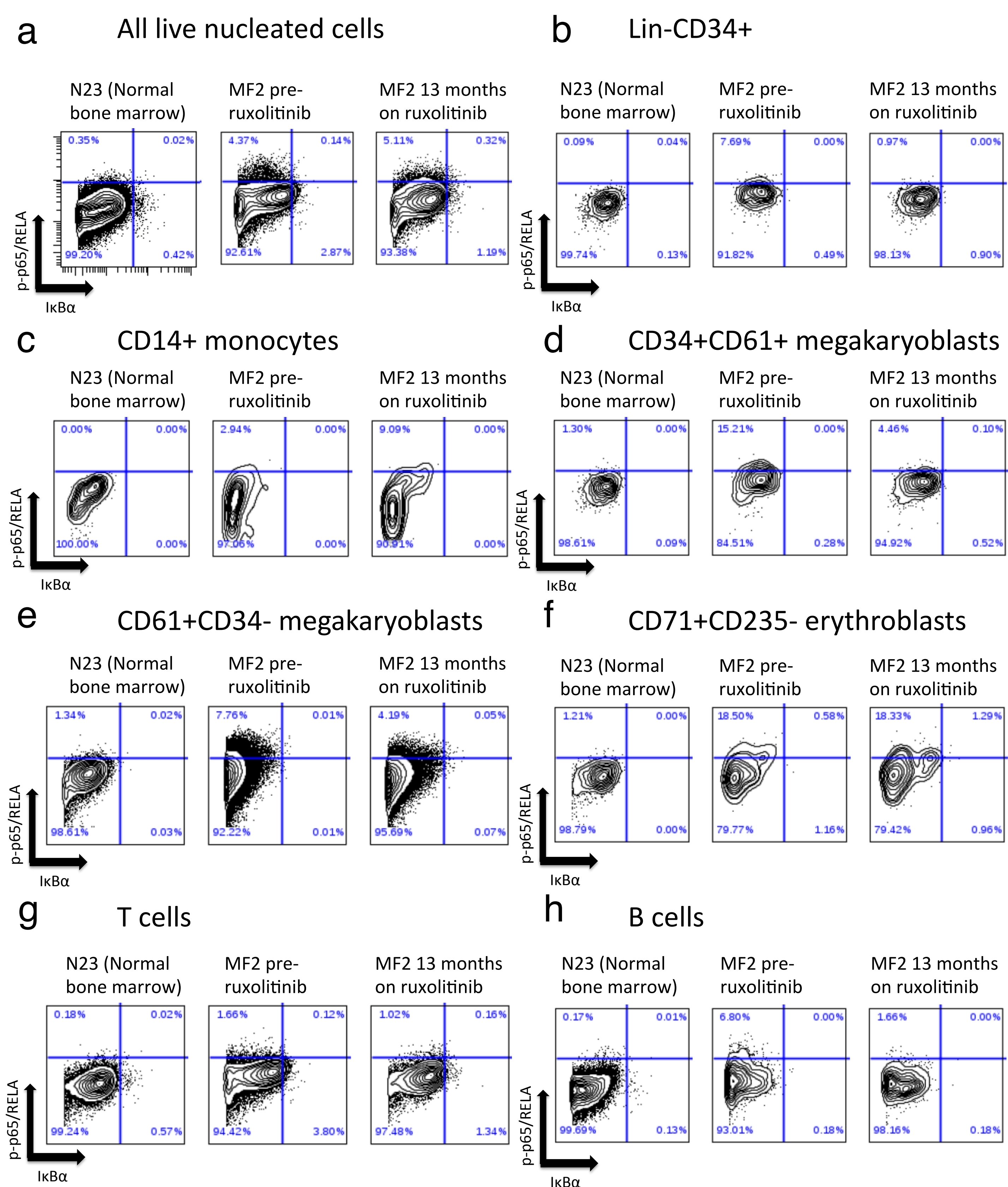
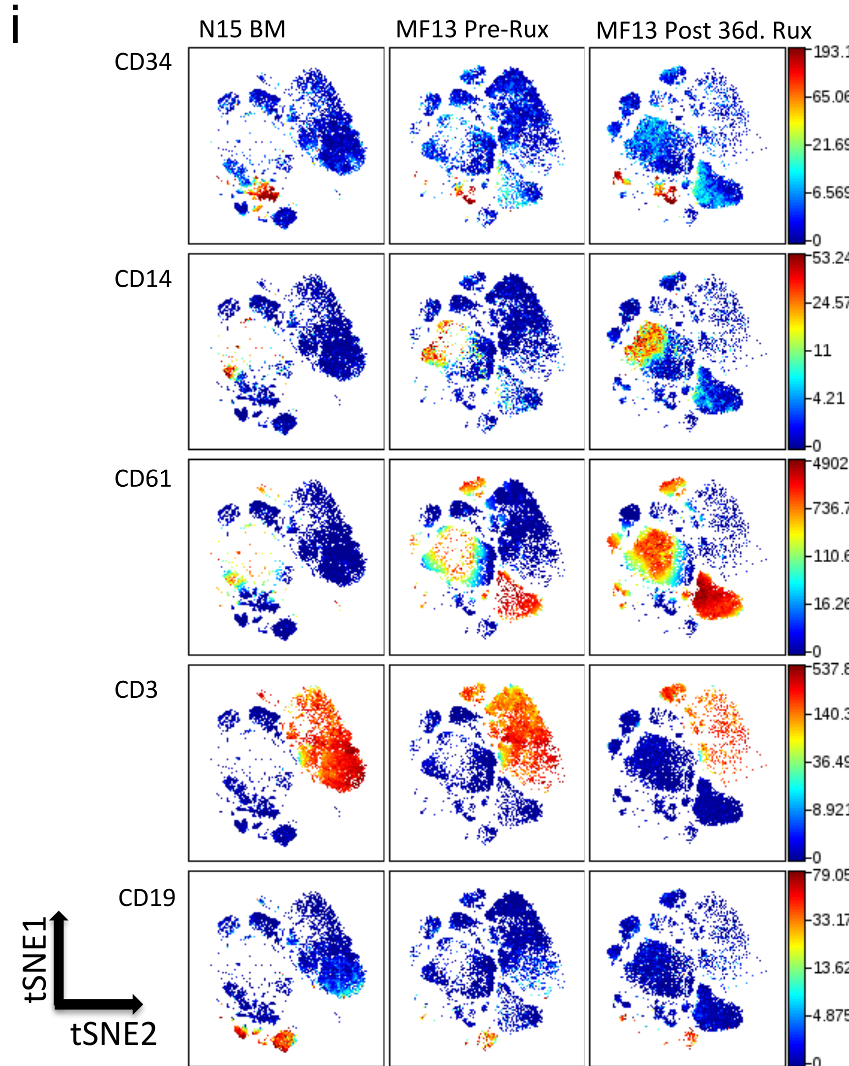
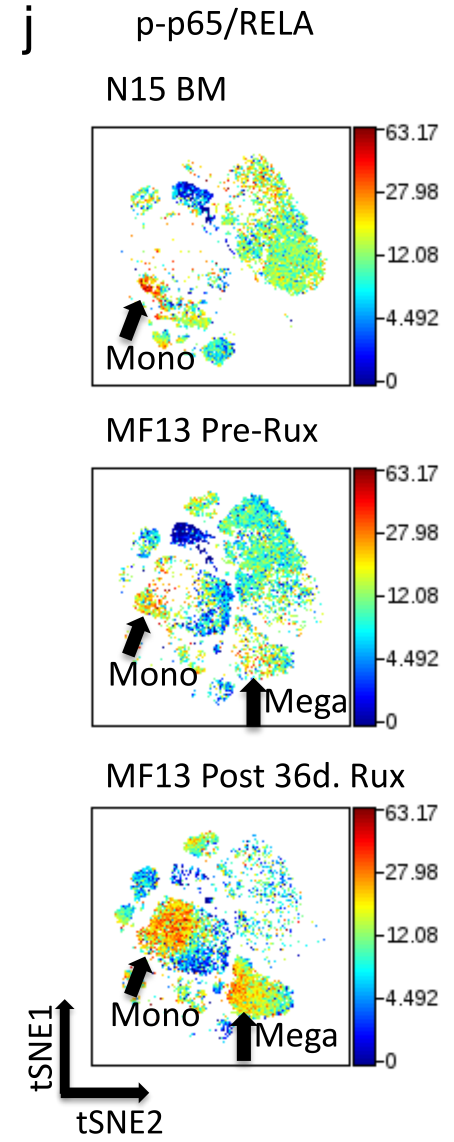
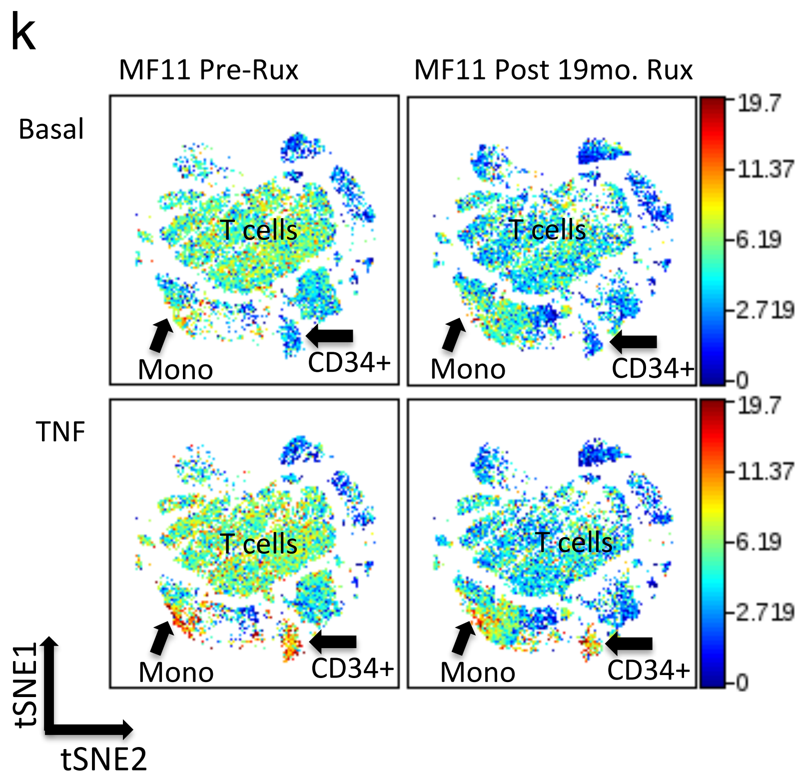
Supplementary Figures



Supplementary Figure S1 (continues on next page): **Patterns of NFκB activity in MF cell populations and persistence with ruxolitinib therapy. a-h:** Biaxial plots showing manually gated cell populations in a basal (unstimulated) state *ex vivo*, plotted for markers of NFκB activity: phosphorylated p-65/RELA (S529, Y axis) and total IκBα (X axis). Quadrant gate was determined by the distribution of 99% of normal control cells in the Lin-CD34+ cell population (**b**) below a threshold of p65/RELA phosphorylation, and by degradation shift of the distribution of IκBα labeling with TNF activation (not shown).[9] Numbers in corners show percent of cells in each quadrant. Individual samples shown are (left to right) normal control bone marrow N23, and blood from patient MF2 both prior to and after 13 months treatment with ruxolitinib. Gated cell populations include all live nucleated cells (**a**), Lin-CD34+ cells (**b**), CD14+ monocytes (**c**), CD34+CD61+ megakaryoblasts (**d**), CD34-CD61+ megakaryoblasts (**e**), CD71+CD235- erythroblasts (**f**), CD3+ T cells (**g**), and CD19+ B cells (**h**). (Continued on following page).

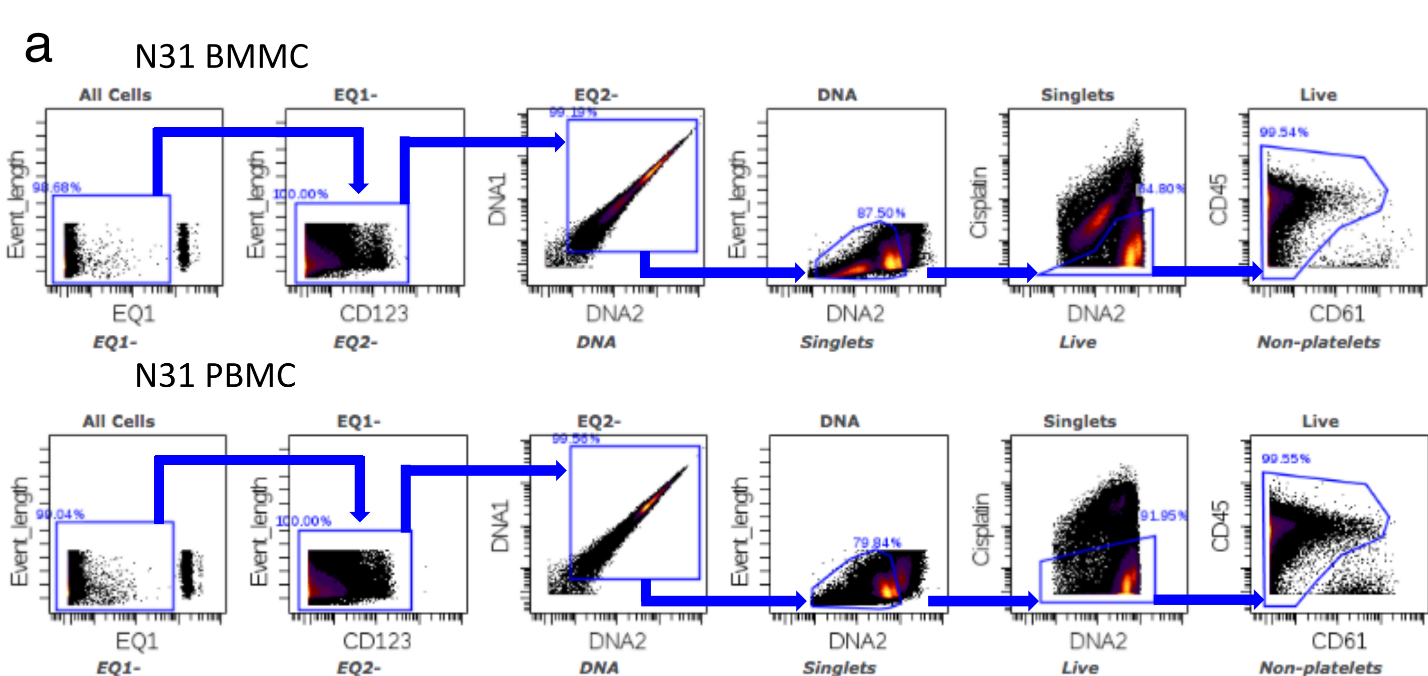






i, j: viSNE analysis[17] of a mass cytometry experiment comparing normal bone marrow control (N15) with blood from MF patient MF13 both prior to and after 36 days of *in vivo* ruxolitinib treatment. **i**. Localization of cell populations labeled for specific surface markers in viSNE plots. **j**: viSNE plots labeled for phosphorylated p65/RELA (S529). Arrows with labels identify position of monocyte (Mono) and megakaryoblast (Mega) cell populations, as identifiable from surface marker labeling in **i**. **k**. viSNE plots labeled for phosphorylated p65/RELA (S529) from blood from MF patient MF11, prior to and after 19 months treatment with therapeutic ruxolitinib. Labeled populations (based on surface marker labeling, not shown but similar to **i)** are monocytes (Mono) , CD34+ cells, and T cells.

a



b

N31 BMMC

Non-platelets | Non-platelets | Non-B cells | Non-T or B cells | Non-T B or NK cells

CD45 | HLA-DR | CD45 | CD45RA | HLA-DR

94.36% | 77.31% | 49.17% | 88.54% | 70.57%

CD235 | CD19 | CD3 | CD7 | CD11c

CD235- | Non-B cells | Non-T or B cells | Non-T B or NK cells | HLADR+ CD11c+

N31 PBMC

Non-platelets | Non-platelets | Non-B cells | Non-T or B cells | Non-T B or NK cells

