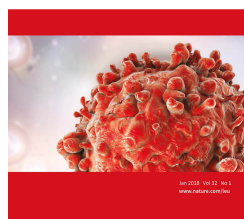Author Version: Published ahead of online first

Leukemia

Autocrine Tnf Signaling Favors Malignant Cells in Myelofibrosis in A Tnfr2-Dependent Fashion

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1 **AUTOCRINE TNF SIGNALING FAVORS MALIGNANT CELLS IN MYELOFIBROSIS IN A TNFR2-DEPENDENT**
2 **FASHION**

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21 **Conflict-of-interest disclosure:**

31 **Abstract**

32 Tumor necrosis factor alpha (TNF) is increased in myelofibrosis (MF) and promotes survival of malignant
33 over normal cells. The mechanisms altering TNF responsiveness in MF cells are unknown. We show that
34 the proportion of marrow (BM) cells expressing TNF is increased in MF compared to controls, with the
35 largest differential in primitive cells. Blockade of TNF receptor 2 (TNFR2), but not TNFR1, selectively
36 inhibited colony formation by MF CD34$^+$ and mouse JAK2^{V617F} progenitor cells. Microarray of mouse
37 MPN revealed reduced expression of X-linked inhibitor of apoptosis (*Xiap*) and mitogen-activated
38 protein kinase 8 (*Mapk8*) in JAK2^{V617F} relative to JAK2^{WT} cells, which were normalized by TNFR2 but not
39 TNFR1 blockade. *XIAP* and *MAPK8* were also reduced in MF CD34$^+$ cells compared to normal BM, and
40 their ectopic expression induced apoptosis. Unlike *XIAP*, expression of cellular IAP (cIAP) protein was
41 increased in MF CD34$^+$ cells. Consistent with cIAP's role in NF-κB activation, TNF induced NF-κB activity
42 was higher in MF versus normal BM CD34$^+$ cells. This suggests that JAK2^{V617F} reprograms TNF response
43 towards survival by downregulating XIAP and MAPK8 through TNFR2. Our results reveal an unexpected
44 pro-apoptotic role for XIAP in MF and identify TNFR2 as a key mediator of TNF-induced clonal expansion.

45

Introduction

Myelofibrosis (MF) is a myeloproliferative neoplasm (MPN) characterized by bone marrow (BM) reticulin fibrosis, anemia and splenomegaly due to extramedullary hematopoiesis. Constitutive activation of JAK/STAT signaling due to somatic mutations in Janus kinase 2 (JAK2), calreticulin (CALR) or the thrombopoietin receptor (MPL) is central to MF pathogenesis and endows neoplastic cells with a competitive advantage over their normal counterparts.[1-6] JAK2 inhibitors, such as ruxolitinib, reduce splenomegaly and symptom burden and may prolong survival in MF patients, but are not curative.[7] JAK/STAT signaling promotes generation of inflammatory cytokines such as tumor necrosis factor (TNF), which cause constitutional symptoms such as weight loss and fever.[8] We have shown that TNF stimulates myeloid colony formation by MF CD34$^+$ cells but reduces colony formation by normal controls and that absence of TNF attenuates disease in mice with JAK2^{V617F}-induced MPN.[2]

The aging BM is characterized by inflammation, a bias towards myelomonocytic differentiation and somatic mutations in genes related to myeloid malignancies, including JAK2.[9-14] This suggests that MPNs such as MF may arise through a process in which clones carrying JAK2 activating mutations are selected in the inflammatory environment, a concept supported by the steep increase of MPN incidence with age.[15] The fact that TNF mediates the proliferative advantage of MPN over normal cells suggests therapeutic utility for TNF antagonists. The TNF neutralizing agent etanercept improved symptoms in a pilot study of MF patients.[16] However, TNF inhibitors have not been developed as MF therapeutics, reflecting concerns about immunosuppression in an already immunocompromised patient population.

TNF signaling involves two distinct receptors, TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2). TNFR1 expression is ubiquitous, while TNFR2 expression is largely confined to cells of hematopoietic origin. Only TNFR1 contains an intracellular death domain (DD) that is required for formation of a pro-apoptotic complex (Complex II) consisting of Fas-associated death domain (FADD), TNFR1-associated death domain (TRADD), receptor-interacting serine/threonine protein kinase 1 (RIPK1) and procaspase-8.[17] Despite TNFR1's ability to form this pro-apoptotic complex, the initial signaling event following TNF engagement is the formation of a membrane bound complex (Complex I) where TRADD associates with TNFR-associated factor 2 (TRAF2), cellular inhibitor of apoptosis (cIAP) and poly-ubiquitinated RIPK1 leading to nuclear factor-κB (NF-κB) activation. Subsequent formation of Complex II occurs after dissociation from TNFR1 and internalization to the cytoplasm.[18] Cell fate is dependent on factors that balance the NF-κB induction of inflammatory and survival signals with caspase-8 dependent apoptosis. Important factors are cIAP-mediated stabilization of Complex I and expression of the long isoform of cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP$_L$), an inactive caspase-8 homolog that competes for binding to FADD, thereby maintaining Complex II in an inactive state.[19, 20] Since TNFR2 lacks the DD, it cannot associate with TRADD and FADD, however TNFR2 is able to bind TRAF2 and coordinate NF-κB signaling in association with cIAP.[21]

The fact that TNF favors growth of MPN over normal cells suggests that JAK/STAT activation shifts TNF signaling outcomes to survival/proliferation, but it is unknown which differences in signaling between MPN and normal cells underlie the differential response to TNF. Here we show that blocking TNFR2 but not TNFR1 selectively inhibits MPN cells over normal controls and implicate X-linked inhibitor of apoptosis (XIAP), cIAP and mitogen-activated protein kinase 8 (MAPK8) as key mediators of differential responses to TNF.

Methods

88 **Human Samples**
89 Peripheral blood (PB) and BM samples were collected according to an IRB-approved protocol (#45880),
90 following informed consent. When isolating specific cell fractions, mononuclear cells (MNCs) were
91 prepared with ficoll-paque (GE Healthcare, Uppsala, Sweden) and positive selection with microBeads
92 (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed on an autoMACS Pro Separator
93 (Miltenyi). Details of MF patient information and normal BM donors are provided in Supplemental
94 Tables 1-2.
95
96 **Intracellular TNF and TNF Receptor (TNFR) Staining**
97 Leukocytes from MF (n=5) or normal BM (n=10) samples were treated with 10 µg/mL brefeldin A (Sigma-
98 Aldrich) and 100 ng/mL lipopolysaccharide (LPS; Sigma-Aldrich), where indicated. Human antibody
99 panels were used to identify stem and progenitor cells as described[22] and mature populations were
100 identified using standard markers. Stem and progenitor cell populations in Balb/c mice (n=8) were
101 identified as reported[23] except for the omission of Sca-1 (low expression in Balb/c mice; complete list of
102 markers and antibodies is provided in the Supplemental Tables 3-6). Cells were stained for surface
103 markers prior to incubation in a paraformaldehyde/saponin buffer (BD Bioscience, Franklin Lakes, NJ),
104 then stained with TNF antibody (BD Bioscience). Samples were analyzed using a FACSCanto cytometer
105 (BD Bioscience) and analyzed with FlowJo analysis software (Treestar, Ashland OR). Graphs show
106 mean/s.e.m.
107
108 **Clonogenic Assays**
109 In murine samples, lineage depletion was performed with an autoMACS Pro Separator (Miltenyi Biotec),
110 prior to sorting for DAPI⁻ Kit⁺ positive cells with a FACSAria Cell Sorter (BD). Human CD34⁺ cells (MF-n=4,
111 CB-n=3) or Lin⁻Kit⁺ mouse cells (n=3) were treated in liquid culture with TNFR blocking antibodies
112 (Supplemental Table 7) at 10 µg/mL for 72 hours, then plated in MethoCult with continued antibody
113 treatment. Liquid culture and colony assays were supplemented with SCF (human-50 ng/mL, murine-100
114 ng/mL) and IL-3 (human-10 ng/mL, murine-50 ng/mL). Inducible shRNA constructs were purchased from
115 Cellecta (Mountain View, CA) in lentiviral vectors containing a GFP expression marker. GFP⁺ cells were
116 sorted 3 days post infection (n=3), and treated ±200 ng/mL doxycycline (Clontech Laboratories Inc.,
117 Mountain View, CA). Cells were maintained in liquid culture for 96 hours, transferred to clonogenic
118 assays as described above, with colonies scored after 10-14 days. Graphs show mean/s.e.m.
119
120 **Microarray Analysis**
121 BM cells were harvested from 3-4 mice for each experiment, cultured for 16 hours ±TNFR1 or TNFR2 BA
122 (10 µg/mL). After treatment, Lin⁻Kit⁺ cells were isolated as described above and further subdivided into
123 GFP⁺ and GFP⁻ cells for a total of 6 distinct groups. Three independent experiments were performed and
124 RNA was isolated using RNeasy Micro Kit (Qiagen, Germantown, MD). RNA was further purified using
125 Clean & Concentrator columns (Zymo Research, Irvine, CA), cDNA was prepared with the Ovation Pico
126 WTA System V2 (NuGEN, San Carlos, CA) and labeled with the Encore Biotin Module (NuGEN). The cDNA
127 target samples were hybridized to an Affymetrix Mouse Expression 430 2.0 array (Thermo Fisher
128 Scientific). Image processing was performed using Affymetrix Command Console (AGCC) v.3.1.1 software
129 (Thermo Fisher Scientific) and expression analysis was performed using Affymetrix Expression Console
130 build 1.4.1.46 (Thermo Fisher Scientific). Data has been uploaded to Gene Expression Omnibus
131 (accession number GSE104792).

