Altered T-cell subset repertoire affects treatment outcome of patients with myelofibrosis

Ivo Veletic,1,* Sanja Prijic,1,2,* Taghi Manshouri,3 Graciela M. Nogueras-Gonzalez,3 Srdan Verstovsek,4 and Zeev Estrov1

1Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, TX, USA; 2Clinical Department of Laboratory Diagnostics, University Hospital Center Zagreb, Zagreb, Croatia and 3Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, TX, USA

*IV and SP contributed equally to the study as co-first authors.

©2021 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2020.249441

Received: February 6, 2020.
Accepted: July 16, 2020.
Pre-published: July 30, 2020.
Correspondence: ZEEV ESTROV - zestrov@mdanderson.org
**Supplementary Data**

**Supplementary Table S1.**
Flow cytometry antibodies and isotype controls.

| Target | Clone | Fluorescent label | Vendor       | Cat #     | RRID     |
|--------|-------|-------------------|--------------|-----------|----------|
| Antibodies |       |                   |              |           |          |
| CD3    | SK7   | APC               | BD Biosciences | 340440    | AB_400513|
| CD4    | RPA-T4| V500              | BD Biosciences | 560768    | AB_1937323|
| CD8    | SK1   | APC-H7            | BD Biosciences | 641400    | AB_1645736|
| CD45   | HI30  | PE-CF594          | BD Biosciences | 562279    | AB_11154577|
| CD45RO | UCHL1 | FITC              | BD Biosciences | 555492    | AB_395883|
| CD62L  | DREG-56| BV421         | BioLegend     | 304828    | AB_2562914|
| HLA-DR | L243  | PerCP-Cy5.5       | BD Biosciences | 339194    | AB_647443|
| PD1    | MIH4  | PE                | BD Biosciences | 557946    | AB_647199|
| TCRγ/δ | 11F2  | PE-Cy7            | BD Biosciences | 655434    | AB_2827402|
| Isotype controls |       |                   |              |           |          |
| IgG1, κ | MOPC-21| APC            | BD Biosciences | 555751    | AB_398613|
| IgG1, κ | X40    | V500              | BD Biosciences | 560787    | AB_1937319|
| IgG1, κ | X40    | APC-H7            | BD Biosciences | 561427    | AB_10714779|
| IgG1, κ | X40    | PE-CF594          | BD Biosciences | 562292    | AB_11207243|
| IgG2a, κ | G155-178| FITC           | BD Biosciences | 555573    | AB_395952|
| IgG1, κ | X40    | BV421             | BD Biosciences | 562438    | AB_11207319|
| IgG2a, κ | MOPC-173| PerCP-Cy5.5| BioLegend     | 400252    | AB_400252|
| IgG1, κ | MOPC-21| PE                | BD Biosciences | 559320    | AB_397218|
| IgG1, κ | MOPC-21| PE-Cy7           | BD Biosciences | 557646    | AB_396763|
**Supplementary Table S2.**
Characteristics of myelofibrosis (MF) patients.