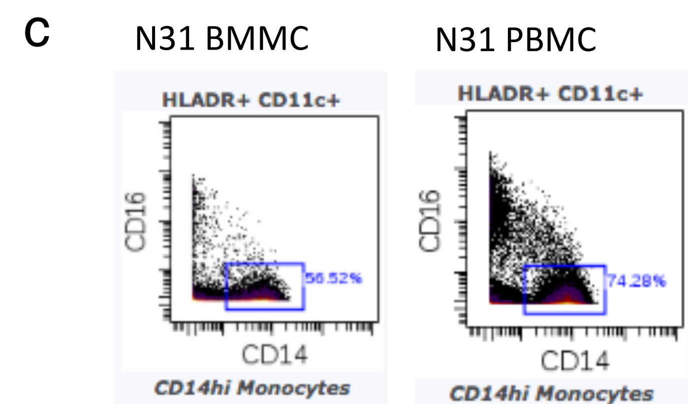
CD45 | HLA-DR | CD45 | CD45RA | HLA-DR

99.99% | 92.32% | 40.62% | 88.38% | 91.07%

CD235 | CD19 | CD3 | CD7 | CD11c

CD235- | Non-B cells | Non-T or B cells | Non-T B or NK cells | HLADR+ CD11c+

c



N31 PBMC

HLADR+ CD11c+

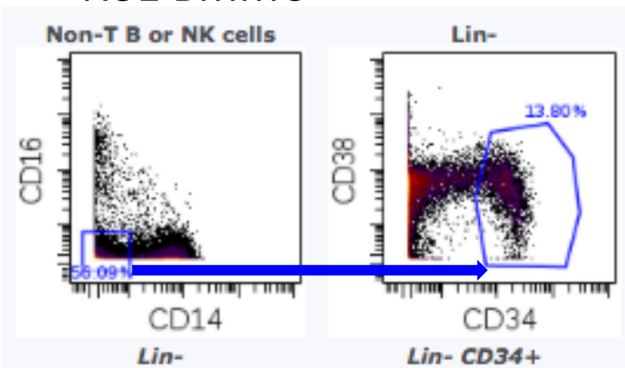
CD16

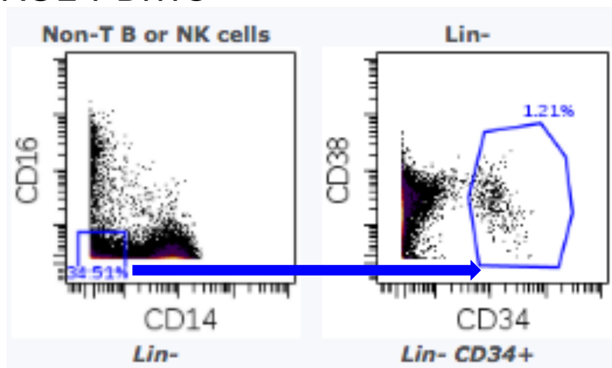
74.28%

CD14

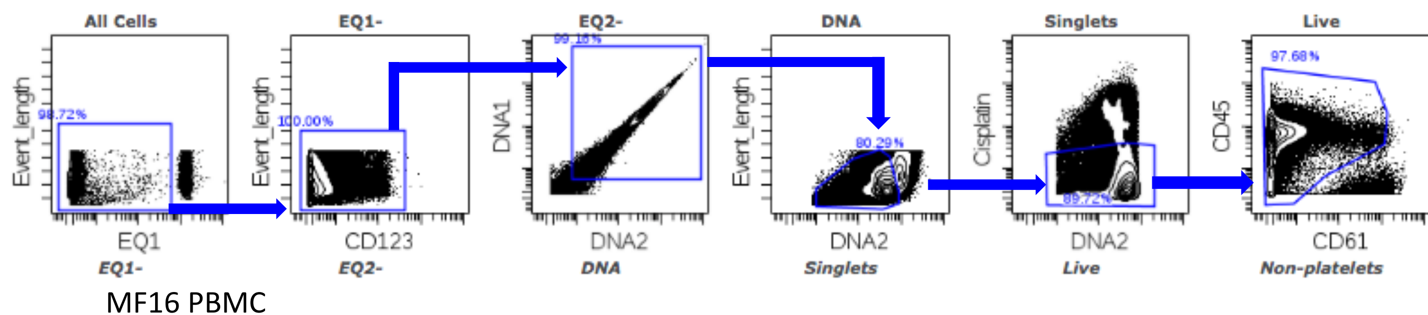
CD14hi Monocytes

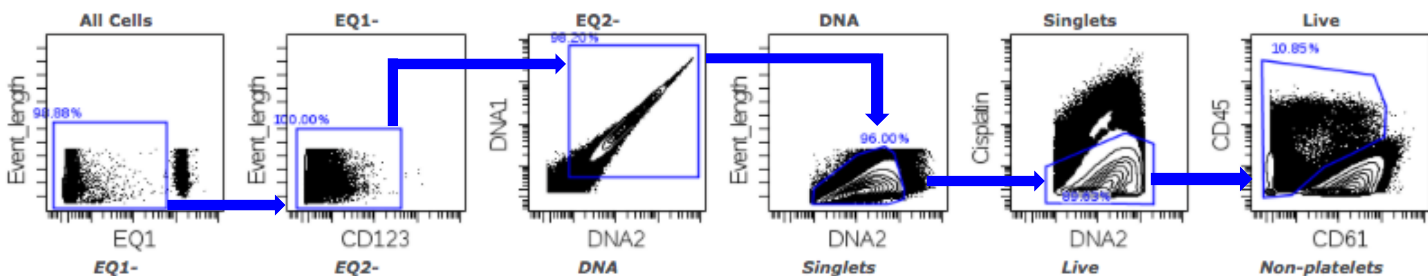
d



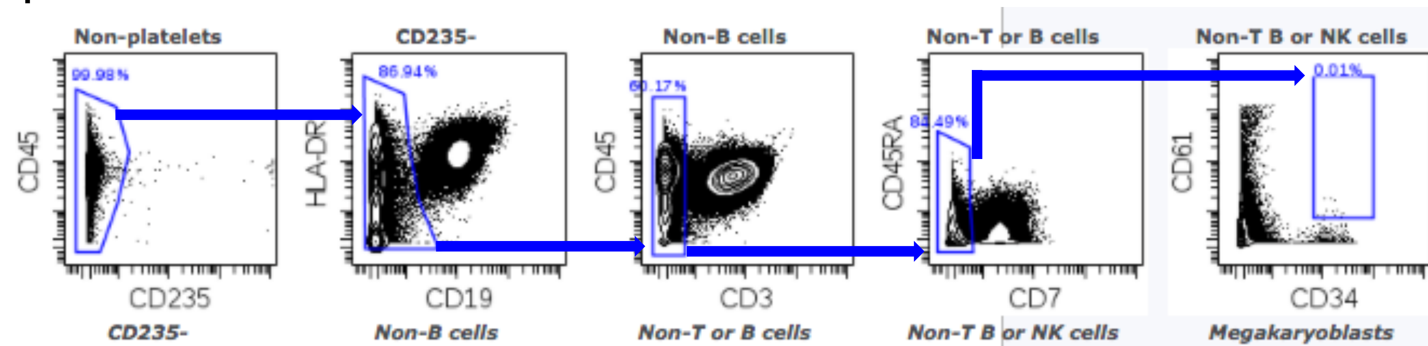


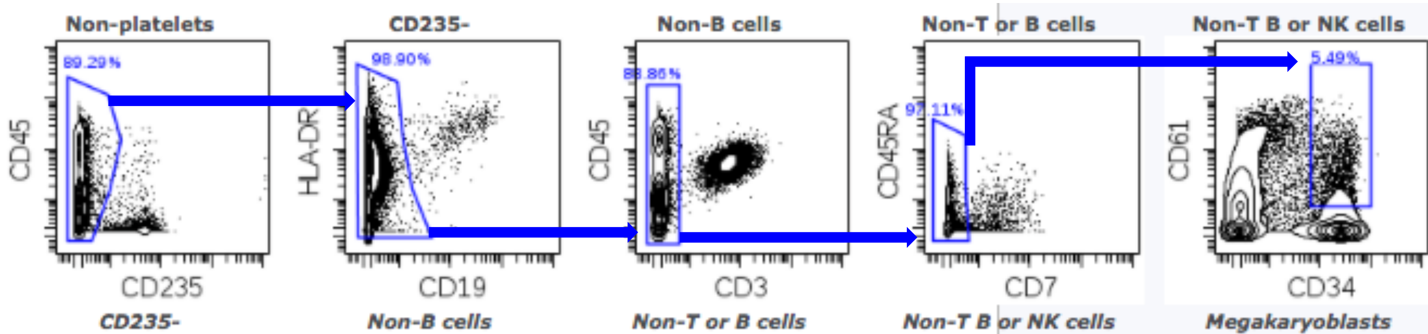
e





f





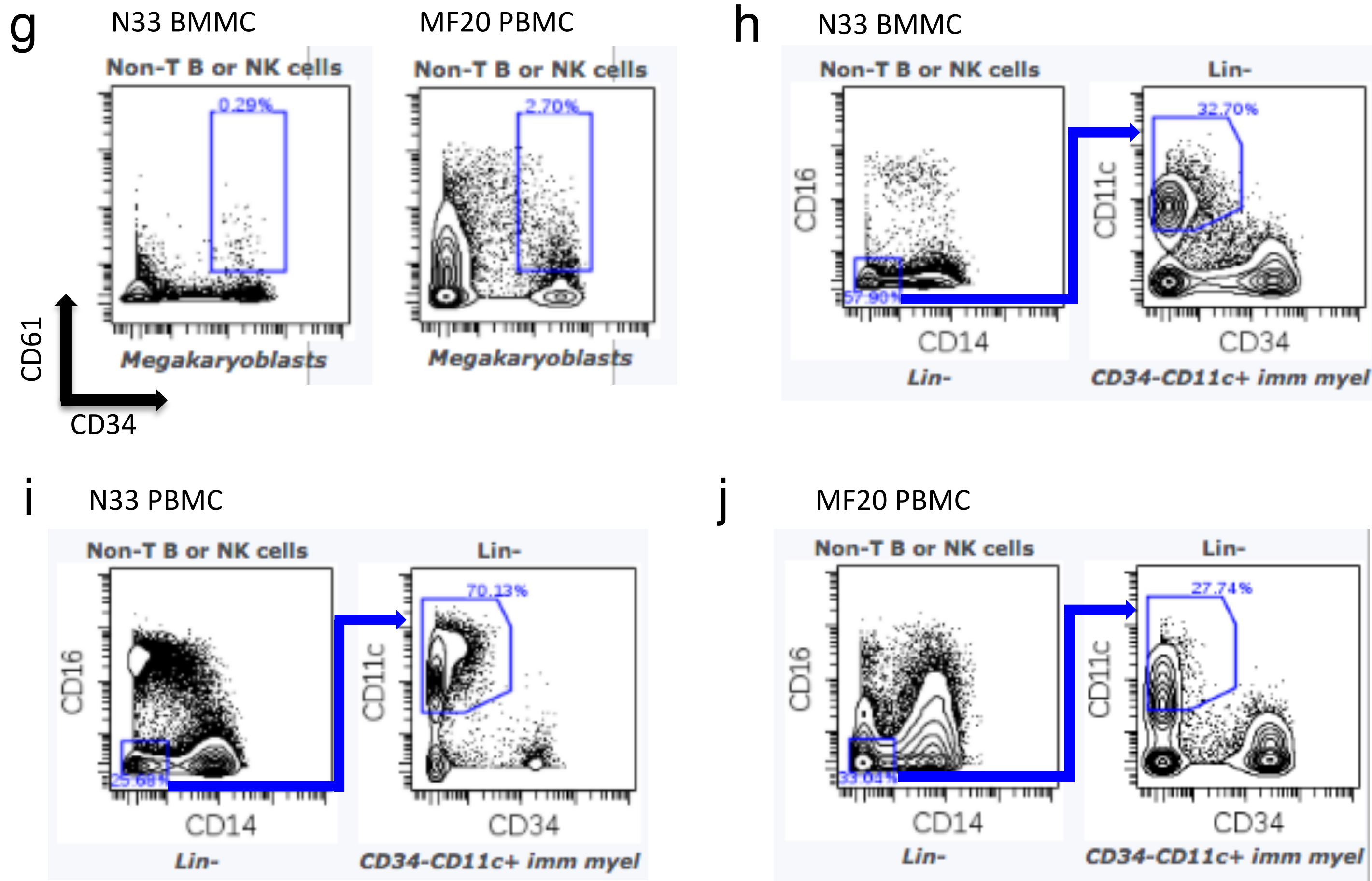
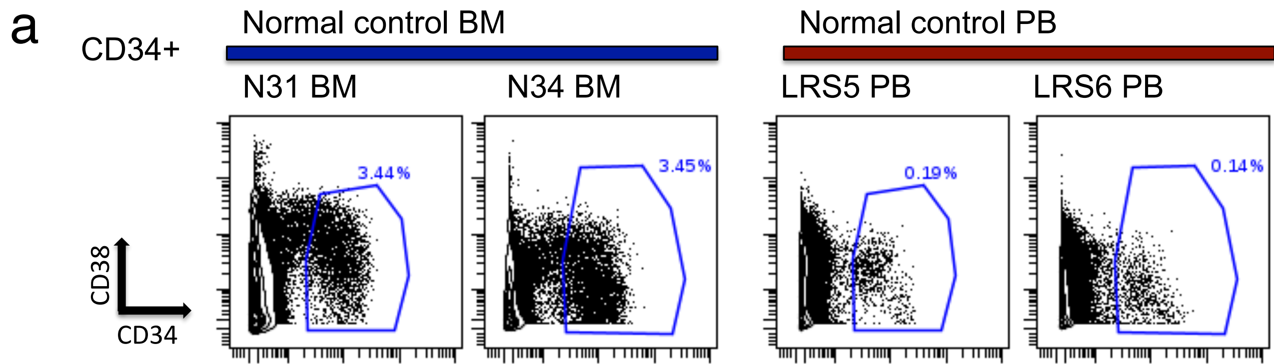
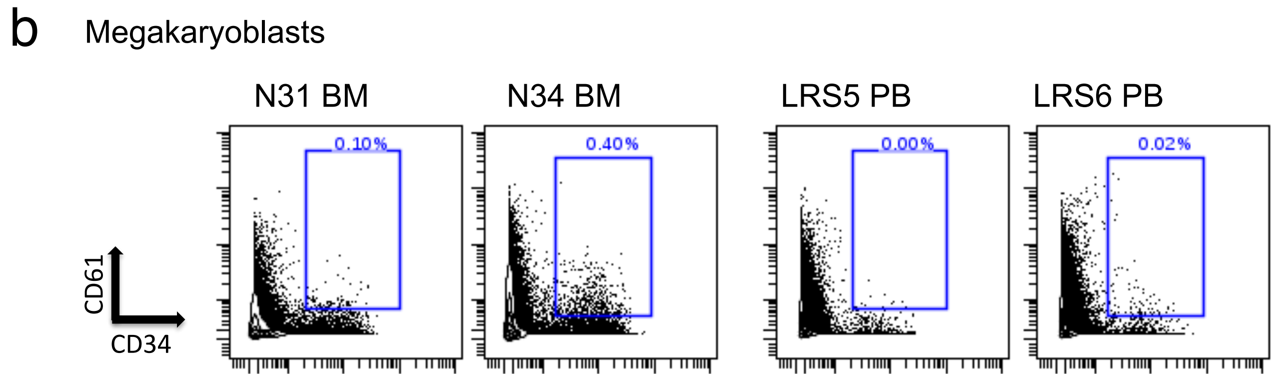
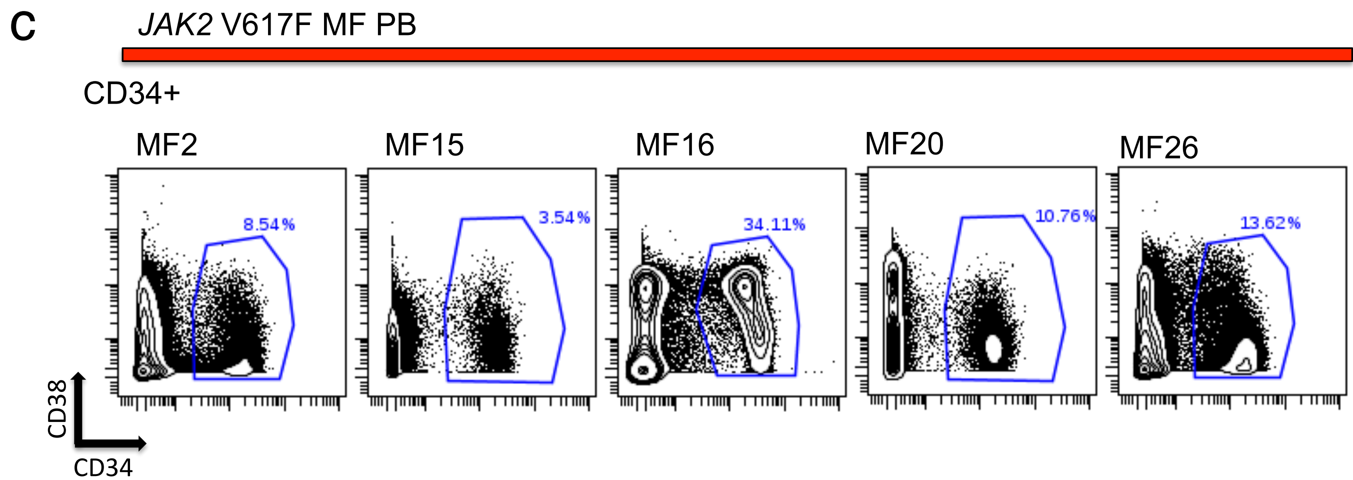
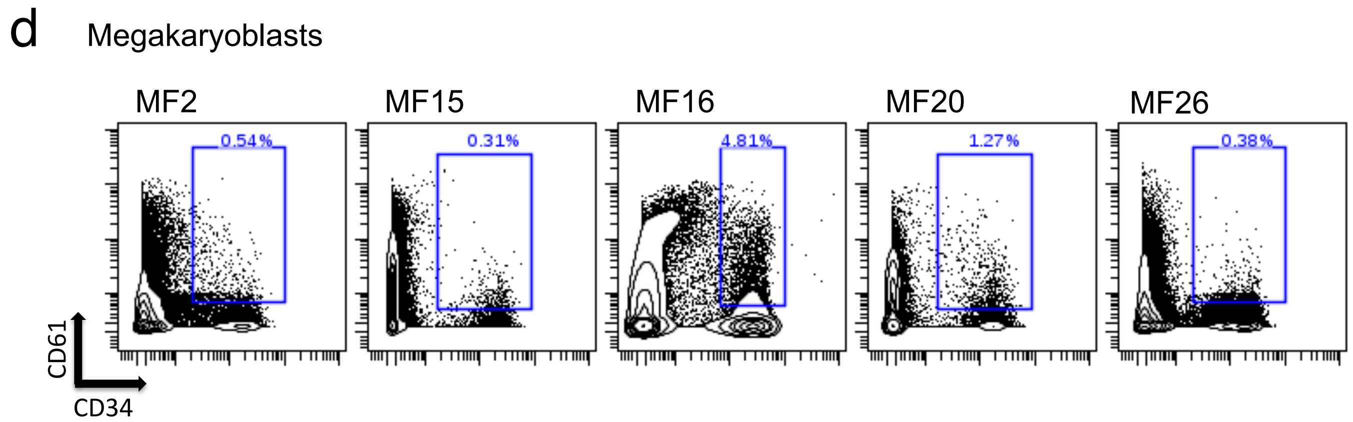
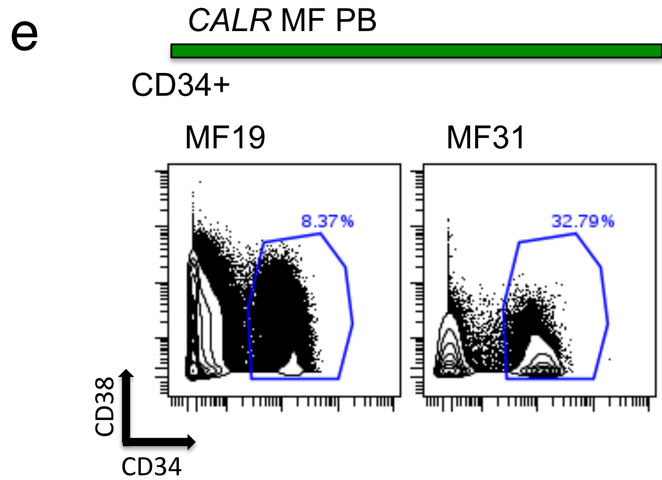
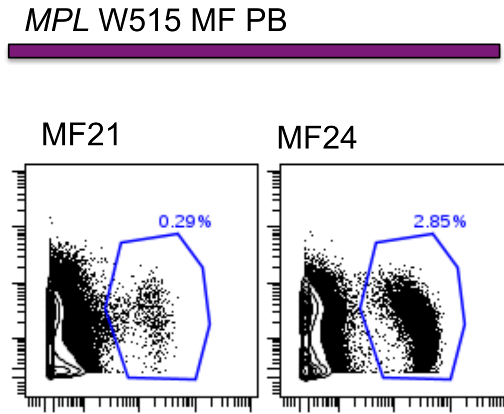
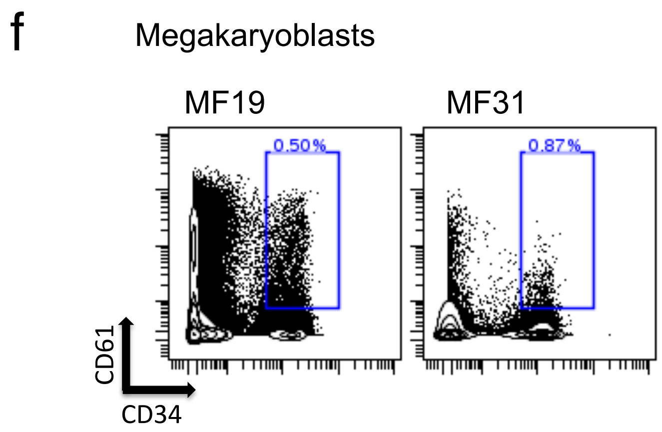


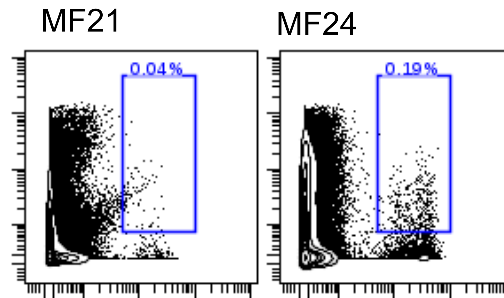
Figure S2: Gating for Lin-CD34+ HSPC, monocytes, megakaryoblasts, and immature myeloid cells. a. Density dot plots showing initial manual gates for obtaining the live nucleated cell population (as in **Figure S1a**). Shown are paired normal control bone marrow (BMMC, above) and peripheral blood (PBMC, below) mononuclear cells from control donor N31 (see **Supplemental Table S1**). Sequential gates are shown with percentage of cells in gate for each plot. The name of the derived gated population is written below each plot, with the parental population written above each plot, in format from Cytobank (cytobank.org). EQ normalization beads are gated out (see **Supplemental Methods**), followed by gating for DNA-positive singlets, live cells, and finally exclusion of platelets. **b**. Gates used to discriminate myeloid versus lymphoid cell populations, and specifically to exclude (left to right) erythroid cells, B cells, T cells, and NK cells, and finally to select for CD11c+HLA-DR+ myeloid cells. N31 control samples are shown as in **a**. **c**. Gating of the CD14+ monocyte population from the parental CD11c+HLA-DR+ myeloid population, for N31 control BMMC and PBMC. **d**. Gates to derive Lin-CD34+ cell population, starting with parental non-lymphoid gated population (as derived in **b**), for N31 control BMMC and PBMC. Rare circulating Lin-CD34+ cells are identifiable in healthy control PBMC (right), as well as among BMMC (left). **e**. Contour plots showing gates for obtaining the live nucleated cell population (as in **a**), for a normal control blood sample (LRS2), and a *JAK2* mutant MF patient (MF16). **f**. Gates for exclusion of (left to right) erythroid cells, B cells, T cells, and NK cells, and identification of CD34+CD61+ megakaryoblasts from normal and MF16 PBMC. **g**. Contour plots identify the CD34+CD61+ megakaryoblasts from N33 control BMMC. **h-j**: Contour plots identify the Lin-CD34-CD11c+ immature myeloid cell population from N33 control BMMC (**h**), matched N33 control PBMC (**i**), and PBMC from *JAK2* mutant MF patient MF20 (**j**). The cell populations derived in this figure are those analyzed in heat maps in **Figure 2e-g**.

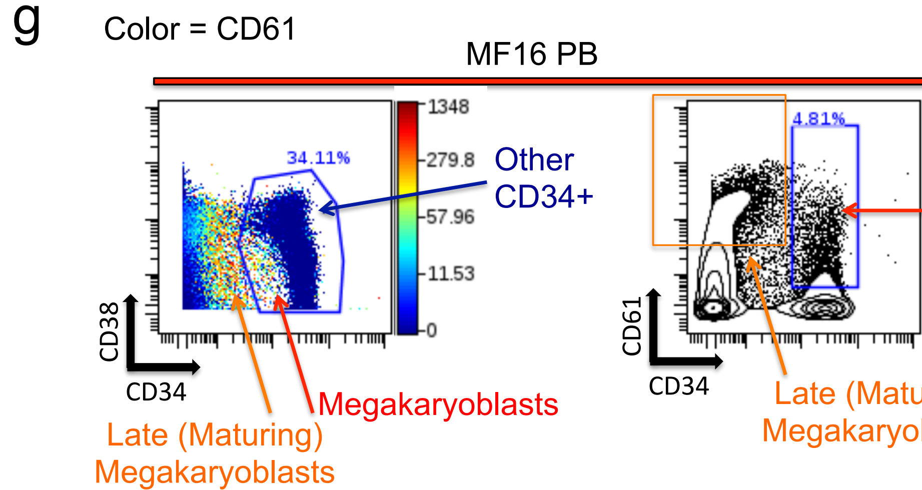

a
CD34+
Normal control BM
Normal control PB
N31 BM
N34 BM
LRS5 PB
LRS6 PB
3.44%
3.45%
0.19%
0.14%
CD38
CD34

b
Megakaryoblasts
N31 BM
N34 BM
LRS5 PB
LRS6 PB
0.10%
0.40%
0.00%
0.02%
CD61
CD34

c
JAK2 V617F MF PB
CD34+
MF2
MF15
MF16
MF20
MF26
8.54%
3.54%
34.11%
10.76%
13.62%
CD38
CD34

d
Megakaryoblasts
MF2
MF15
MF16
MF20
MF26
0.54%
0.31%
4.81%
1.27%
0.38%
CD61
CD34


e
CALR MF PB
CD34+
MF19
MF31
8.37%
32.79%
CD38
CD34


MPL W515 MF PB
MF21
MF24
0.29%
2.85%


f
Megakaryoblasts
MF19
MF31
0.50%
0.87%
CD61
CD34


MF21
MF24
0.04%
0.19%


g
Color = CD61
MF16 PB
1348
279.8
57.96
11.53
0
34.11%
Other CD34+
4.81%
Megakaryoblasts
CD38
CD34
CD61
CD34
Megakaryoblasts
Late (Maturing) Megakaryoblasts
Late (Maturing) Megakaryoblasts

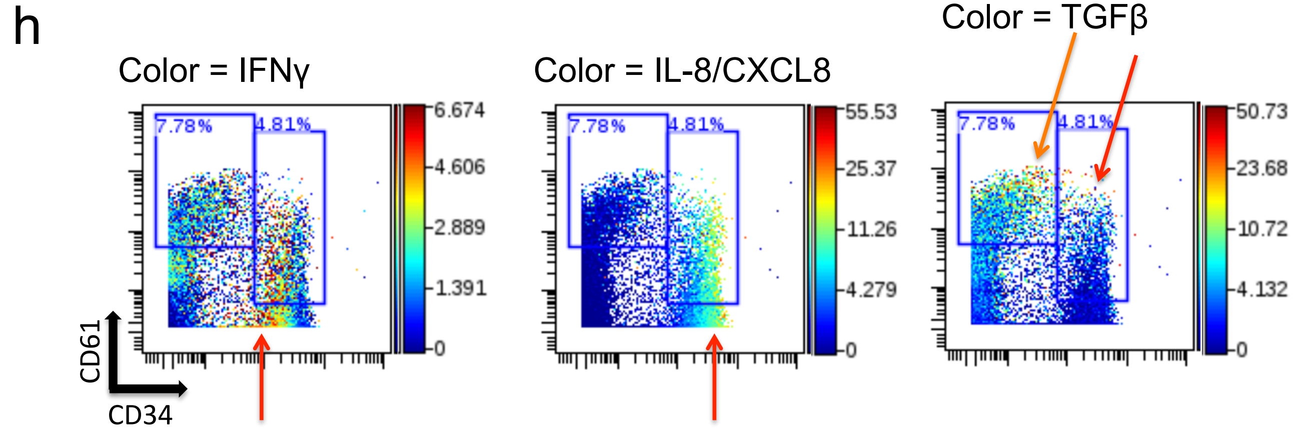

h
Color = IFNγ
Color = IL-8/CXCL8
Color = TGFβ
7.78%
4.81%
6.674
4.606
2.889
1.391
0
55.53
25.37
11.26
4.279
0
50.73
23.68
10.72
4.132
0
CD61
CD34

Figure S3: Gating and prevalence of Lin-CD34+ cells and CD34+CD61+ megakaryoblasts. a. Biaxial plots showing CD34 (X axis) versus CD38 (Y axis), illustrating the gating of the Lin-CD34+ cell population, as derived from the gated Lin- population (similar to **Figure S2d)**. Shown from left to right are plots from two normal control bone marrow samples (N31 and N34) and two normal peripheral blood samples (LRS5 and LRS6). The plots illustrate the total non-platelets population (**Figure S2 a,e**), rather than the more restricted Lin- population (**Figure S2d),** from which the Lin-CD34+ cell population is derived. Therefore the percentage in the gate shown in each plot corresponds to the total percentage of live, non-platelet cells in the sample that are Lin-CD34+. **b.** Biaxial plots illustrating the total non-platelets population (as in **a**), with CD34 (X axis) versus CD61 (Y axis). The gated population comprises CD34+CD61+ megakaryoblasts.(labeled as CD34+CD61+ in Figure **2 c-e** and as "megakaryoblasts" elsewhere). Shown left to right are the same normal control individuals as in **a**. The percent of cells shown labeling the gate in each plot represents the percent of the total non-platelets population in each sample that are CD34+CD61+ megakaryoblasts. **c**. Biaxial plots showing CD34 (X axis) versus CD38 (Y axis), illustrating the gating of the Lin-CD34+ cell population, from peripheral blood samples from five *JAK2* V617F mutant MF patients. The plots illustrate the total non-platelets population. Therefore the percentage in the gate shown in each plot corresponds to the total percentage of live, non-platelet cells in the sample that fall within the Lin-CD34+ gate. **d**. Biaxial plots for the identical five *JAK2* V617F mutant MF patients as in **c**, illustrating the total non-platelets population, with CD34 (X axis) versus CD61 (Y axis). The gated population comprises CD34+CD61+ megakaryoblast, and percent in gate shown illustrates their prevalence within the total non-platelets population in these patient samples. **e**. Biaxial plots showing CD34 (X axis) versus CD38 (Y axis), illustrating the gating of the Lin-CD34+ cell population, in two *CALR* mutant MF patients (left to right, MF19 and MF31), and in two *MPL* W515L/K mutant MF patients (left to right, MF21 and MF24). Plots are otherwise similar to those in **a** and **c**. **f**. Biaxial plots with CD34 (X axis) versus CD61 (Y axis) illustrating the identical non-platelets population from patient samples shown in **d**. Gate and labeled percentage shows the prevalence of CD34+CD61+ megakaryoblasts within this population. **g.** Left: biaxial plot with CD34 on the X-axis and CD38 on the Y axis (similar to **a, c, e**) and colored for intensity of labeling for CD61. Right: contour plot with CD34 on the X-axis and CD61 on the Y axis. Both plots illustrate the total non-platelets population for a *K2* V617F mutant MF patient (MF16) with an abundance of both CD34+ and CD34- megakaryoblasts. Primitive or early (CD34+) megakaryoblasts and late, maturing (CD34-) megakaryoblasts are labeled on both plots. **h**. Biaxial plots CD34 (X axis) versus CD61 (Y axis), for MF patient MF16 (as in **g**). Plots are colored by intensity of label for each of the following cytokines (left to right): IFNγ, IL-8/CXCL8, and TGFβ, all expressed by CD34+ megakaryoblasts (red arrows) and/or CD34- megakaryoblasts (orange arrows).

