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RESEARCH ARTICLE

# Targeting exhausted cytotoxic T cells through CTLA-4 inhibition promotes elimination of neoplastic cells in human myelofibrosis xenografts

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## Abstract

Myeloproliferative neoplasms represent a group of clonal hematopoietic disorders of which myelofibrosis (MF) is the most aggressive. In the context of myeloid neoplasms, there is a growing recognition of the dysregulation of immune response and T-cell function as significant contributors to disease progression and immune evasion. We investigated cytotoxic T-cell exhaustion in MF to restore immune response against malignant cells. Increased expression of inhibitory receptors like

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CTLA-4 was observed on cytotoxic T cells from MF patients together with a reduced secretion of IFNγ and TNFα. CTLA-4 ligands CD80 and CD86 were increased on MF granulocytes and monocytes highlighting a possible role for myeloid cells in suppressing T-cell activation in MF patients. Unlike healthy donors, the activation of cytotoxic T cells from MF patients was attenuated in the presence of myeloid cells and restored when T cells were cultured alone or treated with anti-CTLA-4. Moreover, anti-CTLA-4 treatment promoted elimination of neoplastic monocytes and granulocytes in a co-culture system with cytotoxic T cells. To test CTLA-4 inhibition *in vivo*, patient-derived xenografts were generated by transplanting MF CD34+ cells and by infusing homologous T cells in NSGS mice. CTLA-4 blockade reduced human myeloid chimerism and led to T-cell expansion in spleen and bone marrow. Overall, these findings shed light on T-cell dysfunction in MF and suggest that CTLA-4 blockade can boost the cytotoxic T cell-mediated immune response against tumor cells.

## 1 | INTRODUCTION

Chronic Philadelphia-negative myeloproliferative neoplasms (MPNs) represent a group of clonal hematopoietic disorders originating from a single hematopoietic stem cell (HSC), resulting in excessive production of mature blood cells. Among those, myelofibrosis (MF) is the most aggressive disease, characterized by leukocytosis, thrombocytosis, bone marrow (BM) fibrosis, rapid progression to acute myeloid leukemia, and a median survival of 5 years from diagnosis.[1,2]

Driver mutations, which include JAK2V617F, CALR indel, and MPL point mutations, are central to the pathogenesis of MF and all lead to the overactivation of the JAK–STAT signaling pathway.[2,3] While the JAK1/2 inhibitor Ruxolitinib, introduced in clinical practice as a targeted therapy, effectively mitigates debilitating symptoms and enhances overall survival, it falls short of eradicating the neoplastic clone.[2] HSC transplantation represents the only curative treatment, but it is often unsuitable for elderly MF patients due to its high risk of complications.[4] This underscores the unmet need for treatments that effectively target the malignant clone.

In the context of MPNs, there is a growing recognition that a combination of dysregulation of immune response and altered T-cell function plays a critical role in disease progression and immune evasion.[5] The abnormal clonal expansion of myeloid cells leads to the production of pro-inflammatory cytokines and changes the composition of the BM microenvironment.[6,7] As reported for various solid tumors, these alterations, combined with persistent antigen stimulation within the tumor microenvironment, can induce the differentiation of T effector cells into terminally exhausted T cells.[8,9] Such cells exhibit impaired function in recognizing and eliminating malignant cells, thereby compromising the overall immune surveillance mechanisms against the disease.[8] Specifically, exhausted T cells may express checkpoint inhibitory receptors (IRs), including PD-1 and CTLA-4, which effectively suppress T-cell activation upon ligand binding. This suppressive mechanism is frequently exploited by cancer cells that evade the immune system by upregulating IR ligands, such as PD-L1 and CD80/CD86.[10,11] In line with this, targeting IR pathways can reverse the T-cell dysfunctional state and stimulate the immune response against malignant cells, with FDA-approved anti-PD-1 and anti-CTLA-4 monoclonal antibodies leading the way in the treatment of several cancers.[12]

Because of the remarkable success of immune checkpoint inhibitors in many malignancies, there has been growing attention towards investigating immunology in MF. In particular, MF stands as a representative model for studying the immune response against neoplastic cells since T cells are wild-type and do not belong to the malignant clone.[13] Interestingly, increased expression of PD-L1 has been observed in JAK2V617F-mutant MPN cells,[14] while anti-PD-1 treatment has shown promise for CALR-mutant MPNs by restoring antigen-specific T-cell immunity *in vivo*.[15] Nevertheless, a phase 2 clinical trial evaluating Pembrolizumab (monoclonal antibody targeting PD-1) has been conducted in patients with advanced MF but yielded no objective clinical response.[16] Many factors influence the effectiveness of checkpoint blockade, including tumor mutation burden, antigen processing and presentation, and the tumor microenvironment. These findings underscore the need to deepen our understanding of the crosstalk between wild type T cells and malignant cells, as well as to explore the role of additional immune checkpoint pathways in MF immune escape.

Here, we identify an exhausted cytotoxic T-cell population expressing multiple IRs and with reduced cytokine production in MF patients. Furthermore, we characterize an immunosuppressive interplay between T cells and IR ligand-expressing MF myeloid cells. We demonstrate that anti-CTLA-4 treatment reactivates CD3 + CD8+ T-cell response both in vitro and in a PDX mouse model, promoting T cell-mediated clearance of malignant cells in vivo.

## 2 | METHODS

### 2.1 | Human studies

The present study was approved by local ethical committees: Comitato Etico dell'Area Vasta Emilia Nord (AVEN) for Azienda Ospedaliero-Universitaria (AOU) Policlinico di Modena, University of Modena and Reggio Emilia in Modena, Italy; Comitato Etico Regionale per la Sperimentazione Clinica della Regione Toscana, sezione area vasta centro for AOU Careggi, Florence, Italy and Ospedale ASST Sette Laghi, Varese, Italy; and Comitato Etico di Area Vasta Romagna (CEROM) for IRCCS Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori", Meldola, Italy. Peripheral blood (PB) from patients with a diagnosis of MF were provided by the above-mentioned institutions. MF was diagnosed according to the 2016 World Health Organization criteria.[17] Both male and female patients were included. A total of 57 MF patients were included in the study (median age 68 years, range 44–83), 27 females and 30 males. PB from healthy donors (HDs) was collected from Azienda Ospedaliero-Universitaria (AOU) Policlinico di Modena, University of Modena and Reggio Emilia according to the institutional guidelines for discarded material. A total of 25 gender-matched HDs, 12 males and 13 females, were included (median age 58 years, range 41–68 years).

