**Bone Marrow Stroma–Secreted Cytokines Protect JAK2V617F-Mutated Cells from the Effects of a JAK2 Inhibitor**

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**Introduction**

The myeloproliferative neoplasms (MPN)—polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF)—originate from the clonal transformation of hematopoietic stem cells (HSC)/hematopoietic progenitors (HP), which gives rise to abnormal proliferation of one or several hematopoietic lineages driven by hypersensitivity to regulatory growth factors (1). Deregulation of kinase activity has emerged as a major mechanism by which cancer cells evade normal physiologic constraints on growth and survival. In MPNs, the gain-of-function Janus activated kinase 2-V617F (JAK2V617F) mutation is present in almost all patients with PV and in approximately 50% of patients with ET or PMF (1–5). JAK2V617F activates several signaling pathways crucial for cellular survival and proliferation. The putative role of JAK2V617F in the pathogenesis of MPNs provided the rationale for the development of JAK2 inhibitors for the treatment of patients with MPNs. Clinical trials testing the activity of several JAK2 inhibitors are ongoing, particularly in myelofibrosis (6, 7). Although preliminary results show significant clinical benefit of therapy, these agents have shown no activity in correcting the fibrosis, osteosclerosis, and neoangiogenesis that characterize the bone marrow of patients with myelofibrosis, and, furthermore, there has been no elimination of malignant clone, as evaluated by the continuous presence of JAK2V617F-positive cells in patients on therapy.

Several lines of evidence suggest that, in myelofibrosis, stromal cells are primed by the malignant hematopoietic clone, which, in turn, conditions the stroma to create a "favorable" pathologic microenvironment that nurtures and protects the malignant cells. In myelofibrosis, both cellular and extracellular levels of various fibrogenic and angiogenic cytokines are increased, thus supporting the notion that the bone marrow histologic changes that characterize myelofibrosis are reactive and mediated by cytokines such as TGFβ, platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and VEGF (8). The net result is a tumor niche...
that provides environmental cues contributing to the proliferation, maintenance, and (potentially) resistance to therapy of the malignant clone. Indeed, marrow stromal cells have been shown to protect chronic lymphocytic leukemia (CLL) cells from spontaneous or drug-induced apoptosis in vitro and to confer resistance to therapy in CLL and other B-cell malignancies, such as acute lymphoblastic leukemia (ALL; refs. 9–11). Understanding the mechanisms of information exchange between the malignant clone and the bone marrow milieu may illuminate methods to eliminate malignant MPN cells that reside in a protective stromal niche within the marrow. In this article, we present evidence supporting a protective effect of the stromal bone marrow niche against JAK2 inhibitor therapy via stromal cell–secreted humoral factors. The manipulation of these contextual cues might be potentially exploited in therapeutic applications for the eradication of JAK2V617F–positive clones.

