Supplemental Data

Supplemental methods:

Mouse genotyping:

To verify using mice with correct modified genotype, all mice were controlled by PCR analysis. Cre-recombinase (GAC CAG TTG CTC CAG GGT TA, GCA AAG GGA GAC AAG AA ACG T and TCA CAA GCA TTT GGT TTT GAA T), huTNFR1$_{ecd}$ (huTNFR1$_{ecd}$ TGT CCC ACC ACA CAC AC (for), CTG GCT GTT CCT AGC AT (rev)).

Flow cytometry:

Single cell suspensions from peripheral blood were stained for CD3 and CD19 (both BioLegend, San Diego, CA, USA). For analysis of stem cell compartments, single cell suspension of bone marrow and spleen were stained with lineage markers, c-kit, Sca1, CD16/CD32 and CD34 (all BioLegend, San Diego, CA, USA). All samples were analyzed with a FACS Canto II flow cytometer (BD Biosciences).

Clonogenic assay:

Lin- cells were isolated from total bone marrow of huTNFR1$_{ecd}$ x JAK2$^{+/VF}$ mice by negative selection using MACS beads (Milteny Biotec, Bergisch Gladbach, Germany) and FACS-sorted for Lin-ckit+ cells. Cells were cultured for three days in StemSpanSFEM medium containing mSCF (100ng/mL) and mIL-3 (100ng/mL) and

additional stimulation with αTNFR1 antibody H398 or αTNFR2 antibody (each 10 µg/mL). After this pre-incubation period, 5,000 cells were seeded in MethoCult™ GF M3434 containing mSCF (100ng/mL) and mIL-3 (100ng/mL) and αTNFR1 or αTNFR2 antibody (10 µg/mL). Colony formation was analyzed seven days after start of treatment. Non-anti-TNFR1/2 treated cells were used as control.

Hepcidin ELISA:

Hepcidin concentrations in serum of $JAK2^{+/VF}$ and $JAK2^{WT}$ mice were determined by applying Hepcidin Murine-Compete™ ELISA Kit (Intrinsic LifeSciences, La Jolla, CA, USA). Assay was performed according to the manufacturer's instructions.

Granulocyte migration

Granulocytes were isolated by Ficoll-paque density gradient centrifugation from peripheral blood of JAK2-V617F-positive MPN patients or healthy controls. After isolation, cells were rested for 60 min in starvation medium (0.5% fetal bovine serum) and incubated with and without αTNFR1 H398 or αTNFR2 antibody (Monoclonal Mouse IgG_1 Clone # 22210; R&D Systems, Inc, Minneapolis, MN, USA) (both c = 10mg/mL). Migration was performed along CXCL12 (c = 100 ng/mL), TNFα (c = 10 ng/mL), CXCL12+TNFα (combi), combi + αTNFR1 antibody, combi + αTNFR2 antibody gradients for 3 hours (pore size: 3µm). After migration, cells were labeled with calcein AM (2 µM) at 37°C. Fluorescence of the samples was monitored using a Synergy HT plate reader (BioTek).

TNFR1/2 pathway inhibition

Hel cells or isolated splenocytes from $JAK2^{+/VF}$ mice were incubated with different concentrations of αTNFR1 antibody H398 (Hel cells) or αTNFR2 antibody TR75-54.7 (splenocytes) for 3 hours in serum-reduced medium (RPMI+0.5%FCS) at 37°C and 5%CO_2. After incubation, cells were stimulated with 50 ng/mL TNFα for 10 min at 37°C and 5%CO_2. Reaction was stopped by adding ice-cold PBS. After centrifugation cells were lysed and phosphorylation of p38 and p65 analyzed by immunoblotting.

Immunoblotting

Isolated protein of Hel cells (30µg) or splenocytes (50µg) were separated by SDS-PAGE and transferred tonitrocellulose membranes. Membranes were blocked with 5%BSA in TTBS at RT and incubated with primary antibody overnight at 4°C. After analysis of phospho-protein signals, membranes were stripped and incubated again with antibodies for total protein and GAPDH as loading control. Blots were scanned with a chemoluminescenceimager (Peqlab) and inversed with ImageJ 1.52a software.

