Gain of function in Jak2\textsuperscript{V617F} positive T-cells

Running Title: Functional activation of T-cells by Jak2\textsuperscript{V617F}

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Conflict of interest:
The authors declare no conflict of interest.
Myeloproliferative neoplasms (MPN) are clonal disorders of aging hematopoietic stem cells and early myeloid progenitors. A somatic activating point mutation in the Jak2 tyrosine kinase (Jak2\(^{V617F}\)) is the most prevalent genetic aberration in BCR-ABL-negative MPNs. JAKs are essential for cytokine-induced intracellular signaling and their inactivation leads to impaired immune cell function. Therapeutic inhibition of Janus kinases in MPN-patients causes decreased number and function of immune cells \(^ {1,2}\) and therefore may contribute to increased incidence and re-activation of viral infections \(^ {3}\). Jak2\(^{V617F}\) mutation is detectable in hematopoietic stem and progenitor cells of MPN patients and also been described in lymphoid progenitors and more differentiated lymphocytes \(^ {4,5,6,7}\), including Jak2\(^{V617F}\) mutated T-cells \(^ {4}\). However, depending on the allelic burden, incidence and clone size the number of Jak2\(^{V617F}\) mutated T-cells may have been underestimated and it is currently unclear, whether both CD4\(^{+}\) and CD8\(^{+}\) T lymphocytes may be equally affected by the Jak2\(^{V617F}\) mutation.

Therefore, we aimed to assess for the frequency of Jak2\(^{V617F}\) mutation in CD3\(^{+}\) T lymphocytes of patients with high allelic burden MPN and investigated the impact of Jak2\(^{V617F}\) mutation on T-cell function \textit{in vivo}. A model with T cell-specific Jak2\(^{V617F}\) expression was analyzed under physiological conditions and upon infection with an intracellular pathogen.

In order to address the frequency of JAK2\(^{V617F}\) mutations in human T-cells, we selected 13 MPN patients from our institutional database diagnosed with Jak2\(^{V617F}\)-positive PV, ET or myelofibrosis that exhibited an allelic burden of more than 50% in peripheral blood granulocytes. CD3\(^{+}\) T-cells and granulocytes were FACS-sorted and analyzed for quantitative expression of Jak2\(^{V617F}\) (Figure 1a). 6/13 patients tested positive for Jak2\(^{V617F}\) in sorted CD3\(^{+}\) cells, with an allelic burden between 2% and 47.8% within the T-cell compartment (Figure 1a). When analyzing sorted CD3/CD4 and CD3/CD8 T-cells of three additional patients with a high allelic burden separately, Jak2\(^{V617F}\) burden was comparable in both T cell subsets (Figure 1b). These data suggest that JAK2\(^{V617F}\) mutations may be more frequent in patients with high allelic burden, especially in patients diagnosed with PV.
The presence of JAK2 V617F in both, CD4+ and CD8+ T-cells is consistent with a genetic event that arises at the stem- and progenitor-cell level. To explore the T cell-specific function of Jak2 V617F mutated clones in vivo, we crossed C57BL/6 Jak2 V617F [8] with CD4-Cre mice. The resulting CD4-Cre Jak2 V617F mice (henceforth designated as Jak2 V617F+) exhibited heterozygous expression of Jak2 V617F in CD4+ and CD8+ T-cells but not in the myeloid lineage (e.g. granulocytes, Figure 1c). Those animals were compared to wildtype and Cre-negative littermate controls (henceforth designated as Jak2+/+).