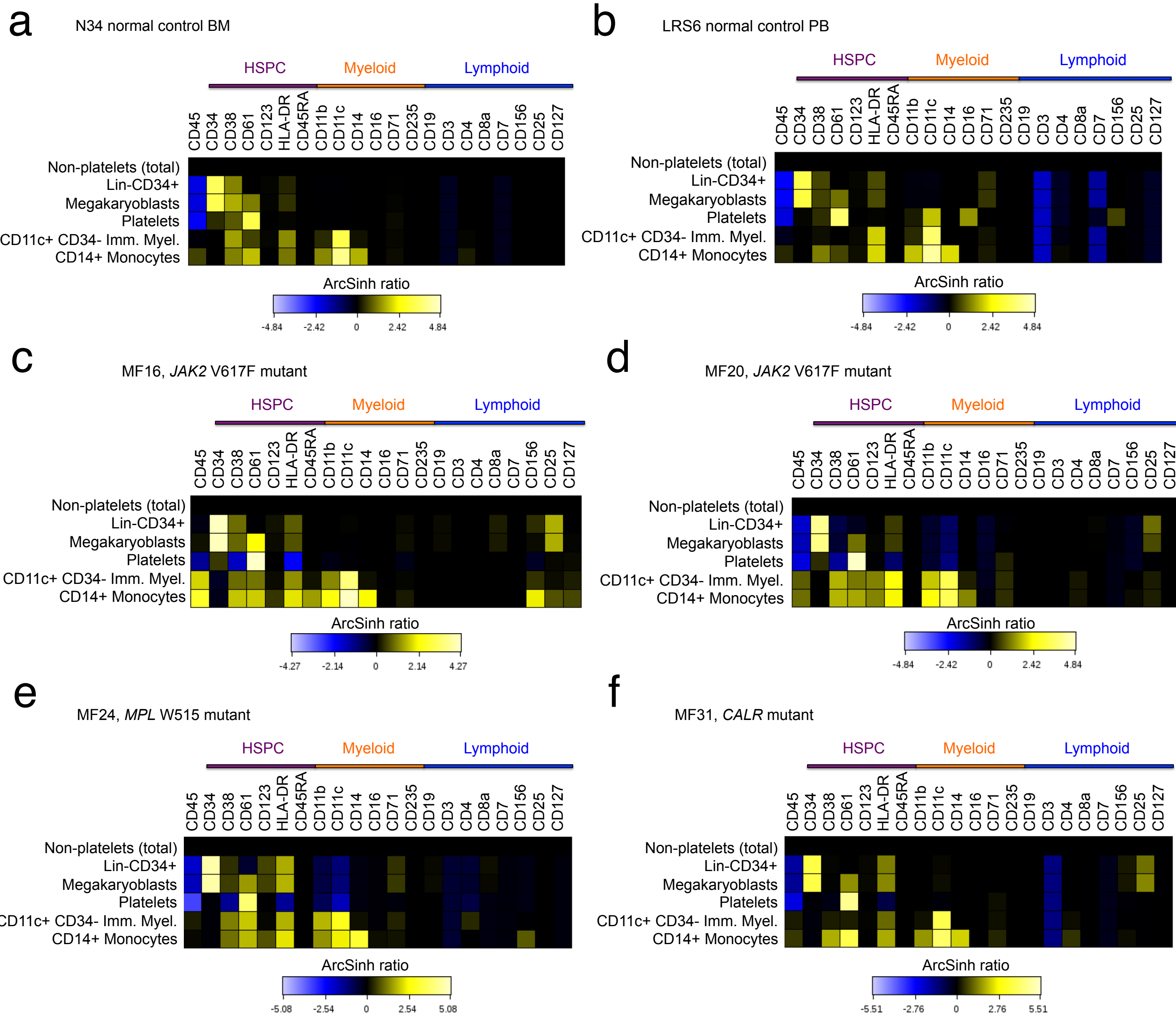


Figure S4: Surface marker expression in gated myeloid cell populations (related to **Figure 2**). Heat maps show relative median levels of antibody labeling for all surface markers, on ArcSinh ratio scale, normalized to the median signal level for each antibody in the total non-platelets cell population (see **Figure S2** for gating). Cell population-specific surface marker signal is displayed in rows for each heat map that correspond to the following cell populations (top to bottom in each heat map): Total non-platelets, Lin-CD34+, CD34+CD61+ megakaryoblasts, platelets, CD11c+CD34- immature myeloid cells, and CD14+ monocytes. Surface markers, in addition to CD45 (pan-leukocyte, left column in each heat map), are grouped into markers typical of hematopoietic stem and progenitor cells (HSPC), myeloid cells, and lymphoid cells (left to right). Heat maps illustrate surface marker labeling of cell populations in the following individual samples: Normal control N34 bone marrow (**a**), Normal control LRS6 peripheral blood (**b**), blood from JAK2 V617F mutant MF patients MF16 (**c**) and MF20 (**d**), blood from MPL W515 mutant MF patient MF24 (**e**), and blood from CALR mutant MF patient MF31 (**f**).

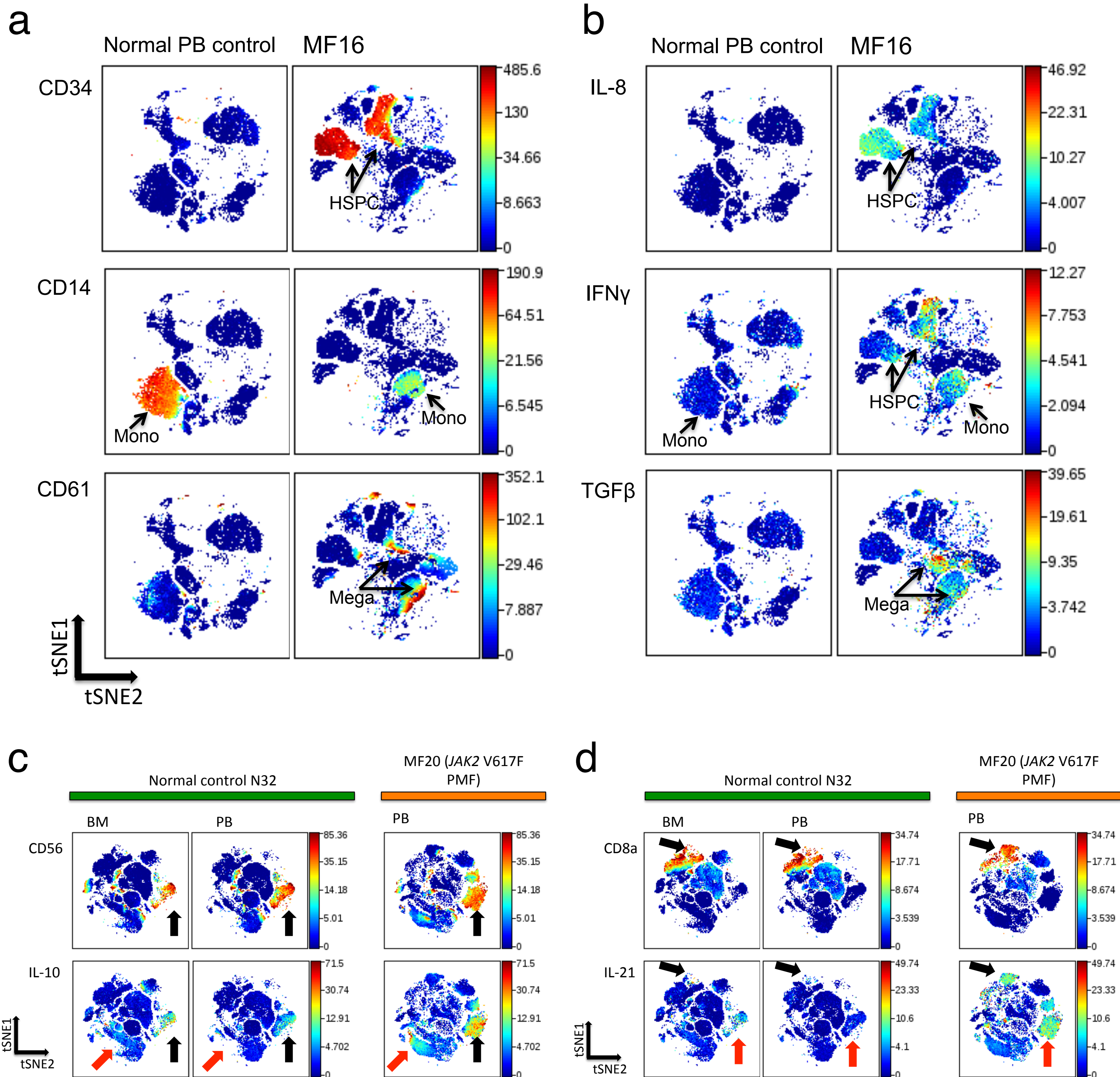
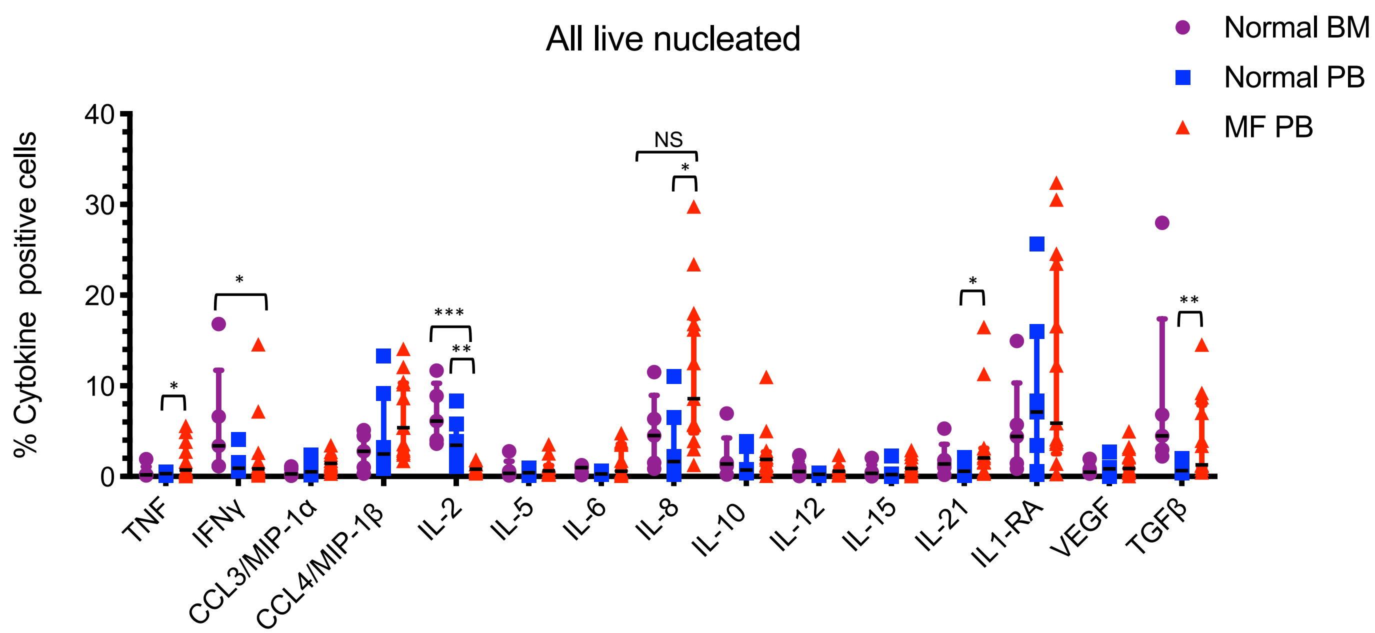
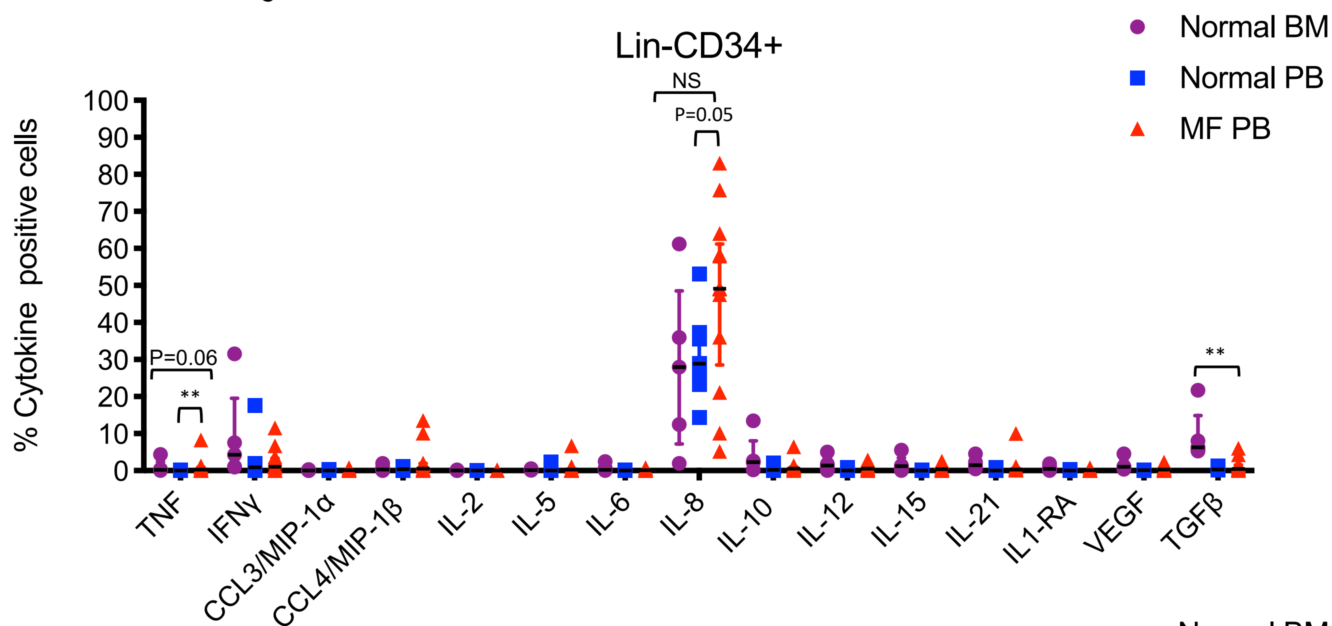
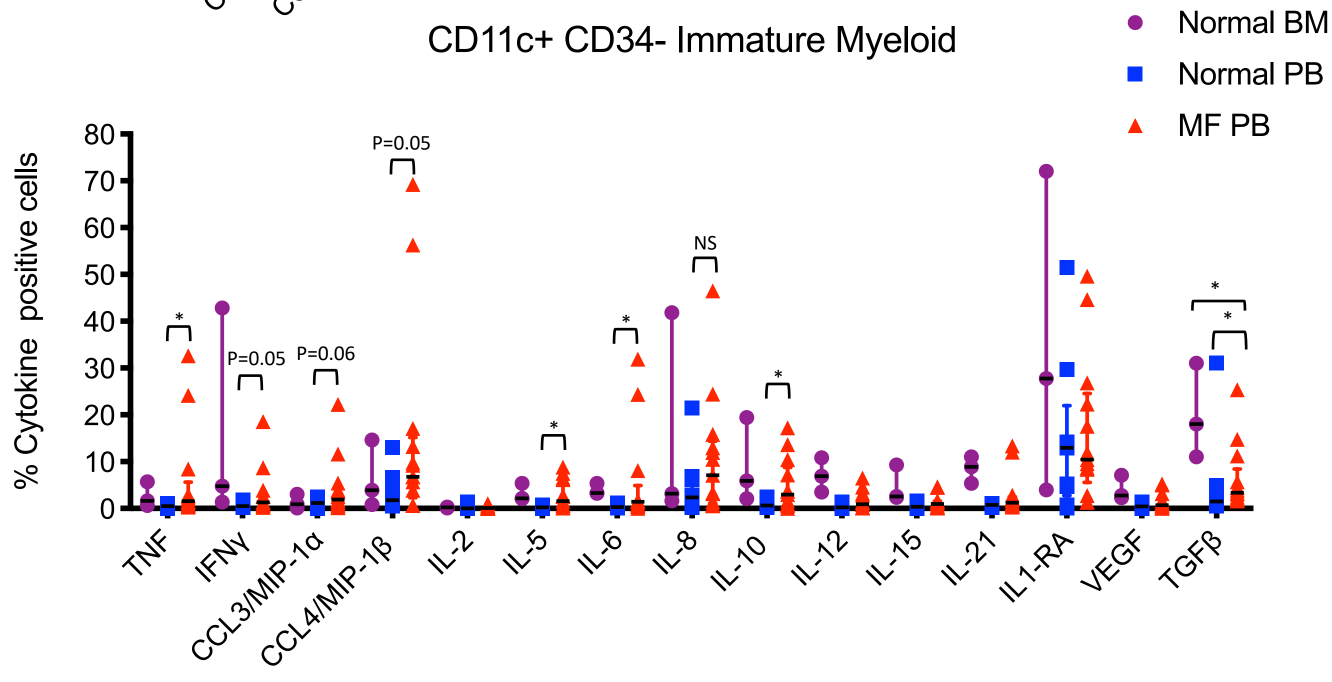


Figure S5: Individual cytokines localized to cell populations in MF versus normal control samples. a, b: ViSNE plots illustrate cell populations identified by surface markers (**a**) and corresponding expressed cytokines (**b**). **A**, surface markers (top to bottom) CD34, CD14, and CD61 respectively identify locations of hematopoietic stem and progenitor cells (HSPC), monocytes (Mono), and megakaryoblasts (Mega) in peripheral blood samples. Mobilized HSPC and megakaryoblasts are much more abundant in blood from *JAK2* V617F mutant MF post PV patient MF16 (right column) than from healthy control blood (left column). **b**, ViSNE plots from MF patient MF16 versus healthy control labeled for cytokines (top to bottom) IL-8/CXCL8, IFNγ, and TGFβ, respectively expressed in HSPC, HSPC and monocytes, and megakaryoblasts. **c, d:** viSNE analysis comparing normal control N32 bone marrow, N32 blood, and blood from MF20 (*JAK2* V617F mutant MF post PV). **c.** CD56 (above) and IL-10 (below), with black arrows indicating NK cells, and red arrows indicating IL-10 expressing monocytes (see **Figure 2**). **d.** CD8a (above) and IL-21 (below), with black arrows indicating CD8a+ T cells, and red arrows indicating NK cells.


a
All live nucleated
Normal BM
Normal PB
MF PB
% Cytokine positive cells
NS
P
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
IL-8
IL-10
IL-12
IL-15
IL-21
IL1-RA
VEGF
TGFβ


b
Lin-CD34+
Normal BM
Normal PB
MF PB
% Cytokine positive cells
NS
P=0.05
P=0.06
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
IL-8
IL-10
IL-12
IL-15
IL-21
IL1-RA
VEGF
TGFβ


c
CD11c+ CD34- Immature Myeloid
Normal BM
Normal PB
MF PB
% Cytokine positive cells
P=0.05
P=0.05
P=0.06
NS
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
IL-8
IL-10
IL-12
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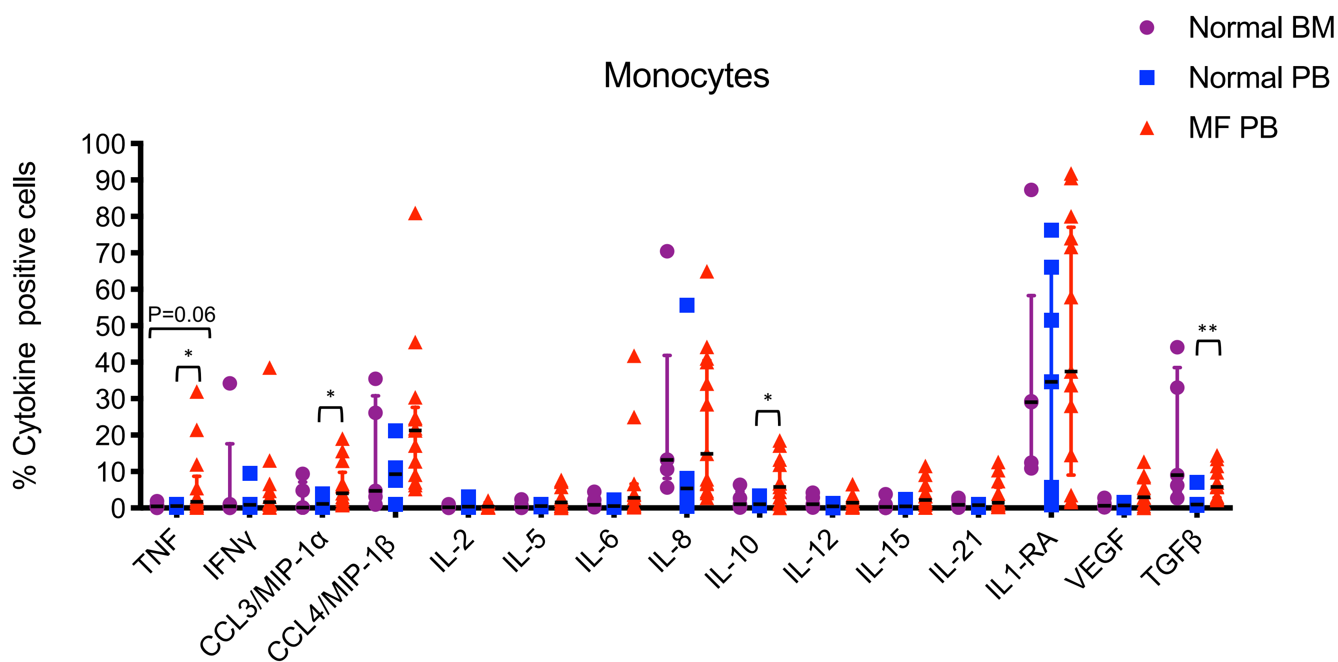
IL1-RA
VEGF
TGFβ


