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CD8$^+$ T cells and IFN-γ induce autoimmune myelofibrosis in mice

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ABSTRACT

Myelofibrosis usually occurs either as a part of a myelodysplastic syndrome or in conjunction with neoplasia. It is not commonly thought of an autoimmune disease. We reported that p40$^{-/-}$IL-2Rα$^{-/-}$ (interleukin-12p40 and interleukin-2 receptor alpha double knockout) mice, a mouse model of human primary biliary cholangitis, exhibited features consistent with autoimmune myelofibrosis, including anemia associated with bone marrow fibrosis, and extramedullary hematopoiesis (EMH) including LSK (Lineage$^-$c-Kit$^+$Sca-1$^+$) cells in spleen, liver and peripheral blood. There were also increased LSK cells in bone marrow but they demonstrated impaired hematopoiesis. Importantly effector memory T cells that infiltrated the bone marrow of p40$^{-/-}$IL-2Rα$^{-/-}$ mice manifested a higher ability to produce IFN-γ. CD8$^+$ T cells, already known to play a dominate role in portal inflammation, were also key for bone marrow dysregulation and EMH. IFN-γ was the key cytokine that induced bone marrow fibrosis, bone marrow failure and EMH. Finally anti-CD8α antibody therapy fully protected p40$^{-/-}$IL-2Rα$^{-/-}$ mice from autoimmune myelofibrosis. In conclusion, our results demonstrate that CD8$^+$ T cells and IFN-γ are associated with autoimmune myelofibrosis, a finding that may allow targeting of CD8$^+$ T cells and IFN-γ as a therapeutic targets.

1. Introduction

Primary myelofibrosis is a myeloproliferative neoplasm (MPN) characterized by stem cell-derived clonal myeloproliferation, bone marrow fibrosis, anemia, splenomegaly, extramedullary hematopoiesis (EMH), cachexia, leukemic progression and shortened survival [1–3]. Most primary myelofibrosis patients have mutations in *JAK2*, *CALR*, or *MPL* [1]. Myelofibrosis is not always malignant [4], and there are benign causes that occur in association with well-defined autoimmune disorders, particularly systemic lupus erythematosus (SLE) [5]. These cases are referred to as autoimmune myelofibrosis, and may accompany Sjögren's syndrome [4], rheumatoid arthritis [6], ulcerative colitis [7], or primary biliary cholangitis [8]. Autoimmune myelofibrosis patients usually have lymphocyte infiltration in BM and can be treated by corticosteroids [9]. Several murine models of primary myelofibrosis as part of a myeloproliferative neoplasia have been described including JAK2^{V617F} knock-in mice [10], Gata-1^{low} mice [11], and SMRT^{mRID} mice [12]. However neither a mechanism nor an autoimmune myelofibrosis model has been described.

Most myelofibrosis patients have the JAK2^{V617F} mutation; constitutive expression of JAK2^{V617F} in mice can also induce myelofibrosis [10]. However most people carrying the JAK2^{V617F} mutation do not develop myeloproliferative disorders [13], and not all subjects with myeloproliferative disorders have the *JAK2*, *CALR*, or *MPL* mutations, indicating that there are other factors that participate in the pathogenesis of primary myelofibrosis. Patients with primary myelofibrosis have reduced frequency of peripheral blood and splenic CD4$^+$IL-2Rα^{bright}CD127^{low}Foxp3$^+$ regulatory T cells [14,15]. Serum soluble interleukin 2 alpha (sIL2Rα) in

Abbreviations: BM, bone marrow; CFU, colony forming unit; EMH, extramedullary hematopoiesis; HCT, hematocrit; HGB, hemoglobin; HSC, hematopoietic stem cell; IFN-γ, interferon γ; IL-2Rα$^{-/-}$, interleukin-2 receptor alpha knockout; JAK, Janus kinase; LSK cells, Lineage$^-$c-Kit$^+$Sca-1$^+$ cells; MNCs, mononuclear cells; p40$^{-/-}$, interleukin-12p40 knockout; PB, peripheral blood; PLT, platelet count; RBC, red blood cell count; Th1, T helper 1; Treg, regulatory T; WBC, white blood cell count.