132

Statistical Analysis

133 **Statistical Analysis**

134 Significance of individual comparisons was determined by Student's t-test with paired comparisons for
135 murine samples (JAK^{V617F+} and JAK2^{V617F-} cells from individual mice) and unpaired comparisons between
136 human MF samples relative to normal controls (with test for variance). Two-tailed tests were used for all
137 comparisons except for intracellular TNF expression (one-tailed) where the distribution was limited by
138 zero values. A two-way ANOVA was used for comparison of the NFκB time-course experiment.

139
140
141

Results

142 **Results**

143 ***TNF expression is increased in MPN cells from multiple hematopoietic cell compartments.*** To
144 determine the cellular origin of TNF, we assessed expression by FACS in BM or blood of MF patients
145 compared to normal controls (Figure 1a). To increase intracellular TNF, allowing detection at the single
146 cell level, cells were treated with brefeldin A (10 μg/mL).[24, 25] The percentage of TNF$^+$ cells was increased
147 in hematopoietic stem cells (HSCs), multipotent progenitor cells (MPPs), common lymphoid progenitor
148 cells (CLPs), monocytes and mature B-cells (Figure 1b upper panel). To determine if cell populations
149 were differentially sensitive to inflammatory stimuli, cells were treated with LPS (100 pg/mL). TNF
150 expression in monocytes from MF patients (72.5% TNF$^+$) and normal individuals (70.5% TNF$^+$) was
151 comparable. In contrast, LPS-induced TNF expression in primitive populations was elevated in MF
152 relative to normal controls, with the largest differential in HSCs (16.7% vs. 1.1% TNF$^+$) and significant
153 differences in MPPs and MEPs (Figure 1b lower panel). Since the average age of the control population
154 used in this study was significantly lower than the MF population (40.0 vs. 59.8 years old, p<0.001), we
155 also stratified normal controls based on age (<50 vs. >50 years old) and compared TNF expression. We
156 found no difference in any of the populations, except for the LPS stimulated granulocytes (p<0.05),
157 although the absolute expression was extremely low in this populations (<50 = 0.1% vs. >50 = 0.7% TNF$^+$;
158 Supplemental Figure 1). Analogous experiments were performed on BM from Balb/c mice with MPN
159 induced by transplantation of donor cells transduced with JAK2^{V617F}, where JAK2^{V617F} cells are identified
160 by GFP expression.[26, 27] TNF expression was generally higher in JAK2^{V617F} cells, with the largest difference
161 in the HSC-enriched population (21.5% vs. 6.4% TNF$^+$ cells; Figure 1c upper panel), irrespective of LPS
162 stimulation. In mature populations, the largest difference in TNF expression between JAK2^{V617F+} and
163 JAK2^{V617F-} cells was in T-cells, while expression in the mature myeloid lineages (Mac1$^+$ and/or GR1$^+$) was
164 comparable (Figure 1c lower panel). These results indicate that the proportion of TNF expressing cells is
165 consistently higher in MF vs. normal controls, particularly in less differentiated cell types. Furthermore,
166 JAK2^{V617F} is sufficient to induce these differences in TNF expression.

167 ***Treatment with pan-TNF inhibitors does not reduce disease burden in murine MPN.*** To test the effects
168 of TNF neutralization *in vivo*, we used two TNF inhibitors in a retroviral mouse model of JAK2^{V617F}-
169 induced MPN. Etanercept is a soluble TNFR2 decoy receptor that binds human or mouse TNF, and has
170 shown efficacy in mouse models of rheumatoid arthritis (RA).[28] CNTO5048 is a modified version of the
171 TNF-neutralizing antibody infliximab with an altered variable region that increases its affinity for mouse
172 TNF.[29] Both agents are used to treat RA and other autoimmune disorders.[30] We confirmed the activity of
173 each of these agents against mouse TNF in an assay of TNF-mediated cytotoxicity (L929 cells;
174 Supplemental Figure 2a,b).[31] In successive studies with etanercept and CNTO5048, mice with established

175 MPN were treated for 8-12 weeks with dosing schedules reported to be effective in murine models of
176 RA.[28, 32] Neutralization of soluble TNF in serum of mice was confirmed with the L929 cytotoxicity assay
177 (Supplemental Figure 2c,d). No significant differences in white blood cell counts, hematocrit, GFP$^+$ cells
178 (Supplemental Figure 2e-j) or spleen weights (Supplemental Figure 2k,l) were observed between
179 treatment groups. These results are consistent with the modest effects observed with etanercept in MF
180 that showed hematologic improvement in 20% of patients.[16] Since the TNF neutralizing agents failed to
181 inhibit disease, we hypothesized that differential signaling from TNFR1 and/or TNFR2 underlies the
182 competitive advantage of MPN cells.

