Supporting Information

Silvescu and Sackstein 10.1073/pnas.1320833111

SI Materials and Methods

Cells. All human cells were obtained and used in accordance with the procedures approved by the Human Experimentation and Ethics Committees of Partners Cancer Care Institutions (Massachusetts General Hospital, Brigham and Women's Hospital, and Dana-Farber Cancer Institute). To ensure consistency in sample preparation and obtain data most relevant to native human biology, cells from blood and marrow samples were uniformly processed for relevant analyses within 2 h of collection. Mobilized leukocytes (MLs) were collected by blood leukapheresis from healthy donors receiving granulocyte-colony stimulating factor (G-CSF) to mobilize hematopoietic progenitors for hematopoietic stem cell transplantation (samples were provided by the Cell Manipulation Core Facility from Brigham and Women's Hospital/Dana-Farber Cancer Institute). Native leukocytes (NLs) were isolated from blood obtained from healthy volunteers. Human bone marrow (BM) cells were separated from filter sets used during bone marrow harvests performed at Massachusetts General Hospital. Febrile leukocyte (FL) cells were collected from hospitalized patients with fever and leukocytosis (white blood cell counts higher than 10×10^6 cells/mL). Cryopreserved myeloblasts from patients with acute myeloid leukemia (AML) were obtained from the Cell Manipulation Core Facility at Brigham and Women's Hospital/Dana-Farber Cancer Institute. Leukocytes were isolated from peripheral blood by centrifugation and collection of buffy-coat cells or by collecting interface cells following density centrifugation using Ficoll Histopaque-1077 (Sigma-Aldrich). In all cases, contaminating red cells were eliminated by brief hypotonic lysis. Isolated cells were washed with PBS without Ca^{2+}/Mg^{2+} in preparation for all procedures.

To evaluate MPO expression on mouse myeloid cells, C57BL/6 and BALB/c mice were injected with 250 μg/kg recombinant human G-CSF (Neupogen; Amgen) or with saline (as control) for 5 consecutive days, and nucleated cells were isolated from peripheral blood. To confirm that G-CSF treatment mobilized the hematopoietic stem cells from the bone marrow, aliquots of peripheral blood cells were seeded in MethoCult methylcellulose (StemCell Technology) and the number of hematopoietic progenitor cells was detected by colony-forming unit (cfu) assay. Also, circulatory hematopoietic progenitors were measured in peripheral blood by flow cytometry analysis of Sca-1, c-kit, and lineage markers.

Antibodies. Western blot analysis was performed with the following antibodies: recombinant mouse E-selectin–human Fc chimera, (R&D Systems), rat anti-mouse E-selectin, horseradish peroxidase (HRP)-conjugated goat anti-rat Ig, (Southern Biotech), mouse monoclonal anti-myeloperoxidase (anti-MPO) clone 3D3 (a generous gift from Carrie Rice, Maine Biotechnology Service, Portland, ME) or clone 2C7 (Abcam), goat anti-mouse IgG HRP-conjugated and streptavidin HRP-conjugated (BD Bioscience), rat anti-human/mouse cutaneous lymphocyte antigen (CLA) clone HECA-452 (BioLegend). Rabbit polyclonal anti-growth factor receptor-binding protein 2 (GRB2) was used for detecting Grb2 levels as loading control. Mouse anti-MPO mAb clone 1A1 (Abcam) was used for immunoprecipitations. Flow cytometry analysis was performed with the following antibodies: HECA-452 mAb (BD Bioscience); goat anti-rat IgM fluorescein isothiocyanate (FITC)-conjugated, rat IgM isotype, and mouse IgG isotype (Southern Biotech); mouse anti-MPO mAb, clone 2C7 (Abcam), and goat anti-mouse IgG phycoerythrin (PE)-conjugated (Santa Cruz Biotechnology), allophycocyanin (APC) anti-human CD34 (clone 581) (BioLegend), biotin anti-human lineage panel (Miltenyi Biotec), FITC anti-mouse Ly-6A/E (Sca-1) (BioLegend), PE anti-mouse CD117 (c-kit), clone 2B8 (BioLegend), biotin anti-mouse lineage panel followed by streptavidin-APC (BioLegend).