d
Monocytes
Normal BM
Normal PB
MF PB
% Cytokine positive cells
P=0.06
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
IL-8
IL-10
IL-12
IL-15
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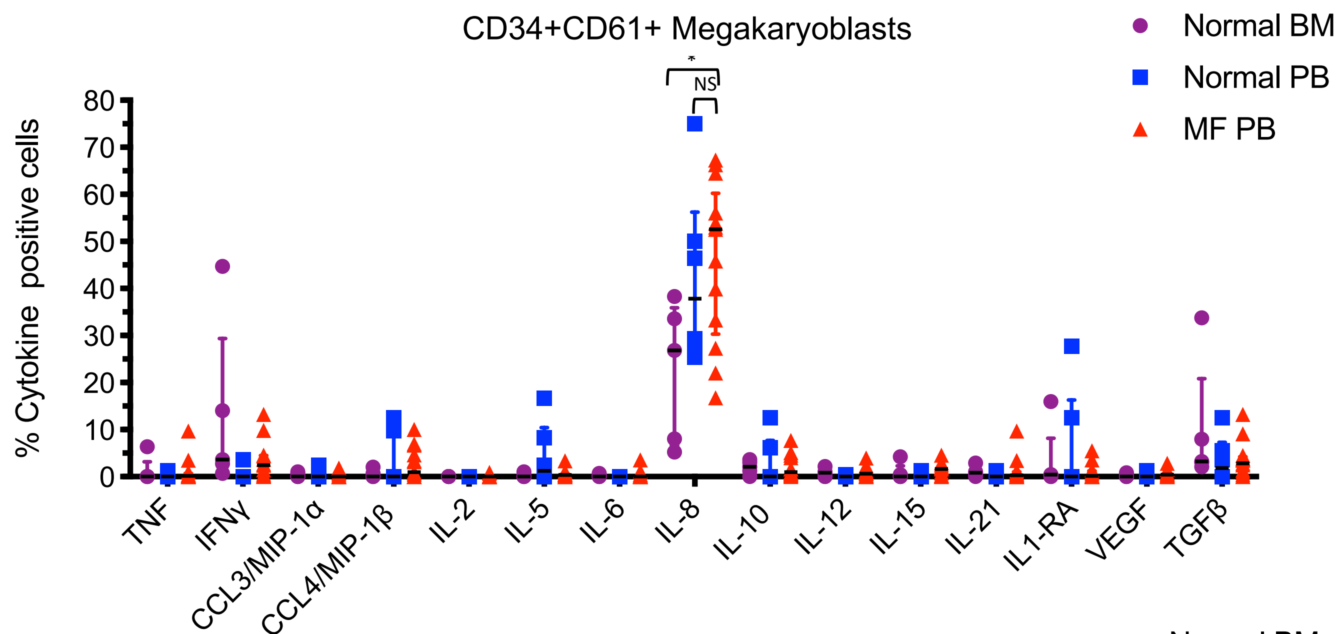
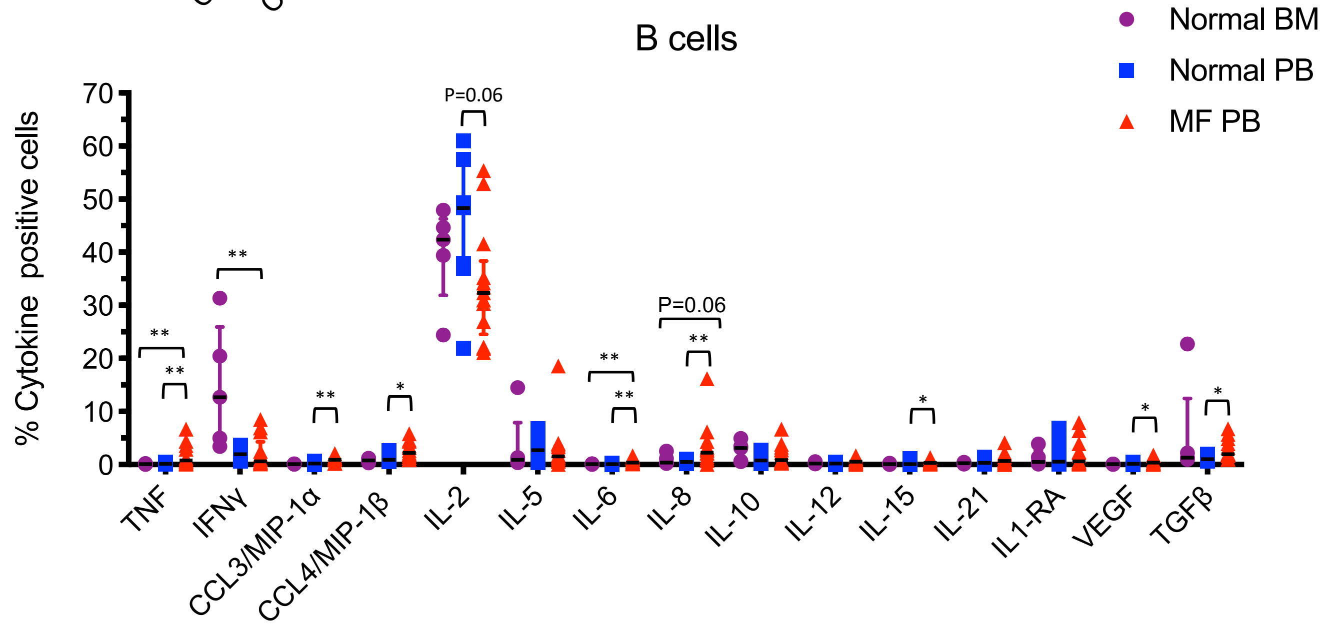
IL1-RA
VEGF
TGFβ


e
CD34+CD61+ Megakaryoblasts
Normal BM
Normal PB
MF PB
% Cytokine positive cells
NS
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
IL-8
IL-10
IL-12
IL-15
IL-21
IL1-RA
VEGF
TGFβ


f
B cells
Normal BM
Normal PB
MF PB
% Cytokine positive cells
P=0.06
P=0.06
TNF
IFNγ
CCL3/MIP-1α
CCL4/MIP-1β
IL-2
IL-5
IL-6
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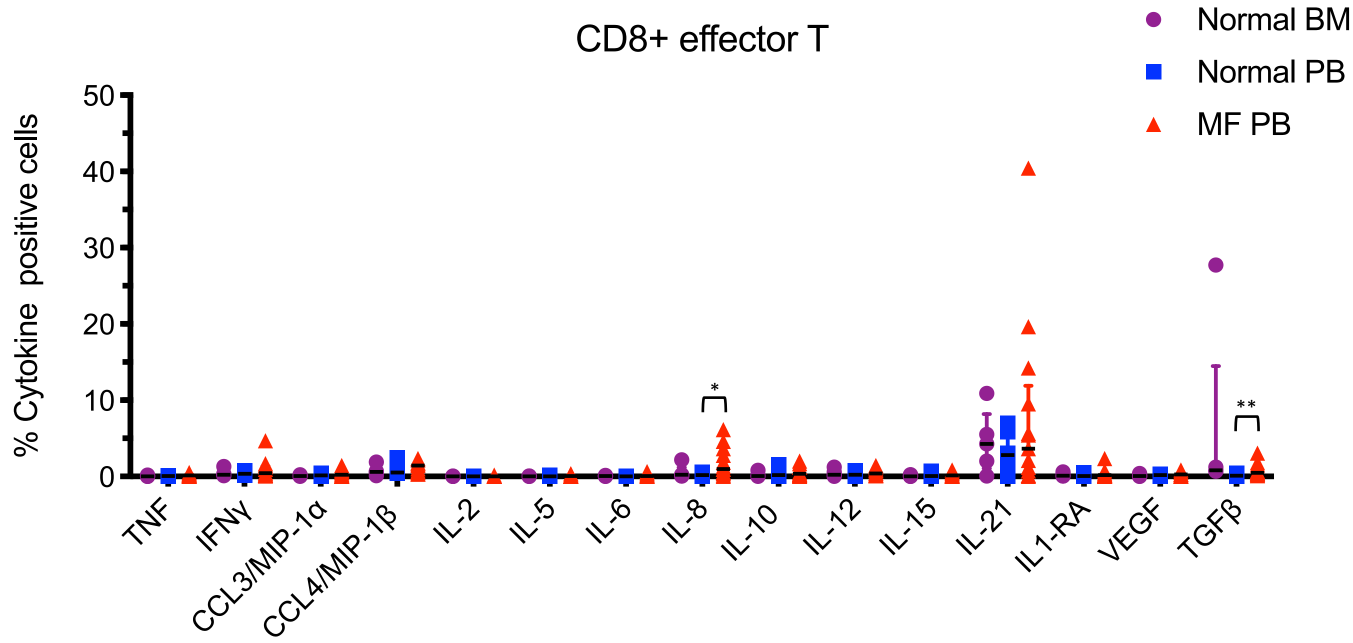
IL1-RA
VEGF
TGFβ



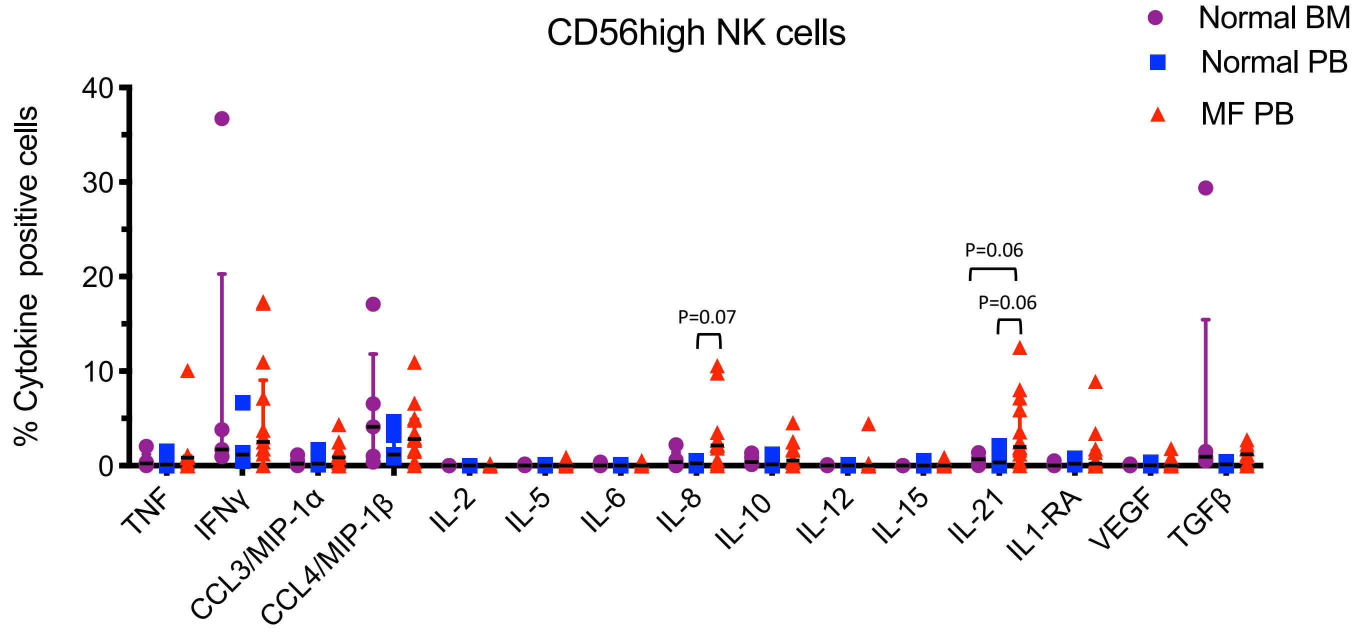
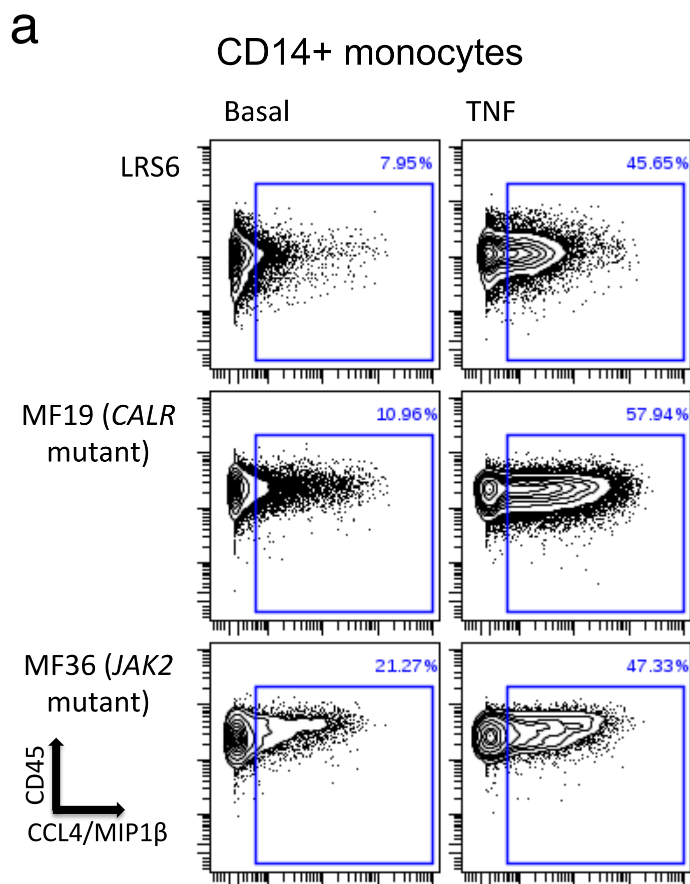
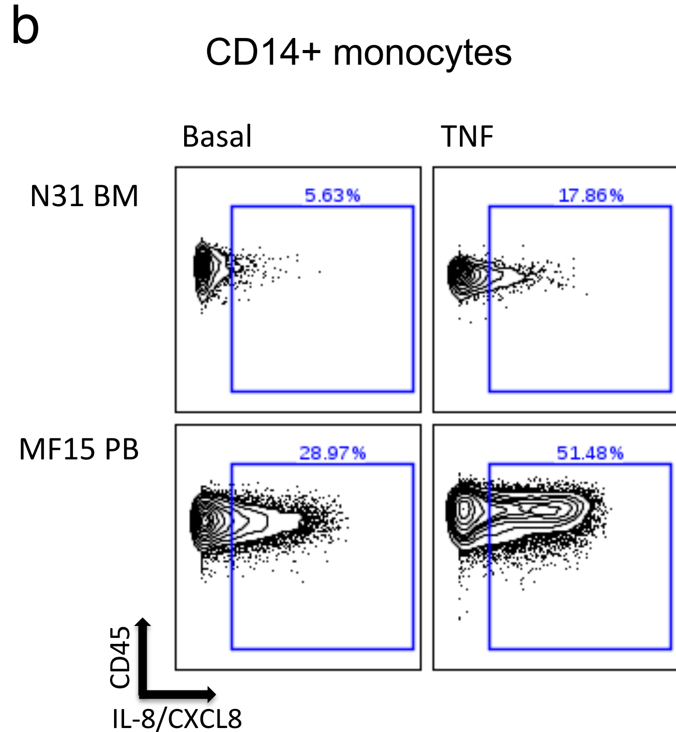
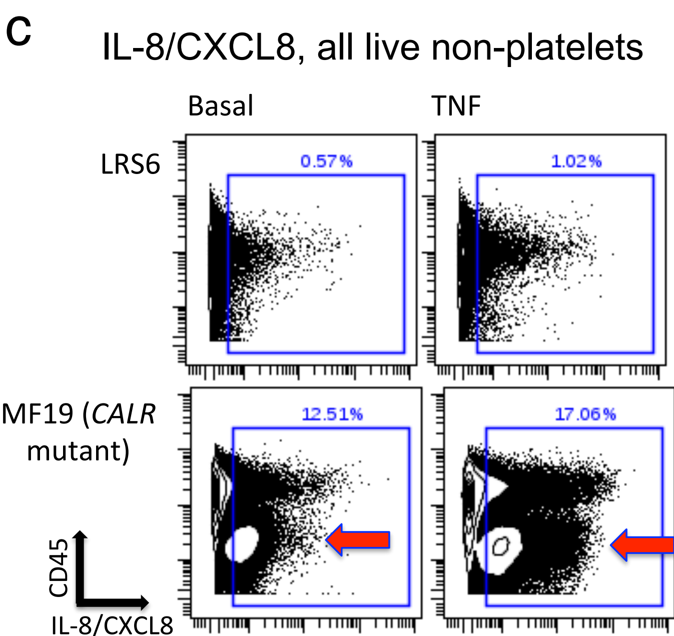
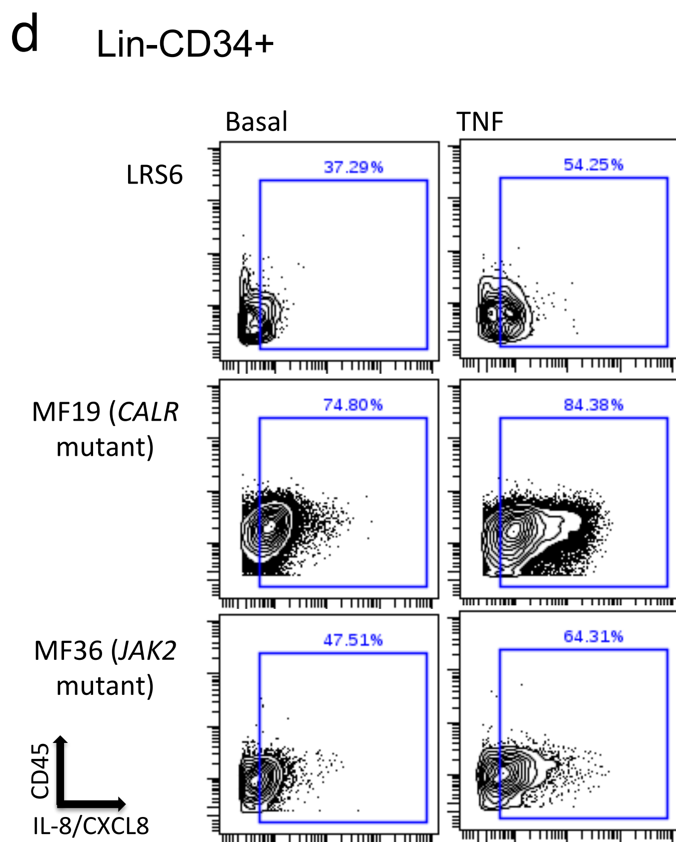


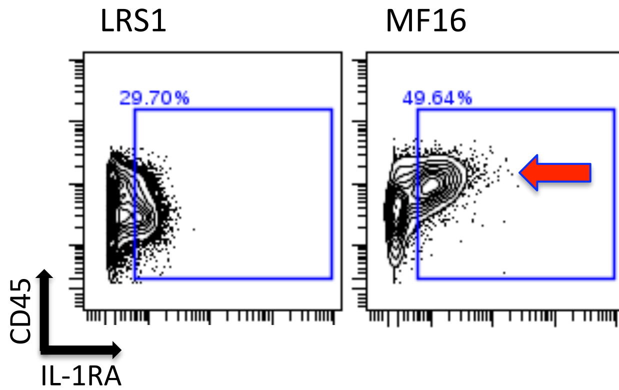
Figure S6: Cytokine positive cells present in MF blood samples versus normal control blood and bone marrow. MF patient and control samples analyzed by mass cytometry (CyTOF) were gated into identifiable cell populations (see **Figure S2**). Basal (unstimulated) cell samples from all individuals were manually gated within identified cell populations, to cytokine-positive and cytokine-negative cells for each cytokine (as in **Figure 2h)**. Percent positive for each cytokine and each cell population were tabulated for each individual. Values from individuals assayed in multiple experiments were averaged (LRS2, LRS3, MF15, MF19, MF20). Values for other individuals were obtained from a single experiment per individual. Individual values are displayed as points in column graphs. Error bars show median +/- interquartile distance. Graphs illustrate percent of cells positive for each cytokine for all live nucleated cells (**a**), Lin-CD34+ HSPC (**b**), CD11c+CD34- immature myeloid cells (**c**, also see **Figure S2** and **Figure 2 c-e**), CD14+ monocytes (**d**), CD34+CD61+ megakaryoblasts (**e**), B cells (**f**), CD8+ effector T cells (**g**), and CD56high NK cells (**h**). Statistical significance between patient and control populations was determined by Mann-Whitney U-test. P values are indicated by brackets above columns: *, P<0.05; **, P<0.01, ***, P<0.001; NS=not significant. No significant difference for any cytokine between patient and control samples was observed for the cell populations not shown: CD4+ T cells, CD16high NK cells.