This study was conducted in accordance with the Declaration of Helsinki under the local Institutional Review Board's approved protocol. All subjects involved in the study provided informed written consent. A list providing characteristics of MF patients is provided in Table S1. Mutation analysis was performed using the Sophia Myeloid Plus Solution™ kit (Sophia Genetics) on genomic DNA extracted from purified circulating granulocytes or whole PB; Illumina MiSeq instrument was used for sequencing.

### 2.2 | Mouse strains

All animal studies were reviewed and approved by the Italian Ministry of Health (approval #686/2018 PR and #996/2023-PR). All mice were maintained under specific pathogen-free (SPF) conditions at the experimental animal facility of the University of Modena and Reggio Emilia (Modena, Italy). The NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Wjl}$ Tg(CMV-IL3, CSF2,KITLG)1Eav/MloySzJ, also known as NSG-SGM3 or NSGS, mouse strain used for the experiments was purchased from The Jackson Laboratory (strain #013062). A live colony of NSGS was maintained at the facility by breeding NSGS female and male mice. Both female and male mice were used experimentally.

### 2.3 | Co-culture system

Mononuclear cells (MNCs) and isolated T cells were used to establish a co-culture system similar to the methods described by Ferraro et al (2021).[18] For every donor, either MF or HD, MNCs and negatively isolated T cells were resuspended in T-cell culture medium (10% FBS (Sigma), 2 mM L-Glu, 50 μM 2-ME, 100 μM NEAA, 25 mM HEPES, P/S 1X in RPMI 1640 medium (Gibco)) supplemented with the following human cytokines: IL-15 10 ng/mL, IL-7 10 ng/mL, Flt3 ligand 10 ng/mL, SCF 10 ng/mL, IL-6 10 ng/mL, TPO 10 ng/mL (Miltenyi Biotec). 0.5*10$^6$ MNCs or T cells were seeded in a 48-well flat bottom plate (Corning). The number of T cells contained in each fraction (MNCs or isolated T cells) was determined by flow cytometry. T cells were then activated by adding 25 μL of CD3/CD28 activating beads (Dynabeads, Invitrogen) for 1*10$^6$ T cells according to manufacturer's instruction. When needed, checkpoint inhibitors or isotype control IgG were added to the cells at the concentration of 10 μg/mL, according to conditions reported in Table S2. Cells were then incubated in a humidified $CO_2$ incubator at 37°C for four days. On the 4th day of culture cells were harvested, CD3/CD28 activating beads were magnetically removed according to the manufacturer's instruction, and cells were stained for flow cytometry analysis.

### 2.4 | *In vivo* treatment

PDX mice were established as described in Supporting Information Methods section by intra femoral transplantation of CD34+ cells from patients Pt_21 and Pt_25. Four weeks after the transplantation, PB was collected from the submandibular vein, PB engraftment was assessed via flow cytometry and used to equally sort mice in three distinct group: (1) Untreated PDX; (2) PDX receiving MF T cells and IgG1k; (3) Mice receiving MF T cells and anti-CTLA-4. Six weeks post transplantation mice belonging to group (2) and (3) received an intravenous (IV) injection of 1–5*10$^6$ expanded T cells (T cell expanded from the same patients donating CD34+ cells for the PDXs) and began IgG1k or anti-CTLA-4 (Ipilimumab clone) (MedChemExpress) treatment at the same time. IgG1k 10 mg/kg or Ipilimumab 10 mg/kg were administered once a week, for 4 weeks, via intraperitoneal (IP) injection. Mice underwent blood withdrawal from the submandibular vein at weeks 4, 8 and 10 following the transplantation to monitor PB engraftment via flow cytometry. Ten weeks after the transplantation, mice were sacrificed and PB, spleen, femurs, hips and tibias were collected. Spleen and bones were processed to obtain cells. PB, spleen and BM cells were used for flow cytometry.

## 3 | RESULTS

### 3.1 | Cytotoxic T cells from MF patients show expression of multiple IRs and reduced cytokine production

To investigate the immune response in MF, we characterized IR expression on cytotoxic CD3+CD8+ T cells from the PB of 57 MF patients naive to JAK-inhibitor treatment and 25 HDs. We assessed the following IRs by flow cytometry: PD-1, CTLA-4, TIM-3, LAG-3, CD160, and CD244 (Figure S1.) This analysis highlighted the presence in MF patients of a population of cytotoxic T cells expressing

