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

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

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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 infections³. 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-cells⁴. 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 *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

61 and PMF. The presence of JAK2^{V617F} in both, CD4⁺ and CD8⁺ T-cells is consistent with a genetic
 62 event that arises at the stem- and progenitor-cell level.

63 To explore the T cell-specific function of Jak2^{V617F} mutated clones *in vivo*, we crossed C57BL/6
 64 Jak2^{V617F} ⁸ with CD4-Cre mice. The resulting CD4-Cre Jak2^{V617F} mice (henceforth designated as
 65 Jak2^{+VF}) exhibited heterozygous expression of Jak2^{V617F} in CD4⁺ and CD8⁺ T-cells but not in the
 66 myeloid lineage (e.g. granulocytes, Figure 1c). Those animals were compared to wildtype and Cre-
 67 negative littermate controls (henceforth designated as Jak2^{+/+}).

68 Activation of Jak2^{V617F} in T-cells could potentially lead to alteration of thymic development and
 69 composition of T-cell subsets. However, T-cell development in the thymus (Supplementary Figure
 70 1a) of Jak2^{+VF} animals remained unchanged and comparable to Jak2^{+/+} littermate controls.
 71 Moreover, numbers of splenic naïve (CD62L⁺ CD44⁻), memory-like (CD62L⁺ CD44⁺) and effector
 72 (CD62L⁻ CD44⁺) CD4⁺ and CD8⁺ T-cells (Supplementary Figure 1b), numbers of Foxp3⁺ CD25⁺
 73 CD4⁺ regulatory T-cells (Supplementary Figure 1c), and expression of the T-cell activation marker
 74 CD69 (Supplementary Figure 1d) were equal in both strains of mice. Importantly, Jak2^{+VF} mice did
 75 not develop any clinical signs or symptoms that could be attributed to immunodefects or
 76 dysregulation of autoimmunity.

77 Activated T-cells play an important role in protecting the organism from infections with intracellular
 78 pathogens, such as intracellular bacteria and viruses. To investigate the function of Jak2^{V617F} T-cell
 79 clones in a relevant model, we analyzed T-cell responses upon infection with *Listeria*
 80 *monocytogenes* (Lm), a Gram-positive facultative intracellular bacterium (Figure 1d). In animals
 81 and humans, Lm causes severe brain, intestine, liver and bloodstream infections. While T-cell
 82 function is crucial for the elimination of Lm, experimental studies in mice revealed that innate
 83 immune cells (dendritic cells, macrophages, inflammatory monocytes and natural killer (NK) cells)
 84 additionally contribute to the control of Lm in liver and spleen. Both, CD4⁺ and CD8⁺ T-cells are
 85 activated in listeriosis and, in particular, CD8⁺ T-cells, which peak at day 7 post infection (p.i.), are
 86 pivotal for final elimination of the pathogen around day 14 p.i.⁹ The cytokines, TNF, G-CSF, GM-

87 CSF and IFN- γ are critically important for control and survival of listeriosis ¹⁰. In our model,
 88 infection with Lm resulted in an increase of CD4⁺ and CD8⁺ T-cells in spleens of Jak2^{+VF} and
 89 Jak2^{+/+} control mice, which was slightly pronounced in Jak2^{+VF} mice (Supplementary Figure 2a).
 90 Importantly, Lm-specific T-cell responses were greatly augmented in Jak2^{+VF} mice (Figure 2a).
 91 Upon infection with Lm OVA, which express a strong CD8 (OVA₂₅₇₋₂₆₄, SIINFEKL) H-2K^b-restricted
 92 T-cell epitope ¹¹, the number of SIINFEKL-specific CD8⁺ T-cells was significantly increased in
 93 Jak2^{+VF} mice (Figure 2a). In addition, numbers of Lm OVA-specific CD8⁺ T-cells and listeriolysin₁₉₀₋
 94 ₂₀₁-specific CD4⁺ T-cells producing interferon- γ , TNF, G-CSF and GM-CSF were significantly
 95 increased in Jak2^{+VF} mice (Figure 2a). Of note, these findings resulted in functional improvement
 96 of pathogen elimination: compared to Jak2^{+/+} controls, numbers of Lm were significantly decreased
 97 in spleen (Figure 2b) and liver (data not shown) of Jak2^{+VF} mice. Despite the augmented T-cell
 98 response in Lm-infected Jak2^{+VF} mice, the expression of activation markers including CD11a (LFA-
 99 1) were comparable between CD4⁺ and CD8⁺ T-cells in Lm-infected mice of both genotypes.
 100 Consistent with elevated G-CSF and GM-CSF production, listeriosis induced a significantly
 101 stronger increase of neutrophils in spleen, blood, liver and lung of Jak2^{+VF} mice (Figure 2c). In
 102 mice, neutrophils only marginally contribute to the control of Lm ¹². Therefore, the generalized
 103 increase of neutrophils does not necessarily contribute to an improved pathogen control but may
 104 rather be the consequence of increased paracrine cytokine stimulation. Abundance of all other
 105 major leukocyte populations was comparable to non-infected and infected Jak2^{+VF} mice and
 106 Jak2^{+/+} littermate controls in spleen and blood (Supplementary Figure 2b).
 107 Jak2^{V617F} promotes chronic inflammation through induction of pro-inflammatory cytokines which
 108 contribute to constitutional symptoms in MPN patients. At present it is unclear whether an
 109 increased activation of Jak2 in affected lymphocytes may add to disease progression and
 110 inflammation in MPN. Production and secretion of cytokines by Jak2^{V617F} positive cells may impact
 111 expansion of the erythroid lineage as well as proliferation of malignant and non-malignant
 112 progenitors ¹³. 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 ¹⁴. 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.