| Parameters                          | Median [range] or n (%) |
|-------------------------------------|-------------------------|
| Total patients                      | 47 (100)                |
| **Demographics**                    |                         |
| Age (years)                         | 65 [43 - 83]            |
| Sex: Male                           | 28 (59.6)               |
| **Disease status at baseline**      |                         |
| Myelofibrosis subtype\(^a\)         |                         |
| PMF                                 | 27 (57.4)               |
| PPV MF                              | 13 (27.7)               |
| PET MF                              | 7 (14.9)                |
| BM fibrosis grade\(^b\)             |                         |
| MF-1                                | 3 (6.4)                 |
| MF-2                                | 24 (51.1)               |
| MF-3                                | 20 (42.6)               |
| Driver mutation                     |                         |
| JAK2 (V617F)                        | 42 (89.4)               |
| MPL (W515L/K)                       | 2 (4.3)                 |
| Not determined                       | 3 (6.4)                 |
| Mutant JAK2 allele burden (%)       | 80.7 [20.5 - 98.2]      |
| Unfavorable karyotype\(^c\)         | 4 (8.5)                 |
| Constitutional symptoms\(^d\)      | 37 (78.7)               |
| Splenomegaly                        | 41 (87.2)               |
| Risk category\(^e\)                 |                         |
| Low risk                            | 2 (4.3)                 |
| Intermediate-1                      | 11 (23.4)               |
| Intermediate-2                      | 31 (66.0)               |
| High risk                           | 3 (6.4)                 |
| **Disease course**                  |                         |
| Time since diagnosis (months)       | 8.2 [0.1 - 218.8]       |
| Previous treatment                  |                         |
| Hydroxyurea                         | 33 (70.2)               |
| IMiDs                               | 10 (21.3)               |
| Steroids                            | 7 (14.9)                |
| Anagrelide                          | 5 (10.6)                |
| Azacitidine                         | 5 (10.6)                |
| Interferon-α                        | 4 (8.5)                 |
| Lestaurtinib (JAK inhibitor)        | 4 (8.5)                 |
| Splenectomy                         | 3 (6.4)                 |
| None                                | 7 (14.9)                |
| Transfusion dependence\(^f\)        | 10 (21.3)               |
| Transformation to AML               | 4 (8.5)                 |
| **Treatment outcome**               |                         |
| Treatment duration (months)         | 38.9 [2.8 - 85.7]       |
| Overall survival (months)           | 53.8 [11.6 - 116.7]     |
| Best clinical response\(^g\)        |                         |
| Clinical improvement (CI)           | 33 (70.2)               |
| No response (NR)                    | 11 (23.4)               |
| Progressive disease (PD)            | 2 (4.3)                 |
| Spleen response\(^h\)               |                         |
| Complete resolution (CR)            | 9 (19.1)                |
| Persistent splenomegaly (PS)        | 29 (61.7)               |
Myelofibrosis subtypes were determined in accordance with the 2008 World Health Organization classification.\(^1\)

Bone marrow (BM) fibrosis grade was established according to the European consensus criteria.\(^2\)

Unfavorable karyotype was defined as a complex karyotype or abnormalities including +8, -7/7q-, i(17q), -5/5q-, 12p-, inv(3) or 11q23 rearrangement.

Constitutional symptoms were defined as either weight loss >10% of baseline in the year preceding diagnosis, unexplained fever, or excessive sweats persisting for more than 1 month.

Risk category was calculated using Dynamic International Prognostic Scoring System (DIPSS) Plus.\(^3\)

Transfusion dependency was defined by a history of transfusions of at least 2 units of red blood cells in the last month.

Clinical response was evaluated based on the International Working Group for Myelofibrosis Research and Treatment (IWG-MRT) criteria.\(^4\)

Spleen response was assessed after 6 months of treatment in patients with ≥5 cm of palpable spleen at treatment baseline.

Abbreviations: PMF, primary myelofibrosis; PPV MF, post-polycythemia vera MF; PET MF, post-essential thrombocythemia MF; IMiDs, immunomodulatory imide drugs (thalidomide, lenalidomide, pomalidomide)
**Supplementary Table S3.**

Model selection for linear mixed effects models.\(^a\)

| Predictors (fixed effects) | Full | -1 | -2 | -3 | -4 | -5 | -6 |
|---------------------------|------|----|----|----|----|----|----|
| Time (years)              | +    | +  | +  | +  | +  | +  | +  |
| Spleen size               | +    | +  | +  | +  | +  | +  | +  |
| BM fibrosis grade\(^b\)   | +    | +  | +  | +  | +  |    |    |
| % JAK2 (V617F)            | +    | +  | +  | +  |    |    |    |
| Leukocyte count           | +    | +  |    |    |    |    |    |
| Platelet count            | +    |    |    |    |    |    |    |
| Monocyte count            |      |    |    |    |    |    |    |

| AIC                       | 563.4| 551.6| 541.5| **536.9**| 554.2| 606.8| 675.5|
| BIC                       | 603.1| 589  | 576.6| **569.6**| 585  | 631.2| 698.4|
| Marginal R\(^2\)\(^c\)    | 0.216| 0.211| 0.204| **0.187**| 0.174| 0.143| 0.128|
| Conditional R\(^2\)       | 0.731| 0.727| 0.734| **0.726**| 0.722| 0.723| 0.709|

| AIC                       | 514.7| 501.7| 491.1| **484.3**| 497.3| 547.3| 614.7|
| BIC                       | 556.4| 540.9| 527.9| **518.7**| 529.7| 572.9| 638.6|
| Marginal R\(^2\)          | 0.225| 0.217| 0.196| **0.182**| 0.141| 0.095| 0.096|
| Conditional R\(^2\)       | 0.381| 0.36  | 0.372| **0.366**| 0.385| 0.338| 0.354|

\(^a\) Selection of optimal mixed model was performed using the “top-down” approach where full model was gradually reduced by eliminating one by one predictor (fixed effect), while keeping the patients (random effect) constant. Highlighted in grey is the best model to explain the variance in data out of 7 possibilities. In addition, interactions between predictors were assessed and were all deemed inferior in explaining the variance in data. The values are reported as a mean of each group of variables.