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myelofibrosis patients is significantly elevated compared to patients with other myeloproliferative neoplasms and normal healthy controls, and sIL2Rα induces $CD8^+$ T cell proliferation in the presence of Treg cells [16]. Serum sIL2Rα levels have clinical significance for predicting survival [17]. Patients with primary myelofibrosis have a significantly higher percentage of effector memory cells in peripheral blood $CD4^+$ T cells and $CD8^+$ T cells than healthy subjects [18]. These studies suggest that autoimmune related factors may also participate in myelofibrosis without significant systemic features of autoimmune diseases.

IL-$2R\alpha^{-/-}$ mice manifest a defect of Treg cell function [19,20]. Our previous work demonstrate that $p40^{-/-}$IL-$2R\alpha^{-/-}$ mice develop severe portal inflammation and bile duct damage with liver fibrosis and model human primary biliary cholangitis [21]. $p40^{-/-}$IL-$2R\alpha^{-/-}$ mice have increased number of $CD4^+$ T and $CD8^+$ T cells, with a higher percentage of effector memory phenotype and a greater ability to produce IFN-γ [21]. To our surprise we found that $p40^{-/-}$IL-$2R\alpha^{-/-}$ mice had several key features of autoimmune myelofibrosis without myeloid metaplasia. We had taken advantage of this model and studied the role of $CD4^+$ T cells, $CD8^+$ T cells and IFN-γ in autoimmune myelofibrosis. Our data demonstrate that $CD8^+$ T cells and IFN-γ stimulate the development of autoimmune myelofibrosis.

2. Materials and methods

2.1. Mice

IL-$2R\alpha^{-/-}$ (B6.129S4-*Il2ra*^{tm1Dw}/J), $p40^{-/-}$ (B6.129S1-*Il12b*^{tm1Jm}/J), $CD4^{-/-}$ (B6.129S2-*Cd4*^{tm1Mak}/J), CD8a$^{-/-}$ (B6.129S2-*Cd8a*^{tm1Mak}/J), IFN-$\gamma^{-/-}$ mice (B6.129S7-*Ifng*^{tm1Ts}/J), Ly5.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) mice on C57BL/6J background were initially obtained from The Jackson Laboratory (Bar Harbor, ME, USA). $Foxp3^{GFP}$ mice (*Foxp3*^{tm2Ayr}) were kindly provided by Dr. A.Y. Rudensky [22]. $p40^{-/-}$IL-$2R\alpha^{-/-}$, $p40^{-/-}$IL-$2R\alpha^{+/-}$, Ly5.1 $p40^{-/-}$IL-$2R\alpha^{-/-}$, $CD4^{-/-}p40^{-/-}$IL-$2R\alpha^{-/-}$, CD8a$^{-/-}p40^{-/-}$IL-$2R\alpha^{-/-}$, IFN-$\gamma^{-/-}p40^{-/-}$IL-$2R\alpha^{-/-}$, $Foxp3^{GFP}p40^{-/-}$IL-$2R\alpha^{-/-}$, and $Foxp3^{GFP}p40^{-/-}$IL-$2R\alpha^{+/-}$ mice used in this article were generated by backcrossing. The mice studied herein were maintained in individually ventilated cages under specific pathogen-free conditions in Laboratory Animal Center, University of Science and Technology of China. All mice were studied from 11 to 13 weeks of age regardless of gender unless mentioned. Animal experiments conformed to the guidelines outlined in the Guide for the Care and Use of Laboratory Animals, University of Science and Technology of China.

2.2. Histology

Liver, spleen, femur, and tibia sections were prepared and immediately fixed with 4% paraformaldehyde for 1–2 days. Then femur and tibia sections were decalcified with 5% hydrochloric acid plus 5% acetic acid for 1 day. Tissues were embedded in paraffin and cut into 4-μm slices. All slices were deparaffinized, stained with hematoxylin and eosin. Reticulin silver staining Kit (Modified Gordon-Sweets) (Solarbio, Beijing, China) was used for visualizing reticular fibers.

2.3. Cell isolation

Liver was first homogenized with phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA), passed through a steel mesh, and resuspended. Mononuclear cells (MNCs) from suspended liver cells were isolated by centrifugation with 40% Percoll (GE Healthcare, Little Chalfont, United Kingdom). Spleen was disrupted between two glass slides, suspended in PBS/0.2% BSA, and passed through a 74 μm nylon mesh. Bone marrow cells were flushed from femurs and tibias using a 2.5 ml syringe fitted with a 25-gauge needle. Red blood cells were depleted by Red Blood Cell Lysis Buffer (NH_4Cl 8024 mg/L, $KHCO_3$ 1001 mg/L, EDTA $Na_2 \cdot 2H_2O$ 3.722 mg/L) [23]. Cells were counted on a hemocytometer in the presence of trypan blue.

