IMMUNOLOGIC MECHANISMS OF MYELOID NEOPLASMS

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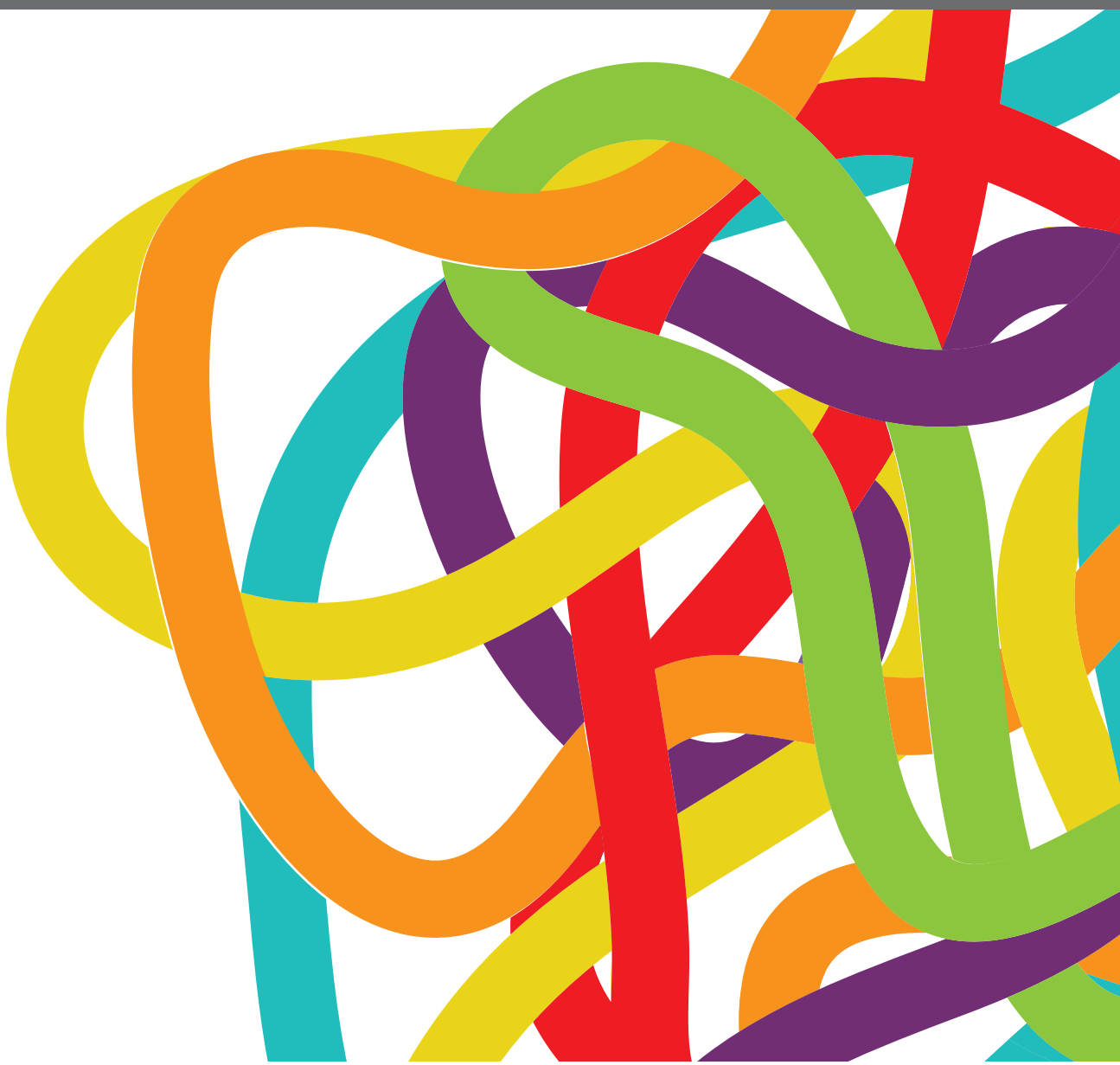

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IMMUNOLOGIC MECHANISMS OF MYELOID NEOPLASMS

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EDITORIAL
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Editorial: Immunologic Mechanisms of Myeloid Neoplasms

Bruno Fattizzo[1,2]*, *Matteo Claudio Da Vià*[1,2] *and Austin G. Kulasekararaj*[3]

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Keywords: myeloid neoplasm, immune system, immunomodulatory, neoplastic hematopoietic stem cell, single cell analysis

Editorial on the Research Topic

Immunologic Mechanisms of Myeloid Neoplasms

Myeloid neoplasms (MN), namely myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), and acute myeloid leukemias (AML) are characterized by disrupted myelopoiesis encompassing increased apoptosis of bone marrow (BM) progenitors, differentiation arrest and increased proliferation (1). This results in either peripheral cytopenia (with fatigue, bleeding, and infectious risk), or in hyperproliferative phenotype (with splenomegaly, high blood counts, and thrombosis). Along with the "first genetic hit" that may happen several years before disease onset in a hematopoietic stem cell (2), the surrounding immunologic niche seems to play a pivotal role in the subsequent disease development. Clinically, the immune system disruption is evidenced by an increased incidence of autoimmune phenomena in MN, which may worsen the degree of cytopenia (particularly anemia and thrombocytopenia) and respond to immunosuppressive therapy (3–5). From a pathogenic point of view, bone marrow hematopoietic stem cells are strongly regulated by the crosstalk with the surrounding microenvironment and its components, including mesenchymal stem cells, lymphocytes, and macrophages (**Figure 1**) (6). Several alterations of these cells have been described in MN, and it is not clear whether they are the cause or consequence of disease development and progression. Furthermore, niche disruption might sustain pancytopenia and promote the accumulation of molecular alterations that lead to leukemic evolution (4, 6, 7). Finally, immunologic alterations might in turn be potential targets for novel biologic drugs (8). In this Research Topic the above-mentioned points have been addressed by eleven articles focusing on pathogenic, prognostic, and therapeutic implications of immune system disruption in MN.

Barcellini and Fattizzo asked themselves the "egg or chicken" question as to whether immune phenomena comes before or after MN. They examined their epidemiological association, and discussed that autoimmunity and immunodeficiency are the two faces of a dysregulated immune tolerance and surveillance possibly resulting in tumor escape and infections. Alterations of the microbiota and of mesenchymal stem cells in MN are also discussed to highlight the importance of a permissive microenvironment for tumor growth. Finally, the authors highlight how novel therapies for MN (including checkpoint inhibitors and chimeric antigen receptor T-cells) may increase autoimmune phenomena.

Cominal et al., focused on Philadelphia chromosome-negative MPN that display inflammatory alterations of BM niche. They studied BM soluble mediator signatures using a multiplex assay and

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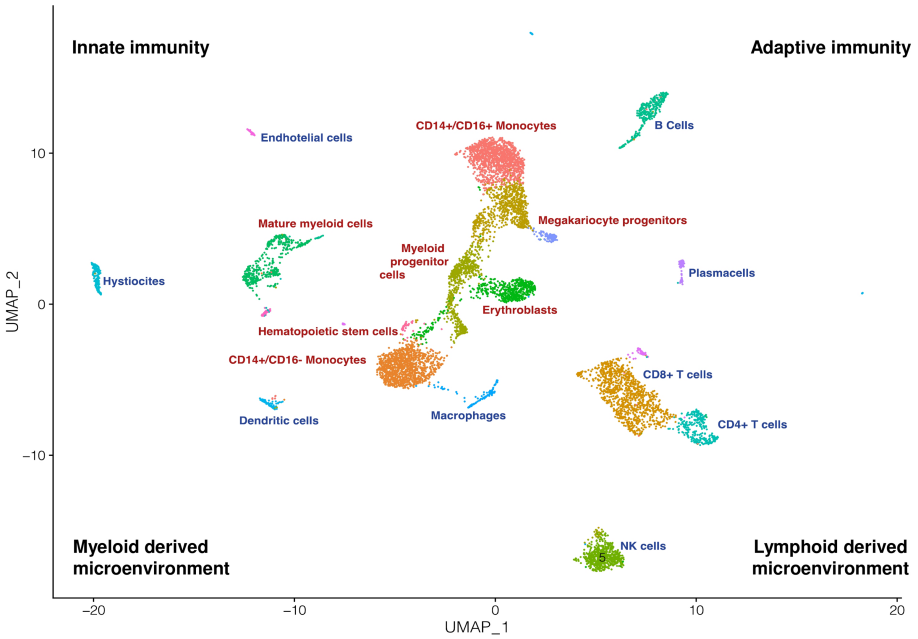

FIGURE 1 | The myeloid compartment and its microenvironment. Bone marrow is a tissue defined by a high cellular and biological complexity. This UMAP recapitulates how myeloid cells (red text) are surrounded and in strict connection with a complex and multifaceted immune microenvironment (blue text).

found a distinctive profile in polycythemia vera with increased levels of chemokines, and growth factors compared to essential thrombocytopenia and primary myelofibrosis. Deregulation of soluble mediators was associated with abnormal blood counts, thrombosis, treatment status and risk stratification and this might represent a therapeutic target. Additionally, JAK inhibitors also affect the levels of inflammatory cytokines in MPN patients, as described by Cattaneo and Iurlo. They also discussed how these drugs affect several components of the innate and adaptive immune systems such as dendritic cells, natural killer cells, T helper cells, and regulatory T cells, resulting in a level of immune deficiency with increased infectious risk.

Sciumè et al., focused on another rare "proliferating" condition: systemic mastocytosis. They described a *KIT D816V* mutated patient who evolved into MN with PDGFRA rearrangement and responded to imatinib therapy; they discuss how immunological mechanisms may play a role in promoting clonal prevalence of one entity (mastocytosis) over the other (MN).

The clinical and prognostic aspects of the concomitant presence of distinct hematological clonal entities was further addressed by Bucelli et al., who described a large series of patients with co-occurrence of myeloid and lymphoid neoplasms. Patients mainly suffered from MPN with associated non-Hodgkin lymphomas; nearly a half required anti-lymphoma therapy and 1/3 experienced a high-grade infection that was significantly associated with mortality.

Whether the myeloid and lymphoid clones share a common origin or develop autonomously is still debated, and another interesting example is the association of large granular lymphocyte (LGL) expansion with MN and BM failure syndromes. Our group performed a literature review and discussed how LGL clones, found in up to 1/3 of MN, are associated with deeper cytopenia (likely through immune mediated apoptosis) and good response to immunosuppression. Far from being innocent bystander, LGL clones may contribute to immunosurveillance, as their depletion after immunosuppression may favor leukemic escape.

Focusing on AML, Li et al., developed and validated an innovative prognostic model based on a novel immune-17 signature derived from transcriptome data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) databases. They confirmed that immune biology processes and transcriptional dysregulations are critical factors in the development of AML. Interestingly, the incorporation of the immune-17 signature to the ELN2017 risk score improved patient stratification. This immune signature may be therapeutically exploited, as described by Sun Yao et al., that treated an AML patient with PD-1 blockade in combination with azacytidine after allogeneic hematopoietic stem cell transplantation; these strategies that reactivate anti-leukemic immune surveillance may in turn result in devastating autoimmune/autoinflammatory responses, as in the case described who developed fatal graft versus host disease.

Moving to innate immunity effectors, Razanamahery et al., described a case of Erdheim–Chester disease (ECD), a rare histiocytosis, characterized by somatic mutations of MAP-kinase pathway in CD14+ monocytes. They found a correlation between disease activity and increased CD14+ +CD16− "classical monocyte" and decreased CD14lowCD16++

"non-classical monocyte" highlighting the contribution of a phenotype switch of innate immunity in this rare disease.

Another very rare condition associated with autoimmunity and MN is paroxysmal nocturnal hemoglobinuria (PNH). Giannotta et al., reported a patient with MPN who developed clinically overt PNH requiring anti-complement therapy. They discuss that the selection and expansion of PNH clones in MPN is likely to be ascribed to the same immunological bottlenecks described in BMF: autoimmunity against BM precursors, toxicity of therapies, and acquirement of cooperative somatic mutations.

Finally, Caprioli et al., described how the use of single-cell technologies represent powerful tools to assess the cellular composition of the complex tumour ecosystem and its immune environment (**Figure 1**), to dissect interactions between neoplastic and non-neoplastic components, and to decipher their functional heterogeneity and plasticity. In addition, recent progress in multi-omics approaches provide an unprecedented opportunity to study multiple molecular layers (DNA, RNA, proteins) at the level of single-cell or single cellular clones during disease evolution or in response to therapy. Applying single-cell technologies to MN holds the promise to uncover novel cell subsets or phenotypic states and highlight the connections between clonal evolution and immune escape, which is crucial to fully understand disease progression and therapeutic resistance.

In conclusion, this Research Topic highlights the multifaceted immunologic aspects of pathogenesis, clinical course, and treatment of MN. This expanding field will increasingly benefit from sophisticated molecular tools to further identify druggable pathways/targets and optimize management of MN and other rare entities.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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CASE REPORT
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Case Report: Combination Therapy With PD-1 Blockade for Acute Myeloid Leukemia After Allogeneic Hematopoietic Stem Cell Transplantation Resulted in Fatal GVHD

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Background: Azacitidine is commonly used in the treatment of relapsed acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) after allogeneic hematopoietic stem cell transplantation (allo-HSCT), but the effectiveness of this monotherapy is still very low. A possible mechanism of resistance to hypomethylating agents (HMAs) is the upregulation of the expression of inhibitory checkpoint receptors and their ligands, making the combination of HMAs and immune checkpoint blockade therapy a rational approach. Although the safety of anti-programmed cell death protein (PD)-1 antibodies for patients with post-allo-HSCT remains a complicated issue, the preliminary clinical result of combining azacitidine with anti-PD-1 antibodies is encouraging; however, the safety and efficacy of this approach need further investigation.

Case Presentation: We reported a case of treated secondary (ts)-AML in a patient who received tislelizumab (an anti-PD-1 antibody) in combination with azacitidine. The patient relapsed after allo-HSCT and was previously exposed to HMAs-based therapy. The patient received tislelizumab for compassionate use. After the combination treatment, the patient achieved complete remission with incomplete hematologic recovery, negative minimal residual disease (MRD) by flow cytometry (FCM), and negative Wilms' tumor protein 1 (WT1). However, the patient successively developed serious immune-related adverse events (irAEs) and graft vs. host disease (GVHD) and eventually died from complications of GVHD.

Conclusion: To our knowledge, this is the first case to report the combined use of tislelizumab and azacitidine to treat relapsed AML posttransplantation. This report highlights the safety concerns of using an anti-PD-1 antibody in combination with

azacitidine after allo-HSCT, especially the risk of GVHD, and provides a basis for future studies.

Keywords: acute myeloid leukemia, post-transplantation relapse, GvHD, immune checkpoint blockade, hypomethylating agents

INTRODUCTION

Although allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for patients with high-risk acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS), the relapse of the disease remains the major cause of treatment failure in these patients and carries a dismal prognosis (1–4). Hypomethylating agents (HMAs), such as azacitidine and decitabine, are the most common, non-targeted pharmacologic agents used to treat and prevent the relapse in posttransplantation AML and MDS in recent times. However, a single-agent HMA therapy in relapsed/refractory (r/r) HMAs-naïve AML has only achieved a low response rate (5–8). Previous studies have shown that, while HMAs promote antitumor immune signaling (9), they concurrently dampen antitumor immunity by increasing the expression of programmed cell death protein (PD)-1 and programmed death-ligand (PD-L)1 in solid tumors (10) and MDS/AML (11). This could be a possible mechanism of resistance to HMAs (8). For patients with relapsed AML after human leukocyte antigen

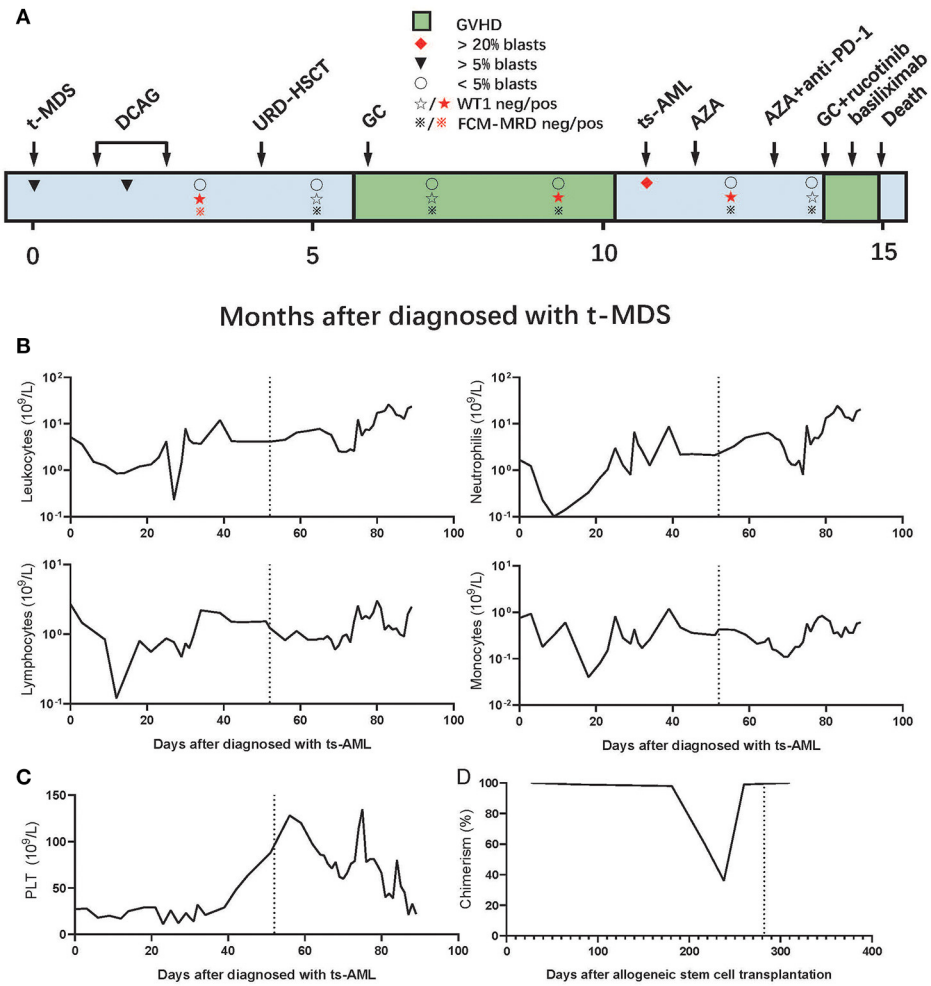

FIGURE 1 | (A) Clinical course of the patient. **(B,C)** Numbers of **(B)** leukocytes, lymphocytes, neutrophils, monocytes, and **(C)** platelets in the peripheral blood after the AML diagnosis. **(D)** Donor cell chimerism in the bone marrow (BM) following allo-HSCT. In **(B–D)**, the dotted vertical line indicates the timing of tislelizumab administration. GC, glucocorticoid; AZA, azacytidine; t-MDS, therapy-related myelodysplastic syndrome; ts-AML, treated secondary-acute myeloid leukemia. URD-HSCT, unrelated donor hematopoietic stem cell transplantation.

(HLA) matching and incompatible transplantation without HLA loss, the mechanism of recurrence after the transplantation is mainly by the downregulation of HLA class two molecules (30–40%) and the upregulation of immune checkpoints (∼20%) at the epigenetic level, which can be treated by HMAs and immune checkpoint blockade (ICB) therapy, respectively (12). Thus, for posttransplantation AML, the combination therapy of azacitidine with anti-PD-1 antibody may be a better approach in comparison to monotherapy. In fact, single-agent anti-PD-1 antibodies exhibit only minimal activity in patients with relapsed AML and high-risk MDS (13–15). ICB therapy after allo-HSCT has been reported to cause severe graft vs. host disease (GVHD) in both preclinical (16–18) and clinical studies (15, 19–22). However, the combination therapy of azacitidine and nivolumab (an anti-PD-1 antibody) showed an encouraging response with no GVHD and moderate immune-related adverse events (irAEs) with respect to the relapse of AML/MDS (prior allo-HSCT-19%) in a clinical trial (8). Given these promising preliminary clinical results, the safety and efficacy of combining azacitidine and anti-PD-1 antibodies in post-allo-HSCT patients should be urgently investigated further. Tislelizumab® (BeiGene, China), an antihuman PD-1 monoclonal IgG4 antibody, has been approved in China for patients with r/r classical Hodgkin lymphoma (HL) after at least a second-line chemotherapy (23). In the present study, we report a case of compassionate use of tislelizumab combined with azacitidine to treat a patient with relapsed AML after allo-HSCT. The report highlights the importance of the prudent use of an anti-PD-1 antibody in patients who are undergoing HSCT.

CASE PRESENTATION

A 56-year-old man was diagnosed with follicular lymphoma [FL; grade IIIA, stage IVA, Follicular Lymphoma International Prognostic Index (FLIPI) stage: high risk] 18 years ago. The patient was cured by four sequential cycles of fludarabine, cyclophosphamide, rituximab (FCR) chemotherapy; four cycles of rituximab, cyclophosphamide, hydroxyldaunorubicin, oncovin, and prednisone (R-CHOP) chemotherapy; and local lymph node radiotherapy. Unfortunately, the patient was diagnosed with therapy-related MDS (t-MDS) in February 2019 according to the WHO classification (**Figure 1A**). The baseline characteristics of the patient diagnosed with t-MDS are presented in the **Supplementary Material**.

The patient received induction chemotherapy with a decitabine, cytarabine, aclacinomycin, and recombinant human granulocyte colony-stimulating factor (G-CSF) (DCAG) scheme in March 2019 and achieved a partial response (PR). Then, the patient received another cycle of consolidation chemotherapy with DCAG and achieved a complete response (CR); at this stage, the patient was positive for minimal residual disease (MRD), confirmed through flow cytometry (FCM). The patient underwent allo-HSCT from a HLA-mismatched unrelated donor (8/10), after preconditioning with decitabine, fludarabine, and busulfan, followed by cyclosporine A, mycophenolate mofetil, basiliximab (a monoclonal anti-CD25 antibody), and short-term methotrexate for prophylaxis of GVHD. The patient achieved CR with MRD negativity (CR_{MRD}-) 1 month after allo-HSCT and developed extensive skin chronic GVHD (cGVHD) and bronchiolitis obliterans with organizing pneumonia (BOOP) 6 months after allo-HSCT but improved after glucocorticoids and antifungal therapy. During the treatment for BOOP, the patient remained CR_{MRD}- but was positive for Wilms' tumor protein 1 (WT1+). In January 2020, the disease progressed to AML, and the evaluation of bone marrow (BM) showed that 34.5% of blasts, 36.14% of donor chimeric,; 28.8% of FCM–MRD; and 7.57% of WT1. The patient was diagnosed with treated secondary (ts)-AML, arising from an antecedent hematologic disorder that was previously treated with chemotherapy or immunomodulatory therapy, an entity known to have an extremely dismal prognosis (24–26). The gene mutation test from a BM sample showed casitas B-lineage lymphoma (CBL) of 5.92% and Kirsten rat sarcoma (KRAS) of 6.3%. The immunosuppressor was immediately withdrawn. We performed the HLA-loss test, but no HLA gene loss was detected.

The patient was counseled on the risks and benefits of azacitidine in combination with tislelizumab. Although the patient did not have any signs or symptoms of GVHD at the time of relapse, we decided to administer anti-PD-1 after one course of azacitidine to ensure the use of tislelizumab for at least 4 weeks after the withdrawal of immunosuppressive agents according to a previous study (15). Thus, the patient received azacitidine monotherapy and achieved 0.611% of CRi_{MRD}- and WT1 1 month later. The patient subsequently developed herpes zoster infection, but the condition of the patient improved with antiviral therapy. The patient also developed a drug-induced liver injury, but the condition of the patient improved after the drugs causing liver injury were discontinued, namely estazolam and zopiclone, which had been prescribed for insomnia. In March 2020, the patient received 100 mg of azacitidine on days 1–7 subcutaneously and 200 mg of tislelizumab on day 1 intravenously. About 20 days later, the patient remained CRi_{MRD}- and was WT1 negative (WT1–) (0.11%, the cutoff value of WT1/ABL in our laboratory is 0.5%). The patient successively developed hypoadrenocorticism, infectious diarrhea, fever, and shock. Although the symptoms of the patient were relieved with symptomatic and antimicrobial treatment, diarrhea continued to worsen. No definite infection was found after repeated etiological examinations, and multiple antibiotic treatments proved to be ineffective.

The patient refused to undergo a colonoscopy and further biopsies, so the diagnosis of G3 gut acute GVHD (aGVHD) was mainly based on history and clinical manifestation. Prednisone, 2 mg/kg/day, combined with ruxolitinib, 10 mg (bid), was prescribed. The patient continued to have diarrhea even after 5 days. Prednisone was tapered and basiliximab was started. The patient subsequently developed delirious behavior, involuntary tremors, decreased muscle strength, and dystonia. A diagnosis of autoimmune-related encephalopathy was hypothesized after consultation with a neurologist, based on history, clinical manifestations, and imaging. CT showed

TABLE 1 | The safety and efficacy of the published clinical trials of immune checkpoint blockade in post-allo-HSCT myeloid malignancies.

References	Immune checkpoint inhibitors/ pathway	HMAs	Study design	Trial regimen	Study population (N)	Efficacy	Safety
ICB THERAPY ONLY							
Bashey et al. (27)	Ipilimumab/CTLA-4	-	Phase 1	Single arm in relapsed Malignancies after allo-HSCT	Total (29) AML (2) CML (2)	No response in the four patients	In all myeloid malignancies after allo-HSCT, no patient developed DLT and GVHD. One patient with AML developed G3 polyarthropathy with nodules clinically consistent with rheumatoid arthritis.
Davids et al. (28)	Ipilimumab/CTLA-4	-	phase 1/1b	Single arm in relapsed HMs After allo-HSCT	Total (28) AML (12) Relapse with extramedullary disease (4) MDS (2) MPN (1)	In myeloid malignancies, four patients with extramedullary and one patient with MDS/AML achieved CR.	In all patients, 6 (21%) developed irAEs including 1 death, 4 (14%) developed GVHD. All of GVHD resolved with glucocorticoids. Other sAEs: acute kidney injury, corneal ulcer, thrombocytopenia, neutropenia, anemia, and pleural effusion. *
Holderried et al. (22)	Nivolumab/PD-1 Ipilimumab/CTLA-4	-	Retrospective study	Disease recurrence after allo-HSCT other than HL	Total (21) AML/MDS (12)	One patient with AML received Niv + DLI survived > 2 years after Niv with ongoing CR. One AML received Niv survived > 2 years after Niv with PD.	2/12 patients with AML/MDS developed GVHD. One received Niv, the other one received Niv + Ipi.
Wong et al. (29)	Nivolumab/PD-1	-	Phase 2a	Single arm in relapsed or persistent HMs after allo-HSCT	Total (6) AML (2)	One patient with AML achieved transient blast reduction but progressed subsequently.	2/6 patents with HMs developed G3 aGVHD 2 weeks after first dose of Niv. *
Davids et al. (15)	Nivolumab/PD-1	-	Phase 1	Single arm in relapsed HMs after allo-HSCT	Total (28) AML (10) MDS (7) CMML (1)	less activity in patients with myeloid malignancies (ORR 21%).	11 HMs pts (39%) developed new or worsening a/c-GVHD (two acute, eight chronic, and one both). Additional sAEs: pneumonitis, transaminitis, respiratory syncytial virus pneumonia, rash, orthostatic hypotension, and lipase elevation. *
Schoch et al. (30)	Nivolumab/PD-1 Pembrolizumab/PD-1 Ipilimumab/CTLA-4	-	Retrospective study	Relapsed cancers after allo-HSCT.	Total (9) AML (1) MDS (1)	*	In all the 9 patients (including two with solid tumors), one developed G2 cutaneous aGVHD when DLI was given for relapsed disease after ipilimumab. *
Liao et al. (31)	Pembrolizumab/PD-1	-		Single arm in relapsed AML after allo-HSCT	AML (8)	No response	Can induce early and severe irAEs.
Wang et al. (32)	Nivolumab/PD-1	-	Cases report	Maintenance therapy after allo-HSCT in myeloid malignancies	AML (3) t-MDS (1)	-	All the 4 patients rapidly developed irAEs, 2 of them ≥G3.
Albring et al. (33)	Nivolumab/PD-1	-	Cases report	Monotherapy in relapsed AML after allo-HSCT	AML (3)	1 CR, 1 SD, 1 NR	Pancytopenia and skin GVHD in one patient, muscle and joint pain in another. No severe GVHD.

(Continued)

TABLE 1 | Continued

References	Immune checkpoint inhibitors/ pathway	HMAs	Study design	Trial regimen	Study population (N)	Efficacy	Safety
HMAs+ICB							
Daver et al. (8)	Nivolumab/PD-1	AZA	Phase 2	single arm in R/R AML	AML (70) Post-allo-HSCT (13) Post-transplantation AML (13)	ORR 33%, CRR 22% in all patients, ORR 58% in HMAs-naïve and 22% in HMAs-pre-treated patients. ORR 13% in post-allo-HSCT r/r AML.	Grade 3–4 irAEs occurred in 8/70 (11%) R/R AML patients. No GVHD was reported.

R/R, Relapsed/refractory; NR, Not reported; ORR, Overall response rate; OS, Overall survival; CR, Complete remission; CRR, Complete remission rate; PR, Partial response; HMAs, Hypomethylating agents; ICB, Immune checkpoint blockade; allo-HSCT, Allogeneic hematopoietic stem cell transplantation; SD, Stable disease; PD, Progressive disease; GVHD, Graft vs. host disease; DLT, Dose-limiting toxicity; AML, Acute myeloid leukemia; MDS, Myelodysplastic syndrome; CML, Chronic myeloid leukemia; CMML, Chronic myelomonocytic leukemia; HL, Hodgkin lymphoma; DLI, Donor lymphocyte infusion; irAEs, immune-related adverse events; AZA, Azacytidine; ITP, Immune thrombocytopenic purpura; HMs, Hematological malignancies; MPN, Myeloproliterative neoplasms.

**Detailed data about the separate disease are unavailable.*

multiple spots and patches of low-density lesions around bilateral lateral ventricles, and MRI showed scattered spots and patchy lesions near both frontal lobes and lateral ventricles that showed equal or long signal on T1 images, a long signal on T2 images, and a high signal on T2WI fluid-attenuated inversion recovery (FLAIR). Gamma globulins were administered, but the nervous system symptoms were not relieved. Gut aGVHD was resistant to steroid and second-line treatment, and the patient subsequently developed hematochezia, enteric infections, septic shock, and metabolic acidosis secondary to gut GVHD and died 6 days later (**Figures 1B–D**).

DISCUSSION AND LITERATURE REVIEW

The patient with MDS mentioned in the study was previously exposed to HMA therapy, which rapidly progressed to ts-AML after allo-HSCT. At the time of relapse, neither HLA loss nor active GVHD was present. First, the patient received azacitidine monotherapy and achieved CRi_{MRD}- but was WT1+. Subsequently, the patient received a combination of azacitidine and tislelizumab and remained CRi_{MRD}- and became WT1-. Unfortunately, the patient developed serious irAEs, including hypoadrenocorticism, autoimmune-related encephalopathy, and fatal gut GVHD. We have summarized the safety and efficacy of using checkpoint inhibitors in post-allo-HSCT myeloid malignancies in **Table 1**. Clinical studies showed that the CTLA-4 blockade induces lower GVHD as compared to anti-PD-1 (14% vs. 39%) (15, 28). Furthermore, CTLA-4 inhibitors as single agents demonstrated activity in patients with high-risk MDS after the therapy of HMAs and relapsed AML post-allo-HSCT, while anti-PD-1 antibodies showed limited efficacy (28, 34). Currently, clinical trials of the combination of HMAs with ICB therapy are ongoing (8, 34). **Table 2** shows a summary of autoimmune complications of the published clinical trials using checkpoint inhibitors in post-allo-HSCT hematologic malignancies other than myeloid malignancies. On the whole, the incidence of autoimmune diseases, including GVHD, after ICB monotherapy is high: 21–39% in AML/MDS (15, 28) and 30%—55% in other hematological malignancies (20, 36).

A possible pathogenic mechanism of GVHD in the patient could involve enteric infection that may have damaged gastrointestinal tissue, favoring T-cell activation against self-antigens. The blockage of PD-1/PD-L1 increases the proliferation, activation, Th1 cytokine-production, and metabolic stress of donor T cells, along with increased homing in the GVHD target tissues such as the gut, due to the loss of intestinal epithelial integrity (47). Moreover, the blockage of PD-1/PD-L1 accelerated donor CD8+ T-cell expansion and exacerbated aGVHD (48). It is challenging to distinguish between gut GVHD and GI-irAEs even after biopsies. We diagnosed a gut aGVHD for the following reasons: first, the patient had a history of cGVHD and was more likely to be susceptible to develop GVHD after the treatment of PD-1 as described in the previous studies (36, 49). Meanwhile, the patient remained completely donor chimeric after anti-PD-1 therapy. However, other studies have suggested that prior a/cGVHD has no significant impact on the development of GVHD after ICB therapy (15, 22). Although this issue is controversial and remains to be clarified, it is a possibility that deserves attention. In addition, the cumulative incidence of gastrointestinal aGVHD might be as high as 60% (50), while the incidence of diarrhea was 11–17% after the treatment of anti-PD-1 (51).

Previous studies showed that 0.5 mg/kg of nivolumab monotherapy for every 3 weeks and 100 mg of nivolumab plus azacitidine for every 2 weeks are considered safe (8, 15). In addition, the low affinity of tislelizumab for the Fc receptor and Fc-γ receptor 1 (FcγRI) may contribute to improved anticancer efficacy as compared to other anti-PD-1 antibodies (52), which means that the dose of tislelizumab may need to be further reduced. It is interesting that the patient developed a delayed

TABLE 2 | Autoimmune complications of the published clinical trials using checkpoint inhibitors in post-allo-HSCT hematologic malignancies other than myeloid malignancies.

References	immune checkpoint inhibitors/pathway	Other drugs	Study design	Trial regimen	Study population (N)	Autoimmune complications
Bashey et al. (27)	Ipilimumab/CTLA-4	-	Phase 1	Single arm in relapsed malignancies after allo-HSCT	Total (29) HL (14) Myeloma (6) CLL (2) NHL (1)	3 patients developed organ-specific irAEs, including G2 hyperthyroidism, recurrent G4 pneumonitis, and G3 dyspnea.
Davids et al. (28)	Ipilimumab/CTLA-4	-	Phase 1/1b	Single arm in relapsed HMs after allo-HSCT	Total (28) HL (7) NHL (4) MM (1) ALL (1)	In all patients, 6 (21%) developed irAEs including 1 death, 4 (14%) developed GVHD. *
Holderried et al. (22)	Nivolumab/PD-1 Ipilimumab/CTLA-4	-	Retrospective study	Disease recurrence after allo-HSCT other than HL	Total (21) ALL (2) NHL (5) MF (2)	4/9 patients with non-myeloid hematologic malignancies developed GVHD, 1 received Niv, 3 received Niv + DLI.
Wong et al. (29)	Nivolumab/PD-1	-	phase 2a	Single arm in relapsed or persistent HMs after allo-HSCT	Total (6) HL (2) tCLL (1) MCL (1)	2/6 HMs patients developed G3 aGVHD 2. *
Khouri et al. (35)	Ipilimumab/CTLA-4	Lenalidomide	Phase ii	Relapsed lymphomas after allo-HSCT and high-risk patients after autologous HSCT	17 pts (10 allo, 7 auto)	Allogeneic: 1 cGVHD of liver, mouth, 1 G2 hypothyroid; Autologous: 1 G2 dermatitis, 1 G1 hypothyroid.
Schoch et al. (30)	Nivolumab/PD-1 Pembrolizumab/PD-1 Ipilimumab/CTLA-4	-	Retrospective study	relapsed cancers after allo-HSCT.	Total (9) HL (4) Dsmoplastic small round cell tumor (1)	In all the 9 patients (including 2 with solid tumors), 1 developed G2 cutaneous aGVHD when DLI was given for relapsed disease after ipilimumab. *
Davids et al. (15)	Nivolumab/PD-1	-	Phase 1	Single arm in relapsed HMs after allo-HSCT	Total (28) HL (5) NHL (3) CLL (1)	11 HMs patients (39%) developed new or worsening a/c-GVHD (2 acute, 8 chronic, and 1 both). *
Herbaux et al. (36)	Nivolumab/PD-1	-	Retrospective study	HL patients relapsing after allo-HSCT.	r/r HL (20)	30% (6/20) patients developed GVHD, all of them had prior history of aGVHD. 1 developed possibly related G2 hepatic cytolysis.
Haverkos et al. (20)	Nivolumab/PD-1 Pembrolizumab/PD-1	-	Retrospective study	Relapsed lymphomas after allo-HSCT	HL (29) Other lymphomas (2)	55% (17/31) patients developed treatment-emergent GVHD (6 acute, 4 overlap, and 7 chronic). 29% developed ≥G3 a/cGVHD. 26% deaths related to GVHD. Only 2 of these 17 achieved CR to GVHD treatment, and 14/17 required ≥2 systemic therapies. The majority experienced cutaneous and hepatic GVHD.
Angenendt et al. (37), Covut et al. (38), and Shad et al. (39)	Nivolumab/PD-1	-	Cases report	Relapsed HL after allo-HSCT	HL (4)	None
Chan et al. (40) and Villasboas et al. (41)	Pembrolizumab/PD-1	-	Cases report	Relapsed lymphoma after allo-HSCT	HL (2) ALCL (1)	None
Singh et al. (19)	Pembrolizumab/PD-1	-	Case report	Relapsed HL after allo-HSCT	HL (1)	Stage IV skin, stage II gut and stage IV liver leading to an overall grade IV aGvHD.

(Continued)

TABLE 2 | Continued

References	immune checkpoint inhibitors/pathway	Other drugs	Study design	Trial regimen	Study population (N)	Autoimmune complications
Kwong et al. (42)	Pembrolizumab/PD-1	-	Cases report	Relapsed or refractory NK/T-cell lymphoma after allo-HSCT	NK/T-cell lymphoma (7)	G2 skin GVHD disease in 1 patient with previous allo-HSCT.
Godfrey et al. (43)	Nivolumab/PD-1	-	Cases report	Relapsed HL after allo-HSCT	HL (3)	G3 polyarthritis in 1 patient, G2 keratoconjunctivits in 2, G1 rash (possibly representing limited-stage chronic GVHD) in 1.
Boekstegers et al. (44)	Pembrolizumab/PD-1	-	Case report	Relapsed ALL after allo-HSCT	ALL (1)	G4 aGVHD of the skin, mucosa, liver, lung, CNS and eyes. A severe lethal inflammatory disease.
El Cheikh et al. (45)	Nivolumab/PD-1	-	Cases report	Relapsed HL after allo-HSCT	HL (2)	G3 aGVHD involving ocular, liver and skin in 1 patient, G3 aGVHD involving skin, GI and liver in the other pt.
Yared et al. (46)	Nivolumab/PD-1	-	Case report	Relapsed HL after allo-HSCT	HL (1)	G2 pneumonitis and hepatitis

NHL, Non-Hodgkin's lymphoma; ALL, Acute lymphoblastic leukemia; CLL, Chronic lymphoblastic leukemia; ALCL, Anaplastic large cell lymphoma; R/R, Relapsed/refractory; CR, Complete remission; CRR, Complete remission rate; allo-HSCT, Allogeneic hematopoietic stem cell transplantation; GVHD, Graft vs. host disease; HL, Hodgkin lymphoma; DLI, Donor lymphocyte infusion; irAEs, immune-related adverse events; DLT, Dose-limiting toxicity; MF, Marrow failure; MM, Multiple myeloma; tCLL, transformed chronic lymphocytic leukemia; MCL, Mantle cell lymphoma; HMs, Hematological malignancies.
**Detailed data about separate disease are unavailable.*

and steroid-resistant GVHD nearly 4 weeks after anti-PD-1 therapy. This could be related to the highest terminal half-life of tislelizumab compared to other ICB (23). Therefore, reducing the dose of tislelizumab or extending the interval of administration should be evaluated to improve safety in future studies on patients with post-allo-HSCT.

Some other factors may also cause the occurrence of GVHD after ICB therapy in posttransplantation patients. Although it is still controversial (22), two studies observed that a shorter interval between the transplantation and the first nivolumab infusion was associated with a higher risk of developing GVHD (15, 36). Extreme caution should be followed during the enrollment of patients with active cGVHD (21). Furthermore, the question remains as to whether anti-PD-L1 is safer than anti-PD-1. Hematopoietic cells upregulate the expression of both PD-L2 and PD-L1 after HSCT, but only PD-L1 is broadly expressed by parenchymal cells in host GVHD target tissues (47). Host PD-L1 is dominant over PD-L2 in regulating GVHD lethality (47), and the PD-L1 expression on donor T cells may drive GVHD lethality (53). Thus, PD-L1 may play a vital role in the development of GVHD. At present, there is still a lack of reliable data on the clinical application of anti-PD-L1 for posttransplantation patients, and the safety and efficacy of PD-L1 inhibitors need to be investigated clinically.

CONCLUSION

Azacitidine in combination with anti-PD-1 seems to be a rational strategy for posttransplantation relapsed AML but needs further urgent clinical investigation. The report highlights the safety issues of an anti-PD-1 antibody in combination with azacitidine after allo-HSCT, especially GVHD. Additionally, we conducted an in-depth discussion around safety issues and provided suggestions for follow-up research. For such patients, the type, dosage, and timing of ICB drugs should be selected with caution.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HL was involved in the identification, selection, and management of the patient and manuscript review. SY was involved in the management of the patient and manuscript drafting. CJ and QZ were involved in the selection and management of the patient and manuscript review. LY, ZB, HJ, NH, and ZB were involved in manuscript editing. HG was involved in the detection of samples. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639217/full#supplementary-material

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Development and Validation of a Novel Prognostic Model for Acute Myeloid Leukemia Based on Immune-Related Genes

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The prognosis of acute myeloid leukemia (AML) is closely related to immune response changes. Further exploration of the pathobiology of AML focusing on immune-related genes would contribute to the development of more advanced evaluation and treatment strategies. In this study, we established a novel immune-17 signature based on transcriptome data from The Cancer Genome Atlas (TCGA) and The Genotype-Tissue Expression (GTEx) databases. We found that immune biology processes and transcriptional dysregulations are critical factors in the development of AML through enrichment analyses. We also formulated a prognostic model to predict the overall survival of AML patients by using LASSO (Least Absolute Shrinkage and Selection Operator) regression analysis. Furthermore, we incorporated the immune-17 signature to improve the prognostic accuracy of the ELN2017 risk stratification system. We concluded that the immune-17 signature represents a novel useful model for evaluating AML survival outcomes and may be implemented to optimize treatment selection in the next future.

Keywords: acute myeloid leukemia, prognostic signature, immune-relate genes, The Cancer Genome Atlas, Least Absolute Shrinkage and Selection Operator

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INTRODUCTION

Acute myeloid leukemia (AML) is one of the most common hematological cancers in adults, characterized by the accumulation of immature myeloblasts in the bone marrow and peripheral blood at the expense of normal blood components (1). Unlike many other cancers, AML has a low tumor mutation burden (TMB) with an average of 10–13 coding mutations per patient (2). Although we have understood the role of mutational genes in driving tumor progression along with an uncomplicated mutational landscape, the overall therapeutic strategy for AML patients has remained the same for the last 30 years (3, 4). The conventional treatment paradigm has a restricted contribution to improve overall survival (OS), especially in the elderly population (4). Although

allogeneic hematopoietic cell transplantation (alloHCT) and chemotherapy regimens allowed a five-year survival rate of 40–70% in younger patients (<40 years of age), survival in the elderly remains poor (5, 6) with a risk of relapse within 5 years from diagnosis as high as 75% (7). Therefore, it is urgent to identify potential biomarkers to inform prognosis and treatment allocation in this setting.

It has been well proved that allogeneic hematopoietic cell transplantation (alloHCT) is successful in treating AML and AML is immune-responsive (8). However, immunosuppression could also be caused by AML blasts, leading to paradoxical immunosuppression in patients with AML (9). Therefore, further understanding of how immune cells battle with AML blasts could lead to more effective therapies for AML.

Previous studies (10–12) have stratified AML patients into low- and high-risk groups based on immune and stromal scores with the ESTIMATE algorithm (13), which is based on single sample Gene Set Enrichment Analysis and then generates stromal and immune scores to predict the infiltration of stromal and immune cells in tumors. The identified differentially expressed genes (DEGs) from low- and high-risk groups were then studied to look for potential prognostic value in AML patients. However, there is a lack of a comprehensive study on the utility of immune-related gene (IRG) expression in predicting AML prognosis and comparing AML with healthy samples. This study aimed to fill the gap by focusing on the relationship between IRG expression and AML patients' prognosis.

MATERIALS AND METHODS

Data Acquisition

Clinical and transcriptome information of TCGA-LAML and GTEx-whole blood cohorts were acquired from the UCSC Xena database (http://xena.ucsc.edu/). The GTEx project is a data resource of the healthy population from organ donation and rapid autopsy settings (14). All the transcriptome data have been normalized according to the description from the UCSC Xena database. The ImmPort database provided a total of 2,498 immune-related genes. We obtained clinical and transcriptome data from the GSE37642 cohort (validation cohort) in the Gene Expression Omnibus (GEO) database to validate our prognostic model. All eligible samples from TCGA and validation cohorts were collected according to the following inclusive criteria: (1) newly diagnosed acute myeloid leukemia specimen; (2) Availability of transcriptome data; (3) Availability of general survival information and related clinical data. The clinical information of inclusive AML samples is detailed in **Table 1**.

Establishment of an Immune-Related Signature

2,516 DEGs were identified between the TCGA-LAML and GTEx-whole blood cohorts (**Table S1**). After integrating 2,498 IRGs, we obtained 199 differentially expressed IRGs (**Table S2**). Univariate Cox regression analysis was performed to evaluate the association between expression levels of individual IRGs and OS and 72 of them were of potential prognostic value (**Table S3**). To minimize the risk of overfitting, LASSO regression analysis was applied to construct a prognostic model (15). We finally established an immune prognostic signature (immune-17 signature, because it contains 17 IRGs). The risk score was calculated using the equation: $\beta1 \times$ gene1 expression + $\beta2 \times$ gene2 expression + … + $\beta n \times$ genen expression, where β was the correlation coefficient generated by LASSO regression analysis.

Evaluation of the Immune-17 Signature

Each patient from the GEO or TCGA database was allocated a risk score derived from the immune-17 signature. These patients were then stratified into low- and high-risk groups using the median risk score as the cutoff value. The Kaplan–Meier analysis was conducted to evaluate the prognostic significance of the immune-17 signature. Model specificity and sensitivity were assessed by calculating the area under the curve (AUC) values. Specific predictive ability was determined when AUC >0.60, while excellent predictive values were determined if AUC >0.75. Univariate and multivariate Cox analysis were used to prove the signature is an independent prognostic model.

Improvement of European LeukemiaNet (ELN) 2017 Risk Stratification System

Patients were stratified into three new groups: ELN favorable/immune-17^{high} and ELN adverse/immune-17^{low} patients were re-assigned to the intermediate-risk group, and ELN intermediate/immune-17^{high} patients were re-assigned to the high-risk group. Through Kaplan–Meier analysis, we evaluated the prognostic significance of the new risk stratification system.

Statistical Analysis

The R software (version 4.0.2, https://www.r-project.org/) was used to perform all statistical analyses. DEGs between healthy individuals and AML patients were identified using the "limma" package with filter criteria (FDR <0.05 and |log FC |>2). Heatmap and clustering were carried out using the "pheatmap" package. Enrichment analysis was performed using the "clusterProfiler" package. Univariate and multivariate Cox regression analysis was conducted using "survival" package. "glmnet" and "survival" packages were used to conduct LASSO regression analysis. "survminer" and "survival" packages were used to perform Kaplan–Meier analysis. "survivalROC" package was used to determine AUC values and construct receiver operating characteristic (ROC) curves. The introduction of packages in R software can be found at the site of (https://cloud.r-project.org/). All statistical tests were two-sided, and $p < 0.05$ was considered to be statistically significant.