Materials and Methods

Cells, monoclonal antibodies, and chemicals

Murine FDCP (factor-dependent cell Patersen) cells transfected with the erythropoietin receptor harboring the human JAK2V617F mutant allele (henceforth, referred to as FDCP-EpoR V617F cells), a kind gift from Dr. Joseph Prchal (University of Utah, Salt Lake City, UT), were cultured at 37°C in a humidified 5% CO2 atmosphere by using RPMI-1640 medium in alpha-minimum essential medium Eagle (α-MEM; Invitrogen) with Earl salts and L-glutamine supplemented with 12.5% FBS (HyClone), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma-Aldrich), and 100 μg/mL amphotericin B (Sigma-Aldrich) and maintained in RPMI-1640 medium supplemented with 20% FBS. Cells from the stromal NK.tert cell line (derived from human bone marrow cells immortalized with human telomerase reverse transcriptase (hTERT]) containing exogenous MFG-tsT-IRES-neo was obtained from the RIKEN Cell Line Bank (Sapporo Medical University, Japan; ref. 12) and cultured in alpha-minimum essential medium Eagle (α-MEM; Invitrogen) with Earl salts and L-glutamine supplemented with 12.5% FBS (HyClone), 12.5% human serum (Cellgro), 1 μmol/L hydrocortisone (Sigma-Aldrich), and 100 μmol/L 2-mercaptoethanol (Sigma-Aldrich). Human stromal cells HS5 [CRL-11882; from the American Type Cell Culture (ATCC)] were maintained in α-MEM medium containing 10% FBS. The primary stromal cell line TM-R1 (Taghi Manshouri-Rob1) was established in our laboratory by culturing bone marrow mononuclear cells from a patient with PMF in α-MEM medium containing 20% FBS. Bone marrow aspirate samples and peripheral blood samples from patients with PV (none of whom were receiving PV-directed therapy) were derived according to an Institutional Review Board (IRB)-approved laboratory protocol, utilizing leftover material obtained from specimens used for clinical purposes: mononuclear cells were isolated as previously published and used in experiments without further isolation of specific cell types (13, 14).

The monoclonal antibodies used include mouse anti- phospho-STAT3 (05-485) and -STAT5 (06-553); mouse anti-phosphotyroline clone-4G10 (05321); rabbit anti-JAK2 (06-255); rabbit anti-total STAT3 (06-596), and rabbit anti-total STAT5 (05–533); all obtained from Upstate Biotechnology. Goat anti-human-interleukin 6 (IL-6; AF-206-NA), -chemokine C-X-C-motif ligand 10 [CXCL-10/IFN-γ-inducible 10-kDa protein (IP-10); AF-266-NA], and -FGF basic/2 (FGF-2; AF233-NA) were obtained from R&D Systems (Minneapolis, MN). Mouse anti-β-actin (A5441) was purchased from Sigma-Aldrich. Conjugated horseradish peroxidase sheep anti-mouse antibodies (NA931V) from GE Healthcare, and horseradish peroxidase donkey anti-rabbit antibodies (711-035-15) from Jackson Immunoresearch were obtained. Cytokines used include recombinant human anti-IL8 (208-IL010), -FGF (hFGF; 233-FA-025), and -VEGF (hVEGF; 4644-VS/CF) from R&D Systems. The JAK2 inhibitor atiprimod (Callisto Pharmaceutical) was dissolved in PBS (Gibco BRL) to a final concentration of 8 mmol/L. The stock solution was stored at 4°C and further diluted in tissue-cultured medium just before use.

Growth-inhibition MTT assay

MTT assays were conducted as previously described (13, 14). Results shown represent the average SD of 3 independent experiments, each done in sextuplets.

Coculture assays

Stromal cell suspensions were prepared at a concentration of 4 × 105 cells/mL, and then seeded on 6-well culture plates (3046; Falcon) at a volume of 2 mL/well, or on 96-well assay black plates (3603; Corning INC) at a volume of 50 μL/well. After overnight incubation, the nonadherent cell fraction was removed and adherent stromal confluent monolayers were washed 3 times with PBS, FDCP-EpoRV617F or SET-2 cells were then added to the prepared adherent stroma (8 × 104 cells/mL/well into 6-well plates; 1 × 105 cells/50 μL/well into 96-well plates) either directly (cell-on-cell) or indirectly (separated by 0.4-μm-thick micropore membranes; Falcon; 35-3493). Atiprimod was added at different concentrations as indicated. FDCP-EpoRV617F or SET-2 were then assayed for cell proliferation in 96-well plates after 72 hours of incubation, as described above (MTT assay). Alternatively, cell aliquots from 6-well plates were collected after 4, 24, and 48 hours of incubation to check for apoptosis or were washed 3 times with cold PBS and frozen at –80°C before Western blot analysis. The same method was carried out using primary bone marrow mononuclear cells, instead of FDCP-EpoRV617F or SET-2 cells, from PV patients.