Activation of Jak2 V617F in T-cells could potentially lead to alteration of thymic development and composition of T-cell subsets. However, T-cell development in the thymus (Supplementary Figure 1a) of Jak2 V617F+ animals remained unchanged and comparable to Jak2+/+ littermate controls. Moreover, numbers of splenic naïve (CD62L+ CD44-), memory-like (CD62L+ CD44+) and effector (CD62L- CD44+) CD4+ and CD8+ T-cells (Supplementary Figure 1b), numbers of Foxp3+ CD25+ CD4+ regulatory T-cells (Supplementary Figure 1c), and expression of the T-cell activation marker CD69 (Supplementary Figure 1d) were equal in both strains of mice. Importantly, Jak2 V617F+ mice did not develop any clinical signs or symptoms that could be attributed to immunedefects or dysregulation of autoimmunity.

Activated T-cells play an important role in protecting the organism from infections with intracellular pathogens, such as intracellular bacteria and viruses. To investigate the function of Jak2 V617F T-cell clones in a relevant model, we analyzed T-cell responses upon infection with Listeria monocytogenes (Lm), a Gram-positive facultative intracellular bacterium (Figure 1d). In animals and humans, Lm causes severe brain, intestine, liver and bloodstream infections. While T-cell function is crucial for the elimination of Lm, experimental studies in mice revealed that innate immune cells (dendritic cells, macrophages, inflammatory monocytes and natural killer (NK) cells) additionally contribute to the control of Lm in liver and spleen. Both, CD4+ and CD8+ T-cells are activated in listeriosis and, in particular, CD8+ T-cells, which peak at day 7 post infection (p.i.), are pivotal for final elimination of the pathogen around day 14 p.i. The cytokines, TNF, G-CSF, GM-
CSF and IFN-γ are critically important for control and survival of listeriosis. In our model, infection with Lm resulted in an increase of CD4+ and CD8+ T-cells in spleens of Jak2+/VF and Jak2+/+ control mice, which was slightly pronounced in Jak2+/VF mice (Supplementary Figure 2a).

Importantly, Lm-specific T-cell responses were greatly augmented in Jak2+/VF mice (Figure 2a). Upon infection with Lm OVA, which express a strong CD8 (OVA257-264, SIINFEKL) H-2Kb-restricted T-cell epitope, the number of SIINFEKL-specific CD8+ T-cells was significantly increased in Jak2+/VF mice (Figure 2a). In addition, numbers of Lm OVA-specific CD8+ T-cells and listeriolysin201-specific CD4+ T-cells producing interferon-γ, TNF, G-CSF and GM-CSF were significantly increased in Jak2+/VF mice (Figure 2a). Of note, these findings resulted in functional improvement of pathogen elimination: compared to Jak2+/+ controls, numbers of Lm were significantly decreased in spleen (Figure 2b) and liver (data not shown) of Jak2+/VF mice. Despite the augmented T-cell response in Lm-infected Jak2+/VF mice, the expression of activation markers including CD11a (LFA-1) were comparable between CD4+ and CD8+ T-cells in Lm-infected mice of both genotypes.

Consistent with elevated G-CSF and GM-CSF production, listeriosis induced a significantly stronger increase of neutrophils in spleen, blood, liver and lung of Jak2+/VF mice (Figure 2c). In mice, neutrophils only marginally contribute to the control of Lm. Therefore, the generalized increase of neutrophils does not necessarily contribute to an improved pathogen control but may rather be the consequence of increased paracrine cytokine stimulation. Abundance of all other major leukocyte populations was comparable to non-infected and infected Jak2+/VF mice and Jak2+/+ littermate controls in spleen and blood (Supplementary Figure 2b).

Jak2V617F promotes chronic inflammation through induction of pro-inflammatory cytokines which contribute to constitutional symptoms in MPN patients. At present it is unclear whether an increased activation of Jak2 in affected lymphocytes may add to disease progression and inflammation in MPN. Production and secretion of cytokines by Jak2V617F positive cells may impact expansion of the erythroid lineage as well as proliferation of malignant and non-malignant progenitors. To investigate potential paracrine effects of Jak2+/VF T-cell mediated cytokine
secretion, we assessed for red blood cell numbers in the spleen. Lm infection increased erythroid progenitor numbers in both strains compared to non-infected controls. This increase, however, appeared 7-fold higher in Jak2^{+/VF} mice compared to Jak2^{+/+} littermate controls (Figure 2d, Supplementary Figure 2c). Consistently, serum erythropoietin concentration was suppressed in Jak2^{+/VF} mice following infection on day 7 (Figure 2e).