RESULTS

IRGs Were Associated With the OS of AML Patients

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses indicated that

TABLE 1 | Baseline characteristics of the patients in the training and validation cohorts.

Clinicopathological variables	Training dataset (n = 151)			Clinicopathological variables	Validation dataset (n = 417)		
	high-risk group	low-risk group	p		high-risk group	low-risk group	p
Age (years)			0.004	**Age (years)**			0.256
<60	33	51		<60	108	119	
≥60	42	25		≥60	101	89	
FAB classification			0.01	**FAB classification**			0.003
M0	10	5		M0	11	3	
M1	20	15		M1	40	44	
M2	15	23		M2	49	68	
M3	4	11		M3	4	15	
M4	10	19		M4	57	47	
M5	12	3		M5	30	17	
M6	2	0		M6	7	8	
M7	1	0		M7	2	0	
Gender			0.467	**Status**			0.001
Female	36	32		Alive	40	69	
Male	39	44		Dead	169	139	
Status			<0.0001	**RUNX1-RUNX1T1**			0.001
Alive	11	43		Negative	205	189	
Dead	64	33		Positive	4	19	
WBC			0.787	**RUNX1 mutation**			0.043
$<10 \times 10^9$/L	28	30		Negative	153	157	
$\geq 10 \times 10^9$/L	47	46		Positive	37	21	
BM blast			0.559				
<70%	31	35					
≥70%	44	41					
Risk (Cytogenetic)			<0.0001				
Good	7	24					
Intermediate	43	38					
Poor	24	12					
ELN2017			<0.0001				
Favorable	7	25					
Intermediate	28	23					
Adverse	36	17					
Transplant			0.161				
Yes	29	38					
No	46	38					
Chemotherapy			0.638				
Yes	72	74					
No	3	2					
Relapse			0.12				
Yes	38	29					
No	36	46					

FAB, French-American-British; WBC, white blood cell; BM, bone marrow; ELN, European LeukemiaNet.

DEGs were mainly enriched in the immune biology processes (**Figure S1A**). The KEGG enrichment analysis results revealed the central role of transcriptional dysregulations in the development of AML (**Figure S1B**). 17 IRGs involved in the model were associated with the OS of AML patients (**Table 2**), and their expression in AML patients is shown in **Table S2**. Representative Kaplan–Meier plots showed TRH, MPO, IGHV4-39 and CLEC11A associated with prolonged survival of AML patients. On the contrary, APOBEC3G, IL1R2, GZMB, ISG20 and HSPA1B correlated with a poor OS (**Figures 1A–I**).

Evaluation of the IRG Signature

The IRG signature was evaluated in the training (TCGA-LAML) and validation cohorts. Kaplan–Meier plots demonstrated that patients allocated to the high-risk group showed a significantly shorter OS (p = 1.321e−14, TCGA-LAML; p = 5.275e−4, validation cohort), (**Figures 2A, B**). This model's AUC value achieved a value of 0.823 in the TCGA-LAML cohort and a value of 0.613 in the validation cohort, respectively (**Figures 2C, D**). To determine whether the immune-17 signature was a stable predictive model, we performed the Kaplan-Meier analysis in patients from the TCGA-LAML cohort stratified by age (**Figure S2A**) and ELN risk system (**Figure S2B**), respectively. The results indicated that the immune-17 signature could discern low-risk patients from high-risk patients in all stratified groups. We performed the univariate and multivariate Cox analysis revealed that the signature was an independent prognostic model (univariate Cox: hazard ratio [HR], 1.768; 95% confidence interval [95% CI], 1.559–2.019; p <0.001; multivariate Cox: HR, 1.631; 95% CI, 1.417–1.878; p <0.001), (**Table 3**). After re-stratification of patients (**Figures 3A**), we found that combined ELN + immune-17 risk stratification

TABLE 2 | Univariate Cox regression analysis of 17 genes from immune-17 model for overall survival of TCGA-LAML patients.

id	HR	HR.95L	HR.95H	p-value	Coef
CALR	0.5621	0.4192	0.7537	0.0001	−0.0376
HSPA1B	1.2640	1.0710	1.4918	0.0056	0.0362
APOBEC3G	1.8348	1.3200	2.5503	0.0003	0.0589
MX1	1.2173	1.0787	1.3736	0.0014	0.0687
ISG20	1.6087	1.2889	2.0077	0.0000	0.1710
MPO	0.8764	0.8274	0.9284	0.0000	−0.0483
CCL4	1.3606	1.0863	1.7041	0.0074	0.1340
FGR	1.1088	1.0253	1.1991	0.0097	0.0032
MIF	1.2278	1.0085	1.4948	0.0410	0.2294
IGHD5.18	1.1075	1.0345	1.1856	0.0033	0.0108
IGHV4.39	0.8980	0.8301	0.9714	0.0073	−0.0794
IGHV5.51	0.8796	0.8042	0.9620	0.0050	−0.0608
PLXNB2	1.2653	1.0672	1.5002	0.0068	0.0676
CLEC11A	0.8809	0.8211	0.9450	0.0004	−0.0324
TRH	0.8622	0.7922	0.9385	0.0006	−0.0645
IL1R2	1.1756	1.0602	1.3035	0.0021	0.0939
GZMB	1.3243	1.1444	1.5324	0.0002	0.0535

HR (Hazard ratio) is intended for overall survival; Coef, correlation coefficient.

system could more accurately define AML patients' prognosis (**Figures 3B, C**).

DISCUSSION

Genetic and clinical factors play increasingly important roles in predicting OS and event-free survival (EFS) for AML patients (16). Patient-related factors, AML-related genetic factors and MRD monitoring are considered as key factors responsible for AML prognosis (17). In the past, immune factors have been largely ignored. This study constructed an immune prognostic signature for AML based on the TCGA-LAML and GTEx-whole blood cohort, which improved the accuracy of ELN2017 risk stratification system.

Among the 17 IRGs involved in the immune-17 signature, we roughly divided them into three categories according to genes' function: 1) innate immunity-related genes; 2) specific immunity-related genes; 3) endocrine-related genes.

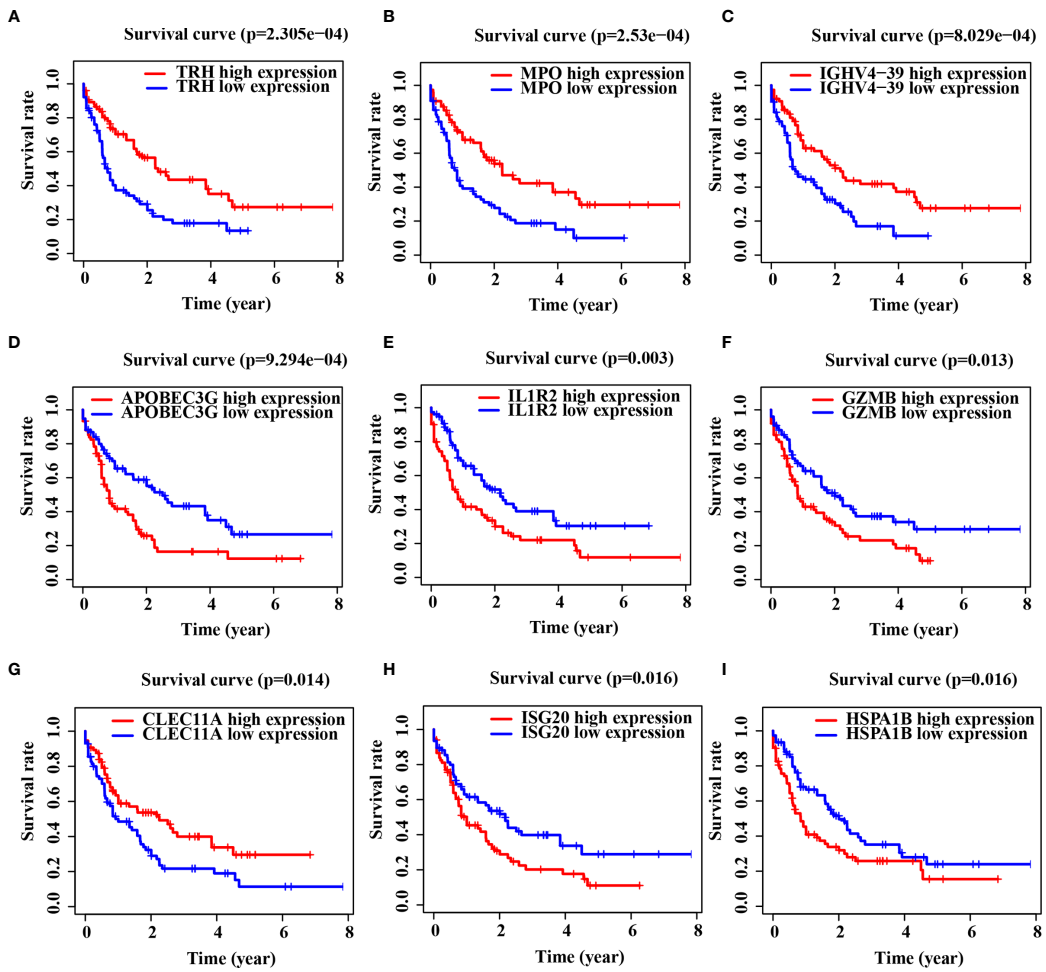

FIGURE 1 | Immune-related genes (IRGs) associated with the overall survival (OS) of acute myeloid leukemia (AML) patients. **(A–I)** Kaplan–Meier curve analysis of nine representative IRGs in TCGA-LAML cohort.

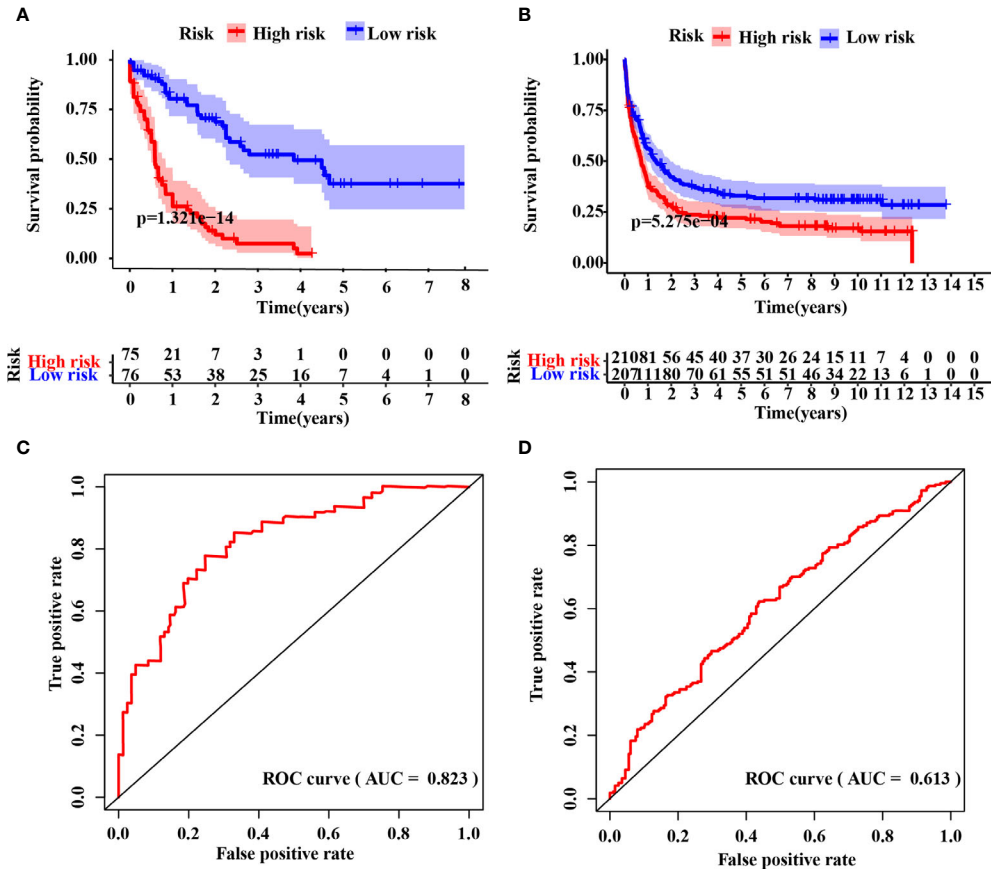

FIGURE 2 | Evaluation of the IRG signature. Kaplan–Meier curve analysis between the high- and low-risk groups in the raining **(A)** and validation **(B)** cohort, respectively. Time-dependent receiver operating characteristic (ROC) curve analysis of the immune-17 signature in the training **(C)** and validation **(D)** cohort, respectively.

APOBEC3G, MIF, MX1, ISG20, MPO, FGR, and IL1R2 are innate immunity-related genes. APOBEC3G is highly expressed in various cancers and plays an essential role in regulating tumor growth and innate immune responses (18, 19). MIF contributes to the immune escape, anti-inflammatory, and immune tolerance in either innate or adaptive immune cells (20). Primary AML highly expressed MIF and MIF drives the bone marrow mesenchymal stromal cells (BM-MSC) to express IL-8, which in turn assists AML cell survival and proliferation (21). Base on this finding, an anti-MIF monoclonal antibody (Imalumab) is being studied in a phase I study (NCT01765790) to assess the safety, pharmacokinetics, tolerability, and antitumor activity against solid cancers (22). FGR is a member of the Src family and contributes to the transition of signals from cell surface receptors and promotes inflammatory cytokines releases. FGR expression is restricted to myeloid lineage and is markedly highly expressed in a subset of AML (23). CALR, CCL4, and GZMB are considered genes of the adaptive immunity. CALR is found in myeloproliferative neoplasms (MPN) and represents an MPN-driver mutation. The presence of this gene is included in the current diagnostic criteria of $Ph^{(-)}$ MPN (24). The AML patients converted from MPN had more CALR mutation rate frequency (25). Moreover, there are reports that the expression of CALR is remarkably higher than other hematologic malignancies, such as ambiguous lineage, ALL, MPN, MDS/MPN (26). CCL4 and GZMB are associated with T-cell immunity. CCL4 is a biomarker of multiple sclerosis and associated with inflammation and T-cell activation (27). AML patients with monocytic differentiation had increased serum levels of CCL4 along with CCL5 and CCL3. The three biomarkers promote an inflammatory state and participated in the progression of suppressing T cell-related immune response (28). GZMB encodes the preprotein secreted by NK cell and CTLs, which is related to the apoptosis of target cells (29). Hypermethylation of the enhancer upstream of GZMB might contribute to an inferior overall survival of AML (30). The mutation in these genes might cause obstacles to the elimination of abnormal cells. Finally, TRH and CLEC11A not only have functions in the endocrine system, but also play a role in immune system. Other genes included in the 17-immune signature have been reported to participate in immune response. These include HSPA1B, IGHD5-18, IGHV4-39, IGHV5-51, and PLXNB2, providing directions and clues for our future research.

TABLE 3 | Univariate and multivariate Cox regression analysis of immune-17 model for overall survival of TCGA-LAML patients.

Characteristics	Univariate Cox				Multivariate Cox			
	HR	HR.95L	HR.95H	p-value	HR	HR.95L	HR.95H	p-value
Age	1.0324	1.0171	1.0479	2.73E−05	1.0258	1.0102	1.0416	0.0011
Sex	1.0141	0.6684	1.5386	0.9474	0.7646	0.4824	1.2118	0.2533
BM Blast (%)	1.0025	0.9920	1.0131	0.6431	1.0058	0.9944	1.0172	0.3201
WBC	1.0019	0.9972	1.0067	0.4242	1.0034	0.9984	1.0083	0.1824
Platelet count	0.9999	0.9964	1.0034	0.9408	0.9983	0.9943	1.0024	0.4199
ELN2017	1.7708	1.3287	2.3600	9.64E−05	1.6412	1.1859	2.2711	0.0028
Immune-17 score	1.7685	1.5490	2.0190	3.35E−17	1.6313	1.4167	1.8784	1.04E−11

HR is intended for overall survival; WBC, white blood cell; BM, bone marrow; ELN, European LeukemiaNet.

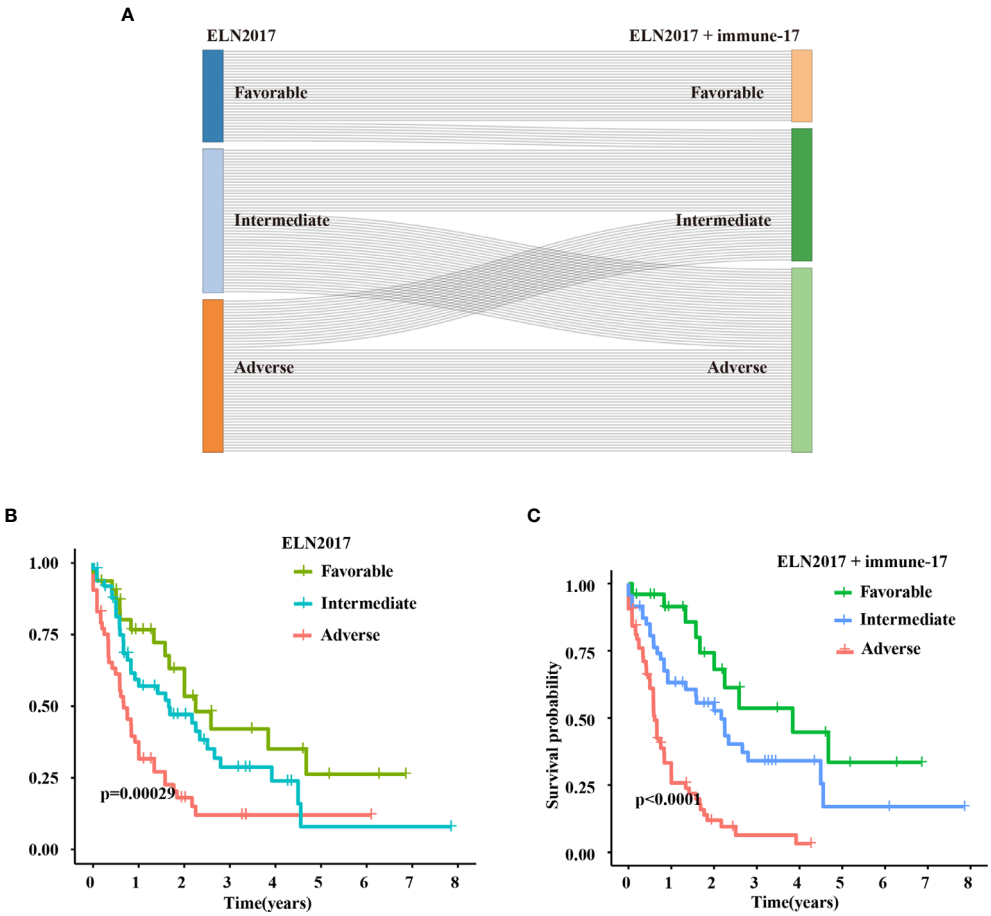

FIGURE 3 | Improved ELN2017 risk stratification system. **(A)** Re-stratification of patients from the three ELN2017 categories to the novel three ELN2017+immune-17 categories. Each line represents a patient. The line's left end means the ELN2017 categories and the line's right end means novel ELN-immune-17 categories. If the line is parallel, the patient's classification is not change. If not, the patient's classification has changed. Kaplan–Meier analysis for AML patients stratified by ELN2017 risk stratification system **(B)** or ELN2017+immune-17 risk stratification system **(C)**.

Although the immune-17 signature has been proved effective in both training and validation cohorts, the AUC value for this model applied in the validation cohorts is not satisfying; it might be due to different data platforms. The training cohort is generated from RNA-sequencing, while the validation cohort is obtained from the microarray platform. In addition, the different ethnicity distribution may contribute to this result. Although the AUC value is lower in the validation cohort than that in the training cohort, the signature still exhibited predictive power in validation cohort evidenced by the Kaplan–Meier survival and ROC curve analysis.

ELN2017 risk stratification system (integrated cytogenetic and mutational status information) has been utilized in general practice (17). However, non-genetic potential mechanisms have

been found to play essential roles in AML patients' survival (31, 32). In this study, from the immunology perspective, we improved the accuracy of ELN2017 risk stratification system by incorporating the immune-17 signature. Different immune signatures in myeloid neoplasms need to be investigated, which will likely improve knowledge on disease pathogenesis and inform novel therapies. Several novel drugs such as anti-TIM3 and anti-CD47 antibodies are macrophage and lymphocyte immune checkpoint inhibitors, which are under active investigation and (33, 34). These drugs reactivate immune response against myeloid blasts, thus blocking leukemia immune escape.

In conclusion, we constructed an immune-related signature, which is a reliable and accurate model to predict AML patients' OS. We also refined the ELN2017 risk stratification system after the incorporation of this model. The immune-17 signature may be implemented to refine AML prognosis and, in the future, to inform treatment with novel immunotherapies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: (https://www.ncbi.nlm.nih.gov/), GSE37642.

AUTHOR CONTRIBUTIONS

RL, ZD, and PJ performed the research. RL designed the research study. SW, GJ, RX, WW, ZJ, and XL analyzed the data. RL, KX, XW, JL and PZ wrote and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2021.639634/full#supplementary-material

Supplementary Figure 1 | Enrichment analysis of differentially expressed genes (DEGs) derived from AML patients and healthy individuals. **(A)** The top 10 Gene Ontology (GO) terms of biological process. **(B)** The top 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) terms.

Supplementary Figure 2 | Stratification analysis. Kaplan-Meier analysis of AML patients from TCGA-LAML cohort stratified by age **(A)** and ELN risk stratification system **(B)**.

Supplementary Table 1 | 2516 DEGs between AML patients and healthy individuals.

Supplementary Table 2 | 199 differentially expressed IRGs between AML patients and healthy individuals.

Supplementary Table 3 | Univariate Cox analysis of 199 differentially expressed IRGs.

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Bone Marrow Soluble Mediator Signatures of Patients With Philadelphia Chromosome-Negative Myeloproliferative Neoplasms

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Background: Essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) are clonal hematological diseases classified as Philadelphia chromosome-negative myeloproliferative neoplasms (MPN). MPN pathogenesis is associated with the presence of somatic driver mutations, bone marrow (BM) niche alterations, and tumor inflammatory status. The relevance of soluble mediators in the pathogenesis of MPN led us to analyze the levels of cytokines, chemokines, and growth factors related to inflammation, angiogenesis and hematopoiesis regulation in the BM niche of MPN patients.

Methods: Soluble mediator levels in BM plasma samples from 17 healthy subjects, 28 ET, 19 PV, and 16 PMF patients were determined using a multiplex assay. Soluble mediator signatures were created from categorical analyses of high mediator producers. Soluble mediator connections and the correlation between plasma levels and clinic-laboratory parameters were also analyzed.

Results: The soluble mediator signatures of the BM niche of PV patients revealed a highly inflammatory and pro-angiogenic milieu, with increased levels of chemokines (CCL2, CCL5, CXCL8, CXCL12, CXCL10), and growth factors (GM-CSF M-CSF, HGF, IFN-γ, IL-1β, IL-6Ra, IL-12, IL-17, IL-18, TNF-α, VEGF, and VEGF-R2). ET and PMF patients presented intermediate inflammatory and pro-angiogenic profiles. Deregulation of soluble mediators was associated with some clinic-laboratory parameters of MPN patients, including vascular events, treatment *status*, risk stratification of disease, hemoglobin concentration, hematocrit, and red blood cell count.

Conclusions: Each MPN subtype exhibits a distinct soluble mediator signature. Deregulated production of BM soluble mediators may contribute to MPN pathogenesis and BM niche modification, provides pro-tumor stimuli, and is a potential target for future therapies.

Keywords: myeloproliferative neoplasms, soluble mediators, inflammation, angiogenesis, cytokines, bone marrow niche

INTRODUCTION

Essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) are classical myeloproliferative neoplasms (MPN) also known as Philadelphia chromosome (Ph)-negative MPN. These clonal diseases are characterized by single or multilineage hyperproliferation of the bone marrow (BM) that results in spontaneous accumulation of mature myeloid cells in the BM and peripheral blood. The erythroid lineage is the mostly affected in PV patients, while the megakaryocytic lineage from ET and PMF patients exhibit hyperplasia and atypia, and BM fibrosis. PMF patients also have increased or decreased number of granulocytes, monocytosis, and erythroid dysplasia (1, 2).

Disease pathogenesis is partially attributed to the presence of acquired driver mutations in Janus Kinase 2 (JAK2), calreticulin (CALR) or myeloproliferative leukemia virus (MPL) genes. These somatic mutations lead to abnormal activation of the JAK pathway, resulting in constitutive activation of their downstream effectors, specially STATs (2, 3). JAK2V617F is the most frequent driver mutation in MPN patients: it is detected in more than 90% of PV patients, and in about 50–60% of ET and PMF patients (4, 5). The CALR mutation is found in about 20–30% of ET and PMF patients, and is the second most frequent MPN-mutation (4, 5). Triple negative and MPL mutation are present in less than 12% of ET and PMF patients (4).

The crosstalk between inflammation and neoplastic cells plays a crucial role in disease development and progression. The BM of MPN patients is rich in inflammatory cytokines and growth factors, which form a pro-tumorigenic microenvironment that supports neoplastic cells and favors specific clinical phenotypes (6–8).

Cytokines and chemokines are key mediators of the immune system that regulate many complex signaling processes, and whose levels reflect the systemic and local immune activation status (9, 10). The co-participation of cytokines may result in activation or inactivation of immune pathways, and one cytokine can be secreted by different cell types in the same environment (10). In addition, cytokines act as important regulatory signals of hematopoiesis by inducing proliferation and/or survival of hematopoietic stem-cells (8, 11). Cytokines can also play a role as extrinsic factors that contribute to BM pathological changes (6, 7).

MPN are considered tumor inflammatory diseases. Over the past years, several studies have investigated how chronic inflammation contributes to MPN pathogenesis (6, 7, 12). We have recently described an altered cytokine profile in peripheral blood of MPN patients (13). PMF patients exhibit high inflammatory profile due overproduction of multiple pro-inflammatory cytokines and chemokines (13, 14); in these patients, the presence of JAK2V617F mutation is associated with high CXCL10 levels (13).

Considering the relevance of cytokines, chemokines, and growth factors (hereafter referred to as soluble mediators) as mediators of inflammation, angiogenesis and hematopoiesis regulation, here we examined the soluble mediator signature in the BM niche of ET, PV, and PMF patients. We also analyzed the correlation between cytokine levels and clinic-laboratory parameters.

MATERIALS AND METHODS

Patients and Samples

The Ethics Committees for Human Research from the School of Pharmaceutical Sciences of Ribeirão Preto, from the University Hospital of the Ribeirão Preto Medical School (HC-FMRP; Ribeirão Preto, Brazil), and from the Euryclides de Jesus Zerbini Transplant Hospital (São Paulo, Brazil) approved the study protocol.

The studied groups consisted of 17 healthy volunteers (CTRL group) and 63 MPN patients (28 ET, 19 PV, and 16 PMF patients). The patients were recruited at the Bone Marrow Transplantation Unit of HCFMRP-USP and at the Euryclides de Jesus Zerbini Transplant Hospital. All MPN patients were diagnosed according to the 2016 World Health Organization criteria (1). Healthy BM donors were recruited at the Bone Marrow Transplantation Unit of HCFMRP-USP.

BM aspirates were collected from the left posterior iliac crest into EDTA tubes at the time of diagnosis. Plasma was separated from the BM samples by centrifugation at 400×*g* for 10 min at 4°C (Eppendorf 5810R centrifuge), and aliquots were stored at −80°C for further cytokine analysis.

Risk-Stratification of MPN Patients

PV patients were classified into two risk categories: high and low. High-risk patients were the ones with age >60 years and/or history of vascular complications (including previous thrombosis, cardiovascular events and/or strokes). The patients who did not present the abovementioned two risk factors were classified as low risk (15).

The revised IPSET-thrombosis (r-IPSET-t) risk score splits ET patients into four risk categories: very low (age <60 years and absence of JAK2 mutation), low (age <60 years and presence of JAK2 mutation), intermediate (age >60 years and absence of JAK2 mutation), and high risk (age >60 years and presence of JAK2 mutation or vascular complication) (16).

The DIPSS-Plus scoring system was used to classify PMF patients into four categories: low, intermediate-1, intermediate-2, and high risk. The prognosis score considered the following risk factors: age, white blood cell and platelet counts, peripheral blood blast percentage, hemoglobin concentration, transfusion dependency, presence of constitutional symptoms, and unfavorable karyotype (17).

Multiplex Assays

Soluble mediator levels were determined using a customized microbeads multiplex assay (Human Magnetic Luminex® Assay, R&D Systems) performed on the Luminex1 MAGPIX1 System (Luminex Corporation). The cytokines and chemokines measured were interleukins (IL) IL-6, IL-1β, IL-12p70, IL-10, IL-17a, and IL-18; interferon gamma (IFN-γ); tumor necrosis factor alpha (TNF-α); IL-6 receptor subunit alpha (IL-6Ra); C–X–C motif ligands (CXC) CXCL8 (also known as IL-8), CXCL12 (also known as SDF-1, stromal cell-derived factor 1), CXCL10 (also known as IP-10, interferon-induced protein 10); and C–C motif chemokine ligands (CCL) CCL2 (known as MCP-1, monocyte chemotactic protein 1) and CCL5 (known as RANTES). The growth factors granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), and VEGF receptor 2 (VEGF-R2) were also quantified. Data were analyzed using the Milliplex Analyst software v3.5 (Millipore; VigeneTech Ind).

Data Analyses

Mann–Whitney test was applied to compare differences in distribution of soluble mediators among MPN groups (ET, PV, and PMF) and patients' JAK2V617F status. Spearman test was used for the correlation analysis of hematological parameters. GraphPad Prism 6.0 software (GraphPad Software) was used for statistical analysis. Significance was set at 0.05.

Cytoscape 3.7.2 software (available at htpp://cytoscape.org, National Institute of General Medical Sciences of the National Institutes of Health, USA) was used to construct the soluble mediator signatures (18), using the *r*-values (correlation coefficient) from the Spearman test that had *p*-value <0.05.

Overall soluble mediator profile was obtained by characterizing the general cytokine pattern of each group (18, 19). Each individual was classified as high or low mediator-producer, based on overall median values, as described previously by Vitelli-Aguiar et al. (19). The complete protocol of overall analyses was adapted from our previous work (13).

RESULTS

Demographic Data and Clinic-Laboratory Profile of Study Cohort

The median age of MPN patients was 65.5 years (20–85), distributed as 63 (31–78), 62 (20–85), and 68.5 (54–80) years for ET, PV, and PMF patients, respectively. The median age of CTRL subjects was 49 (19–83) years. Male–female proportions in the studied CTRL, ET, PV, and PMF groups were 6–11, 5–23, 12–7, and 12–4, respectively. Demographic and clinic-laboratory characteristics of the MPN cohort, including age, gender, mutation *status*, risk stratification, fibrosis rate, transfusion dependency, treatment *status*, hematological parameters, and reticulin rate are summarized in **Table 1**. The individual characteristics of CTRL volunteers and MPN patients are summarized in **Tables S1**–**S4**.

TABLE 1 | Demographic data and clinic-laboratory parameters from polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) patients.

Data	PV (n=19)	ET (n=28)	PMF (n=16)
Age (years/range)	62 (20–85)	63 (31–78)	68.5 (54–80)
Gender (male %)	12 (63.16)	5 (17.86)	12 (75)
Mutation *status*			
JAK2V617F^{+} (%)	19 (100)	13 (46.43)	9 (56.25)
CALR^{+} (%)	0 (0)	7 (25)	5 (31.25)
JAK2V617F^{-} (%)	0 (0)	5 (17.86)	0 (0)
Double negative (%)	0 (0)	3 (10.71)	2 (12.5)
Treatment, n (%)	4 (21.06)	10 (35.71)	5 (31.25)
ASA	2 (10.53)	2 (20)	0 (0)
HU	2 (10.53)	6 (60)	3 (60)
ASA + HU	0 (0)	2 (20)	0 (0)
Anagrelide + ASA	0 (0)	0 (0)	1 (20)
Ruxolitinib	0 (0)	0 (0)	1 (20)
Vascular event, n (%)	7 (36.84)	5 (17.86)	5 (31.25)
NA	0 (0)	4 (14.29)	0 (0)
Transfusion dependency, n (%)	0(0)	2 (7.14)	5 (31.25)
NA	1 (5.26)	3 (10.71)	0 (0)
Fibrosis rate n, (%)			
0	10 (52.64)	20 (71.44)	1 (6.25)
1	5 (26.32)	3 (10.71)	0 (0)
2	1 (5.26)	2 (7.14)	3 (18.75)
3	1 (5.26)	1 (3.57)	8 (50)
4	1 (5.26)	0 (0)	4 (25)
NA	1 (5.26)	2 (7.14)	0 (0)
Reticulin rate n, (%)			
0	0 (0)	14 (50)	0 (0)
1	0 (0)	4 (14.29)	0 (0)
2	0 (0)	1 (3.57)	4 (25)
3	0 (0)	1 (3.57)	8 (50)
4	0 (0)	1 (3.57)	4 (25)
NA	19 (100)	7 (25)	0 (0)
Hematological parameters			
WBC count, ×10^{3}/mm^{3} (range)	12.1 (3.59–21)	7.28 (3.35–15.6)	5.85 (1.46–15.3)
RBC count, ×10^{6}/mm^{3} (range)	6.33 (3.09–7.46)	4.34 (2.98–5.19)	3.66 (2.49–6.01)
Hemoglobin, g/dl (range)	16.3 (11.1–21.5)	13.2 (10.1–22.4)	11.1 (8.62–17.4)
Hematocrit, % (range)	49.1 (33.7–62)	40.65 (34.6–64.7)	35.15 (26.3–55.3)
PLT count, ×10^{3}/mm^{3} (range)	605 (161–1502)	665.5 (304–1293)	305 (71.7–917)

ASA, acetylsalicylic acid; CALR^{+}*, positive for calreticulin mutation; Double negative, negative for JAK2V617F and CALR mutation; HU, hydroxycarbamide; JAK2V617F*$^{+}$*, positive for JAK2V617F mutation; JAK2V617F*$^{-}$*, negative for JAK2V617F mutation; NA, data not available; PLT, platelets; RBC, red blood cells; VE, previous vascular event; WBC, white blood cells.*

Soluble Mediator Levels in the BM Niche of PV, ET and PMF Patients

Compared with CTRL, the BM niche of PV patients presented increased levels of inflammatory cytokines and angiogenesis- and hematopoiesis-related factors, including CCL2, CCL5, CXCL8, CXCL10, CXCL12, GM-CSF, HGF, IFN-γ, IL-1β, IL-6Ra, IL-12p70, IL-17a, IL-18, M-CSF, TNF-α, VEGF, and VEGF-R2 (**Figure 1** and **Table S5**).

ET patients exhibited augmented levels of CCL2, CCL5, CXCL8, CXCL10, GM-CSF, IFN-γ, IL-1β, IL-17a, IL-18, TNF-α, and VEGF, when compared with CTRL. MF patients displayed higher levels of only CXCL8, CXCL10, IL-6Ra, and IL-18, as compared with CTRL (**Figure 1** and **Table S5**).

All MPN categories presented high production of the chemokines IL-18, CXCL10 and CXCL8. Compared with PV patients, ET patients had lower levels of G-CSF, HGF, IFN-γ, IL-10, and IL-17a in the BM niche, while PMF patients presented lower levels of CCL2, CCL5, CXCL12, G-CSF, GM-CSF, HGF, IFN-γ, IL-1β, IL-10, IL-17a, IL-12p70, M-CSF, TNF-α, and VEGF. The BM niche of ET patients exhibited higher levels of CCL5, IL-6 and VEGF than PMF patients.

The present results indicated that the BM levels of IL-17, IFN-γ, G-CSF, and HGF in PV patients were higher than those detected in PMF and ET patients. Hence, PV patients seem those with more unique soluble mediators profile as compared to the other MPN.

Categorical Analyses of Soluble Mediator Production in BM Niche of MPN Subtypes

Categorical analyses were performed to better comprehend the soluble mediator production patterns and the differences among MPN subtypes. Patients were stratified into high and low producers of soluble mediators using the overall median as

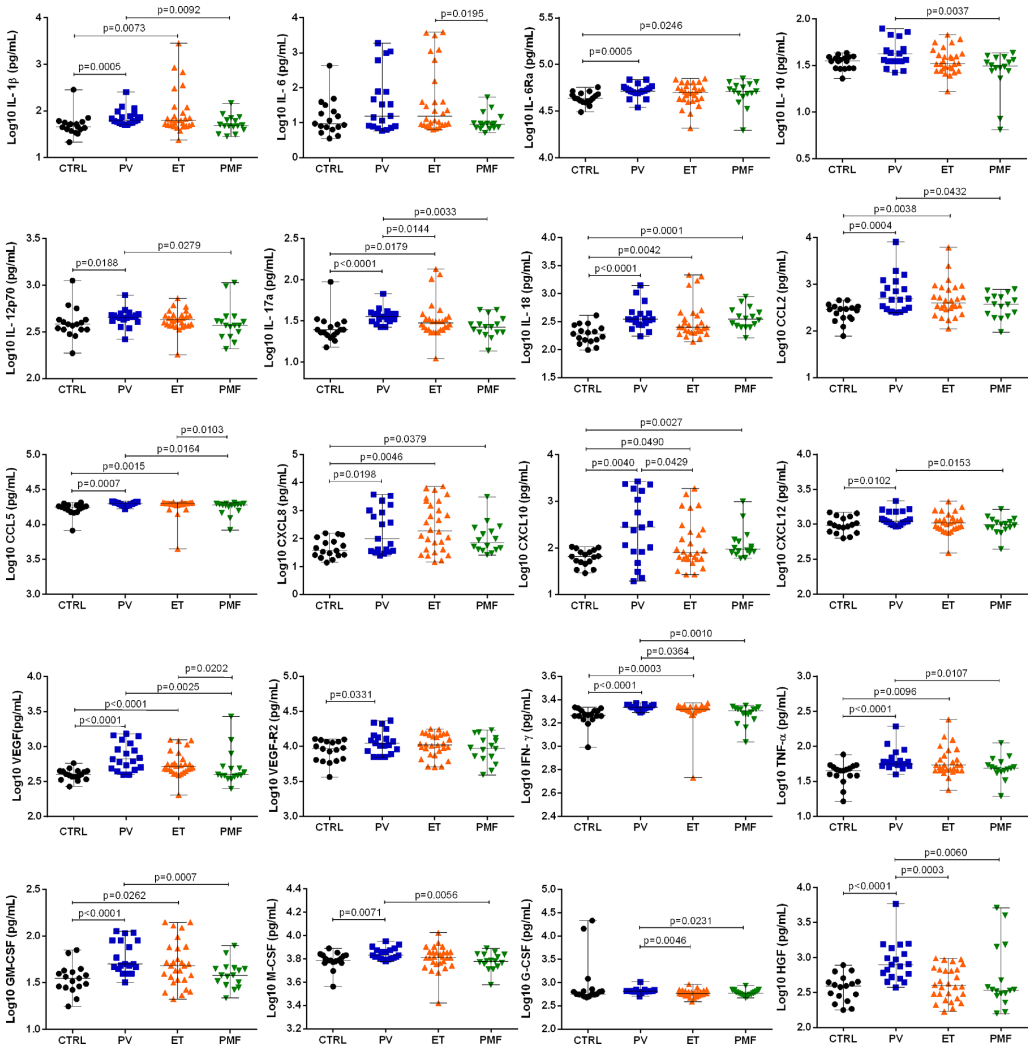

FIGURE 1 | Bone marrow plasma levels of inflammatory soluble mediators in healthy subjects (CTRL; n = 17) and patients with essential thrombocythemia (ET, n = 28), polycythemia vera (PV, n = 19), and primary myelofibrosis (PMF, n = 16). The concentration of all the soluble mediators was determined using a Multiplex assay. Significant differences when p <0.05, Mann–Whitney test.

cut-off point. High producer was the individual whose soluble mediator production value was higher than the overall median, while low producer was the individual whose soluble mediator production level was equal to or lower than the overall median. The frequency of high producers of each mediator was calculated for all disease and CTRL subsets. The production of each mediator was considered relevant when the frequency of high producers exceeded 50% (13, 19, 20).

The CTRL group did not produce any of the mediators in relevant amounts, since less than 50% of the individuals were high producers (**Figure S1**). Soluble mediator production in BM niche differed among MPN subtypes. PV patients exhibited remarkably dysregulated production of soluble mediators, with relevant production of the 20 mediators analyzed. Only PV group showed high producers for IL-10, CXCL12, IFN-γ, G-CSF and HGF in a relevant frequency. Differently from PMF, PV and ET group showed relevant production of IL-6, IL-1β, IL-17a, IL-12p70, CCL5, VEGF, VEGF-R2, TNF-α, GM-CSF and M-CSF.

ET patients produced most of the mediators in relevant amounts, but PMF patients exhibited relevant production of only five mediators (**Figure S1**), among them IL-6Ra, IL-18 and CXCL10 were present in PMF but not in ET patients. Interestingly, all MPN categories presented high production of the chemokines CXCL8 and CCL2 (>50% high producers).

Spider charts summarize the soluble mediator signatures and enable visual comparison among the CTRL, ET, PMF, and PV groups (**Figure S2**).

Soluble Mediator Networks in MPN Subtypes

After identifying the soluble mediator signature for each disease (ET, PV, and PMF) and CTRL group, we analyzed the existence of correlation between the mediator levels (**Figure 2**). Correlations were stratified into negative (r <0), weak (r ≤0.35), moderate (r ≥0.36 and r ≤0.67) or strong (r ≥0.68). The CTRL group exhibited the highest number of strong correlations, and similar findings were obtained in ET patients (IL-1β with IL-17a and IFN-γ; IL-10 with IL-12p70, IFN-γ and M-CSF; IL-17a with CCL5 and IFN-γ; IL-12p70 with CCL5 and IFN-γ; CCL5 with IFN-γ and M-CSF; VEGF with VEGF-R2; and IFN-γ with M-CSF). PMF patients also showed many strong interactions (between IL-17a with IL-6Ra, CCL5, VEGF, GM-CSF, G-CSF and M-CSF; IL-10 with CXCL10 and GM-CSF; CCL5 with IFN-γ and VEGF;

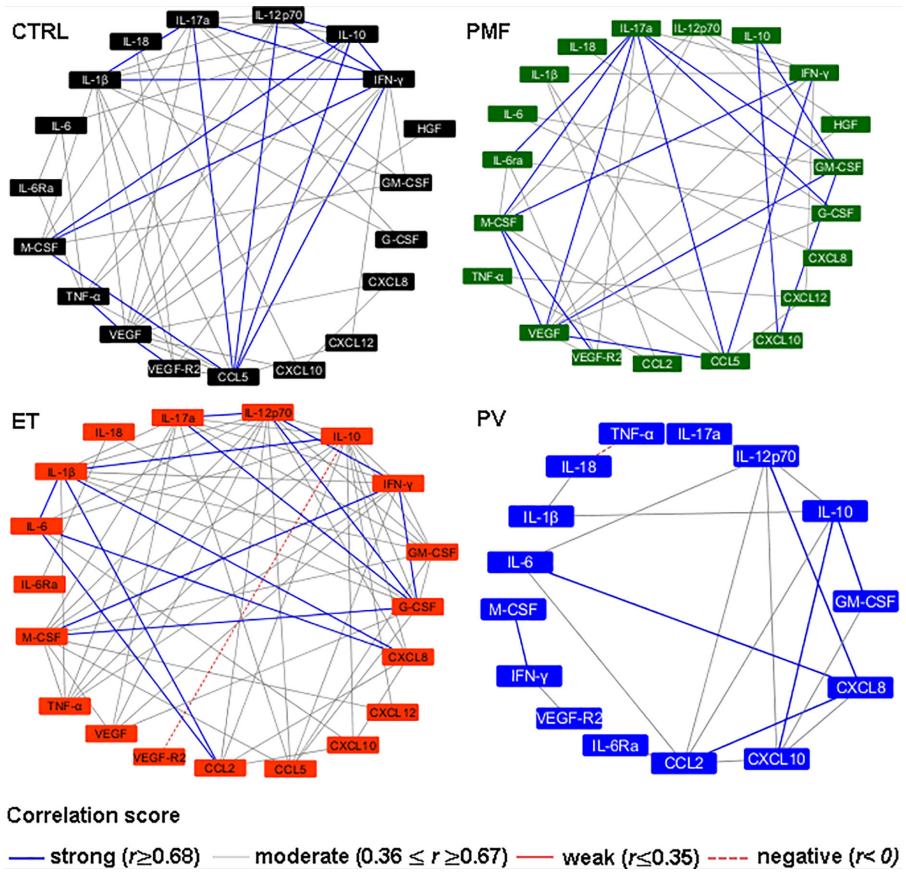

FIGURE 2 | Soluble mediators interaction networks in healthy subjects (CTRL, n = 17) and patients with essential thrombocythemia (ET, n = 28), polycythemia vera (PV, n = 19), and primary myelofibrosis (PMF, n = 16). The correlations were stratified according to r-values: strong (blue lines, r ≥0.68), moderate (gray lines, 0.36 ≤ r ≥ 0.67) and negative (dashed red line, r <0). The correlations depicted in this figure were significant (p <0.05).

CXCL10 with GM-CSF; M-CSF with IFN-γ and VEGF-R2), while PV patients were highly different from the CTRL group and showed the lowest number of strong correlations (CXCL8 with IL-6, IL-12p70 and CCL2; IL-10 with GM-CSF and CXCL10; and M-CSF with IFN-γ). These results highlighted the deregulation of soluble mediator network in PV patients versus other MPN categories and CTRL group.

Correlation Between the BM Soluble Mediator Levels and Patients' Clinic-Laboratory Parameters

We analyzed the potential correlation between soluble mediator levels and the major clinic-laboratory parameters (**Figure 3A**). The patients' hematological parameters analyzed were hemoglobin concentration, hematocrit, and white blood cell, red blood cell, and platelet counts.

The following correlations among soluble mediators and MPN clinical parameters were found:

In PV, hemoglobin concentration positively correlated with VEGF and HGF, and negatively with CCL5 and IFN-γ; red blood cell count positively correlated with IL-6Ra and TNF-α; hematocrit positively correlated with IL-6Ra, and negatively correlated with CCL5 and IFN-γ; white blood cell count positively correlated with IL-6Ra and VEGF, and negatively with CCL5; and platelet counts negatively correlated with CXCL10 and CCL2 (**Table S6**).

ET patients displayed positive correlations between hemoglobin concentration with IL-6Ra, IL-1β, IL-10, IL-18 and G-CSF; red blood cell count with IL-6Ra and VEGF-R2; hematocrit with GM-CSF and IL-1β; white blood cell count with IL-6Ra; and platelet count with VEGF-R2 and CCL5 (**Table S7**).

In PMF, white blood cell count positively correlated with VEGF-R2. Negative correlations among hemoglobin concentration, red blood cell count and hematocrit with IL-6, IL-10, CXCL10 and GM-CSF were also observed (**Table S8**). No similar patterns of correlation between soluble mediator levels and the clinic-laboratory parameter were obtained in PV, ET and PMF.

Risk-stratification analysis showed in very low risk ET patients (n = 6) higher CXCL8 levels than those detected in low risk ET patients (n = 4) (**Figure 4A**). No relationships were found among soluble mediators and PV and PMF risk *status*.

Regarding therapy, treated PV patients (with hydroxycarbamide or acetylsalicylic acid) had lower G-CSF levels than untreated PV patients (**Figure 4B**). There was no association between soluble mediator levels and treatment in ET and PMF patients

Five ET patients with vascular event (including thrombosis, cardiovascular events and/or strokes) exhibited lower levels of IL-17, CCL5, GM-CSF, and VEGF than ET patients with no vascular event (n = 17) (**Figure 4A**). PV patients with vascular event (n = 7) displayed higher levels of CXCL12, TNF-α and VEGF-R2 than PV patients with no vascular event (n = 12) (**Figure 4B**). PMF patients with vascular event (n = 5) had increased levels of IL-6Rα when compared with patients with no vascular event (n = 11) (**Figure 4C**).

