Cytokine Production In Myelofibrosis Exhibits Differential Responsiveness To JAK/STAT, MAP Kinase, And NFκB Signaling

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Supplementary data

Supplementary methods

Antibodies.
Antibodies were obtained from suppliers including Fluidigm (South San Francisco, CA, USA), BD Biosciences (San Jose, CA, USA), Cell Signaling Technology (CST; Danvers, MA, USA), and BioLegend (San Diego, CA, USA). Antibodies used for intracellular cytokine mass cytometry experiments are described in Supplementary Table S3. The panel of antibodies for intracellular signaling mass cytometry experiments (Figure 1a, b; Figure 6d; Supplementary Figure S1) was described in a previous publication.[10] Fluorescent antibodies used for cell sorting experiments and fluorescent flow cytometry are described in Supplementary Table S4.

Cryopreserved patient and control samples
Patient and control samples utilized were obtained from patients seen at Washington University according to a protocol approved by the Washington University Human Studies Committee (WU no. 01-1014); from AllCells (Alameda, CA: Supplementary Table S1); or from StemCell Technologies (Vancouver, BC, Canada: Supplementary Table S1). Mononuclear cells from blood or bone marrow (PBMC or BMMC) were isolated from primary PB or BM samples by equal volume centrifugation over Ficoll-Paque (GE Healthcare, Chicago, IL, USA). Cell samples were preserved in 10% dimethyl sulfoxide in heat-inactivated fetal bovine serum (FBS, Gibco Life Technologies, Carlsbad, CA, USA) and stored in liquid nitrogen. Samples were removed from cryopreservation and incubated in RPMI plus medium (RPMI containing 10% heat-inactivated FBS, heparin (20 U/ml, Sigma, St. Louis, MO, USA), and benzonase (25 mU/ml, Sigma)) prior to stimulation.[10]

HEL cells
HEL cells (figure 7) were obtained from Washington University Tissue Culture Support Center and tested for mycoplasma.

Patient Inclusion Criteria
For intracellular cytokine analysis, MF patients not on ruxolitinib or other JAK inhibitors were selected. For plasma cytokine and signaling analysis, patients were chosen for whom samples were available before and > 1 month after commencement of ruxolitinib treatment.

Replication of Experiments
Due to limited quantities of patient and control samples, most experiments were performed only once on any individual sample. Intracellular cytokine experiments, however, were performed 2-3 times on each of blood controls N32, LRS1, LRS2, and LRS3 (Supplementary Table 1), and MF patients MF2, MF15, MF16, MF20, and MF26 (Supplementary table 2), with similar results.

Statistical tests
Statistical methods are described in the figure legends for each experiment. As a general procedure, paired two-tailed T-tests were used for comparisons of paired samples from identical individuals, as in MF samples before and on ruxolitinib treatment (Figure 1c-h). Otherwise, non-parametric tests were preferred, due to both absence of clear Gaussian distributions, and ranges of cytokine levels or of cytokine positive cells that included zero (e.g. Figure 2f, Figure 8d-g). Mann-Whitney U test was preferred where ranges of values were separable from zero and mean values were compared, and Wilcoxon sign-rank test was preferred where ranges overlapped with zero and medians were compared. Error bars were mean +/- SEM for plasma cytokine measurements (Figure 1d-h), utilizing 3 technical replicates per patient per measurement, but means or medians +/- 95%CI elsewhere (including Figure 1d, for overall plasma cytokine values, hence absence of significance notation in said panel, for sake of clarity: complete statistical data for plasma cytokines, however, is included in Supplementary Table 5). Medians +/- 95%CI were preferred for basal intracellular cytokine

statistics (along with Wilcoxon sign-rank test) due to distributions of values including zero and values >200% of median in some individuals (Figure 2f, Supplementary Figure S6). SD or SEM were used in place of 95%CI only in individual experiments with low N≤6, where 95%CI would be too large to display on an easily legible graph (Figure 3b, g, h; Figure 8d-g).

Proportional contribution of cytokine production

For each cytokine studied, the proportion of total cytokine production derived from each cell population could be calculated, as $\Sigma_{pop}/\Sigma_{total}$ = $((mean)_{pop}$ x (cell number assayed)$_{pop})$/ $((mean)_{total}$ x (cell number assayed)$_{total})$, where pop refers to a given cell population (e.g. monocytes), and total refers to all live cells from a given individual (patient or healthy control) in an experiment. This calculation provided a quantitative metric of the relative contribution of each source population for each cytokine.

Cell stimulations and reagents.