In additional experiments, supernatants were collected from aforementioned coculture assays, plated in new plates, and fresh FDCP-EpoRV617F cells were added with 1 μmol/L atiprimod for 48 hours; induction of apoptosis was then assessed by flow cytometry.

Apoptosis assays

Apoptosis of FDCP-EpoRV617F and SET-2 cells, obtained from coculture assays, was detected by flow cytometry using recombinant human Annexin-V–conjugated with Allophycocyanin (APC; CALTAG; used for FDCP cells) or with fluorescein isothiocyanate (FITC; 51-6710AK; Becton Dickinson Pharmingen; used for SET-2 cells). Analysis was done on a
FACS flow cytometer (Becton Dickinson Systems) and results were analyzed using FlowJo Software version 7.2.5 (Ashland).

**JAK2 immunoprecipitation and Western blotting**

JAK2 immunoprecipitation and Western blot analysis were carried out as previously described (15, 16). Quantitative densitometry has been used to determine fold change in protein content in Western blots.

**Cytokine multiplexed Bio-Plex assay**

FDPC-EpoRV617F and SET-2 cells were washed 3 times with PBS at room temperature and resuspended in serum-free medium (StemCell Technology); 2 × 10^6 cells/mL were dispensed on 6-well plates that were coated or uncoated with stroma (separated by micropore membranes), with or without 1 µmol/L atiprimod, and cocultured for 4, 24, and 48 hours. Supernatants were collected for cytokine measurements. Bio-Plex human cytokine 27-plex panel assay (171-A11127; Bio-Rad) was used for simultaneous quantitation of 27 cytokines, according to the manufacturer's instructions. After washing, beads were resuspended in 125 µL of Bio-Plex Assay Buffer and analyzed on the Bio-Plex system using Bio-Plex Manager software with 5PL curve fitting. The reader was set to read a minimum of 100 beads; results were expressed as median fluorescent intensity (MFI). After centering each treated sample on the corresponding control sample from the same time point, the treated and control groups were compared using 2-sided paired t-test.

**Cytokine neutralization**

Stromal cell monolayers were prepared in 6-well plates as described earlier. After overnight incubation at 37°C, they were washed with PBS 3 times at room temperature and normal goat serum (1 µg/mL as a control), goat anti-human-IL-6 (0.3 µg), CXCL-10/IP-10 (0.3 µg), or -FGF (0.3 µg) antibodies (individually or in combination) were added to culture together with culture medium at 37°C for 4 hours. FDPC-EpoR-JAK2V617F cells (2 × 10^7/mL) were then dispensed onto all wells (separated by micropore membranes from stroma) and cultured in the presence of 1 µmol/L atiprimod for 72 hours. Supernatants were collected for cytokine measurements. Bio-Plex human cytokine 27-plex panel assay (171-A11127; Bio-Rad) was used for simultaneous quantitation of 27 cytokines, according to the manufacturer's instructions. After washing, beads were resuspended in 125 µL of Bio-Plex Assay Buffer and analyzed on the Bio-Plex system using Bio-Plex Manager software with 5PL curve fitting. The reader was set to read a minimum of 100 beads; results were expressed as median fluorescent intensity (MFI). After centering each treated sample on the corresponding control sample from the same time point, the treated and control groups were compared using 2-sided paired t-test.

**Results**

**Stroma attenuated the antiproliferative effect of atiprimod on JAK2 inhibitor therapy on JAK2V617F-positive cells**

We have recently reported that atiprimod is a potent inhibitor of JAK2 tyrosine kinase (13). Atiprimod inhibited the growth and caused apoptosis of JAK2V617F-positive cells, both from cell lines and primary MPN patient blood or bone marrow mononuclear cells, in short-term assays. Furthermore, it suppressed (but not completely abrogated) the growth of blast forming unit-erythroid (BFUE) colonies from primary PV patient blood or bone marrow mononuclear cells in 14-day clonogenic assays (13). Indeed, treatment with atiprimod induced only a moderate reduction in the JAK2V617F allele burden of the proliferating BFUE colonies. Because colonies were grown in the presence of growth factors and accessory bone marrow cells were present in all cultures, we hypothesized that these factors, either alone or in combination, prevented complete growth inhibition of primary JAK2V617F colony-forming cells. To explore this possibility, we designed subsequent experiments.