183 ***TNFR2 but not TNFR1 selectively suppresses myeloid colony formation by MPN progenitor cells.*** To
184 identify the mechanistic basis for the differential effects of TNF on MF vs. normal cells, we quantified
185 TNF receptor expression in primitive (Supplemental Figure 3a) and mature (Supplemental Figure 3b)
186 hematopoietic cells from human MF vs. normal BM and JAK2^{V617F+} vs. JAK2^{V617F-} cells from MPN mice
187 (Supplemental Figure 3c). No significant differences were observed, suggesting that the differential
188 effects of TNF on MPN vs. normal cells are due to modulation of downstream signaling events. To
189 determine the contribution of TNFR1 and TNFR2-mediated effects on MPN clonal dominance, we used
190 receptor blocking antibodies (BAs) to inhibit each receptor. The specificity of all BAs was confirmed
191 using ELISA and L929 cytotoxicity assays (Supplemental Figure 4a-d). CD34$^+$ cells from MF samples or
192 normal cord blood (CB) were cultured for 72 hours ±TNFR1 or TNFR2 BAs (10 µg/mL), then transferred
193 to semisolid medium with continued treatment. Blocking TNFR1 did not significantly change
194 granulocyte-macrophage (GM) colony numbers for MF, BM or CB samples, while TNFR2 block
195 consistently reduced MF colonies by 25-30%, with no effect on BM or CB (Figure 2a; colony counts
196 provided in Supplemental Figure 5a-c). Single colonies were genotyped for JAK2 by BsaXI digestion
197 (Supplemental Figure 6a). The vast majority of samples were homogenous for JAK2^{V617F} colonies. In one
198 MF patient sample (from a patient with a JAK2^{V617F} allele burden of ~85%) with a mixture of JAK2^{V617F} and
199 JAK2^{WT} colonies, blocking TNFR2 selected for JAK2^{WT} over JAK2^{V617F} colonies (Figure 2b). We next tested
200 the effects of TNFR BAs on Lin$^-$Kit$^+$ BM cells from mice with JAK2^{V617F} MPN, using the same experimental
201 design. After enumeration, 20-25 single GM colonies were genotyped using a FACS-based assay
202 measuring GFP (Supplemental Figure 6b, c). Both BAs mildly reduced total colony numbers. While the
203 TNFR1 BA reduced JAK2^{V617F+} and JAK2^{V617F-} colonies to a similar degree, the TNFR2 blocking antibody
204 selectively reduced JAK2^{V617F+} colonies (Figure 2c). For validation, human MF and CB CD34$^+$ cells were
205 infected with doxycycline-inducible shRNAs targeting TNFR1 or TNFR2 (Supplemental Table 8). Effective
206 TNFR1/2 knockdown was confirmed in SET-2 cells (Supplemental Figure 7a, b). Cells were cultured in
207 cytokine containing media (SCF and IL-3, no exogenous TNF) ±doxycycline prior to plating in colony
208 assays with continued treatment. Knockdown of TNFR2 reduced colony formation by MF cells to a
209 similar degree as seen with the TNFR2 BA (~30%) with no effect on CB-derived colonies, while
210 knockdown of TNFR1 had no effect (Figure 2d). Altogether these results suggest that the differential
211 effects of TNF on MPN vs. normal progenitor cells are mediated by TNFR2 and that selective inhibition of
212 TNFR2 may reverse the clonal dominance of MPN cells. To test this, we performed a competitive
213 repopulation assay with BM cells from TNFR$^{+/+}$ (CD45.1_C57BL/6) and TNFR2$^{-/-}$ (CD45.2_C57BL/6) mice.
214 Analogous experiments were done using TNFR$^{+/+}$ vs. TNFR1$^{-/-}$ donors. Engraftment of GFP$^+$ cells was
215 monitored over time for TNFR$^{+/+}$ and TNFR2$^{-/-}$ compartments with CD45.1 and CD45.2 specific antibodies
216 (Supplemental Table 9). While the proportion of TNFR1$^{+/+}$ and TNFR1$^{-/-}$ cells within the GFP$^+$ population
217 remained constant (Figure 2e), JAK2^{V617F} expressing TNFR2$^{+/+}$ outcompeted TNFR2$^{-/-}$ cells (Figure 2f).

218 However, engraftment of GFP$^+$ cells was transient and eventually JAK2^{V617F+} cells were lost irrespective of
219 genotype, precluding further analysis.

220 ***TNFR1 or TNFR2 inhibition partially reverses gene expression differences between JAK2^{V617F+} and***
221 ***JAK2^{V617F-} cells.*** To identify differences in TNF-induced signaling between JAK2^{V617F+} and JAK2^{V617F-} cells,
222 we cultured BM cells from Balb/c mice with established JAK2^{V617F}-induced MPN in media containing SCF
223 and IL-3, ±murine TNFR1 or TNFR2 BA in three independent experiments (Figure 3a). Lin$^-$Kit$^+$ cells were
224 sorted into GFP$^+$ and GFP$^-$ subsets and subjected to gene expression profiling. All but one sample
225 (untreated-JAK2^{V617F+} replicate #3, which was excluded) passed quality thresholds. Unsupervised
226 hierarchical clustering grouped samples according to genotype (JAK2^{V617F+} vs. JAK2^{V617F-}) and treatment
227 (Figure 3b). We hypothesized that genes critical to TNFR2's distinct effects would be differentially
228 expressed in JAK2^{V617F+} vs. JAK2^{V617F-} cells and that blocking TNFR2 should reverse this difference. In
229 addition, we considered genes regulated by TNFR2 in JAK2^{V617F+} cells with minimal effects in JAK2^{V617F-}
230 cells as the highest priority and applied sequential filters to identify genes meeting these criteria for
231 TNFR2 and TNFR1, respectively (Figure 3c). Evaluation of the top 10 up- and down-regulated genes,
232 based on fold-change between JAK2^{V617F+} vs. JAK2^{V617F-} cells, showed that expression of the genes with
233 the highest degree of up-regulation (*Itm2a*; *Prdx2*; *Asb17*) was reversed by either TNFR1 or TNFR2 block,
234 while the two genes with the highest down-regulation, *Xiap* and *Mapk8* (*Jnk1*), were selectively reversed
235 by TNFR2 but not TNFR1 block (Figure 3c). We therefore focused on *Xiap* and *Mapk8*, as genes
236 potentially mediating the differential effects of TNF signaling in JAK2^{V617F+} vs. JAK2^{V617F-} cells. While it is
237 conceivable that other differentially expressed genes such as *Prdx2* are also disease relevant, they were
238 not investigated in the current study since they were not differentially regulated by TNFR2 over TNFR1.

239 To validate the microarray results, we analyzed by qPCR *Xiap* and *Mapk8* expression in Lin$^-$Kit$^+$ cells from
240 an independent group of MPN mice (n=3) and in MF patients and normal BM controls (primer
241 sequences are provided in Supplemental Table 10). Expression of *Xiap* and *Mapk8* was consistently
242 lower in JAK2^{V617F+} compared to JAK2^{V617F-} murine cells and in MF cells compared to normal BM (Figure
243 4a,b). MAPK8 has been implicated as a necessary component of TNF-mediated apoptosis[33], predicting
244 that reduced expression would have an anti-apoptotic effect. Although XIAP inhibits caspase activity,
245 low expression of XIAP is permissive for stabilization of the related family member cIAP. IAPs are known
246 to regulate each other's expression through their E3 ubiquitin ligase activity[34] and *Xiap* null mice show
247 markedly increased cIAP protein[35]. Since cIAP is required for TNF-dependent NF-κB signaling[36], we
248 hypothesized that the growth advantage of MPN cells exposed to TNF could be mediated through an
249 increase in cIAP protein levels. We evaluated cIAP expression in CD34$^+$ cells from MF samples and
250 normal BM using immunofluorescence and found that cIAP expression was higher in MF samples
251 relative to normal BM (Figure 4c). Concordantly, cIAP staining was increased in BM cores from MF
252 patients relative to normal controls (Figure 4d). To validate the inverse relationship between XIAP and
253 cIAP, we overexpressed XIAP in CD34$^+$ cells from MF patient samples and measured cIAP expression.
254 Overexpression of XIAP reduced cIAP levels relative to vector control (Figure 4e).

255 ***Blocking cIAP inhibits survival, while ectopic expression of XIAP or MAPK8 induces apoptosis in***
256 ***primitive MF cells.*** To test whether cIAP is functionally important, we used birinapant at a concentration
257 that selectively inhibits cIAP over XIAP (10 nM).[37,38] CD34$^+$ MF and normal BM cells were treated in liquid
258 culture for 72 hours then transferred to semi-solid media. Colony formation was significantly reduced in
259 MF samples relative to normal BM, suggesting that cIAPs favor survival of MPN over normal cells (Figure
260 5a). In contrast, 100 nM birinapant inhibited colony growth of MF and normal BM to a similar degree.

261 Since cIAPs mediate TNF-induced NF-κB activation[36], we next assessed NF-κB signaling. MF (n=3) or
262 normal BM (n=3) CD34^{+} cells were infected with an NF-κB reporter construct, pretreated ±TNFR1 or
263 TNFR2 BA (10 µg/mL) followed by addition of TNF (1 ng/mL). NF-κB activity was maximal at 8 hours post
264 TNF stimulation with a 41-fold increase in MF cells and a 19-fold increase in normal BM. NF-κB activity
265 was significantly higher in MF cells over the entire time course (p=0.03). Treatment with either the
266 TNFR1 or TNFR2 BA reduced NF-κB activity in MF and normal BM cells. Notably, either blocking antibody
267 reduced NF-κB activity in MF cells to levels seen in the normal BM cells (Figure 5b). We next ectopically
268 expressed XIAP and/or MAPK8 in CD34^{+} cells from MF patient samples (n=4) and measured annexin V
269 after 72 hours. Expression of MAPK8 and XIAP significantly increased annexin V^{+} cells over vector
270 control, suggesting that their downregulation is critical for MPN cells to avoid TNF-induced apoptosis
271 (Figure 5c).

