756 **Figure 6. CSF1R as a potential therapeutic target for ibrutinib resistant patients.** (A)
757 ibrutinib-sensitive (Pt#7, 11, 15, 16) and ibrutinib-resistant (Pt#2, 5, 18, 19) primary MCL
758 cells were cocultured with monocytes and treated with ibrutinib (0.5μM) or GW2580 (0.5μM)
759 for 72h. (B) Ibrutinib-sensitive (Pt#7, 11, 15) and ibrutinib-resistant (Pt#5, 12, 18) primary
760 MCL cells were cocultured with monocytes and treated with low doses of ibrutinib (125 nM)
761 or GW2580 (125 nM) or both for 72h. Cell death was assessed by assessed by Annexin-V
762 staining.



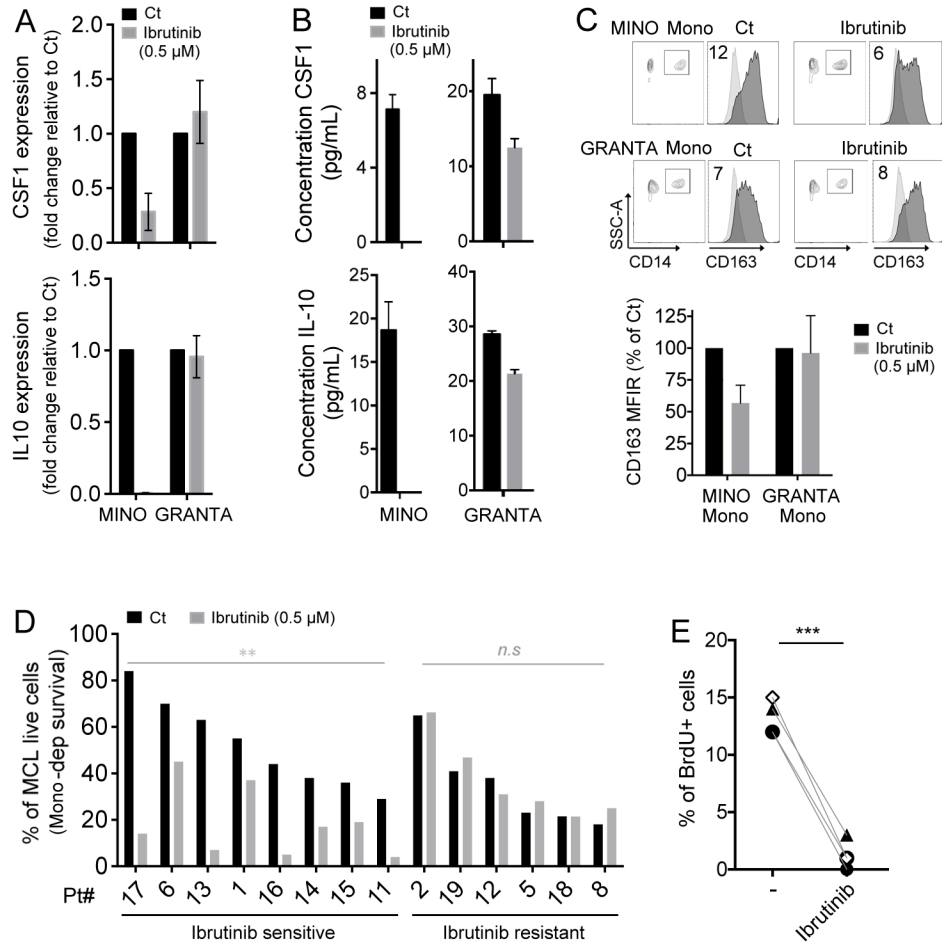
Papin et al. Figure 1



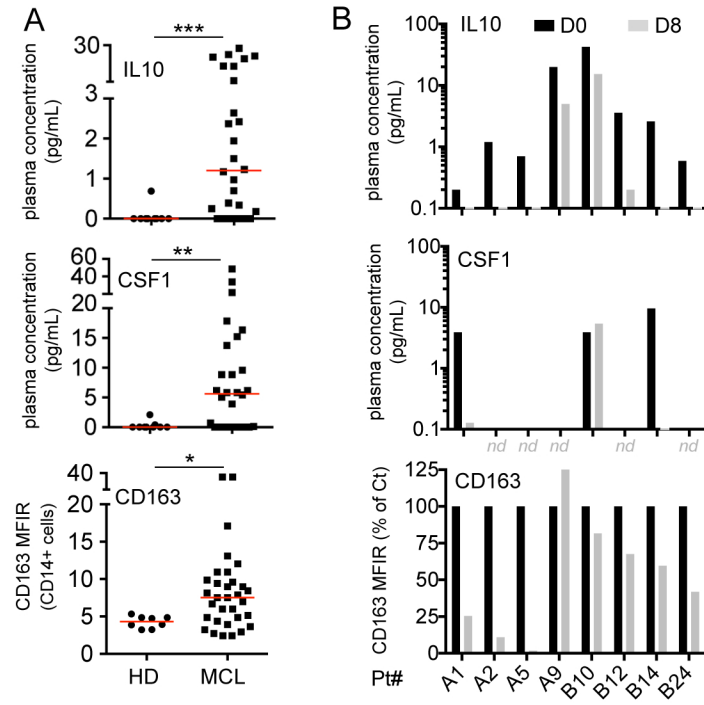
Papin et al. Figure 2



Papin et al. Figure 3



Papin et al. Figure 4



Papin et al. Figure 5