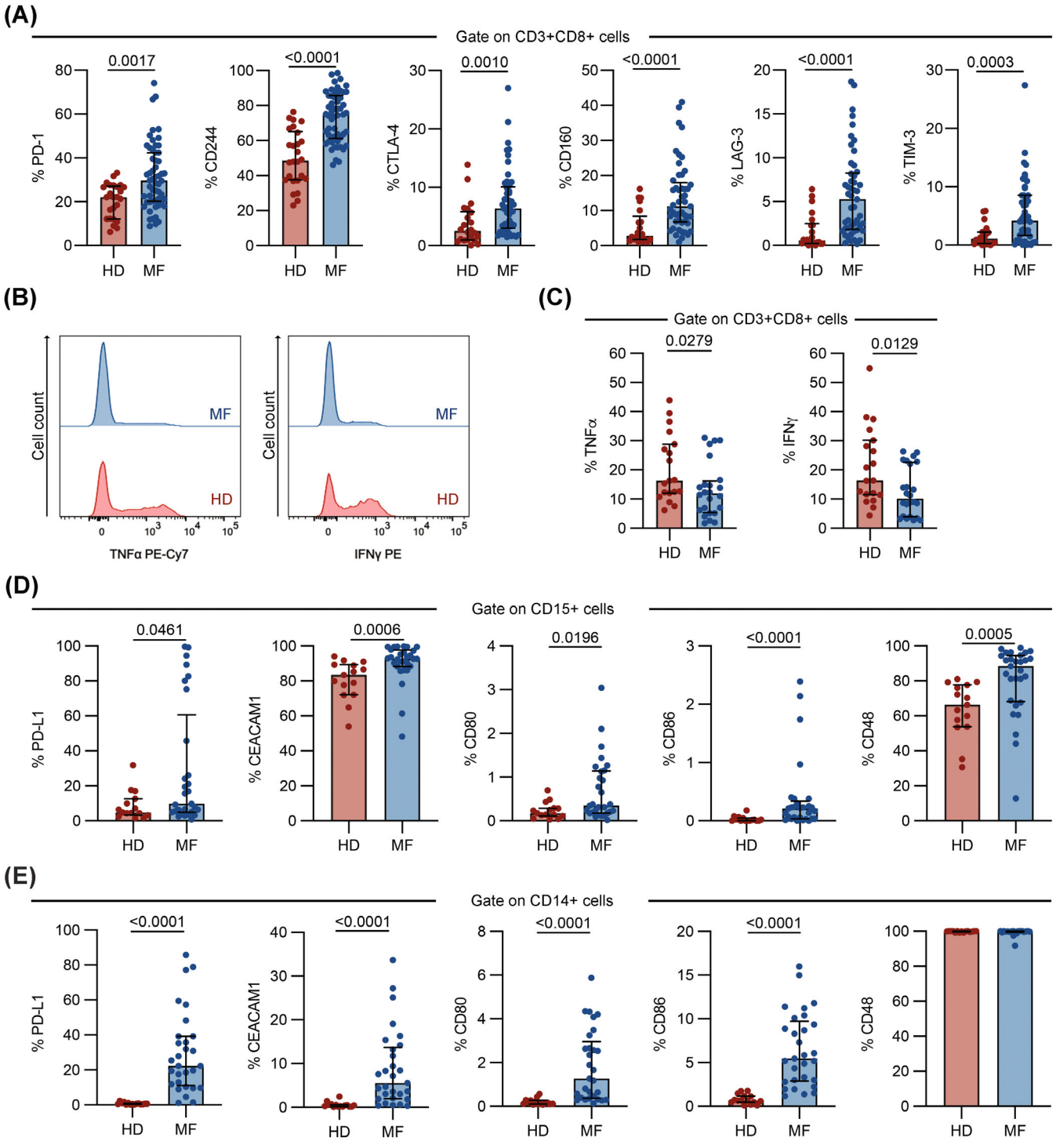


**FIGURE 1** Cytotoxic T cells from MF patients show evidence of phenotypic and functional exhaustion while MF myeloid cells express higher levels of IR ligands. (A), Quantification of IRs expression on CD3+CD8+ T cells, each point represents a subject ($n = 49–56$ for MF and 21–25 for HD), and each subject was analyzed in an individual experiment. Statistical significance was assessed by Mann–Whitney U test, scatter plot reports median with interquartile range. (B), Representative histograms showing the expression of TNFα (left) and IFNγ (right) on cytotoxic T cells from HD (red) and MF patients (blue). (C), Quantification of cytokines produced by cytotoxic T cells in 12 experiments, each point representing a subject ($n = 24$ for MF and 19 for HD). Statistical significance was assessed by Mann–Whitney *U* test, scatter plot reports median with interquartile range. Flow cytometry quantification of IR ligands expression on (D), CD15+ cells and (E), CD14+ cells, each point represents a subject ($n = 29$ for MF and 15 for HD), each subject was analyzed in an individual experiment. Statistical significance was assessed by Mann–Whitney *U* test; scatter plot reports median with interquartile range. HD, healthy donor; MF, myelofibrosis.

increased levels of all the evaluated IRs (Figure 1A). Moreover, the frequency of exhausted CD57-PD-1+ cells was increased within cytotoxic T cells in MF patients compared to HDs (Figure S2A), with a higher proportion of cells expressing the investigated IRs, either alone or in combination (Figure S2B,C).[19–21] We next assessed the residual cytotoxic function of CD3+CD8+ T cells by measuring cytokine production after *in vitro* stimulation. In this setting, CD3+CD8+ T cells exhibited a 0.27 (16.30% vs 11.90%) and 0.38 (16.40 vs 10.15)-fold reduction of TNFα and IFNγ expressing cells respectively when compared to HDs (Figure 1B,C).

## 3.2 | Higher IR expression on MF cytotoxic T cells correlates with detrimental clinical features

The patients' cohort included 40 JAK2V617F-mutant, 12 CALR-mutant, 2 MPL-mutant, and 3 triple negative MF patients (Table S1). Our results demonstrate that within CD3+CD8+ T cells the frequencies of IR-positive and of CD57-PD-1+ cells do not change across the different groups (Figure S3A–C). We next correlated the patients' clinical features with IR expression on CD3+CD8+ T cells regardless of the driver mutation type. Higher frequencies of CTLA-4-positive cells were observed in patients with at least 1 high molecular risk (HMR) mutation and higher BM fibrosis grade. Increased proportion of PD-1-positive cells was correlated with hemoglobin levels <10 g/dL, splenomegaly, transfusion dependency and high Dynamic International Prognostic Scoring System (DIPSS) categories (intermediate-2 and high) (Table S3). Correlations with other prognostic risk factors were observed for all the IRs assessed demonstrating that increased numbers of IR-positive cytotoxic T cells correlate with more aggressive disease phenotype (Table S3).

## 3.3 | IR ligands are increased on granulocytes and monocytes from MF patients

To characterize how the malignant cells affect the immune response, we assessed the expression of IR cognate ligands on myeloid cells belonging to the neoplastic clone. The mRNA expression level of selected IR ligands was first evaluated in granulocytes from 141 MF patients, of which 114 came from an already published dataset[22] and compared with granulocytes from 28 HDs. This analysis revealed the increased expression of multiple IR ligands in MF granulocytes, including *CD274* (encoding PD-L1), *CEACAM1*, *CD80*, and *CD48* mRNA, whose protein products bind PD-1, TIM-3, CTLA-4, and CD244, respectively (Figure S4). These results were then validated by multiparametric flow cytometry on PB myeloid cells, including granulocytes and monocytes.

In MF patients, we observed an increased frequency of granulocytes and monocytes positive for PD-L1, CEACAM1, CD80, and CD86 (another ligand of CTLA-4), with the former population being also positive for CD48 (Figure 1D,E). Interestingly, the frequencies of IR ligands expressing cells were increased within the MF hematopoietic stem and progenitor cells (HSPC) compartment with a significant increase of CD48-positive and PD-L1-positive cells (Figure S5). Collectively, these data suggest the existence of an immune suppressive interplay between CD3+CD8+ T cells and myeloid neoplastic cells.