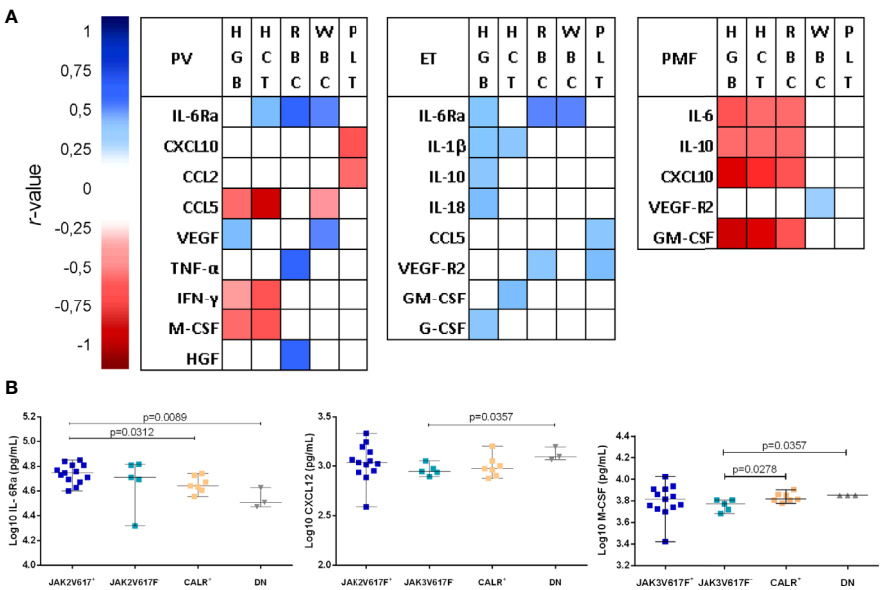

FIGURE 3 | **(A)** Correlation between soluble mediator levels and laboratory parameters of healthy subjects (CTRL, n = 17) and patients with essential thrombocythemia (ET, n = 28), polycythemia vera (PV, n = 19), and primary myelofibrosis (PMF, n = 16). The Spearman coefficient (*r*-value) was represented by a color gradient that ranged from close to 1 (dark blue) to −1 (dark red); white indicates no correlation. The correlations depicted in this figure were significant when p <0.05. HCT, hematocrit; HGB, hemoglobin concentration; PLT, platelet count; RBC, red blood cell count; WBC, white blood cell count. **(B)** Association between driver mutation *status* and IL-6Ra, CXCL12, and M-CSF levels in bone marrow plasma from patients with ET. JAK2V617F^{+} (n = 13 ET patients positive for JAK2V617F mutation). JAK2V617F^{-} (n = 5; ET patients negative for JAK2V617F mutation). CALR^{+} (n = 7; patients positive for calreticulin mutation). DN (n = 3; double negative for CALR and JAK2V617 patients). Statistical difference when p <0.05, Mann–Whitney test.

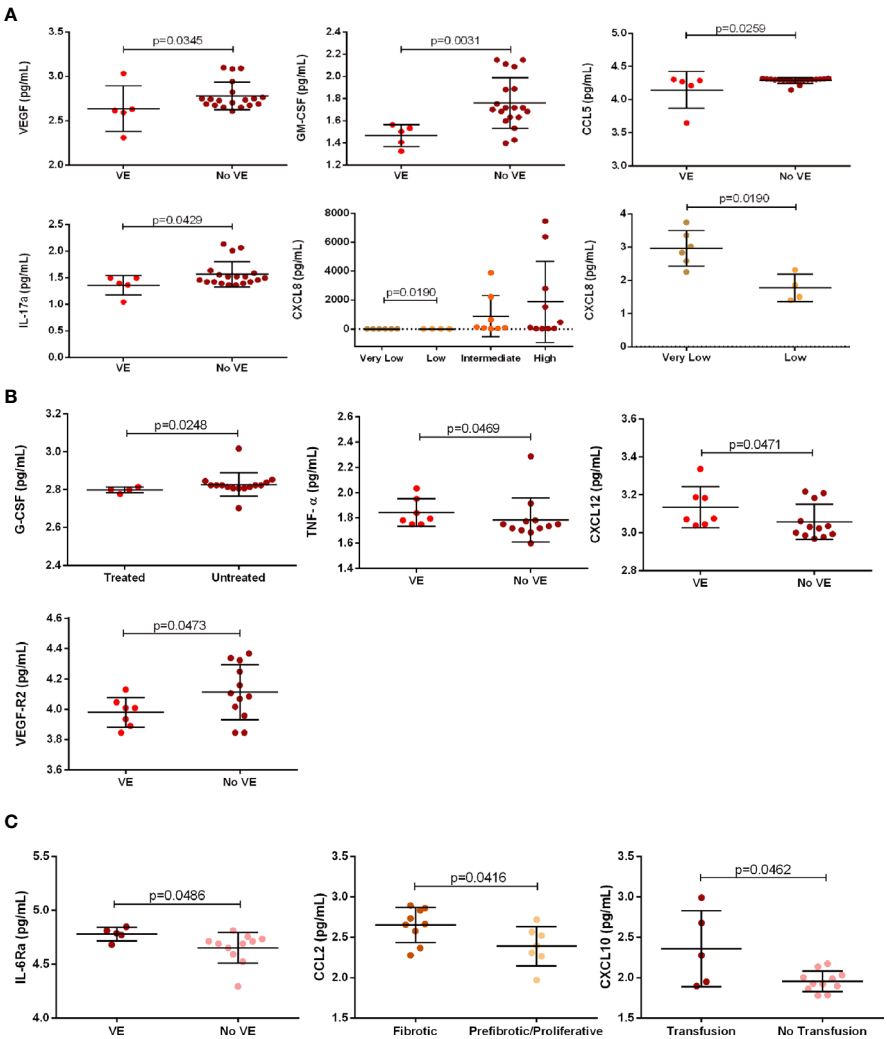

FIGURE 4 | Association between clinical parameters of patients with essential thrombocythemia (ET, n = 28), polycythemia vera (PV, n = 19), and primary myelofibrosis (PMF, n = 16) and soluble mediator levels. **(A)** ET patients: association between VEGF, GM-CSF, CCL5, and IL-17 levels and the presence (n = 5) or absence (n = 19) of VE; and CXCL8 levels and risk-stratification in very low (n = 6), low (n = 4), intermediate (n = 8) and high (n = 10) risk. **(B)** PV patients: association between G-CSF levels and administration (n = 4) or not (n = 15) of drug treatment; and association between TNF-α, CXCL12, and VEGF-R2 levels and the presence (n = 7) or absence (n = 12) of VE. **(C)** PMF patients: association between IL-6Ra levels and the presence (n = 5) or absence (n = 11) of VE; association between CCL2 levels and fibrotic disease (n = 9) or pre-fibrotic/proliferative (n = 7) stage; and association between CXCL10 levels and transfusion dependency (n = 5) or no transfusion necessity (n = 11).

Finally, we analyzed, in PMF patients, the potential association between soluble mediator levels and transfusion dependency, and disease stages (**Figure 4C**). PMF patients with transfusion dependency had higher CXCL10 levels than those with no dependency. In addition, PMF patients in pre-fibrotic/proliferative stage had elevated CCL2 levels when compared with those in fibrotic stage.

JAK2V617F$^+$ ET Patients Have High IL-6Ra Levels

The soluble mediators levels of ET and PMF patients were stratified according to their driver mutation *status*. ET JAK2V617F$^+$ showed higher IL-6Ra levels than those with CALR mutation, and double negative (DN) for JAK2V617F and CALR. ET CALR mutated patients displayed elevated M-CSF levels than DN; and JAK2V617F$^-$ patients showed lower levels of M-CSF and CXCL12 than DN patients (**Figure 3B**). There was no association between soluble mediators levels and mutation *status* in PMF patients.

The extent of production (i.e. high versus low producers) of soluble mediators according to the presence of driver mutations was also analyzed in ET (**Figure S3**) and PMF (**Figure S4**) patients. JAK2V617F$^+$ ET patients were high producers of CCL2, CXCL10, CXCL12, IL-1β, IL-6Ra, IL-18, and TNF-α; while

CALR mutated were high producers of GM-CSF, G-CSF, HGF, IFN-γ, IL-12p70, and IL-17a. The soluble mediators were differentially produced in PMF subgroups, JAK2V617F$^+$ patients were high producers of CXCL8, and low producers of G-CSF, M-CSF, and VEGF-R2, while CALR mutated patients showed the opposite profile of these mediators' production.

DISCUSSION

Both the MPN clone and BM-resident cells maintain the cytokine-mediated inflammatory microenvironment in a feedback loop mechanism that also maintains a pro-tumorigenic environment. Neoplastic cells secrete pro-inflammatory and angiogenic mediators that promote autocrine and paracrine stimulation of fibroblasts, endothelial cells, and stromal cells. In contrast, mediators produced in the BM have the potential to modify the phenotype of resident cells, stimulate angiogenesis and fibrosis, and thereby influence neoplastic cell survival, proliferation, and progression (7, 21, 22).

Little is known about the cytokine milieu in the BM niche of MPN patients. Most of the studies have reported the presence of angiogenesis-related molecules by immunohistochemistry analysis and associated their high levels with the presence of neo-angiogenesis and fibrosis in BM (23, 24).

In physiological state, the control of cytokines and chemokines production include an intricate of regulatory mechanism, with an inhibitory feedback and synergic actions to guarantee the balance of mediators levels, non-inflammatory *status*, and tissue homeostasis (25, 26).

PV patients exhibited a unique soluble mediator signature, as demonstrated by the overall and single analysis of soluble mediators. PV patients had higher levels of IL-17, IFN-γ, G-CSF and HGF, as compared with PMF and ET patients. These cytokines may be a useful tool in differential diagnosis of MPN.

The increased soluble mediator levels in BM niche from MPN patients could be partially explained by chronic inflammation associated with oncogenesis (7, 21). Chronic inflammation may be linked to hypoxia due to cell accumulation in the BM, which in turn was associated with JAK/STAT pathway activation by IL-6, IL-11, VEGF, HGF, PDGF, and TGF-β that mediates cell survival and proliferation, and thereby contributes to MPN pathogenesis (27, 28). Cytokine overproduction could also result from cancer-associated genetic mutations (27). Most of our results demonstrate that soluble mediators are not influenced by driver mutation *status* and corroborate previous studies on MPN patients (13, 28–31).

It is worth to note that IL-6Ra and M-CSF levels, in this study, were associated with JAK2V617F and CALR driver mutations, respectively. JAK2V617F$^+$ ET patients presented high BM IL-6Ra levels, while CALR$^+$ ET patients exhibited high M-CSF levels. Categorical analysis of soluble mediator production according to driver mutation *status* identified that the group of CALR$^+$ ET patients had the lowest number of high producers.

The trans-signaling of IL-6/IL-6R soluble receptor (sIL-6R) complex lead to subsequent activation of JAK/STAT, MAPK and PIK pathways, and it can be activated in all types of cells, including cells not responsive to IL-6 alone (32). An observational epidemiological study reported that polymorphisms that cause loss of IL-6R function are associated with reduced risk of JAK2V617F mutation and MPN (33).

M-CSF-stimulated human macrophages have growth-promoting and proangiogenic phenotype with tissue repair potential under conditions of induced inflammation (34). In the present study, CALR$^+$ MPN patients had higher M-CSF levels than JAK2V617F$^+$ patients. It is well-known that CALR$^+$ patients have better prognosis and higher overall survival than JAK2V617F$^+$ patients (35). Our results, combined with the abovementioned references (32–35), may suggest that: 1) high IL-6Ra levels favor JAK/STAT pathway activation and the oncoinflammatory state in ET patients; 2) high M-CSF levels favor tissue homeostasis and attenuate inflammation in BM from CALR$^+$ patients.

The BM milieu and the peripheral blood systemic profile from MPN patients are distinct. Many authors have reported that MPN patients develop a robust and systemic inflammatory response in peripheral blood (13, 29, 36, 37). In our study, ET and PMF patients had mild BM niche inflammation, while PV patients exhibited the most prominent and diffuse inflammatory response in BM niche, among the studied MPN categories.

PMF is the MPN subtype with higher number of alterations in the BM niche, including the presence of fibrosis and defective hematopoiesis (1). Indeed, BM fibrosis seems to result from continuous and long-lasting shift of the cytokine milieu rather than a specific genetic trigger (38). In our study, the cytokine milieu in PMF patients was similar to the CTRL group, despite the increased levels of CXCL8, CXCL10, IL-18, and IL-6Ra. Indeed, we identified CCL2, CXCL8, CXCL10, and IL-18 as MPN-associated cytokines, due to their prominent levels in BM niche of all MPN subtypes.

IL-18 is considered an inflammasome product whose main function is to promote IFN-γ secretion (39). IL-18 secreted by BM stroma elicits the growth of leukemia blast cells and contributes to progression of T-cell acute leukemia (40). Elevated BM IL-18 levels are also associated with poor overall survival of multiple myeloma patients (41). IL-18 has been implicated in induction of fibrosis in idiopathic pulmonary fibrosis and heart inflammation; blockage of IL-18 activity has antifibrotic effects (42, 43). As described in other hematological malignancies and diseases characterized by accentuated fibrosis, we hypothesize that IL-18 may contribute to tumorigenesis and BM fibrosis process in MPN.

CXCL8 and CXCL10 are important to regulate hematopoietic stem cells (44, 45) and mediate inflammation-driven angiogenesis due to CXCL10 angiostatic activity and CXCL8 angiogenic properties (46). The contribution of CXCL8 to tumorigenesis has been described in patients with acute myeloid leukemia, in which high CXCL8 levels are secreted by BM mesenchymal stromal cells and support the proliferation and survival of leukemic cells (47). Blockage of CXCL8 expression in PMF CD34$^+$ cells promotes cell proliferation and megakaryocyte differentiation (48). Neutralization of the CXCL8 receptors CXCR1 and CXCR2 enhances PMF megakaryocyte cell

proliferation, indicating that CXCL8 and its receptor are involved in megakaryocyte abnormalities and contribute to PMF pathogenesis (48). The two last reports cited (47, 48) confirmed our findings and stressed the importance of these mediators in MPN subtypes, manly by regulating neoplastic cell proliferation, survival, and differentiation.

CXCL8 is a pre-fibrotic cytokine (21) whose levels are increased in BM biopsies of PMF patients, as demonstrated by immunohistochemistry. MF $CD34^+$ CXCL8-secreting clones are associated with patients with high-grade reticulin fibrosis in BM (49). Moreover, elevated CCL2 levels in MPN patients are associated with fibrosis and poor prognosis (28, 29, 36, 37). The expression of inflammatory genes, especially *CCL2* and *CXCL10*, is upregulated in patients with overt fibrosis, indicating that pro-inflammatory gene upregulation is associated with BM fibrosis, independently of the MPN (30). CCL2 and CXCL8 also exert a myelosuppressive effect that can disturb normal hematopoiesis (50).

In summary, these reports corroborate our findings and reinforce the contribution of CCL2, CXCL8, CXCL10, and IL-18 for MPN pathogenesis by promoting hematopoietic niche modifications, activation of angiogenesis, and deregulation of hematopoiesis. Only CXCL8 has been previously reported as a MPN-associated cytokine (21, 22, 51); this could be explained by the distinct cytokine levels detected in peripheral blood and BM.

The network analysis of soluble mediators revealed a distinct integrative system among PV and the other studied groups (ET, PMF and Control). Most of the soluble mediators interaction found in our study presented biological relevance, and resides in their synergistic interactions, which could be observed between: 1) IL-1β and IL-12 inducing IFN-γ secretion (52); 2) IL-1β, TNF-α and IL-6 promoting VEGF secretion (53); 3) GM-CSF interaction with M-CSF/G-CSF resulting in increase of granulopoiesis and monocytopoiesis (54); 4) IFN-γ with IL-1β and TNF-α upregulates CCL5 expression (55). These correlations were observed in ET and PMF patients.

PV patients display very strong positive correlations only between a few cytokine and chemokine molecules (IL-6, CXCL-8, IL-12 and CCL2). This data suggests that the immune imbalance in BM microenvironment is more prominent in PV than in ET and PMF patients. Literature reported that these molecules are associated with a pro-inflammatory *status*, occurrence of vascular events and oncoinflammation.

We demonstrated that soluble mediator levels in the BM niche correlated to clinic-laboratory parameters, including hemoglobin concentration, hematocrit, and white blood cell, red blood cell, and platelet counts. Many studies corroborate our findings and have demonstrated cytokine-phenotype associations related to pro-inflammatory *status* in MPN patients' serum (13, 36, 37). In PMF patients, CXCL8 is associated with leukocytosis, and CXCL10 levels correlate with thrombocytopenia (37). In PV patients, high levels of IL-12 are associated with hematocrit; high IL-1β and HGF levels are associated with leukocytosis; low IL-6 and FGF (fibroblast growth factor) levels are associated with hemoglobin concentration; and low GM-CSF levels are associated with thrombocytosis (36). In PMF patients, the elevation of CXCL10 was associated with thrombocytosis and decreased levels of CXCL10 and IL-17 with erythropenia, while in ET patients the low levels of TNF-α was associated with thrombocytosis (13). The different soluble mediator association patterns among MPN subtypes may explain their distinct clinical features.

In our study, soluble mediator levels were not significantly associated with disease prognosis risk, probably due to the small number of patients enrolled. The reduced soluble mediator levels in treated MPN patients suggest that drug treatment influenced the production of many soluble mediators, although most of the comparisons did not reach statistical significance—except for G-CSF levels in PV patients.

It is well-known that hydroxycarbamide (hydroxyurea), a cytoredutor drug indicated for MPN treatment, is capable of lowering serum inflammatory markers such as TNF-α, IL-6, CXCL8, and IL-1β in sickle cell disease patients (56, 57). This drug suppresses production of pro-inflammatory cytokines in monocytes from sickle cell anemia patients (57); however, its effect on cytokine levels of MPN patients is poorly studied. The anti-inflammatory action of hydroxycarbamide relies on the hematological remission resulting from myelosuppression, reduction of leukocyte counts (58), and the drug effects on monocytes, as pointed out in sickle cell anemia.

The frequency of vascular events was associated with different setups of soluble mediators among MPN subtypes. PV patients exhibited increased TNF-α, CXCL12, and VEGFR2 levels; ET patients displayed increased IL-17, CCL5, GM-CSF, and VEGF levels; and PMF patients had increased IL-6Ra levels. Elevated GM-CSF and IL-12 serum levels are associated with the lack of vascular complications in ET and PV patients; these cytokines may also help to select the treatment regimen (29). In addition, CCL5 levels are associated with microvascular manifestations in PV patients (36). Augmented levels of angiogenic cytokines as VEGF, soluble vascular endothelial growth factor receptors 1 and 2, and placenta growth factor, as well as the increased number of endothelial cells and endothelial precursors are associated with high risk of thrombotic events in ET and PV patients (59). The levels of coagulation activation markers did not differ with respect to the JAK2V617F mutational status, but the association between endothelial cells and leukocytes may contribute to thrombosis (59).

Activated platelets are the mainly secretors of CXCL12. Upregulated CXCL2 expression and secretion may favor the development of cardiovascular diseases, while the fast increase of CXCL12 in peripheral blood platelets can be used as biomarker for cardiac injury (60). Patients with ischemic stroke have increased TNF-α serum levels and higher risk for cardiovascular diseases, compared with healthy volunteers (61). The studies reported in the two last paragraphs (26, 33, 53, 54) corroborate our findings on the influence of soluble mediators on vascular events and support the concept that the inflammatory environment is a crucial stimulus for the initiation and development of thrombo-hemorrhagic events and cardiovascular diseases (7).

In PMF patients, there were associations between high CXCL10 levels and transfusion requirement, and high CCL2 levels and pre-fibrotic/proliferative disease stage. These results are supported by the association between upregulated *CXCL10* and *CCL2* gene expression and increased fibrosis in BM niche (30). Our findings revealed a potential utility of monitoring CCL2 levels during PMF course, and provide a new tool to measure BM fibrosis evolution.

CONCLUSIONS

Taken together, our findings demonstrate the existence of different soluble mediator signatures for each MPN subtype, among which PV patients present the highest levels of inflammatory and angiogenic soluble mediators. We identified CXCL8, CXCL10, IL-18, and CCL2 as MPN-associated soluble mediators; IL-17, IFN-γ, and HGF as biomarkers for PV; and CCL2 as biomarker for monitoring the BM fibrosis. In addition, specific mediators are potential targets for developing future therapies to prevent BM transformation in MPN patients. The molecular mechanisms involved in cellular malignant transformation by the inflammatory/angiogenic BM milieu in MPN patients are currently unknown and further investigations are underway.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Suppelementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by The Ethics Committee for Human Research of the School of Pharmaceutical Sciences of Ribeirão Preto, the University Hospital of the Ribeirão Preto Medical School, and the Euryclides de Jesus Zerbini Transplant Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JC, KM, and FC conceived and designed the study. JC, MC, MB-C, IP, and FF prepared the material and collected and analyzed data. LF-P, ES, and MO selected and recruited the patients and performed their clinical evaluation. DC, FC, and KM acquired funding and provided the resources. JC and MC wrote the first draft of the manuscript, while FC, KM, and MO revised and edited it. All authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2021.665037/full#supplementary-material

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REVIEW
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Immune Phenomena in Myeloid Neoplasms: An "*Egg or Chicken*" Question

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Immune phenomena are increasingly reported in myeloid neoplasms, and include autoimmune cytopenias/diseases and immunodeficiency, either preceding or complicating acute myeloid leukemia, myelodysplastic syndromes (MDS), chronic myeloproliferative neoplasms, and bone marrow failure (BMF) syndromes. Autoimmunity and immunodeficiency are the two faces of a dysregulated immune tolerance and surveillance and may result, along with contributing environmental and genetic factors, in an increased incidence of both tumors and infections. The latter may fuel both autoimmunity and immune activation, triggering a vicious circle among infections, tumors and autoimmune phenomena. Additionally, alterations of the microbiota and of mesenchymal stem cells (MSCs) pinpoint to the importance of a permissive or hostile microenvironment for tumor growth. Finally, several therapies of myeloid neoplasms are aimed at increasing host immunity against the tumor, but at the price of increased autoimmune phenomena. In this review we will examine the epidemiological association of myeloid neoplasms with autoimmune diseases and immunodeficiencies, and the pivotal role of autoimmunity in the pathogenesis of MDS and BMF syndromes, including the paroxysmal nocturnal hemoglobinuria conundrum. Furthermore, we will briefly examine autoimmune complications following therapy of myeloid neoplasms, as well as the role of MSCs and microbiota in these settings.

Keywords: myelodysplastic syndromes, acute myeloid leukemia, myeloproliferative neoplasms, microbiome, autoimmunity, immunodeficiencies

INTRODUCTION

The immune system is broadly involved in maintaining homeostasis, either by fighting infectious agents or controlling tumor growth. Autoimmunity and immunodeficiency are the two faces of a dysregulated immune tolerance and surveillance and may result, along with contributing environmental and genetic factors, in an increased incidence of tumors (1). Autoimmunity is primarily the consequence of an improper self-directed immune reaction, whilst immunodeficiency

is the inability to efficiently eliminate infectious pathogens or neoplastic cells, and both may result in severe and life-threatening diseases. There is a delicate balance between immune-defense mechanisms and autoimmune reactivity, as recently highlighted by the autoinflammatory response and autoimmune complications following therapy with checkpoint inhibitors (CPI) and chimeric antigen receptor (CAR) T-cells (2). The association between lymphoproliferative disorders and peripheral immune-mediated cytopenias is well known, along with the underlying pathogenic mechanisms (3). The presence of autoimmune phenomena/diseases is less investigated in myeloid neoplasms, although reported in bone marrow failure (BMF) and myelodysplastic syndromes (MDS), as well as in chronic and acute myeloproliferative diseases (2). Of note, autoimmune phenomena may be a spurious serologic finding without clinical consequences, or even represent a favorable response aimed at eliminating damaged/harmful self-structures (1). On the other hand, the association of immunodeficiency with tumors is well known, together with the role of consequent chronic/relapsing infections that may fuel both autoimmunity and immune activation. The latter may be exaggerated, ineffective and potentially harmful, triggering a vicious circle among infections, tumors and autoimmune phenomena. More generally, there is increasing interest on the role of the immune system in the generation of a permissive or hostile microenvironment for tumor growth, which has recently involved also the microbioma and the mesenchymal stem cells (MSCs) (4–6). This review will examine immune phenomena (autoimmunity and immunodeficiency) in myeloid neoplasms, including MDS, BMF syndromes, acute myeloid leukemia (AML), and chronic myeloproliferative neoplasms (MPN). We will also focus on the several overlapping conditions, highlighting the continuous and mutual cross-talk between the immune effectors and the neoplastic cells (**Figure 1**).

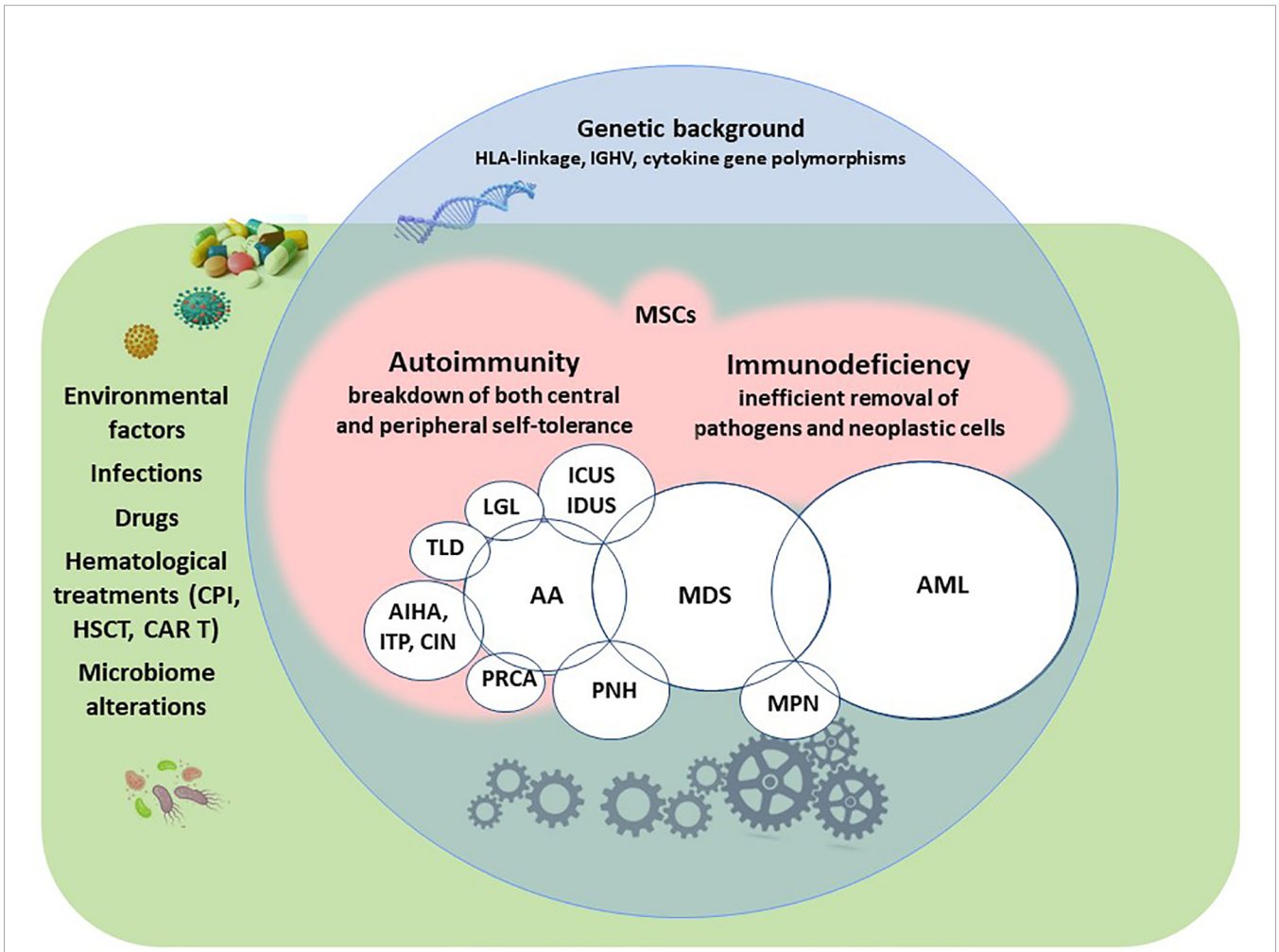

FIGURE 1 | Autoimmunity and immunodeficiency in myeloid neoplasms and associated conditions. AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; AA, aplastic anemia; ICUS, idiopathic cytopenia of undetermined significance; IDUS, idiopathic dysplasia of undetermined significance; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red cell aplasia; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia, CIN, chronic idiopathic neutropenia; TLD, telomeres diseases; LGL, large granular lymphocyte lymphoproliferative syndromes; CPI, checkpoint inhibitors; HSCT, hematopoietic stem cell transplantation; CAR T, chimeric antigen receptor T-cells; MSCs, mesenchymal stem cells.

EPIDEMIOLOGICAL ASSOCIATION OF AUTOIMMUNE DISEASES AND MYELOID NEOPLASMS

Several autoimmune diseases (AID) and, less frequently, autoimmune cytopenias (AIC) have been described in myeloid neoplasms. These include systemic and organ specific disorders, such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), vasculitis, thyroid autoimmune diseases, Sjogren syndrome (SS), autoimmune hemolytic anemia (AIHA), immune thrombocytopenia (ITP), pure red cella aplasia (PRCA), and immune-mediated hemostatic disorders (2, 7–10). The diagnosis may be challenging due to the great clinical heterogeneity and variable organ involvement of AID, whose signs/symptoms may be confounded with those of the hematologic malignancy. Likewise, diagnosis of AIC may be complicated by overlapping conditions like chemotherapy, bone marrow infiltration, and transfusion support (2). Additionally, straightforward diagnostic tests are lacking, particularly for AID, and several diagnostic pitfalls exist for AIC as well. Among myeloid neoplasms, MDS and chronic myelomonocytic leukemia (CMML) are complicated in up to 20-30% by vasculitis subtypes, more commonly Behçet's-like syndrome, relapsing polychondritis, polyarteritis nodosa and giant-cell arteritis (2, 9). CMML is also frequently complicated by ITP either concomitant or preceding its diagnosis, while AIHA and PRCA are occasionally observed (9). Regarding MPN, including myelofibrosis (MF), various cases of RA, dermatomyositis, polyarteritis nodosa, multiple sclerosis, inflammatory bowel disease, and primary biliary cirrhosis have been reported (11, 12). AML is also occasionally associated with AID as well as with AIC (13, 14). Finally, case-reports/small series of immune-mediated hemostatic disorders have been described in MDS, CMML, MPN, and AML. These included acquired hemophilia A, thrombotic thrombocytopenic purpure, and anti-phospholipid syndrome, that may be life-threatening (2). On the other hand, there is evidence that patients with prior systemic autoimmune rheumatic diseases have an increased risk for the development of hematological malignancies, particularly lymphomas and MDS. This has been reported for RA, SS, SLE, ITP, myasthenia gravis, and giant cell arteritis, suggesting that the immune dysregulation underlying the autoimmune disease may be involved in the generation of a "tumor permissive" soil, although the contribution of treatment with immunosuppressive/cytotoxic drugs cannot be excluded (15–17).

IMMUNODEFICIENCY AND MYELOID NEOPLASMS

Immunodeficiency is a broad concept that may involve the deficiency of one of the several arms of the immune system (1). Primary immunodeficiency syndromes (PID) represent a complex and heterogeneous category comprising more than three-hundred distinct disorders, mostly congenital, increasingly diagnosed through genetic and immunologic tests. They are grouped according to the predominant deficiency, including T-B severe combined defects, antibody, complement, neutrophils, and cytokine deficiencies, and also encompass hematologic conditions such as Fanconi Anemia, Diamond-Blackfan anemia, Familial Hemophagocytic Lymphohistiocytosis, and Wiskott-Aldrich syndrome (immunodeficiency with congenital thrombocytopenia). Other immunodeficiencies are associated with somatic mutations, such as the autoimmune lymphoproliferative syndrome and the RAS-associated autoimmune leukoproliferative disease (18–20). An increased risk of developing acute leukemias (mostly T-cell derived), lymphomas and MDS, as well as other solid cancers have been described in most of the PID listed above, underlying the concept that an efficient immunosurveillance is pivotal in preventing tumorigenesis. Moreover, continuous/relapsing infections consequent to immunodeficiency may sustain a state of chronic hyper-inflammation, which in turn may favor tumor growth (18–20). An interesting player in controlling neoplastic expansion is the complement system that may be exploited by monoclonal antibodies directed against tumor antigens, increasingly used as therapeutic tools in various cancers (21). An example of interplay among immunodeficiency, autoimmunity and cancer is the Kabuki syndrome, a rare genetic disorder with specific facial features, intellectual disability, and increased frequency of infections, autoimmune diseases and neoplasias. The syndrome is caused by mutations in the KDM6A or KMT2D genes, which are involved in the early differentiation of mesenchymal cell lineage and in the development of tolerance and immune system maturation (22, 23). Another example is the MIRAGE syndrome (Myelodysplasia, Infection, Restriction of growth, Adrenal hypoplasia, Genital problems, and Enteropathy) which is caused by mutation in the SAMD9 gene, a regulator of inflammatory response acting as a downstream target of TNF-alpha signaling (24). Finally, the group of telomeropathies comprises several heterogeneous defects in the telomere maintenance machinery, characterized by a variable clinical phenotype and genetic penetrance, and by great susceptibility to environmental factors. Among the several, Dyskeratosis Congenita, idiopathic pulmonary fibrosis, and familial liver cirrhosis, show variable overlap with autoimmune diseases and BMF syndromes, as well as increased risk of infections and hematologic neoplasms (25, 26).

AUTOIMMUNITY IN BMF SYNDROMES: MDS, HYPOPLASTIC MDS, AND AA

Several lines of evidence support the relationship between BMF/MDS and autoimmunity, i.e. their epidemiologic association, the response to similar immunosuppressive therapies, and the existence of common immune-mediated physiopathologic mechanisms (27–29). The latter include bone marrow suppression by T-cells, cytokine dysregulation, and apoptosis of hematopoietic precursors. Aplastic anemia is the prototype of an immune-mediated attack against BM, with several effectors involved, such as activated cytotoxic T cells, increased production of type 1 cytokines, reduced T reg, and enhanced apoptosis *via* Fas/FasL. Moreover, defects of the innate immunity

and of the hematopoietic niche are also reported as important pathogenic mechanisms. The most relevant evidence supporting the autoimmune pathophysiology is the response to immunosuppressive therapy (IST) and the requirement, in most cases, of continuous immunesuppression to maintain response (30). At the boundaries of the classic BMF syndromes, and with frequent overlap, there are other diseases in which several immunologic abnormalities are increasingly reported. These include pure red cell aplasia, pure white cell aplasia, amegakaryocytic thrombocytopenia, and the telomere diseases, all definitely cytopenic; however, other diseases, such as large granular lymphocyte lymphoproliferative (LGL) syndromes, and hemophagocytic lymphohistiocytosis (HLH), may manifest with autoimmune cytopenias along with proliferative features (31–35). Additionally, the landscape of BMF syndromes has been enriched with the recently described idiopathic cytopenia/dysplasia of undetermined significance (ICUS/IDUS) and the hypoplastic MDS, which again share common immune-mediated pathogenic mechanisms and a cytopenic phenotype (27, 32, 36, 37).

There is growing interest about the presence of somatic mutations in autoimmune/autoinflammatory conditions. It is known that mutational burden increases with age, as described in the so called clonal hematopoiesis of indeterminate potential (CHIP) or clonal cytopenia of undetermined significante (CCUS). Recently, DNMT3A, TET2 and ASXL1 mutations were found in 29.5%, 15.0% and 3.5% of studied patients, with a striking association with autoimmune diseases (38). Regarding BMF, a variable combination of somatic mutations has been largely described: in MDS the most frequently observed involve the splicing genes SF3B1, SRSF2, U2AF1, ZRSR2, the DNA methylation genes DNMT3A, TET2, IDH1, IDH2, and the chromatin modification genes ASXL1, EZH2, KDM6A (39). In AA the genomic landscape is more shaded, with less MDS-associated and more frequent typical paroxysmal nocturnal hemoglobinuria (PNH)-related PIGA mutations (40, 41), while in hypoplastic MDS the picture is somehow in between MDS and AA (32). More recently, mutations have been detected even in lymphoid cells of BMF patients. For instance, JAK-STAT and MAPK pathways mutations have been described by single-cell sequencing in CD8+ T cells of AA patients, and the mutational burden was associated with CD8+ T-cell clonality (42). It may be speculated that mutations accumulate in the pathogenic immune effectors, which are the most activated and replicating cell types. Finally, STAT3 somatic mutations have been reported in CD8+ T cells in LGL, and in other autoimmune "benign" conditions, such as rheumatoid arthritis/Felty's syndrome, multiple sclerosis, and celiac disease (43).

THE PNH CONUNDRUM

One of the key mechanisms of PNH pathogenesis is the escape of GPI-negative hematopoietic precursors from a GPI-directed autoimmune attack. This hypothesis is supported by the known association of PNH and AA, and by the reported increase of the PNH clone after IST in AA (44, 45). However, inactivating mutations of PIG-A do not "per se" confer a selective growth advantage to hematopoietic precursors, since they are found in several conditions including healthy subjects, without causing overt disease. Thus, further events (additional cooperating mutations)? are thought to be necessary for the expansion of the PIG-A mutant clone. Additionally, the bone marrow environment (dominated by an auto-immune signature) seems to play an important role in a further growth advantage of the PNH clone (46). Bone marrow microenvironment and autoimmune phenomena may play a role also in MDS, where mainly small PNH clones are described, without an overt hemolytic disease (45). Consistently, anti-erythroblast antibodies have been demonstrated in about 2/3 of early MDS together with increased values of the pro-apoptotic protein Bax and decreased levels of Bcl-2 levels, and their BM culture supernatants induced dyserythropoietic signs, erythroblastic clustering, and increased overall in cultured normal BM (47, 48). Furthermore, small PNH clones have been demonstrated in hypomegakaryocytic thrombocytopenia (49, 50) and chronic idiopathic neutropenia (51), two conditions hardly distinguishable from ICUS and with autoimmune reactivity against BM precursors. Finally, small PNH clones have been reported also in a considerable proportion of AIHAs, conferring a prominent hemolytic pattern and a higher thrombotic risk to the disease. The presence of a PNH clone was also associated with a different cytokine signature (reduced levels of IFN-γ and IL-17) as compared to PNH-negative AIHA cases (52). In AIHA the clinical picture is dominated by an immune attack directed against peripheral erythrocytes; however, in cases with reticulocytopenia and severe onset the immune attack is also directed against bone marrow precursors. Additionally, chronic/relapsing AIHA show a possible evolution to ICUS, IDUS or BMF syndromes over time, suggesting a shift from "peripheral" to "central" autoimmunity (27, 28, 53, 54). Altogether these findings support the idea that the PNH clone may be the "immunological scar" of an immune attack directed against BM precursors.

AUTOIMMUNE COMPLICATIONS FOLLOWING THERAPY OF MYELOID NEOPLASMS

Historically IFN-alpha has been used in MPN, including chronic myeloid leukemia (CML), systemic mast cell disease, and hypereosinophilic syndrome, and has been associated with the occurrence of several AID and AIC. Several autoimmune side effects have been described, ranging from spurious autoantibody formation to overt diseases, such as AIHA, ITP, thrombotic thrombocytopenic purpura, hypo- or hyper thyroid disorders, SLE, RA, and Behçet's disease (55). Tyrosine kinase inhibitors (TKIs) have certainly changed the therapeutic approach to MPN, and their risk-benefit balance is well established, with mainly infectious concerns (56). However, most of their effects on the complex regulations of the immune system are far from being fully elucidated. They have immunosuppressive effects on monocyte/

macrophage functions, dendritic cell maturation, and lymphocyte subsets, but also exert an immunomodulatory activity inducing monocyte type 1 polarization, increased NK function and T-lymphocyte activation. This has provided a rationale for investigating a possible therapeutic use in autoimmune diseases (57). On the other hand, imatinib has been associated with the occurrence of aplastic anemia, and nilotinib and dasatinib implicated in the development of immune-mediated liver injury, SLE, panniculitis, and neurologic demyelinating disease. The harmful effects of potent immunomodulation are even more marked with aggressive therapies such as BM transplantation, where several autoimmune complications are thought to be the consequence of the "immunological storm" elicited by the battle against tumor cells (58–60). The complexity of the immunological perturbation following allogenic BM transplantation is maximally reflected in the well known graft-versus-host-disease, which resembles progressive systemic sclerosis and vasculitis of the skin, gastrointestinal tract, liver, lungs, and kidneys. Additionally, CAR T-cells, although mainly studied in lymphoid conditions, are recently considered also for acute myeloid leukemia (61). Well known toxicities of CAR T-cells also derive from a complex immune dysregulation and include the cytokine release syndrome, the immune effector cell-associated neurotoxicity syndrome, and the hemophagocytic lymphohistiocytosis; later toxicities comprise prolonged cytopenias and hypogammaglobulinemia (62). Finally, the difficulty of a balanced immune stimulation is also highlighted by the autoimmune complications following therapy with check point inhibitors in solid and hematologic tumors (63, 64).

MESENCHYMAL STEM CELLS IN AUTOIMMUNE DISEASES AND MYELOID NEOPLASMS

Mesenchymal stem cells are key constituents of the BM niche able to differentiate in various tissues and to exert several immunomodulatory activities (5, 6). They appear to play a pivotal role against tumors and infections through a variety of properties including anti-inflammatory, regenerative, angiogenic, anti-fibrotic, anti-apoptotic, and anti-oxidative stress activities. Conversely, there is growing evidence that MSCs from patients with myeloid neoplasms may differently support leukemic growth and protect the leukemic cell from apoptosis or chemotherapy-induced cell death. MSCs behavior may be due to the bidirectional crosstalk between the leukemic cell and the BM niche, that realizes through several cytokines, chemokines and other soluble factors (CXCR2, CXCR4, IL6R, LFA, VLA4, RANK and FAT/CD36) as described in AML (65). On the other hand, MSCs may harbor intrinsic alterations, as recently reported for MDS and AA. These included dysregulated proliferation/apoptosis (prevalent in MDS), decreased angiogenesis (prevalent in AA), and immunosuppressive functions, with a shift from type 1 (pro-inflammatory) to type 2 (anti-inflammatory/tumor-educated) MSC profile (5). Finally, MSCs may be involved in resistance even to novel treatments, as observed in CML. In this setting, MSCs favor the immune escape of residual leukemic cells causing resistance to TKIs, that may overcome by interferon-α (66). On the whole, the immunomodulatory properties of MSCs and their possible allogeneic/unmatched use have promoted several clinical trials in various autoimmune disorders, including aplastic anemia, Crohn's disease, multiple sclerosis, inflammatory liver and pulmonary diseases, neurodegenerative disorders, as well as in graft rejection and graft-vs-host-disease (67). Limitations of MSCs as cell therapy include handling difficulties, safety issues, and high economic cost. Thus, the use of MSC-derived secretome products is increasingly pursued, although the choice of the ideal MSC type and the standardization of production strategies need to be defined.

MICROBIOME IN AUTOIMMUNE DISEASES AND MYELOID NEOPLASMS

There is growing evidence on the role of microbiome in regulating several homeostatic processes, such as metabolic pathways, synthesis of vitamins and fat storage, as well as self-tolerance, immune surveillance for tumors, and host defense against pathogens. Alterations of the microbiome have been associated with the development of autoimmune diseases, neoplasms, and infections and their treatments (68). Beyond data on BM transplantation, it has been shown that a permissive microbiota, i.e. the microorganisms that colonize various districts of the body, is associated with acute leukemias, lymphoma, and multiple myeloma (4). Of note, microbiome changes may be induced by the leukemic cell itself, by chemotherapy or antibiotics, or by superimposed infections, adding further complexity to the topic. Alterations of the microbiome have been reported also in autoimmune cytopenias and aplastic anemia (69–71). Helicobacter pylori colonization has long been associated with ITP, although it is not strictly considered a microbiome alteration (4). More recently, in ITP and chronic idiopathic neutropenia, a peculiar microbiota composition has been identified, possibly predictive of response to therapy (72). Similarly, alterations of the microbiota have been reported in SLE, RA, Multiple Sclerosis and Type-1 diabetes (73). Moreover, Parvovirus B19, hepatitis viruses, CMV and EBV have been associated with transitory forms of AA, probably due to a molecular mimicry between foreign and self-antigens or polyclonal immune stimulation. In addition, the presence of chronic inflammation, the alteration of epithelial barriers, and the dissequestration of self-antigens driven by an altered microbioma are also thought to contribute to the development of autoimmune diseases and neoplasms (4). In the next future the availability of NGS techniques will improve the ability to analyze the microorganisms, providing new insights in this fascinating area, and improving the knowledge of disease pathogenesis, complications, and therapy outcome.

AUTOIMMUNE PHENOMENA

By definition autoantibodies are antibodies that react with self-antigens (1), although not invariably associated with AID and AIC. It is largely known that low-affinity IgM and, occasionally, low titer IgG autoantibodies are detected in healthy individuals. They comprise rheumatoid factors, antinuclear-, and even anti-RBC and anti-platelets antibodies, without a clinically overt disease. Additionally, natural autoantibodies, mainly polyreactive IgM with a moderate affinity for self-antigens, may provide a first line defense against infections and have housekeeping functions by recognizing apoptotic cells and promoting their phagocytic clearance (74). This phenomenon has been extensively studied in thalassemias and congenital hemolytic anemias, hypothesizing a physiologic role in the clearance of debris of lysed cells (75). In this view natural autoantibodies may concur to the opsonization and removal of potentially harmful elements, including tumor cells. The role of natural autoantibodies is not known in hematologic neoplasms, with the isolated exception of chronic lymphocytic leukemia (CLL). Autoimmunity and immunodeficiency are a hallmark of CLL, with a relentless accumulation of anergic, self-reactive CD5+ B cells, which produce several polyreactive natural autoantibodies (76). As regards myeloid neoplasms, it may be hypothesized that natural autoantibodies may contribute (or not) to the first-line defense of innate immunity that has been found deficient in most of these disease (77–79).

CONCLUSION

Immune phenomena, both autoimmunity and immunodeficiency, are definitely an egg and chicken question in cancer, including myeloid neoplasms. The complexity of the issue is further increased by the heterogeneity of myeloid disorders that encompass truly malignant cells (as in acute leukemias) and more subtle diseases (as MDS and BMF syndromes). Likewise, immune-mediated phenomena are broadly represented, including immune-mediated cytopenias and hemostatic disorders, classic autoimmune diseases, and immunodeficiencies, as summarized in **Table 1**. The immune system is undoubtedly pivotal in preventing and controlling tumor growth and in direct killing of neoplastic cells. These functions are characterized by pleiotropism (several different activities are performed by a single effector depending on the setting) and redundancy (the same result is accomplished by different effectors). In this complex scenario, two main tasks are required to maintain homeostasis: tolerance versus self and surveillance against potential harmful noxae, i.e., infectious agents and tumors. However, microorganisms are not always dangerous and autoimmunity may be potentially useful in removing damaged/badly functioning cells. Autoimmunity occurs in peripheral blood and, less investigated, in bone marrow and lymphoid organs, and may be fundamental in maintaining homeostasis, provided its tight control and absence of over activation. Stretching the concept, the removal of a

TABLE 1 | Main evidences of immune system involvement in myeloid neoplasms.