The effect of atiprimod on the viability of FDCP-EpoR cells, transduced with either mutant (JAK2V617F) or wild-type (JAK2WT) human JAK2, and on the JAK2V617F-positive SET-2 cell line was investigated using the MTT assay. Exposure to increasing concentrations of atiprimod for 72 hours resulted in a dose-dependent inhibition of proliferation (IC50 of 397 nmol/L for FDCP-EpoRV617F, 544 nmol/L for SET-2, and 810 nmol/L for FDCP-EpoRWT cells; fig. Fig. 1A). To test the effect of stroma presence in the culture on the activity of JAK2 inhibitor, we cocultured FDCP-EpoRV617F and SET-2 cells with the human stromal cell lines HS5 or NK.tert, with or without atiprimod, which had no effect on the proliferation of either stromal cell line (data not shown). The activity of atiprimod on the proliferation of FDCP-EpoRV617F or SET-2 cells was significantly reduced in the presence of either HS5 or NK.tert stromal cells (Fig. 1A). The same protective effect was seen when primary JAK2V617F-positive mononuclear blood cells from PV patients were exposed to atiprimod in coculture with stromal cells; they continued proliferating over time even in the presence of 1 µmol/L atiprimod (Fig. 1B).

**Stroma protects JAK2V617F-positive cells from atiprimod-induced apoptosis**

To understand the protective effect of the stroma on the JAK2V617F-positive cells, we first determined whether atiprimod-mediated growth inhibition of JAK2V617F-positive cells was due to drug-induced apoptosis and whether stroma could prevent it. Treatment of FDCP-EpoRV617F cells with 1 µmol/L atiprimod resulted in significant apoptosis; however, almost no atiprimod-induced apoptosis of FDCP-EpoRV617F cells was observed in the presence of HS5 stromal cells (Fig. 2A). The experiment was then expanded to include both FDCP-EpoRV617F and SET-2 cells treated with atiprimod in cocultures with 3 different stromal cell lines—HS5, NK.tert, or TM-R1 (Fig. 2B). Coculture of each JAK2V617F-positive cell line on any monolayer of different stromal cells almost completely abrogated the proapoptotic activity of atiprimod on JAK2V617F-positive cells.

**Stroma impedes atiprimod-induced inhibition of JAK2, STAT3, and STAT5 phosphorylation in JAK2V617F-positive cells**

FDCP-EpoRV617F cells were exposed to atiprimod in the presence or absence of stroma and after 4, 24, and 48 hours of culture and then cell aliquots were collected and subjected to immunoprecipitation and Western blot analysis. Whereas untreated FDCP-EpoRV617F cells consistently, at every time point, showed phosphorylation of JAK2, atiprimod exposure resulted in significant reduction of JAK2 phosphorylation without altering total JAK2 levels; JAK2 remained dephosphorylated.

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even 48 hours post–atiprimod treatment (Fig. 3A). However, if FDCP-EpoR-V617F were grown with HS5 or NK.tert stromal cells while being treated with atiprimod, this pattern was significantly altered. In the presence of stroma in culture, after 24 hours, phosphorylation of JAK2 was partially restored and, after 48 hours, the phosphorylation of JAK2 returned to a level similar to that of untreated cells (Fig. 3).