## 3.4 | T-cell cytotoxic activity was curbed by neoplastic myeloid cells and restored by anti-CTLA-4 treatment *in vitro*

To confirm the hypothesized dysfunctional interaction, we developed a co-culture system to test the activation of cytotoxic T cells. PB derived-CD3+ T cells from HDs or MF patients were cultured alone or by plating total MNCs, which contain a mixture of T cells and myeloid cells. T-cell activation was achieved by using anti-CD3/CD28 microbeads and assessed on day 4 by flow cytometric analysis measuring the percentage of OX40-positive CD3+CD8+ T cells (Figure 2A).[18] We found that CD3+CD8+ T cells from HDs showed equally effective activation when stimulated by anti-CD3/CD28 microbeads either alone or in co-culture with myeloid cells (29.90% vs 23.05%) (Figure 2B). Conversely, the microbead-induced activation of MF CD3+CD8+ T cells was attenuated in the presence of myeloid cells belonging to the neoplastic clone as shown by reduced frequencies of OX40-positive cytotoxic T cells (31.60% vs 5.75%) (Figure 2C). Strikingly, cytotoxic T cells obtained from MF patients with higher grade of BM fibrosis were less responsive to microbead-induced activation when co-cultured with malignant cells (12.00% vs 4.44%) (Figure 2D).

Since this co-culture system recapitulates the immune suppressive interaction between T cells and myeloid cells occurring *in vivo*, we exploited it to test the ability of different immune checkpoint inhibitors to restore the defective activation of MF cytotoxic T cells. Among the tested monoclonal antibodies, anti-CTLA-4 (Ipilimumab), anti-PD-1 (Nivolumab), and anti-TIM-3 (Sabatolimab) showed efficacy in reactivating MF T-cell response in the presence of myeloid cells, as demonstrated by the increased percentage of OX40-positive CD3+CD8+ T cells compared to isotype control-treated samples (Figure 2E–G). Conversely, antibodies against CD244 and CD160 proved poorer or no efficacy, respectively (Figure S6A,B).

Since anti-CTLA-4 monoclonal antibody was the most effective in restoring CD3+CD8+ T-cell activation (Figure 2H), we decided to further focus on CTLA-4 inhibition. To this end, we assessed changes in the numbers of lymphoid and myeloid cells in the co-culture system. While anti-CTLA-4 treatment failed to induce significant changes in CD3+, CD3+CD8+, and CD19+ cell numbers, as well as in malignant CD34+ cell numbers, it elicited a significant T cell-mediated killing of CD14+ and CD15+ myeloid populations (0.41 and 0.27 fold reduction respectively) (Figures 2I and S7). Interestingly, we observed a heterogeneous response to anti-CTLA-4 treatment with some patients showing a poor T cell-mediated killing of monocytes and granulocytes. Linear regression analysis showed that

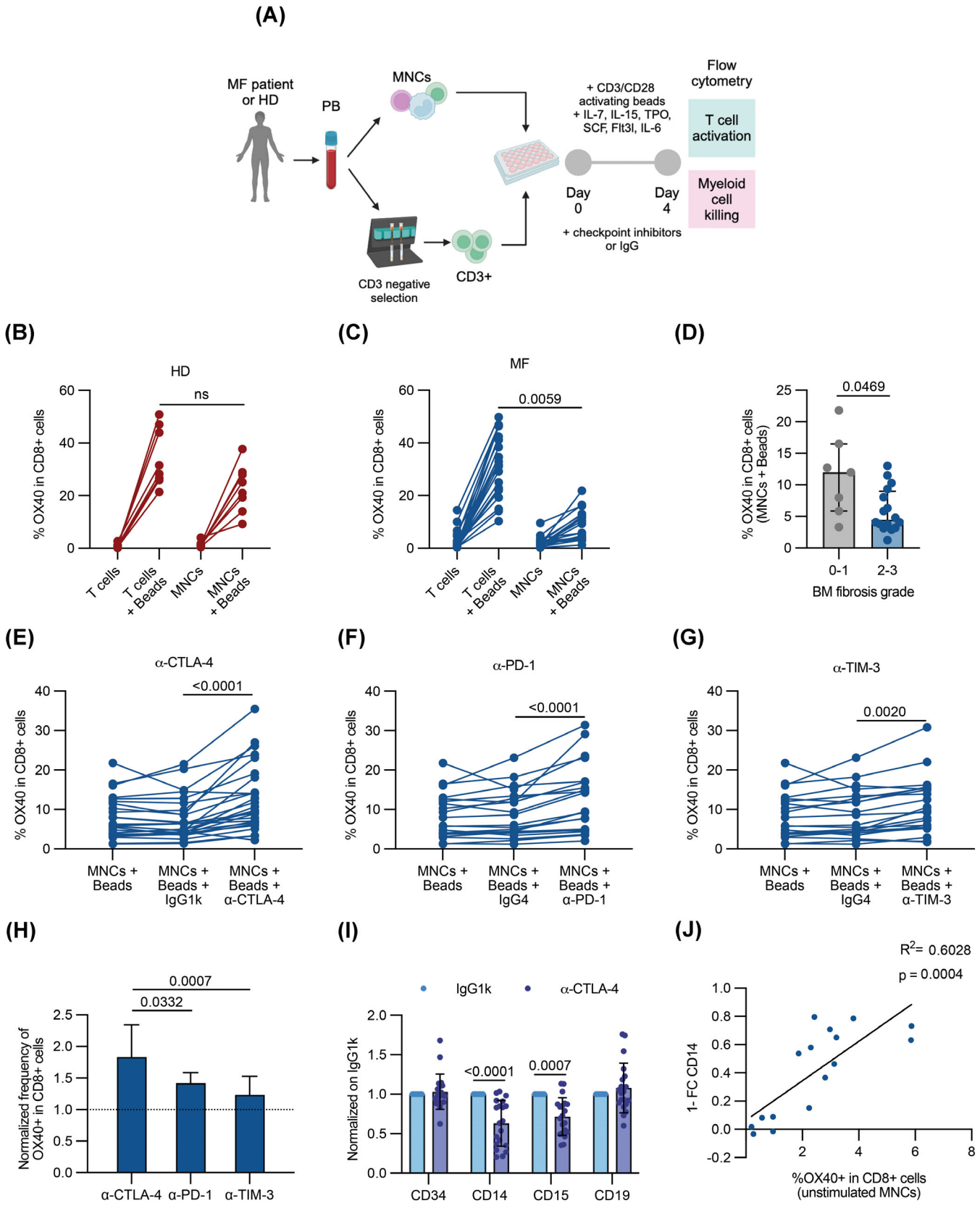


**FIGURE 2** Legend on next page.

low basal frequencies of OX40+ cells within CD3+CD8+ T cells correlated with poor cytotoxic activity *in vitro* ($R^2 = 0.6028$, *p*-value = .0004) (Figure 2J).