Epidemiological associations with autoimmunity	- MDS is associated with systemic and organ specific disorders, such as RA, SLE, vasculitis, thyroid autoimmune diseases, SS, AIHA, ITP, PRCA, and immune-mediated hemostatic disorders in about 20% of cases. - CMML are complicated in up to 30% by vasculitis and ITP. - MPN and AML are occasionally complicated by autoimmune cytopenias and diseases.
Epidemiological associations with immunodeficiency Autoimmunity in BMF syndromes	- AML and MDS are observed in patients with primary immunodeficiencies, including T-B severe combined defects, antibody, complement, neutrophils, and cytokine deficiencies. - MDS, particularly low-grade hypoplastic type, is marked by autoimmune phenomena promoting apoptosis of hematopoietic precursors. - AA, which may evolve to MDS/AML, has a well-defined autoimmune pathogenesis against BM precursors (cytotoxic T cells, increased production of type 1 cytokines, and reduced T reg). - MDS and AA share common somatic mutations (mainly splicing genes, DNA methylation genes, and chromatin modification genes), although with different frequencies.
Overlapping syndromes	- MDS presenting with cytopenia and autoimmunity displays overlapping features with ICUS/IDUS, PRCA, white cell aplasia, amegakaryocytic thrombocytopenia, telomere diseases, LGL, and HLH.
PNH clones	- MDS, MPN and AML may display PNH clones, usually small or very small. - AA and peripheral autoimmune cytopenias are also associated with PNH clones.
Autoimmune complications following therapy	- MPN, particularly CML, has been historically treated with IFN-alpha, which induced several autoimmune complications (AIHA, ITP, TTP, and other autoimmune diseases). - MPN treated with TKIs (imatinib, nilotinib and dasatinib) may be complicated by immune-mediated disorders (AA, SLE, liver injury, and neurologic demyelinating disease). - AML and MDS subjected to more aggressive therapies (HSCT, CAR T, and CPI) may be complicated by several autoimmune cytopenias and other immune-mediated disorders (cytokine release, neurotoxicity syndrome, HLH).
Mesenchymal stem cells Microbiome	- AML, MDS, and MPN show alterations of MSCs that may be implied in disease pathogenesis and resistance to therapy. - AML is associated with alterations of the microbiome, either *per se* or because of chemotherapy, antibiotics and HSCT. Alterations of the microbiome have been reported in various autoimmune diseases.

AML, acute myeloid leukemia; MDS, myelodysplastic syndromes; MPN, myeloproliferative neoplasms; CMML, chronic myelomonocytic leukemia; AA, aplastic anemia; ICUS, idiopathic cytopenia of undetermined significance; IDUS, idiopathic dysplasia of undetermined significance; PNH, paroxysmal nocturnal hemoglobinuria; PRCA, pure red cell aplasia; LGL, large granular lymphocyte lymphoproliferative syndromes; HLH, hemophagocytic lymphohistiocytosis; AIHA, autoimmune hemolytic anemia; ITP, immune thrombocytopenia, TTP, thrombotic thrombocytopenic purpura; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus, SS, Sjogren syndrome; IFN, interferon; TKIs, tyrosine kinase inhibitors; CPI, checkpoint inhibitors; HSCT, hematopoietic stem cell transplantation; CAR T, chimeric antigen receptor T-cells; BM, bone marrow; MSCs, mesenchymal stem cells.

neoplastic cell may be considered a self-directed "autoimmune" reaction. Additionally, autoimmune phenomena, although not directly causative, may be deeply involved in the pathogenesis of myeloid neoplasms, particularly MDS and BMF. At variance, in AML, autoimmunity takes a second place in pathogenesis, whilst it can be clinically harmful. Finally, there are escape phenomena, like PNH clones, which can guarantee hematopoiesis, albeit deficient. More than 30 years ago a visionary scientist, J. Edwin Blalock, pioneer of neuroimmunoendocrinology, defined the immune system as our "circulating brain" (80), and this notion is still up to date and far away from being fully understood.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Large Granular Lymphocyte Expansion in Myeloid Diseases and Bone Marrow Failure Syndromes: Whoever Seeks Finds

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Large granular lymphocytes (LGL) are lymphoid cells characterized by either a T-cell or a natural killer phenotype whose expansion may be reactive to toxic, infectious, and neoplastic conditions, or result from clonal selection. Recently, the higher attention to LGL clones led to their detection in many clinical conditions including myeloid neoplasms and bone marrow failures. In these contexts, it is still unclear whether LGL cells actively contribute to anti-stem cell autoimmunity or are only a reaction to dysplastic/leukemic myelopoiesis. Moreover, some evidence exists about a common clonal origin of LGL and myeloid clones, including the detection of STAT3 mutations, typical of LGL, in myeloid precursors from myelodysplastic patients. In this article we reviewed available literature regarding the association of LGL clones with myeloid neoplasms (myelodysplastic syndromes, myeloproliferative neoplasms, and acute myeloid leukemias) and bone marrow failures (aplastic anemia and pure red cell aplasia, PRCA) focusing on evidence of pathogenic, clinical, and prognostic relevance. It emerged that LGL clones may be found in up to one third of patients, particularly those with PRCA, and are associated with a more cytopenic phenotype and good response to immunosuppression. Pathogenically, LGL clones seem to expand after myeloid therapies, whilst immunosuppression leading to LGL depletion may favor leukemic escape and thus requires caution.

Keywords: large granular lymphocyte, myelodysplastic syndromes, acute myeloid leukemia, myeloproliferative neoplasm, aplastic anemia

INTRODUCTION

Large granular lymphocytes (LGL) are lymphoid cells characterized by either a T-cell or a natural killer (NK) phenotype that physiologically participate in innate immunity and immunosurveillance. Their expansion may be a response to toxic, infectious, and neoplastic conditions, or result from clonal selection (1). The latter may rarely lead to the development of a lymphoproliferative disorder, namely a T-cell or NK lymphoma with variable aggressiveness. Beyond overt lymphoproliferative disease, the increasing awareness about LGL cells and their phenotype led to the discovery of many clinical associations including idiopathic cytopenias and hematologic malignancies (2, 3).

The former are part of a spectrum ranging from peripheral autoantibody mediated cytopenias (autoimmune hemolytic anemia, immune thrombocytopenia, and autoimmune neutropenia) to bone marrow failures (aplastic anemia, AA, and low risk myelodysplastic syndromes) characterized by central immune attack towards stem cells (2). In this context it is still unclear whether LGL cells actively contribute to anti-stem cell autoimmunity or are only part of the proinflammatory microenvironment. Regarding hematologic malignancies, LGL clones have been recently detected in myeloid neoplasms such as myeloproliferative neoplasms and acute myeloid leukemia (1, 2). Whether LGL expansion is only a reactive phenomenon or has a common clonal origin with the myeloid clone is object of open investigation. In this review we collect more recent literature about the association of LGL with myeloid neoplasms and bone marrow failures focusing on evidence of pathogenic, clinical, and prognostic relevance.

DEFINITION AND DETECTION OF LGL CLONES

Morphologically, LGL are more than twice the diameter of erythrocytes, and are characterized by mature chromatin, excessive cytoplasm, with or sometimes without prominent cytoplasmic granules. Normally, LGLs comprise 10 to 15% of blood mononuclear cells which may be either surface CD3+ (T-cell) or surface CD3– (NK cell). Most normal LGLs in the peripheral blood are NK cells, whilst some are T lymphocytes (2). As mentioned before, LGLs may configure heterogeneous disorders comprising non-clonal reactive processes, indolent clonal proliferative disorders and highly aggressive neoplasms. World Health Organization (WHO) divides clonal LGL expansions into three disorders: T-cell LGL leukemia (T-LGLL), chronic lymphoproliferative disorders of NK cells (CLPD-NK), and aggressive NK-cell leukemia (ANKL) which is associated with Epstein-Barr virus (EBV) infection of the neoplastic NK cells. In contrast to ANKL, both T-LGLL and CLPD-NK are clinically indolent and have a low risk of transformation into an aggressive malignancy (4). In the last decade, European and US Registry are actively studying LGL leukemias and accumulating evidence on clinical features and outcome. Overall, incidence of LGL leukemia is reported as 0.2-0.72 per million persons per year, with no gender effect, and more than 85% of cases are the T-LGL subtype. Median age at diagnosis is 60 years and the disease is only rarely observed in the infancy (5–7).

Flow cytometry is the gold standard for LGLs detection and is usually based on the expression of NK-associated markers CD16 and CD57. CD56 is another marker, constitutively expressed by circulating normal NK cells and usually downregulated in CLPD-NK; its expression in T-LGLL may be associated with a less-favorable prognosis (2, 3). T-LGLs usually express CD3+, TCR αβ+, CD4–, CD5dim, CD8+, CD16+, CD27–, CD28–, CD45R0–, CD45RA+, and CD57+ phenotype, representing a constitutively activated phenotype. Less commonly, T-LGLs mount CD4 with variable expression of CD8. The rare CD3+/CD56+ T-LGL leukemias may show higher clinical aggressiveness. T-LGL usually harbor the T-cell receptor (TCR) αβ+ heterodimer, rarely γδ TCR heterodimer. NK-LGLs are characterized by CD2+/sCD3-/CD3ε+/TCRαβ-/CD4-/CD8+/CD16+/CD56+ phenotype. Evidence of T-LGL clonality is assessed using TCRγ-polymerase chain reaction analyses (PCR) and deep sequencing of TCR has demonstrated a restricted diversity of TCR repertoire. Vβ TCR gene repertoire analysis can also be ascertained using flow cytometry, although this is not routinely performed (8). NK-LGLs do not express TCR so it is difficult to assess their clonality. However, they often show abnormal killer immunoglobulin-like receptor (KIR) expression with complete absence of surface KIR or restricted expression. Restricted KIR expression is often seen in both in T- and NK-LGL leukemia (2).

Regarding other markers, LGL leukemia patients show increased serum levels of interferon-γ 2, monocyte chemoattractant protein-1 (attractive factor for monocytes, T, and NK cells to sites of inflammation), epidermal growth factor, and various interleukins (IL) including IL-6, IL-8, and IL-18. Rheumatoid factor and antinuclear antibody are detected in 60% and 40% of patients, respectively (2). Serum protein electrophoresis usually shows polyclonal hypergammaglobulinemia. Defects in downregulation of Ig secretion in LGL leukemia could explain part of association with autoantibodies malignancies (3).

PATHOGENESIS OF LGL EXPANSION

From a pathogenic perspective, it is generally thought that normal LGLs acquire a defect of apoptosis that leads to their accumulation. An interesting explanation for this phenomenon is the expansion of an oligoclonal LGL population under chronic stimulation from an unknown antigen. LGL cells may then acquire a molecular lesion promoting monoclonal proliferation, and release cytokines and toxic granules that contribute to bone marrow failure (2). Concerning apoptosis, LGL cells strongly express Fas (CD95) and Fas-ligand (Fas-L) (CD178). Moreover, RAS and ERK constitutive activation and G12 KRAS mutation are often found in NK-LGL leukemia, and their blockade may restore Fas sensitivity in leukemic LGLs. Although not routinely performed, increased soluble Fas-L is a good surrogate marker of LGL leukemia (9). From a cytogenetic point of view, karyotype is normal in most cases. Recurrent somatic mutations in the Src homology 2 (SH2) domain of the signal transducer and activator of transcription 3 (STAT3) gene have been found in 27-40% of patients with T-LGL leukemia and 30% of patients with CLPD-NK. These mutations lead to constitutive activation of STAT3, with consequent dysregulation of genes downstream of STAT3 (10). Once dimerized, STAT3 shuttles from the cytoplasm to the nucleus, where it ultimately binds to DNA, mediating growth and survival. Disease manifestations such as cytopenias and autoimmune diseases may result from the production of proinflammatory cytokines mediated by STAT3 hyperactivation, as well as from a direct attack on bone marrow by the STAT3-activated LGL (11–13).

Another relevant member of STAT protein family is STAT5b which has been reported to carry gain-of-function mutations in 15–55% of CD4+ T-LGLL, and in 19% of TCRγδ LGLL (14, 15). STAT5b N642H has been identified as an oncogenic driver in innate-like lymphocytes, and a mouse model expressing human N642H mutated STAT5b developed severe CD8+ T-cell neoplasia. IL-15 is an upstream factor of STAT5b and seems crucial for neoplastic transformation. In fact, IL-15 transgenic mice developed the aggressive variant of T or NK cell leukemia (15). The requirement of additional cytokine signals on STAT5b genetic lesions to lead neoplastic evolution suggests the importance of the immunological microenvironment. STAT3 and STAT5b mutations have been included in the 2017 WHO classification of LGLL and STAT5b mutation is associated with a more aggressive clinical course (16). Another gene recurrently mutated in LGLL is TNFα-induced protein 3 (TNFAIP3), a tumor suppressor encoding A20, a negative regulator of nuclear factor kappa B (NFkB) (17). Other genes occasionally mutated in T-LGLL, mainly linked to STAT3 signaling pathway and cytotoxic T lymphocyte activation, are PTPRT, BCL11B, PTPN14, PTPN23 (15). Moreover, it has been shown that patients lacking STAT mutations may harbor other lesions involving genes connecting STAT with Ras/MAPK/ERK and IL-15 signaling, such as FLT3, ANGPT2, KDR/VEGFR2, and CD40LG (18). Finally, whole exome sequencing (WES) on 3 STAT-mutation negative CLPD-NK patients found somatic mutations including KRAS, PTK2, NOTCH2, CDC25B, HRASLS, RAB12, PTPRT, and LRBA (15). Altogether, these data shows that LGL clonal selection and expansion result from a complex interplay among genetic and environmental factors that may be heterogeneously combined.

LGL CLONES REACTIVE TO AUTOIMMUNE, INFECTIOUS, AND OTHER CONDITIONS

LGL clones can be identified in different conditions such as autoimmune diseases, infections, and transplant, likely representing an unbalanced response to systemic infections and/or immune deregulation. It is difficult to differentiate primary LGL leukemia from reactive LGL expansions. Flow cytometry patterns, together with the molecular lesions, are important tools to assess "quantity and quality" of LGL populations and establish clonality.

Autoimmune Diseases

Concerning LGL in the context of autoimmune diseases, rheumatoid arthritis is the most common association, being present in up to 18% of patients with LGL expansion (19). This association may be difficult to distinguish from Felty syndrome (FS) that is characterized by chronic arthritis, splenomegaly, and neutropenia, in the setting of longstanding seropositive rheumatoid arthritis. Clonal proliferations of LGLs have been observed in patients with FS, and it has been proposed that these patients may in fact have T-LGLL (20). Clonality tests may be useful, although the patient is generally managed according to the prevailing phenotype (autoimmune versus proliferative). Systemic lupus erythematosus, Sjogren syndrome, autoimmune thyroiditis, autoimmune coagulopathies, vasculitis with cryoglobulinemia, and inclusion body myositis have also been reported as associated with LGLs (2, 19, 21). Overall, autoimmune diseases should be taken into account in the workup of patients with LGL expansion and vice versa.

Infections

Infections, particularly viral and chronic ones, represent a persistent trigger stimulating lymphocytes with the possible development of lymphoproliferative disorders (22). Cases of LGL expansion secondary to Epstein Bar virus (EBV), cytomegalovirus (CMV), Hepatitis B virus (HBV), Hepatitis C virus (HCV), and Human immunodeficiency virus (HIV) have been reported (2). Moreover, some case reports described untreated strongyloidiasis as cause of chronic inflammation and consequent LGL expansion (23). History of infection and serology for hepatitis and herpetic viruses and HIV have to be investigated when approaching patients with LGL expansion and lymphoproliferative disorders in general.

Transplant

LGL clones may also arise after both solid and hematopoietic stem cell transplant (HSCT) (24, 25). These procedures induce an immunological storm encompassing the host and the donor immune system. Moreover, the occurrence of viral infections (CMV, EBV, etc.) and the immunosuppressive drugs administered may favor autoimmunity (26). In the case of HSCT, the graft shows immune competence and may mount a response against persistent self-antigens. Graft versus host disease (GVHD) is a typical manifestation, and other autoimmune conditions may develop during immune reconstitution. It has been reported that up to 20% patients show increased LGLs after HSCT, with a median onset of 312 days from transplant, and CMV reactivation and acute GVHD as prominent risk factors (24, 26). Post-transplant LGL expansion, although mainly chronic and indolent, deserves proper investigation in patients with new-onset persistent cytopenia following transplant, since may require adjustment of ongoing immunosuppressive therapy.

Other Associations

LGL clones are not only associated to autoimmunity but even to immunodeficiency. Although pediatric cases of LGL disorders are rare, a phenotypic overlap may occur with primary immunodeficiencies characterized by increased susceptibility to infections, autoimmunity, and development of lymphoproliferative disorders. Interestingly, LGL clones have been reported in patients with adenosine deaminase 2 deficiency (27). Their presence was related with an activation of phosphatidylinositol-3-phosphate kinase pathway, whose disruption has been implied in the apoptosis imbalance typical of LGL.

Many drugs induce immune/inflammatory perturbations and LGL expansion after the tyrosine kinase inhibitor (TKI) dasatinib (a drug used in chronic myeloid leukemia and Philadelphia positive acute lymphoblastic leukemia) has been reported.

Although most LGL clones developing upon dasatinib treatment are asymptomatic, some cases of fever, colitis, and pleural effusions have been reported, suggesting an aberrant immune response (28). Finally, LGLs have been reported after solid tumors and hematologic diseases, particularly myeloid malignancies and bone marrow failure syndromes, as discussed thereafter, and may be associated with autoimmune/autoinflammatory phenomenon such as livedoid vasculopathy, urticarial vasculitis, or complex recurrent aphthous stomatitis in these patients (2).

LGL EXPANSION IN MYELODYSPLASTIC SYNDROMES

Various evidence exists about LGLs expansion in patients with myelodysplastic syndromes (MDS). Some studies only reported the prevalence of LGLs clones in patients affected by MDS without the development of an overt lymphoproliferative syndrome, whilst other also described a "true" LGL chronic expansion in these subjects (13, 29–33). These findings are summarized in **Table 1**. The prevalence of LGL clones in MDS was highly variable across studies and ranged from 1.4% to 49%. Conversely, in a study by Huh et al., 9 out of 28 patients with T-LGLL also had MDS, and all of them had monoclonal TCR gene rearrangement. Clinically, LGL clones were associated with more marked cytopenias, mainly anemia and thrombocytopenia (30). In particular, patients with T-LGLL/MDS showed lower median hemoglobin and lymphocyte counts when compared with the subgroup affected by T-LGLL alone, whilst platelets levels and neutrophil count were similar (33). Contrarily, in another case series, 9 patients with LGL expansion/MDS from a group of 100 cytopenic subjects showed no significant differences in the grade of cytopenia as compared to patients with MDS or T-LGLL alone (29). These features are consistent with the variable and multifaceted factors contributing to cytopenias in subjects with MDS. In fact, MDS patients are usually elderly, with reduced stem cell reserve, and with pro-inflammatory and pro-apoptotic bone marrow milieu as compared to T-LGLL patients. On the other hand, the presence of a T-LGL infiltrate may contribute to the immune imbalance typical of MDS pathogenesis. In this view, various studies reported the pathogenic role of LGL clones in bone marrow failures and also showed the possibility of a common origin of the two clones. In particular, Durrani et al. analyzed 240 patients with LGL leukemia and found that 5.4% of them was affected by MDS (11/13 with TCR gene rearrangement) (33). They showed that somatic STAT3/STAT5 mutations can be found in up to 15% of LGLL/MDS patients versus 39% of those with LGL clones only. More recently, STAT3-mutated clones were reported in up to 37.5% patients with MDS harboring LGL clones and in 2.5% of MDS alone (13). The detection of LGL-related mutations in MDS cases supports a common pathogenic origin of the two conditions. Interestingly, constitutive STAT5 activation is observed in various myeloid diseases, including chronic myeloid leukemia and JAK2 mutated myeloproliferative syndromes. In fact, JAK/STAT pathway is downstream of many growth factor receptors including those of erythropoietin and thrombopoietin. STAT5b mutations have been associated with more aggressive LGLL phenotype, and recent evidence suggests their unique distribution in T-LGL cells of advanced myeloid neoplasms (35). In another study including 1177 patients with MDS, a LGL clone was found in 322 subjects (27%), and LGL leukemia in 36 (2%). They observed that mutations in certain genes associated with myeloid disorders (e.g., TET2, SF3B1 and ASXL1) had same frequencies in LGL/MDS and MDS alone, whilst U2AF1 mutations were more common among the former (32). Very recently, STAT3 and TET2 mutations were found in 27% and 34% of patients with CLPD-NK, respectively. TET2-mutated CLPD-NK was preferentially associated with MDS, and whole-exome sequencing of sorted cells found that TET2 mutations were

TABLE 1 | Large granular lymphocytes in myelodysplastic syndromes.

Study Type	Relevance	Main Findings	Ref.
Clinical study 76 MDS, 15 T-LGLL, 9 T-LGLL/MDS.	Clinical and therapeutic	11.8% of patients with MDS showed LGL/MDS association and had lower LGL counts and lower response rate to immunosuppression compared to patients with T-LGLL alone.	29
Case series 28 T-LGLL patients, 9 had MDS (32%)	Clinical	Patients with T-LGLL/MDS showed lower median Hb level and lymphocytes compared with patients with T-LGL alone.	30
Clinical study 367 MDS, 24 with LGL clones (9,2%).	Pathogenic	Somatic STAT3 mutations may be found in 2.5% of patients affected by MDS, the frequency reaches 37.5% in patients with MDS/LGLL association.	13
Clinical study 71 MDS, 35 with MDS/LGL (49%)	Clinical and prognostic	85% of LGL/MDS had a TCR gene beta or/and gamma rearrangement by PCR and mainly showed bone marrow hypocellularity. LGL/MDS had similar OS as MDS alone.	31
Clinical study 1177 MDS, 322 with LGL clonal expansion (27%)	Pathogenetic/Prognostic	LGL clonal expansion was associated with similar survival and frequency of AML evolution. TET2, SF3B1 and ASXL1 were the most frequently mutated genes among both groups. U2AF1 mutations more common among LGL/MDS than MDS alone.	32
Clinical study 240 LGLL, 13/240 (5.4%) had also MDS	Pathogenic and clinical	5.4% of patients with LGLL had concomitant MDS and were more thrombocytopenic. 15% showed somatic mutations of STAT3/STAT5 versus 39% with LGLL only.	33
Clinical study 721 MDS, 10 (1.38%) with LGLL	Pathogenic	LGLL/MDS patients were characterized by lower Hb levels and erythroid dysplasia and mostly showed mutations in ASXL1 (30%) and STAG2 (30%).	34

LGL, large granular lymphocytes; MDS, myelodysplastic syndromes; T-LGLL, T cell large granular lymphocyte leukemia.

shared by myeloid and NK cells indicating that they occurred in early hematopoietic progenitors (36).

From a therapeutic point of view, in the large study by Komrokij et al. MDS patients harboring an LGL clone showed a lower response rate to immunosuppression with anti-thymocyte globulin and cyclosporine as compared to the MDS group (28% *vs* 41%), whilst no difference was observed regarding hypomethylating and erythroid stimulating agents (32). This finding was also confirmed by a different group that showed LGLL/MDS subjects had lower responses to immunosuppression compared to those with T-LGLL alone, possibly due to older age and likely decreased stem cell reserve in those with LGLL/MDS (29). Additionally, it may be speculated that inhibiting two clones may be harder than targeting a single one. Finally, Olson et al. showed that patients with CLPD-NK harboring TET2 mutation show prominent thrombocytopenia and resistance to immunosuppressive treatments (37).

From a prognostic perspective, the presence of an LGL clone did not seem to impact MDS outcome. A study including 71 MDS patients, 49% of whom harbored a T-LGL expansion, did not find substantial differences in OS between the two groups (83 months in the LGL/MDS group versus 65 months in the MDS one) (31). These data were more recently confirmed by Komrokji et al., who found similar median OS (24 months *vs* 27 months) and acute myeloid leukemia (AML) transformation rates (19% in both groups) among patients affected by MDS and LGL/MDS (32).

Concerning overt LGL lymphoproliferative diseases, 3 studies described an association with MDS (30, 33, 34). In particular, Ai et al. evaluated 721 patients with MDS and identified 7 T-LGLL, 2 mixed-phenotype LGLL, and 1 CLPD-NK, resulting in a prevalence of 1.38%. Lower hemoglobin levels, neutropenia and thrombocytopenia were a common finding, as well as a higher frequency of erythroid dysplasia in patients with MDS/LGLL. This condition was mostly associated with mutations in ASXL1 (30%) and STAG2 (30%) genes, and TCR gene rearrangement was present in 9/10 patients (34).

On the whole, LGL expansion may be found in more than 1/3 of MDS patients, is associated with a more cytopenic phenotype, and does not seem to markedly impact on outcome. LGL/MDS cases responded worse to immunosuppression as compared to MDS or LGL alone, so that the same approach as for primary disease is suggested. Conversely, overt LGLL and MDS are rarely associated.

LGL EXPANSION IN OTHER MYELOID NEOPLASMS

LGL clones have been also described in myeloproliferative neoplasms (MPN) and AML, even though this association is rarer (**Table 2**). There are case reports describing the association of LGL clones with AML, acute promyelocytic leukemia (APL), essential thrombocythemia, chronic myeloid leukemia (CML), and chronic myelomonocytic leukemia (38–44). Again, a hallmark of this association is cytopenia. For instance, Reda et al. reported a case of an association between T-LGLL and APL in the same patient, who initially presented with prolonged neutropenia, due to different causes (autoimmunity, APL and LGL expansion). After the APL diagnosis, induction chemotherapy was started, leading to a complete response. Despite chemotherapy, LGLL clone continued to increase, possibly due to a growing advantage after leukemic depletion. Interestingly, severe neutropenia persisted and also interfered with chemotherapy maintenance (44). Finally, even for LGL/AML, it has been speculated that the two clones may origin from the same progenitor (38) and this association did not lead to a worse prognosis. In another experience, Costello et al. reported a 60-year-old man diagnosed with AML, treated with chemotherapy and hematopoietic stem cell transplant with response. Thereafter, the patient developed an LGL expansion requiring therapy with low-dose modified mini-CHOP and methotrexate. Later, AML

TABLE 2 | Large granular lymphocytes in acute myeloid leukemia and myeloproliferative neoplasms.

Study Type	Relevance	Main Findings	Ref.
Case report (1 MPN, 1 MDS, 1 HCL)	Pathogenic and prognostic	Clinically, the concomitant existence of LGL proliferation and other leukemia doesn't seem to be responsible for a worse prognosis on patients.	38
Case report	Pathogenic	LGLs have a significant spontaneous cytotoxicity against autologous leukemia and hematopoietic cells.	39
Case report	Clinical	First reported case of concomitant presentation of T-LGLL with acute myeloid leukemia in an eldery patient who was treated with combination AML chemotherapy and remained alive and well seven months after initial diagnosis.	40
Clinical study 46 CML patients (20 on dasatinib, 14 on imatinib, 12 healthy volunteers	Pathogenic	In the subgroup of patients on dasatinib, clonal lymphocytes increased, both CD8+ cytotoxic cells and NK- and gamma/delta T-cell fractions. These clones may help in the elimination of the residual CML cells.	41
Case report	Pathogenic	A patient with essential thrombocythemia treated with hydroxyurea. developed a clonal proliferation of cytotoxic T-cells with consequent BM failure	42
Case report	Pathogenic	Concomitant existence of CMML and T-LGL clone may be due to a common pathogenic pathway, linked to immune-dysregulation mediated by expanded cytotoxic T-cells clones.	43
Case report	Clinical	In a patient affected by acute promyelocytic leukemia and concomitant LGL, LGL clones continued to increase despite leukemia chemotherapy. Leukemia treatment may have given a growing advantage to clone expansion of LGL cells.	44

LGL, large granular lymphocytes; AML, acute myeloid leukemia; MPN myeloproliferative syndromes; T-LGLL, T cell large granular lymphocyte leukemia; HCL, hairy cell leukemia; T-LGLL, T cell large granular lymphocyte leukemia; CML, chronic myeloid leukemia; NK, natural killer cells; BM, bone marrow; CMML, chronic myelomonocytic leukemia.

relapsed, and the patient died (39). Finally, Malani et al. reported a case of concomitant presentation of T-LGLL with AML in an elderly patient who was treated with combination chemotherapy with good outcome (40). Overall, these experiences suggest a relationship among LGL and AML clones: chemotherapy-induced leukemia depletion leads to LGL expansion, whilst immunosuppression reduces immunosurveillance and favors leukemic escape.

Regarding myeloproliferative diseases, treatment with dasatinib for CML has been associated with an increase in clonal T-/NK-LGL. Kretuzman et al. observed 34 CML patients on treatment with either dasatinib (N=20) or imatinib (N=14): 83% had clonal BCR/ABL-negative lymphocytes (mostly with TCR rearrangement) and this percentage increased during tyrosine kinase therapy with dasatinib but not with imatinib. The Authors speculated that these clones may inhibit the proliferation of residual CML cells and facilitate remission (41). Selvan et al. described a patient with essential thrombocythemia treated with hydroxyurea who eventually developed a clonal proliferation of cytotoxic T-cells and consequent BM failure (42). Finally, concomitant existence of CMML and T-LGLL clone has also been reported with no therapeutic insights (43).

LGL EXPANSION IN APLASTIC ANEMIA

Many Authors described the association of LGL clones with AA and pure red cell aplasia (PRCA), as summarized in **Table 3**. Some case reports addressed the clinical association only, whilst others speculated about the pathogenic implications. For instance, Handgretinger et al. reported a case of PRCA associated with clonal expansion of T-LGLs of γδ-type. They commented that LGL cells were able to selectively destroy erythroid progenitors that lack HLA class I expression and are unable to inhibit TCR/KIR activation. LGLs contribute to cytopenia by direct toxicity (Fas-FasL interaction) and cytokine production (46). Consistently, Saitoh et al. described polyclonal T-LGLs along with high serum-soluble FasL in a patient who developed severe AA 10 years after Hodgkin lymphoma remission. The patient responded to steroids and cyclosporine, but HL relapsed leading to death. As already discussed for AML, this data indicate that LGL depletion might have impaired immunosurveillance on HL clone (50). More recently, Li et al. analyzed the quantitative and functional changes of CD56bright NK cells in peripheral blood of patients with moderate AA. They found that CD56bright NK were higher than in normal controls,

TABLE 3 | Large granular lymphocytes in aplastic anemia and pure red cell anemia.

Study Type	Relevance	Main Findings	Ref.
Case series 10 NK-LGL, 1 PRCA	Therapeutic	One patient had PRCA and obtained response with immunosuppressive therapy.	45 45
Case series 47 PRCA, 9 T-LGLL	Therapeutic	PRCA/T-LGLL association predicts superior response to immunosuppressive therapy, but is not correlated with improved survival.	45
Case report	Pathogenic	A case of PRCA with clonal expansion of T-LGLs of γ δ-type in which the malignant LGLs were shown to carry functional inhibitory MHC class I receptors.	46
Case series 9 AA/LGL	Clinical	AA can be a presenting manifestation of T-LGLL, and T-LGLL should be considered in the differential diagnosis of acquired aplastic anemia.	47
Case series 203 T-LGLL patients	Pathogenic Therapeutic	14% had pancytopenia at presentation and some fit the diagnostic criteria for AA. LGLL-associated PRCA was observed in 7% of cases and effectively treated with immunosuppression.	48
Case report	Therapeutic	T-LGLs of γ δ-type of pure red cell aplasia with low-dose alemtuzumab in a patient with T-LGLs of γ δ-type refractory to cyclosporine and methotrexate	49
Case report	Pathogenic	This case shows a rare instance of a patient who had aplastic anemia associated to polyclonal LGL as the first manifestation of a relapse of Hodgkin lymphoma.	50
Case series 14 LGLL/PRCA	Therapeutic	LGLL with pure red cell aplasia responded well to continuous treatment with cyclophosphamide or cyclosporine A.	51
Case series 367 MDS, 140 AA	Pathogenic Clinical Therapeutic	STAT3 clones can be found in 7% AA and 2.5% MDS and are associated with better responses to immunosuppressive therapy and with HLA-DR15.	13
Case series 42 T-LGLL, 19 with PRCA (45%), 11 CLPD-NK, 3 PRCA (27%)	Pathogenic	PRCA associated with LGL frequently displays STAT3 mutations.	12
Case series 36 LGLL, 18 PRCA (50%)	Therapeutic	PRCA/LGLL was associated with response to methotrexate. Response was shorter in patients with STAT-3 mutation.	10
Case series 41 AA, 46 hypoplastic-MDS	Pathogenic	the proportions of NK and T-LGL in the hypoplastic-MDS group were higher than those in the AA group.	52
Case series 54 AA, 21 MDS, 7 PNH, and 42 PRCA	Pathogenic	STAT3 mutations in 18 of 42 PRCA patients (43%) with or without T-LGLL. STAT3-mutated CD81 T cells may be closely involved in the selective inhibition of erythroid progenitors in PRCA patients.	11
Case series 50 AA	Pathogenic	CD56bright NK cells in newly diagnosed AA patients was higher than in normal controls	53
Case series 24 AA	Pathogenic	Somatic mutations in T cells, particularly JAK-STAT and MAPK are frequent in AA patients and may have a pathogenic role.	54

LGL, large granular lymphocytes; AA, aplastic anemia; PRCA, pure red cell anemia; T-LGLL, T cell large granular lymphocyte leukemia; NK, natural killer cells; BM, bone marrow.

and displayed higher expression of NKG2D and CD158a, likely contributing to disease pathogenesis (53). Zhang et al. compared T lymphocyte subsets in AA and hypoplastic MDS (hypo-MDS) and showed that the proportions of NK- and T-LGL cells in the hypo-MDS group were higher than those in the AA group. These findings indicate that the dysplastic clone (present in hypo-MDS but not in AA) may be a trigger for LGL expansion (52). Finally, PRCA complicated LGL leukemia in 7% of cases only, whilst neutropenia is the leading cytopenia in these patients (48).

At a molecular level, Ishida et al. investigated STAT3 in an Asian cohort of T-LGLL and CLPD-NK of whom a proportion had concomitant PRCA (19/42 and 3/11, respectively). They found STAT3 mutation in 47.6% of T-LGLL and 27.2% of CLPD-NK patients (12). Furthermore, Jerez et al. studied STAT3 mutation in a large series of patients with acquired BMF syndromes, including 140 AA, and identified 16 mutated patients of whom 6 with an LGL clone (13). Other Authors found a STAT3 mutation in 43% of 42 PRCA patients, of whom 13 had associated LGL clones, but not in the 82 patients with AA/MDS (11). More recently, Lundgren et al. showed that CD8+ T cells from AA patients frequently show somatic mutations of JAK-STAT and MAPK pathways, that are associated with CD8+ T-cell clonality and alter CD8+ phenotype (54). On the whole, these studies suggest that STAT3 mutations, may play a pathogenic role, particularly in PRCA, but also in AA, by increasing the production of proinflammatory/proapoptotic cytokines.

Immunosuppression is the backbone therapy for both AA/PRCA and LGL lymphoproliferative disorders, although with heterogeneous outcome (45, 47, 51). In STAT3-mutated AA patients a better response to immunosuppressive therapy has been described in some studies, whilst others reported that STAT3 mutated patients were less responsive to cyclosporine. As regards other immunosuppressants, Lacy et al. reported good response to steroids and azathioprine in an LGLL/PRCA patient (45) and Go et al. described bad responses with cyclophosphamide in LGLL/AA cases (47). More recently, Fujishima et al. analyzed 185 patients with PRCA, of whom 14 had an LGLL clone and responded well to continuous treatment with cyclophosphamide or cyclosporine (51). Finally, methotrexate produced good long-lasting responses in 18 subjects with LGLL/PRCA, similarly to those observed in patients with LGL expansion only (10). Moreover, Schutzinger et al. described successful treatment of LGLL/PRCA with low-dose alemtuzumab, a monoclonal antibody against CD52, in a patient refractory to cyclosporine and methotrexate (49). Finally, only one study addressed the impact of LGL clones on survival of 9 AA/PRCA patients and showed that superior response to immunosuppression did not correlate with improved survival (45). Beyond cytotoxic immunosuppressants, the role of steroids is still controversial, as they may be effective in patients with autoimmune cytopenias associated with LGL-expansion. However, their use in primary LGL, AA, and PRCA is usually not sufficient to revert the phenotype, and association with cytotoxic immunosuppressants is suggested.

In conclusion, this evidence confirms that immunosuppression, particularly with cyclosporine but also with methotrexate, are good options in bone marrow failures associated with LGL clones. Since AA/PRCA are already treated with cyclosporine combinations, patients with LGL expansion may be effectively treated as the primary disease. A warning persists about infectious risk that represents an important cause of morbidity and mortality in these patients and may be increased by immunosuppression. This may also account for the absence of a favorable effect on survival of LGL/AA association, despite a better response to treatment. Further insights in the pathogenetic mechanisms of LGL/AA associations will possibly enable the development of more targeted and less toxic treatments in the next future (10).

DISCUSSION AND CONCLUSIONS

LGL clones are increasingly recognized in patients with bone marrow failures (AA/MDS), but also, although rarely, in AML and MPN. **Figure 1** depicts the features and intersections among myeloid neoplasms and LGL disorders. LGL clones are relatively easy to be demonstrated but the communication between the clinician and the pathologist is pivotal for a correct interpretation. In fact, depending on the method utilized (morphology, flow cytometry, TCR/KIR clonality by molecular analysis) the prevalence of LGL clones in these conditions ranges from less than 5% to more than 30%. Their detection is usually associated with a more cytopenic phenotype, likely due to a more pro-inflammatory and pro-apoptotic bone marrow milieu, where LGL clones may induce direct toxicity against stem cells or produce a variety of immunoregulatory cytokines. Moreover, LGL clones are associated with autoimmune phenomena, frequently described in myeloid neoplasms (55). When peripheral lymphocytosis and splenomegaly are present, overt lymphoproliferative disease should be assessed and classified as per WHO criteria. The latter may in fact require specific immunosuppressive treatment. To avoid overtreatment, LGL-specific therapies are indicated based on clinical features of cytopenia (symptomatic anemia, thrombocytopenia, and neutropenia), constitutional symptoms, and lymphoproliferative progression (2). Moreover, it is worth mentioning that splenomegaly, although considered a classic association, has been reported in about 1/5 of patients only, and may be a common finding in several hematologic diseases, including MPN.

Immunosuppression is the mainstay treatment of LGL disorders as well as of AA and hypo-MDS. In these contexts, the presence of an LGL clone seems associated with better outcome, particularly if cyclosporine or methotrexate are used. At variance, patients with MDS/LGL showed a worse treatment outcome as compared with those with LGLL, likely due to an older age and reduced stem cell reserve in the former. An intriguing feature of LGL clones is that they tend to expand after therapy, particularly if they are associated with myeloid disease. This phenomenon is common to other clonal entities such as paroxysmal nocturnal hemoglobinuria (PNH). Here, a multi-step pathogenesis is postulated encompassing the acquisition of a somatic mutation of PIG-A gene, the autoimmune attack to normal stem cells, and the selection/expansion of the PNH clone favored by immunosuppression and/or acquisition of co-

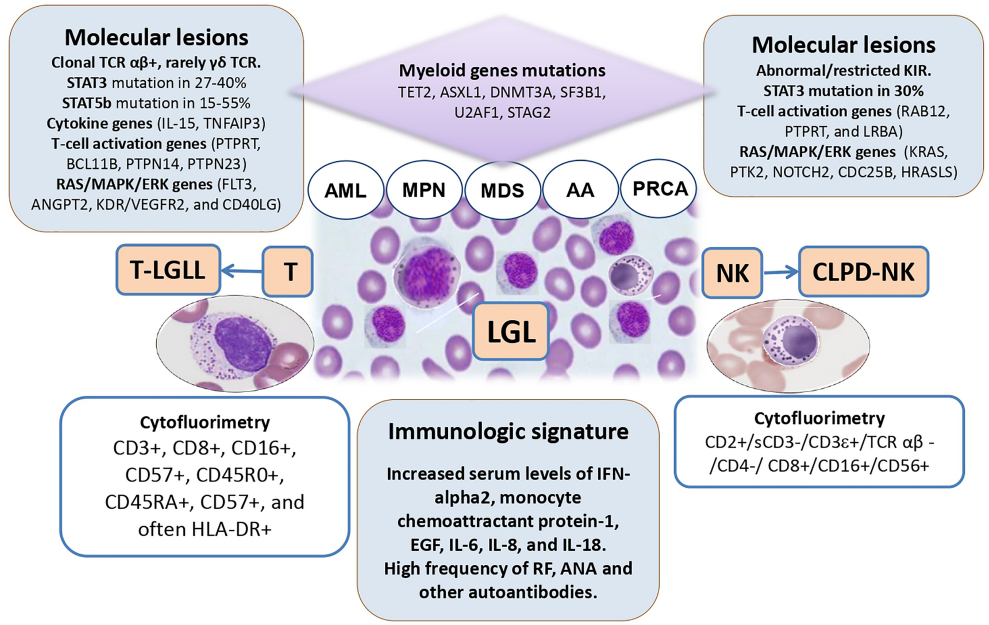

FIGURE 1 | The intersections among large granular lymphocyte (LGL) disorders and myeloid neoplasms. TCR, T-cell receptor; AML, acute myeloid leukemia; MPN, myeloproliferative neoplasm; MDS, myelodysplastic syndromes; AA, aplastic anemia; PRCA, pure red cell aplasia; NK, natural killer cells; T-LGLL, T-cell large granular lymphocyte leukemia; CLPD-NK, chronic lymphoproliferative disorder of NK-cells; IL, interleukin.

mutations (56). Similarly, in LGL clones, somatic mutations of STAT3 and STAT5b have been demonstrated, that seem however not sufficient to cause the disease, without additional contribution of environmental factors, cytokine dysregulation, and therapies. To make this picture even more confusing, the possible common origin of the two clones has been reported, since STAT mutations have been found in myeloid precursors of LGL/MDS patients and myeloid genes mutations have been detected in LGL cells. Overall, although regarded as an important pathogenic player, the detection of a STAT mutation does not inform treatment for LGL that is still based on the severity of cytopenia, B symptoms, and lymphoproliferative progression (2).

The relationship of LGL clones with AA/MDS prognosis is less clear. In fact, some studies indicated a better outcome for LGL/MDS association as compared with MDS alone, whilst other showed no differences despite a better response to immunosuppression. A possible explanation may be the increased infectious risk that represents an important cause of morbidity and mortality after immunosuppression. Furthermore, it may be speculated that LGL depletion after immunosuppression may reduce immunosurveillance on leukemic landscape and counteract the advantage obtained with a better response to immunosuppression. This has been observed even in patients with more aggressive diseases, such as acute myeloid leukemia, where immunosuppression improved LGL signs/symptoms but led to leukemia relapse.

In conclusion, LGL clones may be detected in myeloid diseases in a "whoever seeks finds" fashion. The use of cytofluorimetric and molecular techniques, although the analysis of somatic mutation is not routinely available, will likely allow us to investigate small lymphoid clonalities in myeloid diseases, but even in the general population. This might unveil the presence of a "clonal lymphopoiesis of indeterminate potential" that may precede overt lymphoproliferative diseases, including LGL, but also favor the development of autoimmune diseases still called "idiopathic".

AUTHORS CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

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Co-Occurrence of Myeloid and Lymphoid Neoplasms: Clinical Characterization and Impact on Outcome. A Single-Center Cohort Study

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The co-occurrence of myeloid neoplasms and lymphoproliferative diseases (LPDs) has been epidemiologically described, particularly in myeloproliferative neoplasms (MPNs). However, the clinical features of these patients are poorly known. In this study, we evaluated a single-center cohort of 44 patients with a diagnosis of myeloid and LPD focusing on clinical features, therapy requirement, and outcome. The two diagnoses were concomitant in 32% of patients, while myeloid disease preceded LPD in 52% of cases (after a median of 37 months, 6–318), and LPD preceded myeloid neoplasm in 16% (after a median of 41 months, 5–242). The most prevalent LPD was non-Hodgkin lymphoma (50%), particularly lymphoplasmacytic lymphoma (54.5%), followed by chronic lymphocytic leukemia (27%), plasma cell dyscrasias (18.2%), and rarer associations such as Hodgkin lymphoma and Erdheim–Chester disease. Overall, 80% of *BCR-ABL1*-negative MPN patients required a myeloid-specific treatment and LPD received therapy in 45.5% of cases. Seven subjects experienced vascular events, 13 a grade >/= 3 infectious episode (9 pneumonias, 3 urinary tract infection, and 1 sepsis), and 9 developed a solid tumor. Finally, nine patients died due to solid tumor (four), leukemic progression (two), infectious complications (two), and brain bleeding (one). Longer survival was observed in younger patients ($p = 0.001$), with better performance status ($p = 0.02$) and in the presence of driver mutations ($p = 0.003$). Contrarily, a worse survival was significantly associated with the occurrence of infections ($p < 0.0001$). These data suggest that in subjects with co-occurrence of myeloid and lymphoid neoplasms, high medical surveillance for infectious complications is needed, along with patient education, since they may negatively impact outcome.

Keywords: myeloproliferative neoplasms, lymphoproliferative syndromes, myelodysplastic syndromes, infections, secondary malignancies

INTRODUCTION

The potential for myeloid neoplasms to evolve one into each other is largely known [i.e., leukemic evolution of myeloproliferative neoplasms (MPNs) and myelodysplastic syndromes (MDSs)], and the same occurs for lymphoproliferative disorders [(LPDs), i.e., chronic lymphocytic leukemia, (CLL), which may evolve to aggressive non-Hodgkin lymphomas (NHLs)]. Less is known about the permutation of a myeloid into a lymphoid neoplasm and vice versa, and about their co-occurrence. Some reports describe epidemiological associations of myeloid and lymphoid cancers, and a large Italian study involving 820 MPN patients reported an increased risk for LPD (3.4-fold greater for CLL and 12.4-fold for NHL) and solid tumors compared to the general population (1). From a lymphoid perspective, CLL patients are known to be at higher risk for secondary neoplasms, mostly cutaneous ones (2); however, hematological neoplasms are rarely observed. Irrespective of the former neoplasm (either myeloid or lymphoid), important concerns have been raised about the possible causal effect of hematological treatments on the development of second tumors, and it is still an unanswered question. Moreover, little is known about the clinical characteristics of patients with co-occurrence of myeloid and lymphoid neoplasms, and their outcome in terms of infectious and thrombotic complications, and survival. In this study, we evaluated a single-center cohort of patients with a double diagnosis to assess their clinical features, therapy requirement, and outcome.

PATIENTS AND METHODS

We retrospectively evaluated a cohort of 1,351 myeloid neoplasms (930 MPNs and 421 MDSs) diagnosed within the period 1987–2020 and followed up at a tertiary hematological center in Milan, Italy.

All patients displaying the association of a myeloid and lymphoid neoplasm were included (either presenting with myeloid, lymphoid, or concomitant diseases).

MPN diagnoses, including polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myeloid leukemia (CML), and myeloproliferative neoplasms, unclassified (MPN, U), were made according to the most recent WHO classification and current guidelines (3). The same was performed for MDS cases.

Lymphoid diseases included NHL, Hodgkin lymphomas (HLs), CLL, and plasma cell dyscrasias (PCDs), with a histological diagnosis according to current guidelines (4).

For each patient, we evaluated clinical features at first diagnosis, including demographics, performance status according to the Eastern Cooperative Oncology Group scale (ECOG), hematological parameters, cytogenetic abnormalities, and the presence of driver mutations (*JAK2*V617F, *CALR*, and *MPL*) for MPN cases.

The time from initial diagnosis to LPD development and LPD type was collected and all therapies performed for both myeloid and lymphoid disorders. For LPDs, the time from diagnosis to first treatment was also calculated.

Concerning outcome, the occurrence of thrombosis, infections, and death was registered and overall survival (OS) analyzed.

For statistical analysis, Student's t-test or Wilcoxon test was used for continuous variables where appropriate. Chi-squared or Fisher's exact tests were used for the comparison of categorical variables, where appropriate. Analysis of variance was performed by using mean, median, ranges, and standard errors. Once identified, variables associated with occurrence of complications and OS hazard ratios for 95% confidence intervals were calculated by Cox regression models. Overall survival was evaluated by Kaplan–Meier method.

RESULTS

Baseline Features

During a median follow-up of 9 years (range, 0.8–35), a total of 44 of the 1,351 patients (3.25%) were diagnosed with both a myeloid and lymphoid neoplasm. The two diagnoses were concomitant in 32% of patients, while myeloid disease preceded LPD in 52% of cases after a median time of 37 months (range, 6–318) from myeloid disease, and LPD preceded myeloid neoplasm in 16% after a median time of 41 months (range, 5–242). **Table 1** shows clinical and laboratory characteristics: patients were mainly male, elderly (61% aged >65 years), with a good performance status (96% ECOG 0–1), and all but three had a diagnosis of MPN (3 out of 158 CMLs and 38 out of 772 *BCR-ABL1*-negative MPNs). The remaining subjects were low-risk MDS with multilineage dysplasia (3/421 total MDS patients). Cytogenetic aberrations, excluding t(9;22), were reported in nine patients, comprising one complex karyotype. Among *BCR-ABL1*-negative MPN patients, *JAK2*V617F mutation was found in 30 (79%) cases, while 8% and 3% were *CALR* and *MPL* mutated, respectively. In those first presenting with a myeloid disease, mutated patients showed a longer time to LPD development (mean 120 ± 92 vs. 31 ± 17 months in triple-negative cases; $p = 0.01$). As shown in **Table 2**, the most prevalent LPD was NHL (50%), all but one of B-cell origin, particularly lymphoplasmacytic lymphoma (54.5%). Most NHL were indolent, except for one diffuse large B-cell lymphoma (DLBCL). The second most frequent LPD was CLL (27%), followed by plasma cell dyscrasias (18.2%), and rarer associations such as HL and Erdheim-Chester disease.