\(^b\) Bone marrow (BM) fibrosis grade was established according to the European consensus criteria.\(^2\)

\(^c\) R-squared (R\(^2\)) value was calculated as marginal (considers only the variance of the fixed effects) and conditional (considers both the fixed and random effects).\(^5\)

Abbreviations: AIC, Akaike information criterion; BIC, Bayesian information criterion
## Supplementary Table S4.

Mixed model used in the longitudinal analyses of T-cell subsets and PD1⁺ fractions.

### Total T cells

| Predictors | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  |
|------------|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|
| (Intercept) | 62.1   | 38.41 - 85.79 | <0.001 | 35.62  | 10.96 - 60.28 | 0.006 |
| Year 1     | 0.73   | -0.94 - 3.91 | 0.864 | -0.28  | -2.91 - 8.85 | 0.95  |
| Year 2     | 0.88   | -4.45 - 2.22 | 0.741 | 0.07   | -0.58 - 5.82 | 0.08  |
| Year 3     | -3.56  | -8.86 - 1.93 | 0.183 | 3.84   | -1.89 - 9.36 | 0.167 |
| Year 4     | -3.65  | -9.82 - 2.33 | 0.225 | 3.69   | -2.92 - 9.81 | 0.237 |
| Year 5     | -6.43  | -15.29 - 2.34 | 0.15 | 6.71   | -2.51 - 15.93 | 0.149 |
| Year 6     | -7.26  | -22.52 - 7.99 | 0.341 | 9.32   | -6.55 - 25.18 | 0.242 |
| Spleen size | 0.1    | -0.32 - 0.53 | 0.631 | -0.2    | -0.64 - 0.25 | 0.374 |
| MF-1       | -3.79  | -27.34 - 19.75 | 0.746 | 3.78   | -20.73 - 28.28 | 0.757 |
| MF-2       | -5.42  | -26.26 - 14.51 | 0.601 | 4.93   | -16.73 - 26.61 | 0.647 |
| MF-3       | -8.28  | -29.47 - 12.91 | 0.433 | 8.71   | -13.34 - 30.75 | 0.428 |
| % JAK2 (V617F) | 0.06  | -0.06 - 0.18 | 0.293 | -0.08  | -0.21 - 0.04 | 0.197 |

### CD4⁺ T cells

| Predictors | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  |
|------------|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|
| (Intercept) | 5.92   | -9.41 - 25.98 | 0.917 | 8.06   | -1.80 - 25.53 | 0.071 |
| Year 1     | -0.44  | -3.85 - 2.77 | 0.786 | -1     | -6.88 - 4.66 | 0.725 |
| Year 2     | 0.27   | -1.54 - 2.39 | 0.797 | 1.14   | -2.64 - 4.92 | 0.55  |
| Year 3     | -0.61  | -2.83 - 1.62 | 0.368 | -0.86  | -4.85 - 3.13 | 0.668 |
| Year 4     | 0.66   | -1.73 - 3.04 | 0.584 | -2.22  | -4.92 - 0.00 | 0.268 |
| Year 5     | -0.09  | -3.88 - 3.49 | 0.959 | 0.56   | -0.74 - 9.61 | 0.861 |
| Year 6     | 7.86   | 10.67 - 14.85 | 0.004 | 5.24   | -3.41 - 13.62 | 0.088 |
| Spleen size | 0.04   | -0.07 - 0.19 | 0.438 | 0.1    | -0.09 - 0.29 | 0.279 |
| MF-1       | 2.75   | -7.15 - 12.64 | 0.581 | 0.19   | -0.79 - 17.96 | 0.983 |
| MF-2       | 3.94   | -8.43 - 13.31 | 0.404 | 1.82   | -1.87 - 5.05 | 0.031 |
| MF-3       | 4.89   | -4.80 - 14.37 | 0.307 | 1.46   | -18.55 - 15.83 | 0.865 |
| % JAK2 (V617F) | -0.02  | -0.04 - 0.01 | 0.245 | -0.03  | -0.08 - 0.02 | 0.199 |