Collectively, our *in vitro* characterization demonstrated that neoplastic myeloid cells expressing IR ligands may be responsible for the impaired activation of cytotoxic T cells in MF patients. Administration of immune checkpoint inhibitors such as anti-CTLA-4 monoclonal antibody can significantly restore cytotoxic T-cell function promoting the elimination of myeloid cells.

### 3.5 | MF patient-derived xenograft (PDX) mice showed IR ligand-positive myeloid cell production but lacked human T cells

To investigate the *in vivo* effect of anti-CTLA-4 treatment, we developed a PDX mouse model by performing intrafemoral injection of human MF CD34+ cells in sub-lethally X-ray irradiated NSGS mice (Figure 3A). To assess human cell engraftment, we evaluated the expression of hematopoietic cell-specific human CD45 (hCD45) by flow cytometry in the PB, spleen (SP) and BM at terminal readout. Within hCD45-positive cells, we then identified cells belonging to the myeloid, B lymphoid and T lymphoid lineages together with HSPCs through the expression of human CD33, CD19, CD3, and CD34, respectively (Figures 3B–D and S8A,B). Results showed that MF CD34+ cells were able to engraft in PDX mice and demonstrated a clear myeloid bias at 12 weeks post transplantation, with a nearly exclusive production of myeloid cells and failure to generate B cells as well as CD3+ T cells in all the assessed compartments (Figure 3B–D). Given the prevalence of human myeloid cells generated in the PDX mice, we next asked whether these cells retained the expression of CTLA-4 ligands *in vivo*. Our findings revealed that PDX mice consistently generated human CD14-positive cells expressing CD80 and CD86 (Figures 3E,F and S8C). Overall, generation of IR ligand-positive myeloid cells indicates that MF PDX mice constitute a suitable model to assess the efficacy of anti-CTLA-4 treatment *in vivo*.

### 3.6 | Anti-CTLA-4 treatment promoted T-cell mediated killing of neoplastic myeloid cells in vivo

To test the effect of anti-CTLA-4 treatment *in vivo*, we generated PDX mice by transplanting CD34+ cells as above mentioned. To reconstitute the immune system in these mice, we expanded human homologous CD3+ T cells *in vitro* and infused them intravenously into the corresponding PDX mice 6 weeks later. Interestingly, we were able to successfully expand *in vitro* T cells only from MF patients with a non-severe disease phenotype (defined as patients with a low or intermediate-1 DIPSS score and/or BM fibrosis grade 0–2) (Figure S9). Following this, PDX mice were treated with an anti-CTLA-4 antibody, an isotype control, or left untreated for 4 weeks (Figure 4A).

At sacrifice, we first assessed whether the injected immune cells lingered *in vivo* and detected human CD3+ T cells only in the SP and BM of anti-CTLA-4 receiving animals (Figure 4B,C). To further investigate the effects of immune checkpoint inhibition on CD3+CD8+ T-cell cytotoxic activity *in vivo*, we analyzed the human neoplastic compartment and observed reduced myeloid chimerism in anti-CTLA-4 treated mice. Anti-CTLA-4 monoclonal antibody treatment dramatically decreased granulocyte numbers in SP and BM as well as monocyte numbers in the BM (Figure 4D,E). Strikingly, HSPCs, already detectable in the BM of PDX mice 10 weeks post-transplantation, were reduced by the anti-CTLA-4 treatment (Figure 4F).

Collectively, our results demonstrate that CTLA-4 blockade stimulates CD3+CD8+ T-cell activation promoting eradication of the neoplastic clone *in vivo*.

## 4 | DISCUSSION

In this study, we comprehensively investigate the functional state of cytotoxic T cells in MF patients and explore whether their impairment could depend on the interaction with malignant cells. Our findings uncover novel mechanisms of immune evasion in MF that can be

**FIGURE 2** *In vitro* co-culture system evidences an immunosuppressive interplay targetable by CTLA-4 inhibition. (A) Schematic outline of co-culture system. (B, C) Flow cytometry evaluation of OX40+ activated CD8+ T cells in HDs (B) and MF patients (C). A total of 8 HDs and 21 MF patients were analyzed in 8 independent experiments. Statistical significance was assessed by Kruskal-Wallis test. (D) Clinical correlation analysis of OX40+ activated CD8+ T cells from MF patients with BM fibrosis grade. Each point represents a patient ($n = 23$), statistical significance was assessed by Mann–Whitney U test, scatter plot reports median with interquartile range. (E–G) Flow cytometry evaluation of OX40+ activated CD8+ T cells in our *in vitro* co-culture system. Total MNCs from MF patients were treated with isotype control immunoglobulins or monoclonal antibodies. A total of 27 (E), 21 (F) and 21 (G) MF patients were analyzed in 7 independent experiments. Statistical significance was assessed by Friedman uncorrected Dunn's test. (H) Flow cytometry measurement of OX40+ activated CD8+ T cells in MF patients normalized on IgG1k or IgG4 control samples. The dashed line in the graph indicates the normalized frequency of OX40+ cells within CD8+ cells in IgG treated samples. Statistical significance was assessed by Kruskal-Wallis uncorrected Dunn's test, scatter plot reports median with interquartile range. $n = 20–27$ (I) Flow cytometry measurement of CD34+, CD14+, CD15+ and CD19+ cells, in the CD3- live cells fraction, normalized on IgG1k control samples. Each point represents a patient, a total of 18 patients were analyzed in 6 independent experiments. Statistical significance was assessed by paired t-test, scatter plot reports median with interquartile range. (J) Simple linear regression analysis between OX40 expression level of unstimulated cytotoxic T cells and 1-FC CD14 (representing killing capacity against CD14+ cells by the same cytotoxic T cells treated with anti-CTLA-4), each point represents a patient, a total of 16 patients were analyzed in 6 independent experiments. Beads, CD3/CD28 activating beads; HD, healthy donor; MF, myelofibrosis; MNCs, mononuclear cells; ns, not statistically significant.

targeted and reversed through checkpoint inhibitor treatment. Previous reports have highlighted a compromised T-cell phenotype in MPNs, describing an increased PD-1 expression on MF T cells[15,23,24] and elevated expression of CTLA-4 and other IRs in CALR-mutant MPNs.[15] In malignant myeloid cells, the expression of PD-L1 is positively correlated with JAK2V617F mutational burden and is elevated in various cell types, including monocytes, granulocytes, myeloid-derived suppressor cells, and platelets.[14,24]