Therapy Requirement

The clinical characteristics and therapy sequences of patients divided according to the first presenting neoplasm are detailed in the **Supplementary Table**. Overall, 80% of *BCR-ABL1*-negative MPN patients required myeloid-specific treatment, including hydroxyurea in most cases (71%), followed by ruxolitinib in three patients, and pipobroman in two. Of note, 14 cases had been treated before LPD diagnosis (11 hydroxyurea, 1 hydroxyurea and pipobroman, and 2 ruxolitinib), 11 cases started therapy concomitantly (10 hydroxyurea, 1 ruxolitinib, and 1 imatinib), and 11 after LPD diagnosis (9 hydroxyurea, 1 hydroxyurea and imatinib, and 1 hydroxyurea and pipobroman). All CML patients received imatinib either before, concomitantly, or after LPD diagnosis (one case each). Moreover, 29 patients

TABLE 1 | Clinical and hematological characteristics of patients with concomitant myeloid and lymphoid neoplasms.

Table 1	All patients (N = 44)
Median age, years (range)	70 (21–93)
Males	25 (56)
PS ECOG	
0	32 (73)
1	10 (23)
2	1 (2)
3	1 (2)
Laboratory data at diagnosis, median(range)	
Leukocytes, ×10^9/L	9.56 (1.8–116)
Hemoglobin, g/dl	14 (7–20)
Platelets, ×10^9/L	489 (20–1482)
Lymphocytes, ×10^9/L	1.94 (0.69–25)
Sequence of neoplasm	
First myeloid	23 (52)
Concomitant	14 (32)
First lymphoid	7 (16)
Myeloid type	
ET	8 (18)
PV	12 (27)
PMF	13 (30)
MPN, U	5 (11)
CML	3 (7)
MDS	3 (7)
Splenomegaly	22 (50)
Driver mutations	
***JAK2*V617F**	30 (79)
Median allele burden, % (range)	28.7 (1.2-97.3)
CALR	3 (8)
MPL	1 (3)
Triple-negative	4 (10)
Cytogenetic aberrations*	9 (21)
LPD type	
NHL	24 (55)
CLL	11 (25)
HL	1 (2)
PCD	8 (18)
Complications	
Thrombosis	7 (16)
Infections	13 (30)
Solid tumors	9 (21)
Death	9 (21)

Values are given as N (%) unless otherwise specified.
**Excluding CML.*
PS ECOG, performance status according to the Eastern Cooperative Oncology Group; ET, essential thrombocytemia; PV, polycytemia vera; PMF, primary myelofibrosis; MPN U, myeloproliferative neoplasm unclassified; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome; LPD, lymphoproliferative disorder; NHL, non-Hodgkin lymphoma; CLL, chronic lymphocytic leukemia; HL, Hodgkin lymphoma; PCD, plasma cell dyscrasia.

were on antiplatelet prophylaxis (27 aspirin and 2 ticlopidine). Finally, one MDS subject received recombinant erythropoietin and steroids (concomitant MDS and LPD diagnosis). Lymphoid diseases required specific treatment in 45.5% of cases, after a median time from the first diagnosis of 8 months (range, 0–115). The following therapies were administered: seven anti-CD20 monoclonal antibody plus chemotherapy, one rituximab-ibrutinib, three chemotherapies, two radiotherapies, one pegylated interferon, one parotidectomy, one phototherapy, two steroids only, one lenalidomide-dexamethasone, and one allogeneic hematopoietic stem cell transplant. Notably, four patients had received LPD treatment before myeloid diagnosis.

Complications

Seven patients (4 ETs and 3 PMFs) experienced a total of 10 vascular events (5/7 occurring before diagnosis of the second hematological neoplasm), including 7 venous thromboses (3 pulmonary embolisms, 1 cerebral vein thrombosis, 1 retinal vein thrombosis, and 2 portal vein thromboses), and 3 arterial events (1 myocardial infarction, 1 stroke, and 1 ileal infarction). Of note, three patients experienced more than one events. All venous events were managed with low molecular weight heparin, and four were switched to long-term oral anticoagulants (three warfarin and one on direct oral anticoagulant). Unexpectedly, *JAK2* resulted unmutated in more than half of the cases (57.1%), and no associations were observed with LPD type.

Concerning infections, 13 (30%) patients experienced a grade ≥3 episode, including 9 pneumonias (of whom 2 fatal), 3 urinary tract infection, and 1 sepsis, all due to bacterial agents (12/13 occurring after the second neoplasm diagnosis). Infections were mainly diagnosed in elderly subjects (12/13, $p = 0.006$), in CLL patients (54%), and only one after a recent LPD therapy with rituximab plus chemotherapy. Importantly, no patients were on ruxolitinib at the time of infection.

After a median time from hematological diagnosis of 106 months (range, 0–301), nine subjects (five PMFs, two CMLs, one PV, and one ET) developed a solid tumor (two lung, two gastric, one liver, one kidney, one cutaneous, one bladder carcinoma, and one seminoma); six occurred after the second hematological diagnosis (**Supplementary Table**). Of note, one patient had a concomitant diagnosis of PMF, follicular lymphoma, CLL, and lung cancer. Overall, cases developing a solid tumor had a shorter time to first LPD therapy as compared to those without solid cancer (7 ± 10 vs. 37 ± 42 months; $p = 0.02$).

Survival

Nine (20%) patients died due to solid tumor (four), leukemic progression (two), infectious complications (two), and brain bleeding (one). Fatalities were more frequent in patients with lymphoid treatment requirement (35% vs. 9%; $p = 0.04$). As shown in **Figure 1**, a longer OS was observed in younger patients ($p = 0.001$), with better performance status ($p = 0.02$) and in the presence of driver mutations ($p = 0.003$). Contrarily, a worse survival was significantly associated with the occurrence of infections ($p < 0.0001$). Multivariate analysis by Cox regression model showed that the occurrence of infections was the only independent predictor of worse survival within the cohort (HR, 3.18; 95%CI, 2.9–19; $p = 0.003$).

DISCUSSION

Here, we describe a single-center series of associated myeloid and lymphoid neoplasms and contribute to delineate some peculiar clinical features and their impact on outcome. In particular, these patients, despite being mainly chronic/indolent neoplasms (i.e., MPN, low-risk MDS, and indolent LPD), display a dismal outcome, mostly related to infectious complications. Importantly, the detrimental impact of infections on survival

TABLE 2 | Overview of the associated myeloid and lymphoid malignancies.

	PV	ET	PMF	CML	MPN-U	MDS	Total
Chronic lymphocytic leukemia	2	3	5	1	1	–	12
Lymphoplasmocytic lymphoma	4	2	2	–	3	–	11
Follicular lymphoma	1	1	3	–	–	–	5
Marginal zone lymphoma	–	–	2	–	–	2	4
Diffuse large B-cell lymphoma	1	–	–	–	–	–	1
Multiple myeloma	3	1	1	1	–	1	7
Plasmocytoma	–	1	–	–	–	–	1
Erdheim–Chester disease	1	–	–	–	–	–	1
Mycosis fungoides	–	–	–	–	1	–	1
Hodgkin lymphoma	–	–	–	1	–	–	1
Total	12	8	13	3	5	3	44

ET, essential thrombocytemia; PV, polycytemia vera; PMF, primary myelofibrosis; MPN U, myeloproliferative neoplasm unclassified; CML, chronic myeloid leukemia; MDS, myelodysplastic syndrome.

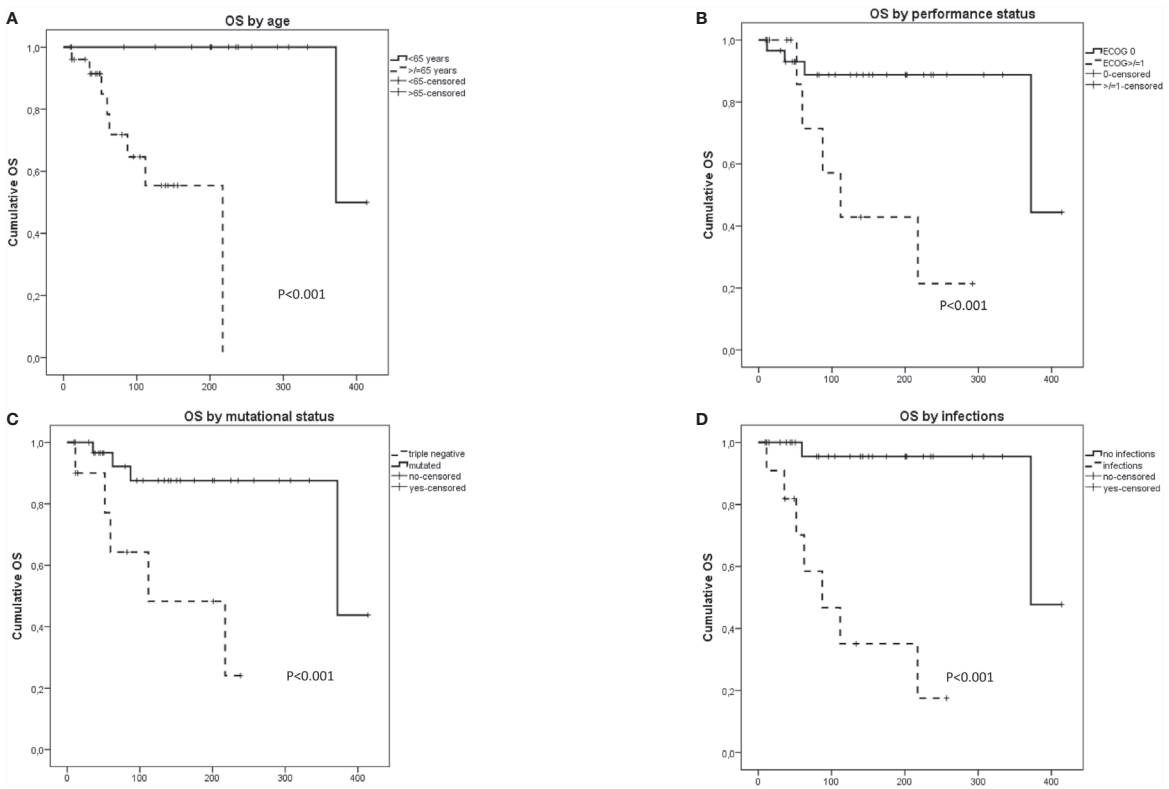

FIGURE 1 | Overall survival in patients with concomitant myeloid and lymphoid neoplasms according to **(A)** age, **(B)** performance status, **(C)** presence of driver mutation, and **(D)** infectious complications.

seems independent from LPD aggressiveness or therapy. In fact, only one infection was related to recent rituximab plus chemotherapy and none to ruxolitinib. This might hint that the infectious risk is more disease intrinsic than iatrogenic, is possibly favored by the presence of a double myeloid/lymphoid clonality, and should be considered even in untreated patients. Interestingly, infections had a higher impact on survival than thrombotic episodes that are the major cause of morbidity and mortality in MPNs. In this study, thromboses were not associated with *JAK2* mutation in MPN-LPD cases, contrarily to what was reported for classic MPNs (5, 6). Moreover, most events occurred in patients presenting with myeloid neoplasm, before the development of the LPD, and did not have an impact on survival. Additionally, together with expected dismal outcome in elderly patients with poorer performance status, triple negative MPN-LPD subjects also showed shorter OS, as already described for isolated PMF (7). Triple negative MPN-LPD cases also displayed a shorter time to LPD development compared to mutated ones, although this observation requires further investigation.

From an epidemiological point of view, an Italian population study has reported that MPN patients (excluding CML) have a 3.44-fold increased risk of LPD compared with the general population (1). This was confirmed by a further review of 1,915 MPN patients of whom 22 displayed coexistent LPD, with a calculated risk 2.79-fold higher than the general Italian population (8). Our results well compare to these data, with a prevalence of 3.25%, although a direct comparison with the general population has not been performed. At variance, other studies reported a higher prevalence of myeloid/lymphoid associations (up to 15%), possibly due to the inclusion of pre-neoplastic conditions such as monoclonal gammopathy of uncertain significance (MGUS) and monoclonal B-cell lymphocytosis (MBL) (9–11).

In our series, a minority of patients had LPD preceding myeloid neoplasm, mostly CLL or indolent NHL, while plasma cell dyscrasias and aggressive neoplasms occurred all concomitantly or after myeloid diagnosis. This finding is in accordance with a recent meta-analysis, where aggressive forms were rarer (14%), mostly concomitant or subsequent to myeloid cancer diagnosis, and required chemotherapy in 7% of cases only (12).

Biologically, it has been speculated that the co-occurrence of myeloid and lymphoid neoplasms may be sustained by a common clonal progenitor (13, 14). This could be assumed particularly in MPN patients with a demonstrated driver mutation, preceding LPD onset. In our series of MPN-LPD cases, *JAK2*V617F was overrepresented as compared to MPN patients not developing lymphoid diseases (79% vs. 65%). Consistently, it has been shown that patients harboring the *JAK2*V617F mutation display an increased risk (5.46-fold) of LPD development (1, 15). Moreover, Vannucchi *et al.* demonstrated the *JAK2*V617F mutation in lymphoid tumor cells in two out of three evaluated cases (1). Conversely, the presence of a "true" double clonality might be hypothesized, with the myeloid and lymphoid clones facilitating each other's selection/expansion in a vicious circle. This could be in line with our study and that of others, reporting the possible coexistence or subsequent development of the two diseases and with the hardly demonstrable common origin of the two clones in most cases. Finally, the possible contribution of germline mutations, including DDX41, RUNX1, ETV6, ANKRD26, and POT1, which have been associated with the occurrence of both myeloid and lymphoid neoplasms, is an intriguing point to be explored in future studies (3, 16).

External triggers, such as hematological therapies, may also favor the emergence of the second clone. In our series, the only therapies administered before LPD development were hydroxyurea, ruxolitinib, and pipobroman. It has been speculated that these drugs may contribute to a reduced immune surveillance on the development of second tumors (17, 18). This immunological derangement is already present in untreated MPN patients and encompass the increase in myeloid-derived suppressor cells and the defective function of T-regulatory and NK cells, showing impaired degranulation and killing capacity. Ruxolitinib may further exert a negative influence on the innate and the adaptive immune system (19). Accordingly, it has been reported that its use may disclose a latent clonality, including lymphoid one, promoting its clinical emergence (20).

Finally, 20% of patients developed a solid tumor, six after the second hematological malignancy and three in between. These cases had three neoplasms, and four of nine patients died. Although we did not find any association with previous therapies or hematological disease pattern, it may be speculated that an underlying genomic instability may be present in these cases (21).

We reckon that our study carries several limitations, particularly regarding the retrospective nature of the study, the relatively small number of patients, and the inclusion of diagnoses dating back to 30 years ago. However, only patients with overt clinical association and available detailed clinical data have been included, and all subjects have been followed at a single center and their diseases restaged/reclassified as per the state of the art. Larger multicenter studies including a control population would be important to confirm our observations.

In conclusion, our data suggest that in subjects with co-occurrence of myeloid and lymphoid neoplasms, high medical surveillance for infectious complications is needed, along with patient education, irrespective of treatment requirement, since they may negatively impact outcome.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

CB and BF followed patients, conceived the study, collected and analyzed data, and wrote the manuscript. DC, JG, KB, RP, EB, MC, LP, FR, GR, RC, WB, and LB followed patients and revised the article for important intellectual content. AI conceived and designed the study and supervised the analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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CASE REPORT
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Paroxysmal Nocturnal Hemoglobinuria in the Context of a Myeloproliferative Neoplasm: A Case Report and Review of the Literature

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Paroxysmal nocturnal hemoglobinuria (PNH) is characterized by intravascular hemolytic anemia and thrombosis and is notoriously associated with aplastic anemia and myelodysplastic syndromes. Rarer associations include myeloproliferative neoplasms (MPNs), which are also burdened by increased thrombotic tendency. The therapeutic management of this rare combination has not been defined so far. Here, we describe a 62-year-old man who developed a highly hemolytic PNH more than 10 years after the diagnosis of MPN. The patient started eculizumab, obtaining good control of intravascular hemolysis but without amelioration of transfusion-dependent anemia. Moreover, we performed a review of the literature regarding the clinical and pathogenetic significance of the association of PNH and MPN. The prevalence of PNH clones in MPN patients is about 10%, mostly in association with *JAK2V617F*-positive myelofibrosis. Thrombotic events were a common clinical presentation (35% of subjects), sometimes refractory to combined treatment with cytoreductive agents, anticoagulants, and complement inhibitors. The latter showed only partial effectiveness in controlling hemolytic anemia and, due to the paucity of data, should be taken in consideration after a careful risk/benefit evaluation in this peculiar setting.

Keywords: paroxysmal nocturnal hemoglobinuria, myeloproliferative neoplasm, complement inhibitors, thrombosis, bone marrow failure, case report

INTRODUCTION

Paroxysmal nocturnal hemoglobinuria (PNH) is a rare acquired disorder caused by the somatic mutations of phosphatidylinositol glycan A (*PIGA*). The consequent defect of glycosyl phosphatidylinositol (GPI)-anchored proteins on red blood cell (RBC) surface increases the susceptibility of PNH cells to complement-mediated destruction, leading to intravascular hemolytic anemia, which is the main clinical feature of the disease (1, 2). The natural history of

PNH was burdened by high morbidity due to chronic anemia and considerably increased mortality, mainly related to fatal thrombotic events (3). With the advent of complement inhibitors, PNH patients significantly ameliorated their quality of life and survival (4). PNH has been described in the context of bone marrow failure (BMF) syndromes, namely, aplastic anemia (AA) and myelodysplastic syndrome (MDS) (4). However, with the development of more sensitive cytofluorimetric techniques (5), PNH clones of various sizes are increasingly being detected in various onco-hematologic and autoimmune disorders (6–9). The coexistence of PNH and myeloproliferative neoplasms (MPNs) has been reported, but its clinical/prognostic significance and therapeutic management are still poorly known. Moreover, these two conditions share an overlapping clinical presentation, represented by thrombotic events at usual and unusual sites (3, 10–13). Here, we provide the description of a patient with MPN who was subsequently diagnosed with PNH and required specific treatment for hemolytic anemia. In addition, we searched for the available evidence in literature about the association of PNH and MPN, collecting data over the last 40 years in MEDLINE *via* PubMed and the National Library of Medicine. In detail, we reviewed data about the coexistence of clinically overt PNH and MPN, the prevalence of PNH clones in MPN patients, and the prevalence of MPN driver mutations in PNH subjects.

CASE DESCRIPTION

A 62-year-old Caucasian male was diagnosed with Janus kinase (JAK)2-negative essential thrombocythemia (ET) in May 2007 due to isolated asymptomatic thrombocytosis (**Table 1**). His medical history was unremarkable, except for moderate arterial hypertension on regular treatment; no previous thrombotic events were registered. Bone marrow (BM) evaluation showed normocellularity (40%), increased mature megakaryocytes, slight increase of reticulin fibers (MF-1), and normal karyotype. Once-daily acetylsalicylic acid (ASA) and low-dose hydroxyurea (HU) were started with adequate control of platelet count. From March 2016, a trend to increased lactate dehydrogenase (LDH) levels was noticed, and from February 2019, a mild macrocytic anemia [hemoglobin (Hb) 10.2 g/dL, mean corpuscular volume (MCV) 103 fL; normal iron and vitamin status] developed (**Figure 1**). Direct antiglobulin test (DAT) was negative, with mild elevation of unconjugated bilirubin (UB, 1.3 mg/dL), consumption of haptoglobin, and increased reticulocytes. HU dose was decreased, but anemia worsened (Hb nadir 7 g/dL), LDH rose to 4× upper limit of normal (ULN), and peripheral CD34-positive cells increased. The patient became strongly symptomatic for anemia, requiring about 1–2 RBC units/month. His physical examination was unremarkable. In October 2019, BM reevaluation showed increased cellularity (>95%) with dystrophic megakaryocytes and increased fibrosis (MF-2) and was therefore interpreted as fibrotic evolution of ET. Molecular tests on peripheral blood revealed the presence of a type-2 calreticulin (*CALR*) mutation at exon 9. Abdomen ultrasonography displayed normal spleen and liver size. HU and ASA were stopped, and an attempt with steroids (oral prednisone 50 mg/day) was made with a transient response and reappearance of transfusion dependency during tapering (2–3 packed RBC units/month). In February 2020, danazol was administered, again without anemia improvement. In November 2020, due to persistent intravascular hemolytic anemia and referred dark-colored urines, a flow cytometry for PNH was made and turned positive with a clone size of 95%/94% on neutrophils/monocytes. Low-molecular weight heparin (LMWH) prophylaxis (enoxaparin 4,000 units/day) was started, and the patient was referred to our center for treatment indications. No major PNH-related symptoms were registered. In December 2020, the patient started eculizumab after recommended vaccinations. In the subsequent months, Hb stabilized at 7.5–8 g/dL, LDH progressively lowered to 1.1×–1.2× ULN, and transfusion need returned to 1–2 units/month, with subjective amelioration of the quality of life and disappearance of hemoglobinuria. LMWH was stopped. In June 2021, due to a minor response to eculizumab, a re-evaluation was performed: DAT result turned positive for complement without evidence of cryoagglutinins (as frequently observed under eculizumab treatment), and UB levels slightly increased, indicative of extravascular hemolysis (EVH) during terminal complement inhibitor treatment. BM biopsy confirmed increased age-adjusted cellularity, granulocytic hyperplasia, and numerous megakaryocytes in loose and dense clusters, including both mature and atypical, dystrophic cells; fibrosis was stable with diffuse increase in

TABLE 1 | Laboratory parameters at different time points of the clinical history of the patient.

	ET diagnosis (May 2007)	Post-ET MF (Oct 2019)	PNH diagnosis (Nov 2020)	Month +6 after ECU start (June 2021)	Normal ranges
Hb (g/dL)	13.5	7*	7.3*	8*	13.5–17.5
PLT (×10^3/μL)	830	396	450	540	130–400
WBC (×10^3/μL)	7.5	4.5	6.0	8.5	4.8–10.8
LDH (× ULN)	0.8	4	4	1.1	-
UB (mg/dL)	0.5	1.3	1.3	2.0	0–0.8
Retics (×10^9/L)	40	140	200	155	20–100
Haptoglobin (mg/dL)	70	<10	<10	<10	30–200
CD34+ cells (n/μL)	7	56	56	60	-

*ET, essential thrombocythemia; MF, myelofibrosis; PNH, paroxysmal nocturnal hemoglobinuria; ECU, eculizumab; Hb, hemoglobin; PLT, platelet; WBC, white blood cell; LDH, lactate dehydrogenase; ULN, upper limit of normal; UB, unconjugated bilirubin; Retics, reticulocytes. *Pre-transfusion Hb levels.*

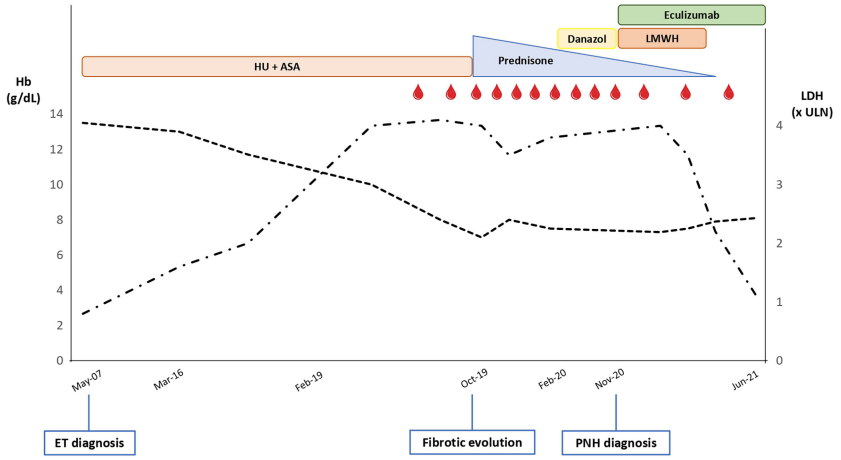

FIGURE 1 | Trend of hemoglobin (Hb; dotted black line) and lactate dehydrogenase (LDH; dashed-dotted black line) levels along the patient's clinical journey. ULN, upper limit of normal; HU, hydroxyurea; ASA, acetylsalicylic acid; LMWH, low-molecular weight heparin; ET, essential thrombocythemia; PNH, paroxysmal nocturnal hemoglobinuria. Red drops represent red blood cell transfusions.

reticulin and focal bundles of thick collagen fibers (MF-2); of note, hyperplasia of the erythroid lineage in the absence of dysplastic features was described. Karyotype analysis was normal. Analyzed by an expert hemopathologist, the BM trephine was consistent with MPN unclassifiable (MPN-U). A targeted next-generation sequencing (NGS) myeloid panel confirmed the presence of type-2 *CALR* mutation [variant allele frequency (VAF), 45%] and showed an additional somatic mutation in ten-eleven translocation 2 (*TET2*) gene (VAF, 9.7%). The patient is continuing regular fortnightly eculizumab infusions, with subjective benefit, although with persistent transfusion dependence.

REVIEW OF THE LITERATURE

Clinical Association of Paroxysmal Nocturnal Hemoglobinuria and Myeloproliferative Neoplasm

Since the 1970s, several case reports about the coexistence of PNH and MPN have been described (**Table 2**). A total of 23 cases have been reported so far, mainly in association with myelofibrosis (MF; n = 12), followed by polycythemia vera (PV; n = 3), MPN-U (n = 2), and only one case each of ET (20) and chronic myeloid leukemia (CML) (19). Nine cases were *JAK2*-positive, while only two *CALR*- and one *MPL*-mutated (24). In 12 cases, the diagnosis of MPN preceded that of PNH, and the clinical suspicion for the latter (when indicated) was the development of hemolytic or iron-deficient anemia (15, 17, 20). The diagnoses were concomitant in seven patients, whose main clinical presentation included atypical thromboses or the coexistence of hemolytic anemia and thrombocytosis (18, 21, 24, 25). In the remaining four subjects, the diagnosis of MPN followed that of PNH and derived from recurrent thrombosis or development of thrombocytosis/leukocytosis (14, 18, 19). Ten patients harbored PNH clones >10% (8/10 were >50%), while in four cases, it was <10%. PNH clone size showed a complessively wide distribution, as reported by Richards et al. (23) in a recent large retrospective study analyzing the clinical presentation of 1,081 PNH patients. The five patients with a previous MPN diagnosis had a PNH clone from 0.7% to 96.3%, while cytopenic/myelodysplastic patients generally harbored smaller clones, and hemolytic/thrombotic subjects harbored larger ones (23). With regard to treatments, eculizumab was administered in four patients (notably, all with *JAK2V617F*-positive MPN). In one patient, eculizumab monotherapy was able to resolve anemia (18); in another, the concomitant administration of eculizumab with acenocumarol and HU resolved a severe case of visceral thrombosis (25). In the remaining two cases, hemolytic anemia was refractory, and one experienced visceral thrombosis recurrence notwithstanding eculizumab plus HU and anticoagulation (18). A retrospective series of 55 PNH patients undergoing hematopoietic stem cell transplantation (HSCT) included two cases associated with MF. The reported overall survival at 5 years was 70%, although the outcome of these two patients is not specified (22). Regarding outcome, four out of the total 23 patients reported (17.4%) died due to acute lymphoblastic/myeloid leukemia progression (16, 17), infections, or liver failure secondary to refractory Budd–Chiari syndrome (18).

Prevalence of Paroxysmal Nocturnal Hemoglobinuria Clones in Myeloproliferative Neoplasm Patients

Some cross-sectional studies on MPN patients evaluated the association with PNH clones. Tanasi et al. (26) tested 32 patients with MPN and concomitant hemolysis or unexplained anemia and found a PNH clone in three (9.3%) with MF (two *JAK2*- and one *CALR*-mutated). Two of them harbored a PNH clone >90% but did not require specific PNH therapy (26). In a recent large

TABLE 2 | Association of MPNs and PNH.

Type of study, year	N. of patients (sex, age)	Timing of PNH and MPN presentation (delay)	PNH clone size	MPN diagnosis (driver mutation)	Thrombosis	Treatment	Outcome	Notes	Ref.
Case report, 1979	1 (M, 60)	PNH first	-	Not specified	No	Oxymetholone for hypoplastic PNH	-	-	(14)
Retrospective, 1992	47 PNH patients, four of whom with MPN	MPN first	-	PMF	-	-	-	PNH succeeded the development of PMF, while it preceded the diagnosis of MDS.	(15)
Case report, 1993	1 (M, 58)	Concomitant	-	PMF	No	HU, splenic irradiation	Died for AML progression 6 years after diagnosis	AML blasts were PNH+.	(16)
Case report, 2005	1 (M, 53)	MPN first (2 years before PNH)	-	PMF	3 AMI	IFN, HU	Died 6 months after PNH diagnosis due to BCP-ALL progression	PNH diagnosed for iron-deficient anemia.	(17)
Case series, 2012	#1 M, 51 #2 M, 65 #3 M, 78	#1 Concomitant #2 PNH first (2 years before MPN) #3 PNH first (1 year before MPN)	#1 99% G, 13% R #2 40% R #3 73% G, 53% R	#1 and #2 MPN-U (JAK2) #3 PMF (JAK2)	#1 Multiple thromboses (stroke, BCS) #2 splenic infarction under anticoagulation; BCS under HU and ecu #3 No	#1 Ecu #2 added HU and ecu to anticoagulation #3 ecu, danazol, steroid	#1 Transfusion-free with ecu, but variceal bleeding due to BCS #2 died in 10 months for liver failure and iron overload #3 died for clostridiosis 1 year after	#1 and #2: JAK2V617F detected in PNH+ granulocytes, but not in those PNH- → the JAK2V617F mutation coexists within the PNH clone #3: PMF diagnosed for thrombocytosis; anemia refractory to all treatments.	(18)
Case report, 2015	1 (M, 52)	PNH first (11 years before MPN)	12% CD55-, 24% CD59-G	CML	No	Cyclosporin A, prednisolone, erythropoietin, and Andriol	PNH responsive to IST, CML responsive to imatinib	Disappearance of PNH clones at the time of CML diagnosis.	(19)
Case report, 2016	1	MPN first (6 years before PNH)	73% M, 60% G, 14% R	ET (CALR)	No	No	Alive	Diagnosis of PNH because of hemolytic anemia	(20)
Case report, 2017	#1 F, 72 #2 M, 75	#1 Concomitant #2 MPN first (24 years before PNH)	#1 88.6% G, 86.9% M, 71% R #2 <1%	#1 Post-ET MF (JAK2) #2 PV (JAK2V617F)	#1 Portal vein thrombosis #2 multiple arterial and venous thromboses	#1 HU, anticoagulation #2 anticoagulation, anti-platelets	#1 Recanalization within 2 months #2 Alive	In #1, cell sorting showed that JAK2+ subclone arose within the PNH population.	(21)
Retrospective, 2019	55 PNH patients, two of whom with MPN	Concomitant	-	PMF	-	HSCT	-	Indication for HSCT was association with MF. Complessive 5-year-OS in the cohort: 70%.	(22)
Retrospective, 2020	1081 PNH patients, five of whom with MPN (71, M; 65, F; 55, M; 71, F; 74, M)	MPN first	#1 0.7% #2 93.3% #3 1.2% #4 3.4%	#1 MF (CALR) #2 not specified (JAK2) #3 PV (JAK2) #4 not	#1 DVT/PE #2 BCS	-	-	Severe hemolysis was evident only in Pt #5	(23)

(Continued)

TABLE 2 | Continued

Type of study, year	N. of patients (sex, age)	Timing of PNH and MPN presentation (delay)	PNH clone size	MPN diagnosis (driver mutation)	Thrombosis	Treatment	Outcome	Notes	Ref.
			#5 96.3%	specified (JAK2) #5 not specified (JAK2)					
Case report, 2020	1 (M, 49)	Concomitant	99%	PMF (MPL)	No	-	-	The patient presented with anemia, thrombocytosis, elevated LDH, dark-colored urine.	(24)
Case report, 2021	1 (F, 51)	Concomitant	>90%	Masked PV (JAK2V617F)	Venous (hepatic, splenic, kidney)	Anticoagulation, HU, ecu	Clinical resolution of ascites in 2 months	No signs of hemolysis	(25)

MPN, myeloproliferative neoplasm; PNH, paroxysmal nocturnal hemoglobinuria; PMF, primary myelofibrosis; MDS, myelodysplastic syndrome; HU, hydroxyurea; AML, acute myeloid leukemia; AMI, acute myocardial infarction; IFN, interferon; B-ALL, B-acute lymphoblastic leukemia; G, granulocyte; R, red blood cell; MPN-U, MPN unclassifiable; BCS, Budd–Chiari syndrome; ecu, eculizumab; CML, chronic myeloid leukemia; IST, immunosuppressive treatment; M, monocyte; ET, essential thrombocythemia; HSCT, hematopoietic stem cell transplantation; PV, polycythemia vera; DVT, deep vein thrombosis; PE, pulmonary embolism; LDH, lactate dehydrogenase.

monocentric study, more than 3,000 patients were tested for PNH because of unexplained cytopenia/thrombosis, including 92 patients diagnosed with MPN (27). A PNH clone was found in 16 patients (17.4%), mainly MF, and was generally smaller than 1% (except for one patient with a clone of 5%). It was associated with increased frequency of thrombosis without impact on overall survival (28). In a large cross-sectional study including 197 MPN, 14.2% of subjects had CD55/CD59-negative red cells; this prevalence rose to 21.3% in the subgroup of ET patients (29). At variance, in a series of 98 MPN subjects, PNH clones greater than 1% were detected in only two patients (2%) (30), one of whom with recurrent thrombotic complications. Finally, two studies failed to detect PNH clones in MPN patients. In detail, Nazha et al. (31) tested 62 MF patients with significant anemia (Hb <10 g/dL) and elevated LDH, but none of them harbored a PNH clone. Likewise, a study on 136 patients with myeloid disease, including five MF and 15 MDS/MPN overlap, found GPI-negative cells in 8% of low-risk MDS, but none in MPN subjects (32).

Prevalence of Myeloproliferative Neoplasm Driver Mutations in Paroxysmal Nocturnal Hemoglobinuria Patients

There are isolated reports of PNH patients harboring mutations in MPN-related driver genes, namely, *JAK2*. Shen et al. (33) performed targeted NGS in a cohort of 36 PNH patients and found *JAK2V617F* homozygous mutations in two patients (5.5%). Langabeer et al. (34) reported a case of AA-PNH with a concomitant *JAK2V617F* mutation at low allele ratio (1.8%) without any clinical feature of MPN; intriguingly, the *JAK2*-positive clone disappeared after cyclosporin therapy, while the PNH clone remained stable. More recently, Santagostino et al. (35) described a case of hemolytic PNH occurring 10 years after HSCT for acute myeloid leukemia (AML); concomitantly, somatic mutation analysis revealed the presence of *JAK2* mutation with an allele ratio of 44% and *TET2* with a VAF of 34%. Soon after, the patient relapsed for AML.

DISCUSSION

Our patient, along with the others described, allows several clinical and pathogenic considerations about the rare coexistence of PNH and MPN. Besides AA and MDS, PNH clones have been detected also in the context of lymphoid disorders, such as acute lymphoblastic leukemia and lymphomas (9, 36), and in autoimmune/idiopathic cytopenias (6–8, 37). The review of the literature highlighted that about 10% of MPN patients harbor a PNH clone (26, 29, 30), and this frequency rises up to 17% if clones smaller than 1% are considered (27). More importantly, the disregarded association of these two conditions may cause a significant delay in PNH diagnosis, as observed in our case. Additionally, in the MPN setting, the differential diagnosis of hemolytic anemia may be hampered by several confounders: haptoglobin can be decreased in more than 30% of MF (38), LDH is often elevated as a consequence of disease burden (31), and reticulocytosis can be observed in case of myeloid metaplasia (39). Furthermore, the appearance of anemia in MPN should prompt the exclusion of fibrotic evolution, which was in fact observed in our patient, who met the diagnostic criteria for post-ET MF (40). However, at variance with post-ET MF, BM trephine revealed erythroid hyperplasia, which may be attributed to the concomitant peripheral hemolytic process and indicative of bone marrow compensation. Consultation with an expert hemopathologist may be thus advised when morphological findings are not fully consistent with a clear diagnosis and confounding factors coexist.

An important clinical issue is the thrombotic risk in MPN-PNH patients and its management. In our literature review, 35% of MPN-PNH subjects had a severe thrombotic presentation,

often refractory to combined anticoagulant, cytoreductive, and anti-complement treatments. This frequency appears higher than that reported for isolated untreated PNH (18.8%) (41) and for MPN (complessively 20%, higher in PV vs. ET/MF and *JAK2*-mutated patients) (12, 13, 42, 43), possibly due to the association of two thrombophilic conditions. With regard to therapy, it is well established that cytoreductive and anti-coagulant/platelet therapies are the cornerstones of thrombosis treatment in MPN, although recurrencies interest about 20% of treated patients (44, 45). In PNH, complement inhibition (Ci) has proven to significantly reduce the thrombotic risk, while anticoagulation alone is poorly effective (4, 46). Whether primary thromboprophylaxis is indicated in untreated PNH is still debated. Given the higher risk observed in patients with a larger clone size (i.e., >50%), they are generally candidates to primary prophylaxis if there are no contraindications. Prophylaxis may be then discontinued once complement inhibitors are started, as in the case described (47). With regard to secondary prophylaxis in patients on complement inhibitors, some experts discontinue anticoagulants when Intravascular hemolysis (IVH) is well controlled by anti-complement therapy (4, 48, 49). Finally, anti-platelet prophylaxis had been stopped in our patient when *CALR*-positive post-ET MF diagnosis was made. In fact, *JAK2*-negative ET is apparently associated with lower rates of thrombosis (50, 51); additionally, the indication to anti-thrombotic primary prophylaxis in MF is not clear-cut (52).

Another relevant clinical issue in MPN-PNH patients may be the infectious risk due to the treatment with anti-complement therapy and the known infectious diathesis observed in MPN (53). Despite the known risk of capsulated bacterial infections under Ci (54), no infectious complications occurred in our patient and in only one out of 23 patients (4%) in the literature (18).

With regard to therapy, in our patient, eculizumab showed effectiveness in controlling IVH but was not able to resolve transfusion-dependent anemia. Accordingly, the review of the literature showed that only a fraction of MPN-PNH patients responded to eculizumab (**Table 2**) (18, 25). In classic PNH, persistent anemia under Ci treatment can be caused by residual IVH, concomitant BMF, and EVH. The management of the latter still remains an unmet need, but promising results are coming from clinical trials with proximal complement inhibitors that avoid deposition of C3 on RBC surface (55).

Many speculations can be made regarding the pathogenic significance of the association of MPN and PNH clones. The evidence of MPN driver mutations, particularly *JAK2*, selectively in GPI-deficient cells (18, 21) has raised the hypothesis that they may confer an intrinsic growth advantage to PNH cells. This cooperative effect has also been proposed for other somatic mutations, such as *TET2* (33, 35, 56, 57), also present in our patient. This view would provide a different explanation to the more common notion that PNH cells have an extrinsic growth advantage secondary to an autoimmune, GPI-selective process against bone marrow precursors (58, 59). Interestingly, MPN diagnosis preceded that of PNH in more than half of cases, and Shen et al. (33) demonstrated that *PIGA* mutation was subclonal to *JAK2*-mutated clone in their report. It is tempting to speculate that the MPN-associated inflammatory microenvironment (60–63) can impair normal hematopoiesis, exerting an "immune pressure" that favors PNH clone expansion, similarly to what happens in AA.

In conclusion, the association of MPN and PNH deserves attention because of the unpredictable clinical course often affected by dramatic thrombotic complications. Since PNH diagnosis is based on highly sensitive and cost-effective techniques, testing for PNH clones should be prompted in any MPN patient showing unexplained anemia with/without frank hemolysis, recurrent thrombosis, and/or atypical BM morphologic findings. Anti-complement treatment in the setting of MPN relies on a careful case-by-case evaluation, weighing the contribution of intravascular hemolysis to anemia and the thrombotic risk.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary files. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comitato Etico Milano Area 2. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JG, BF, and WB followed the patient, described his clinical history, and revised the literature and the paper for intellectual content. All authors contributed to the article and approved the submitted version.

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CASE REPORT
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Case Report: Evolution of a Severe Vascular Refractory Form of ECD Requiring Liver Transplantation Correlated With the Change in the Monocyte Subset Analysis

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Erdheim–Chester disease is a rare histiocytosis characterized by iconic features associated with compatible histology. Most patients have somatic mutations in the MAP-kinase pathway gene, and the mutations occur in $CD14^+$ monocytes. Differentiation of the myeloid lineage plays a central role in the pathogenesis of histiocytosis. Monocytes are myeloid-derived white blood cells, divided into three subsets, but only the $CD14^{++}CD16^-$ "classical monocyte" can differentiate into dendritic cells and tissue macrophages. Since most mutations occur in $CD14^+$ cells and since ECD patients have a particular monocytic phenotype resembling CMML, we studied the correlation between disease activity and monocytic subset distribution during the course of a severe vascular form of ECD requiring liver transplantation. During early follow-up, increased $CD14^{++}CD16^-$ "classical monocyte" associated with decreased $CD14^{low}CD16^{++}$ "non-classical monocyte" correlated with disease activity. Further studies are needed to confirm the use of monocyte as a marker of disease activity in patients with ECD.

Keywords: monocyte, histiocytosis, Erdheim–Chester disease, transplantation, vascular diagnosis

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HIGHLIGHTS

- This case report raises the question of whether an increase in $CD14^{++}CD16^{-}$ "classical monocytes" associated with a decrease in $CD14^{low}CD16^{++}$ "non-classical monocytes" correlates with ECD activity.
- Monocyte immunophenotyping could be a simple and reproductible tool to assess disease activity in ECD patients.
- Monocyte immunophenotyping can be repeated frequently unlike metabolic evaluation

INTRODUCTION

Monocytes are myeloid-derived white blood cells divided into three subsets (classical, intermediate, non-classical) based on the level of expression of surface chemokines (1) (CD14 and CD16). The functions of the three subsets (classical, intermediate, and non-classical) are different, but only the classical monocyte can differentiate into dendritic cells and tissue macrophages (2, 3).

The involvment of myeloid lineage differentiation, particularly monocytes, has been highlighted in neoplasia and plays a central role in the genesis of histiocytoses (4). Histiocytoses are orphan diseases characterized by the proliferation of dendritic cells and various monocyte-macrophage (histiocytic) cell types infiltrating tissues and causing organ damage (5). Among the histiocytoses, Erdheim–Chester disease (ECD) is a rare clonal histiocytosis characterized by iconic features (long bone, retroperitoneal, and vascular involvement) associated with compatible histology (CD68+, CD1a−, S100− histiocyte infiltration with various degrees of fibrosis) (6). In most biopsies, histiocytes express a phosphorylated extracellular signal-regulated kinase (p-Erk) testifying mitogen-activated protein kinase (MAP-kinase) pathway gene activation (7, 8). This activation of p-ERK is the final nuclear traduction of somatic mutations on the MAP-kinase pathway genes present in most ECD patients (6). Few other patients harbor a mutation in *PI3K/AKT/mtor* pathway, and unmutated patients represent <15% of ECD patients (9). Mutations in the MAP-kinase pathway genes (particularly *BRAF*) occur mainly in $CD14^{++}$ $CD16^{low}$ monocytes (10, 11).

Treatment is based on conventional agents [pegylated interferon, mammalian target of rapamycin (mTOR) inhibitors], biological agents [interleukin (IL)-1 or tumor necrosis factor (TNF)-alpha inhibitors], or targeted therapies (*BRAF/MEK* inhibitors) depending on staging and molecular status (6). Staging evaluation is based on metabolic response using PRECIST criteria (12) by analogy to solid tumors. However, these assessments, repeated every 6 months or every year, are not accurate in deciphering the specific activity of ECD in relation to infectious or other inflammatory processes.

To date, no biological marker of the disease activity has been so far validated.

Papo et al. recently demonstrated that a particular monocyte phenotype, resembling chronic myelomonocytic leukemia (CMML), was found in ECD patients (13). Moreover, $CD14^{++}$ $CD16^{low}$ monocytes play a central role in ECD pathogenesis, as they carry somatic mutations in most cases (10, 11). Based on these points, our case report presents (14) a correlation between clinical activity and monocytes subset distribution during the follow-up of a patient presenting a severe vascular ECD requiring liver transplantation.

CASE DESCRIPTION

We report a case of a patient with a severe vascular form of ECD requiring liver transplantation for whom a correlation between monocytes subset distribution and disease activity was shown.

METHODS

Histology was performed on 4-µm thick tissue sections after staining with hematoxylin & eosin and immunohistochemistry, including at least CD1a, S100, and (CD68, CD163) primary antibodies (7). Tumor DNA was extracted from formalin-fixed and paraffin-embedded tissues. Detection of MAP-kinase pathway genes' mutations was performed using targeted next-generation sequencing. Samples were analyzed using MiSeq (Illumina) after preparing the Custom Amlicon Low Input Kit libraries. The targeting genes were *AKT1*, *ALK*, *ARAF*, *ASXL1*, *BRAF*, *CALR*, *CBL*, *CDK4*, *CDKN1B*, *CDKN2A*, *CEBPA*, *CSF3R*, *CTNNB1*, *DNMT3A*, *EGFR*, *EZH2*, *FLT3*, *GATA2*, *GNA11*, *GNAQ*, *GNAS*, *HERC1*, *HRAS*, *IDH1*, *IDH2*, *JAK2*, *JAK3*, *KIT*, *KRAS*, *KTM2D*, *MAML3*, *MAMLD1*, *MAP2K1*, *MAP2K2*, *MAP2K3*, *MAP2K4*, *MAP2K6*, *MAP3K1*, *MAP3K8*, *MAP3K9*, *MAP3K10*, *MAP3K19*, *MAP4K4*, *MAPK1*, *MAPK11*, *MAPK9*, *MPL*, *NF1*, *NOTCH1*, *NOTCH2*, *NPM1*, *NRAS*, *PDGFRA*, *PIK3CA*, *PP6C*, *PTEN*, *PTPN11*, *RAC1*, *RAF1*, *RIT1*, *RUNX1*, *SETBP1*, *SRSF2*, *STAG2*, *STK19*, *SYNGAP1*, *TAOK1*, *TAOK2*, *TET2*, *TP53*, *U2AF1*, *WT1*, and *ZRSR2* as described by Melloul et al. (15)

The gene panel analyzed by next-generation sequencing (NGS) on bone marrow were *ASXL1*, *BCOR*, *BCORL1*, *CALR*, *CBL*, *CSF3R*, *DNMT3A*, *ETV6*, *EZH2*, *FLT3, GATA2, IDH1, IDH2*, *JAK2*, *KIT*, *KRAS*, *MPL*, *NIPBL1*, *NPM1*, *NRAS*, *PHF6*, *PTPN11*, *RAD21*, *RIT1*, *RUNX1*, *SETBP1*, *SF3B1*, *SMC1A*, *SMC3*, *SRSF2*, *STAG2*, *TET2*, *TP53*, *U2AF1*, *WT1*, and *ZRSR2*. Variant allele frequency (VAF) was significant over a threshold of 1%. Variants between 0% and 1% were confirmed with another NGS technology with a library preparation using the Haloplex Target Enrichment System (Agilent Technologies) and run on MiSeq (Illumina). The variant interpretation was performed according to their absence in public databases of polymorphisms (especially GnomAD) and their status in our "in-house" database of more than 8,000 samples validated (including AML samples from the Acute Leukemia French Association and MDS samples from the Groupe Français des Myélodysplasies)

Whole-blood samples were stained with dot plot CD45-KO (clone J33) to isolate the monocyte population. The three subsets of monocytes are gated with the combination CD16/CD14.