To further analyze the effect of T-cell-specific Jak2^{V617F} in a pathogen-independent context, we stimulated isolated naïve CD4\(^+\) and CD8\(^+\) T-cells with anti-CD3/CD28 in vitro. As illustrated in Supplementary Figures 3a and b, both IFN-γ and TNF production and proliferation of Jak2^{+/VF} CD4\(^+\) and CD8\(^+\) T-cells were significantly increased. In addition, we treated mice with the plant lectin Concanavalin A (ConA), which activates T-cells via crosslinking of the CD3/T-cell receptor complex and other immune cells by binding to receptors containing mannose carbohydrates. ConA stimulation resulted in a significantly stronger increase of Jak2^{+/VF} CD4\(^+\) and CD8\(^+\) T-cells (Supplementary Figure 4a), increased production of IFN-γ, TNF, and GM-CSF by Jak2^{+/VF} CD4\(^+\) and CD8\(^+\) T-cells (Supplementary Figure 4b), and augmented incorporation of bromodeoxyuridine in proliferating Jak2^{+/VF} CD4\(^+\) and CD8\(^+\) T-cells (Supplementary Figure 4c). Functionally important, ConA stimulation also caused a significantly stronger increase of neutrophils and erythroblasts in Jak2^{+/VF} mice (Supplementary Figures 4d and e).

As outlined above, Jak2^{+/VF} mice did not develop any clinical signs of an inflammatory syndrome in the absence of an infectious stimulus. Both uninfected Jak2^{+/VF} mice and Jak2^{+/+} littermate controls revealed minor numbers of TNF, GM-CSF, and IFN-γ producing CD4\(^+\) and CD8\(^+\) T-cells (Fig. 2C), low levels of EPO in serum (Fig. 2J) and normal histology of liver, spleen and lung (not shown). Thus, activating stimuli are probably required to induce the augmented inflammatory phenotype in Jak2^{V617F} mutated CD4\(^+\) and CD8\(^+\) T-cells.

In conclusion, almost 50% (9/16) of patients with high allelic burden Jak2^{V617} MPN that were investigated in our study had in addition to granulocytes Jak2^{V617F} mutated T-cell clones and the mutation affected both CD4\(^+\) and CD8\(^+\) T-cells. Due to limited number of patients in this study and
a high variability of therapeutic approaches (including hydroxyurea, JAK-inhibitor treatment and irradiation of the spleen), significant differences in clinical responses or disease phenotype were not detectable between patients with and without Jak2^{V617F} mutated T-cells. T-cell-specific expression of Jak2^{V617F} in mice did not only result in improved pathogen-specific CD4^+ and CD8^+ T-cell responses and clearance of Listeria infection but additionally increased neutrophil numbers and extramedullary erythropoiesis, two hallmarks in patients with MPN. Therefore, it is tempting to speculate on activation of T-cell clones by Jak2^{V617F} that may - to a minor extent - contribute to the myeloproliferative phenotype through paracrine stimulation of myelopoiesis. Future prospective clinical trials will need to clarify, to which extent Jak2^{V617F} positive T-cell clones may enable improved control of specific infections while contributing to the chronic inflammatory phenotype upon challenge by pathogens and environmental antigens.
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Authorship Contributions: GN, CF, DW, TS, FP, and SJ conducted experiments and analyzed data. AM, TF and FH provided experimental techniques and material. FH and DS analyzed data and wrote the manuscript.

References:


Figure Legends:

Figure 1. Presence of Jak2^{V617F} in T-cells of MPN patients and in mice with T cell-specific Jak2^{V617F} activation.