In this study, we extended these insights by characterizing the immune response of a broad cohort of triple-negative, JAK2V617F-, CALR-, and MPL-mutant MF patients ($n = 57$). For the first time, we provide a concurrent holistic overview of the interplay between

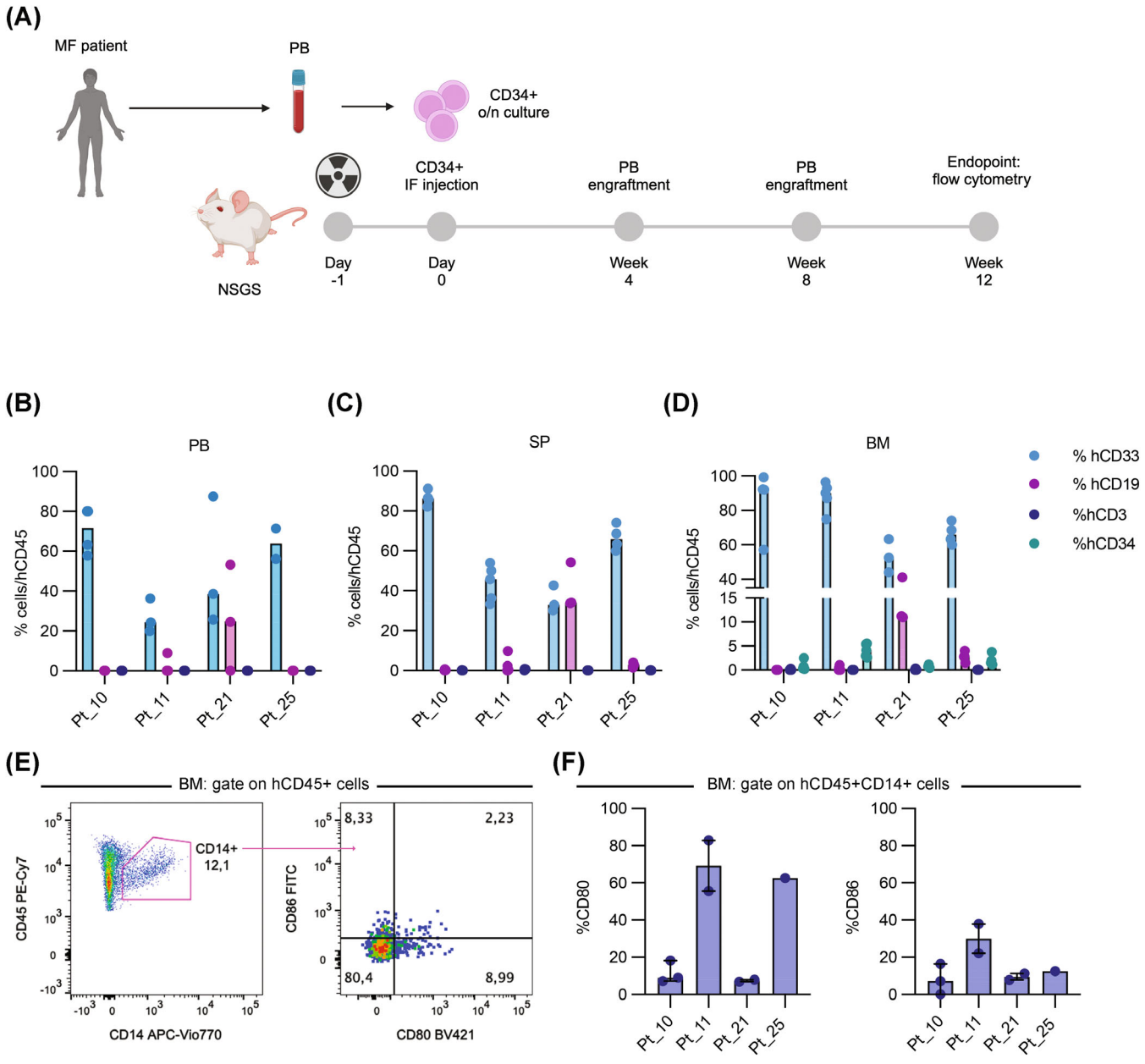


**FIGURE 3** PDX mice transplanted with MF CD34+ cells develop myeloid-based hematopoiesis and fail to generate human T cells. (A) Schematic overview of the generation of PDX *in vivo* model with primary CD34+ cells isolated from the PB of MF patient. (B–D) Flow cytometry evaluation of CD33+, CD19+, and CD3+ human cells in (B) PB and (C) SP and of CD33+, CD19+, CD3+, and CD34+ human cells in (D) BM of PDX mice. Four patients were transplanted in four independent experiments. Each point represents a different mouse (B) $n = 2–4$, (C) $n = 3–5$; and (D) $n = 3–5$ for each group. (E) representative flow cytometry scatter plots of stained cells from BM of PDX mice, CD14+ cells gate was designed on human CD45+ cells (plots not shown) and CD80 and CD86 expression was assessed on CD14+ gated cells. (F) Flow cytometry quantification of CD80 and CD86 expression on human CD45+ CD14+ cells in the BM of PDX mice. Four patients were transplanted in four independent experiments. Each point represents a different mouse, for each group $n = 1–3$, and bar plots represent the median. BM, bone marrow; h, human; IF, intra-femoral; MF, myelofibrosis; o/n, overnight; PB, peripheral blood; SP, spleen.