Table: Immunoblotting antibodies

Antibody	Supplier / Catalog number	Dilution
Phospho-p38 (Tyr180/182)	Cell Signaling Technology, Danvers, MA, USA; 4631	1/1000
p38	Cell Signaling Technology; 9212	1/1000
Phospho-p65 (Ser536)	Cell Signaling Technology; 3033	1/1000
p65	Cell Signaling Technology; 8242	1/500
GAPDH	Meridian Bioscience, Cincinnati, OH, USA; H86504M	1/5000
Anti-rabbit IgG, HRP-linked	Cell Signaling Technology; 7074	1/2000
Anti-mouse-IgGκ BP-HRP	Santa Cruz Biotechnology, Dallas, TX, USA; sc-516102	1/2000

Table S1. Serum concentrations of cytokines/chemokines in JAK2$^{+/VF}$ mice upon αTNFR2 treatment

Mean cytokine concentrations of serum samples (pg/mL) measured by Eve Technologies Corporation (Mouse Cytokine Array/Chemokine Array 31-Plex; Calgary, AB, Canada), standard error of mean and fold change of mean cytokine concentrations in JAK2$^{+/VF}$ versus JAK2^{WT}, JAK2$^{+/VF}$ versus αTNFR2-treated JAK2$^{+/VF}$, and JAK2$^{+/VF}$ versus IgG treated JAK2$^{+/VF}$ mice. Untreated JAK2^{WT} mice (n = 4-5), untreated JAK2$^{+/VF}$ mice (n = 4-10), αTNFR2 antibody treated JAK2$^{+/VF}$ mice (n = 3) and IgG treated JAK2$^{+/VF}$ mice (n = 3). Figures indicated by Eve Technologies to be out of standard range and interpolated values were excluded. A minimum of n = 3 measurements of cytokine concentrations to calculate the mean was required. n.e.: not evaluable.

	$JAK2^{WT}$		$JAK2^{+/VF}$		$JAK2^{+/VF}$ vs $JAK2^{WT}$	αTNFR2-treated		$JAK2^{+/VF}$ vs αTNFR2-treated	IgG-treated		$JAK2^{+/VF}$ vs IgG-treated
Cytokines	Mean [pg/mL]	Std error of mean [pg/mL]	Mean [pg/mL]	Std error of mean [pg/mL]	Fold change	Mean [pg/mL]	Std error of mean [pg/mL]	Fold change	Mean [pg/mL]	Std error of mean [pg/mL]	Fold change
CCL2	41.47	5.85	99.94	22.19	2.41	48.37	6.95	0.48	71.67	8.70	0.72
CCL3	110.79	12.00	122.94	14.79	1.11	153.52	27.49	1.25	167.95	25.53	1.37
CCL5	27.33	3.07	44.27	5.77	1.62	133.28	92.33	3.01	97.86	52.10	2.21
CCL11	1105.20	133.02	3303.04	1034.51	2.99	1488.52	642.91	0.45	n.e	n.e.	
CXCL1	673.16	208.88	1176.29	332.81	1.75	972.42	277.88	0.83	1201.76	77.78	1.02
CXCL2	140.92	32.67	173.37	16.84	1.23	1826.04	642.25	10.53	118.50	20.96	0.68
CXCL5	2860.92	1845.00	10853.52	1377.54	3.79	4048.80	667.15	0.37	11199.59	3789.51	1.03
CXCL9	138.75	74.46	545.04	219.70	3.93	163.83	40.47	0.30	447.84	88.06	0.82
CXCL10	110.68	8.59	299.25	24.16	2.70	222.24	31.16	0.74	365.42	32.95	1.22
G-CSF	594.46	138.32	836.14	208.19	1.41	626.33	169.60	0.75	1070.28	411.73	1.28
IFNγ	2.82	0.44	2.65	1.06	0.94	9.49	2.24	3.59	7.38	2.44	2.79
IL-1α	139.32	26.01	675.27	165.04	4.85	1234.85	663.88	1.83	773.48	269.12	1.15
IL-2	24.52	6.81	16.65	1.07	0.68	28.79	8.97	1.73	53.27	7.98	3.20
IL-5	33.90	9.47	14.06	3.61	0.41	9.82	0.65	0.70	73.83	35.64	5.25
IL-6	18.68	8.92	121.92	102.25	6.53	75.56	55.30	0.62	30.11	9.45	0.25
IL-7	6.07	1.32	6.16	0.92	1.02	7.65	2.68	1.24	12.48	0.59	2.03
IL-9	24.34	0.59	24.53	5.02	1.01	19.00	3.15	0.77	40.88	4.86	1.67
IL-12 (p40)	14.65	3.22	25.20	8.06	1.72	752.58	466.36	29.87	36.29	4.36	1.44
IL-13	46.21	6.99	58.57	4.59	1.27	59.67	3.05	1.02	91.77	6.88	1.57
M-CSF	17.03	5.28	15.63	5.04	0.92	726.92	375.54	46.51	49.63	0.00	3.18