272

273 **Discussion**

274 Chronic low-level inflammation is a feature of the aging hematopoietic system.[9,14,39-43] The BM
275 concentrations of key inflammatory cytokines such as TNF and IFNγ increase with age and induce a
276 myelomonocytic differentiation bias.[9] Concentrations of inflammatory cytokines are elevated in the
277 plasma of most MPN patients. The highest levels are found in MF and correlate with symptom burden
278 and shorter survival.[44] We have previously shown that TNF mediates the clonal dominance of JAK2[V617F]
279 over JAK2[WT] cells, implicating TNF as a disease driver.[2] Although many inflammatory cytokines are
280 elevated in MF, these data suggest a central role for TNF in MPN.

281 We show that TNF expression is higher in most cellular subsets of MF compared to controls under basal
282 conditions, with differences enhanced by LPS in most hematopoietic progenitor cells. This is consistent
283 with recent data showing that different hematopoietic cells have distinct cytokine production profiles
284 including TNF.[8] These results suggest that MPN HSCs express TNF to inhibit the growth of normal HSCs
285 competing for the same niches, especially under systemic inflammatory stress (LPS stimulation). These
286 studies included samples from MF patients with either JAK2[V617F] or CALR mutations, without appreciable
287 differences between genotypes (patient profiles are provided in Supplemental Table 9).

288 TNF scavengers, such as etanercept and infliximab are associated with increased risk for opportunistic
289 infections, giving rise to concerns about their use in immunocompromised patients, such as MF.[45] Data
290 on anti-TNF therapeutics in MF are limited to one small study and anecdotal cases.[16, 46] Consistent with
291 their modest effects in MF patients, etanercept and infliximab failed to reduce MPN disease in mice with
292 JAK2[V617F] MPN (Supplemental Figure 2). These results are at odds with the attenuation of MPN in a
293 previous study using TNF deficient mice[2] and suggest that additional, unintended effects, of pan-TNF
294 antagonists may neutralize differences in TNF responses between MF and normal cells.[2, 47] Several
295 studies have implicated TNF as a negative regulator of HSCs.[48-50] Conflicting data were reported
296 regarding the requirement for TNFR1 to mediate the suppressive TNF effects on HSCs cultured ex vivo.[49,
297 50] In competitive repopulation experiments, both TNFR1^{-/-} and TNFR2^{-/-} HSCs were shown to outcompete
298 TNFR[WT] cells to a similar degree, with a more pronounced advantage for double null cells.[51] To
299 understand how JAK2[V617F] reprograms TNF signaling in hematopoietic progenitors from suppression to
300 stimulation, we asked whether this involves altered responses through TNFR1 and/or TNFR2. Our results
301 support a model in which signaling through TNFR2, but not TNFR1, mediates the differential effects of

302 TNF on myeloid colony formation of JAK2^{V617F} vs. JAK2^{WT} progenitor cells. Although the MF-specific
303 reduction in colony formation with TNFR2 BA or knockdown was relatively modest (~30%; Figure
304 2a,b,d), this difference may be sufficient to promote clonal dominance during the slow evolution of
305 MPN. For unknown reasons, TNFR2 block reduced the JAK2^{V617F+} colonies much more profoundly in
306 assays of mouse progenitor cells (by 89%, Figure 2c). It is conceivable that the co-culture of JAK2^{V617F} and
307 JAK2^{WT} cells is necessary to potentiate the differential effect and thus the difference was less dramatic in
308 the human samples where MF and normal cells were cultured individually, in line with the observation
309 that cytokine expression is altered both in mutant and normal cells isolated from MPN mice.[8] It is also
310 possible that the over-expression of JAK2^{V617F} induced by the transduction/transplantation model may
311 have amplified the effect in the mouse cells relative to the human samples. We selected this model
312 because plasma TNF concentrations are elevated over controls to a similar degree as in MF (~10-15-
313 fold). [26, 47]

314 The fact that TNFR1 and TNFR2 expression is comparable between MPN and normal BM cells excludes
315 JAK/STAT regulation of TNFR1/2 expression as the cause of differential TNF responses, instead
316 implicating differences in TNF signaling cascades. We found that expression of *Xiap* and *Mapk8*, two
317 regulators of TNF signaling, is reduced in JAK2^{V617F+} mouse progenitor cells and that blocking TNFR2
318 restores their expression to the levels in JAK2^{WT} cells (Figure 3a-c). Consistent with the mouse
319 experiments, expression of *XIAP* and *MAPK8* was lower in human MF vs. normal BM CD34$^+$ cells (Figure
320 4b). Reduced expression of *Xiap* in murine JAK2^{V617F+} cells could be considered counterintuitive as XIAP
321 inhibits caspases and hence apoptosis.[52] However, there is evidence for mutual regulation between XIAP
322 and cIAP. Thus cIAP expression is increased in *Xiap* null mice, and this has been linked to the ability of
323 XIAP to promote cIAP ubiquitination and proteasomal degradation, and vice versa.[34, 35] Indeed, cIAP
324 expression was higher in MF compared to normal CD34$^+$ cells and BM biopsies. Attempts to assess XIAP
325 protein levels in hematopoietic cells were unsuccessful, but consistent with above predictions, ectopic
326 expression of XIAP induced apoptosis in MF CD34$^+$ cells (Figure 5c). Similarly, in many cancer cell lines,
327 IAP proteins inhibit apoptosis induced by TNF.[53, 54] Downregulation of cIAPs or treatment with a cIAP
328 inhibitor allows TNF-dependent apoptosis to proceed. This is reminiscent of our findings in MF cells,
329 which are more sensitive than control to inhibition with the IAP inhibitor birinapant, at concentrations
330 that block cIAP, but not XIAP; while the differential is lost at higher concentrations that block both
331 (Figure 5a). cIAP promotes activation of NF-kB and, as predicted, NF-kB reporter activity was shown to
332 be higher in MF cells relative to normal BM cells (Figure 5b) as reported by others.[55] This is consistent
333 with data for the cIAP inhibitor LCL161, which is demonstrating activity in early clinical testing for the
334 treatment of MF.[56] A second entry point into TNFR signaling involves reduction of MAPK8 expression to
335 inhibit apoptotic signaling through TNFR1 Complex II. As MAPK8 promotes degradation of c-FLIP$_L$[20],
336 reduced MAPK8 should stabilize the c-FLIP$_L$ heterodimer with procaspase-8, favoring the inactive form
337 of TNFR1 Complex II (Figure 5d).

338 Altogether our findings are consistent with a model, in which MF cells downregulate XIAP and MAPK8
339 through a TNF/TNFR2-dependent autocrine loop to escape an apoptotic response and enhance NF-κB
340 signaling (Figure 5d). Since NF-κB is also a key mediator of inflammatory cytokine expression [57], this
341 creates a positive feedback loop where TNF functions as a master regulator of inflammatory cytokine
342 production in MPN cells. Studies to determine exactly how JAK2^{V617F} and other MF-associated mutations
343 modulate TNFR2 signaling to limit the expression of XIAP and MAPK8 compared to their normal

344 competitors are in progress. Our work supports the further clinical development of cIAP inhibitors and
345 implicates selective TNFR2 inhibitors as potential therapeutics in the treatment of MF.

346

347 **Authorship**

348 Contribution: W.L.H designed experiments, carried out experiments, analyzed data and wrote the
349 manuscript; A.V.S. carried out animal experiments, A.D.P designed experiments and analyzed data;
350 M.E.S. carried out experiments and analyzed data; P.M.C. processed patient samples; D.Y. carried out
351 experiments; R.N.B. analyzed microarray data; J.M.G. and J.T.P. provided BM and blood samples; T.O.
352 reviewed the manuscript and provided critical feedback; M.W.D. designed experiments and wrote the
353 manuscript.