Because coculturing with stroma prevented the long-lasting inhibitory effect of atiprimod on JAK2-V617F phosphorylation and, further, because in vitro expression of JAK2-V617F results in constitutive activation of the downstream signaling effectors STAT3 and STAT5, we next examined the status of the latter under the same culture conditions. FDCP-EpoR-V617F and SET-2 cells were exposed to 1 μmol/L atiprimod in the presence or absence of HS5 or NK.tert (Supplementary Fig. S1A) or NK.tert (Supplementary Fig. S1B) stromal cells for 4, 24, and 48 hours. Atiprimod caused complete, long-lasting inhibition of STAT3 and STAT5 phosphorylation in both FDCP-EpoR-V617F and SET-2 cells. However, if FDCP-EpoR-V617F and SET-2 cells were treated with atiprimod in the presence of stroma, the atiprimod-induced dephosphorylation of STAT3 and STAT5 was transient, with phosphorylation fully restored within 48 hours (Supplementary Fig. S1A and B). Therefore, atiprimod inhibits signaling through the JAK–STAT axis in JAK2-V617F-positive cells (causes dephosphorylation of JAK2, STAT3, and STAT5), but this effect is abrogated over time (i.e., 48 hours) if JAK2-V617F-positive cells are cocultured with stroma.

Next, we repeated the aforementioned experiments by using peripheral blood and bone marrow mononuclear cells from PV patients instead of JAK2-V617F-positive cell lines. Treatment of PV cells with 1 μmol/L atiprimod for 48 hours resulted in their apoptosis (Fig. 4A). However, their treatment in the presence of HS5 stroma completely abrogated the proapoptotic activity of atiprimod (Fig. 4A). When primary PV cells were treated with 1 μmol/L atiprimod in the presence of HS5, NK.tert, or TM-R1 stroma, STAT3 phosphorylation was preserved (Fig. 4B), whereas it was significantly reduced if stromal cells were absent.

Stroma abrogates atiprimod-induced apoptosis of JAK2V617F-cells in a cell contact–independent manner

Given the protective effect of stroma on atiprimod-induced apoptosis and JAK2–STAT pathway dephosphorylation, we next explored the nature of the molecular cross-talk between JAK2-V617F-positive cells and stromal cells. To determine whether the protective effect of stroma on JAK2-V617F–carrying cells in coculture assays was dependent on cell-to-cell interactions, we carried out cocultures in which JAK2-V617F–positive cells (FDCP-EpoR-V617F and SET-2) were cultured either in direct contact with stromal cell monolayers (i.e., HS5, NK.tert,
TM-R1) or were separated from stroma by a 0.4-µm-thick micropore membrane that interrupted cell-to-cell contact but allowed them to be bathed by the same culture medium. Atiprimod (1 µmol/L) induced apoptosis in approximately 90% of JAK2V617F-positive cells but caused minimal or no apoptosis of any of the stromal cell lines (data not shown).

We exposed FDCP-EpoRV617F and SET-2 cells, which were cocultured either directly onto HS5, NK.tert, or TM-R1 stromal cell monolayers or separated from them by 0.4-µm-thick micropore membranes to prevent direct cell-to-cell contact, to atiprimod for 48 hours. Atiprimod failed to induce significant apoptosis regardless of whether JAK2V617F-positive cells were cocultured in direct contact with stroma or separated from stromal cells by a membrane (Fig. 5; Supplementary Fig. S2).

**Quantitation of secreted cytokines in coculture systems**

The fact that stroma protects JAK2V617F-positive cells from apoptosis without the requirement for direct cell-to-cell contact led us to hypothesize that humoral factors secreted by stromal cells may mediate such phenomenon. To investigate this hypothesis, the levels of 27 cytokines were measured in serum-free supernatants from coculture assays involving FDCP-EpoRV617F or SET-2 cells and HS5 or NK.tert stromal cells, separated by a membrane, in the absence or presence of 1 µmol/L atiprimod (Fig. 6). IL-6 levels changed significantly over time in the coculture system ($P < 0.00002$) in the presence of atiprimod; furthermore, CXCL-10/IP-10 levels changed ($P < 0.05$), although the fold increase observed was more modest. In addition, we noticed that FGF showed a marked upward trend in some of the cultures, but this varied depending on the cell types involved (Fig. 6). We therefore, examined the role of these 3 cytokines in subsequent experiments as representative of the changes in cytokine levels observed in our experiments.