RESULTS

A 25-year-old woman was hospitalized for progressive abdominal pain. Regarding her medical history, Budd–Chiari syndrome was diagnosed at the age of 14 years, which led to long-term treatment with vitamin K antagonists. During this episode, the search for hereditary thrombophilia (factor V Leiden mutation, prothrombin gene mutation, protein C and S deficiency, antithrombin-III deficiency) or acquired thrombophilia (antiphospholipid syndrome, paroxysmal nocturnal hemoglobinuria) was negative. The search for *JAK2* mutation was negative. Bone marrow examination was not performed because the cells in the blood count were normal.

The current medical history started with recurrent abdominal pain responsible for weight loss (13 kg in 4 months). The physical examination was unremarkable. Biological tests showed an inflammatory syndrome (C-reactive protein, 300 mg/L). CT scan showed mesenteric ischemia secondary to the superior mesenteric artery sheath and celiac trunk (**Figure 1**) associated with bilateral nephromegaly. The patient underwent thrombectomy of the superior mesenteric artery associated with a secondary stent graft with successful reimplantation on the abdominal aorta associated with heparin-anticoagulation therapy. Despite the anticoagulation treatment, she presented with extensive digestive ischemia that required partial surgical removal of the jejunum. Investigations for hereditary or acquired thrombophilia were again negative. Mesentery artery biopsy showed normal intima and media, without atherosclerosis or vasculitis. The arterial lumen was normal without any sign of recent or ancient thrombosis. The adventitia contained abundant fibrosis with a mild leukocyte infiltration including lymphocyte and histiocyte but only rare plasma cells. The immunohistochemical analysis showed $CD68^{+}$, $CD1a^{-}$ histiocytes with strong phospho-ERK expression (**Figure 1**). NGS analysis on the biopsy tissue showed no mutation in the MAP-kinase pathway gene. Extensive analysis of the bone marrow showed no dysplasia and no reported mutations in clonal hematopoiesis. Thus, there was no evidence of hematological malignancy, solid tumor (on body CT scan), and infectious or rheumatological disease (connective tissue disease, vasculitis).

Technetium bone scan showed radiotracer uptake by long bones highly suggestive of ECD (**Figure 1**).

In the early postoperative period, the patient received an interleukin-1 receptor antagonist (Anakinra 100 mg/day) for 2

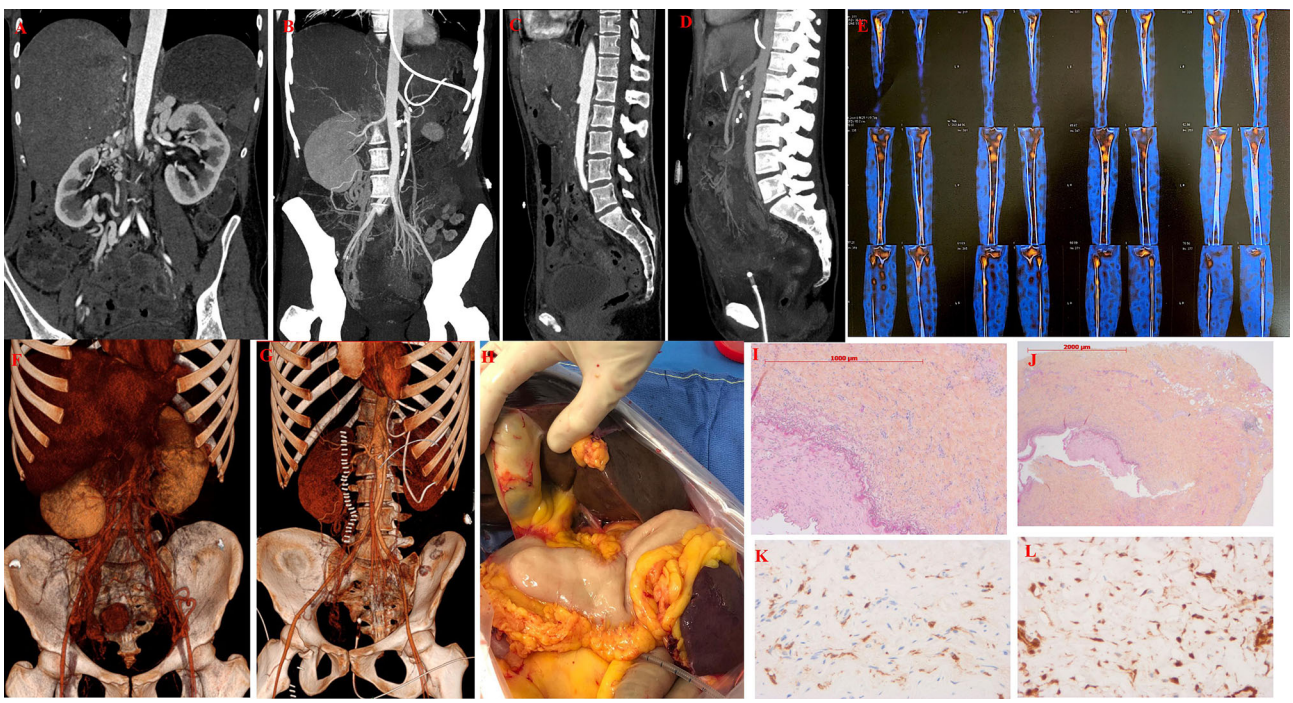

FIGURE 1 | Clinical, radiological, and histological features of Erdheim–Chester Disease. **(A)** Sagittal computed tomography of the patient showing severe stenosis of superior mesenteric artery responsible for mesenteric ischemia in a patient with ECD. **(B)** Sagittal computed topography of the patient after thrombectomy and stenting of superior mesentery artery. **(C)** Coronal view of severe stenosis of superior mesenteric artery responsible for mesenteric ischemia in a patient with ECD. **(D)** Coronal view after thrombectomy and stenting of superior mesentery artery. **(E)** Bone scintigraphy showing radiotracer uptake on long bones characteristic of ECD. **(F)** Maximum intensity sagittal and angio3D projection showing vascular stenosis of coeliac trunk and superior mesentery artery. **(G)** Maximum intensity sagittal and angio3D projection demonstrates extensive digestive. **(H)** Maximum intensity sagittal and angio3D projection after digestive removal and vascular stenting. **(I)** Tissue biopsy of mesentery artery showing fibrosis of vessels adventitia without signs of vasculitis (×100). **(J)** Same sample showing adventitia fibrosis without signs of vasculitis (×200), **(K)** Same samples showing tissue infiltration with multinucleated histiocytes with CD163 (brown staining) expression on immunostaining.(HES; immunohistochemistry, ×200) consistent with ECD. **(L)** Same samples showing tissue infiltration with multinucleated histiocytes expressing phosphor-Erk (brown staining) HES; immunohistochemistry, (200×).

weeks, followed by an *MEK* inhibitor (Cobimetinib) 40 mg/day (20 mg twice daily, for 21 days of a 28-day cycle). Cobimetinib induced partial metabolic remission with decreased radiotracer uptake by long bones on ^{18}fluorodeoxyglucose PET-CT. After Cobimetinib induced partial metabolic remission, monocyte subset analyses showed 92.9% "classical" MO1 monocytes ($CD14^{++}CD16^{-}$), 5.7% "intermediate" MO2 monocytes ($CD14^{+}CD16^{+}$), and 1.2% "non-classical" MO3 monocytes ($CD14^{low}CD16^{+}$). The total white blood cell count was 7.5 G/L. The monocyte count was 0.62 G/L or 7% of the total white blood cell count. Unfortunately, the patient developed a new celiac trunk thrombosis causing acute liver failure that required an emergency liver transplant. Prevention of graft rejection included high-dose steroids (500 mg/day in pulses for 3 days and then 1 mg/kg/day followed by a gradual decrease in steroids), a calcineurin inhibitor (tacrolimus, 0.2 mg/kg/day), mycophenolate mofetil (2 g/day), and basiliximab (20 mg/day for 4 days after transplantation). Cobimetinib was stopped after transplantation. One month later, despite immunosuppressive agents and curative anticoagulant therapy, the patient developed new thrombotic events and immunosuppressant-related infections and died. During this ECD flare, the blood cell count reached a total of 8.87 G/L, with a monocyte count of 0.33 G/L (3.7% of total white blood cells). Monocyte subset analyses showed an increase in "classical" monocytes (97.5%) and a decrease in "nonclassical" monocytes (1%) with 0.7% "intermediate" monocytes (**Figure 2**). The entire clinical course of the patent is reported in **Figure 3**.

DISCUSSION

We present a case report evaluating the correlation between monocyte subset and disease activity in the early follow-up of

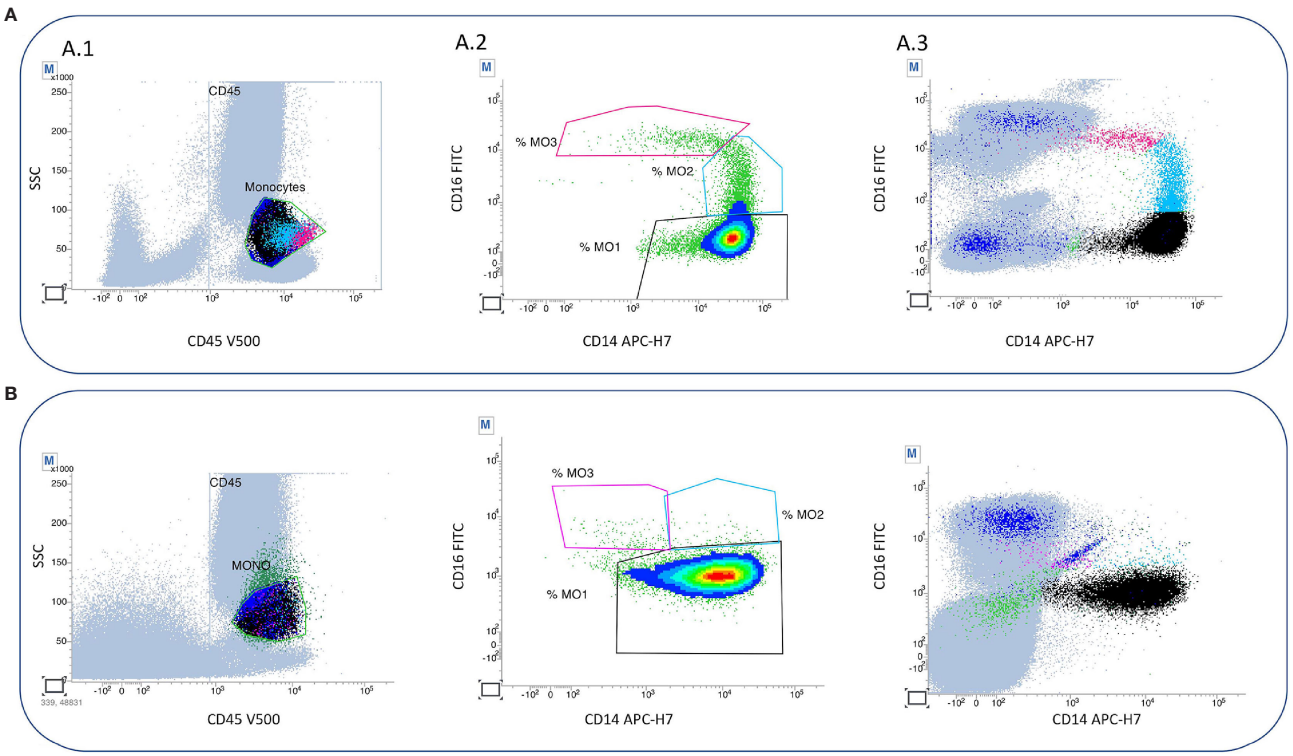

FIGURE 2 | Flow cytometry analysis of monocyte subset on peripheral blood samples of a patient with ECD. **(A)** Flow cytometry analysis of monocyte subset in ECD remission. A.1 Monocyte population (green) is gated in dot plot SSC/CD45, and the zone is purified residual population (blue) by exclusion of T and NK lymphocytes with expression of CD7, granular cells with expression of $CD16^{+}$ and $CD14^{+}$) and B lymphocytes without $CD16^{-}$ but with expression of $CD24^{+}$. A.2 The three subtypes of monocytes are gated with the combination CD16/CD14: classical monocytes are MO1 (black) $CD16^{-}CD14^{+}$ representing 92.9% of total monocytes. Intermediate monocytes are MO2 (blue) $CD16^{+}$ $CD14^{+low}$ and represent 5.7% of total monocyte. Non-classical monocytes are MO3 (pink) expressing $CD16^{+}$ without CD14 and representing 1.2% of total monocytes. A.3 In order to define the subtype, the gate is positioned with the help of all cells. **(B)** Flow cytometry analyses of monocyte subset in ECD flare The three monocytes subsets are gated with the combination CD16/CD14: classical monocytes are MO1 ((black) $CD16^{-}$ $CD14^{+}$ representing 97.5% of total monocytes. Intermediate monocytes are MO2 (blue) $CD16^{+}$ $CD14^{+low}$ representing 0.7% of total monocytes. Non classical monocytes are MO3 (pink) $CD16^{+}$ $CD14^{-}$ representing 1% of total monocytes. $CD45^{V500}$, $CD7^{V450}$, $CD16^{FITC}$, $CD24^{PE}$, $CD14^{APC\text{-}H7}$, and $CD56^{PC7}$. BC, Beckman Coulter Immunotech, Miami, FL, USA; BD, BD Biosciences, San Jose, CA, USA; V500, Horizon V500; APC-H7, allophycocyanin-H7; PC7, phycoerythrin-cyanin-7; PE, phycoerythrin; V450, Horizon V450; FITC, fluorescein isothiocyanate. Peripheral blood mononuclear cells (PBMC) were stained with CD15, CD 14, CD13 PE-CF594, CD33, CD34, and CD45 KO, and monocytes subsets were sorted. PBMC were centrifugated on a microscope slide, dried at room temperature for 30 min, and stained using May–Gruenwald–Giemsa stain. Monocyte population is gated in dot plot SSC/CD45, and the zone is purified of residual population by exclusion of T and NK lymphocytes with expression of CD7, granular cells with expression of $CD16^{+}$ and $CD14^{+}$ and B lymphocytes without $CD16^{-}$ but with expression of $CD24^{+}$.

ECD. Papo et al. studied the distribution of monocyte subsets in patients with ECD without monocytosis (13). Patients with active ECD had an increase in "classical" monocytes and a decrease in "non-classical" monocytes compared with treated patients (considered to have controlled disease). This distribution resembles that of patients with CMML (16).

This distribution seems logical since most mutations in the MAP-kinase pathway genes occur in $CD14^+$ monocytes (10, 11). Based on this consideration, we believe that uncontrolled expansion of $CD14^+$ cells could be a cornerstone of the pathogenesis of histiocytosis. Even though mutated $CD14^+$ monocytes represent a marginal part of blood cells, their expansion leads to classical monocytes and to the production of dendritic cells and homing tissue macrophages in histiocytic disorders. Our result regarding the level of classical monocytes in controlled ECD is comparable to the study of Papo et al. (13). Furthermore, our work reports the repeat analysis of monocyte subsets in a patient with ECD, and the expansion of classical monocytes appears to be associated with disease activity. $CD14^{++}CD16^{low}$ monocyte expansion was primarily assessed in CMML when the monocyte count was >1 G/L (16). CMML is a hybrid myelodysplastic/myeloproliferative disease that may overlap with histiocytosis (17, 18) and whom sometimes involve the MAP-kinase pathway (19, 20). By analogy with the CMML flare in proliferative patients, we believe that the expansion of $CD14^+$ monocytes is associated with the flare in ECD, independent of the monocytes count in histiocytosis.

It should be noted that non-classical monocytes ($CD14^{low}$, $CD16^{++}$) are involved in the wound healing process (21) and promote endothelial adhesion of neutrophils *via* TNF-alpha secretion (22). In patients with atherosclerosis, their decrease correlates with coronary plaque progression (23). Our case calls into question the role of "non-classical" monocytes as potential markers of vascular disease because their decrease was associated with thrombosis.

This case also reports an aggressive vascular presentation of ECD that led to organ transplantation at an early age. ECD usually occurs in middle-aged patients (5, 6, 9), but pediatric patients have been reported previously (24). The current observation is that ECD is not a disease of the heart. In the current observation, Budd–Chiari syndrome at 14 years of age may be the first vascular event related to ECD. ECD should be sought in unusual site thrombotic events, including Budd–Chiari syndrome. On the other hand, transplantation has been reported only twice in patients with ECD-related organ failure (25, 26). It was associated with favorable outcomes in patients with ECD-related organ failure. It was associated with favorable outcomes in both cases, but the ECD was in remission at the time of transplantation, which was not the case in our patient. Neither immunosuppressive drugs (calcineurin inhibitors, mycophenolate mofetil) nor *MEK* inhibitor induced remission of ECD in this patient. The fact that the disease is not controlled by a *MEK* inhibitor in non-mutated ECD patients calls into question another dominant activation pathway rather than MAP-kinase activation (possibly PI3K/AKT/mTOR) (27). Other pathways have recently been described in patients with histiocytosis, (28, 29) suggesting new therapeutic targets in refractory patients. Inhibition of *RANKL* may be a promising therapeutic approach because *RANKL* is expressed in active ECD lesions along with p56 *NF-kB* activation (29, 30). *CSF1-R* inhibitor can also suppress dendritic cell differentiation and migration in histiocytosis (31). This approach may decrease the local tumor infiltrate and may reduce disease activity in refractory patients.

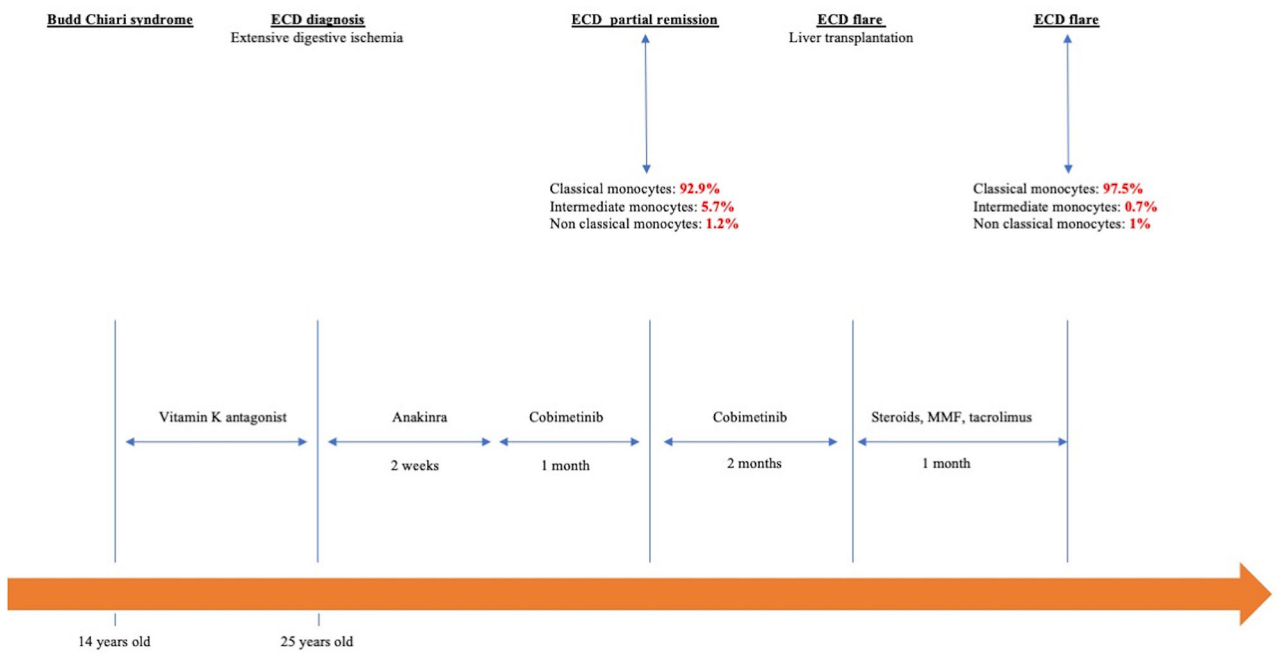

FIGURE 3 | Chronological history of the patient from Budd–Chiari syndrome to ECD.

Treatments may interfere with monocyte assays; patient-administered immunosuppressive drugs (calcineurin inhibitors, mycophenolate mofetil) have no effect on monocyte subsets (32), but steroids in renal transplant patients may increase the ratio of classical monocytes and decrease that of non-classical monocytes (32). Indeed, the subset distribution results may have been biased by steroid use. However, in the cohort of transplanted patients, the change in monocyte distribution was not associated with clinical events. The change in the classical/non-classical monocyte ratio resembles what is observed in uncontrolled vascular disease. From our point of view, we cannot exclude a unfavorable role of steroid use in patients with ECD.

The main strength of our presentation is the use of monocyte subset analysis in two consecutive samples from an ECD patient, which shows an increase in classical monocytes correlated with the flare-up. This presentation may provide reassurance of the central role of monocytes in the pathophysiology of histiocytosis. The main limitation of the study is a case report, and the results need to be validated in a prospective cohort of patients. Thus, the functional role of monocyte subsets in histiocytosis is not fully elucidated, and basic science studies are mandatory to compare monocyte polarization to major pathologies described in the literature and broadly explore their pivotal role in histiocytosis.

Nevertheless, this bioassay may be useful in specific situations where ^{18}FDG PET may be misleading (infection, postoperative period). Physicians may repeat it more frequently than ^{18}FDG PET in patients with ECD.

In conclusion, this case supports the hypothesis that monocyte subset analysis can be a simple tool to assess ECD activity. However, further studies are needed to confirm this hypothesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

JR collected the data and wrote the initial draft. J-FE confirmed histological features of ECD. AR and FG-O performed the cytometry analysis. All authors participated in the primary care of the patient and provided clinical, radiographic, and histological data of the patients. All authors contributed to the article and approved the submitted version.

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REVIEW
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Immune Dysregulation and Infectious Complications in MPN Patients Treated With JAK Inhibitors

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BCR-ABL1-negative myeloproliferative neoplasms are burdened by a reduced life expectancy mostly due to an increased risk of thrombo-hemorrhagic events, fibrotic progression/leukemic evolution, and infectious complications. In these clonal myeloid malignancies, *JAK2*V617F is the main driver mutation, leading to an aberrant activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Therefore, its inhibition represents an attractive therapeutic strategy for these disorders. Several JAK inhibitors have entered clinical trials, including ruxolitinib, the first JAK1/2 inhibitor to become commercially available for the treatment of myelofibrosis and polycythemia vera. Due to interference with the JAK-STAT pathway, JAK inhibitors affect several components of the innate and adaptive immune systems such as dendritic cells, natural killer cells, T helper cells, and regulatory T cells. Therefore, even though the clinical use of these drugs in MPN patients has led to a dramatic improvement of symptoms control, organ involvement, and quality of life, JAK inhibitors–related loss of function in JAK-STAT signaling pathway can be a cause of different adverse events, including those related to a condition of immune suppression or deficiency. This review article will provide a comprehensive overview of the current knowledge on JAK inhibitors' effects on immune cells as well as their clinical consequences, particularly with regards to infectious complications.

Keywords: myeloproliferative neoplasms, JAK inhibitors, ruxolitinib, infections, immune system

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1 INTRODUCTION

The *BCR-ABL1*-negative myeloproliferative neoplasms (MPNs) are a heterogenous group of clonal disorders of the hematopoietic stem cell, mainly characterized by hyperproliferative bone marrow with varying degrees of reticulin/collagen fibrosis, extramedullary hematopoiesis, abnormal peripheral blood counts, and constitutional symptoms. They include polycythemia vera (PV), essential thrombocythemia (ET), and myelofibrosis (MF). The latter may present as a primary disorder (PMF) or evolve from another pre-existing *BCR-ABL1*-negative MPN, such as PV or ET, globally identified as secondary MF (SMF) (1).

Somatic mutations in MPNs are classified into "driver" and "other" mutations; the former include *JAK2*, *CALR*, and *MPL*; and the latter, *ASXL1*, *EZH2*, *IDH1/2*, *SRSF2* and *U2AF1*,

among others (2–4). It is generally believed that driver mutations are essential for MPN phenotype, whereas the "other" mutations might contribute to fibrotic progression and leukemic evolution (5, 6).

*JAK2*V617F is the main driver mutation in MPNs, leading to an aberrant activation of the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway. Therefore, its inhibition represents an attractive therapeutic strategy for these disorders.

Numerous JAK inhibitors have entered clinical trials, including ruxolitinib, the first JAK1/2 inhibitor to become commercially available for the treatment of MPNs. Indeed, it was initially approved in both the US and Europe for the treatment of splenomegaly and/or constitutional symptoms in MF patients (7, 8); subsequently, it was also licensed for PV subjects with an inadequate response to or an unacceptable toxicity from hydroxyurea (HU) (9, 10).

Despite its efficacy, ruxolitinib-related loss of function in JAK-STAT signaling pathway can be a cause of different adverse events (AEs), including those related to a condition of immune suppression or deficiency. Accordingly, the increased risk of infections already inherent to even untreated MPNs is further augmented due to the immunomodulatory and immunosuppressive effects of JAK inhibitors.

This review article will provide a comprehensive overview of the current knowledge on JAK inhibitors' effects on immune cells, as well as their clinical consequences particularly with regards to infectious complications.

2 IMMUNOSUPPRESSIVE ACTIVITY OF JAK INHIBITORS

The JAK-STAT pathway—based on four non-receptor protein tyrosine kinases, JAK1, JAK2, JAK3, and Tyk2, and seven STAT proteins—regulates proliferation, differentiation, and survival of a variety of cells and is crucially relevant for hematopoiesis as well as for immune cell development and function (11–14). As an example, JAK1 is involved in type I IFNs, IFN-γ, IL-2, IL-7, IL-15, and IL-21 signaling; it also cooperates with Tyk2 for type I IFN and with JAK2 for IFN-γ signal transduction (15–17). JAK2 is activated by several cytokines and growth factors including erythropoietin, thrombopoietin, IL-3, IL-6, G-CSF, and IFN-γ (18, 19), while JAK3 is associated with the Υc chain family of cytokines (20) and Tyk2 is triggered by cytokines such as type I IFNs, IL-6, IL-10, and the IL-12 and IL-23 families (21).

Due to interference with the JAK-STAT pathway, JAK inhibitors affect several components of the innate and adaptive immune systems such as dendritic cells (DCs), natural killer cells (NKs), T helper cells, and regulatory T cells (Tregs), thus resulting in a significant immunosuppressive activity (**Table 1**) (27).

2.1 Dendritic Cells

Dendritic cells are crucial antigen-presenting and phagocytic cells responsible for presenting antigens to T lymphocytes, initiating therefore the process of adaptive immunity (28, 29). The pivotal study by Heine et al. on ruxolitinib-induced alterations of DCs function laid the preliminary bases for understanding the increased infection rate recorded in MPN patients treated with this JAK inhibitor as well as its anti-inflammatory and immunomodulating activity. The *in vitro* development of human monocyte-derived DCs was almost completely blocked, and DCs activation was inhibited as shown by decreased IL-12 production. Dendritic cell migration both *in vitro* and *in vivo* in mice was also reduced, resulting in impaired T-cell activation (22).

The impact of ruxolitinib on DCs migration and the identification of target molecules mediating this effect were further investigated by means of an *ex vivo* assay. Dendritic cell migration turned out to be heavily depressed *via* interference with Rho-associated coiled-coil kinase (ROCK) that controls non-muscle myosin activity regulating reorganization and contraction of cellular actin–myosin filaments. This DCs loss of mobility may lead to a reduced T cell activation in draining lymph nodes and might explain the increased number of these proinflammatory blood cells in ruxolitinib-treated MPN patients (30).

Similar to ruxolitinib, momelotinib impacts on DCs' functions. In a mouse model of atopic dermatitis, besides inhibiting mRNA expression of IL-4, IL-5, IFN-γ and STAT1, STAT3 and STAT5 phosphorylation in topically treated skin

TABLE 1 | Immune system components and JAK inhibitors' effects.

	Physiological function	JAK inhibitors' effects	References
Dendritic cells (DCs)	Antigen-presenting/phagocytic cells responsible for presenting antigens to T lymphocytes	Ruxolitinib: inhibition of DCs development, activation, and migration Momelotinib: reduced CD80/CD86 expression involved in T-cell activation, expansion, and differentiation	(22) (23)
Natural Killer cells (NKs)	Cytotoxic lymphocytes that play a critical role in antiviral and antitumor responses	Ruxolitinib: impaired maturation as reflected by an increased ratio in immature to mature NKs; reduced capacity to form lytic synapses with NK target cells	(24)
CD4+ T cells	Heterogeneous cell population involved in adaptive immunity, inflammatory response, and protection against a wide range of both intracellular and extracellular pathogens	Ruxolitinib: severe, long-lasting reduction of circulating regulatory T cells not reversible upon drug reduction or withdrawal Ruxolitinib/fedratinib: "silence" CD4+ T cells and decrease cytokine secretion; polarize the immune profile toward a "Th17 type" response	(25) (26)

lesions, momelotinib reduced in activated DCs *in vitro* the expression of the co-stimulatory molecules CD80 and CD86 involved in T-cell activation, expansion, and differentiation (23). On the contrary, in a more recent paper, another JAK inhibitor, pacritinib, exhibited only a mild suppressive effect on DCs. Pacritinib, at concentrations reflecting patients' plasma levels, reduced IL-12 secretion, whereas IL-6 and TNF-α levels were unchanged, thus producing an immunosuppressive effect on DCs significantly less pronounced than that of ruxolitinib (31).

2.2 Natural Killer Cells

Natural Killer cells are cytotoxic lymphocytes of the innate immune system that play a critical role in antiviral and antitumor responses. The coordinated action of multiple cytokines is crucial for NKs development and maturation, and many of these cytokines such as IL-2, IL-7, IL-12, IL-15, IL-21, IL-27, and IFNs signal *via* the JAK-STAT pathway (32). The effects of JAK inhibition on human NKs was extensively investigated in a study comparing 28 MPN patients with or without ruxolitinib treatment and 24 healthy subjects. In ruxolitinib-treated subjects, a decrease in NKs number associated with clinically relevant infections mostly of viral origin was recorded likely due to impaired maturation as reflected by an increased ratio in immature to mature NKs. Also, the endogenous defect in NKs function of MPN patients was further worsened by ruxolitinib. These *in vivo* findings were supported by *in vitro* data showing that the cytokine-mediated NKs activation was inhibited by ruxolitinib as suggested by a reduced expression of NKs activation markers such as CD16, CD69, NKG2D, NKp46, and granzyme B. A diminished killing activity was also reported due to an impaired capacity to form lytic synapses with NK target cells. The *in vitro* ruxolitinib effects on NKs' function were restored upon drug removal, indicating reversibility of this action (24).

The impact of ruxolitinib and fedratinib, a JAK2-specific inhibitor, on NKs activation and function were then compared *in vitro* using γc cytokines and human DCs subtypes. While ruxolitinib completely blocked IL-2, IL-15, and DC-mediated STAT5 phosphorylation, along with the capacity of NKs to secrete IFNγ and lyse NK-sensitive targets, fedratinib inhibited only soluble IL-15-mediated STAT5 phosphorylation, which Langerhans-type DCs, presenting membrane-bound IL-15 *in trans*, could salvage, demonstrating that a selective JAK2 inhibitor better preserves NKs activity (33).

Ruxolitinib effect on NKs was evaluated also in the context of host immune response against gene therapy viral vectors by means of a co-culture system with human NKs line, macrophages, and airway epithelial cells. The increased IFN-γ cytokine expression induced by NKs co-cultured with helper-dependent adenoviral (HD-Ad) vector-activated macrophages as well as the kill of HD-Ad vector-transduced bronchial epithelial cells by activated NKs were both significantly reduced by ruxolitinib due to a block of IL-12 and IL-15 production (34).

2.3 CD4+ T Cells

CD4+ T cells, a heterogeneous cell population differentiating into multiple effector subsets such as T-helper and Tregs, play a central role in adaptive immunity, mediate inflammatory response, and protect against a wide range of both intracellular and extracellular pathogens by releasing cytokines and chemokines that induce and/or recruit target cells (35, 36). The effect of JAK inhibition on CD4+ T cells was firstly investigated by Massa et al. on 18 MF patients: the administration of ruxolitinib resulted in a severe, long-lasting reduction of circulating Tregs not reversible upon drug reduction or withdrawal. It was suggested that decreased levels of Tregs by disrupting the immune response might explain the increased frequency of infections, such as tuberculosis, Herpes zoster, and pneumonia of viral origin reported in ruxolitinib-treated MF patients (25). Similar results were obtained in a study on nine MPN patients. After 3 weeks of ruxolitinib treatment, a decrease in total CD3+ cells, number of Tregs, Th1, and Th17 was observed; moreover, in T cells isolated from these patients, TNF-α, IL-5, IL-6, and IL-1B production was downregulated (37).

Frequency and function of CD4+ T cell subsets at baseline and during treatment with either ruxolitinib or fedratinib were further investigated in 50 MPN subjects. At baseline, Tregs in MPN patients were significantly lower than in 23 healthy controls, and all subgroups of Tregs as defined by CD45RA expression were further decreased by JAK inhibitors with no targeting of specific subpopulations. After 6 months of treatment in responsive subjects, a significant increase in Th17 cells compared to baseline and an expansion of "dual positive" IFNγ+/IL-17+ cells were recorded, suggesting a polarization from a "Th1" to a "Th17" phenotype. A functional "silencing" of T helper cells both *in vivo* and *in vitro* and a significant decrease of pro-inflammatory cytokines secretion by CD4+ T cells were also observed, thus representing a possible explanation for the increased rate of atypical infections reported in JAK inhibitors-treated subjects. It was suggested that JAK inhibitors may display a dual effect on number and function of CD4+ T cells, the early one being to "silence" CD4+ T cells and decrease cytokine secretion and the long-term one being to polarize the immune profile toward a "Th17 type" response (26).

The role of JAK inhibition specificity on T-cell proliferation and function was also investigated by evaluating the effects of ruxolitinib and momelotinib as JAK1/2 inhibitors and of BSK805 as selective JAK2 inhibitor. In T-cells derived from ruxolitinib-treated MPN patients, CD69 expression and proliferative capacity were almost abrogated in CD8+ and significantly impaired in CD4+ T cells, confirming that JAK1/2 inhibitors significantly decrease T-cell reactivity and proliferation *in vivo*. Ruxolitinib, together with momelotinib and BSK805, was also assessed *in vitro* on healthy donors T cells. In a mixed lymphocyte culture assay, while JAK1/2 inhibition significantly decreased T-cell reactivity, JAK2 specific blockage did not have any inhibitory effect, indicating that the T-cell function impairment is strictly dependent upon JAK1 inactivation (38).

3 JAK INHIBITORS-ASSOCIATED INFECTIOUS COMPLICATIONS IN MPN PATIENTS

Myeloproliferative neoplasms are burdened by a reduced life expectancy mostly due to an increased risk of thrombo-

hemorrhagic events, fibrotic progression/leukemic evolution, and infections. Indeed, an intrinsic MPN propensity to infectious complications was firstly suggested by Swedish investigations performed before JAK inhibitors' introduction. In a cohort of 9.285 MPN subjects, the 10-year probability of death from infections was 4.6% in PV, 2.5% in ET, and 10.4% in PMF compared to 2.3% in 35.769 matched controls (39). In a further study including 8.363 MPN cases and 32.405 controls, the hazard ratio (HR) of any infection was 2.0. According to MPN subtypes, the HR was 3.7 in PMF, and 1.7 in both PV and ET, with no significant difference between untreated patients and subjects treated during the years 2006–2013 with HU, IFN-α, or anagrelide. During the follow-up, however, the rate of infections raised in patients subsequently treated with ruxolitinib (40).

The issue that the inherent MPN susceptibility to infections might be increased by JAK inhibitors was addressed in clinical trials, retrospective series, case reports, and reviews (**Table 2**).

3.1 Clinical Trials and Retrospective Series

3.1.1 Myelofibrosis

Due to the availability of more long-term safety reports, most of the data on JAK inhibitors-associated infections in MPNs concern MF patients. In the COMFORT-I trial, at a median 3-year follow-up, the incidence of urinary tract and Herpes zoster infections, the most common ones during randomized treatment with ruxolitinib, were not increased by long-term therapy, being respectively 10.5% in 0–12 months, 6.7% in 12–24 months, 7.7% in 24–36 months, 6.0% after ≥36 months, and 2.1% in 0–12 months, 3.5% in 12–24 months, 3.4% in 24–36 months, and 0% after ≥36 months. No other opportunistic infections occurred with long-term ruxolitinib therapy (41). According to the final analysis at 5-year follow-up, while grade 1/2 Herpes zoster infections were recorded at higher rate in ruxolitinib-treated subjects, other infections, including pneumonia, sepsis, upper respiratory, and urinary tract infections, displayed similar rates between ruxolitinib- and placebo-treated patients (54). In the COMFORT-II trial, the only grade 3/4 infectious AE was pneumonia, 1% in the ruxolitinib group *vs.* 5% in the best available therapy (BAT) group, being all other infections of grade 1/2 (8). At 5-year follow-up the longer exposure to ruxolitinib did not determine a significant increase in incidence of infections (65). In the JUMP trial, a ruxolitinib single-arm expanded-access study enrolling 2.233 MF patients, all-grade infections included urinary tract infection (6.0%), pneumonia (5.3%), Herpes zoster (3.6%), influenza (3.0%), and oral Herpes (1.6%). Tuberculosis occurred in three patients and legionella pneumonia in one; no HBV reactivation was instead observed (42). The ROBUST phase II study evaluated safety and efficacy of ruxolitinib in 48 intermediate-1, intermediate-2, and high-risk MF patients. The most common infections were of the urinary tract (16.7%), lower (14.6%) and upper (10.4%) respiratory tract, and nasopharyngitis (6.3%), with no reports of Herpes zoster, HBV, or tuberculosis (43).

In a further real-life investigation, 507 MF patients, 128 treated with ruxolitinib and the remaining with cytoreductive agents, were retrospectively evaluated to investigate incidence and risk factors of infectious complications. Overall, 112 (22%) patients experienced 160 infectious events, most being bacterial (78%) and affecting mainly the respiratory tract. While the rate of infections was higher in ruxolitinib-treated subjects (44 *vs.* 20%), attention was raised on the possible impact of confounding factors such as high IPSS risk category and splenomegaly, both being the main risk factors for infections and prevailing in the JAK inhibitor cohort (55). In another series of 446 MF subjects retrospectively investigated, after a median ruxolitinib exposure of 23.5 months, 28% of patients experienced 161 infectious events, involving the respiratory tract in 50% of cases. While viral (14.9%) and fungal (2.5%) infections were also observed, bacteria were the most frequent etiological agent (68.9%). Previous infections and high IPSS risk score still correlated with higher infectious risk whereas splenomegaly reduction was associated with a decreased risk of subsequent infectious complications (56). In 70 MF patients treated with ruxolitinib according to clinical practice, after a median time of 8 months from therapy start, 12 subjects experienced 17 grade ≥2 infections, mainly bacterial, including a life-threatening tuberculosis (58).

Data on momelotinib-related infectious complications can be retrieved mainly from the phase III randomized SIMPLIFY-1 and SIMPLIFY-2 trials. In the SIMPLIFY-1 study, grade ≥3 infections occurred in 7% of cases treated with momelotinib

TABLE 2 | JAK inhibitors-associated infectious complications.

	MPN subtype	References
Urinary tract infection	MF	(41–47)
Herpes zoster	MF	(41, 42, 48, 49)
	PV	(9, 10, 50–53)
Herpes simplex reactivation	MF	(42)
Pneumonia/Upper respiratory tract infection	MF	(8, 42, 43, 48, 54–57)
Influenza	MF	(42)
Tuberculosis	MF	(42, 49, 58–61)
	PV	(61)
Hepatitis B reactivation	MF	(59, 62, 63)
	PV	(62, 64)
Pneumocystis jirovecii pneumonia	MF	(62)
	PV	(62)

MPN, myeloproliferative neoplasm; MF, myelofibrosis; PV, polycythemia vera.

and in 3% of patients who received ruxolitinib (66). In the SIMPLIFY-2 trial, grade 3 urinary infection frequencies were 2% in the momelotinib and 0% in the BAT groups (44).

As far as fedratinib is concerned, in a double-blind, randomized phase III study enrolling 289 intermediate-2 or high-risk MF subjects assigned to fedratinib at a daily dose of 400 mg or 500 mg or to placebo, infections occurred in 42 and 39% of patients, respectively, in the 400 and 500 mg groups compared with 27% in the placebo group, the urinary tract infections being the most reported AE (45). In a phase II open-label randomized study on 31 MF patients treated with fedratinib, infections of any grade were recorded in 11 (35%) patients and of grade 3/4 in six (19%) subjects, the most frequent being of the urinary tract (46). Urinary tract infections, usually of grade 1/2, were the most common infectious complication (12 cases) also in the JAKARTA-2 trial, a single-arm phase II study assessing fedratinib in 97 MF patients (47).

In the PERSIST-1 trial enrolling 327 patients with higher-risk MF randomly assigned to pacritinib or BAT, the incidence of serious opportunistic infections in the pacritinib group was low: Herpes zoster infections were reported in 1% of patients, whereas pneumonia (three cases) was among the most frequent AEs leading to death (48). Also, in a further phase III clinical trial on 311 MF subjects randomized to pacritinib 400 mg once daily, pacritinib 200 mg twice daily, or BAT, pneumonia was a serious AE with 4, 7, and 3% of cases, respectively, in the pacritinib once daily, twice daily, and BAT groups (57).

3.1.2 Polycythemia Vera

Data on ruxolitinib-associated infectious complications in PV are derived from the RESPONSE, RESPONSE-2, and RELIEF clinical trials (67). In the RESPONSE study addressed to HU-resistant or -intolerant PV patients with splenomegaly, the rate of any grade infections was 41.8% in the ruxolitinib group and 36.9% in the standard-therapy arm, while grade 3/4 infections were respectively 3.6 and 2.7%. Herpes zoster infections, all of grade 1/2, were recorded in seven (6.4%) patients in the ruxolitinib group as compared with no cases on standard therapy (9). At 80-week follow-up the rate of all infections was 29.4 per 100 patient-years of exposure in the ruxolitinib group, 27.8 in the cross-over cohort and 58.4 in the BAT arm. The rate of Herpes zoster infection was higher in patients originally randomized to ruxolitinib or treated with ruxolitinib after crossover than in BAT (50). The long-term ruxolitinib safety was then evaluated at 5 years. In the ruxolitinib arm, except Herpes zoster, which was more frequent in this group, the rate of infections was lower, 18.9 per 100 patient-years of exposure *vs.* 19.1 in the crossover population and 59.8 in the BAT cohort (51). The RESPONSE-2 study randomized to ruxolitinib or BAT PV patients resistant or intolerant to HU without splenomegaly. In the ruxolitinib group, grade 3/4 infections were reported in 3% of patients and grade 1/2 Herpes zoster infection in 1% of subjects while in the BAT cohort occurred respectively in 1% and in none of the cases (10). At week 80 the rates of all grade and grade 3/4 infections per 100 patient-years of exposure were 24.9 and 2.3, respectively, in the ruxolitinib arm *vs.* 33.7 and 3.7 in the BAT cohort. Frequency of Herpes zoster infections was higher in patients receiving ruxolitinib; in this group, however, no pneumonia or tuberculosis reactivation was reported (52). In the RELIEF trial, recruiting patients with an adequate hematocrit control on HU but still experiencing PV-related symptoms randomized to ruxolitinib or HU, the ruxolitinib safety profile was similar with that reported in the RESPONSE and RESPONSE-2 studies. Infectious complications were generally grade 1/2, with Herpes zoster infection being observed in only one patient in the ruxolitinib arm (53).

3.1.3 Unselected MPNs

In a retrospective study enrolling 202 cases treated with ruxolitinib and a control cohort of 73 ruxolitinib-naïve MPN subjects, infections usually of grade 1/2 occurred in 38.4% of ruxolitinib-naïve and 42.6% of ruxolitinib-treated patients, with upper respiratory infections, urinary tract infections, and pneumonia being the most frequent ones. Rate of Herpes zoster infection was 3.9 and 2.7%, respectively, in the ruxolitinib-treated and ruxolitinib-naïve groups. After propensity score weighting, there was no difference in risk of infection between the ruxolitinib-treated and ruxolitinib-naïve cohorts (68).

3.2 Reviews, Case Reports, and Registries Database

Being the JAK inhibitor with the longest time since approval, both exhaustive systematic reviews (27, 62) and numerous case reports have been published on ruxolitinib-associated infectious complications. Together with a review of the literature, Lussana et al. also performed a meta-analysis of interventional phase III studies on MF and PV patients. In the PV trials RESPONSE, RESPONSE-2, and RELIEF, as well as in the pooled analysis of the extended COMFORT-I and COMFORT-II studies, ruxolitinib turned out to be associated with a statistically significant increased risk of Herpes zoster infection. In the 28 case reports collected in the same review, the most frequent ruxolitinib-associated infections were tuberculosis, followed by HBV reactivation and *Pneumocystis jirovecii* pneumonia (62).

In a literature and institutional records search on ruxolitinib-treated subjects, 32 cases of opportunistic infection mainly in MF patients were retrieved, the most common being tuberculosis followed by cryptococcal infection and HBV reactivation (59). The issue of ruxolitinib-associated Mycobacterial tuberculosis infections was also investigated in a retrospective pharmacovigilance review based on the FDA Adverse Events Reporting System. Between January 2011 and December 2018, out of 4.666 reports of typical Mycobacterial tuberculosis recorded in the database, 91 were due to ruxolitinib compared with 4.575 cases due to all other drugs with a significant odds ratio (OR) at 9.2. Also, for atypical mycobacterial infections, the OR for ruxolitinib compared to all other drugs was significant at 8.3, indicating that patients on ruxolitinib were at increased risk of developing mycobacterial infections (60). In a similar retrospective study based on the French Pharmacovigilance database, between August 2012 and August 2017, in 24 MPN subjects and two GVHD cases all treated with ruxolitinib, 30 cases of infections were reported: nine were bacterial, five mycobacterial, ten viral, four fungal, one protozoan, and one

non-specified opportunistic infection, being Herpes zoster the most frequently identified pathogen (49). A retrospective study on 65 MF/PV patients treated with ruxolitinib between July 2011 and June 2018 recorded two mycobacterial infections (3% of patients), one due to Mycobacterial tuberculosis and one to Mycobacterium avium complex, a higher rate than reported in the original randomized studies (61). The several cases of tuberculosis reported in clinical trials as well as in registries database and in case reports indicate an increased risk of developing this infectious complication in ruxolitinib-treated subjects. Therefore, before starting ruxolitinib, a tuberculosis history and, in the presence of significant risk factors, a screening with Tuberculin Skin Test or preferably IFN-γ Release Assay should always be performed. During ruxolitinib treatment, a regular follow-up aimed at early diagnosis of tuberculosis is also advisable, followed by an appropriate therapy when required (69).

Also, the risk of HBV reactivation in ruxolitinib-treated MPN patients, ranging from asymptomatic virus replication to severe hepatitis, was highlighted in several case reports (63, 64, 70, 71). Screening procedures including HBsAg, anti-HBs, and anti-HBc, and HBV-DNA if anti-HBc is positive are recommended (72, 73). HBV-seropositive patients should be treated with antiviral drugs such as entecavir and tenofovir due to their low viral resistance rate. Patients negative for HbsAg, anti-HBs, and anti-HBc should instead be considered for immunization (69). Recommendations of the German Standing Committee on Vaccinations (STIKO) also suggest vaccinations against influenza, Herpes zoster, and *Streptococcus pneumoniae* for individuals beyond the age of 60 and *Neisseria meningitidis* for those with a pre-existing disorder of the immune system (74).

3.3 JAK Inhibitors and COVID-19

The management of the potential immunosuppressive effects and the relative risk of infectious complications during therapy with JAK inhibitors represent an even more pressing problem at a time like the current one dominated by the COVID-19 pandemic.