354

360

361 **Conflict-of-interest disclosure:**

362 M.W.D. is on the advisory board and is a consultant for Incyte, Novartis and Pfizer, and serves on the
363 advisory board for Ariad, Blueprint and Galena BioPharma. His laboratory receives research funding
364 from Novartis and Pfizer.

365

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581 **Figure Legends**

582 **Figure 1. TNF expression is higher in HSCs from MF patients compared to normal BM and in cells from**
583 **JAK2^{V617F+} compared to JAK2^{V617F-} mice.** (a) Primary cells were immunophenotyped with surface markers
584 and intracellular TNF staining was performed to identify expression in hematopoietic lineages, with HSCs
585 defined as CD45$^+$CD34$^+$CD38$^-$CD45RA$^-$CD90$^+$. (b) Leukocytes were treated *ex vivo* for 16 hours with the
586 protein transport inhibitor brefeldin A (10 μg/mL). The percentage of TNF$^+$ cells was higher in MF
587 patients (n=5; PB=4, BM=1) compared to normal BM (n=10) in HSCs, MPPs, CLPs, monocytes and B cells.
588 (c) Addition of LPS (100 ng/mL) increased the differences in TNF expression between MF specimens
589 (n=5; PB=4, BM=1) and normal BM (n=10) for HSCs, MPPs and MEPs while expression in monocytes was
590 equivalent. (d,e) Similar experiments were performed with BM cells isolated from JAK2^{V617F} MPN mice
591 (n=8), including brefeldin A treatment. (d) TNF expression was significantly higher in JAK2^{V617F+} Lin$^-$
592 Kit$^+$CD48$^-$CD150$^+$ HSC-enriched cells and Lin$^-$Kit$^+$CD34$^-$FcγRII/RIII^{Lo} MEPs. (e) In the mature compartments,
593 JAK2^{V617F+} T-cells (CD4$^+$ and CD8$^+$) expressed higher TNF than JAK2^{V617F-} T-cells, while expression in B-cells
594 (B220$^+$) and myeloid cells (Mac-1$^+$/GR-1$^-$ and Mac-1$^+$/GR-1$^+$) was similar. *p<0.05, **p<0.005

595

596 **Figure 2. Blocking TNFR2 selectively inhibits colony formation by MF vs. normal BM CD34$^+$ cells and**
597 **mouse JAK2^{V617F+} compared to JAK2^{V617F-} progenitor cells.** (a) Human CD34$^+$ cells from MF blood (n=4),
598 normal BM (n=3) or CB (n=3) were treated with TNFR BAs at 10 μg/mL for 72 hours in liquid culture,
599 then plated in clonogenic assays with continued treatment. TNFR2 inhibition reduced colony numbers in
600 MF samples without affecting normal BM or CB control samples. (b) In MF samples, the JAK2 genotype
601 was analyzed for each colony. Of the 4 samples tested, 3 were found to have 100% JAK2^{V617F} allele
602 burden in all conditions tested, while one sample with a mixed JAK2 genotype had a reduction in
603 JAK2^{V617F+} colonies and increase in JAK2^{WT} colonies with TNFR2 inhibition and to a lesser degree with
604 TNFR1 inhibition. (c) Lin$^-$Kit$^+$ BM cells from MPN mice (n=3) were treated in liquid culture with TNFR BAs
605 at 10 μg/mL for 72 hours, then plated in clonogenic assays with continued treatment. Colonies were
606 enumerated after 10 days and isolated colonies genotyped for JAK2 status by analyzing GFP expression.
607 TNFR2 inhibition selectively reduced colony formation of JAK2^{V617F+} cells without affecting total colony
608 numbers, while TNFR1 inhibition had no effect. (d) MF (n=4) and CB (n=4) CD34$^+$ cells were infected with
609 doxycycline-inducible TNFR shRNAs, in liquid culture ±200 ng/mL doxycycline for 96 hours and then
610 plated in clonogenic assays with continued treatment. Induction of the TNFR2 shRNAs reduced colony
611 formation in MF samples without affecting normal controls, while induction with a TNFR1 shRNA had no
612 effect. (e-f) BM cells from 5-FU treated CD45.1 and (e) TNFR1$^{-/-}$ or (f) TNFR2$^{-/-}$ CD45.2 mice were infected
613 with MSCV-IRES-JAK2^{V617F}-GFP retrovirus. Equal numbers of wild type and null GFP$^+$ cells were injected
614 into lethally irradiated TNFR$^{+/+}$ recipients (n=8 per group). TNFR2$^{+/+}$ GFP$^+$ cells increased over TNFR2$^{-/-}$
615 GFP$^+$ cells, while TNFR1$^{+/+}$ GFP$^+$ cells remained at the same level as TNFR1$^{-/-}$ cells. However, GFP$^+$
616 engraftment was not maintained. *p<0.05

617

618 **Figure 3. Expression of Xiap and Mapk8 is downregulated in JAK2^{V617F+} relative to JAK2^{V617F-} cells, but**
619 **this differential is abolished by TNFR2 inhibition.** (a) BM cells from mice with JAK2^{V617F}-induced MPN
620 was cultured for 16 hours ±TNFR BAs (10 μg/mL). Cells were then sorted for Lin$^-$Kit$^+$ expression, then
621 subdivided based on GFP, resulting in 6 groups. Three independent experiments were performed. RNA
622 was extracted for all 18 samples and subjected to microarray analysis using Affymetrix mouse 430 2.0
623 arrays. One sample, Untreated-JAK2^{V617F+} replicate #3 failed quality standards and was removed from

624 further analysis. (b) Unsupervised clustering of the remaining 17 samples grouped all samples according
625 to genotype (JAK2^{V617F}) and treatment. (c) Fold change (FC) and p-values were generated for each
626 condition relative to the JAK2^{V617F-} (Untreated) group. Sequential filters were applied to identify genes
627 whose expression is dysregulated in JAK2^{V617F+} cells and restored with TNFR BA treatment. The set was
628 limited to genes that had a p-value of <0.05 when compared between any 2 treatment groups. Then
629 genes were limited to those that had a FC >|1| in JAK2^{V617F+} relative to JAK2^{V617F-} cells. Genes were then
630 subdivided into those whose expression was reversed with either TNFR1 or TNFR2 BA in JAK2^{V617F+}
631 toward JAK2^{V617F-} by ≥75%. To further identify those that were selectively regulated in JAK2^{V617F+} cells,
632 those with a FC >|1| in JAK2^{V617F-} cells were eliminated. For each of these gene sets we ranked the top
633 10 up- or down-regulated genes. Xiap and Mapk8 were the 2 top differentially expressed genes between
634 JAK2^{V617F+} and JAK2^{V617F-} whose expression was normalized with TNFR2 BA treatment but not TNFR1 BA
635 treatment.

636

637 **Figure 4. XIAP and MAPK8 mRNA expression is down-regulated in mouse JAK2^{V617F+} cells and MF cells.**
638 (a) *Xiap* and *Mapk8* mRNA expression was measured by qPCR, using glyceraldehyde 3-phosphate
639 dehydrogenase (*Gapdh*) as a control gene, in Lin$^-$Kit$^+$ cells from mice with JAK2^{V617F}-induced MPN (n=3).
640 *Xiap* expression was significantly lower and *Mapk8* expression trended lower (p=0.087) in JAK2^{V617F} cells.
641 (b) *XIAP* and *MAPK8* expression was measured by qPCR, using β-glucuronidase (*GUS*) as a control gene,
642 in MF (n=5) and normal BM CD34$^+$ cells (n=3). Expression of both genes was significantly lower in MF. (c)
643 Immunofluorescent images of CD34$^+$ cells stained with a cIAP1/2 antibody. Fluorescence intensity was
644 higher in MF (n=4) cells compared to normal BM (n=3). (d) Core BM biopsy sections from normal
645 controls (n=5), JAK2^{V617F} positive MF (n=4) or CALR positive MF (n=4) samples were stained with a
646 cIAP1/2 antibody to evaluate expression. cIAP staining was stronger in both JAK2^{V617F} and CALR positive
647 MF samples compared to the normal controls, particularly in the megakaryocytes (indicated by the black
648 arrows). (e) Immunofluorescent images of MF cells (n=3) with ectopic XIAP expression showed reduced
649 cIAP1/2 fluorescence intensity relative to vector control. *p<0.05