**Neutralization of selected cytokines abrogates the protective effect of stroma on JAK2V617F-positive cells**

To determine whether elevated cytokines detected in supernatants from coculture assays were stroma derived and to confirm the potential paracrine protective effect of stroma on JAK2V617F-positive cells, we carried out several experiments.

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Figure 2. The effect of stromal cells on the atiprimod-induced apoptosis of cells expressing mutant JAK2V617F. A, FDCP-EpoRV617F cells were cultured alone (control), or with atiprimod, with or without HS5 stromal cells. Following 48-hour culture, cells were harvested, stained with Annexin-V, and the percentage of apoptotic cells was determined by flow cytometric analysis. Data from representative experiment are shown. Atiprimod-induced apoptosis was dramatically impaired when JAK2V617F-positive cells were cocultured with stromal cells. B, FDCP-EpoRV617F and SET-2 cells were cultured without (control) or with 3 different stromal cell monolayers (i.e., HS5, NK.tert, TM-R1) in the absence or presence of atiprimod for 48 hours. The induction of apoptosis was then assessed by flow cytometry. Results represent the mean ± SD from 3 independent experiments. GFP, green fluorescent protein.
First, given that IL-6, CXCL-10/IP-10, and FGF appeared elevated in supernatants from coculture assays, stromal monolayers (HS5, NK.tert, and TM-R1) were treated for 4 hours with monoclonal antibodies against IL-6, CXCL-10/IP-10, and/or FGF. Then, FDCP-EpoRV617F cells were exposed to 1 μmol/L atiprimod for 48 hours and cultured with each of the 3 pretreated stromal monolayers separated by 0.4-μm-thick micropore membranes. Pretreatment of stroma with IL-6, p-JAK2

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Figure 3. Stromal cells prevent phosphorylation of the JAK–STAT axis by atiprimod. FDCP-EpoRV617F cells were exposed to 1 μmol/L atiprimod alone (control), in the presence of HS5 stromal cells (top), or NK.tert stromal cells (bottom) for 4, 24, and 48 hours. Cells were then lysed and whole-cell lysates were immunoprecipitated with a rabbit anti-JAK2 antibody for detection of p-JAK2 (phosphorylated JAK2). Western blot analysis using antiphosphotyrosine antibody was carried out. Then, membranes were stripped and reprobed with anti-JAK2.

Figure 4. The effect of stromal cells on primary bone marrow and peripheral blood cells treated with atiprimod. A, peripheral blood mononuclear cells from PV patients were cultured alone (control), with atiprimod, with or without HS5 stromal cells. Following 48-hour culture, cells were harvested, stained with Annexin-V–FITC, and the percentage of apoptotic cells was determined by flow cytometric analysis. Data from representative experiment is shown. B, ex vivo bone marrow (BM) and peripheral blood (PB) mononuclear cells from patients with PV were cocultured with or without HS5, NK.tert, or TM-R1 stromal cells, with or without of 1 μmol/L atiprimod, for 48 hours. Total protein was extracted and subjected to 4%–12% NuPAGE Gel electrophoresis and immunoblotting membranes were stained with phospho-STAT3 (p-STAT3). Membranes were then stripped and reprobed with rabbit anti-total STAT3. PI, propidium iodide.
CXCL-10/IP-10, or FGF antibodies diminished the protective effect of stroma on FDCP-EpoRV617F cells against atiprimod-induced apoptosis (Fig. 7; Supplementary Fig. S3A and B). Pretreatment of stroma with a combination of 2 or 3 monoclonal antibodies resulted in an additive effect, with the highest neutralizing effect observed when all 3 antibodies were used simultaneously. We replicated these results using IL-6, CXCL-10/IP-10, and/or FGF antibodies in cocultures of human SET-2 and TM-R1 cell lines (data not shown).