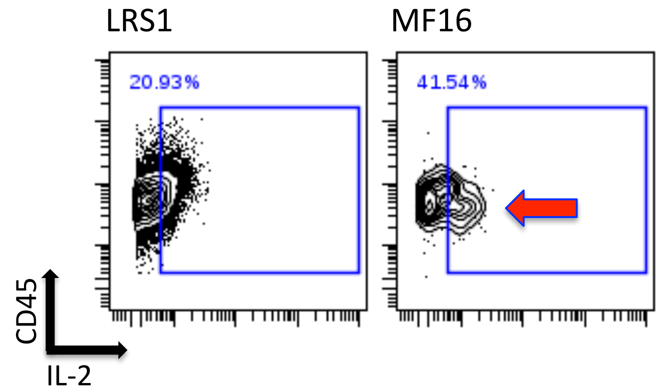

a
CD14+ monocytes
Basal
TNF
LRS6
7.95%
45.65%
MF19 (*CALR* mutant)
10.96%
57.94%
MF36 (*JAK2* mutant)
21.27%
47.33%
CD45
CCL4/MIP1β


b
CD14+ monocytes
Basal
TNF
N31 BM
5.63%
17.86%
MF15 PB
28.97%
51.48%
CD45
IL-8/CXCL8


c
IL-8/CXCL8, all live non-platelets
Basal
TNF
LRS6
0.57%
1.02%
MF19 (*CALR* mutant)
12.51%
17.06%
CD45
IL-8/CXCL8


d
Lin-CD34+
Basal
TNF
LRS6
37.29%
54.25%
MF19 (*CALR* mutant)
74.80%
84.38%
MF36 (*JAK2* mutant)
47.51%
64.31%
CD45
IL-8/CXCL8





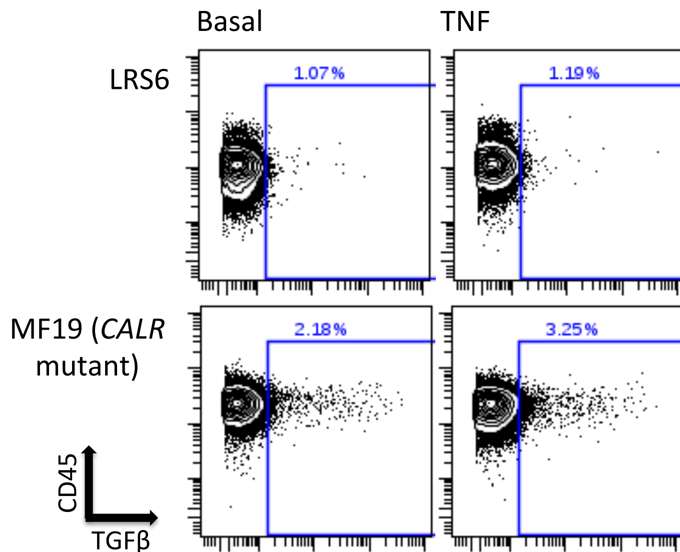


Figure S7: Positive versus negative gating of cytokine expression. a. Illustration of positve versus negative gating illustrated by stimulation producing a bimodal distribution. Biaxial plots show CD14+ monocytes (for population gating, see **Figure S2**) in control peripheral blood (PB) sample LRS6 versus MF patients MF19 (*CALR* mutant) and MF36 (*JAK2* V617F mutant). Contour graphs place CD45 on the Y axis and CCL4/MIP-1β on the X axis, with basal (left) and TNF-treated (right) cells shown. CCL4/MIP-1β positive monocytes are gated in rectangle gate with percent shown. **b**. Second example of positve versus negative gating illustrated by stimulation producing a bimodal distribution. Biaxial contour plots show CD14+ monocytes labeled for CD45 (Y axis) and IL-8/CXCL8 (X axis), with basal (left) and TNF-treated (right) cells shown from bone marrow (BM) control N31 and PB from *JAK2* V617F mutant MF patient MF15. **c**. Gating based on the presence of a distinct positive cell population. Biaxial contour plots show all live non-platelets from control LRS6 and MF15, labeled for CD45 (Y axis) and IL-8/CXCL8 (X axis). Basal (left) and TNF-treated (right) cells are shown, with a distinct CD45low, IL-8/CXCL8+ population marked by arrows. This population can be identified as consisting mainly of Lin-CD34+ cells, as shown in **d**: prevalent IL-8/CXCL8 expression in Lin-CD34+ cells. Lin-CD34+ cells from LRS6 control, MF19, and MF36 (as in **a**) are shown in contour plots for CD45 (Y axis) versus IL-8/CXCL8 (X axis). Extent of IL-8/CXCL8+ positivity is shown by gate (as defined in **c**) with percent of cells, with basal (left) and TNF-treated (right) cells shown. **e-g:** Examples of positive versus negative gating by bimodal distribution of basal cells present in MF but not in normal control. **e**. CD11c+CD34- immature myeloid cells (for population gating, see **Figure S2**) labeled for CD45 (Y axis) and IL-1RA (X axis). Basal unstimulated cells are shown for PB control LRS1 and *JAK2* V617F mutant MF patient MF16. IL-1RA+ cell population evident in MF16 is shown by arrow. **f**. IL-2 (X axis) versus CD45 (Y axis) shown for B cells from LRS1 and MF16 (basal). IL2+ population evident in MF16 is shown by arrow. **g**. CD14+ monocytes from LRS6 control and MF19 labeled for TGFβ (X axis) versus CD45 (Y axis). Basal (left) and TNF-treated (right) cells are shown, with TGFβ+ population identified by gate with percent of cells.

a

Normal PB control | *JAK2* V617F mutant MF

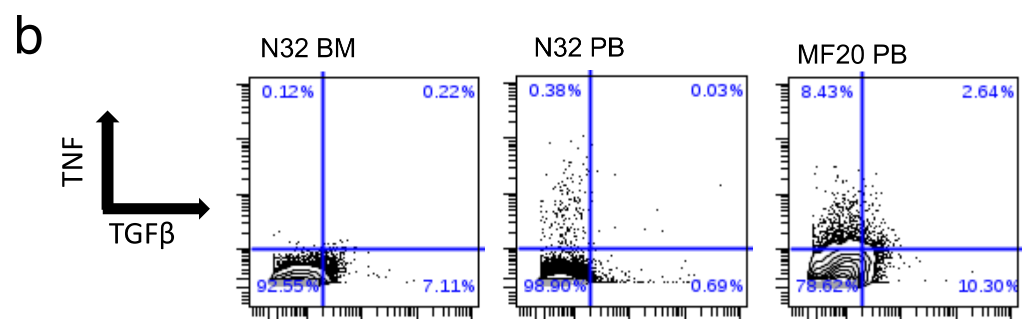
LRS3 | MF2 | MF26

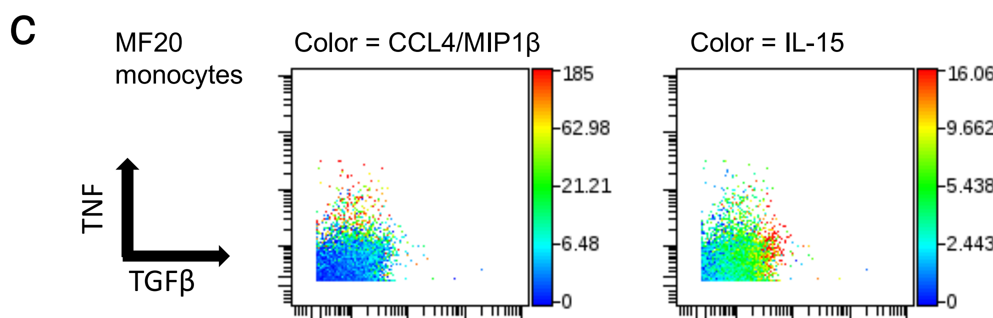
TNF / CCL4/MIP1β
LRS3: 0.03% 0.52% 85.30% 14.15%
MF2: 0.13% 18.86% 53.36% 27.65%
MF26: 0.22% 27.81% 17.59% 54.37%

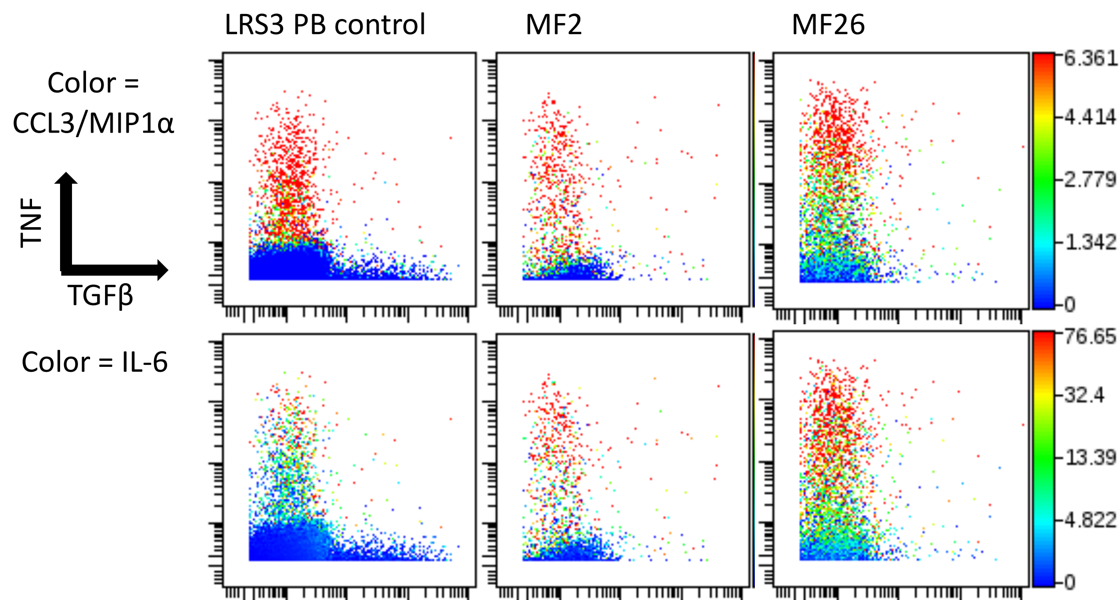
TNF / IL-6
LRS3: 0.33% 0.22% 99.25% 0.21%
MF2: 4.86% 14.13% 71.29% 9.72%
MF26: 6.65% 21.38% 54.28% 17.68%

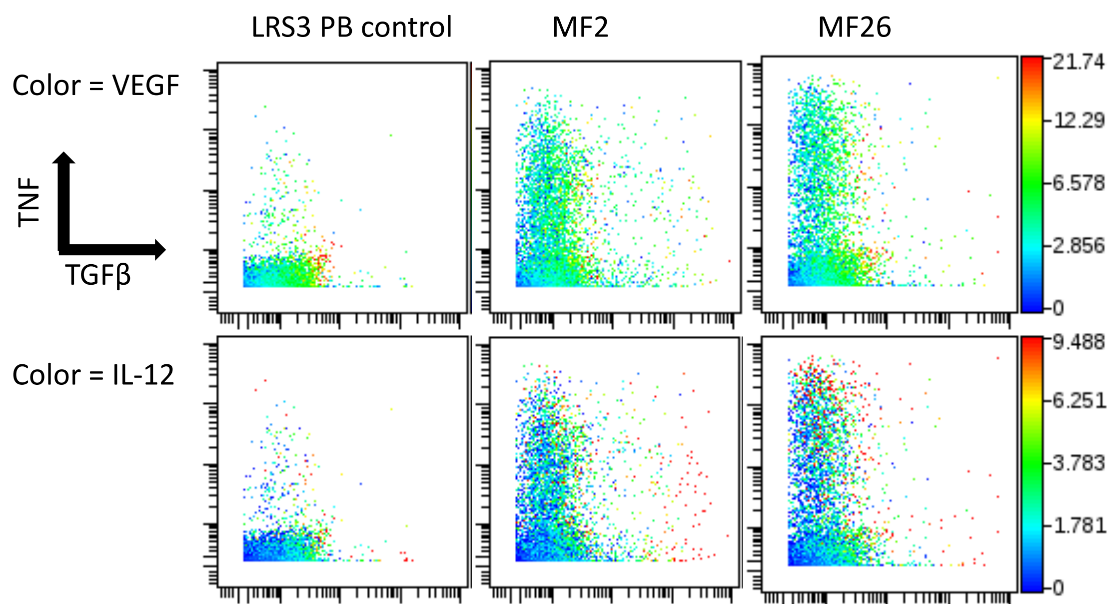
TNF / IL-8/CXCL8
LRS3: 0.08% 0.46% 94.48% 4.99%
MF2: 3.51% 15.45% 39.78% 41.26%
MF26: 14.18% 13.57% 57.23% 15.02%

TNF / TGFβ
LRS3: 0.53% 0.02% 98.68% 0.77%
MF2: 17.77% 1.22% 75.35% 5.66%
MF26: 27.05% 0.99% 70.17% 1.79%






d
Monocytes
LRS3 PB control
MF2
MF26
Color = CCL3/MIP1α
TNF
TGFβ
Color = IL-6
6.361
4.414
2.779
1.342
0
76.65
32.4
13.39
4.822
0


e
Lin-CD34-CD11c+ Immature myeloid
LRS3 PB control
MF2
MF26
Color = VEGF
TNF
TGFβ
Color = IL-12
21.74
12.29
6.578
2.856
0
9.488
6.251
3.783
1.781
0

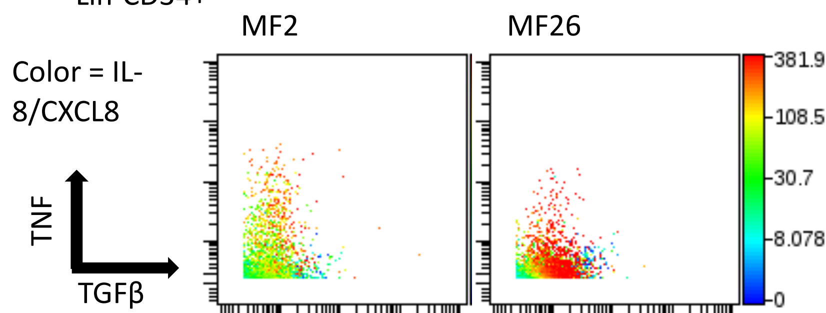
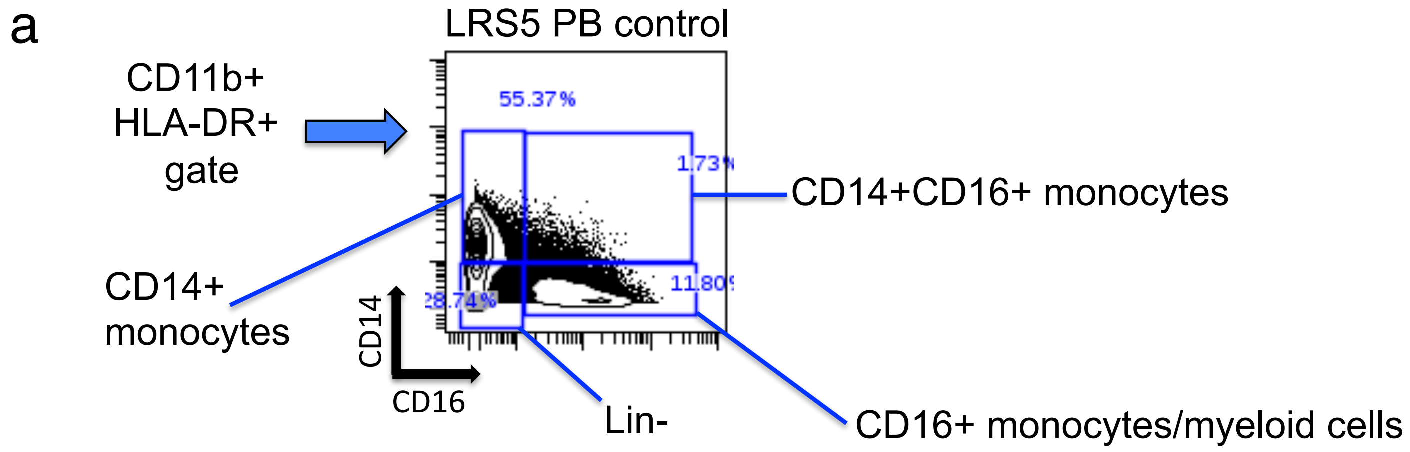
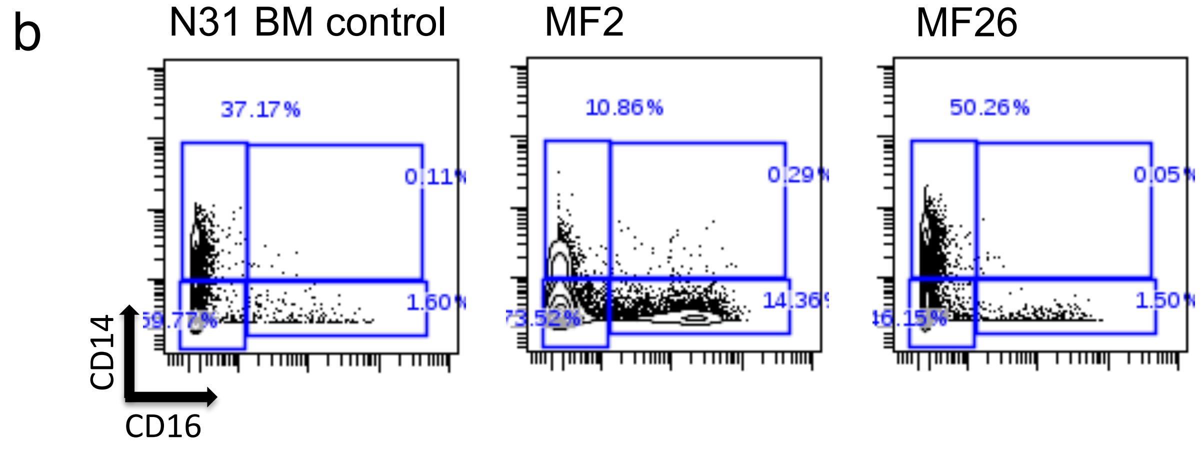

f
Lin-CD34+
MF2
MF26
Color = IL-8/CXCL8
TNF
TGFβ
381.9
108.5
30.7
8.078
0

Figure S8: Coexpression of basally overproduced myeloid cytokines. a. Biaxial plots from CD14high monocytes (for gating, see **Figure S2**) in a healthy control peripheral blood sample (LRS3) and two *JAK2* V617F mutant MF patients (MF2 and MF26). TNF is shown on the Y axis and each of CCL4/MIP1β, IL-6, IL-8/CXCL8, and TGFβ, on the X axis. Quadrant gate shows percent of cells coexpressing each pair of cytokines in the upper right quadrant. **b.** Biaxial plots from CD14high monocytes from an experiment with matched normal bone marrow and peripheral blood controls (from control donor N32) versus blood from *JAK2* V617F mutant MF patient MF20. Quadrant gate shows percent of cells expressing TNF (Y axis) and/or TGFβ (X axis). **c**. Biaxial plots showing monocytes from patient MF20 (as in **b**) showing TNF (Y axis) versusTGFβ (X axis), and colored for expression level of CCL4/MIP1β (left) or IL-15 (right). **d**. Biaxial plots showing monocytes from control LRS3 and patients MF2 and MF26 (as in **a**), with TNF (Y axis) versusTGFβ (X axis), and colored for expression level of cytokines CCL3/MIP1α (above) or IL-6 (below). **e**. Biaxial plots showing Lin-CD34-CD11c+ immature myeloid cells (for gating, see **Figure S2**) from control LRS3 and patients MF2 and MF26 with TNF (Y axis) versusTGFβ (X axis), and colored for expression level of cytokines VEGF (Above) and IL-12 (below). **f**: Biaxial plots of Lin-CD34+ HSPC from patients MF2 and MF26 with TNF (Y axis) versusTGFβ (X axis), and colored for expression level of IL-8/CXCL8.





c Cytokine medians:

LRS3 | MF2 | MF26

Non-platelets
CD14+ Mono
CD16+ Mono/Myeloid
CD14+CD16+ Mono

TNF
IFNγ
CCL3/MIP1α
CCL4/MIP1β
IL2
IL5
IL6
IL8
IL10
IL12
IL15
IL21
IL1RA
VEGF
TGFβ

ArcSinh ratio

-3.55 -1.78 0 1.78 3.55

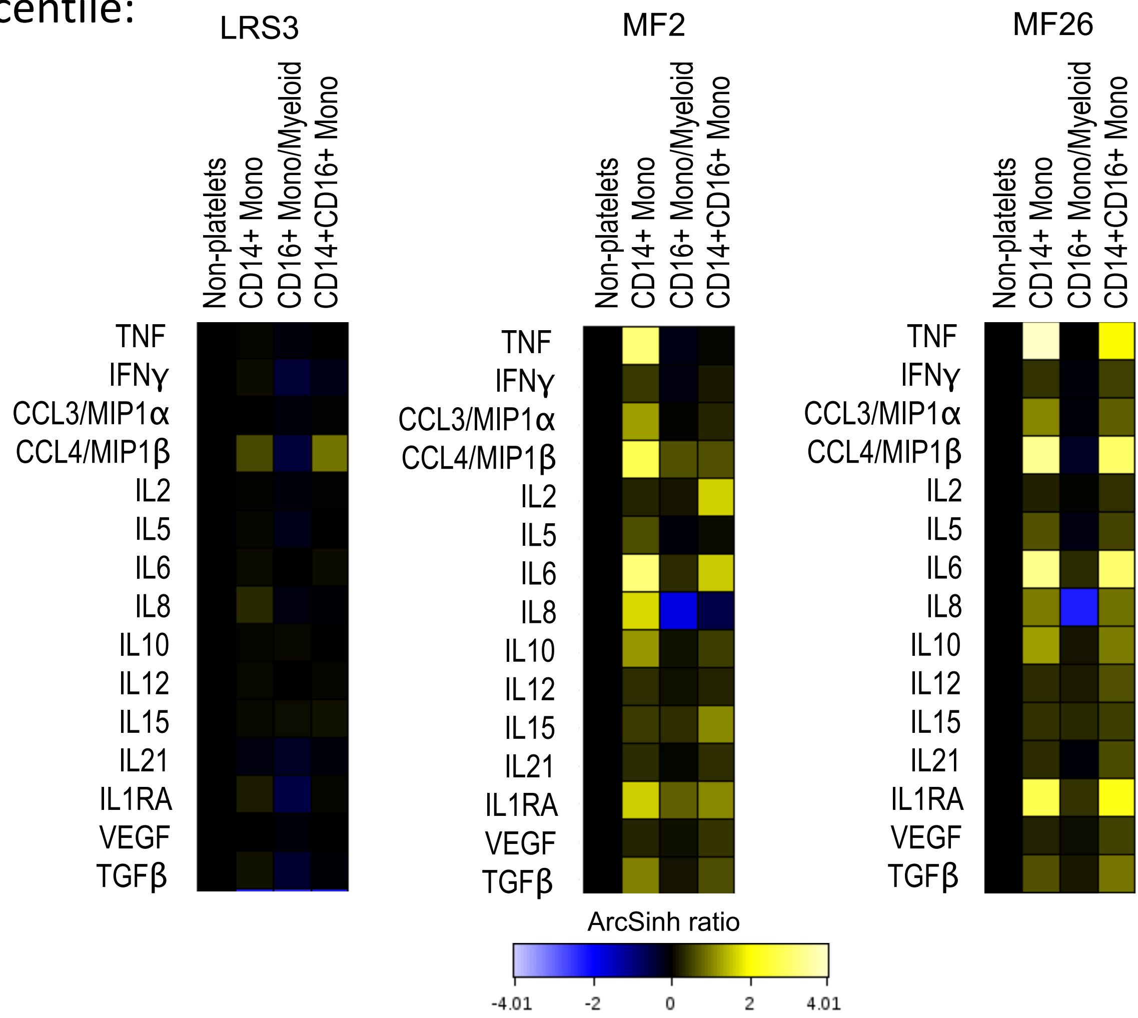
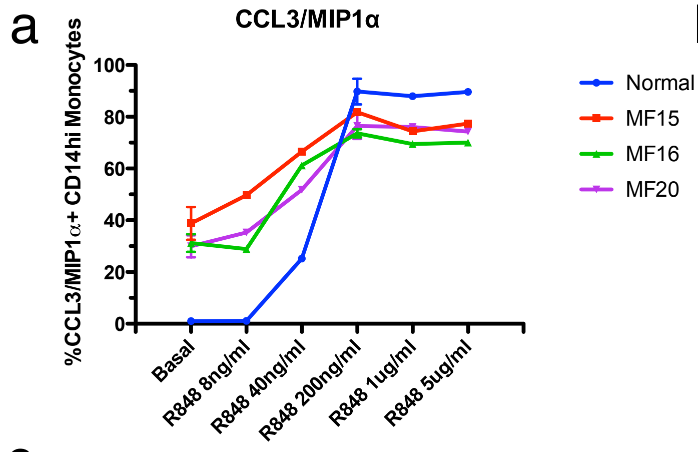
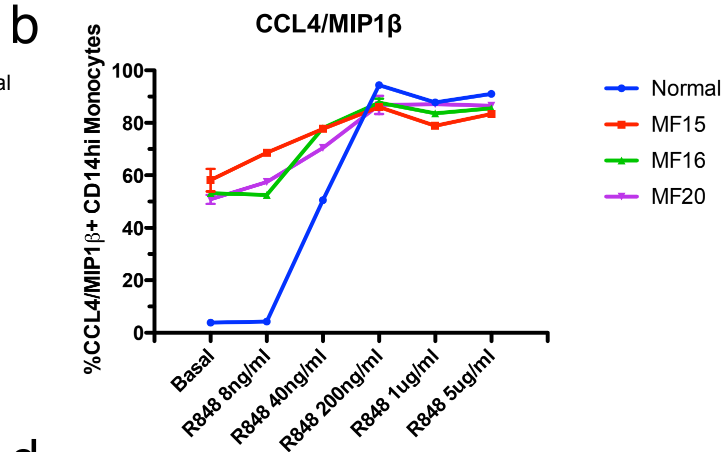


Figure S9: Cytokine production by monocyte subpopulations. **a**. Subdivision of the CD11b+HLA-DR+ parent myeloid population (derived as shown in **Figure S2b**) into subpopulations by gating, as illustrated for normal peripheral blood control LRS5. A quadrant on plots of CD14 (Y axis) versus CD16 (X axis) divides CD11b+HLA-DR+ myeloid cells into CD14+CD16- "classical" monocytes, CD14+CD16+ "inflammatory" monocytes, CD16+CD14low/- monocytes and myeloid cells, including "non-classical" monocytes (Wong, KL et al., 2011 *Blood*),[32] and Lin- gate, as shown by labels and percentages in plot. **b**. Plots of gating of CD11b+HLA-DR+ myeloid cells identical to that in **a**, shown for (left to right) normal control bone marrow N31, and *JAK2* V617F mutant MF patients MF2 and MF26. **c**. Heat maps showing median levels of cytokine labeling in the monocyte and myeloid cell populations defined by gating in **a** and **b**, in (heat maps, left to right) normal peripheral blood control LRS3, and *JAK2* V617F mutant MF patients MF2 and MF26. Median levels of cytokine labeling are normalized on ArcSinh ratio scale to the median level of each cytokine in the total non-platelets population (left column of each heat map). Populations illustrated are (left to right in each heat map): total non-platelets population, CD14+ monocytes ("classical"), CD16+ monocytes ("non-classical") and myeloid cells, and CD14+CD16+ monocytes ("inflammatory"). **d.** Heat maps similar to those in **c**, for identical individual samples, illustrating 90th percentile values of cytokine labeling in each population.