650

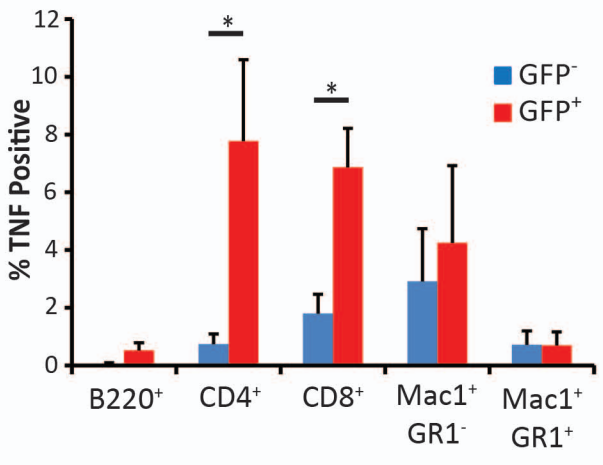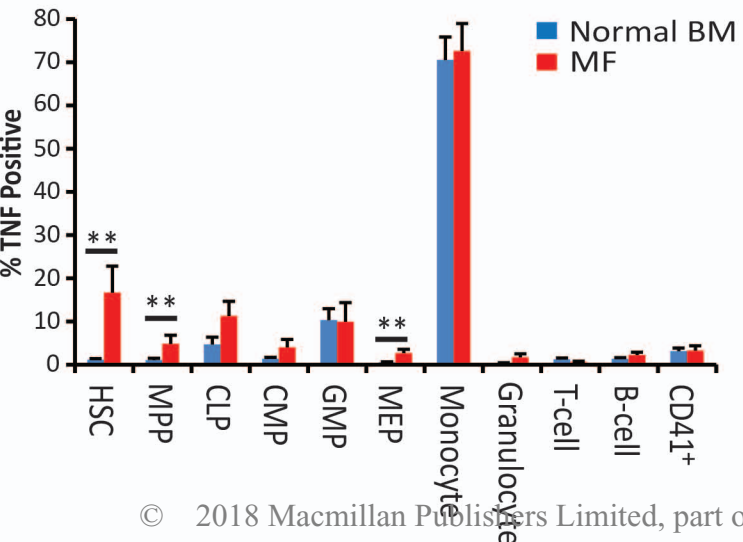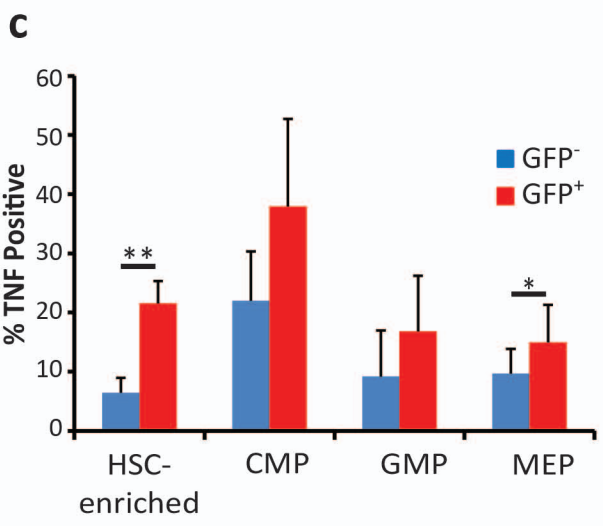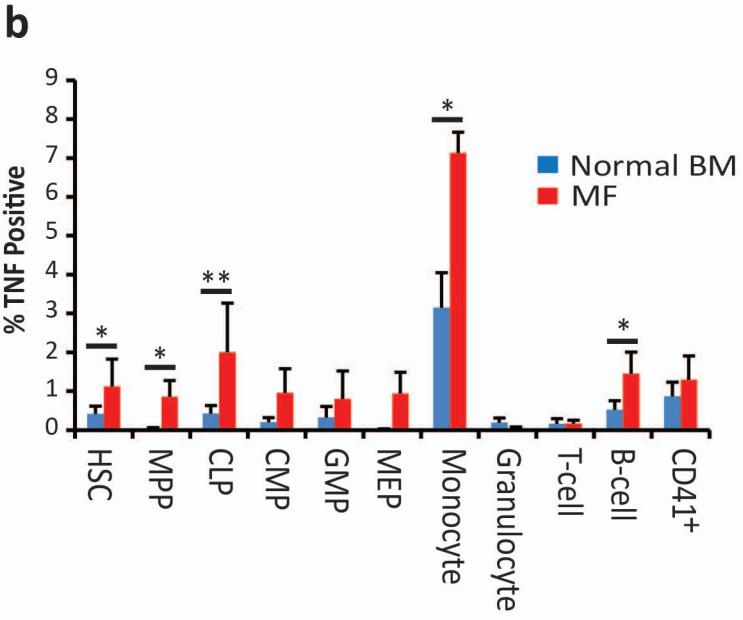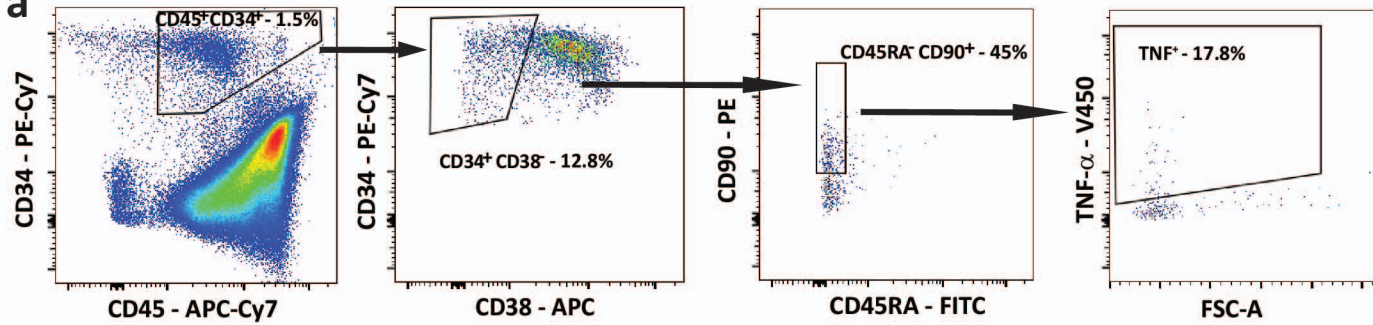651 **Figure 5. JAK2^{V617F} and TNFR2 cooperate to increase NF-κB signaling and reduce apoptosis in MF cells.**
652 (a) Human CD34$^+$ cells isolated from MF patient (n=3) or normal BM (n=3) were treated with birinapant
653 at 10 nM or 100 nM for 72 hours in liquid culture and then plated in clonogenic assays with continued
654 treatment. Colony inhibition was significantly different for normal vs. MF samples at 10 nM, while 100
655 nM birinapant inhibited both. (b) Human CD34$^+$ cells isolated from MF patient (n=3) or normal BM (n=3)
656 were infected with an NF-κB luciferase reporter construct 72 hours prior to evaluation. Cells were
657 treated with TNFR1 or TNFR2 BA (10 µg/mL) prior to stimulation with TNF (1 ng/mL). The fold increase in
658 reporter activity was significantly higher in MF cells at 4 hours post stimulation and over the complete