(a, b) Written informed consent was obtained from all patients participating in this study, approved by the institutional review board and conducted in accordance with the Declaration of Helsinki. Samples have been stored in the ‘Hematology Tumor Bank Magdeburg’ after approval by the respective local ethics committee (Protocol No. 08/115). (a) Top: Sorting strategy for CD3-positive T-cells and for CD13-positive granulocytes. Bottom: Allelic burden (% of Jak2 wildtype allele) in T cell and granulocyte fractions of the respective patients, investigated by qPCR. (b) Top: Sorting strategy for CD3/CD4- and CD3/CD8-positive T-cells. Bottom: Allelic burden in sorted CD3/CD4 and CD3/CD8 positive T-cell fractions as determined by qPCR. (c) Expression of Jak2^{V617F} in MACS-isolated CD4^+, CD8^+ T and granulocytes, respectively, was analyzed by PCR according to previously published protocols. (d) Experimental design of *Listeria* infection: Jak2^{+/VF} and Jak2^{+/+} mice were infected with Lm and were analyzed at day 7 p.i.

Figure 2. Improved control of *Listeria monocytogenes* by Jak2^{+/VF} T-cells.

Mice were infected intravenously with 1x10^4 wildtype Lm (EGD strain) or 5x10^4 ovalbumin-expressing Lm (Lm OVA), respectively. (a) Lm-specific T-cell responses. Left: Absolute number of H2-K\(^b\) SIINFEKL (OVA\(_{257-264}\)) pentamer^+ CD8^+ T-cells in the spleens of uninfected and Lm OVA-infected Jak2^{+/VF} and Jak2^{+/+} mice were determined by flow cytometry at day 7 p.i. (n = 6 per group, * p < 0.05, methodology described in \(^{15}\)). Right: Frequencies of *Listeria*-specific, INF-\(\gamma\)-, TNF-, GM-CSF- and G-CSF-producing CD4^+ and CD8^+ T-cells were determined in splenocytes of Lm-infected Jak2^{+/VF} and Jak2^{+/+} mice after stimulation with (LLO)\(_{190-201}\) (10^6 M) for CD4^+ T-cells and OVA\(_{257-264}\) (10^8 M) for CD8^+ T-cells, respectively, by flow cytometry at day 7 p.i. as described before \(^{15}\). Cytokine-producing cell were identified by gating on live cells expressing CD45, CD3 and CD4 or CD45, CD3 and CD8 (n = 6 per group, * p < 0.05). (b) Jak2^{+/VF} and Jak2^{+/+} mice (n=5 per group)
were infected with $1 \times 10^4$ wildtype Lm and CFUs were determined in the spleens at day 7 p.i. (* p < 0.05) according to a standard protocol. (c) Absolute numbers of neutrophils ($\text{Ly6G}^{\text{high}} \text{Ly6C}^{\text{high}}$), were determined by flow cytometry in spleens and blood of Jak2$^{+/VF}$ and Jak2$^{+/+}$ mice before and at day 7 p.i. (n = 6 per group) (* p < 0.05). Histopathology in the liver, spleen and lung of Jak2$^{+/VF}$ and Jak2$^{+/+}$ mice were analyzed by H&E staining at day 7 p.i. as described before. (d) Absolute numbers (n=6 per group) of extramedullary erythroblasts in the spleens of uninfected and Lm OVA-infected Jak2$^{+/VF}$ and Jak2$^{+/+}$ mice were determined by flow cytometry at day 7 p.i. CD71 and TER119 was used to identify erythroblasts (*p<0.05). (e) Serum EPO levels of uninfected and Lm OVA-infected (day 7 p.i) Jak2$^{+/VF}$ and Jak2$^{+/+}$ mice were determined in mouse serum using a commercial mouse EPO ELISA (R&D Systems, MN) (N=6 per group, *p<0.05). (a-e) All mice were housed under pathogen-free conditions in the accredited Animal Research Facility at the Medical Faculty, OvGU, Magdeburg. We conducted all experiments following approval by the Landesverwaltungsamt Saxony-Anhalt. Statistical significance was determined using the two-tailed Student $t$ test and p < 0.05 was considered as significant.
Figure 1

(a) Flow cytometry analysis of CD19+ B cells and CD3+ T cells.