Second, we addressed the question whether supernatants from cocultures of stromal and JAK2V617F cells would still be protective for JAK2V617F cells in the absence of stromal cells. FDCP-EpoR\textsuperscript{V617F} cells were cocultured indirectly (separated by 0.4-μm-thick micropore membranes) with HS5, NK.tert, or TM-R1 stromal cells, with or without 1 μmol/L atiprimod for 48 hours. Induction of apoptosis was assessed by flow cytometry to compare the direct or indirect effect of stromal monolayers.

**Discussion**

Given that forced expression of JAK2\textsuperscript{V617F} in cell lines confers constitutive STAT5 activation, cytokine-independent cell proliferation and enhanced cytokine sensitivity, targeted pharmacologic inhibition of JAK2\textsuperscript{V617F} kinase is an attractive therapeutic strategy for patients with MPNs (2) However, experimental evidence suggests that MPNs do not evolve simplistically from HSC intrinsic defects. Rather, they are heavily influenced by genetic and epigenetic events affecting the bone marrow niche (17). Alterations of the extrinsic regulation of HSC function have been shown to be capable of overriding intrinsic cues in vivo and giving rise to MPNs as observed in mice deficient for retinoic acid receptor gamma and in those null for the cell-cycle-regulator retinoblastoma protein (18). Therefore, in addition to genetic events in the malignant clone, nongenetically determined alterations of the bone marrow niche in patients with MPNs may be responsible for response and resistance to treatment (17). Clonal MPN cells engage in a constant cross-talk with the surrounding marrow niche directly via adhesion molecules (e.g., N-cadherin, CD44) or indirectly via humoral factors (17, 19). Among the latter, several circulating cytokines and chemokines involved in HSC/HP proliferation and mobilization, as well as promotion of fibrosis and angiogenesis (e.g., VEGF, TGFβ, IL-6), have been shown to be elevated in patients with PMF (8, 17). Although disrupting the cross-talk between the malignant clone and its milieu is an attractive therapeutic strategy in MPNs, little is known about the role of the abovementioned humoral factors in the pathogenesis of MPNs or in the response to MPN-directed therapy. To gain further insights into the biology of the MPN–stroma cross-talk, we explored the interaction between the bone marrow stromal cells and JAK2\textsuperscript{V617F}-positive cells in the context of JAK2 inhibitor therapy. First, we established culture conditions to model the effect of the marrow microenvironment on therapy response.
in MPNs by using coculture assays that employed different marrow stromal cells involving both murine cell lines and human primary cells from patients with MPNs. Potent JAK2 inhibition with atiprimod very effectively suppressed the growth and induced apoptosis of murine FDCP-EpoR^V617F- and JAK2^V617F-carrying human SET-2 cells while exerting negligible inhibition on stromal cells. However, when JAK2^V617F-positive cells were cultured on monolayers of stroma, the atiprimod-induced antiproliferative and pro-apoptotic effects were dramatically impaired, suggesting a protective effect of the stroma on JAK2^V617F-positive cells. It is important to emphasize that our coculture assays obliterated the potential confounding bias of using cells of murine origin as well as that of using stromal cell lines, in light of the fact that we observed similar results when primary MPN cells and stromal cells derived from patients with MPNs were employed in the assays. In addition, similar results were observed when cocultures of malignant and stromal cells were conducted in a species-mismatched manner.