a
CCL3/MIP1α
%CCL3/MIP1α+ CD14hi Monocytes
0
20
40
60
80
100
Basal
R848 8ng/ml
R848 40ng/ml
R848 200ng/ml
R848 1ug/ml
R848 5ug/ml
Normal
MF15
MF16
MF20


b
CCL4/MIP1β
%CCL4/MIP1β+ CD14hi Monocytes
0
20
40
60
80
100
Basal
R848 8ng/ml
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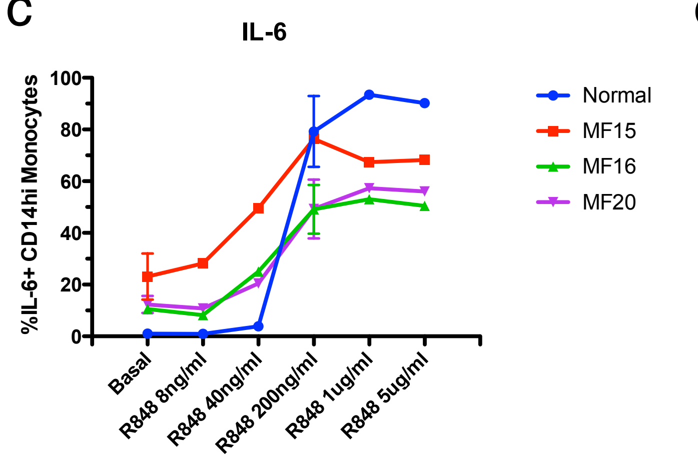
R848 200ng/ml
R848 1ug/ml
R848 5ug/ml
Normal
MF15
MF16
MF20


c
IL-6
%IL-6+ CD14hi Monocytes
0
20
40
60
80
100
Basal
R848 8ng/ml
R848 40ng/ml
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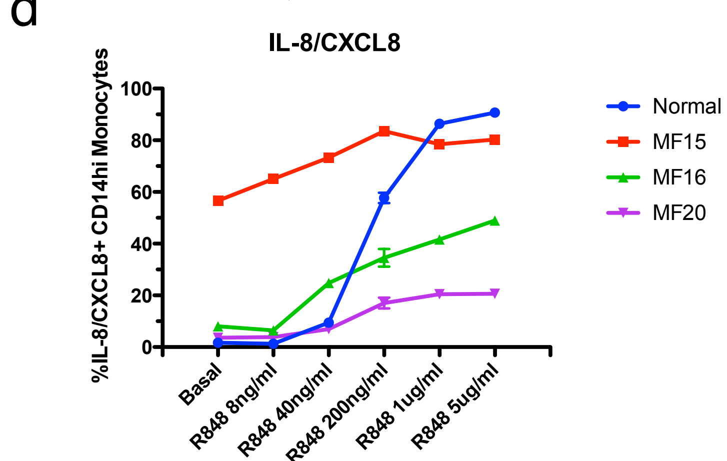
R848 1ug/ml
R848 5ug/ml
Normal
MF15
MF16
MF20


d
IL-8/CXCL8
%IL-8/CXCL8+ CD14hi Monocytes
0
20
40
60
80
100
Basal
R848 8ng/ml
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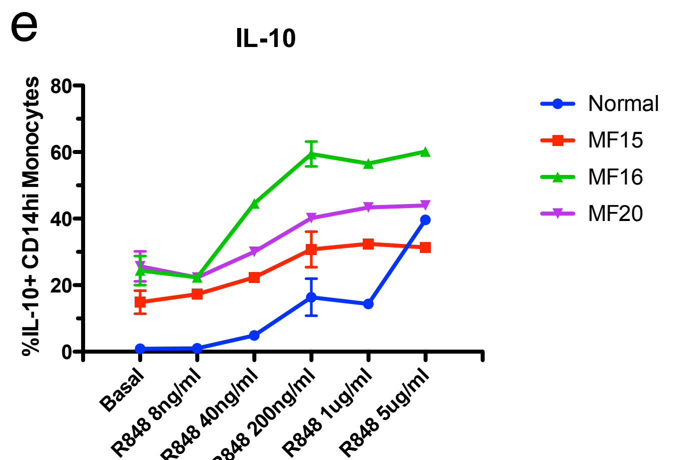
R848 1ug/ml
R848 5ug/ml
Normal
MF15
MF16
MF20


e
IL-10
%IL-10+ CD14hi Monocytes
0
20
40
60
80
Basal
R848 8ng/ml
R848 40ng/ml
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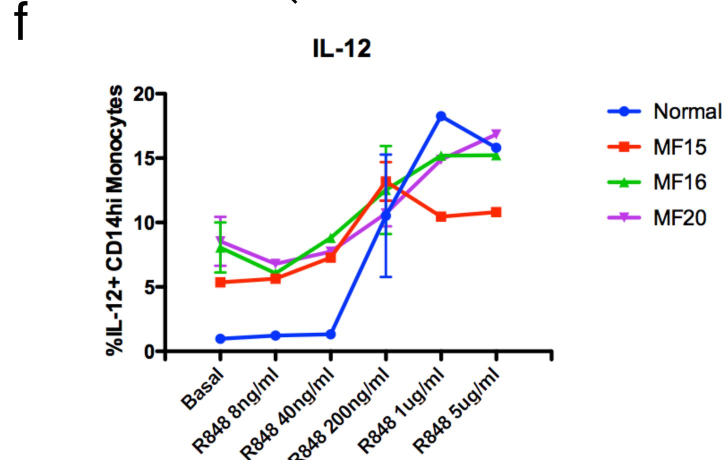
R848 5ug/ml
Normal
MF15
MF16
MF20


f
IL-12
%IL-12+ CD14hi Monocytes
0
5
10
15
20
Basal
R848 8ng/ml
R848 40ng/ml
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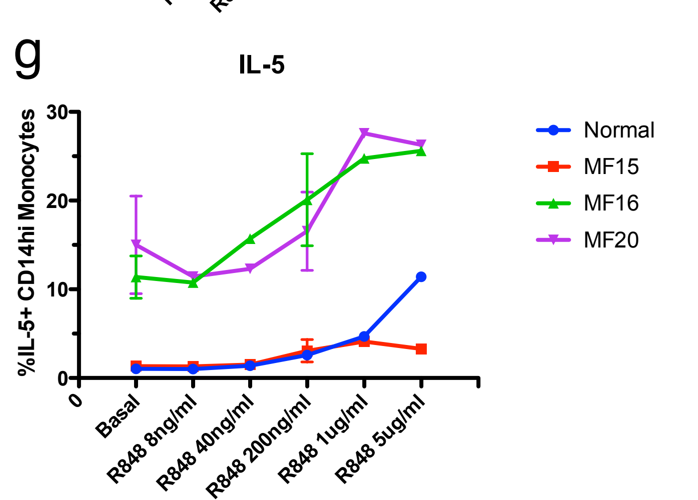
R848 5ug/ml
Normal
MF15
MF16
MF20


g
IL-5
%IL-5+ CD14hi Monocytes
0
10
20
30
0
Basal
R848 8ng/ml
R848 40ng/ml
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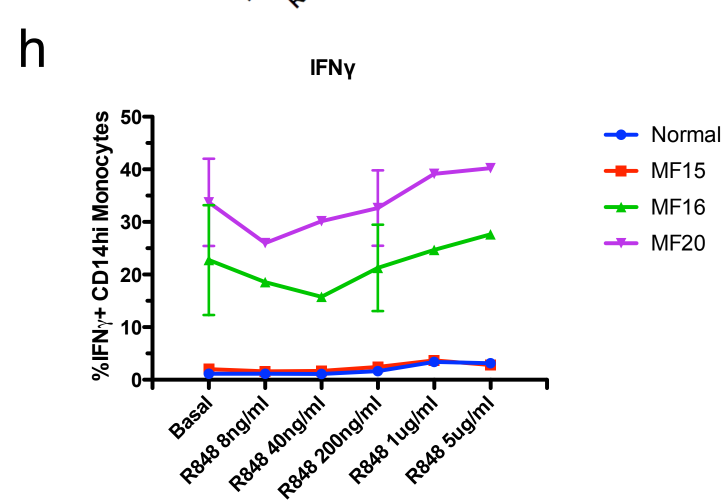
Normal
MF15
MF16
MF20


h
IFNγ
%IFNγ+ CD14hi Monocytes
0
10
20
30
40
50
Basal
R848 8ng/ml
R848 40ng/ml
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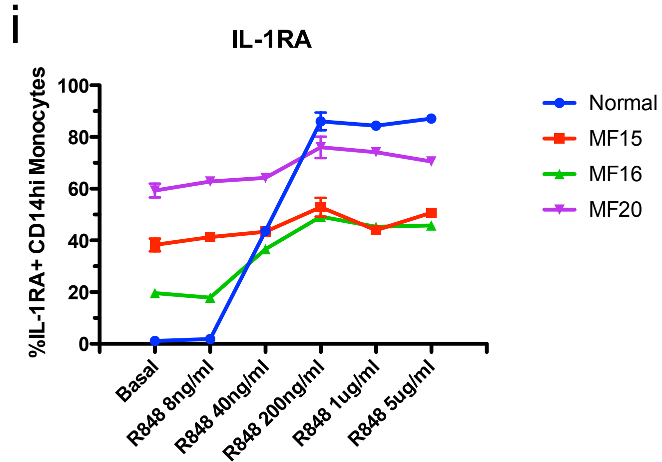
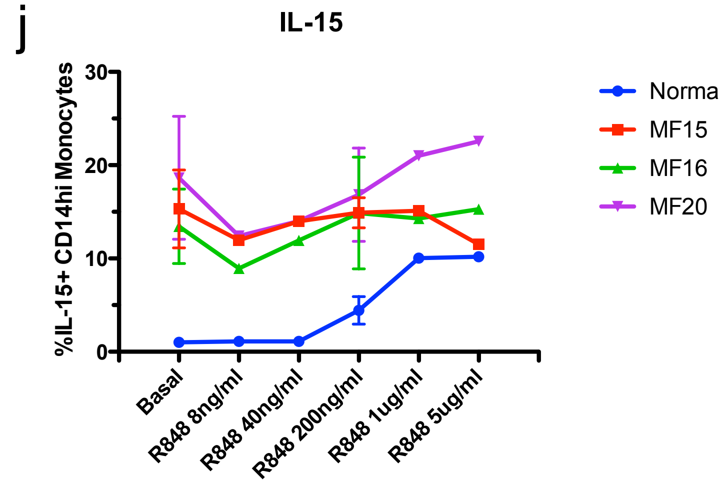
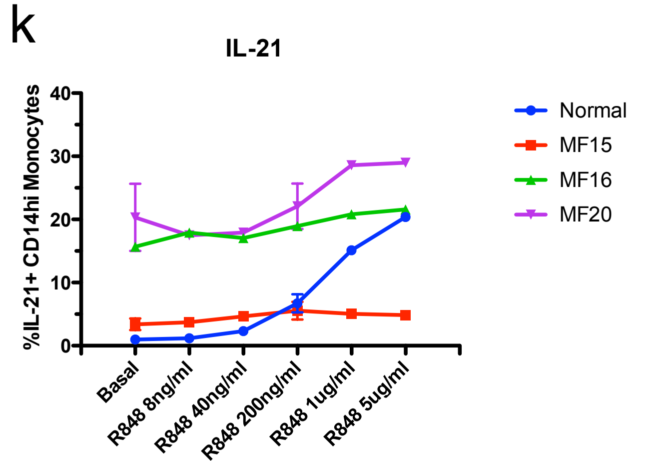
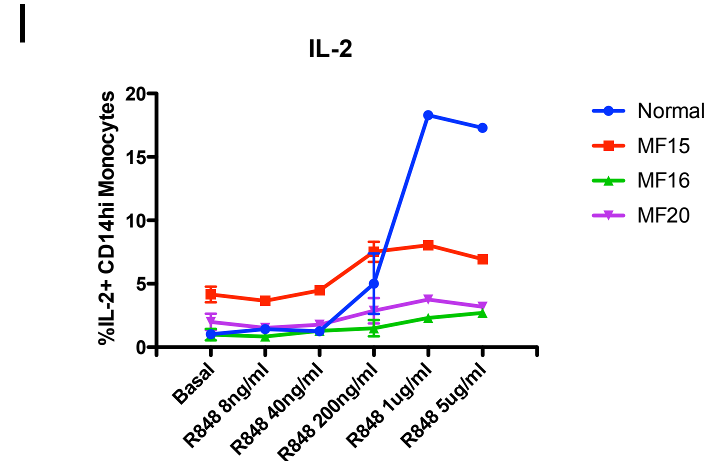
Normal
MF15
MF16
MF20









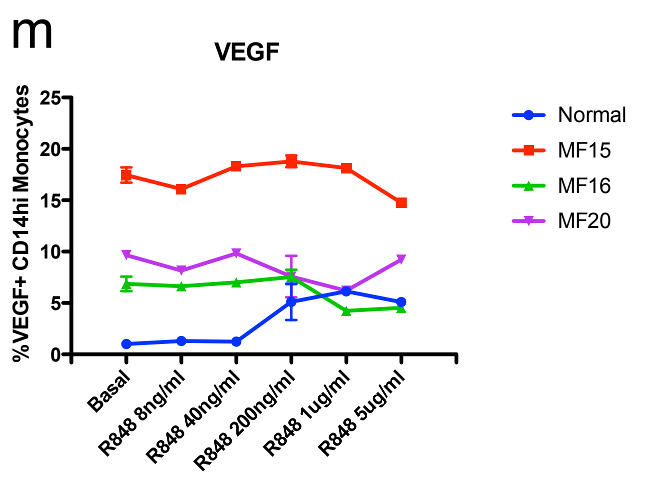
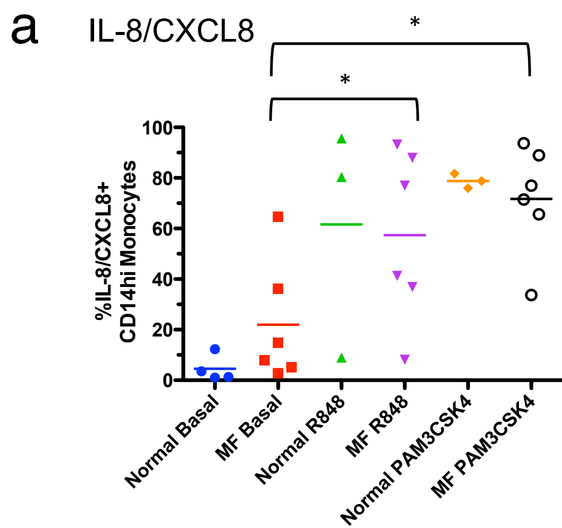
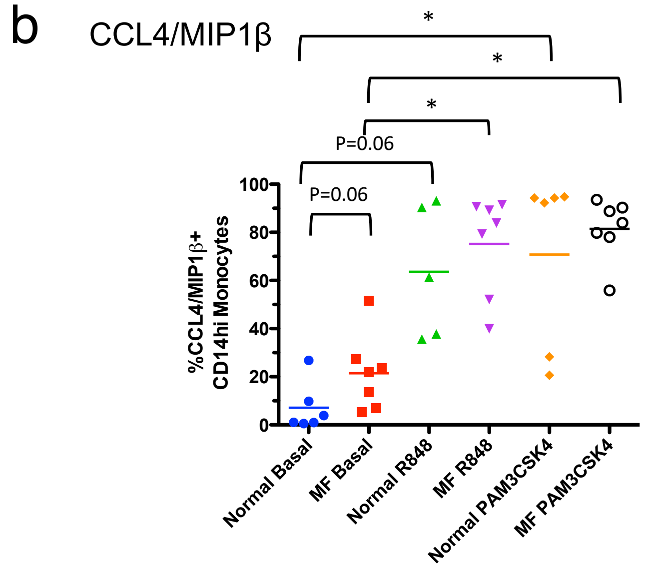
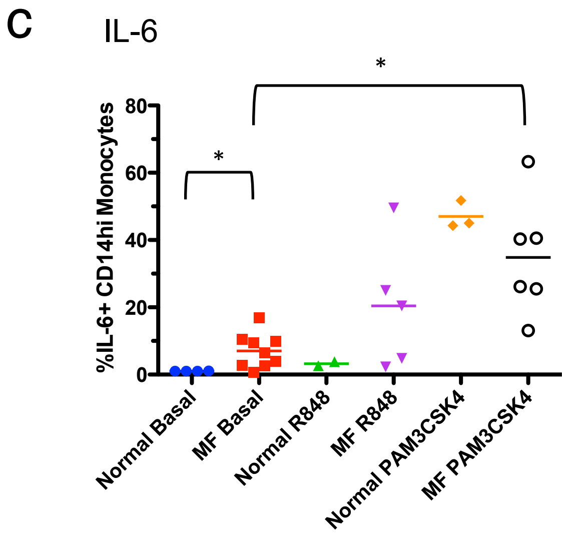
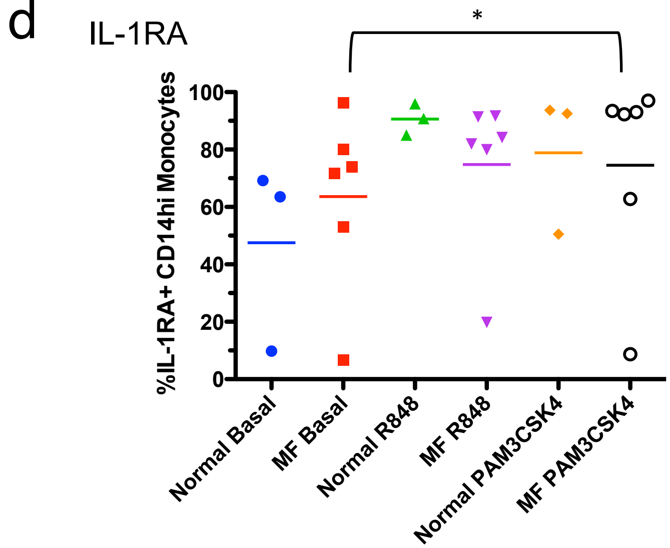


Figure S10: Dose-response of cytokine induction by R848 in normal and *JAK2* mutant MF monocytes. Dose-response plots show percent of monocytes positive for each cytokine for a normal blood control and three *JAK2* V617F mutant MF patients. N=2 experiments, error bars = mean +/- SD. Equivalent data for the cytokines TNF and TGFβ are in **Fig. 3g, h.** Shown here are data for cytokines CCL3/MIP-1α (**a**), CCL4/MIP-1β (**b**), IL-6 (**c**), IL-8/CXCL8 (**d**), IL-10 (**e**), IL-12 (**f**), IL-5 (**g**), IFNγ (**h**), IL1-RA (**i**), IL-15 (**j**), IL-21 (**k**), IL-2 (**l**), and VEGF (**m**).









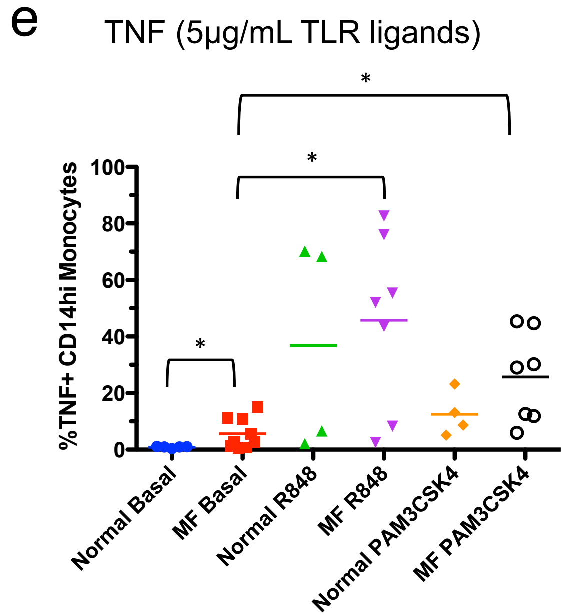


Figure S11: Induction of cytokines by TLR ligands in monocytes. Column graphs show percent of normal or MF blood CD14+ monocytes expressing each cytokine, basally or with cells treated with TLR ligands R848 or PAM3CSK4. Cells were treated with TLR ligands at doses producing maximal response in MF versus normal monocytes (see **Figure S10**): 40ng/mL for IL-8/CXCL8 (**a**), CCL4/MIP1β (**b**), IL-6 (**c**), and IL-1RA (**d**), and 5μg/mL for TNF (**e**). Statistical significance between patient and control populations was determined by Mann-Whitney U-test. Statistical significance between basal and induced paired samples was determined by Wilcoxon matched-pairs signed rank test. P value: *, P<0.05. Cytokines significantly induced by TLR ligands matched those significantly induced by TNF (see **Figure 3b and 5d**).