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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 ⁸. **(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 1x10⁴ wildtype Lm (EGD strain) or 5x10⁴ ovalbumin-expressing Lm (Lm OVA), respectively. **(a)** Lm-specific T-cell responses. Left: Absolute number of H2-K^b SIINFELK (OVA₂₅₇₋₂₆₄) 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-γ-, 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 OVA₂₅₇₋₂₆₄ (10⁻⁸ M) for CD8⁺ T-cells, respectively, by flow cytometry at day 7 p.i. as described before ¹⁵. Cytokine-producing cells 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)

259 were infected with 1×10^4 wildtype Lm and CFUs were determined in the spleens at day 7 p.i. (* $p <$
260 0.05) according to a standard protocol ¹⁵. **(c)** Absolute numbers of neutrophils (Ly6G^{high} Ly6C^{high}),
261 were determined by flow cytometry in spleens and blood of Jak2^{+/-} and Jak2^{+/+} mice before and at
262 day 7 p.i. (n = 6 per group) (* $p <$ 0.05). Histopathology in the liver, spleen and lung of Jak2^{+/-} and
263 Jak2^{+/+} mice were analyzed by H&E staining at day 7 p.i. as described before ². **(d)** Absolute
264 numbers (n=6 per group) of extramedullary erythroblasts in the spleens of uninfected and Lm OVA-
265 infected Jak2^{+/-} and Jak2^{+/+} mice were determined by flow cytometry at day 7 p.i. CD71 and
266 TER119 was used to identify erythroblasts (* $p <$ 0.05). **(e)** Serum EPO levels of uninfected and Lm
267 OVA-infected (day 7 p.i) Jak2^{+/-} and Jak2^{+/+} mice were determined in mouse serum using a
268 commercial mouse EPO ELISA (R&D Systems, MN) (N=6 per group, * $p <$ 0.05). **(a-e)** All mice were
269 housed under pathogen-free conditions in the accredited Animal Research Facility at the Medical
270 Faculty, OvGU, Magdeburg. We conducted all experiments following approval by the
271 Landesverwaltungsamt Saxony-Anhalt. Statistical significance was determined using the two-tailed
272 Student *t* test and $p <$ 0.05 was considered as significant.

Figure 1

Figure 2

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 $\pm$ 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 $\pm$ 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 ($Ly6G^{intermediate} Ly6C^{high}$), 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^{+VF} T cells *in vitro* **(a and b)** CD4⁺ and CD8⁺ T-cells were isolated from spleen by MACS and stimulated with 5 μ g/ml anti-CD3 and 2 μ 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 (CFSE)-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 μ 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^{+VF} and Jak2^{+/+} mice were determined by flow cytometry (n = 6 per group, * $p < 0.05$). **(b)** The numbers of IFN- γ -, TNF-, GM-CSF- and G-CSF-producing CD4⁺ and CD8⁺ T-cells were determined in splenocytes of Jak2^{+VF} 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^{+VF} 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 $\pm$ 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^{intermediate} Ly6C^{high}), and dendritic cells

52 (CD11c⁺ CD3⁻) were determined by flow cytometry in spleens (left) and blood (right) of Jak2^{+[/]VF} and
53 Jak2^{+[/]+} (n = 6 per group, * p < 0.05). **(e)** Relative numbers of extramedullary erythroblasts after
54 ConA-stimulation (left) and absolute numbers (right) of extramedullary erythroblasts in the spleens
55 of Jak2^{+[/]VF} and Jak2^{+[/]+} mice were determined by flow cytometry. Left: Representative contour plots
56 of individual mice. CD71 and TER119 were used to identify pro-erythroblasts (I), basophilic-
57 erythroblasts (II), polychromatophilic-erythroblasts (III), and orthochromatophilic-erythroblasts (IV).
58 Right: The mean $\pm$ SD of erythroblasts is shown (n = 6 per group, * p < 0.05).
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Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

Supplementary Figure 4