### CD8⁺ T cells

| Predictors | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  | $\beta$ | CI | P  |
|------------|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|--------|----|----|
| (Intercept) | 0.52   | -2.91 - 4.15 | 0.971 | 8.06   | -0.80 - 25.53 | 0.017 |
| Year 1     | -0.44  | -3.85 - 2.77 | 0.786 | -1     | -6.88 - 4.66 | 0.725 |
| Year 2     | 0.27   | -1.54 - 2.39 | 0.797 | 1.14   | -2.64 - 4.92 | 0.55  |
| Year 3     | -0.61  | -2.83 - 1.62 | 0.368 | -0.86  | -4.85 - 3.13 | 0.668 |
| Year 4     | 0.66   | -1.73 - 3.04 | 0.584 | -2.22  | -4.92 - 0.00 | 0.268 |
| Year 5     | -0.09  | -3.88 - 3.49 | 0.959 | 0.56   | -0.74 - 9.61 | 0.861 |
| Year 6     | 7.86   | 10.67 - 14.85 | 0.004 | 5.24   | -3.41 - 13.62 | 0.088 |
| Spleen size | 0.04   | -0.07 - 0.19 | 0.438 | 0.1    | -0.09 - 0.29 | 0.279 |
| MF-1       | 2.75   | -7.15 - 12.64 | 0.581 | 0.19   | -0.79 - 17.96 | 0.983 |
| MF-2       | 3.94   | -8.43 - 13.31 | 0.404 | 1.82   | -1.87 - 5.05 | 0.031 |
| MF-3       | 4.89   | -4.80 - 14.37 | 0.307 | 1.46   | -18.55 - 15.83 | 0.865 |

### Abbreviations:
- TN: T-naive cells;
- TCM: T-central memory cells;
- TEM: T-effector memory cells;
- TEFF: T-effector cells;
- $\beta$: beta-coefficient;
- CI: 95% confidence interval.

5
Supplementary Figure S1

**i**

- **CD4**: Total survival probability for **CD4** cells over time since treatment (years), showing survival probability with high and low percentages. Details include the cutoff values and statistical significance (n.s. for not significant).

- **CD8**: Total survival probability for **CD8** cells over time since treatment (years), showing survival probability with high and low percentages. Details include the cutoff values and statistical significance (n.s. for not significant).

**ii**

- **CD4**: Survival probability for different subtypes (TN, CM, EM, EFF) with survival probability for each subtype over time since treatment (years). Details include the cutoff values and statistical significance (P<0.001).

- **CD8**: Survival probability for different subtypes (TN, CM, EM, EFF) with survival probability for each subtype over time since treatment (years). Details include the cutoff values and statistical significance (n.s. for not significant).

**iii**

- **CD4**: Survival probability for different subtypes (TN, CM, EM, EFF) with survival probability for each subtype over time since treatment (years). Details include the cutoff values and statistical significance (P<0.001).

- **CD8**: Survival probability for different subtypes (TN, CM, EM, EFF) with survival probability for each subtype over time since treatment (years). Details include the cutoff values and statistical significance (n.s. for not significant).
Supplementary Figure S1.

Association between baseline T-cell subsets and ruxolitinib-treated MF patients' clinical outcome. Kaplan-Meier analysis of survival of ruxolitinib-treated MF patients based on T-cell differentiation and activation subsets. Subsets of total (i), CD4+ (ii) and CD8+ T cells (iii) were analyzed at the treatment baseline. Cutoff values for dichotomization of each subset into high and low groups were determined using the maximally selected rank statistic. P-values for differences in OS were calculated using the log-rank test. P<0.05 was considered statistically significant. T_N, T-naïve cells; T_CM, T-central memory cells; T_EM, T-effector memory cells; T_EFF, T-effector cells; n.s., not significant.
Supplementary Figure S2