2.4. Flow cytometry

For flow cytometry, cell preparations were incubated with purified anti-CD16/CD32 antibody (BioLegend, San Diego, CA, USA) for 15 min at 4 °C, then stained for 20 min at 4 °C with cocktails containing combinations of fluorochrome-conjugated monoclonal antibodies for cell surface markers. For intracellular cytokine staining, cells were resuspended in RPMI-1640 with 10% fetal bovine serum and stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (eBioscience, San Diego, CA, USA) at 37 °C for 4 h. After surface marker staining, cells were fixed and permeabilized with Cytofix/Cytoperm ™ Fixation/Permeabilization Kit (BD biosciences, San Jose, CA, USA), then stained for intracellular anti-IFN-γ (BioLegend). Normal IgG isotype controls (BioLegend) were used in parallel. A FACSVerse flow cytometry (BD Immunocytometry Systems, San Jose, CA, USA) was used to acquire data, which were analyzed with FlowJo software (Tree star, Inc., Ashland, OR, USA). Monoclonal antibodies against CD3 (17A2), B220 (RA3-6B2), Ter119 (TER119), Gr-1 (RB6-8C5), NK1.1 (PK136), CD11c (N418), CD11b (M1/70) (Lineage markers), c-Kit (2B8), Sca-1 (D7), CD25 (PC61), IFN-γ (XMG1.2), CD4 (GK1.5), CD8b (YTS156.7.7), CD19 (6D5), CD62L (MEL-14), CD44 (IM7), CD45.1 (A20), CD45.2 (104), Ly6C (HK1.4), Ly6G (1A8) were purchased from BioLegend. Antibody against CD8a (53–6.7) was purchased from BD Biosciences.

2.5. Clinical hematology

Peripheral blood was collected in anticoagulant tubes. Red blood cell count (RBC), white blood cell count (WBC), platelet count (PLT), hemoglobin (HGB), hematocrit (HCT) were analyzed using a XT-1800i Automated Hematology Analyzer (Sysmex, Japan). The percentage of $CD4^+$ T cells ($CD3^+NK1.1^-CD4^+$), $CD8^+$ T cells ($CD3^+NK1.1^-CD8^+$), B cells ($CD19^+CD3^-$), NK cells ($CD3^-NK1.1^+$), monocytes ($CD11b^+Ly6C^+Ly6G^-$), neutrophils ($CD11b^+Ly6C^+Ly6G^+$) in $CD45.2^+$ white blood cells were analyzed by FACSVerse flow cytometer.

2.6. Generation of bone marrow chimeric mice

Ly5.1/5.2 wild type mice at 6–8 weeks old were used as recipient mice, fed sterile mouse chow and water containing 1 g/L ampicillin, 1 g/L metronidazole and 1 g/L neomycin beginning one week before irradiation with 10Gy. Ly5.2 $p40^{-/-}$IL-$2R\alpha^{+/-}$ mice and Ly5.1 $p40^{-/-}$IL-$2R\alpha^{-/-}$ mice at 12 weeks old regardless of gender were used as donors. Ly5.1/5.2 mice at 6–8 weeks old were used to provide total bone marrow cells as supporter cells. For total bone marrow cell chimera, bone marrow cells after red blood cell lysis were mixed at a 1:1 ratio (5×10^5: 5×10^5) then transferred into recipients by intravenous injection within 24 h after irradiation. For bone marrow LSK cell chimera, bone marrow LSK cells were first enriched with a Lineage Cell Depletion Kit (Miltenyi Biotec Inc., Bergisch Gladbach, Germany) by magnetic-activated cell sorting, then stained with antibodies against lineage markers, c-Kit, Sca-1. Cells were sorted by MoFlo® Astrios^{EQ} (Beckman Coulter, Brea, CA, USA). Sorted bone marrow LSK cells were mixed at a 1:1 ratio (2.5×10^3: 2.5×10^3) with 1×10^6 supporter cells then transferred into recipients by intravenous injection within 24 h after

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