Immunoprecipitations, SDS/PAGE, and Western Blotting. Cells were lysed in buffer containing protease inhibitors [0.5 mM Tris, pH = 8, 150 mM NaCl, 20 μg/mL PMSF, 0.02% (wt/vol) sodium azide] supplemented with 2% (vol/vol) Nonidet P-40 (Sigma-Aldrich) and protease inhibitor mixture (Roche Diagnostics). Total lysate protein was quantified with BCA Protein Assay kit (Pierce). Cell lysates were precleared with rProtein G agarose beads (Invitrogen). Immunoprecipitations were performed at 4 °C for 16 h by incubating the precleared lysates with 2 μg antibody and a fresh batch of agarose beads blocked in advance with 1 mg/mL BSA (Sigma-Aldrich). After extensive washes, the beads were boiled with Laemmli buffer and the released antigens or cell lysates were resolved on 7.5% Tris·HCl SDS/PAGE (Criterion Precast gel; Bio-Rad Laboratories). In Western blot experiments, separated proteins were transferred to Sequi-BlotPVDF Membrane (Bio-Rad Laboratories), which was blocked for 2 h with a solution of 5% (wt/vol) nonfat milk (LabScientific). Proteins were detected with E-selectin–Ig chimera or with anti-MPO mAbs clone 2C7 (Abcam) and clone 3D3 (a generous gift from Carrie Rice of Maine Biotechnology Services, Portland, ME). HRP-conjugated secondary antibodies goat anti-rat IgG, goat anti-rat IgM, and goat anti-mouse IgG were detected with Lumi-Light Western blotting substrate, (Roche Diagnostics).

Lectin Chromatography and Mass Spectrometry Analysis. Wheat germ agglutinin (WGA) lectin chromatography was used to purify the glycoprotein pool of selectin ligands from MLs. Cell lysates were incubated with WGA immobilized to agarose beads (Pierce) and, after extensive washes, the glycoproteins were released and dialyzed. Two 7.5% SDS/PAGE were run in parallel to resolve the selectin ligands. The proteins from one gel were transferred to a PVDF membrane, which was stained in Western blot with E-selectin Ig. The migration pattern of selectin ligands, revealed by Western blot was used to locate the relevant proteins in the second gel. Thin slices were excised from the gel area where the selectin ligands migrated. Protein in-gel digestion was performed with proteomics grade trypsin (Sigma-Aldrich) by covering the gel slices with trypsin solution [40 mM ammonium bicarbonate in 9% (vol/vol) acetonitrile] and incubating at 37 °C for 16 h. Digested proteins were submitted to peptide mass fingerprinting by mass spectrometry.

Cell Surface Biotinylation. Cells were washed with PBS and incubated with NHS-PEO_4-biotin (Pierce) or DMSO for negative control, as recommended in the manufacturer specifications. After 15 min at room temperature, cells were washed with PBS supplemented with nonessential amino acids followed by extensive washes with PBS. Biotinylation efficiency was monitored by cell surface staining with PE-conjugated streptavidin followed by flow cytometry analysis.

Flow Cytometry. For human cells, membrane expression of E-selectin ligands and MPO was determined by indirect single-color immunostaining with HECA-452 and anti-MPO (2C7) mAbs, respectively. Cells were incubated with primary antibodies and their matched isotype controls in PBS with 2% (vol/vol) FBS for 20 min, on ice. After successive washes with PBS + 2% (vol/vol)

FBS, cells were stained with FITC-conjugated secondary antibody for HECA-452 and PE-conjugated secondary antibody for MPO. Stained cells were then washed, resuspended in PBS, and analyzed using the Cytomics FC 500 MPL flow cytometer (Beckman Coulter). For mouse cells, surface expression of MPO was evaluated with anti-MPO polyclonal antibody H-300 (Santa Cruz Biotechnology).

In Vitro G-CSF Treatment. Myeloid cells (10^6 cells/mL) isolated from different sources were cultured with RPMI medium 1640 (Mediatech) with 10% (vol/vol) FBS, 1% (vol/vol) pen/strep, and recombinant human G-CSF (Neupogen; Amgen); unless otherwise specified, 10 ng/mL G-CSF was used. Cells were maintained in culture for 48 h at 37 °C, and G-CSF aliquots were added after each 24-h period.

Sialidase and DMJ Treatment. Cells were isolated from the buffy coat of bone marrow aspirates after centrifugal sedimentation and red blood cell lysis. Purified nucleated cells (10^7/mL) were incubated with *Vibrio cholerae* sialidase (Roche Diagnostics) for 1 h at 37 °C. Efficiency of sialic acid removal was confirmed by absence of cell surface staining with HECA-452 mAb as assessed by flow cytometry. After extensive washes, cells were divided in equal numbers and cultured in RPMI medium 1640 for 48 h, with or without G-CSF treatment. In parallel, a subset of cells cultured with G-CSF was treated with 1 mM deoxymannojirimycin (DMJ). The effect(s) of G-CSF and of DMJ treatments were assessed by flow cytometry analysis of surface expression of HECA-452 determinants and MPO.

Detection of Membrane MPO Activity. Aliquots of NLs and MLs were surface biotinylated, lysed, and membrane proteins were precipitated with streptavidin-conjugated agarose beads. The beads were incubated with a chromogenic peroxidase substrate, *o*-phenylenediamine dihydrochloride (OPD) (Sigma) and the activity of surface MPO was monitored spectrophotometrically.