A i

% Total PD1+ T cells

CD4+  CD8+

Bsl  Ruxo  Bsl  Ruxo

A ii

% PD1+ CD4+ T cells

Bsl  Ruxo  Bsl  Ruxo  Bsl  Ruxo  Bsl  Ruxo

A iii

% PD1+ CD8+ T cells

Bsl  Ruxo  Bsl  Ruxo  Bsl  Ruxo  Bsl  Ruxo

B i

% PD1+ Total T cells

CD4+  CD8+

Bsl  Bsl  T N  T CM  T EM  T EFF

B ii

% PD1+ CD4+ T cells

Bsl  Bsl  T N  T CM  T EM  T EFF

B iii

% PD1+ CD8+ T cells

Bsl  Bsl  T N  T CM  T EM  T EFF

B

Mean

Linear prediction

CD4+  CD8+

Bsl  Bsl  T N  T CM  T EM  T EFF

CD4+  CD8+

Bsl  Bsl  T N  T CM  T EM  T EFF

CD4+  CD8+

Bsl  Bsl  T N  T CM  T EM  T EFF
Supplementary Figure S2.
Effects of ruxolitinib treatment on PD1-expressing T-cells in patients with MF. PD1+ T-cell fractions from MF patients (n=26) were analyzed at baseline (Bsl; red) and over the course of treatment with ruxolitinib (Ruxo; light blue). (A) Quantitation of PD1+ fractions in CD4/CD8 differentiation subsets (i) and activation subsets (ii-iii) before treatment and during the overall follow-up period. Follow-up values were calculated as a mean of all the analyzed time points over the course of treatment for each patient. (B) Longitudinal analysis of T-cell subsets over 6 years of ruxolitinib treatment. Depicted are the mean percentages (green) and linear predictions (dark blue) based on mixed-effects model with repeated measures in differentiation (i) and activation subsets (ii-iii). The statistical difference between the 2 groups was assessed using the paired t-test. In the longitudinal plots, error bars denote standard error, \( P \)-values represent significance of change from baseline over time, and asterisks indicate significance of change in each year of treatment. Linear mixed models were corrected for spleen size, fibrosis grade, and \( \text{JAK2}^{V617F} \) allele burden, and the \( P \)-values were computed using Kenward-Roger adjusted F-test. \( P<0.05 \) (*) was considered statistically significant; **, \( P<0.01 \); ***, \( P<0.001 \); \( T_N \), T-naïve cells; \( T_{CM} \), T-central memory cells; \( T_{EM} \), T-effector memory cells; \( T_{EFF} \), T-effector cells; n.s., not significant.
Supplementary Figure S3.

Comparison of T-cell subsets and PD1-expressing fractions based on specimen type and MF patients’ characteristics at the treatment baseline. CD4+ and CD8+ total T-cell, T_{EM} subsets, and PD1+ fractions of MF patients were compared based on the specimen type and clinical parameters (age, MF subtype, and cell counts) at the treatment baseline. Shown are healthy control (CTRL; grey), baseline (Bsl; red), and ruxolitinib-treated groups (Ruxo; light blue). Follow-up values were calculated as a mean of all the analyzed time points over the course of treatment. Patients were separated by age based on the cutoff value of 65 years. MF subtypes were determined in accordance with the 2008 World Health Organization classification as primary myelofibrosis (PMF), post-polycytemia vera (PPV) MF or post-essential thrombocytemia (PET) MF. Cutoff values for leukocyte, platelet, and monocyte counts were 30×10^9/L, 200×10^9/L, and 1,500×10^9/L, respectively. Welch’s or paired t-test was used to compare the 2 groups. P<0.05 (⁎) was considered statistically significant; **, P<0.01; ***, P<0.001; T_{EM}, T-effector memory cells; BM, bone marrow; PB, peripheral blood.
References

1. Vardiman JW, Thiele J, Arber DA, et al. The 2008 revision of the World Health Organization (WHO) classification of myeloid neoplasms and acute leukemia: rationale and important changes. Blood. 2009;114(5):937-951.

2. Thiele J, Kvasnicka HM, Facchetti F, Franco V, van der Walt J, Orazi A. European consensus on grading bone marrow fibrosis and assessment of cellularity. Haematologica. 2005;90(8):1128-1132.

3. Passamonti F, Cervantes F, Vannucchi AM, et al. A dynamic prognostic model to predict survival in primary myelofibrosis: a study by the IWG-MRT (International Working Group for Myeloproliferative Neoplasms Research and Treatment). Blood. 2010;115(9):1703-1708.

4. Tefferi A, Barosi G, Mesa RA, et al. International Working Group (IWG) consensus criteria for treatment response in myelofibrosis with myeloid metaplasia, for the IWG for Myelofibrosis Research and Treatment (IWG-MRT). Blood. 2006;108(5):1497-1503.

5. Nakagawa S, Schielzeth H. A general and simple method for obtaining R2 from generalized linear mixed-effects models. Methods Ecol Evol. 2013;4(2):133-142.