Table S2. Clinical characteristics of JAK2-V617F-positive MPN patients

UPN	Sex	Age	Diagnosis	Treatment
P1	m	55	PV	HU + Anticoagulant
P2	m	48	PV	ASA + Phlebotomy
P3	f	47	ET	ASA
P4	f	49	PV	HU + Phlebotomy
P5	f	61	ET	HU
P6	f	80	ET	HU

UPN Unique Patient Number
HU Hydroxyurea
ASA Acetylsalicylic acid

Supplemental Figures

Figure S1.: Serum concentration of hepcidin

(A) Hepcidin ELISA using serum of $JAK2^{WT}$ mice (n = 5), $JAK2^{+/VF}$ mice (n = 6), αTNFR2 treated $JAK2^{+/VF}$ mice (n = 3) and IgG treated $JAK2^{+/VF}$ mice (n = 3)

(B) Hepcidin ELISA using serum of $huTNFR1_{ecd}$ x $JAK2^{WT}$ mice, $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice, αTNFR1 treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice and IgG treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice (n = 3 in each group)

Figure S2. MCV and RBC in $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice upon αTNFR1 treatment and in $JAK2^{+/VF}$ mice upon αTNFR2 treatment

(A) MCV analysis of untreated $JAK2^{+/VF}$ (n = 15), untreated $JAK2^{WT}$ (n = 14), αTNFR2 antibody treated $JAK2^{+/VF}$ (n = 5) and IgG treated $JAK2^{+/VF}$ (n = 4).

(B) RBC analysis of untreated $JAK2^{+/VF}$ (n = 15), untreated $JAK2^{WT}$ (n = 14), αTNFR2 antibody treated $JAK2^{+/VF}$ (n = 5) and IgG treated $JAK2^{+/VF}$ (n = 4).

(C) MCV analysis of untreated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 10), untreated $huTNFR1_{ecd}$ x $JAK2^{WT}$ (n = 8), αTNFR1 antibody treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 8) and IgG treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 9).

(D) RBC analysis of untreated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 10), untreated $huTNFR1_{ecd}$ x $JAK2^{WT}$ (n = 8), αTNFR1 antibody treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 8) and IgG treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 9).

Statistical significance is given as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

The MCV of both groups similarly declined (αTNFR1: mean 36.0 to mean 32.7 fl, IgG: mean 36.2 to mean 33.0 fl) to comparable levels of 12 weeks old untreated $JAK2^{+/VF}$ mice (mean 31.3 fl) (Suppl. Figure 2C). RBC increased during treatment in both groups, but this effect was much stronger and homogeneous in IgG treated mice (IgG: 16.9 – 19.9×10^{12} cells/L; p = 0.0012; αTNFR1: 17.3 – 18.0×10^{12} cells/L; p = 0.4418). Nevertheless, there was no significant difference between the means at the end of treatment (p = 0.1589) (Suppl. Figure 2D).

Figure S3. CFU-GM and BFU colonies; CD3 and CD19 positive cells.

(A-B) Colony forming unit assays of untreated and αTNFR1/αTNFR2 treated Lin-ckit+ bone marrow cells of $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice. Numbers of CFU-GM and BFU were counted and depicted (n = 3).

(C-D) Total T- and B-cell numbers in peripheral blood of IgG (n = 4) and αTNFR2 antibody treated mice (n = 4).

(E-F) Total T- and B-cell numbers in peripheral blood of IgG (n = 5) and αTNFR1 antibody treated mice (n = 5).