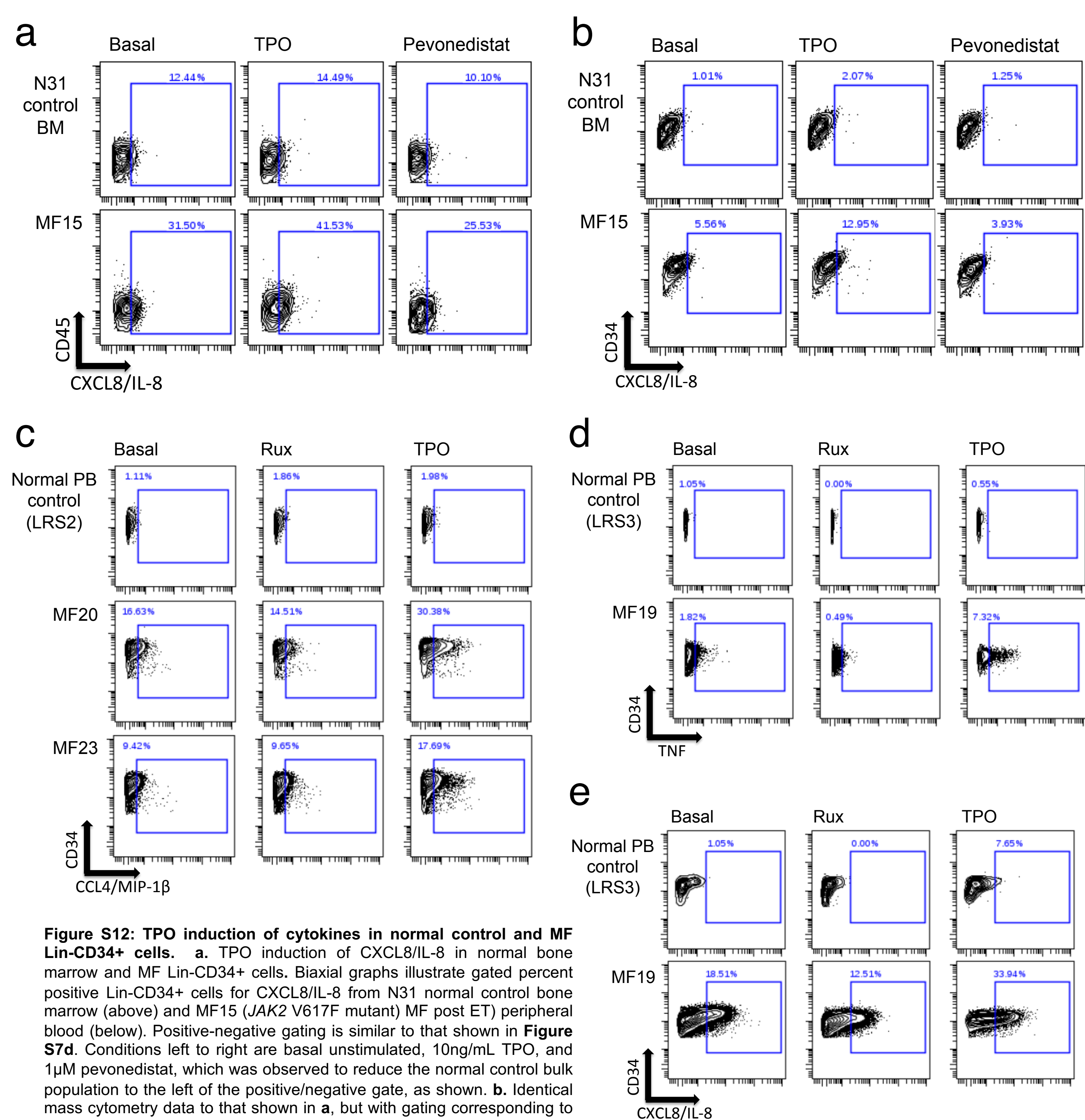
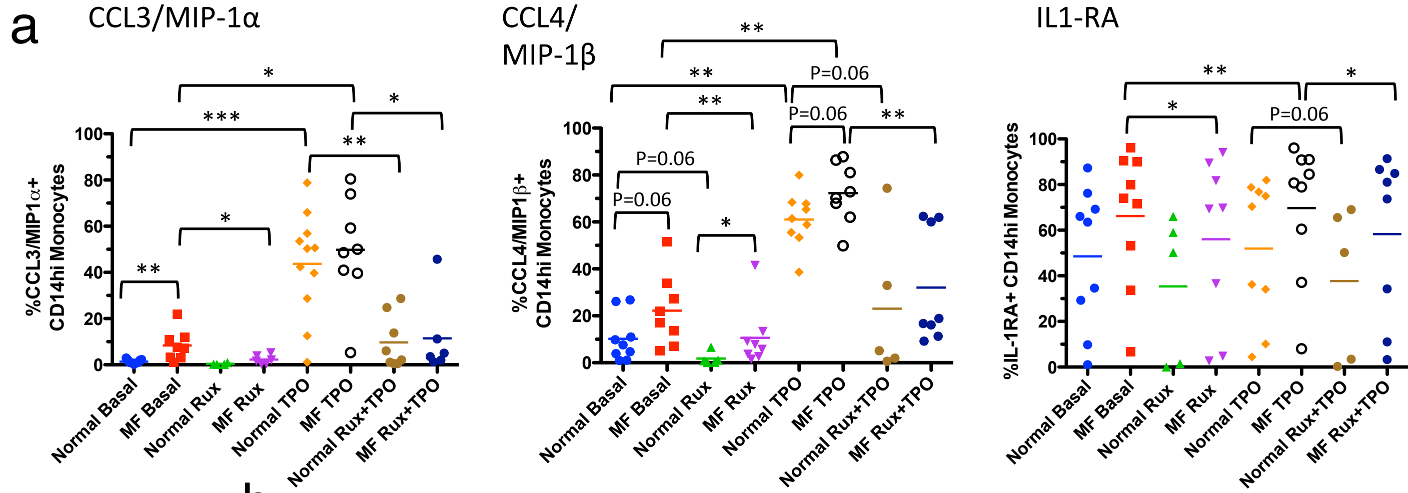
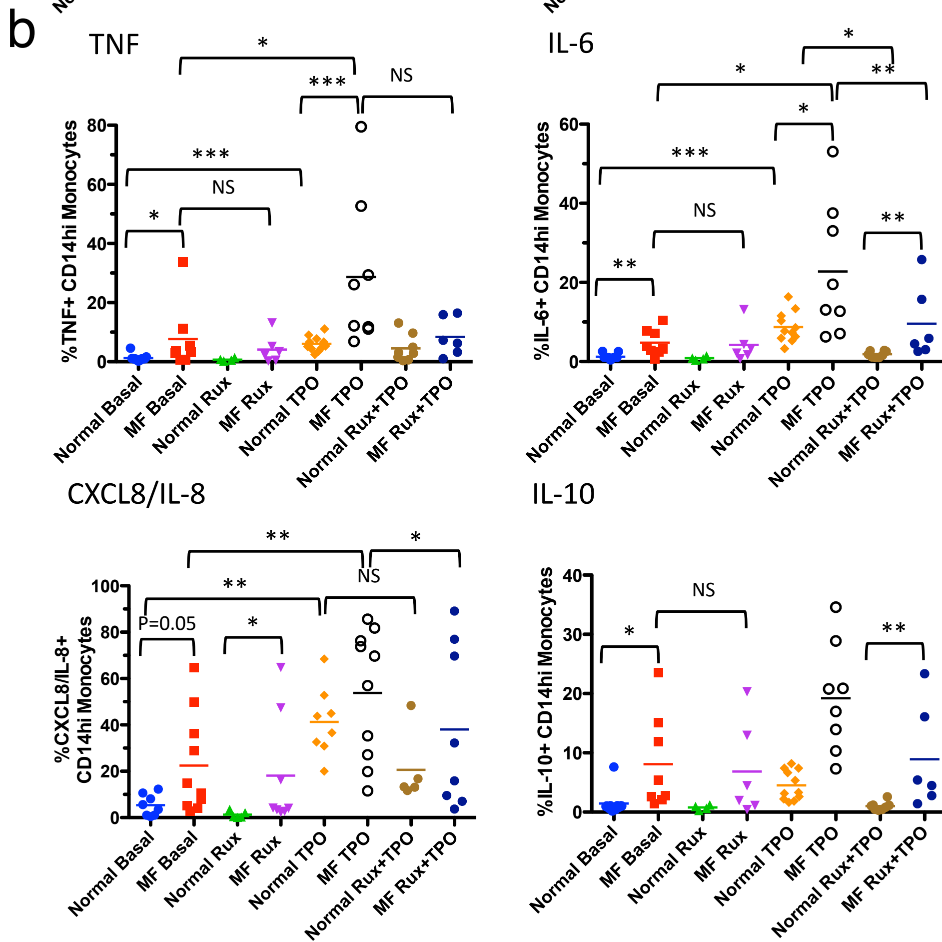
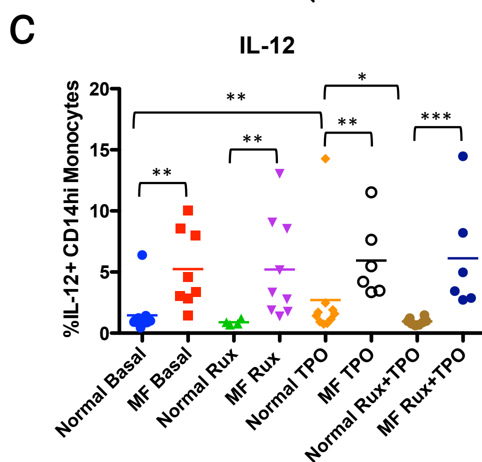
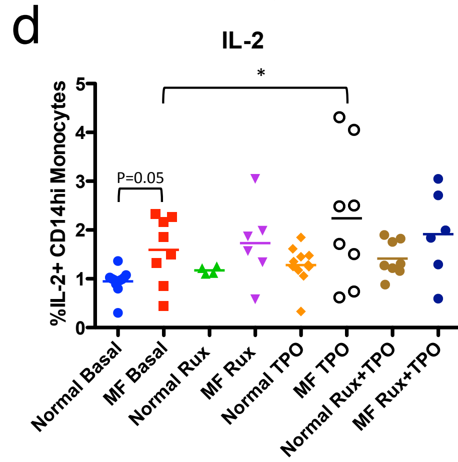


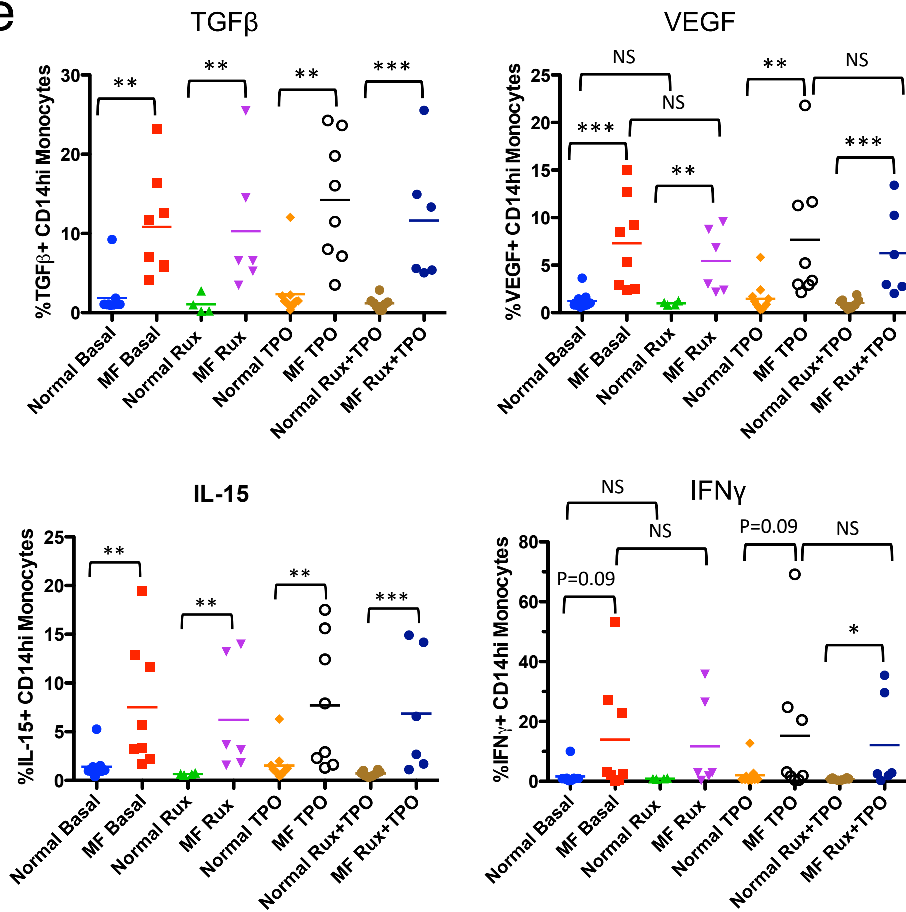
Figure S12: TPO induction of cytokines in normal control and MF Lin-CD34+ cells. **a.** TPO induction of CXCL8/IL-8 in normal bone marrow and MF Lin-CD34+ cells**.** Biaxial graphs illustrate gated percent positive Lin-CD34+ cells for CXCL8/IL-8 from N31 normal control bone marrow (above) and MF15 (*JAK2* V617F mutant) MF post ET) peripheral blood (below). Positive-negative gating is similar to that shown in **Figure S7d**. Conditions left to right are basal unstimulated, 10ng/mL TPO, and 1µM pevonedistat, which was observed to reduce the normal control bulk population to the left of the positive/negative gate, as shown. **b.** Identical mass cytometry data to that shown in **a**, but with gating corresponding to the top 1% of the normal basal unstimulated distribution (as in **Figure 4a**), and with CD34 instead of CD45 as the Y axis parameter. **c**. Data similar to that in **Figure 4a,** with gating corresponding to the top 1% of the normal basal unstimulated distribution, in biaxial graphs illustrating CCL4/MIP-1β production by Lin-CD34+ cells from MF patients MF20 and MF23 (both *JAK2* V617F mutant) versus circulating Lin-CD34+ cells from normal control LRS2 peripheral blood. Conditions left to right are basal unstimulated, 5µM ruxolitinib, and 10ng/mL TPO. No bone marrow sample was included in this experiment. **d, e**: Biaxial plots comparing production of TNF (**d**) and CXCL8/IL-8 (**e**) between mobilized Lin-CD34+ cells from normal control LRS3 peripheral blood versus from patient MF19 (*CALR* 5bp insertion mutant MF post ET). Percent in gate corresponds corresponding to the top 1% of the normal basal unstimulated distribution (as in **Figure 4a**). Conditions left to right are basal unstimulated, 5µM ruxolitinib, and 10ng/mL TPO. No bone marrow sample was included in this experiment.


a
CCL3/MIP-1α
CCL4/
MIP-1β
IL1-RA
%CCL3/MIP1α+
CD14hi Monocytes
%CCL4/MIP1β+
CD14hi Monocytes
%IL-1RA+ CD14hi Monocytes
P=0.06
Normal Basal
MF Basal
Normal Rux
MF Rux
Normal TPO
MF TPO
Normal Rux+TPO
MF Rux+TPO


b
TNF
IL-6
CXCL8/IL-8
IL-10
%TNF+ CD14hi Monocytes
%IL-6+ CD14hi Monocytes
%CXCL8/IL-8+
CD14hi Monocytes
%IL-10+ CD14hi Monocytes
NS
P=0.05
Normal Basal
MF Basal
Normal Rux
MF Rux
Normal TPO
MF TPO
Normal Rux+TPO
MF Rux+TPO


c
IL-12
%IL-12+ CD14hi Monocytes
Normal Basal
MF Basal
Normal Rux
MF Rux
Normal TPO
MF TPO
Normal Rux+TPO
MF Rux+TPO


d
IL-2
%IL-2+ CD14hi Monocytes
P=0.05
Normal Basal
MF Basal
Normal Rux
MF Rux
Normal TPO
MF TPO
Normal Rux+TPO
MF Rux+TPO



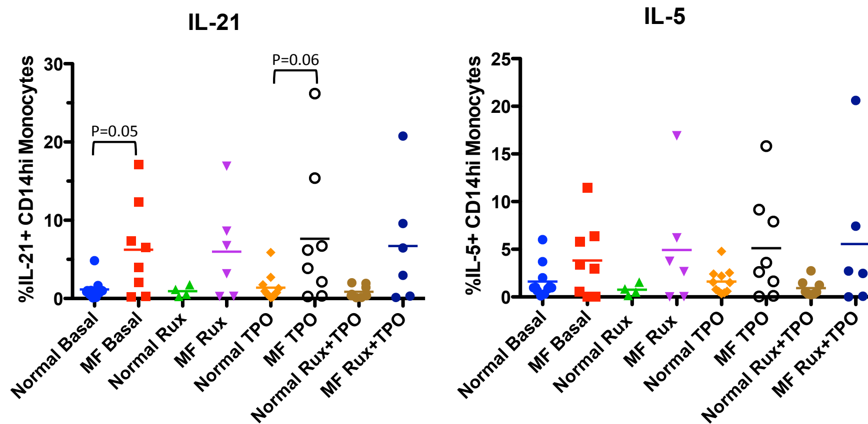
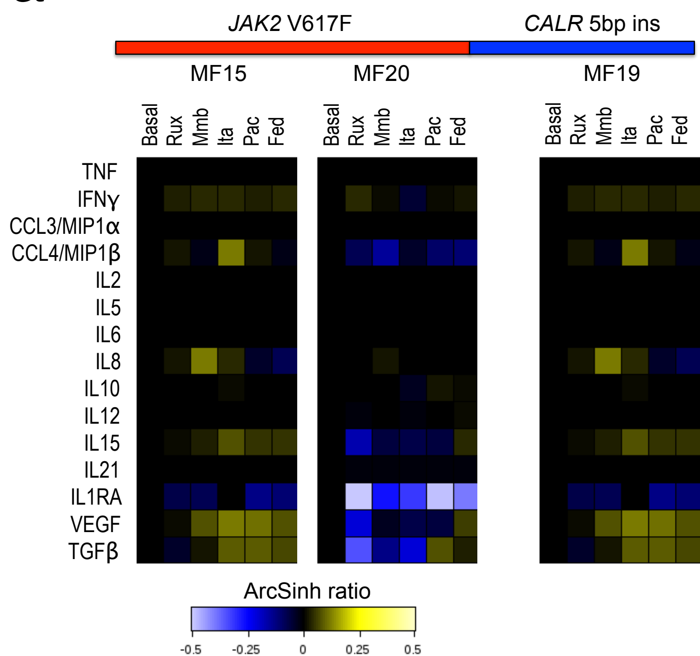
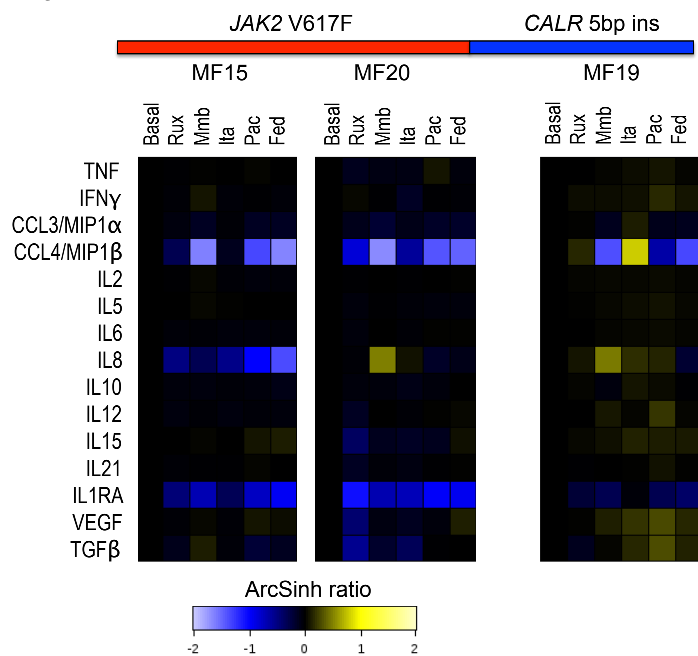


Figure S13: Effects of TPO and ruxolitinib on cytokine expression in MF and normal control monocytes. Plots show cytokine-positive percentages of monocytes from MF patients and healthy controls (left to right in each graph) basally, and treated with 5μM ruxolitinib, 10ng/mL TPO, or TPO plus ruxolitinib. Horizontal bar shows mean value for each group. Significance was determined by Mann-Whitney U-test for MF versus normal comparisons, and by Wilcoxon sign-rank test for comparisons between conditions within the MF or control groups (since the individuals were identical): *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. **a.** Monocytes positive for (left to right) CCL3/MIP-1α, CCL4/MIP-1β, and IL1-RA, with significant effects of both TPO and ruxolitinib versus the unstimulated basal. **b.** Monocytes positive for TNF, IL-6, IL-8/CXCL8, and IL-10, with ruxolitinib-sensitive response to TPO but not significant reduction of basal cytokine levels by ruxolitinib. **c**. Monocytes positive for IL-12, showing significant responses to TPO and ruxolitinib among normal control but not MF samples. **d.** Monocytes positive for IL-2, with a significant stimulation by TPO only among MF samples. **e**. Monocytes positive for TGFβ, VEGF, IL-15 and IFNγ, lacking significant effects of TPO or ruxolitinib. **f.** Monocytes positive for IL-21 and IL-5, for which no significant difference between MF and normal control was observed in any condition.





c

N21 BM basal 35.43%

LRS5 PB basal 19.30%

CD45

CCL4/MIP1β

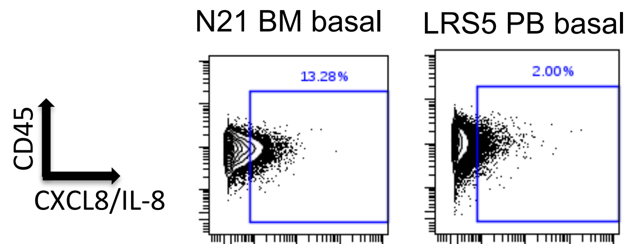
Basal Rux Mmb Ita Pac Fed

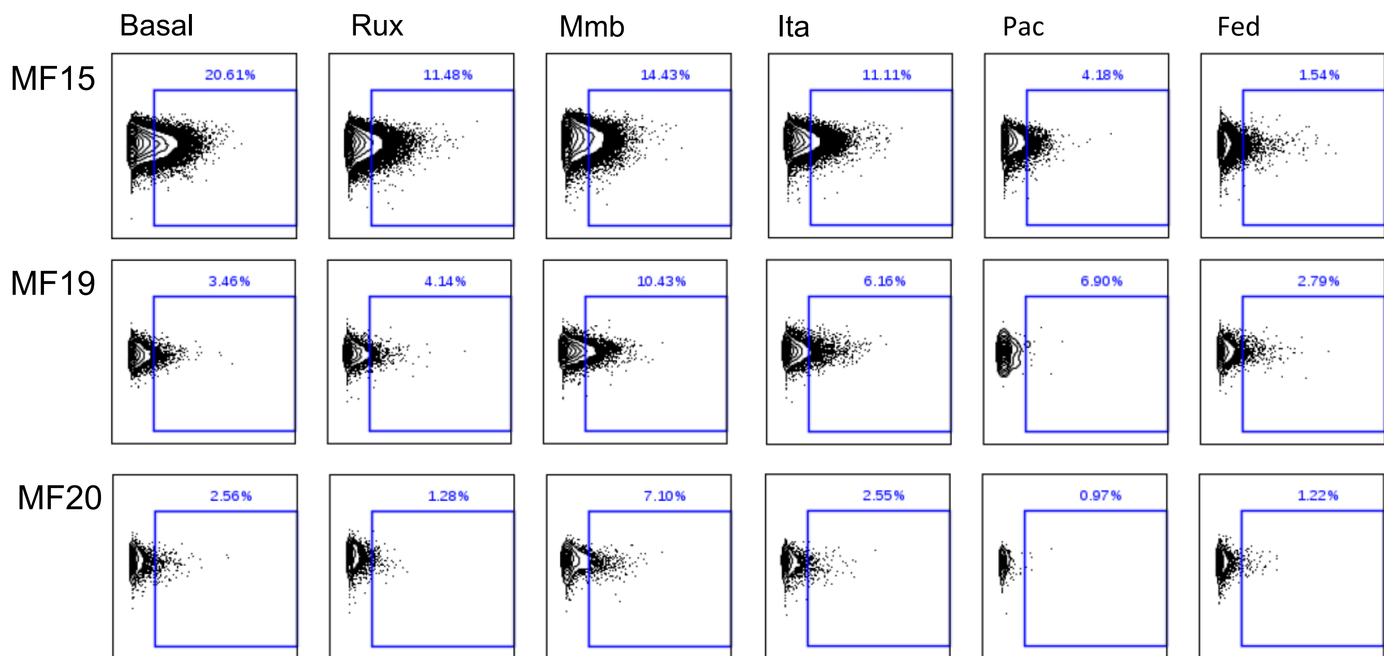
MF15 20.27% 17.89% 1.46% 20.31% 5.75% 2.34%