The SARS-CoV-2 coronavirus infection causing the coronavirus disease 2019 (COVID-19) is a highly contagious and life-threatening disease. The latter is critically associated with a high rate of respiratory failure, thrombo-hemorrhagic complications, and death, mainly due to an abnormal inflammatory response. Recent data have indeed highlighted a pivotal role for pulmonary immuno-thrombosis in the pathogenesis of severe COVID-19. SARS-CoV-2 enters airway epithelial cells *via* ACE2 receptors and subsequently triggers monocytes, macrophages, and T cells infiltration into the alveoli (75). This is then accompanied by local cytokine and chemokine generation, leading to elevated systemic levels of cytokines, including TNF-α, IL-1β, IL-6, and IL-8 (75).

Considering its pronounced anti-inflammatory properties, ruxolitinib was, therefore, hypothesized to be an effective therapy for COVID-19 (76). In a prospective study of 34 aged and high-risk comorbidity patients with severe COVID-19 infection, ruxolitinib was shown to be safe and associated with significant clinical improvement, especially in the lung function (77).

As MPN patients are prone to both thrombosis and bleeding, they call for special care during COVID-19. With this aim, the GIMEMA (Gruppo Italiano Malattie Ematologiche dell'Adulto) group conducted a survey of 34 Italian centers to study the prevalence of infections in this specific setting (78). A total of 1,095 patients were treated with ruxolitinib, 829 for MF (75.7%) and 266 for PV (24.3%), with 36 of them found positive for COVID-19: 13 (36%) were asymptomatic, 13 (36%) had flu-like symptoms, and 10 (27.8%) were affected by COVID-19-related pneumonia. Eight COVID-19-positive patients died with a death rate of 22%. As a result of this survey, it was found that the incidence of COVID-19 infection in MPN patients is rather low, and a certain protective function of ruxolitinib could not be ruled out.

A subsequent study by the European LeukemiaNet collected 175 MPN patients with COVID-19 during the first wave of the pandemic, from February to May 2020, in 38 international hematologic centers (79). Among the MPN phenotypes, patients with MF were the great majority (44%). Furthermore, they were at higher risk of mortality (48%) in comparison with both ET (25%) and PV (19%). When compared with the general COVID-19 population, the mortality ratio in this study was at least two to three times higher than the mortality rates reported by Johns Hopkins University in the same period and comparable to that reported in other hematologic malignancies (80–82). With regards to therapy ongoing at COVID-19 diagnosis, HU did not show significant correlations. In contrast, multivariable and propensity score matching analyses found an increased risk of death in patients who abruptly discontinued ruxolitinib treatment (79). Accordingly, JAK inhibitors should not be adjusted or discontinued in MPN patients to reduce the risk of COVID-19. On the contrary, stopping ruxolitinib in the event of COVID-19 infection may be harmful and should be avoided if clinically feasible. Otherwise, if ruxolitinib needs to be stopped, it should be tapered cautiously (27, 83).

Due to the immunomodulatory properties of ruxolitinib, the question arises whether response to SARS-CoV-2 vaccination might be impaired in MPN patients, in particular those under ruxolitinib therapy.

A recent study has already demonstrated that only a low proportion (17%) of solid organ transplant recipients mounted a positive antibody response to the first dose of SARS-CoV-2 mRNA vaccines, with those receiving anti–metabolite maintenance immunosuppression less likely to respond (84). In a subsequent study from the same authors, most of the patients had detectable antibody responses after the second dose, although participants without a response after dose 1 had generally low antibody levels. Consequently, a substantial proportion of transplant recipients likely remain at risk for COVID-19 after two doses of mRNA vaccine (85).

Focusing on hematological malignancies, different studies have already demonstrated a substantially reduced seroconversion rate post-COVID-19 vaccination in these patients, particularly in

heavily treated patient groups, those with aggressive disease, marked cytopenias, and B-cell neoplasms (86–88).

On the contrary, patients with chronic myeloid neoplasms, including MPN, chronic myeloid leukemia, and myelodysplastic syndromes, seemed to show higher seroconversion rates than those reported in the former groups: more in details, reasonably high seroconversion rates following a single vaccine dose were observed in patients with CML and in MPN patients receiving interferon. However, humoral responses in certain MPN patients, especially in those receiving ruxolitinib, were found to be substantially impaired as compared to healthy adults of a similar age group (89–92). Even though the exact mechanism for this is not yet known, it could be suggested to be the result of both disease- and treatment-mediated immune dysfunction.

4 JAK INHIBITORS' POSITIVE EFFECTS ON INFLAMMATION IN MPNs AND GRAFT-*VERSUS*-HOST DISEASE (GVHD)

The majority of MPN patients harbor mutations of the genes encoding for *JAK2*, *CALR*, or *MPL*, which result not only in the constitutive activation of the JAK-STAT signaling pathway but also of other pro-inflammatory signaling, in particular tumor necrosis factor (TNF)/nuclear factor k-light-chain-enhancer of activated B cells (NF-kB) pathways, in mutated hematopoietic stem cells and their progeny (93–95). *In vitro* studies have shown that the increased production of pro-inflammatory cytokines results from both an increase in the percentage of cytokine-secreting cells, as well as augmented cytokine secretion per *cell* (93). In addition to production of inflammatory cytokines by the MPN clone, immune dysregulation also results from paracrine/endocrine effects on non-clonal hematopoietic and stromal cells (96–99).

Consequently, MPN patients, particularly those with MF, exhibit both uncontrolled myeloproliferation and abnormally elevated levels of circulating pro-inflammatory cytokines causing disease-related systemic symptoms (100).

More precisely, plasma cytokine profile, especially in the setting of PMF or post-PV/ET myelofibrosis have already been shown to be significantly altered (101), and high levels of IL-8, IL-2 receptor, IL-12, and IL-15 were suggested as prognostic indicators of inferior survival and increased rate of leukemic transformation (102). Interestingly, it has also been shown that TNF-α, a pro-inflammatory cytokine, can facilitate clonal expansion of *JAK2*V617F-positive cells in MPNs (103). Besides their direct influence on the neoplastic clones, it is well known that cytokines can profoundly influence the bone marrow microenvironment, in MPNs as well as in other myeloid neoplasms.

In such a context, although potentially responsible for the immunosuppressive effect of ruxolitinib, its anti-JAK1 inhibitory action also leads to a reduction of pro-inflammatory cytokines, with a consequent improvement of symptoms, quality of life, and ultimately, bone marrow fibrosis (104, 105). The clinical response has already been shown to be independent of the *JAK2* mutational status, but it was linked to suppression of increased serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α (37).

In addition, JAK inhibitors' immunomodulatory properties may also be beneficial in other specific settings, as highlighted using ruxolitinib in GVHD (106–110). A recent multicenter retrospective analysis of ruxolitinib as salvage treatment in patients with steroid-resistant (SR) acute or chronic GVHD found an overall response rates (ORR) of >80% and 6-month survival rates ranging from 79% (acute disease) to 97% (chronic disease) (108). Based on the evidence supporting a role in preventing GVHD, ruxolitinib received in 2016 Breakthrough Therapy Designation from the US FDA for the treatment of GVHD (111).

In a more recent phase II study (REACH1) involving patients with grades II to IV SR acute GVHD, an overall response was achieved by 54.9% of the patients at day 28, including 26.8% with complete responses. Best ORR at any time was 73.2%, with a median duration of response of 345 days, thus producing durable responses and encouraging survival compared with historical data in patients with an otherwise dismal prognosis (112). In a subsequent randomized, open-label, phase III trial (REACH2) comparing the efficacy and safety of ruxolitinib with the investigator's choice of therapy in the same patients' setting, ORR at day 28 was higher in the ruxolitinib group than in the control group (62 *vs.* 39%; $p<0.001$), with a median overall survival of 11.1 *vs.* 6.5 months, respectively (113).

Accordingly, even though the treatment of acute GVHD has remained disappointing for decades, ruxolitinib and a few other agents seem to finally offer better therapeutic options, thus leading to a paradigm shift in the treatment of SR GVHD (114, 115).

5 CONCLUSIONS

The clinical use of JAK inhibitors in MPNs has led to a dramatic improvement of symptoms control, organ involvement, and quality of life. While JAK inhibitors are usually well tolerated, the issue of an increased risk of infections was raised by several clinical trials, retrospective series, and case reports (62). Indeed, JAK inhibitors may impair the immune response by mechanisms involving DCs, NKs, and CD4+ T cells as previously summarized.

TABLE 3 | Proposal for the most frequent antiviral and antibiotic prophylaxis for ruxolitinib-treated MPN patients.

	Proposed prophylaxis	References
Herpes zoster	To evaluate a case-by-case basis (acyclovir)	(62)
Hepatitis B virus	Specialist referral (lamivudine *vs.* entecavir or tenofovir)	(116)
Tuberculosis	Specialist referral (isoniazid)	(117)

Even considering the intrinsic MPNs' propensity to infectious complications, and other possible contributing factors such as previous treatments, concurrent immunosuppressive therapies, or comorbidities, available data suggest that incidence, and sometimes severity, of bacterial and viral infections in JAK inhibitors-treated MPN subjects are remarkable. Owing to the crucial therapeutic role of these drugs, their use must therefore be coupled with specific preventive measures (**Table 3**). Before JAK inhibitors therapy, screening for chronic HBV infection should be performed together with antiviral prophylaxis during treatment for suitable subjects. Monitoring of anti-HBc positive, HBsAg-negative patients is also indicated. Screening for latent tuberculosis is necessary, followed by therapy when needed. Antiviral and anti-pneumocystis prophylaxis could be considered in patients with specific risk factors (117). An adequate infections risk control is mandatory to fully exploit the JAK inhibitors' therapeutic efficacy in MPNs.

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DC and AI wrote the paper and gave their final approval.

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CASE REPORT
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Case Report: Evolution of *KIT* D816V-Positive Systemic Mastocytosis to Myeloid Neoplasm With *PDGFRA* Rearrangement Responsive to Imatinib

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Systemic mastocytosis (SM) is a rare neoplasm resulting from extracutaneous infiltration of clonal mast cells (MC). The clinical features of SM are very heterogenous and treatment should be highly individualized. Up to 40% of all SM cases can be associated with another hematological neoplasm, most frequently myeloproliferative neoplasms. Here, we present a patient with indolent SM who subsequently developed a myeloid neoplasm with *PDGFRA* rearrangement with complete response to low-dose imatinib. The 63-year-old patient presented with eosinophilia and elevated serum tryptase level. Bone marrow analysis revealed aberrant MCs in aggregates co-expressing CD2/CD25 and *KIT* D816V mutation (0.01%), and the *FIP1L1-PDGFRA* fusion gene was not identified. In the absence of 'B' and 'C' findings, we diagnosed an indolent form of SM. For 2 years after the diagnosis, the absolute eosinophil count progressively increased. Bone marrow evaluation showed myeloid hyperplasia and the *FIP1L1-PDGFRA* fusion gene was detected. Thus, the diagnosis of myeloid neoplasm with *PDGFRA* rearrangement was established. The patient was treated with imatinib 100 mg daily and rapidly obtained a complete molecular remission. The clinical, biological, and therapeutic aspects of SM might be challenging, especially when another associated hematological disease is diagnosed. Little is known about the underlying molecular and immunological mechanisms that can promote one entity prevailing over the other one. Currently, the preferred concept of SM pathogenesis is a multimutated neoplasm in which *KIT* mutations represent a "phenotype modifier" toward SM. Our patient showed an evolution from *KIT* mutated indolent SM to a myeloid neoplasm with *PDGFRA* rearrangement; when the

eosinophilic component expanded, a regression of the MC counterpart was observed. In conclusion, extensive clinical monitoring associated with molecular testing is essential to better define these rare diseases and consequently their prognosis and treatment.

Keywords: systemic mastocytosis, myeloid neoplasm with *PDGFRA* rearrangement, imatinib, *KIT* D816V mutation, clonal evolution

INTRODUCTION

Systemic mastocytosis (SM) is a heterogeneous group of neoplasms characterized by abnormal expansion of clonal mast cells (MCs) in the bone marrow (BM) and other extracutaneous organ-systems (1).

According to the World Health Organization (WHO) classification, the diagnosis of SM is established in presence of the major criterion and one minor criterion or at least three minor criteria. The major criterion is fulfilled by the detection of multifocal clusters of MCs (aggregates ≥15) in one or more extracutaneous organs (usually BM), while the minor criteria include aberrant MC expression of CD25 and/or CD2, abnormal morphology of MCs, KIT mutation D816V, and a persistent serum tryptase level ≥20 ng/ml (1, 2).

There are five subtypes of SM: indolent SM (ISM), smoldering SM (SSM), SM with an associated hematological (non-MC lineage) neoplasm (SM-AHN), aggressive SM (ASM), and mast cell leukemia (MCL) (1).

The diagnosis of ISM can be established if <2 B findings and no C findings are detected; SSM is defined by ≥2 'B' findings and no 'C' findings. ASM is characterized by one or more C findings, while MCL is defined by MCs ≥20% on marrow smears (1, 2).

There are three types of 'B' findings: MC infiltration >30% on bone marrow biopsy and serum total tryptase >200 ng/mL; hepatomegaly with normal liver function, palpable splenomegaly without hypersplenism, and/or lymphadenopathy; signs of dysplasia or myeloproliferation in non-MC lineage. The six 'C' findings are cytopenias; hepatomegaly with impairment of liver function, ascites, and/or portal hypertension; palpable splenomegaly with hypersplenism; malabsorption with weight loss due to gastrointestinal MC infiltrates; large osteolytic lesions (1, 2).

The goals of ISM treatment are symptom control, severe anaphylaxis prophylaxis, and osteoporosis treatment, while the advanced forms may require cytoreductive therapy. Historically, cytoreductive agents include interferon-α and cladribine. Allogeneic stem cell transplant could be considered in SM-AHN when the associated hematologic neoplasm has an indication of transplantation and in relapsed/refractory ASM or acute MCL (2). With the advent of the tyrosine kinase inhibitors, many efforts have been made to find a proper inhibitor of SM *KIT*–driver mutation. Imatinib still plays a role in the treatment of rare SM cases that are *KIT* D816V-unmutated, while more recently midostaurin has been shown to induce major clinical responses in advanced SM regardless of *KIT* mutational status (2, 3).

Among the myeloid neoplasms, the WHO classification recognizes the family of the myeloid/lymphoid neoplasms with eosinophilia and rearrangement of *PDGFRA*, *PDGFRB*, and *FGFR1*, or with *PCM1-JAK2*. These are rare diseases characterized by a fusion gene or a mutation resulting in the expression of aberrant tyrosine kinases. Eosinophilia (≥1.5x10^9/L) is one of the most common features of these neoplasms. In the subgroup associated with *PDGFRA* rearrangement, the most common genetic abnormality is the *FIP1L1-PDGFRA* gene fusion, caused by 4q12 deletion (**Figure 1**) (1, 4–6). Patients frequently complain of fatigue, pruritus, and symptoms related to eosinophilic infiltrates in different organs; splenomegaly and hepatomegaly are common findings (4–6).

The natural history of *PDGFRA*-rearranged neoplasms has been dramatically altered by imatinib and the dosage of 100 mg daily could be sufficient to elicit a complete molecular response in most of the patients (7–9).

Herein, we report the clinical features and the management of a patient with *KIT* D816V-positive SM who subsequently developed a myeloid neoplasm with *PDGFRA* rearrangement with a complete molecular response to low-dose imatinib.

All procedures were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2013.

CASE REPORT

A 63-year-old Caucasian male patient was referred to hematological investigation because of eosinophilia.

His medical history was unremarkable except for post-infectious glomerulonephritis in childhood and flushing episodes that started one year before the hematological evaluation. No pathological findings were reported at physical examination. Previous blood tests revealed a gradual increase of absolute eosinophils count over two years. Recent laboratory examinations showed white blood count (WBC) 8.2x10^9/L with absolute eosinophil count 2.2x10^9/L (27%), serum tryptase level 26 ng/mL (normal level <5 ng/mL), normal serum lactate dehydrogenase (LDH), and normal liver and renal function.

A bone marrow biopsy detected 60% of cellularity, with myeloid hyperplasia, increased eosinophils and reticulin, and the presence of multifocal clusters of spindle-shaped MCs co-expressing CD25 and CD2 (>15 cells). Karyotype analysis was normal. Polymerase chain reaction (PCR) detected the D816V *KIT* mutation on bone marrow (variant allele frequency – VAF - 0.01%), while the *FIP1L1-PDGFRA* fusion transcript was absent.

Overall, these findings were consistent with a diagnosis of SM for the presence of the major criterion (BM MCs infiltrates) and

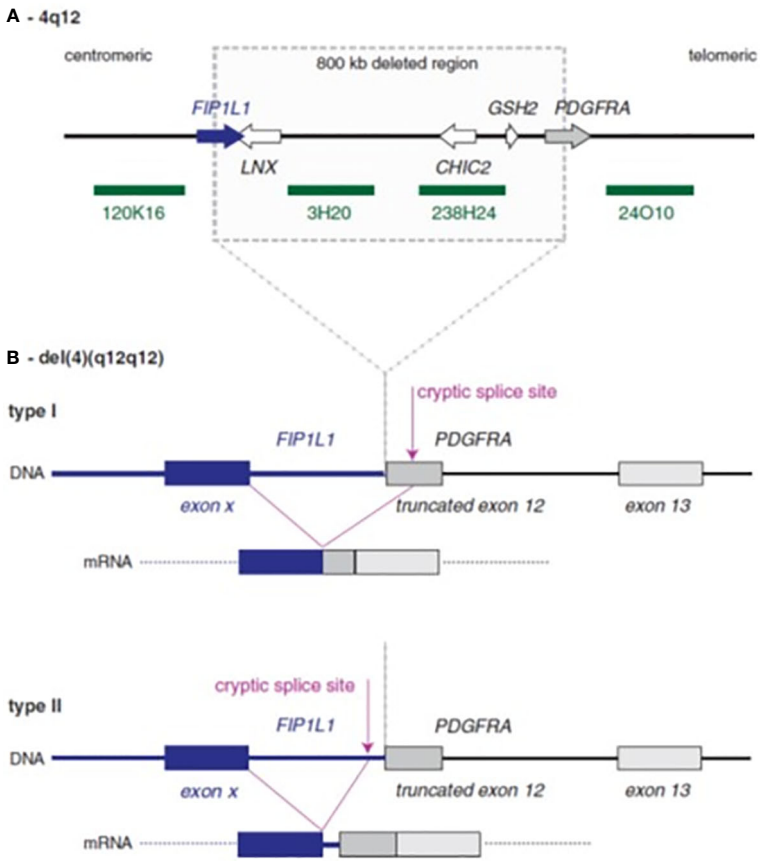

FIGURE 1 | Schematic representation of *FIP1L1–PDGFRA* rearrangement. **(A)** Normal 4q12 region with sites of deletion in cases with the *FIP1L1–PDGFRA* fusion. The four green bars denote probes that can be used to detect the deletion by fluorescence *in situ* hybridization. **(B)** The consequences of deletion: *FIP1L1* usually breaks within an intron, while *PDGFRA* always breaks within exon 12. To obtain splicing between *FIP1L1* and *PDGFRA*, cryptic splice sites need to be used, because the normal splice site in the exon 12 is removed by the deletion. This cryptic splice site could be located either within exon 12 of *PDGFRA* (type I fusion) or within the intron of *FIP1L1* (type 2 fusion).

3 minor criteria (aberrant MCs, *KIT* D816V mutation, tryptase level >20 ng/mL). Abdominal ultrasound showed normal liver and spleen dimensions and an absence of lymphadenopathy. Dual-energy X-ray absorptiometry scan detected osteoporosis (lumbar T score -3.3, lumbar Z-score -2.6, femoral neck T-score -1.5, femoral neck Z-score -0.5).

In the absence of 'B' and 'C' findings, we concluded with an indolent form of SM. The patient did not receive specific therapy other than osteoporosis treatment.

Two years later the patient complained of a worsening of flushing and a recurrent headache. Blood examinations revealed an increase of WBC ($13x10^9$/L) with an absolute eosinophil count of 5.1 x 10^9/L. Suspecting a myeloid neoplasm with eosinophilia, a new bone marrow evaluation was performed. The histological analysis confirmed myeloid hyperplasia with a marked increase of eosinophils and 3% of MCs in rare aggregates. Aspirate smear revealed 36% of eosinophils and 6% of MCs. No aberrant MCs were detected with flow cytometry. Cytogenetic analysis showed normal male karyotype and a digital PCR was negative for *KIT* D816V mutation. Real-time PCR performed on peripheral blood detected the FIP1L1-PDGFRA fusion transcript and serum tryptase was 20 ng/ml. Thus, the final diagnosis was myeloid neoplasm with *PDGFRA* rearrangement.

Imatinib 100 mg daily was started, and after 3 months all symptoms resolved and blood tests showed WBC $5.96x10^9$/L with normal eosinophil count ($0.09x10^9$/L).

BM analysis revealed the absence of eosinophils and the presence of rare MCs with normal morphology, corresponding to 4% of cellularity at the aspirate smear. Flow cytometry showed a normal MCs phenotype. *FIP1L1-PDGFRA* and *KIT* D816V mutations were negative on BM and peripheral blood.

A subsequent BM analysis, performed after 12 months of imatinib treatment, confirmed complete remission of the myeloid neoplasm, absence of aberrant MCs, and negative *FIP1L1-PDGFRA* and *KIT* D816V mutations.

Table 1 and **Figure 2** report the main hematological findings at diagnosis of SM, diagnosis of myeloid neoplasm with *PDGFRA* rearrangement, and after 3, 6, and 12 months of imatinib treatment.

TABLE 1 | Relevant blood and bone marrow parameters in the main time-points from diagnosis of systemic mastocytosis.

	SM Diagnosis	Myeloid neoplasm with *PDGFRA* rearrangement Diagnosis	+3 mo of Imatinib Treatment	+6 mo of Imatinib Treatment	+12 mo of Imatinib Treatment
WBC count, $x10^9$/L	8.2	13.03	5.96	6.48	6.48
Eos. in blood, $x10^9$/L (%)	2.2 (27%)	5.1 (39.6%)	0.09 (1.5%)	0.15 (2.3%)	0.18 (2.8%)
Serum tryptase, ng/mL	26	20			5
MCs in bone marrow, histological analysis, %	CD25+ CD2+, in aggregates, not quantified	3%	2%	1%	1%
MCs in bone marrow, aspirate smear, %	Not performed	6%	4%	0%	0%
MCs in bone marrow, flow cytometry, %	Not performed	0.3%	2%	1%	0.2%
Genetic markers (mononuclear cells of peripheral blood or bone marrow)	Bone marrow: *PDGFRA* - *KIT* D816V + (0.01%)	Peripheral blood: *PDGFRA* + Bone marrow: KIT D816V -	Bone marrow and peripheral blood: *PDGFRA* - KIT D816V -	Bone marrow: *PDGFRA* - *KIT* D816V -	Bone marrow: *PDGFRA* - *KIT* D816V -

DISCUSSION

The clinical features of SM are very heterogenous, ranging from indolent forms to more aggressive diseases which require cytoreductive therapy (1, 2).

MCs derive from CD34+/CD117+ pluripotent hematopoietic progenitor cells in BM. They are normal residents in mucosal tissues and skin with a key role in IgE-associated disorders and acquired or innate immunity (10). More than 90% of SM patients carried a somatic mutation in different regions of *KIT* which led to structural alteration of the protein with a constitutive activation of the receptor. The *KIT* D816V mutation is the most common (11). *KIT* median VAF strongly correlates with disease activity as represented by serum tryptase level, disease subtype (indolent *versus* advanced), and survival (12).

Up to 40% of all SM cases are associated with another hematological disease, which rarely can be a myeloid/lymphoid neoplasm with eosinophilia (2, 4).

Myeloid/lymphoid neoplasms with eosinophilia and rearrangements of *PDGFRA*, *PDGFRB*, and *FGFR1* were recognized as a standalone category in the 2008 WHO classification. Subsequently, *PCM1-JAK2* was added to this family as a new provisional entity in the 2016 WHO classification (1). Besides the rare cases of SM associated with a myeloid/lymphoid neoplasm with eosinophilia, peripheral eosinophilia may affect up to 28% of all SM patients (2). A paper from 2007 compared D816V-positive SM and *FIP1L1/PDGFRA*-positive chronic eosinophilic leukemia (13). The distinguishing features for chronic eosinophilic leukemia included the degree of eosinophilia in relation to the tryptase level, the absence of dense MC aggregates, and pulmonary and cardiac symptoms. The authors concluded that the *FIP1L1/PDGFRA* gene fusion and D816V-*KIT* mutation cause different clinical syndromes and a distinction is essential for therapeutic decisions (13).

Herein, we reported a case of indolent *KIT* D816V-positive SM with eosinophilia at diagnosis, which after two years showed an evolution to myeloid neoplasm with *PDGFRA* rearrangement.

At disease onset, the clinical scenario was characterized by a moderate increase of eosinophils and serum tryptase. Genetic data revealed *KIT* D816V mutation, while rearrangements for eosinophilia were not identified. SM and myeloid neoplasms with eosinophilia were considered in the differential diagnosis.

Similar to SM, myeloid neoplasms with eosinophilia and specific rearrangements can show dysplastic eosinophils and spindle-shaped MCs. The MCs can also be CD25 positive, but do not form compact aggregates, express CD2, or carry the *KIT* D816V mutation (14). Our patient displayed all these characteristics; therefore a diagnosis of SM seemed to be the most likely despite a low *KIT* VAF.

During the follow-up, the patient complained of a worsening of flushing, headache, and eosinophilia. Detection of the *FIP1L1-PDGFRA* mutation and absence of diagnostic criteria for SM led us to a diagnosis of myeloid neoplasm with *PDGFRA* rearrangement.

When the *PDGFRA* rearrangement was identified the eosinophilic component became predominant either in the peripheral blood and bone marrow; Pardanani et al. described 12 cases of SM and chronic eosinophilic leukemia with *FIP1L1-PDGFRA*. These patients with eosinophilia were more likely to be males and exhibit a "loose" pattern of MC infiltration in BM trephines; 8 of them were also screened for *KIT* D816V and all tested negative (15).

A clonal relationship between the MC and the associated hematologic non-MC component has been sought using *KIT* and other mutations as markers of clonality (13, 14). Various studies conducted in patients with concomitant diagnosis of acute myeloid leukemia and SM demonstrated evidence that neoplastic MC and myeloid leukemic blasts are likely to develop from common hematopoietic progenitors (16, 17). Subsequent studies supported the concept of advanced SM pathogenesis as a multimutated neoplasm, in which *KIT* D816V mutation represents a "phenotype modifier" of clonal hematopoietic stem cell disorders toward SM (18). These findings challenged the concept that the SM and the associated non-MC-hematological disease arise uniformly from a pre-

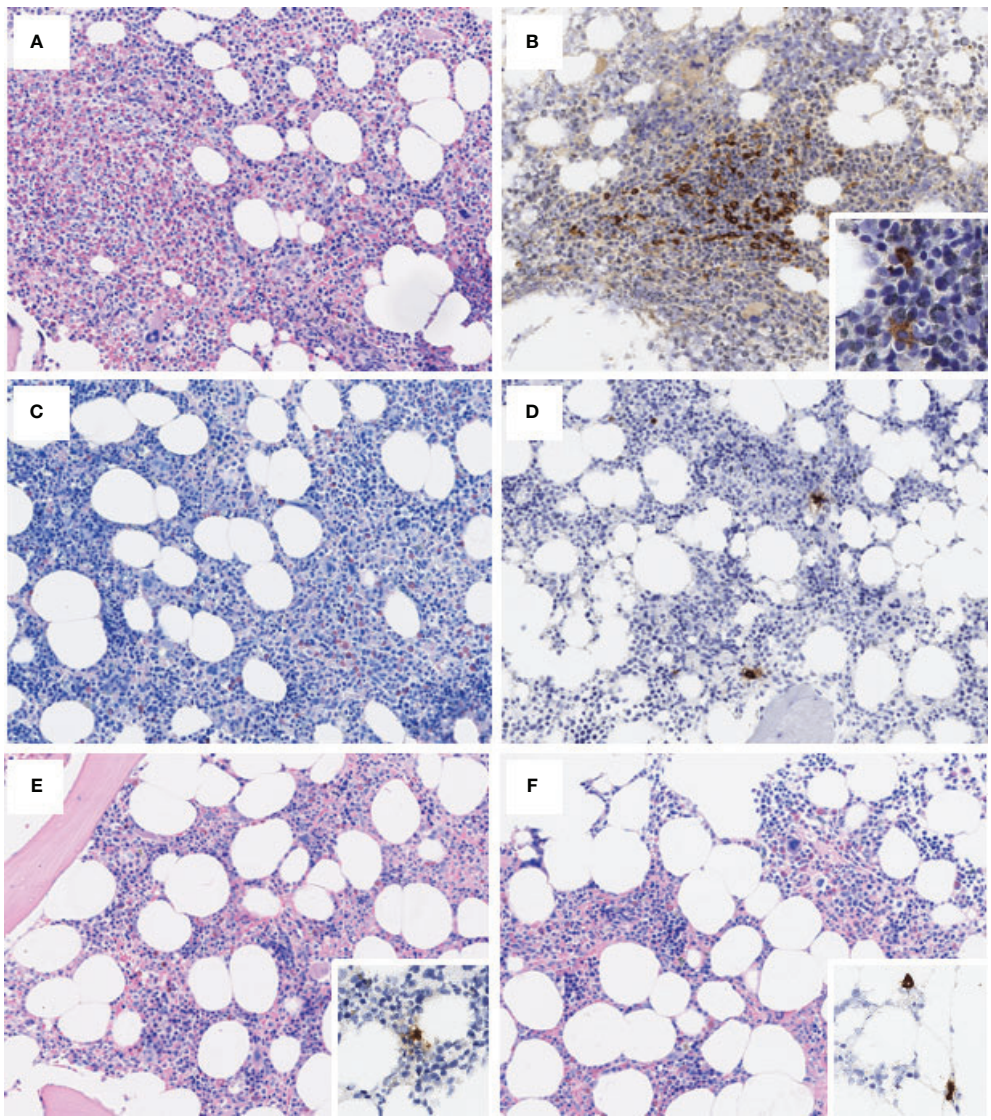

FIGURE 2 | **(A)** (Giemsa, 200x) depicts a hypercellular bone marrow, featuring expansion of the eosinophilic lineage, comprising maturing to fully mature eosinophils, while tryptase stain [**(B)**; 200x] delineates the presence of scattered aggregates of epitheliod to spindled mast cells, featuring at least partial CD25-positivity (inset, 400x). Bone marrow biopsy at 3 months from Imatinib initiation [**(C)**; Giemsa, 200x] shows a reduction of cellularity, eosinophilic compartment, and mast cells [**(D)**; tryptase, 200x], which appear scattered. Restitutio ad integrum of the hematopoiesis is steadily apparent in subsequent biopsies [**(E, F)**; Giemsa, 200x], with only scattered tryptase+ cells [**(E, F)** insets].

committed neoplastic progenitor cell harboring a *KIT* mutation and suggest that this category is highly heterogeneous (18).

Our case corroborates data by Maric and Pardanani (13, 15) who considered eosinophilic disorders and systemic mastocytosis as clinically distinct entities with different therapeutic needs. On a molecular level, further studies of next-generation and single-cell sequencing may be of benefit to clarify whether or not *KIT* mutation and *PDGFRA* rearrangement should be considered as different clones.

At the time of identification of the *FIPL1-PDGFRA* transcript, our patient did not meet the diagnostic criteria for SM: on bone marrow, MCs formed rare aggregates, no cytofluorimetric abnormal markers or *KIT* D816V mutation were identified, and serum tryptase was 20 ng/ml.

According to the literature, only skin diseases in adult patients with SM could regress, in approximately 10% of the cases, while there is no evidence of spontaneous disappearance of bone marrow findings (19). In our case, the expansion of the eosinophilic component was associated with the regression of the MC counterpart, which could be masked or really disappeared during the two years after the SM diagnosis.

ISM has a nearly normal life expectancy; symptom-directed treatment should be considered in all symptomatic patients. These therapies are directed at MC degranulation symptoms,

symptomatic skin disease, and osteopenia/osteoporosis. Cytoreductive therapy could be required for advanced SM; in this patient setting, novel agents with potent inhibitory activity against KIT demonstrated significant clinical benefit and reduction of bone marrow MC burden (2).

Initially, our patient received only symptomatic therapy directed towards osteoporosis treatment. When the myeloid neoplasm with *PDGFRA* rearrangement was diagnosed, low-dose imatinib was started to avoid organ damage related to eosinophilia.

The durable hematologic and molecular remissions induced by imatinib in *FIP1L1-PDGFRA*–positive myeloid neoplasms have been corroborated by many studies; generally, 100 mg daily may be sufficient to achieve and maintain a long-term molecular response (7–9).

In our case report, after 3 months from the start of imatinib treatment bone marrow evaluation and peripheral blood tests showed normal findings with complete molecular remission of the *FIP1L1-PDGFRA*–associated neoplasm. Therapy was well-tolerated, and the patient is still in complete molecular response after 18 months of therapy.

In conclusion, SM is a rare disease with an unpredictable clinical course, especially when another associated hematological disease is diagnosed. Little is known about the underlying molecular and immunological mechanisms that can promote one entity prevailing over the other one. Extensive clinical monitoring associated with molecular testing is essential to better define the disease and consequently its prognosis and treatment.

PATIENT PERSPECTIVE

With the diagnosis of SM, the patient seemed very concerned. He struggled to accept that only symptomatic therapy was necessary for his rare medical condition; his concern increased even more once flushing worsened, headache became recurrent, and eosinophilia led to a diagnosis of myeloid neoplasm with *PDGFRA* rearrangement. The patient's perspective completely changed when the low dose imatinib was started; the treatment was well-tolerated and a complete molecular response with symptom remission was obtained.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the responsible committee on human experimentation (institutional and national). The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MS, GC, LB, and FIG provided the patient data regarding the hematological disease. MS, GC, and FIG wrote the manuscript. CE-V provided patient data for what concerns endocrinological aspects. GAC performed the histological examinations of bone marrow, provided iconographic materials, interpreted these data, and integrated them in the manuscript. SF and SL provided patient data regarding molecular aspects. All authors critically read and approved the final manuscript.

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REVIEW
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Single-Cell Technologies to Decipher the Immune Microenvironment in Myeloid Neoplasms: Perspectives and Opportunities

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Myeloid neoplasms (MN) are heterogeneous clonal disorders arising from the expansion of hematopoietic stem and progenitor cells. In parallel with genetic and epigenetic dynamics, the immune system plays a critical role in modulating tumorigenesis, evolution and therapeutic resistance at the various stages of disease progression. Single-cell technologies represent powerful tools to assess the cellular composition of the complex tumor ecosystem and its immune environment, to dissect interactions between neoplastic and non-neoplastic components, and to decipher their functional heterogeneity and plasticity. In addition, recent progress in multi-omics approaches provide an unprecedented opportunity to study multiple molecular layers (DNA, RNA, proteins) at the level of single-cell or single cellular clones during disease evolution or in response to therapy. Applying single-cell technologies to MN holds the promise to uncover novel cell subsets or phenotypic states and highlight the connections between clonal evolution and immune escape, which is crucial to fully understand disease progression and therapeutic resistance. This review provides a perspective on the various opportunities and challenges in the field, focusing on key questions in MN research and discussing their translational value, particularly for the development of more efficient immunotherapies.

Keywords: single-cell sequencing, myelodysplastic syndromes, acute myeloid leukemia, clonal hematopoiesis, immunotherapies, immune microenvironment

INTRODUCTION

Myeloid neoplasms (MN) consist of a heterogeneous group of hematological cancers, arising from the hematopoietic stem cell (HSC) or progenitors in the bone marrow (BM) and sharing phenotypic features of the myeloid lineage (1). They include myeloproliferative neoplasms (MPN), which are featured by the hyperproliferation of near-normal maturing blood-cells; myelodysplastic syndromes (MDS), characterized by ineffective hematopoiesis, abnormalities in cell maturation and cytopenias; and acute myeloid leukemia (AML), which represents the most aggressive clinical phenotype, whose prominent features are the uncontrolled proliferation of immature hematopoietic precursors (i.e., blasts) and life-threatening BM failure (1).

The pathogenesis of MN is driven by the progressive selection of multiple genetic mutations (clonal evolution) (2–4). Somatic mutations can be identified in the peripheral blood of healthy subjects, a phenomenon known as clonal hematopoiesis (CH) that reflects the expansion of mutated HSC; by years or decades, CH may evolve to AML, eventually involving clinically recognizable pre-leukemic syndromes, such as MDS or MPN (5–10). In parallel, growing evidence also points to a prominent role of the immune system in shaping the evolution and clinical pictures of MN (11–13). The tumor immune microenvironment consists of multiple players, including adaptive and innate immune cells and stromal components, which may either antagonize or promote tumor progression; cancer cells themselves exhibit immunomodulatory properties and interact with microenvironmental components of the tumor niche (11–14). The connections between genetic evolution, changes in the immune microenvironment and clinical correlations, however, are poorly understood.

Single-cell sequencing technologies appear as ideal tools to investigate the highly-connected and plastic immune system. These technologies overcome the limited resolution of DNA and RNA sequencing of entire cell-populations ("bulk" sequencing), allowing deconvolution of heterogeneous populations and identification of rare cell types. Importantly, this is achieved by analyses of individual-cell transcriptional states, thus enabling the characterization of functional states while avoiding the bias of predefined lineage-markers (15, 16). Applications of single-cell technologies is continuously expanding with improving throughput, accuracy and reproducibility, thus making them widely adopted in cancer research, and being currently exploited for precision oncology (16, 17).

This review covers state-of-the-art single-cell technological applications with associated analysis methods; we aim to provide a perspective on the various opportunities to study the immune system and tumor microenvironment - for both experimental research and clinical translation - with a focus on specific issues relevant to MN.

CURRENT STATE-OF-THE-ART IN MYELOID NEOPLASMS AND OPEN CHALLENGES

The immune microenvironment shapes MN through different branches of the immune system. A large body of pre-clinical and clinical studies indicate a key role of innate-immune cells and inflammation in the establishment of preleukemic states and their progression toward AML (13, 14, 18). For instance, the epigenetic reprogramming of aged HSC influences their response to inflammatory and immune-mediated signals, directly impacting on their division rate, myeloid-lineage skewing and survival advantage (5, 6). Adaptive immunity also plays a major role, as the presence of T cells at the tumor site is mandatory for recognition and elimination of transformed cells. Interestingly, its function changes according to the disease phase: in low-risk MDS, anti-leukemia cytotoxic (CD8+), helper (Th17) T cells and NK cells are expanded, in the presence of low counts of pro-leukemia T-regulatory lymphocytes (Treg); in high-risk MDS and AML, instead, Treg prevails over CD8+, Th17 and NK cells, suggesting that tumor clones acquire immune tolerance during disease progression (19, 20). In established AML, higher percentages of BM CD3+ and CD8+ T cells correlate with improved survival (21, 22) and response to the checkpoint inhibitor nivolumab (23). Importantly, expression of the checkpoint inhibitory receptor PD-1 and its ligand PD-L1 increases with disease progression of MN and AML relapse, as an immune-escape mechanism (24). Finally, leukemic blasts themselves modulate T- and NK-cell responses and are implicated in multiple mechanisms of immune evasion (25–28), in the context of an immunosuppressive microenvironment (**Figure 1**).

Prognosis and treatment of MN patients are extremely varying, depending on the disease entities and their associated clinical and molecular characteristics. Beyond achieving clinical control of hyperproliferation or cytopenias, the focus of management and research in MPN and MDS resides in predicting - possibly preventing - the evolution to AML. This is because, once leukemia is established, most AML patients ultimately succumb to their disease, despite some recent implementation of available treatments beyond the backbone of 7 + 3 chemotherapy. In fact, primary chemoresistance and relapse are the major causes of poor survival in high-risk MDS and AML patients (29–31).

The dynamics of leukemic progression, resistance to treatments and relapse have been mostly described in terms of genetic and epigenetic events, as associated to diverse synergistic combinations of mutations (2, 32–34); however, it is increasingly appreciated that genetic/epigenetic alterations do not entirely explain the complexity and heterogeneity of MN (35–37), as also inferred from the limited success of drugs targeting single genomic variants [e.g., FLT3 (38, 39) or IDH inhibitors (40, 41)] or epigenetic traits [e.g., hypomethylating agents, HMA (42, 43)]. Indeed, as featured above, there is increasing evidence of multiple mechanisms of immune-evasion during MN development; as a clinical correlate, the immunological eradication of therapy-resistant leukemia stem cells (LSC) by allogeneic hematopoietic stem cell transplant (alloHSCT) is the only strategy to overcome chemoresistance and obtain sustained remission (29, 44, 45). Therefore, a first major challenge in MN research is understanding the molecular mechanisms of immune-tolerance and the cellular relationships between the immune microenvironment and tumor clones during leukemic progression and therapeutic resistance; highlighting the connections between genetic evolution and immune escape seems particularly meaningful.

Although alloHSCT is an effective treatment, post-transplant relapse is a common occurrence, due to several leukemia-driven immune-escape mechanisms (12); also, its anti-tumor activity is rather poor in patients with active disease and, in general, it comes at the cost of high morbidity and mortality (46). These observations point toward the strong need of developing more potent, specific and possibly less toxic immunotherapeutic strategies for MN patients. These include harnessing T and NK-cell-mediated tumor clearance by checkpoint inhibitors,

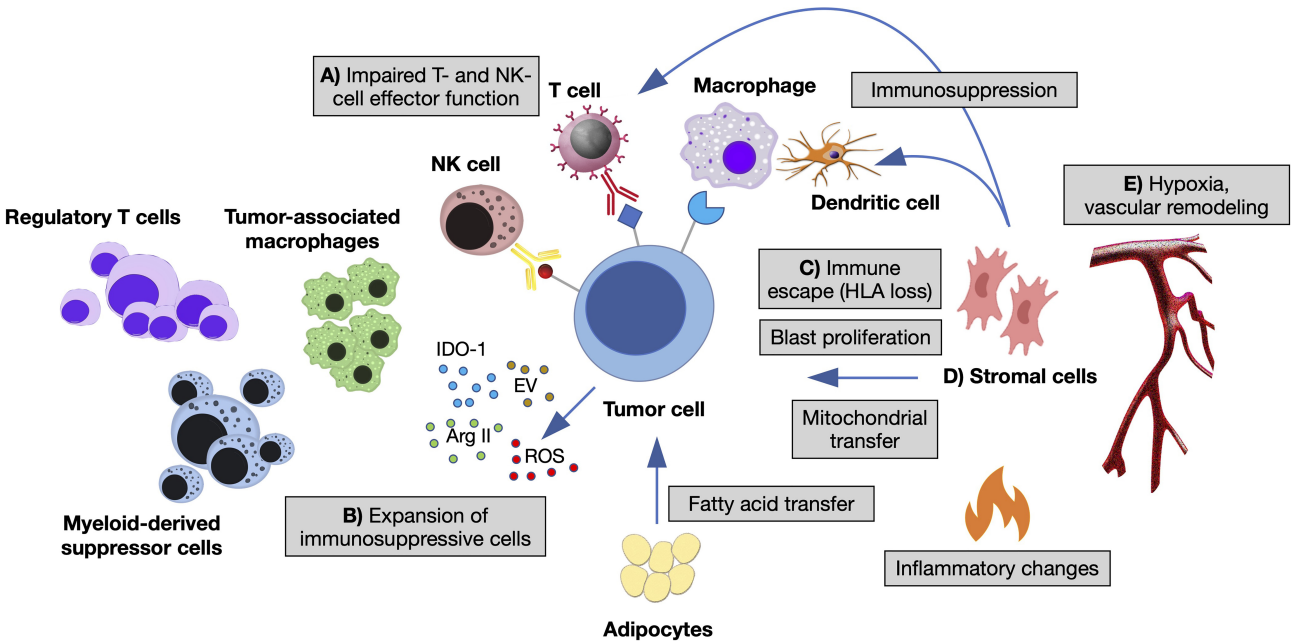

FIGURE 1 | The immune microenvironment of myeloid neoplasms. Summary of the main interactions occurring between neoplastic cells and immune microenvironment in the bone marrow (BM) niche. **(A)** Impaired T- and NK-cell effector function by overexpression of inhibitory ligands (PD-L1, Gal-9, CD155, CD112, CD86, NKG2DL) and interaction with their respective receptors (TIGIT, TIM-3, PD1, CTLA-4, NKG2A); T-cell exhaustion and apoptosis driven by cytokine changes. **(B)** Expansion of immunosuppressive cells (regulatory T cells and myeloid-derived suppressor cells), switch of macrophages to tumor-associated macrophages by altered cytokine milieau and release within the BM niche of other soluble factors, such as reactive oxygen species (ROS), indoleamine 2,3- dioxygenase-1 (IDO1), arginase II (ArgII), and extracellular vesicles (EV). **(C)** Escape from macrophages and dendritic cells by decreased expression of antigen presentation molecules (HLA I and HLA II). **(D)** Stromal cells inhibiting the function of dendritic and T cells, influencing tumor proliferation and metabolic properties. **(E)** Vascular remodeling and hypoxia modifying immune cells' homing and adhesion (11, 12, 14).

monoclonal antibodies, bispecific antibodies or chimeric antigen receptor (CAR) T cells; however, their effect has been less successful in MN than in other cancers (12, 47), despite the clear involvement of the immune system in MN pathogenesis. Reasons for this failure include a limited power of the currently used immunological markers to predict clinical response, the absence of a suitable target antigen and elusive resistance mechanisms. Thus, ongoing research efforts are committed to the discovery of druggable targets or mechanisms and more effective therapeutic combinations, which would benefit from a better understanding of the various cellular and functional components of the immune microenvironment.

OVERVIEW OF STATE-OF-THE-ART SINGLE-CELL TECHNOLOGIES

Limits of Bulk Sequencing and Promises of Single-Cell Technologies to Deconvolve the Immune Microenvironment of Myeloid Neoplasms

Traditional "bulk"-sequencing approaches rely on the analysis of whole samples through next-generation sequencing platforms, which generate multiple sequencing reads covering individual RNA or DNA molecules. Genomic and transcriptomic bulk data from the Cancer Genome Atlas (TCGA) Research Network have been crucial for our initial understanding of the tumor microenvironment and tumor-immune interactions. For example, Thorsson et al. (48) performed an extensive immunogenomic analysis of more than 10,000 tumors comprising 33 cancer types, and identified six immune subtypes that span cancer tissue and molecular subtypes, and differ by somatic aberrations, microenvironment, and survival; results from this study are available for exploration through the interactive Cancer Research Institute iAtlas portal (49). However, such resource is poorly applicable to MN research, as no MDS or MPN patients were included and limited data are available for AML, with no direct clues on tumor microenvironment composition, lymphocyte infiltration, immune features/modulators, immuno-oncology targets and associations with driver mutations (48). Furthermore, the output of bulk sequencing represents an "average" of the transcriptomic or genomic features of all sample cells, which poses a challenge in the precise deconvolution of intra-tumor heterogeneity. Dedicated bioinformatic tools have been developed to determine the composition of cancer microenvironments, including CIBERSORT, a method for estimating the relative proportions of cell types of interest in complex tissues from their gene expression profiles (50). However, this tool systematically over- or underestimates some cell types and requires a reference of gene

expression signatures, which might bias the imputation of cells undergoing phenotypic plasticity or disease-induced dysregulation. Also, low intensity signals from rare cell populations might result undetectable with bulk sequencing approaches, which precludes the identification of rare (yet possibly functionally-relevant) cell populations. Therefore, deconvolution of the immune microenvironment can't be comprehensively achieved from bulk studies.

Conversely, single-cell approaches allow the characterization of individual cells, thus providing a more faithful representation of the heterogeneity of tumor ecosystems (15, 51, 52). The use of single-cell technologies for research purposes is rapidly spreading, favored by combined academic and industrial efforts to improve standardization, develop several different applications and technological platforms and decrease costs. Some key aspects offer relevant advancement in the characterization of the immune tumor microenvironment: tumor and immune cells can be acquired in parallel without prior marker-based sorting, the high resolution of the approach allows the analysis of even small groups of cells with shared features, while the throughput of some sequencing platforms (up to thousands of cells) provides unprecedented statistical power. Moreover, cells can be investigated for both their phenotypic traits (e.g., surface markers, cell types) and functional states (e.g., over-expressed pathways, genomic features, activation of signalling pathways), which potentially opens perspectives on new mechanistic hypotheses (16, 52).