Figure S4. Stem and progenitor cell distribution in spleen of in $JAK2^{+/VF}$ mice upon αTNFR2 treatment and $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ mice upon αTNFR1 treatment.

Presented cell numbers of compartments investigated were normalized to 10^6 live cells.

(A) Analysis of spleen derived stem and progenitor cells from untreated $JAK2^{WT}$ (n = 4), untreated $JAK2^{+/VF}$ (n = 5), αTNFR2 antibody treated $JAK2^{+/VF}$ (n = 3) and IgG treated $JAK2^{+/VF}$ (n = 3) mice.

(B) Analysis of spleen derived stem and progenitor cells from untreated $huTNFR1_{ecd}$ x $JAK2^{WT}$ (n = 3), untreated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 2), αTNFR1 antibody treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 3) and IgG treated $huTNFR1_{ecd}$ x $JAK2^{+/VF}$ (n = 3) mice.

(C) Schematic representation of gating schemes using representative dot plots.

Figure S5. Human Granulocyte migration along CXCL12/TNFα gradient upon αTNFR1 or αTNFR2 inhibition

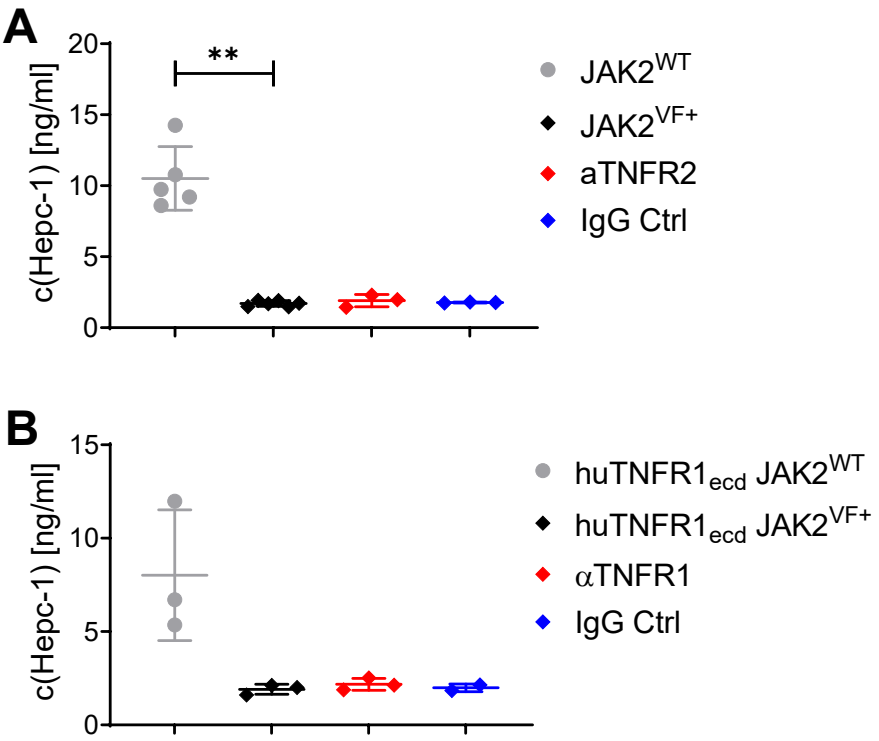
Depicted are number of migrated cells after 3 hours of migration: Light grey: healthy controls: no chemoattractant (n = 6); CXCL12 only (n = 6); TNFα only (n = 6); CXCL12 + TNFα (n = 6); CXCL12 + TNFα + α TNFR1 Ab (n = 6); CXCL12 + TNFα + αTNFR2 Ab (n = 5). Dark grey JAK2-V617F patients: no chemoattractant (n = 6); CXCL12 only (n = 6); TNFα only (n = 6); CXCL12 + TNFα (n = 6); CXCL12 + TNFα + α TNFR1 Ab (n = 6); CXCL12 + TNFα + αTNFR2 Ab (n = 5).