MF19 16.11% 17.84% 1.23% 26.02% 9.85% 2.88%

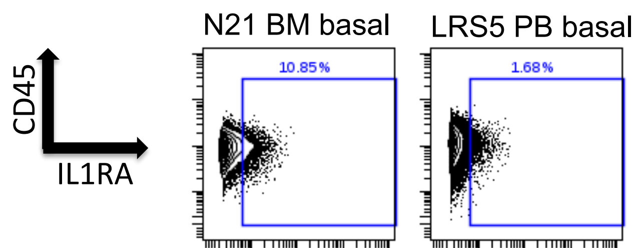
MF20 23.18% 14.43% 2.05% 17.03% 5.32% 5.35%

d





e



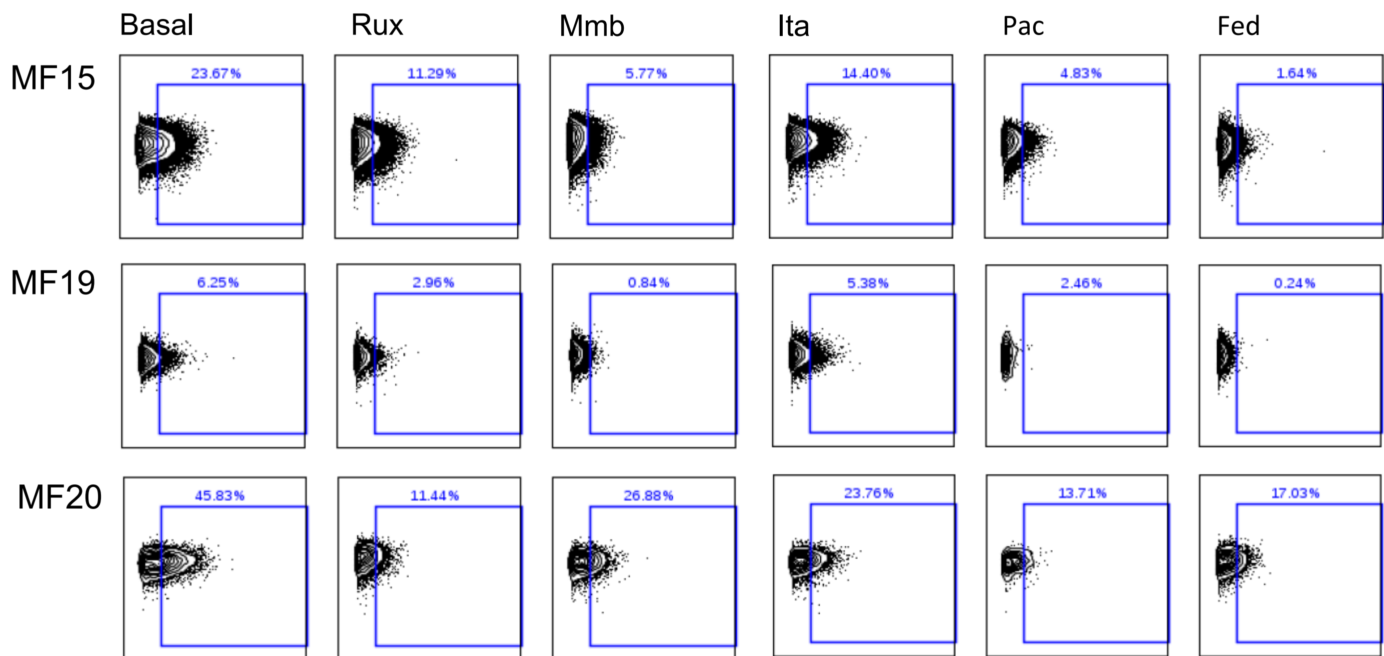


Figure S14: Effects of JAK inhibitors on basal cytokine production in MF. Intracellular cytokines were assayed by mass cytometry, following 4 hour *ex vivo* incubation with no inhibitor (basal), or 5µM concenration of ruxolitinib (Rux), momelotinib (Mmb), itacitinib (Ita), pacritinib (Pac), or fedratinib (Fed). **a**. Heat map illustrates median levels of cytokines (rows) in CD14+ monocytes from each of three MF patients: MF15 and MF20, *JAK2* V617F mutant; and MF19, *CALR* mutant. Cytokine levels after incubation with inhibitors are normalized to basal levels (leftmost column) on ArcSinh ratio scale. Therefore yellow tile color indicates elevation from basal level, and blue color indicates reduction below basal level. **b**. Heat map illustrates 90th percentile levels of cytokines, normalized to basal levels as in **a**. Magnitudes of change versus basal are demonstrated with different scale bars in **a** and **b**: 90th percentile levels show a greater range of change with JAK inhibitors than median levels, and more prominently in the direction of reduction from basal. **c-e**: Biaxial plots showing positive versus negative gating for individual cytokines in CD14+ monocytes, with cytokine on the X axis and CD45 on the Y axis. Plots are shown for normal control bone marrow and peripheral blood basal monocytes (top two plots in each of **c-e**), and for basal and inhibitor treated CD14+ monocytes from each of the three MF patients shown in **a** and **b** (rows below). **c**. Levels and positive versus negative gating for CCL4/MIP1β. **d**. Levels and positive versus negative gating for CXCL8/IL-8. **e**. Levels and positive versus negative gating for IL1RA.

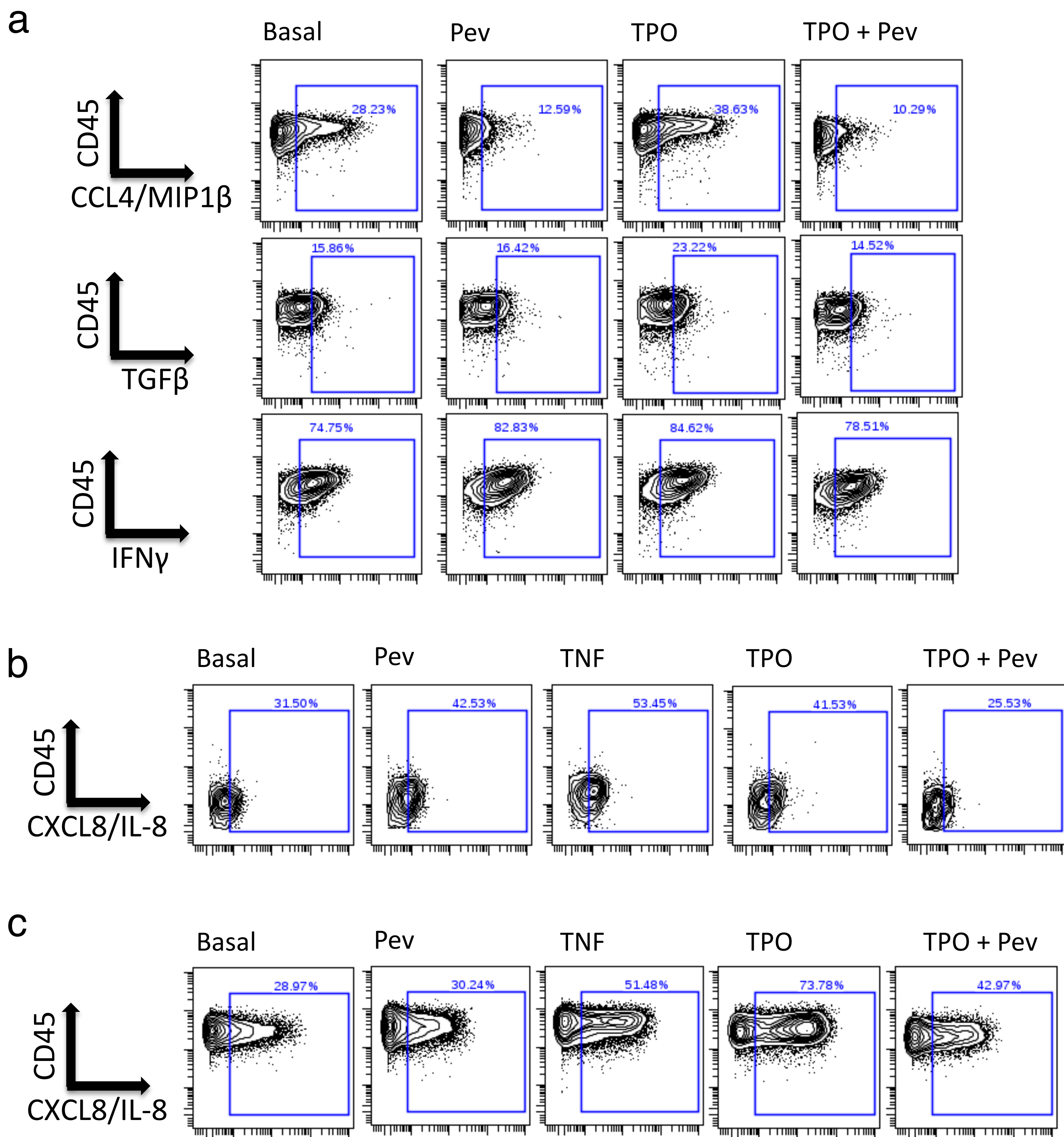


Figure S15: Differential sensitivity of MF cytokines to suppression by pevonedistat. a. Biaxial plots illustrating cytokine production by monocytes from *JAK2* V617F mutant MF patient MF20. Cytokine production is shown (left to right) basally, or in the presence of 1μM pevonedistat, 10ng/mL TPO, or TPO plus pevonedistat. Cytokines illustrated are (top to bottom) CCL4/MIP-1β, TGFβ, and IFNγ. **b**. Biaxial plots illustrate CXCL8/IL-8 production by Lin-CD34+ cells from *JAK2* V617F mutant MF patient MF15. Conditions shown are (left to right) basal unstimulated, 1μM pevonedistat, 20 ng/mL TNF, 10ng/mL TPO, and TPO plus pevonedistat. **c**. Biaxial plots illustrate CXCL8/IL-8 production by monocytes from *JAK2* V617F mutant MF patient MF15. Conditions shown are (left to right) basal unstimulated, 1μM pevonedistat, 20 ng/mL TNF, 10ng/mL TPO, and TPO plus pevonedistat.

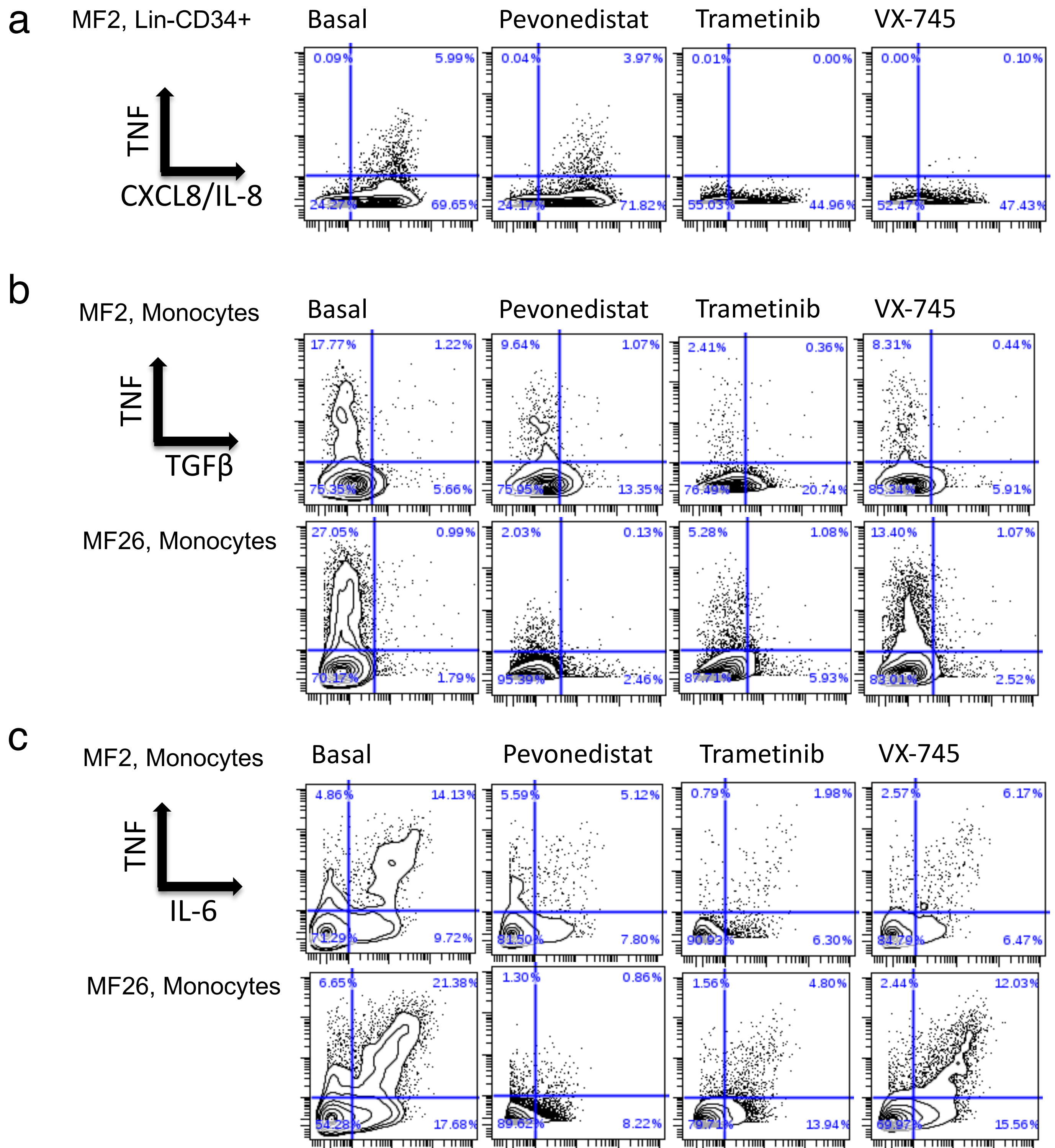
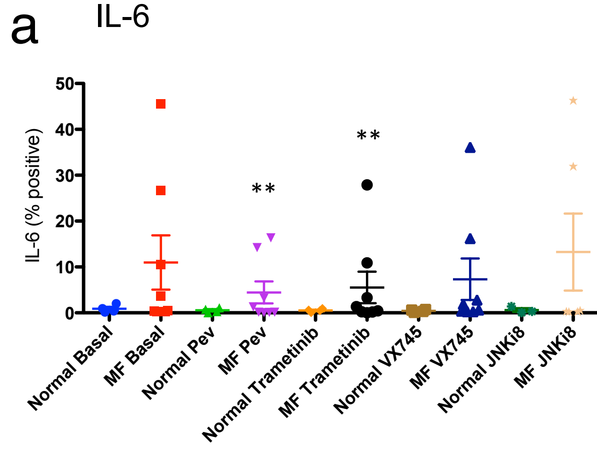
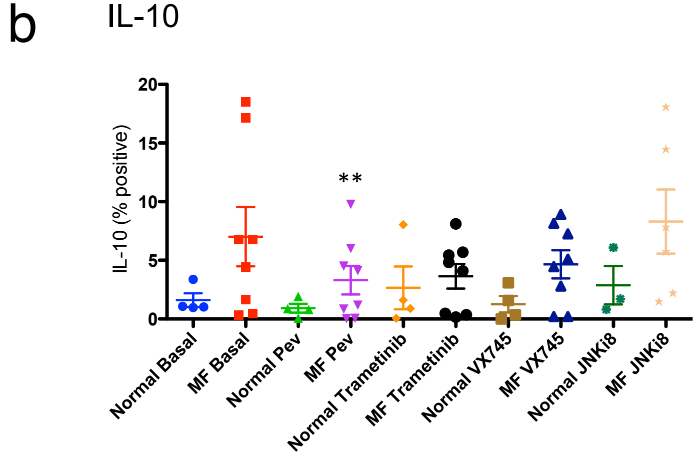
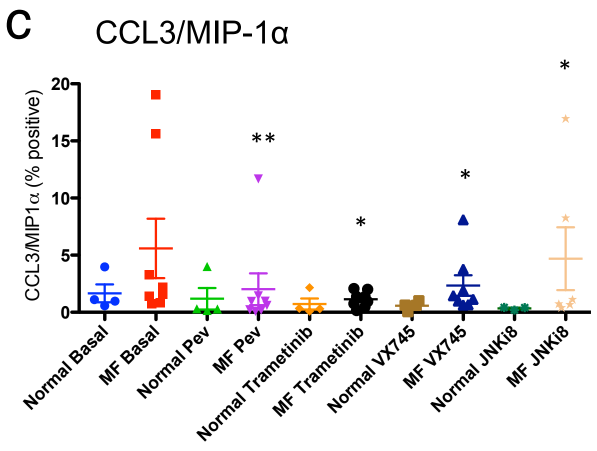


Figure S16: Inhibition of MF cytokine production by pevonedistat, trametinib, and VX-745. a. Biaxial plots illustrating cytokine production by Lin-CD34+ cells from *JAK2* V617F mutant patient MF2. TNF is shown on the Y axis and CXCL8/IL-8 is shown on the X-axis, with quadrant gate denoting percent of cells positive for either or both cytokines. Conditions shown are (left to right) basal, 1μM pevonedistat, 10μM trametinib, 1μM VX-745. **b**. Inhibition of TNF (Y axis) but not TGFβ (X axis) in monocytes from *JAK2* V617F mutant patients MF2 and MF26. Quadrant gate denotes cells identified as positive for each cytokine. Treatment conditions are identical to **a**. **c**. Inhibition of TNF (Y axis) and IL-6 (X axis) production in heavily co-expressing monocytes from *JAK2* V617F mutant patients MF2 and MF26. Quadrant gate denotes cells identified as positive for each cytokine. Treatment conditions are identical to **a** and **b**.


a
IL-6
IL-6 (% positive)
50
40
30
20
10
0
**
**
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8


b
IL-10
IL-10 (% positive)
20
15
10
5
0
**
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8


c
CCL3/MIP-1α
CCL3/MIP1α (% positive)
20
15
10
5
0
**
*
*
*
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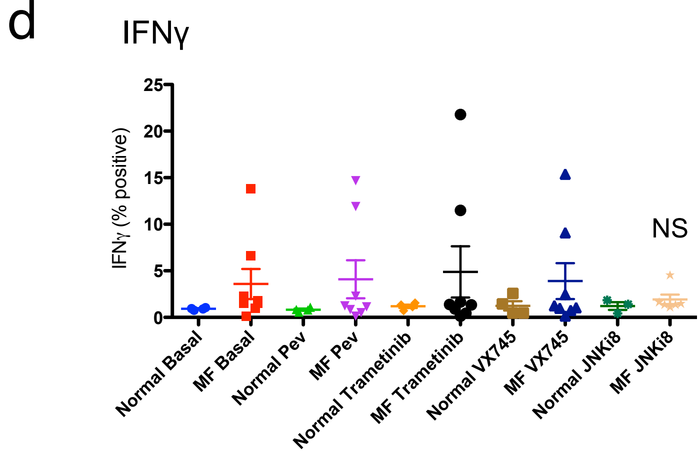
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8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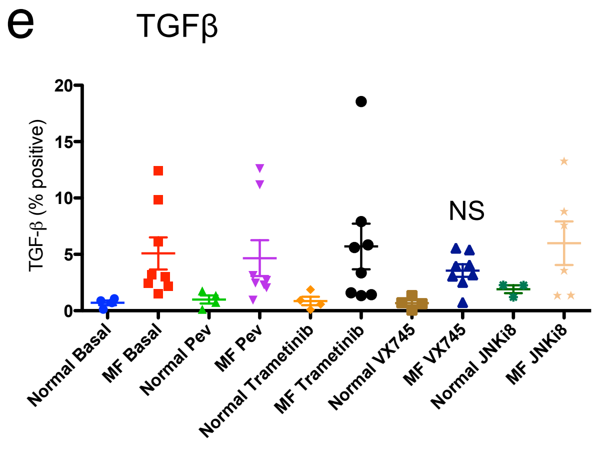
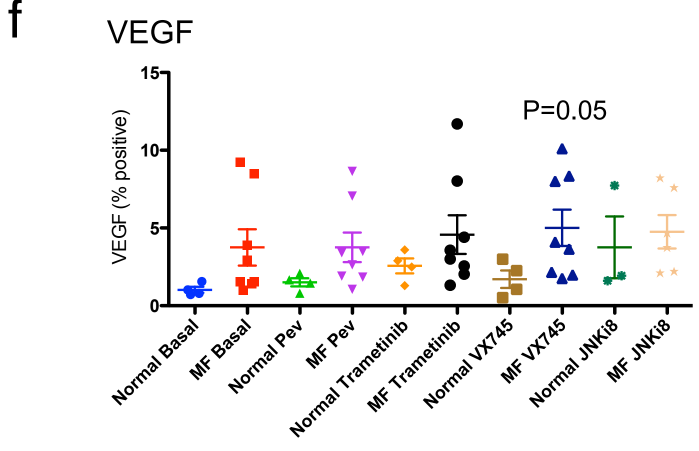
IFNγ
IFNγ (% positive)
25
20
15
10
5
0
NS
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8


e
TGFβ
TGF-β (% positive)
20
15
10
5
0
NS
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8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VEGF
VEGF (% positive)
15
10
5
0
P=0.05
Normal Basal
MF Basal
Normal Pev
MF Pev
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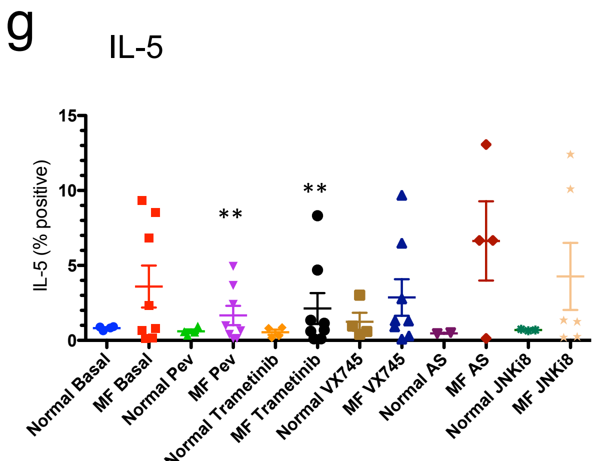
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8


g
IL-5
IL-5 (% positive)
15
10
5
0
**
**
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal AS
MF AS
Normal JNKi8
MF JNKi8

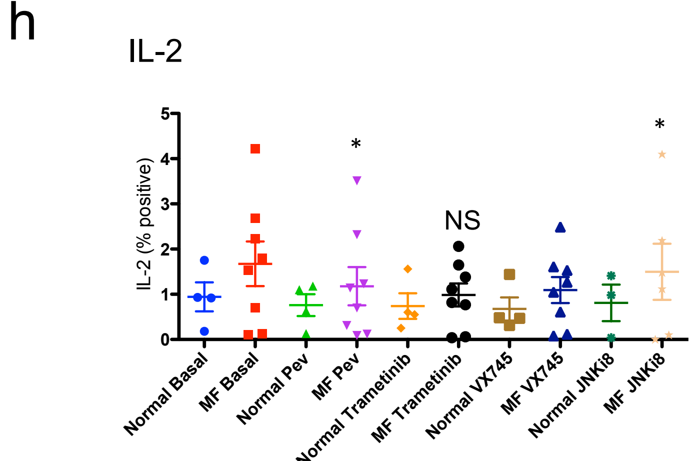

h
IL-2
IL-2 (% positive)
5
4
3
2
1
0
*
NS
*
Normal Basal
MF Basal
Normal Pev
MF Pev
Normal Trametinib
MF Trametinib
Normal VX745
MF VX745
Normal JNKi8
MF JNKi8

Figure S17: Extended analysis of MF monocyte cytokine suppression by non-JAK/STAT inhibitors. Statistical representation of suppression of basal cytokine production by signaling inhibitors in monocytes, as extension of data represented in **Figure 8d-g**. Percent of monocytes identified as expressing each cytokine shown (from biaxial plots, as in **Figure 8a** and **Figure S16**) from healthy control (N=4) or MF patient (N=8; 6 *JAK2* V617F mutant and 2 *CALR* mutant) blood samples. Blood samples were subjected to the following four-hour incubations: basal, 1μM pevonedistat, 10μM trametinib, 1μM VX-745, 1μM JNKi8. Error bars = mean +/- SEM. Statistical significance is shown between basal and inhibitor treated samples where identified by Wilcoxon sign-rank test (*, $P<0.05$; **, $P<0.01$, NS=not significant). **a.** Percent of monocytes identified as positive for IL-6, for both normal control and MF blood samples. **b**. Percent of monocytes positive for IL-10. **c**. Percent positive for CCL3/MIP-1α. **d**. Percent positive for IFNγ. **e**. Percent positive for TGF-β. **f**. Percent positive for VEGF. **g**. Percent positive for IL-5. **h**. Percent positive for IL-2. The following cytokines were measured but are not shown, as no statistically significant difference was observed between basal and any inhibitor treatment, for either MF or normal control: IL-12, IL-15, IL-21.