Main Applications of Single-Cell Technologies

A comparative summary of single-cell methods for genomic studies is provided in **Table 1**.

- **Single-cell transcriptomics (scRNA-seq).** Recent advances in cell isolation methods and automated micro-fluidics techniques have improved tremendously the accuracy, sensitivity, reproducibility, and throughput of scRNA-seq, by which it is now possible to measure and model gene expression profiles from thousands of cells (64–67). A few scRNA-seq platforms are available on the market, differing by protocol complexity, costs, number of output cells, sequencing depth and full or partial coverage of transcripts (67). Such elements, as well as downstream analysis-pipelines, should be considered in view of the specific research-question. For instance, library construction methods that allow full transcript coverage (54, 68) are optimal for scoring expressed mutations, splicing isoforms (69) and T/B-cell receptor sequence (70–73), while molecular-counting methods based on the sequence the 5' or 3' end transcripts are better suited for cost-effective profiling of high numbers of cells and transcripts (64, 74). The introduction of unique molecular identifiers during library preparation allows counting and grouping of specific mRNA molecules prior to PCR amplification, thus increasing accuracy and reducing technical artifacts (75). High-dimensional scRNA-seq data need to be processed with specific computational algorithms, which incorporate various steps of quality control, normalization and dimensionality reduction to enable spatial representation (76–78). Opportunities from downstream analyses include unbiased clustering to identify groups of transcriptionally related cells, differential gene expression, and reconstructing dynamic biological processes, such as cellular differentiation and immune response, by inferring developmental 'trajectories' to reveal transitional states and cell fate decisions of distinct cell subpopulations (79–81).
- **Single-cell DNA sequencing (scDNA-seq).** scDNA-seq overcomes the limits of bulk sequencing allowing the direct identification of intratumoral genetic subclones - as defined by mutations co-occurring within the same cell - including rare clones, which may significantly impact tumor evolution and the acquisition of therapeutic resistance (3, 4, 82). The technique's core involves whole-genome amplification (WGA) of single cells, which allows detection of single nucleotide variations, chromosomal copy number alterations or more complex genomic rearrangements. Droplet-based platforms currently enable high-throughput and cost-effective characterization of hundreds of amplicons in thousands of cells (59). However, a drawback of scDNA methods is the high rate of false negative and false positive hits, due to artifacts introduced during genomic amplification, non-uniform genome-coverage and allelic dropout events.
- **Single-cell epigenomics.** Bulk epigenomic techniques have been recently adapted to single-cell applications; analyses of chromatin organization and regulation enable to elucidate cell lineage and differentiation state in even thousands of individual cells simultaneously. Reported technologies allow scoring DNA-methylation patterns (by bisulfite-based sequencing such as scRRBS, scBS-seq, scWHBS) (60, 83, 84), chromatin regions available for transcription factors activity (scATAC-seq) (61), chromosomal conformations (scHi-C) (63) and histone modifications/binding sites (scChIC-seq) (85). However, these methods are limited by the low coverage of specific regulatory regions (such as enhancers).
- **Single-cell proteomics.** Though multiparameter flow-cytometry allows characterization of individual cells with multiple antibodies, the design of specific antibody-panels can be laborious and implicitly prevents unbiased and system-wide analyses. Predicting protein expression through scRNA-seq data, however, might be unreliable due the great extent of regulation of mRNAs and proteins at post-translational level. Recent technologies [such as CITE-seq (86) and REAP-seq (87)] partially overcame this limitation by combining oligonucleotide-labeled antibodies against cell surface proteins, thus enabling the simultaneous detection of gene expression patterns and protein levels in thousands of single cells in parallel. More than this, significant improvements were recently introduced into mass cytometry techniques (52). Mass cytometry uses antibodies labeled with heavy metals, whose presence and abundance are detected by a mass spectrometer; a key advantage over flow cytometry resides in the simultaneous detection of around 40 parameters per cell, for up to millions of cells, with significantly less spectral overlap. Thus, this high-dimensional assay enables a more thorough characterization and higher resolution of cellular sub-populations and individual cells, which can be especially

TABLE 1 | Overview of selected single-cell technologies for genomic studies.

Method	Overview	Library construction	Features
Single-cell transcriptome sequencing			
Chromium (10× Genomics)	3'-end mRNA transcripts	GemCode (53)	Microdroplet-based method Advantages • automatic cell isolation, cDNA synthesis, and amplification • high number of cells (500-10,000/run) • cell size up to 40 μm • suitable to study individual cells in a large population • use of UMIs mitigates amplification bias Drawbacks • libraries of selected cells cannot be reanalyzed because libraries are mixed after barcoding • diverse coverage across cells (5,000-10,00 reads/cell)
C1 Single-Cell Auto Prep system (Fluidigm)	Full-length cDNA 5′end mRNA transcripts	Smart-seq2 (54) C1-CAGE (55)	Microwell-based method Advantages • automatic cell isolation, cDNA synthesis, and amplification • can perform additional sequencing of libraries in user-selected wells • stable coverage across cells (100-1,000 x 10^6 reads/cell) • suitable to study individual cells in detail Drawbacks • limited number of cells (96-800/run) • limited cell size (up to 25 μm) • no UMI
RamDA-seq (56)	Total RNA (full-length transcripts, long noncoding RNAs and enhancer RNAs)	RamDA retrotranscription	Advantages • information on splicing events and enhancers • possibility of automation with C1 Fluidigm platform Drawbacks • no UMI • high coverage requested • high fraction of ribosomial RNA
Single-cell genome sequencing			
MDA (57)	SNVs	WGA; isothermal amplification	Advantages • >99% genome coverage • reduced representation bias as compared to PCR-based methods Drawbacks • few tens of cells • high rate of allelic dropouts
MALBAC (58)	SNVs, CNVs	preamplification + WGA	Advantages • quasi-linear preamplification reduces WGA amplification bias • 93% genome coverage • 25x mean sequencing depth Drawbacks * few hundreds of cells
Tapestri (MissionBio) (59)	SNVs, CNVs on targeted loci	target amplification + barcoding for parallel processing	Microdroplet-based method • uniform amplification across amplicons • 20x mean sequencing depth • thousands of cells • suitable for clonal architecture reconstruction
Single-cell epigenomics			
scRRBS (60)	DNA methylation		• hundreds/thousands of single-cells • up to 1.5 million CpG sites (10% of genome) • high rate of DNA degradation during bisulfite conversion

(Continued)

TABLE 1 | Continued

Method	Overview	Library construction	Features
scATAC-seq (61)	Chromatin accessibility		• possibility of automation with C1 and Chromium platforms • thousands of cells
Drop-ChIP (62)	Histone modification		Hundreds of cells
Single-cell Hi-C (63)	Chromatin structure		Few tens of cells

UMI, unique molecular identifier; SNV, single-nucleotide variant; WGA, whole-genome amplification; MDA, multiple displacement amplification; MALBAC, multiple annealing and looping-based amplification cycles; CNV, copy-number variation; scRRBS, single-cell reduced representation bisulfite sequencing; scATAC, single-cell assay for transposase-accessible chromatin; ChIP, chromatin immunoprecipitation; Hi-C, high-throughput chromosome conformation capture.

useful when the total number of cells for evaluation is limited. Mass cytometry is particularly suitable for the study of tumor immune microenvironments, because it can characterize novel subpopulations of immune cell subsets and previously unrecognized aberrancies. Markers can be studied in combination, also through unsupervised clustering, and generate signatures that may incorporate relative abundance of different cell subsets, expression levels of different proteins, and/or activation states of various cellular signalling pathways. This can be exploited to discover biomarkers for disease classification and prognostication, and for predicting response to therapy. Mass cytometry also uniquely offer the important opportunity of unbiased identification of HLA-presented neoantigens (88), which are attractive target for immunotherapy as they are expected to drive highly specific and effective anti-cancer immune responses.

Challenges

Along with scientific opportunities, the adoption of single-cell technologies implies dealing with specific experimental and computational/statistical challenges, which are often shared across the different single-cell applications (89).

From the experimental point of view, the generation of single-cell data from a biological sample typically requires some common key steps (67, 89), including dissociation of cells from the tissue of interest, cell purification and isolation, library construction and sequencing. Each step impacts significantly the output results for downstream analyses. For instance, in scRNA-seq protocols, sample preparation and handling have to be carefully planned to avoid unnecessary stressful conditions, which are known to induce extensive cellular responses, thus introducing artifactual modifications of transcriptional states (90). The emergence of microfluidics techniques for cell isolation and combinatorial indexing strategies scaled up the number of cells being sequenced in one experiment and recently enabled multiplexing of different samples. Experimental steps, however, may result in considerable batch effect during later analysis and become the source of technical noise; this might be the case with protocols that use whole genome amplification, or the with carrying over of empty droplets during library preparation, cell doublets or dying cells.

In parallel, recurring computational challenges exist, due to inherent features of the sequencing data. The amount of material sequenced from single cells is considerably less than that available from bulk experiments, which leads to high levels of missing data. Missings may be due to technical dropouts (depending on platform and sequencing depth) or reflect true biological signal (as for variations in expression levels of a gene). This condition requires strategies to impute missing values, which have been more successful for genotype data than for transcriptomic data (89, 91). Conversely, any increase in the number of analyzed cells and features translates in the need of scalable data analysis models and methods. As a further complication, high-dimensional single-cell data have to be processed for easier tractability, while preserving the salient biological signals of the overall dataset.

Another common challenging task is the integration of multiple datasets for comparative analyses across multiple samples (even from different experiments or experimental conditions) (92–94). Computational approaches have been devised to score pairwise correspondences between single cells across datasets, enabling batch-effect correction and identification of populations with common sources of variation. This procedure, however, brings the inherent risk of overcorrection (95) and should be applied cautiously.

Finally, combining multiple types of information (such as DNA, RNA, proteins, epigenomics) on the same cell is crucial to get a more holistic view of cellular processes, but it requires the development of specific experimental settings and dedicated computational strategies to integrate complementary, possibly interdependent measurements. These approaches will be treated in a separate paragraph (see "*Integrating Complementary Cellular Information by Single-Cell Multi-Omics*").

UNRAVELLING THE CELLULAR COMPOSITION OF INTRA-TUMORAL HEALTHY AND PATHOLOGICAL IMMUNE MICROENVIRONMENTS

The intra-tumoral immune microenvironment contains many different cell types, which exert their functions both independently and within cooperative networks (**Figure 1**). Hematopoietic cells include the adaptive (e.g., CD4+ and CD8+ T lymphocytes, B lymphocytes) and innate (e.g., NK lymphoid cells and macrophages) compartments of the immune system along with dendritic and myeloid-derived suppressor cells. Non-hematopoietic cells, instead, comprise mesenchymal stromal cells, adipocytes, osteoblasts, and cells from the vascular and neural niche. Malignant myeloid cells are themselves part of the

immune microenvironment, as they crosstalk with other immune-competent cells (11, 13, 18). A better understanding of the immune tumor microenvironment requires the deconvolution of its cellular composition, which is preliminary for many secondary analyses.

The isolation of blood cancer cells for single-cell analysis is relatively simple as compared to solid tumors, since MN samples are most commonly collected as fresh mononuclear cells (MNC) isolated by Ficoll density gradient-centrifugation of the BM, thus not requiring tissue dissociation and preserving intra-tumoral hematopoietic cells. Non-hematopoietic cells, instead, are by far less abundant in the BM and peripheral blood and may require processing of larger samples (undigested BM or enzymatically digested bones) or specific purification steps, including depletion of the more abundant hematopoietic cells or positive selection using predefined lineage-markers (96). For these reasons, while these procedures have been used for the murine BM (97–99), the human counterpart currently remains uncharacterized.

Innovation Given by Single-Cell Technologies

Single-cell technologies - mostly single-cell transcriptomics and proteomics - allow the analysis of both cell types (defined by phenotypic markers) and functional states, which spares the bias of using predefined lineage-markers (15, 16, 52) and opens innovative perspectives on our ability to classify the cellular components of immune processes. Indeed, emerging evidence from scRNA-based studies suggests that the physiological stages of hematopoietic differentiation, so far identified as discrete and homogenous subpopulations, are instead functionally heterogeneous and display lineage markers that overlap across different cell types, upon different biological conditions (100, 101). Also, the high resolution of single-cell transcriptional and proteomic data enables the recognition of intermediate or transitioning cell states, highlighting the continuity of biological processes. Given the plasticity of the immune system, these features are particularly attracting when applied to study the tumor microenvironment. The possibility of multimodal characterization, as obtained by quantifying both RNA and surface protein abundance (86), is especially promising for the discovery of previously unknown cell-subtypes and associated markers or gene-signatures (94).

Challenges

Different computational approaches have been developed for the reconstruction and imputation of cell identities within both tumor and normal immune populations. A first, reference-free method consists in unsupervised clustering of scRNA-seq data followed by manual cell-type annotation according to cluster-level expression profiles (92, 102, 103). This approach, however, is time-consuming, limited in reproducibility and suffers from limited scalability to large datasets (104).

Specific cell-states might be more easily identified through a supervised analysis guided by an appropriate reference dataset. Such approach relies on mapping the query dataset onto an existing reference from pre-annotated and purified cell types, ideally characterized *via* the same technology (105). To this end, efforts to characterize the landscape of each human tissue and cell type at single-cell level are under the way, converging on the Human Cell Atlas project (106). Cell atlases are reference 'coordinates' that allow for the systematic mapping of cell types and states; for instance, comprehensive single-cell reference datasets are being developed for the human healthy BM and immune system, including both steady-state and perturbed conditions (107–113) (**Table 2**). The creation of a detailed "table of immune elements" including all immune types and states would be particularly useful to the purpose of classifying tumors according to immune subtypes, to make prognostic correlations and guide therapeutic assignment (16).

However, this is currently hard to achieve, due to the inherent plasticity of the immune system and the high variability between individuals. Also, tumor and microenvironment cells may show several and dynamically changing aberrancies, as compared to the healthy tissue counterpart. This poses a limit to our ability to recognize rigidly distinct cell types. In fact, as less-characterized disease entities and large patient cohorts are being studied, many yet-uncharacterized immune cells and pathways will emerge, further challenging current models of immune identity. Analytical pipelines should account for the uncertainty of mapping unknown cell type/state; for instance, a recently published tool for cell-type annotation (CellAssign) (116) leverages prior knowledge of lineage-specific marker genes to annotate scRNA-seq data into predefined or novel cell types, based on a probabilistic model. Orthogonal validation with flow and mass cytometry data, as well as integrating transcriptional data with protein expression and scDNA-seq, are expected to further refine current single-cell classifications.

Application in the Current MN Research

The ability to assign cell identity in hematopoietic tissues has been validated for both scRNA-seq and mass spectrometry, although the latter is more precise in distinguishing immune cells with closely overlapping transcriptional profiles, such as T and NK cell subsets (108). In a recent work, such a technology has been used to classify subsets of NK cells in 48 newly diagnosed AML and 18 healthy subjects (117). AML samples showed an accumulation of aberrant CD56−CD16+ NK cells, which was associated with an adverse clinical outcome and decreased overall survival. High-dimensional characterization of this NK subset highlighted a decreased expression of some receptors required for antileukemic activation, such as NKG2D, DNAM-1, and CD96; the Authors concluded that the accumulation of CD56−CD16+ NK cells, combined with the reduced frequency of conventional NK subtypes, may be the consequence of escape from innate immunity during AML progression. Subsets of monocytes were found to be decreased in MDS BM, which mediated the expansion of a specific T cell pool (118). Mass spectrometry also enables recognizing aberrant myeloid differentiation patterns, as recently demonstrated on MDS samples compared to healthy donors (119).

Importantly, dissecting the cellular composition of the immune microenvironment can be applied to highlight changes across disease and/or treatment phases. For instance, the seminal study

TABLE 2 | Selected scRNA-seq datasets for the healthy and pathological human immune microenvironment.

Dataset	Tissue and cell populations	Condition	N cells/N individuals	Core features
Human Cell Atlas (107)	BM MNC	Healthy	103,000/8	• Marker genes for cellular classification and trajectories • Interactive web portal available
GSE120221, GSE120446 (108)	BM MNC	Healthy	76,645/20	• Largest number of individuals • Broad age range of donors • Orthogonal validation by flow and mass cytometry • Discrepancies in T and NK subsets
Human Cell Landscape (113)	BM MNC PB MNC	Pathologic (cytopenias) Healthy	8,704/2 17,331/4	• Atlas for cell-type identification • Interactive web portal available • Low sequencing depth
TMExplorer (114)	BM MNC	Pathologic [AML (102), CML (115)]	AML: 38,410/40CML: 2,287/20	• Collection of microenvironment datasets from 12 different cancer types • R package interface to access datasets and metadata • Provides gene expression data, cell type annotations and gene-signature information
GSE126030 (110)	T cells (lungs, lymph nodes, BM and PB)	Healthy (resting and activated)	50,000/4	• Reference map of human T cells functions related to tissue site vs PB • Applied to score distinct tumor-associated phenotypes

BM, bone marrow; MNC, mononuclear cells; AML, acute myeloid leukemia; CML, chronic myelogenous leukemia; PB, peripheral blood.

from van Galen et al. (120) employed scRNA-seq to characterize BM MNC from 16 AML patients at diagnosis and during treatment. Results showed great variations in the proportions of cell types during the clinical course, consistently with immunohistochemistry; AML BM generally presented with fewer cytotoxic T cells than healthy donors, yet greater numbers of Tregs, which confirmed previous findings and established the existence of an immunosuppressive tumor microenvironment in AML. Further mechanistic studies are needed to link changes in immune subsets or immune targets to dynamics of relapse. As LSC are deemed to be responsible for AML relapse, their identification and characterization is particularly critical for the development of efficient immunotherapies. In this regard, Levine et al. published PhenoGraph, a software for analyzing mass cytometry data that enabled better identification and characterization of LSC (102). Moreover, one recent paper used mass cytometry and RNA-seq to feature CD200 as a LSC–specific immune checkpoint overexpressed in AML LSC (121).

DISCOVERING FUNCTIONAL PHENOTYPES, MOLECULAR MECHANISMS AND BIOMARKERS

Immune responses are plastic and can be extremely heterogenous, depending on tissues, environmental contexts, healthy or pathological conditions (122–124). Commonly-used small sets of markers fail to describe the full spectrum of functional states and inherent gene expression programs, which, instead, can be optimally captured by high-dimensional single-cell analyses (16, 125, 126). In contrast to marker-based methodologies that seek for rigid separation of defined entities, single-cell technologies allow to set broadly inclusive experiments without *a priori* marker selection, enabling data-driven analyses on all cell populations involved in a given condition. The first large-scale 'ecosystem-wide' scRNA-seq study was performed by Tirosh et al. on melanoma patients (127). In the context of MN research, Van Galen's paper is a paradigmatic example for this approach.

In order to define functional subsets among AML-associated immune cells, Guo et al. (128) re-analyzed the scRNA-seq dataset from the aforementioned study (120), focusing on non-blasts AML cells and 4 healthy BM donors. The study concluded that AML coexists with highly heterogeneous immune effectors and suppressive subsets, which showed common features of functional aberrancy and exhaustion of possible prognostic significance. To the same aim, one group developed an integrated functional approach coupling mass cytometry coupled to cytokines profiles (129) and applied it to 49 AML patients, confirming functional impairment of AML-associated T cells mediated by immune checkpoints (130). Single-cell transcriptomic has been applied in both animal models (131) and cancer patients (132, 133) to investigate changes in the tumor microenvironment upon treatment with immune checkpoint inhibitors, to the end of finding response-associated signatures. Following the same approach, one small study used mass cytometry on serially collected samples from 9 AML patients treated with HMA and avelumab, a PD-L1 inhibitor; the ratio of CD4/CD8 and composition of residual T cells emerged as the most important predictors of response to treatment, and AML cells expressed a variety of other immune checkpoints (such as PD-L2, OX40, TIM3) that might be considered for future combination therapy (134).

Regarding the direct role of malignant cells in shaping the immune microenvironment, van Galen et al. found that AML cells exhibited marked intra-tumoral heterogeneity, with "primitive-like" cell-types showing dysregulated co-expression of stemness and myeloid commitment genes, and more differentiated "monocyte-like" cell-types showing immunomodulatory properties linked to T-cell suppression (120). These two different cell states were obtained by classifying malignant cells according to their similarity to normal hematopoietic cell types and resulted

associated to specific gene signatures. Specifically, the direct comparison of leukemic versus normal cells revealed 296 genes that were preferentially expressed in malignant monocyte-like cells from one or more AML samples, including genes associated with myeloid-derived suppressor cells, antigen presentation components and leukocyte immunoglobulin-like receptors, such as tumor necrosis factor and interleukin-10 pathway genes or regulators of reactive oxygen species. Although expression of these genes markedly varied among patients, most samples expressed high levels of CD206/MRC1 and CD163, two surface markers associated with immunosuppressive myeloid cells (132), whose expression was also found to be associated with poor outcome in the TCGA AML-cohort (135). Thus, though highly heterogenous, the different expression programs identified by scRNA-seq might converge on common functional pathways of prognostic and therapeutic interest. Tightly correlated gene modules can reveal how specific pathways and cellular functions (e.g., proliferation, antigen presentation, exhaustion, differentiation, etc.) are distributed across cell types, thus defining specific immunomodulatory patterns. Thereafter, detailed analyses can be restricted to cells expressing common transcriptional modules, an approach that may lead to the identification of new surface markers, immunoregulatory molecules or tumor-specific antigens for therapeutic exploitation. A catalogue of AML-specific antigens and corresponding HLA ligands has been previously obtained by mass spectrometry characterization (88).

Additional molecular mechanisms for tumor-related immune changes include epigenetic dysregulation, which may affect T cell differentiation and functions by remodeling active-enhancer landscape and transcription factor binding (136–141). One notable example is the documented increased chromatin accessibility at the enhancer site of PDCD1, the gene encoding the checkpoint inhibitor PD-1 (142). A proper T cell functionality is needed to convey the effect of many immunotherapeutics; in this context, a recent study applied scATAC-seq to characterize chromatin profiles of ~200,000 single cells in both peripheral blood and basal cell carcinoma samples before and after PD-1 blockade therapy, which identified chromatin regulators of therapy-responsive T cell subsets at the level of individual genes and regulatory DNA elements (143). This is a critical field of investigation in MN research, since studies have shown that during disease progression the adaptive immune microenvironment switches from cytotoxic to regulatory, suggesting the appearance of immune tolerance (19, 20) and immune-escape mechanism (24); also, T cell exhaustion has been recognized as a cause of failure of autologous CARTs (136).

SHAPING THE IMMUNE MICROENVIRONMENT BY CELL-TO-CELL INTERACTIONS

In either the physiological or tumor microenvironments, immune cells should not be considered as functionally separate entities, as immune processes are mediated by networks of tissue-resident and/or circulating cell types. These interactions respond dynamically to environmental stimuli, possibly driving disease progression and sensitivity or resistance to immunotherapies (137). Thus, identifying critical signalling pathways underlying the network of immune-cell interactions is critical to predict cancer phenotypes, identify druggable genes or manipulate the immune system for therapeutic purposes (138) (for example, by genome editing (139) and cell engineering to control how pairs of cells interact). A critical starting point is the analysis of the coordinated expression of known ligands and their cognate receptors across different cell types. This can be achieved, for example, by combining information from protein–protein interaction databases (140, 141, 144, 145) and single-cell technologies. Alternative strategies for deciphering cell–cell interactions incorporate downstream signalling, gene regulatory networks and metabolite secretion coupled with advanced statistical methods [reviewed by Armingol et al. (137)].

A further application of single-cell transcriptomic is represented by the integration of transcriptomic profiles of single cells with their spatial position in tissue contexts, an approach that allows mapping tumor cells with respect to other cell types or relevant tumor areas, such as vessels or the tumor edge, and that can be used as a guide for refining cell type identification, monitoring cell abundance, behavior and interactions upon different disease or treatment phases (146–148). Various technologies and computational tools exist to profile hundreds to thousands of transcripts at different resolutions, which have been mostly applied to generate spatial transcriptomic maps of solid tumors (149–157). Of note in the context of MN, Baccin et al. developed LCM-seq, a laser-capture microdissection and sequencing protocol specifically designed to capture the three-dimensional organization of BM cell populations and their location within distinct niches (98). Alternative approaches are based on the recovery of specific neighboring cells, as in the PIC-seq (158) and NICHE-seq (159). In the PIC-seq, tissues are mildly dissociated to retain *in situ* cellular structures, physically interacting cells (PICs) are then recovered by FACS-sorting using specific markers and subjected to scRNAseq (158). In the NICHE-seq, instead, cells interacting with specialized niches within organs are identified in model systems using photoactivatable fluorescent reporters (159).

Finally, alternative single-cell technologies are emerging to overcome the limits of RNA analyses, e.g. the lack of information on post-transcriptional and post-translational processing (160). Spatial resolution, in fact, can also be achieved by immunohistochemistry coupled to mass spectrometry, a technology that allows the detection of up to 40 proteins with a subcellular resolution of 1μm (161, 162). One recent study, as an example, applied multispectral imaging to understand the spatial relationship between CD34+ hematopoietic cells and immune cell subpopulations in the BM of MDS and secondary AML samples. CD8+ and FOXP3+ T cells were regularly seen in close proximity of CD34+ MDS/AML, yet not in controls; this finding correlated to blast counts but not to genetics, and the frequencies of immune cell subsets also differed in MDS and sAML when compared to controls, providing novel insights in the dynamics of immune deregulation during MN evolution (163). Methods that allow an accurate view of intercellular communication include Nativeomics (164), which

detects intact ligand–receptor assemblies using mass spectrometry, single-cell proteomics (165) and INs-seq (166), which couple scRNA-seq with intracellular protein measurements to simultaneously profile transcription factors, signalling activity and metabolism. In all cases, mechanistic hypotheses generated by computational inferences of single-cell data should undergo careful validation using orthogonal technologies, including confirmation of the expression of candidate proteins (e.g., with proteomics, enzyme-linked immunosorbent assay, western blot or immunohistochemistry), or direct visualization of interacting cells.

INTEGRATING COMPLEMENTARY CELLULAR INFORMATION BY SINGLE-CELL MULTI-OMICS

Several emerging single-cell technologies are committed to recording complementary types of cellular and molecular information from the same cell, including its transcriptome, genome, epigenome, proteome and spatial localization (**Table 3**). The application of multi-omics approaches enables the integration of different molecular layers within single cells at the same time and, possibly, with respect to their surrounding environment, thus providing an unprecedent description of the cancer ecosystem.

Genomic Data Combined With Transcriptome/Proteins

Because of the prominent role of genetics in cancer biology and clinical management, most efforts have converged on the development of technologies that jointly capture a single cell's genomic profile along with its phenotypes defined by either surface markers or functional features. A number of strategies have been published, each with its own strengths and limits (**Table 2**), which hold enormous potential for the study of the immune microenvironment in MN. Direct approaches analyzing genomic DNA along with mRNA are technically limited by the low DNA sequencing coverage that can be achieved at single-cell level, and are consequently hampered in their sensitivity (167, 175). This limit can be circumvented using indirect approaches, which aim at identifying expressed genomic variants in scRNA-seq data and allow the analysis of high numbers of cells, thus preserving the biological heterogeneity of the sample (115, 120, 169–171, 176, 177). Experimental and computational methods are under continuous development to achieve the broadest applicability. Another approach was featured in the seminal paper by Miles et al. and consists in combining scDNA-seq with cell-surface protein expression, which the Authors exploited to characterize CH, MPN and AML patients (3).

A first application of combined genomic/phenotypic approaches is the distinction of neoplastic from non-neoplastic cells within tumors, which remains inaccurate when solely based on the expression of specific genes or surface markers, due to the occurrence of technical artifacts in scRNA-seq or aberrant expression in either cell-populations. Mapping single-nucleotide variants and/or copy-number variations across phenotypically defined cells can enhance the confidence of such imputation (51). In principle, the acquisition of thousands of unselected cells (e.g., total CD34+ or BM/PB MNCs) would allow the characterization of both neoplastic and non-neoplastic/immune compartments in parallel, to study the functional properties specific to each compartment and clone. Only a few studies have exploited such approaches in MN, focusing on the mapping of single mutations. Giustacchini et al. obtained scRNA-seq profiling of BCR-ABL positive vs negative HSC from patients with chronic myeloid leukemia, and found restricted expression in BCR-ABL negative HSC of inflammatory genes with suppressor functions on HSC (i.e., IL6 and its downstream mediators, TGF-β and TNF-α pathways) (176). Another study used transcriptional and mutational single-cell data to feed a machine-learning model for the identification of malignant vs non-malignant AML cells, and found heterogenous malignant cell-types whose abundance correlated with genotypes and survival (120). Indeed, future studies employing multi-omics single-cell strategies will be instrumental to detail the molecular mechanisms by which tumor cells harboring specific genomic alterations interact with their own immune microenvironment, potentially driving immune escape and response to immune-therapies. Preliminary evidence supports this perspective with different mechanisms, such as the expansion of specific immune populations [e.g. in MDS, where chromosome 8 trisomy and consequent WT1 overexpression fuel CD8+ expansion (178)]; the up/downregulation of immune effectors activity [e.g., fusion proteins PML-RARα and AML1-ETO impair NK cytolytic activity by downregulating their receptor's ligand CD48 on AML cells (179)]; enhancement of specific signalling and immune activation pathways [such as for mutations in JAK2 (180–182) or spliceosome genes (12, 183), which are early genetic events in MN, or for signalling effector mutations, which occur in late AML subclones (3)].

Immunomodulation by either tumor or micro-environment cells has been recognized as a further mechanism that influences the dynamics of clonal expansion in MN. Dysregulation of innate immune and inflammatory cells and signalling contributes to the competitive advantage of CH-mutant HSC during aging, particularly in the context of TET2, DNMT3A and JAK2 mutations (5, 6, 182, 184–186). In addition, mutations associated with CH are nearly always present in circulating innate immune cells and, less frequently, in the T and B lymphoid compartment, which might affect immune surveillance against emerging tumor cells and response to immune therapies (187). Understanding the molecular and cellular relationships between the immune microenvironment and preleukemic clones remains a crucial step to efficiently track - and possibly intercept - the evolution to AML, as the risk of leukemic transformation varies significantly across CH-individuals and pre-leukemic patients and is associated to diverse synergistic combinations of mutations. Although not specifically focusing on the immune microenvironment, Miles et al. observed differential skew to the myeloid, B or T cell

TABLE 3 | Overview of selected single-cell multi-omics methods.

Method	Overview	Throughput (N cells with multiomics characterization)	Features	Limits
Genome + Transcriptome				
G&T-seq (167)	Experimental methodPhysical separation of RNA and DNA with subsequent parallel amplification and sequencing	-/+	• CNV (direct scoring) • SNV (direct scoring) • Full-length transcriptome (including fusions)	• Low throughput • Low coverage
HoneyBADGER (168)	Computational methodIntegration of normalized scRNA-seq profiles as compared to:- putative diploid reference of comparable cell type- allelic frequency of heterozygous germline SNP		• CNV (inferred from scRNA-seq) • LOH (inferred from scRNA-seq) • Transcriptome	• No information on DNA alterations smaller than 10 megabases • Best performance with scRNA-seq protocols that achieve full-transcript coverage
Scmut (115)	Computational methodVariant calling implemented to both scRNA-seq and WES data		• Expressed SNV (inferred from scRNA-seq) • Transcriptome	• Relies on quality of the alignment and transcript annotation • Detection sensitivity of a mutation depends on the corresponding gene expression • High rate of false positives and negatives
Van Galen et al. (120)	Experimental methodTarget amplification of transcript and locus of interest, integration with long-read sequencing	+	• Expressed SNV (inferred from scRNA-seq), insertions, deletions and fusions • Transcriptome	• Depends on expression for mutation detection
Petti et al. (169)	Experimental methodVariants scored in WGS and then detected in scRNA-seq data	++	• Expressed SNV (inferred from scRNA-seq), indels • Transcriptome • High-throughput that preserves biological complexity • General applicability	• 5'-end bias • Heavily depends on expression for mutation detection • No clonal reconstruction (wild-type status not defined)
GoT (170)	Experimental methodTarget amplification and circularization of transcript and locus of interest	++	• Expressed SNV (inferred from scRNA-seq) • Transcriptome • Overcomes end bias by transcripts circularization	• Depends on expression for mutation detection (mitigated by target amplification)
TARGET-seq (171)	Experimental methodRelease of gDNA and mRNA followed by target amplification	+	• SNV, indels • Transcriptome • Parallel information from coding and non-coding DNA • Clonal reconstruction • Low allelic dropout	• End-bias with 'high-throughput' protocol
Genome + Proteins				
Tapestri (Mission Bio, Inc) (3, 59)	Experimental methodMicrofluidic workflow for target amplification of DNA amplicons and proteins	++	• SNV • CNV • Cell-surface proteins • Standardized commercial platform • Customizable gene and antibody panel • Clonal reconstruction at single-cell level • Integrated pipeline for multi-omics analysis	• No information on gene expression and regulatory networks
Transcriptome + Epigenome				
scM&Tseq (172)	Experimental methodPhysical separation of RNA and DNA, which allows for bisulfite conversion of DNA without affecting the transcriptome	-/+	• Transcriptome • Methylome	• Low sequencing depth • Low throughput

(Continued)

TABLE 3 | Continued

Method	Overview	Throughput (N cells with multiomics characterization)	Features	Limits
Paired-seq (173)	Experimental methodLigation-based tagging of both open chromatin fragments and cDNA	+++	• Transcriptome • Chromatin accessibility • Extremely high throughput (up to millions of cells)	• Non optimal library complexity
Transcriptome + Proteins				
CITE-seq (86)	Experimental methodAntibody-bound oligos act as synthetic transcripts that are captured during most large-scale oligodT-based scRNA-seq library preparation protocols	++	• Transcriptome • Surface proteins • Adaptable to RNA interference assays, CRISPR, and other gene editing techniques. • No upper limit in number of antibodies	• No spatial information • No intracellular proteins
PLAYR (174)	Experimental methodLabelling of RNA and proteins with isotope-conjugated probes andantibodies for mass spectrometry detection	+	• Transcriptome • Surface and intracellular proteins	• No spatial information-Limited number of proteins
Transcriptome + T cell receptor				
Tessa (111)	Computational methodBayesian model trained on bulk and scRNA-seq of TCR and T cells		• TCR sequences • Transcriptome	• No information on splicing isoforms
RAGE-seq (73)	Experimental methodCombined targeted capture and long-read sequencing of full-length transcripts	++	• TCR/BCR sequences • Transcriptome • Splicing isoforms • Accurate antigen receptor sequences at nucleotide resolution • Information on splicing isoforms • Adaptable to any scRNA-seq platform using 3′ or 5′ cell-barcode tagging	• Low recovery of cell barcodes due to low accuracy of long-read sequencing • Possible PCR artifacts

CNV, copy number variation; LOH, loss of heterozygosity; SNP, single nucleotide polymorphism; SNV, single nucleotide variant; WES, whole exome sequencing; WGS, whole genome sequencing; gDNA, genomic DNA; cDNA, coding DNA; PCR, polymerase chain reaction; TCR, T cell receptor; BCR, B cell receptor.
-/+, tens of cells; +, tens of cells; ++, hundreds of cells; +++, thousands of cells.

lineages, depending on which CH gene was mutated; genotype-driven changes in cell-surface protein expression were also reported in the leukemic phase, with signaling effector mutations leading to increased CD11b expression (3). In established AML, the same information might instead aid in understanding the molecular basis of chemoresistance and the jeopardized response to various immunotherapeutic strategies. In this context, common AML-associated translocations (AML1-ETO, DEC-CAN, PML-RARα, BCR-ABL) or mutations (FLT3-ITD, NPM1, $IDH1^{R132H}$, mutations in spliceosome genes and some TP53 hotspots, JAK2, CALR) produce MN-specific immunogenic proteins that may become ideal antigen targets for the development of immunotherapies (12, 188).

Transcriptomic Data Combined With T Cell Receptor Information

Finally, the T- or B-cell receptor repertoire of individual lymphocytes can be scored in parallel with their gene expression profiles, using properly devised experimental and computational methods on scRNA-seq data (73, 111), thus providing connections between lymphocyte clonality and functional responses, which can inform the discovery of antigen-reactive antibody candidates, antigen targeting efficiency of T cell clonotypes, and evolution and response to various immunotherapies.

Transcriptomic Data Combined With Proteomic Data

Technologies are also available that allow concomitant analyses of protein and transcripts at single-cell levels. They are particularly useful to investigate post-translational regulatory events and to relate functionally-defined phenotypes to protein markers, which might assist tumor classification, biomarker assessment for prognostic purposes, and development of therapeutic targets. Surface proteins can be detected by implementing gene-expression libraries with oligonucleotide-labeled antibodies, as for the above-mentioned CITE-seq (94) and REAP-seq (87). Notably, the CITE-seq workflow is compatible with the most frequently used commercial platforms for scRNA-seq, and there's no upper limit to the number of antibodies that can be used. PLAYR, instead, relies on mass spectrometry and allows the detection of up to 40 proteins (174). This technique might be critical when high-quality antibodies are unavailable; also, it can be deployed for index sorting and imaging approaches to enable spatial

resolution. Using other techniques, intracellular proteins can be accessed as well with scaling throughput (189, 190).

Transcriptomic Data Combined With Epigenomic Data

Various single-cell technologies are becoming available for the simultaneous analyses of expression, DNA methylation or chromatin accessibility (**Table 2**). This level of investigation would be particularly important to characterize MN, as epigenomic changes occurring in either tumor or immune cells are relevant to aberrant hematopoietic differentiation (191), genetic-independent disease progression (32) and immune functions (132, 142, 192–194). Moreover, HMA [which are typically used in older MDS or AML patients (29)] have been found to potentiate the immunogenicity and the immune recognition of neoplastic cells by up-regulating the expression of molecules that are crucial in host-tumor immune interactions (195–197), which makes them an ideal partner for combination with immunotherapeutic agents (23).

Triple-Omics

Finally, although preliminary, recent studies have reported the development of single-cell triple-omics sequencing techniques, such as for the joint capture of the transcriptome, genome and DNA methylome [scTrio-seq (198)]; transcription, DNA methylation and chromatin accessibility [scNMT-seq (199)]; or transcription, chromatin accessibility and surface proteins (200).

OPEN PERSPECTIVES AND FUTURE DIRECTIONS

Despite the number of single-cell approaches that have been developed in the last few years, and the fewer proof-of-concept applications, most of the relevant questions in the field of MN remain to be addressed (**Figure 2**).

So far, single-cell studies aiming to describe the immune microenvironment in MN have mainly focused on AML, while a thorough characterization of CH, MDS and MPN samples is currently lacking. To formally address questions about disease progression, therapeutic resistance and relapse, more informative research should be performed by prospective monitoring of MN evolution. Following the history of MN patients at multiple time-points should allow tracing of evolving cellular clones across different disease stages, as well as residual disease [scored by immunophenotypic markers, genetic markers or both (6)] after treatment and donor chimerism after alloHSCT, in the context of surrounding immune cells. It is envisioned that such prospective biobanks for single-cell characterization might uncover immune-related pathways that can be targeted for reducing the selective advantage of the CH or MDS transforming clones, an approach supported by proof-of-concept studies in murine models (185, 186). Also, the same strategy could detail the molecular mechanisms of resistance and immune evasion and monitor variability in treatment response. Finally, given the association of immunomodulatory features with both disease progression and survival, there is also a rationale for studying the inclusion of immunologic parameters to refine prognostic models currently used for MDS (201, 202) and AML (22, 29, 203) patients.

Resistance to treatment (including chemotherapy or HMA, target therapies and immunotherapies) represents the main cause for poor survival in AML, which is the final stage of the MN's natural history (29, 30, 46). Resistance and relapse involve genetic and epigenetic dynamics of cell clones in parallel with changes in the immunomodulatory properties of both tumor and immune cells (204–207), whose interplay can be best understood by single-cell multi-omics approaches. Novel single-cell approaches to tackle therapy-resistant cells in model systems include the use of expressed barcodes, which enable the simultaneous recording of clonal evolution and transcriptional phenotypes, eventually coupled to genetic perturbations (74, 208, 209), to study mechanisms of immune evasion. Similarly, other methods can score specific cell clones (including HSC, preleukemic and leukemic stem cells) *via* lineage barcoding and tracing (101, 210, 211), while pulse-chase, inducible lineage tracing methodologies can record past events, such as cell divisions, enabling analyses of cell cycle properties (212, 213). LSC are more frequently quiescent (i.e., not proliferating) than normal HSC, a state that may mediate chemoresistance and relapse; regulation of quiescence can be driven by cell-autonomous genetic or epigenetic changes, but also interactions with the BM immune microenvironment (11, 214, 215), which provides another important hint for clinical translation.

Finally, a further major challenge in MN-related research is the development of effective immunotherapeutic approaches. As discussed above, scRNA-seq and mass cytometry have the capability to identify cell populations with specific functional properties in both tumor and immune compartments. Describing associated molecular markers might aid the process of selecting target antigens in the design of immunotherapies, especially when scRNA-seq is coupled to surface proteins detection in CITE-seq (86) or other platforms for the analyses of cell-to-cell and spatial interactions (137). Since MN are not featured by a single and common surface-antigen with druggable characteristics, as it is, for example, CD19 in B lymphoblastic leukemia (216), multi-omics represent promising strategies to identify different combinations of candidate targets and/or involved pathways.

With the advancement of innovative methodologies, the number and scale of publicly available datasets are continuously increasing (217–220); this offers the opportunity to integrate and interrogate multiple datasets for the validation of previous discoveries or, conversely, the generation of new hypotheses to be experimentally validated, and will possibly allow the construction of a specific cell-type atlas for both cancer and immune cells. Proper curation, quality control and reliable computational strategies for integration are essential to the full exploitation of available data. However, comprehensive integration is challenging because datasets are typically generated through a variety of different approaches and heterogeneous study designs

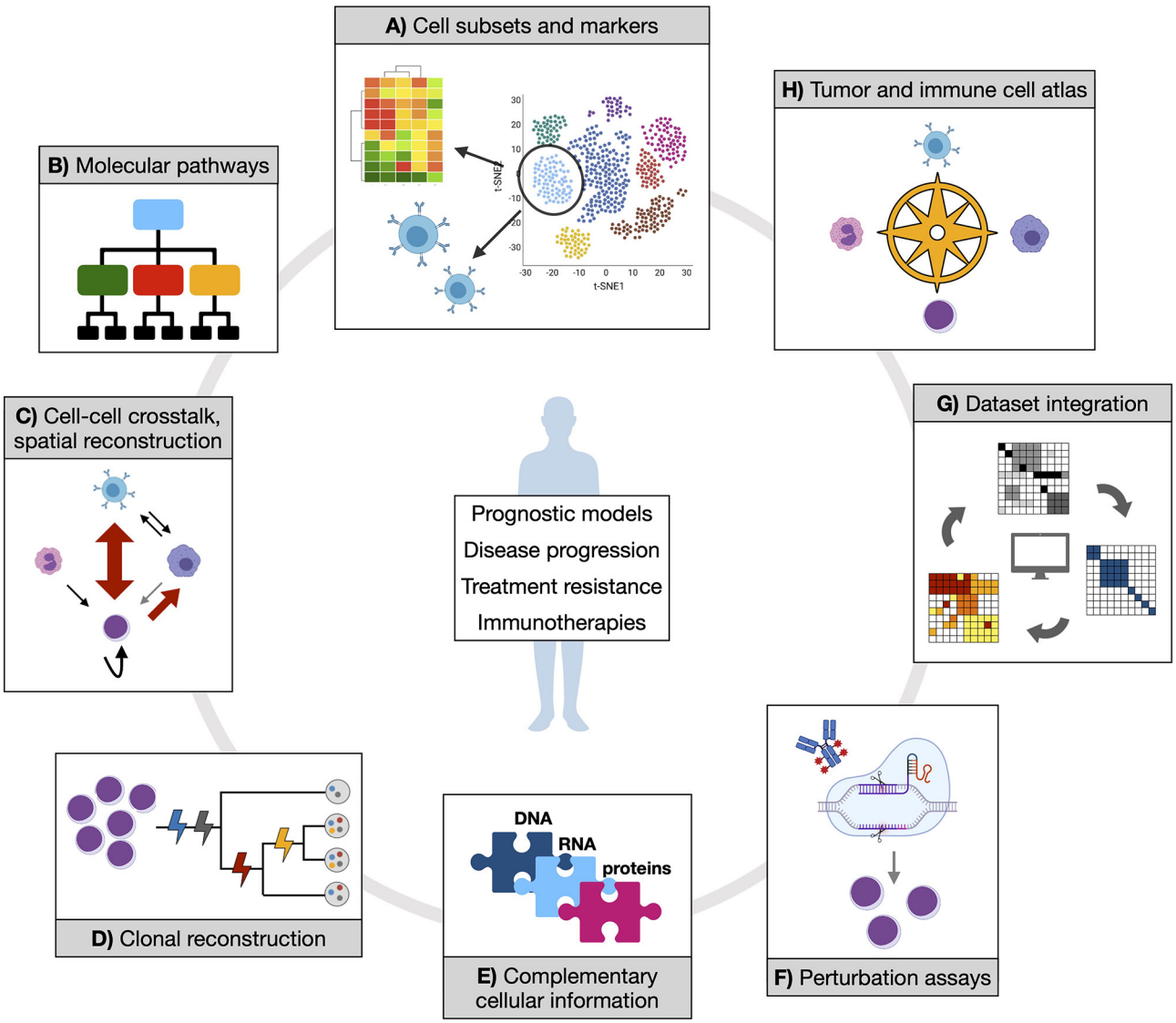

FIGURE 2 | Opportunities of applying single-cell technologies to characterize myeloid neoplasms. Established and novel single-cell technologies can provide manifold information to address clinically relevant questions and contribute to therapy development. **(A)** Isolating cell subsets from transcriptional data could score functional populations (whose markers can be defined in the same context by either gene expression or proteomic data) that might be associated to prognostic features or treatment response. Also, T or B cell receptor clonality can be studied in parallel with associated transcriptome, which would shed light on expansion dynamics of T and B populations in physiology and tumor or upon treatment. **(B)** Inferring molecular pathways (at gene expression or epigenetic level) from such populations might reveal distinct or convergent functional modules, potentially simplifying disease heterogeneity with implications for therapeutic exploitation. **(C)** Cell-cell crosstalk and spatial reconstruction by transcriptomics are fundamental notions to score cancer and immune cells interactions in their proper environmental context, enabling more precise mechanistic and regulatory insights. **(D)** Clonal reconstruction is one core objective of single-cell DNA analysis in myeloid neoplasms, and a mainstay to understand (and potentially prevent) disease evolution. **(E)** Different coexisting molecular layers can be complemented, experimentally and/or computationally, to uncover previously hidden information and mechanistic hypotheses. **(F)** Perturbation assays offer experimental ways to tackle specific functional processes (such as drug response), which can be further dissected by coupling experimental read-out with omics. **(G)** Integration of different datasets are expected to increase statistical power and accuracy of previous observations. **(H)** All of the generated knowledge might enable the creation of an atlas for tumor and immune cell types and states, which would represent a comprehensive reference resource for future studies.

(95). To this aim, achieving standardization of experimental protocols will play an important role. Ongoing and future efforts are committed to identify and benchmark optimal computational methods for data integration, and to improve data sharing and accessibility (221).

CONCLUSIONS

In conclusion, although very few data exist specific to MN, single-cell technologies - especially those providing multi-omic measurements of the same single cell - hold the promise to yield

comprehensive insights into how pre-leukemic and leukemic cells interact with the different players of the associated immune microenvironment. The spreading availability and scaling of the various single-cell approaches is expected to enable the characterization of large clinical cohorts involving patients with different MN types, upon different treatment conditions, as well as more focused experimental models. Despite many challenges to solve, these efforts will build a detailed ecosystem-level picture of MN to help highlight new hypotheses and research directions, inform dynamics of progression, select targeted drugs and rational combinations, and predict efficacy of immunotherapy.

AUTHOR CONTRIBUTIONS

CC and PGP designed the research. All authors contributed to writing and reviewing the manuscript. All authors approved the final version of the manuscript.

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