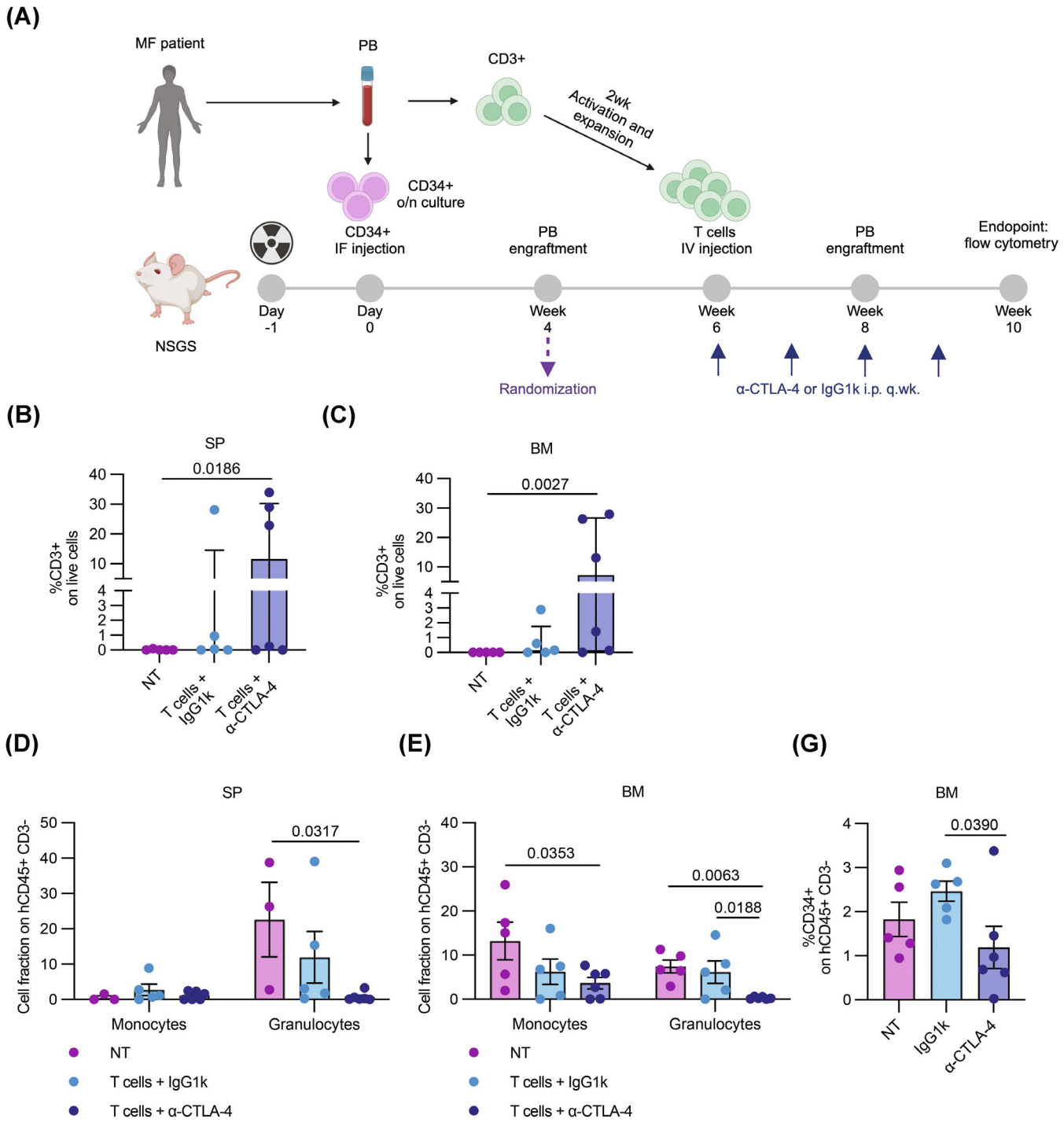


**FIGURE 4** Anti-CTLA-4 treatment efficiently expands infused T cells and kills MF myeloid cells in a PDX mouse model. (A) Schematic overview of MF PDX *in vivo* model generated by transplanting primary CD34+ cells in the femur and infusing expanded CD3+ cells from the same patient 6 weeks later for anti-CTLA-4 treatment. (B, C) Flow cytometry quantification of human CD3+ cells among live cells in the SP (B) and BM (C) of PDX mice. (B, C) Each point represents a mouse transplanted with CD34+ cells from patients Pt_21 or Pt_25 in three independent experiments, for each group $n = 5–6$. Statistical significance was assessed by Kruskal-Wallis uncorrected Dunn's test, scatter plot reports median with interquartile range. (D, E) Flow cytometry quantification of monocytes and granulocytes among human CD45+ CD3- cells in (D) SP and in (E) BM of PDX mice. Each point represents a mouse transplanted with CD34+ cells from patients Pt_21 or Pt_25 in three independent experiments, for each group $n = 3–6$ in (D) and $n = 5–6$ in (E). Statistical significance was assessed by ordinary one-way ANOVA, scatter plot reports mean ± standard error of the mean (SEM). (F) Flow cytometry quantification of CD34+ cells among human CD45 + CD3- cells in BM of PDX mice. Each point represents a mouse transplanted with CD34+ cells from patients Pt_21 or Pt_25 in three independent experiments, for each group $n = 5–6$. Statistical significance was assessed by ordinary one-way ANOVA, scatter plot reports mean ± standard error of the mean (SEM). BM, bone marrow; IF, intra-femoral; i.p., intra-peritoneal; IV, intra-venous; MF, myelofibrosis; o/n, overnight; PB, peripheral blood; q.wk., once a week; SP spleen; wk weeks.

increasingly exhausted T cells and MF malignant cells. Moreover, to gain a clear understanding of the immune landscape in MF, we analyzed samples from patients naïve to JAK inhibitor treatment, as this is known to impair T-cell function in MPNs.[25]

Regardless of their mutational status, all MF patients carry cytotoxic T cells expressing multiple IRs, such as PD-1, CTLA-4, LAG-3, TIM-3, CD160, and CD244. In particular, PD-1 expression cannot be considered a *bona fide* exhaustion marker per se as it serves a physiological immunoregulatory role allowing the immune system to strike a balance between activation and self-antigen tolerance.[26] Therefore, we herein report the co-expression of multiple IRs, which is considered a hallmark feature indicating a more definitive cytotoxic T-cell exhaustion state.[19,27] Since an in-depth characterization of CD4+ T-cell exhaustion has yet to be provided, in this work we focused our attention on the investigation of the CD8+ T-cell population.[28] We show that MF CD3+CD8+ T cells present increased frequencies of cells expressing multiple IRs and exhibit diminished production of TNFα and IFNγ compared to HD cells, suggesting a functional impairment. Moreover, correlation analysis between patients' clinical features and IR expression on CD3+CD8+ MF T cells suggests a more aggressive disease phenotype in patients with increased expression of IRs. Although we did not detect any statistically significant differences, we cannot exclude that the expression of some IRs may be affected by the presence of different driver mutations. Ad hoc studies will be necessary to elucidate whether the type of driver mutation may impact the effectiveness of antitumor immune response in MF patients.