We noted that the protective effect of stroma on the malignant clone during JAK2 inhibitor therapy relates to the fact that the former prevents the sustained inhibition of phosphorylation of JAK2, STAT3, and STAT5 observed when MPN cells are cultured in the absence of stroma. The full extent of the stromal-mediated abrogation of JAK2 inhibitor-induced apoptosis of MPN cells was observed when JAK2^V617F-positive cells were separated from stroma by micropore filters that prevented cell-to-cell contact while allowing both cell types to be exposed to the same medium in coculture assays. Thus, cell-to-cell contact does not appear essential for marrow stroma to extend its protective effect on the malignant clone. These results led us to hypothesize that the protection against therapy-induced apoptosis exerted by bone marrow stroma could be paracrine in nature. To test this hypothesis, we profiled a large panel of soluble factors in serum-free supernatants generated in cocultures of JAK2^V617F-positive cells on a variety of stromal cells, separated by membranes, both before and at different time points during atiprimod treatment. Although the levels of some cytokines varied depending on the cell types involved in the coculture, 3 cytokines—IL-6, CXCL-10/IP-10, and FGF—exhibited increasing levels over time in the presence of atiprimod. IL-6 is a pleiotropic cytokine that, on binding to its receptor, causes activation of the JAK2–STAT3 and ERK-1/2 pathways (20). Serum levels of IL-6 were elevated during progression of patients with ET and PV to myelofibrosis (21). FGF, a potent proangiogenic factor, has been implicated in the pathogenesis of MPNs (8, 22). Although the role of the chemokine CXCL-10/IP-10 in MPNs is yet to be defined, it has been shown to be constitutively secreted by acute myeloid leukemic blast cells (23). To support a putative role of these soluble factors in treatment resistance in our experiments, we utilized neutralizing monoclonal antibodies in coculture assays. Notably, pretreatment of stromal monolayers with neutralizing antibodies against IL-6, FGF, or CXCL-10/IP-10 markedly diminished the protective stromal effect and restored atiprimod-induced apoptosis of JAK2^V617F-positive cells. Our experiments showed a role for stroma-secreted cytokines (of which IL-6, FGF, and CXCL-10/IP-10 are representative from our experiments) in response to JAK2 inhibitor therapy. It is hypothesized that the inhibition of JAK2 in our coculture systems altered its intracellular pathway activation and that alternative pathways might have been engaged, as a result of which cellular interaction and cytokine production may have been modified.

In summary, we have standardized an in vitro culture system that allows the interrogation of the cross-talk between the malignant MPN clones and the stroma, as well as the influence of the latter on the former during therapy. This system has allowed us to identify a role for stroma-derived cytokines in protecting the malignant clones against JAK2-directed therapy. The therapeutic implications of these findings are of importance and may apply to other myeloid hematologic malignancies, as has already been shown for lymphoid malignancies such as CLL and ALL (9–11). Further research is warranted to
dissect the interactions between the nonhematopoietic marrow niche and the malignant hematopoietic clones in MPD. The development of a suitable *in vivo* model would be of immense importance due to limitations of *in vitro*/*ex vivo* laboratory evaluations.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**References**


Figure 7. Antibody-mediated neutralization of IL-6, CXCL-10/IP-10, and FGF restores atiprimod-induced apoptosis of FDCP-EpoR JAK2V617F cells in cocultures. HSS stromal cells were plated and cultured for 24 hours. Then, stromal cell monolayers were pretreated with normal goat serum as a control (1 μg), or with goat anti-hIL6, anti-hCXCL-10/IP-10, and/or anti-hFGF antibodies (0.3 μg), for 4 hours at 37°C. Then, 2 × 10^5/mL mouse FDCP-EpoRV617F cells were seeded in the presence of 1 μmol/L atiprimod. Control culture without atiprimod was also done. After 48 hours, JAK2V617F-expressing cells were collected and apoptotic cells were detected by flow cytometry by using recombinant human Annexin-V conjugated-APC. Data from representative experiment of 3 independent experiments are presented; summary results of all 3 experiments are also shown.


Bone Marrow Stroma–Secreted Cytokines Protect JAK2 V617F-Mutated Cells from the Effects of a JAK2 Inhibitor

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