659 time course (p=0.03). Both TNFR1 and TNFR2 BAs reduced TNF stimulated NF-κB activity in MF and
660 normal BM cells. (c) Annexin V was measured in MF CD34$^+$ cells (n=4) 72 hours after infection with XIAP,
661 MAPK8 or vector control expression constructs. Ectopic expression of XIAP or MAPK8 significantly
662 increased Annexin V staining relative to vector control. (d) Downregulation of MAPK8 by JAK2^{V617F} and
663 TNFR2 inhibits apoptotic signaling through TNFR1 by preventing MAPK8 from promoting the active form
664 of TNFR1 Complex II. Downregulation of XIAP by JAK2^{V617F} and TNFR2 is associated with increased cIAP
665 protein levels. Either TNFR1 (with TRADD) or TNFR2 can form a signaling complex through association
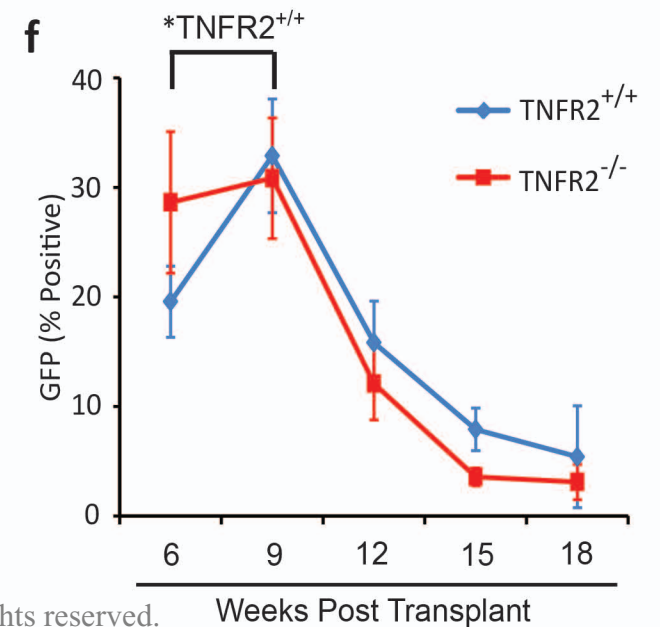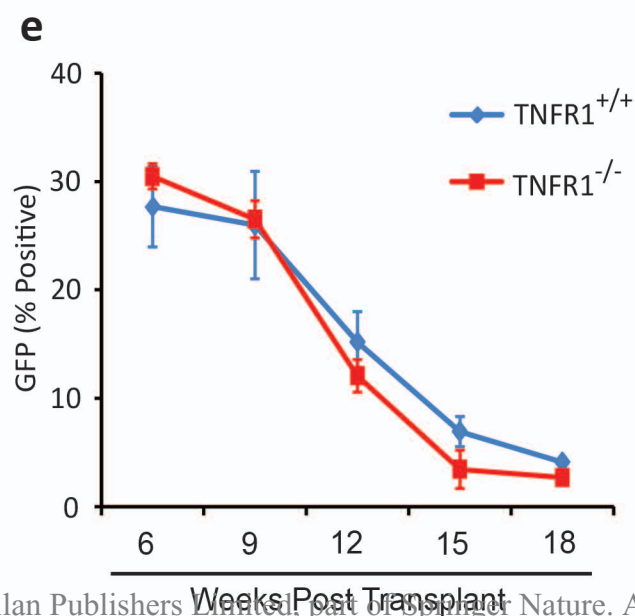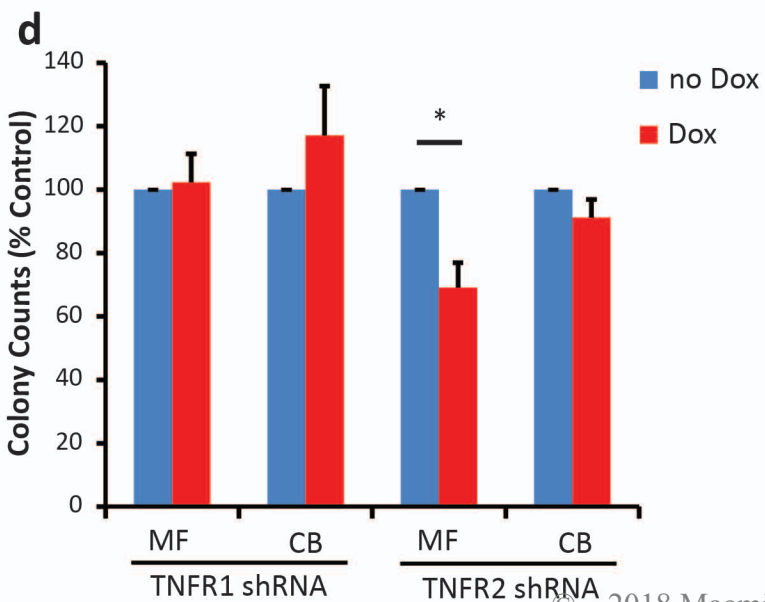
666   with TRAF2 and cIAP to activate NF-κB transcription of pro-survival and inflammation associated genes.