Figure S6. Intracellular pathway inhibition upon treatment using TNFR2 or TNFR1 antibodies in Hel cells and murine splenocytes upon TNFα stimulation

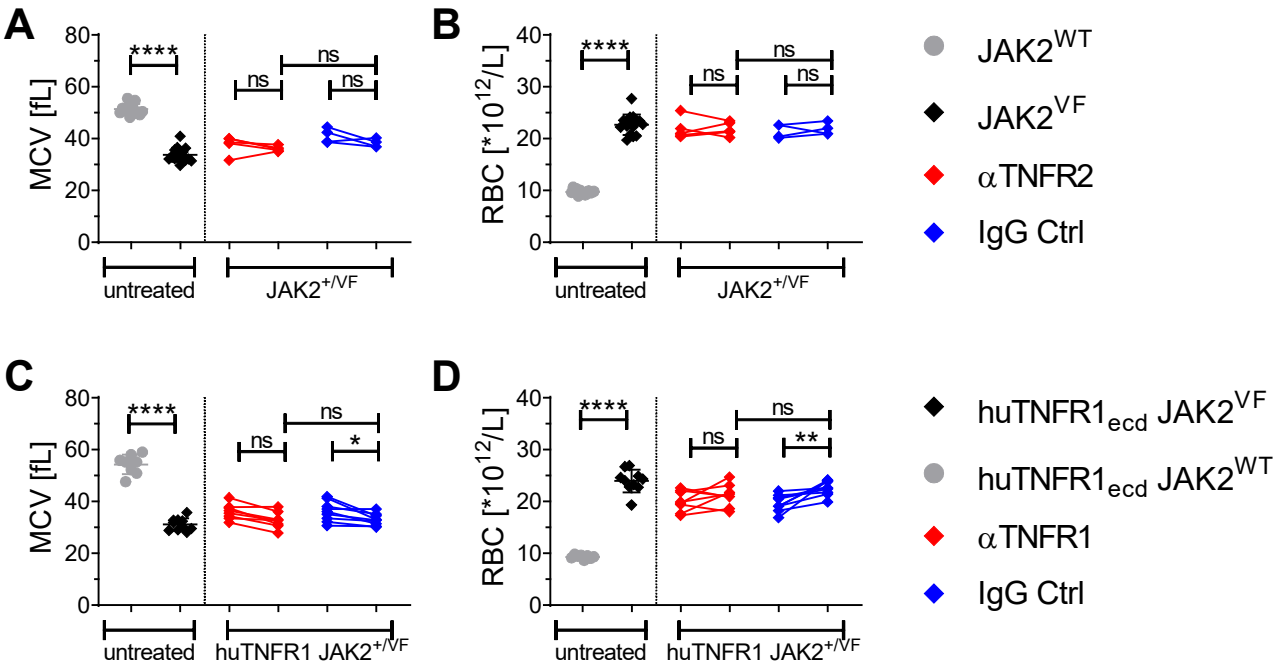
(A) Immunoblot of isolated splenocytes from $JAK2^{+VF}$ mice depicting phosphorylation of p38 and of p65 (NFκB) after αTNFR2 incubation and TNFα stimulation.

(B) Immunoblot of Hel cells depicting phosphorylation of p38 and of p65 (NFκB) after αTNFR1 incubation and TNFα stimulation.