(b) Flow cytometry analysis of CD8+ T cells and CD4+ T cells.

(c) Western blot analysis of Jak2 WT and Jak2 FV in CD4+ T-cells, CD8+ T-cells, and granulocytes.

(d) Schematic representation of Jak2 WT and Jak2 FV in CD4+ T-cells and CD8+ T-cells before and after Lm infection.
Figure 2

(a) SI/NF-κB T-cells (spleen) and cytokine T-cells (spleen) for Jak2<sup>−/−</sup> and Jak2<sup>+/+</sup> mice at d0 and d7.

(b) Colony forming units (spleen) for Jak2<sup>−/−</sup> and Jak2<sup>+/+</sup> mice at d0 and d7.

(c) Neutrophils in spleen and blood for Jak2<sup>−/−</sup> and Jak2<sup>+/+</sup> mice at d0 and d7.

(d) Images of tissues (Liver, Spleen, Lung) for Jak2<sup>−/−</sup> and Jak2<sup>+/+</sup> mice at d7.

(e) EPO levels (pg/ml) for Jak2<sup>−/−</sup> and Jak2<sup>+/+</sup> mice at d0 and d7.
Supplementary Informations

Legends to Supplementary figures

Supplementary Figure 1. Normal T-cell differentiation and activation in naïve Jak2+/VF mice.

(a) Relative (left) and absolute (right) numbers of single and double-positive CD4+ and CD8+ thymocytes in Jak2+/VF and Jak2+/+ mice were determined by flow cytometry. In the left panel, representative data of one of five mice and in the right panel mean values ± SD of five mice are shown. (b) Absolute numbers of naïve (CD62L+ CD44−), memory-like (CD62L+ CD44+) and effector (CD62L− CD44+) CD4+ and CD8+ T-cells in spleens of Jak2+/VF and Jak2+/+ mice were determined by flow cytometry. (c) Absolute numbers of Foxp3+ CD25+ regulatory CD4+ T-cells in spleens of Jak2+/VF and Jak2+/+ mice were determined by flow cytometry. (d) The mean fluorescence intensity (MFI) of CD69 was determined by flow cytometry on splenic CD4+ and CD8+ T-cells of Jak2+/VF and Jak2+/+ mice. (b-d) Mean ± SD of five mice per group is shown.

Supplementary Figure 2. Leukocyte and erythrocyte reactions in Lm-infected mice.

(a) Absolute number of CD4+ and CD8+ T-cells in the spleens of uninfected and Lm OVA-infected Jak2+/VF and Jak2+/+ mice at day 7 p.i were determined by flow cytometry (n = 6 per group). (b) Absolute numbers of CD45+ leukocyte subtypes including B-cells (CD19+ B220+), macrophages (CD68+ F4/80+), inflammatory monocytes (Ly6Gintermediate Ly6Chigh), and dendritic cells (CD11c+ CD3+) were determined by flow cytometry in spleens (left) and blood (right) of Jak2+/VF and Jak2+/+ mice before and at day 7 p.i. (n = 6 per group) (* p < 0.05). (c) The percentage of extramedullary erythroblasts in the spleens of uninfected and Lm OVA-infected Jak2+/VF and Jak2+/+ mice were determined by flow cytometry at day 7 p.i. CD71 and TER119 were used to identify pro-erythroblasts (I), basophilic-erythroblasts (II), polychromatophilic-erythroblasts (III), and
orthochromatophilic-erythroblasts (IV) (*p<0.05). Representative dot plots are shown (n=6 per group).