667   These combined effects favor survival of JAK2^{V617F} cells.*p<0.05, **p<0.005

668

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a Balb/c MPN mice → Harvest BM cells → 16 hr. culture ± TNFR BAs → Sort Lin⁻Kit⁺ → GFP+ GFP- V617F +/- → RNA isolation hybridization QC → Mouse 430 array

	V617F-	V617F+
Unt.	n=3	n=2
TNFR1 BA	n=3	n=3
TNFR2 BA	n=3	n=3

b

JAK2^V617F+ : #2 #1 (Unt.) #1 #3 #2 (TNFR1 BA) #3 #2 #1 (TNFR2 BA)

JAK2^V617F- : #3 #2 #1 (TNFR1 BA) #3 #1 #2 (Unt.) #3 #1 #2 (TNFR2 BA)
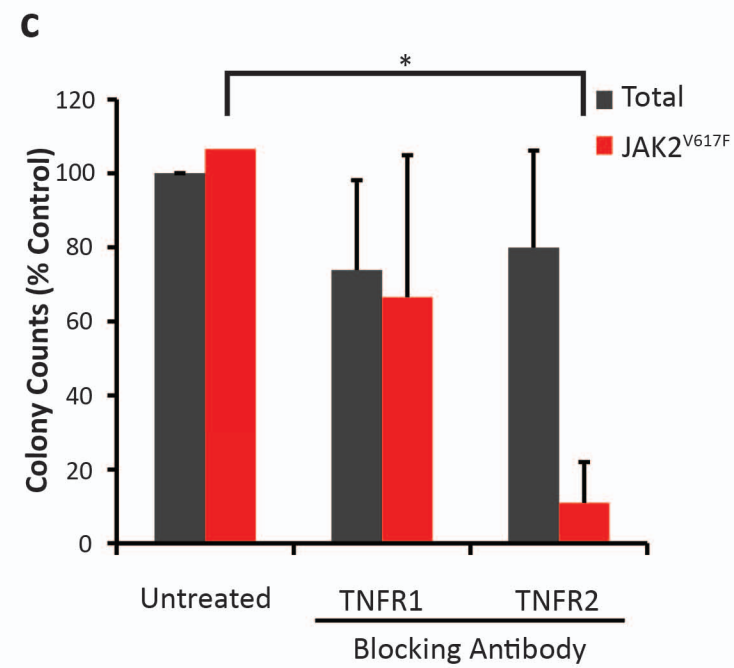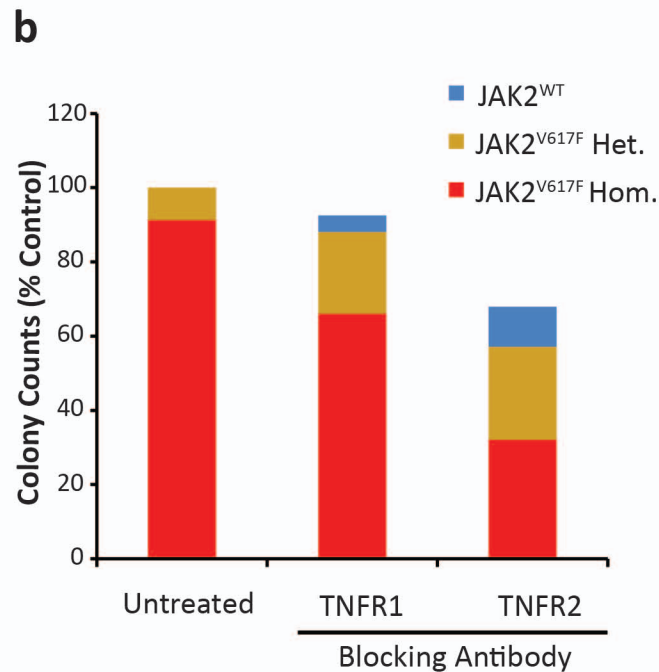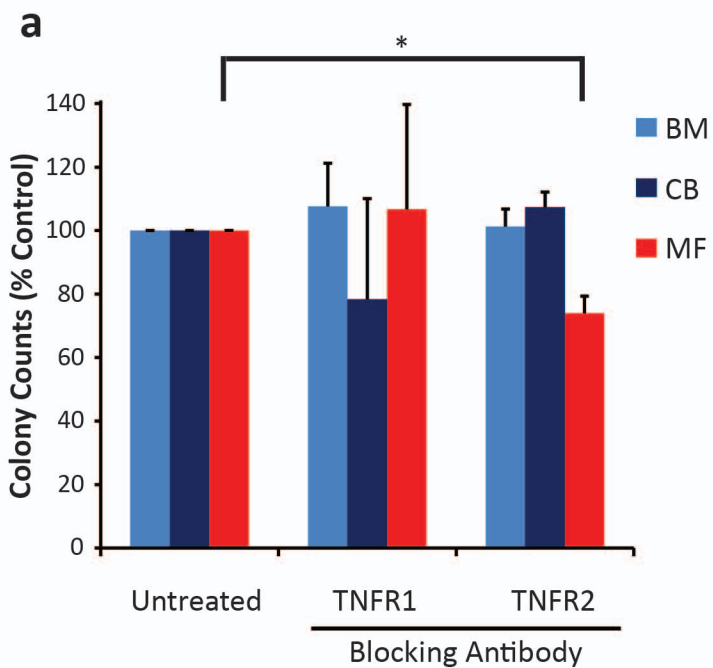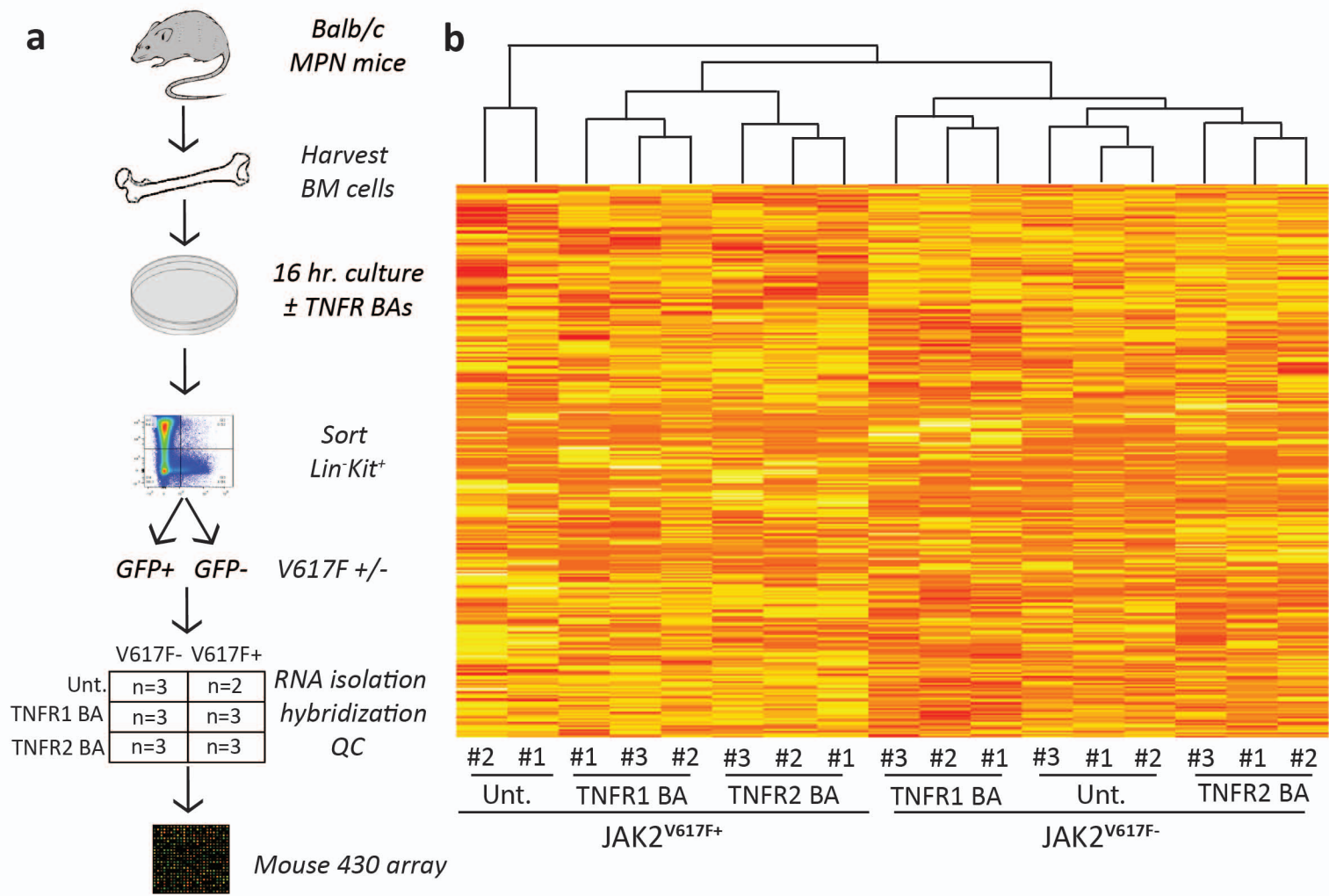
c Microarray analysis criteria - all p-value and Log2 FC comparisons relative to JAK2^V617F- (untreated)

p < 0.05 for any comparison
(1784 genes)
↓
-1 > JAK2^V617F+ FC >1
(1291 genes)

TNFR1 BA reverse of JAK2^V617F+ FC by >75%
(509 genes)
↓
-1 < TNFR1 BA FC < 1 in JAK2^V617F-
(304 genes)
↓
Rank list for top 10
up / down regulated genes

TNFR2 BA reverse of JAK2^V617F+ FC by >75%
(399 genes)
↓
-1 < TNFR2 BA FC < 1 in JAK2^V617F-
(252 genes)
↓
Rank list for top 10
up / down regulated genes

log2 FC versus JAK2^V617F- (untreated)
6 4 0 -4 -6

TNFR1 BA	–	+	+
JAK2^V617F	+	+	–

Itm2a
Prdx2
Asb17
1700017B05Rik
Cxxc1
Def8
Vsp18
Dnlz
Osbpl7
Afp142
HoxB4
Stk38l
Kctd12
Lcor
Fanci
Fubp1
Ddb2
Tm9sf3
Cry2
Lin7c

TNFR2 BA	–	+	+
JAK2^V617F	+	+	–

Itm2a
Prdx2
Asb17
Slco2b1
Prmt2
Avil
Slc16a13
Wnk1
Dhx30
Gpn1
Ccng1
B630005N14Rik
Nsmaf
Commd7
Hspa4l
Ddb2
Cry2
Lin7c
Mapk8
Xiap

a

Relative RNA Expression

JAK2^V617F-
JAK2^V617F+

*

XIAP   MAPK8

b

Relative RNA Expression

Normal BM
MF

*   *

XIAP   MAPK8

c

Normal BM
cIAP1/2
DAPI

MF Sample
cIAP1/2
DAPI

cIAP1/2 Fluorescence Intensity

*

Norm. BM   MF

d

Mega.
Normal BM

Mega.
MF (JAK2^V617F)

Mega.
MF (CALR)

e

Vector Control

XIAP

cIAP1/2 Fluorescence Intensity

*

Vector   XIAP

cIAP1/2
DAPI

cIAP1/2
DAPI