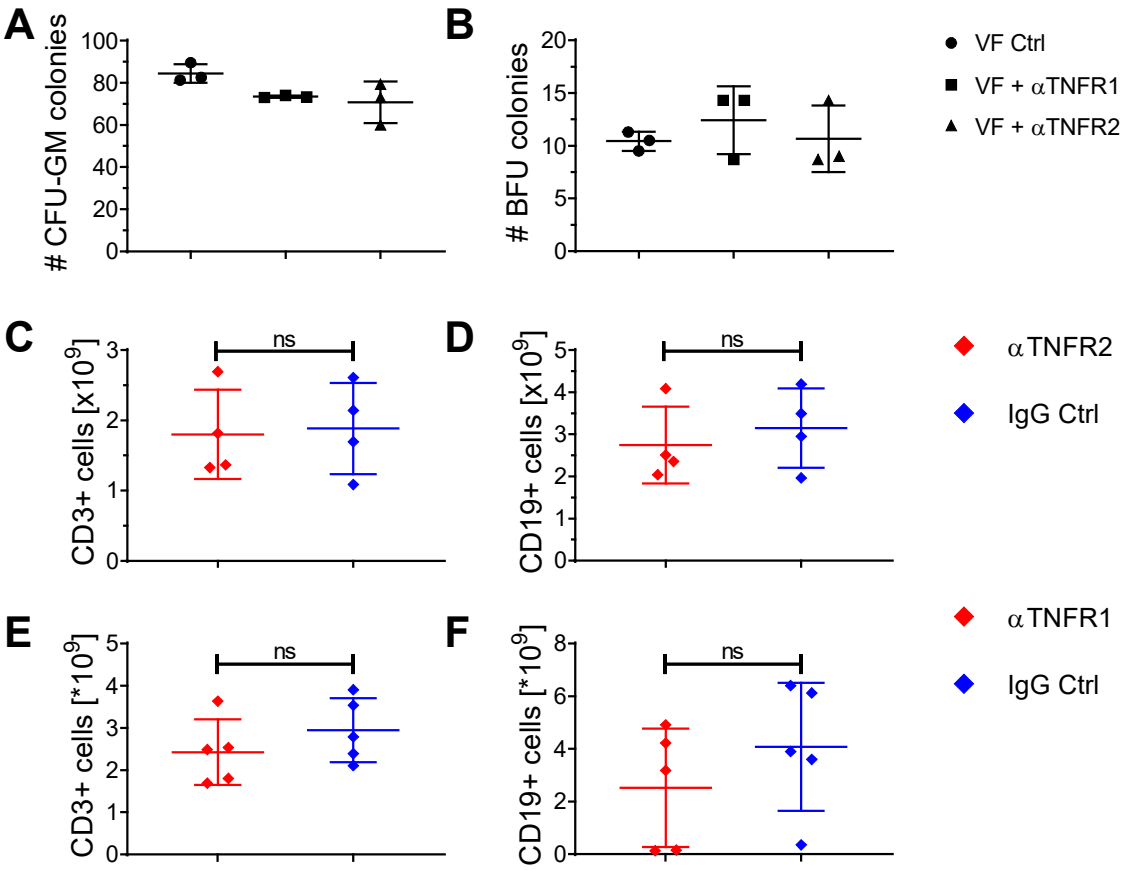
Suppl. Figure 1



Suppl. Figure 2



Suppl. Figure 3



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