Supplementary Figure 3. Increased cytokine production and proliferation of Jak2^{V617F} T cells in vitro (a and b) CD4\(^+\) and CD8\(^+\) T-cells were isolated from spleen by MACS and stimulated with 5 \(\mu\)g/ml anti-CD3 and 2 \(\mu\)g/ml anti-CD28 for 72 hours. (a) Cytokine production was analyzed by flow cytometric bead assay (n = 5 mice per group; * p < 0.05). (b) Proliferation of carboxyfluorescein succinimidyl ester (CSFE)-labelled CD4\(^+\) and CD8\(^+\) T-cells was determined flow cytometry. Left: representative histogram of CFSE dilution in proliferating CD4\(^+\) and CD8\(^+\) T-cells. Right: The percent of proliferating CD4\(^+\) and CD8\(^+\) T cells is shown (n =5 per group, * p < 0.05).

Supplementary Figure 4. T-cell-specific Jak2^{V617F} augments CD4\(^+\) and CD8\(^+\) T-cell responses and increases neurophilia and extramedullary hematopoiesis in Concanavalin A-stimulated mice (a-e) Mice were injected intravenously with 20 \(\mu\)g ConA or were left untreated. ConA-treated mice were analyzed 48 h after stimulation. (a) Absolute numbers of CD4\(^+\) and CD8\(^+\) T-cells in the spleens of Jak2^{V617F} and Jak2\(^{+/+}\) mice were determined by flow cytometry (n = 6 per group, * p < 0.05). (b) The numbers of IFN-\(\gamma\)-, TNF-, GM-CSF- and G-CSF-producing CD4\(^+\) and CD8\(^+\) T-cells were determined in splenocytes of Jak2^{V617F} and Jak2\(^{+/+}\) mice by flow cytometry. Cytokine-producing T-cells were identified by gating on live cells expressing in combination CD45, CD3 and CD4 or CD45, CD3 and CD8 (n = 6 per group, * p < 0.05). (c) Bromodeoxyuridine (BrdU) (1.5 mg/mouse) was administered i.p to Jak2^{V617F} and Jak2\(^{+/+}\) mice 1 h before ConA application. The mice were sacrificed 48 hours after ConA stimulation and BrdU incorporation of CD4\(^+\) and CD8\(^+\) T-cells were determined by flow cytometry. Left: Representative contour plots for CD4\(^+\) and CD8\(^+\) T-cells of individual mice. Right: The mean + SD of BrdU\(^+\) CD4\(^+\) and CD8\(^+\) T-cells is shown (n = 6 per group, * p < 0.05). (d) Absolute numbers of CD45\(^+\) leukocyte subtypes including B-cells (CD19\(^+\)B220\(^+\)), macrophages (CD68\(^+\) F4/80\(^+\)), inflammatory monocytes (Ly6G\(_{\text{int}}\) Ly6C\(_{\text{high}}\)), and dendritic cells
(CD11c⁺ CD3⁻) were determined by flow cytometry in spleens (left) and blood (right) of Jak2⁺/VF and Jak2⁺/⁺ (n = 6 per group, * p < 0.05). (e) Relative numbers of extramedullary erythroblasts after ConA-stimulation (left) and absolute numbers (right) of extramedullary erythroblasts in the spleens of Jak2⁺/VF and Jak2⁺/⁺ mice were determined by flow cytometry. Left: Representative contour plots of individual mice. CD71 and TER119 were used to identify pro-erythroblasts (I), basophilic-erythroblasts (II), polychromatophilic-erythroblasts (III), and orthochromatophilic-erythroblasts (IV). Right: The mean ± SD of erythroblasts is shown (n = 6 per group, * p < 0.05).
Supplementary Figure 1

a

b

c

d
Supplementary Figure 3

(a) Cytokines (ng/mL) for Jak2^{−/−} and Jak2^{+/−} conditions.

(b) Flow cytometry analysis showing CFSE proliferation and CD4^+ and CD8^+ cell counts.