Thawed (formerly cryopreserved) cells were resuspended in RPMI plus medium (see above) and placed in incubator for 30 min. recovery period prior to stimulation. Stimulations for intracellular signaling mass cytometry experiments (Figure 1a, b; Figure 6d; Supplementary Figure S1) were previously described.[10] Stimulations for intracellular cytokine experiments were followed by four-hour incubation and subsequent cell staining procedure, as previously described.[27, 28] Conditions used for cell stimulations included reagents among the following: 20ng/mL TNF (Peprotech, Rocky Hill, NJ, USA), 8ng/mL-5µg/mL R848 (Invivogen, San Diego, CA, USA), 40ng/mL or 5µg/mL PAM3CSK4 (R&D Systems, Minneapolis, MN, USA), 10ng/mL TPO (Peprotech), 5µM ruxolitinib (Chemie-Tek, Indianapolis, IN, USA; or SelleckChem, Houston, TX, USA), 0.2-1µM pevonedistat (Active Biochem; Kowloon, Hong Kong, PRC), 10µM trametinib (SelleckChem), 1µM VX-745 (SelleckChem), 1µM JNKi8 (JNK inhibitor 8, MedChem Express, Monmouth Junction, NJ, USA), 5µM momelotinib (SelleckChem), 5µM itacitinib (SelleckChem), 5µM pacritinib (SelleckChem), 5µM fedratinib (SelleckChem). Ruxolitinib used for experiments comparing JAK inhibitors (Figure S14) was from SelleckChem, in order to compare a set of reagents from a common supplier. Ruxolitinib used for all other experiments was from Chemie-Tek. Ruxolitinib from both suppliers gave identical dose-response in growth inhibition assay on HEL cells (data not shown): therefore 5µM ruxolitinib from either supplier is interpreted as equivalent to that from the other. After 2 hours of incubation, cells were treated with secretion inhibitor cocktail (eBioscience Protein Transport Inhibitor Cocktail (500X), Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10µM Brefeldin A and 2µM monensin, for remaining 2 hours of incubation. Following incubation, cells were resuspended in CyFACS buffer,[27] and kept on ice for cell staining with surface antibody cocktail described in Supplementary Table S3. Surface stained cells were labeled for viability with 2.5µM cisplatin (Enzo Life Sciences, Farmingdale, NY, USA) for 1 min. This was followed by fixation and intracellular cytokine staining according to previously published method, [27, 28] briefly described below.

Intracellular cytokine staining for mass cytometry.

Surface stained and cisplatin labeled cells (see above) were resuspended in 1x eBioscience Perm Buffer (Invitrogen). Intracellular cytokine antibodies (Supplementary Table S3) were diluted in 1x eBioscience Perm Buffer, and cells were treated with antibody cocktail for 1 hour at 4°C. Cells were washed in CyFACS buffer. Cells were barcoded for batch run on CyTOF2 mass cytometer (Fluidigm), using metal barcode solutions diluted in 1x Maxpar Barcode Perm Buffer (Fluidigm), according to manufacturer's instructions. Barcoded cells were fixed in 2% paraformaldehyde and labeled with Ir-intercalator (Fluidigm), to allow DNA-positive cell labeling (Supplementary Figure S2). Mass-channel data was recorded on a CyTOF2 mass cytometer (Fluidigm). Data were decoded using single cell debarcoder software (https://github.com/nolanlab/single-cell-debarcoder), and normalized with software from Fluidigm. Further analysis of data was performed in Cytobank (cytobank.org).

Cell sorting for isolated monocytes.

Cryopreserved PBMC or BMMC were thawed in RPMI plus medium, then transferred to live cell buffer (LCB) consisting of calcium/magnesium-free phosphate buffered saline plus 2% heat inactivated FBS and 1mM EDTA. Cells were blocked in 5% human Fc-R binding inhibitor (eBioscience, Life Technologies, Carlsbad, CA, USA) in LBC. Cells were stained in LCB, in an antibody panel consisting of CD34-FITC, CD38-APC, CD3-PE, CD16-PE, CD19-PE, and CD14-BV421 (Supplementary Table S4). Following staining, cells were stained with 1:100 7-AAD (BD) to determine cell viability. Cells were sorted on a MoFlo flow sorter (Beckman Coulter, Brea, CA, USA). Monocytes were sorted as 7AAD-, lineage negative (CD3-, CD16-, CD19-), CD34- (or low, as

discernible), and CD14+ by sequential gating. Isolated monocytes were resuspended in RPMI plus medium, and incubated for 4h +/- 10ng/mL TPO (Peprotech), followed by fixation in 1.6% formaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 10 minutes at room temperature, resuspension in CyFACS buffer,[27] and intracellular cytokine mass cytometry staining as previously described.[27, 28] This experiment was done with one normal blood control and two MF patient samples (Figure 5).

Fluorescent flow cytometry.

Cryopreserved PBMC or BMMC were thawed in RPMI plus medium, then transferred to live cell buffer (LCB). Cells were blocked in 5% human Fc-R binding inhibitor (eBioscience) in LBC, and subsequently stained in LCB in 100µL volume for ≤3M cells, with an antibody panel consisting of CD34-Alexa 700, CD38-APC, CD14-BV421, CD33-PECy7, CD61-FITC, and CD110/MPL-PE. Monocytic lineage cells were gated as CD14+CD61-, to exclude cells with platelets attached, and megakaryocytic lineage cells as CD61+CD14-. These cells could be separated into CD34-CD110/MPL-, CD34low-int (generally CD110/MPL+), and rare CD34high (CD110/MPL+) populations (Figure 6b, c). For details of antibodies see Supplementary Table S4. Staining of HEL cells (Figure 7) utilized p-p65/RELA (S529)-Alexa 647 and total IκBα-Alexa 488. Cells were stained for viability with 1:100 7-AAD (BD). Data was recorded on an LSR Fortessa X-10 analyzer (BD) and analyzed in Cytobank (Cytobank.org). This experiment utilized three MF patient samples and two normal bone marrow controls (Figure 6a-c).