To suppress T-cell function, IRs need to be engaged by membrane-bound ligands, which are often expressed by cancer cells to avoid detection by immune cells. Previous studies have indeed recognized JAK2V617F-driven expression of PD-L1 as a mechanism mediating immune escape in MPNs.[14] As MF T cells express various IRs associated with an exhausted state, we carried out further analysis on the expression of their corresponding IR ligands on mature myeloid cells, to unveil cell–cell interactions contributing to T-cell dysfunction. Our findings reveal that MF monocytes and granulocytes exhibit various cognate IR ligands, suggesting multiple receptor-ligand interactions that may play a role in immune suppression in MF. Interestingly, we establish that MF T-cell impairment is strictly dependent on the interaction with myeloid cells in an *in vitro* co-culture system, with MF cytotoxic T cells able to display activation features only when cultured alone. Additionally, T cells from patients with a more severe phenotype exhibited a limited response to activating stimuli, in line with existing evidence linking an extensive exposure to malignant antigens to a more severe T-cell exhaustion.[8] Overall, the establishment of this *in vitro* system allowed us to test different immune checkpoint inhibitors, including antibodies against CTLA-4, PD-1, TIM-3, CD244, and CD160. Although anti-PD-1 and anti-TIM-3 antibodies were somewhat effective *in vitro* in stimulating T-cell activity, we chose anti-CTLA-4 treatment for further validation, as it resulted in the strongest T-cell activation and has never been tested in MF patients in a clinical trial, unlike the other immune checkpoint inhibitors. In particular, the anti-PD1 monoclonal antibody Pembrolizumab failed to provide a clinical benefit in MF patients enrolled in a phase 2 clinical trial (NCT03065400),[16] while results from another clinical trial on Sabatolimab (an anti-TIM-3 monoclonal antibody, NCT04097821) in MF patients have yet to be reported. In addition, a new clinical trial (NCT05393674) investigating the effects of Nivolumab (anti-PD-1 monoclonal antibody) in combination with Fedratinib in patients with MF is currently recruiting. Further characterization of our established *in vitro* system revealed that anti-CTLA-4-treated MF T cells showed both immunophenotypic and functional reactivation features with selective killing of myeloid cells.

As the *in vitro* system cannot accurately mimic several factors affecting immune cell functions, our aim was to investigate whether anti-CTLA-4 treatment could reinstate immune surveillance against cancer cells *in vivo*. To explore this, we generated a PDX mouse model by transplanting human HSPCs from MF patients. As MF HSPCs have historically proven difficult to transplant, we employed a recently established xenograft system using NSGS mice, which present a "humanized" environment with expression of human cytokines stem cell factor, granulocyte-macrophage colony-stimulating factor, and interleukin-3, promoting myeloid differentiation.[29] Interestingly, myeloid cells expressed IR ligands in engrafted NSGS mice, thus serving as an ideal platform for the preclinical evaluation of immune checkpoint blockade. Given the presence of these cytokines and the inherently biased differentiation of MF HSPCs towards the myeloid lineage, we further refined this system by introducing at a later stage MF T cells, which are not generated in this PDX model. *In vivo*, anti-CTLA-4 treatment led to T-cell expansion with significant infiltration in the SP and the BM. Strikingly, we observed a marked reduction of neoplastic HSPCs and of myeloid chimerism with a nearly complete eradication of human monocytes and granulocytes.

Collectively, these data suggest an effective anti-CTLA4-mediated reactivation of cytotoxic T-cell activity against cancer cells *in vivo*.

It is important to note that the established mouse model system relies on the prior expansion of T cells that will be infused *in vivo*. Terminally exhausted T cells respond poorly to Interleukin-7 and Interleukin-15 and require continual interaction with antigens to persist.[27] Our results show that patients with severe MF (defined as patients with an intermediate-2 or high DIPSS score and/or BM fibrosis grade 3) present "inactive" T cells that were not able to be expanded enough *ex vivo* suggesting the presence of terminally exhausted T cells. On the contrary, MF patients displaying a non-severe disease phenotype have more responsive T cells and have therefore been selected as donors for *in vivo* experiments. Moreover, it is known that checkpoint inhibitor treatment is able to reinvigorate T lymphocytes, only when they are not terminally exhausted.[8] Consistently, a recent clinical trial evaluating PD-1 blockade that failed to prove treatment efficacy was carried out in patients with advanced disease (classified as "Intermediate-2" or "High" according to DIPSS plus), hence suggesting that T cells may have entered an irreversible terminally exhausted state.[16] Our results from anti-CTLA-4 treatment

*in vivo* experiments support the idea that immune checkpoint inhibitors could be effective in reactivating the immune response in MF, thus favoring clearance of the tumor bulk, when not-terminally exhausted T cells are still present in the immune repertoire of MF patients.

The inhibitory interplay between cancer cells and T lymphocytes is a well-known mechanism of immune escape common to many solid tumors but still poorly investigated in myeloid neoplasms, including MF.[30] Our work for the first time provides a comprehensive overview of how neoplastic myeloid cells interfere with T-cell anti-tumor activity and demonstrates the central role of CTLA-4-driven immune evasion in MF. Anti-CTLA-4 monoclonal antibody Ipilimumab was the first approved immune checkpoint inhibitor in solid tumors[31] and has recently gained momentum in the onco-hematologic field, with several phase 1 trials assessing its safety in myeloid malignancies, mainly as adjuvant therapy for vaccine-based or donor lymphocyte-based treatments (NCT05444530, NCT03912064).

The results presented here provide a rationale to exploit the intrinsic tumor-killing ability inherent to the immune system of MF patients and pave the way for future use of CTLA-4 blockade in the clinical management of the disease.

## AUTHOR CONTRIBUTIONS

R.M. conceptualized the study and supervised the manuscript; L.T., S.R., R.N. performed in vivo experiments and flow cytometry, analyzed data, and wrote the manuscript; M.M. and E.G.; performed in vitro experiments; M.M., B.M., M.T.B., P.G., L.P., A.L., F.P., M.L., and A.M.V. provided human samples; S.P. and C.C. performed cell isolation from human samples; E.B., M.B., and F.P. provided support for in vivo procedures; E.T. and S.M. performed mutation analysis, and E.T. performed analysis and interpretation of the data.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

## DATA AVAILABILITY STATEMENT

Data generated in this study are available within this paper and upon request from the Lead Contact. The Gene Expression Data generated during this work have been deposited to GEO (accession number GSE159514). Any additional information required to reanalyse the data reported in this study is available from the Lead Contact upon request.

## PATIENT CONSENT STATEMENT

This study was conducted in accordance with the Declaration of Helsinki under the local Institutional Review Board's approved protocol. All subjects involved in the study provided informed written consent.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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