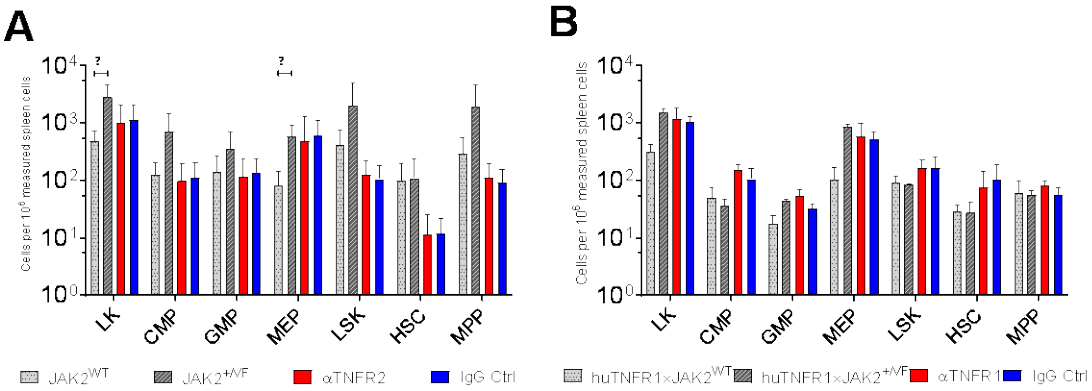
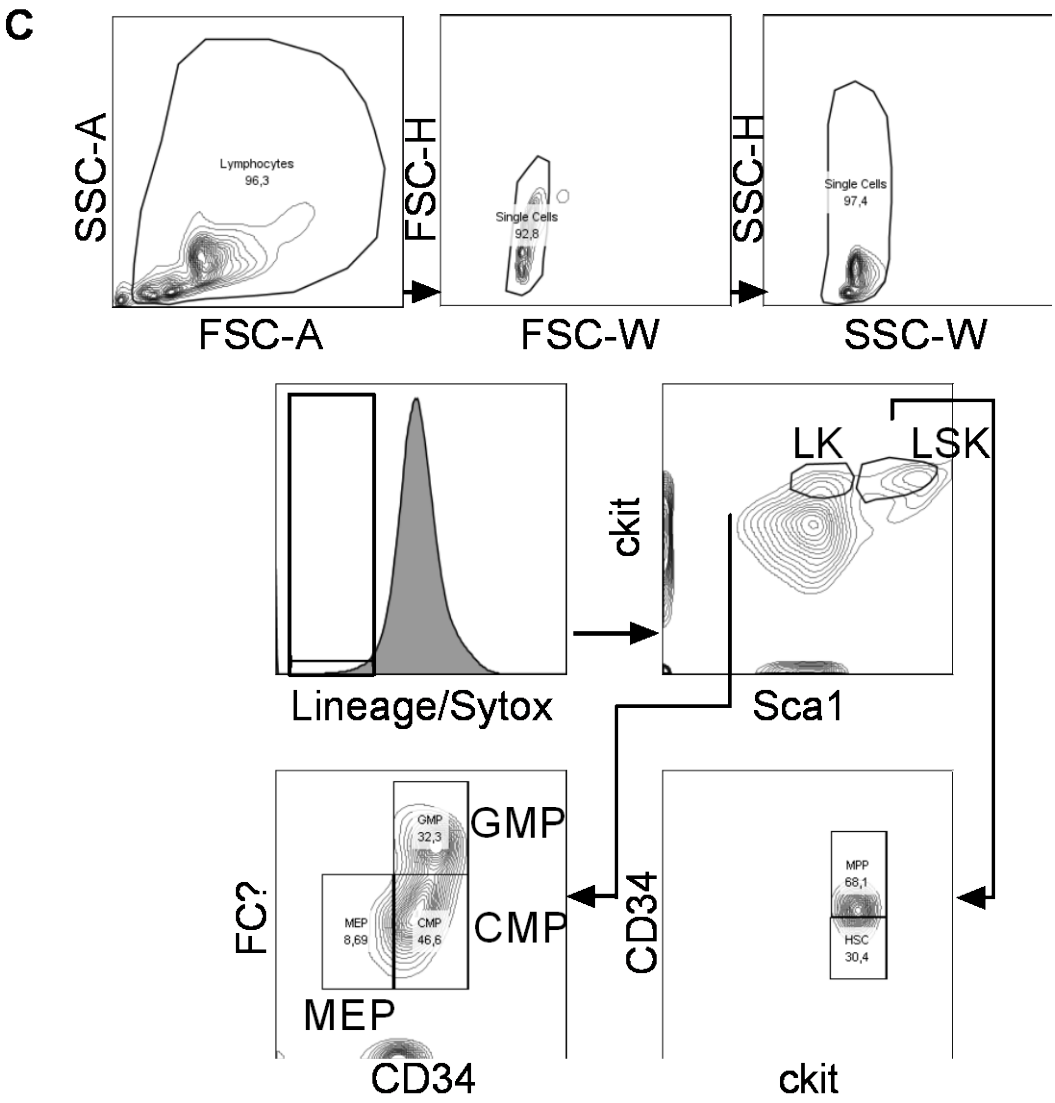
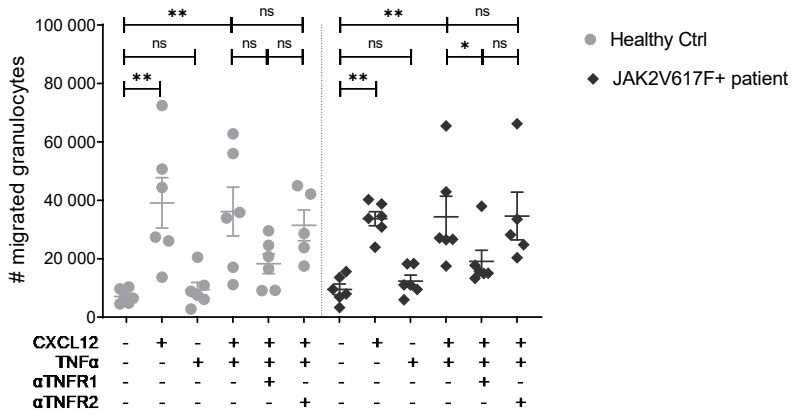




Suppl. Figure 5



Suppl. Figure 6