Endothelial Cell Death Evaluation and Inhibition of MPO Activity. Human umbilical vein endothelial cell (HUVEC) monolayers were obtained from the Vascular Biology Core Facility of the Department of Pathology of Brigham and Women's Hospital and were grown on fibronectin (BD Bioscience)-coated plates (20 μg/mL) with Medium 199 (Cambrex), supplemented with 20% (vol/vol) FBS, 2 mM L-glutamine (Invitrogen), 1% (vol/vol) pen/strep, 100 μg/mL heparin (Sigma), and 50 μg/mL endothelial cell growth supplement (Biomedical Technologies). Endothelial cells were activated for 6 h in culture with 40 ng/mL TNFα to express E-selectin, and, where indicated, incubation with 10 μg/mL mouse anti-human CD62E antibody (BD Pharmingen) was used to block E-selectin function. Myeloid leukocytes from the various sources were treated with G-CSF, or G-CSF and DMJ, and cocultured with activated HUVECs for 48 h. To inhibit MPO activity, leukocytes were incubated with 100 μM 4-aminobenzoic acid hydrazide (4-ABAH) (Sigma-Aldrich) in culture media for 48 h at 37 °C. Endothelial cell death was assessed by Trypan blue exclusion assay and reported as percentage of total cells counted per squared unit of HUVEC monolayer.

Phosphatidylinositol-Specific Phospholipase C Cell Treatment. MLs (1×10^6) were incubated in PBS with 1 unit of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus cereus* (Molecular Probes) for 30 min at 4 °C. To verify the enzyme efficiency, cells were stained with anti-CD55 mAb clone JS11 (BioLegend) because CD55 is known to be attached to the hematopoietic cell membrane by a glycosylphosphatidylinositol (GPI) anchor. Cells were stained with anti-MPO mAB clone 2C7 to verify whether MPO is GPI anchored or with anti-CD44 mAb clone 515 as negative control. Anti-mouse IgG-FITC was used as secondary antibody staining and cells were monitored by flow cytometry.

Washes with High Salt Solutions. Cells were washed with 0.5, 1.0, or 1.5 M NaCl by vigorous agitation at room temperature for 2 min and then quickly resuspended in PBS. Cell surface expression of MPO was monitored by two-step staining with anti-MPO mAb (clone 2C7), followed by anti-mouse IgG-FITC secondary antibody and flow cytometry analysis.

Statistical Analysis. The compared values represent mean ± SD of cell subsets isolated from random human clinical samples of multiple donors. Statistical analysis was performed using a two-tailed, unpaired Student *t* test of the means. $P < 0.05$ were considered statistically significant.

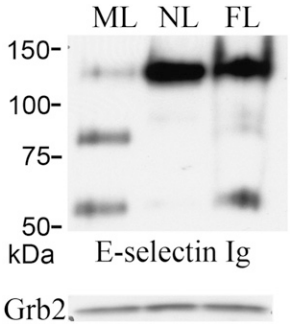

Fig. S1. Expression of the ~65-kDa E-selectin ligand is specific to the mobilized leukocytes (MLs) and febrile leukocytes (FLs). Lysates of MLs, native leukocytes (NLs), and circulating myeloid cells of patients with febrile leukocytosis (FL), normalized for input protein quantity, were resolved by SDS/PAGE and stained in Western blot with E-selectin Ig chimera (E–Ig). As loading control, the level of growth factor receptor-bound protein 2 (Grb2) was evaluated by Western blot staining with anti-Grb2 mAb. E–Ig reactivity of the ~65-kDa glycoprotein is characteristic of G-CSF–primed cells (MLs and FLs), but not NLs.

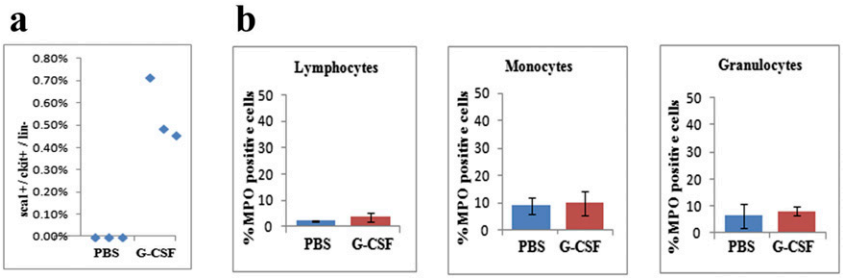

Fig. S2. Analysis of MPO expression on the cell surface of murine leukocytes. (*A*) The efficiency of G-CSF treatment to mobilize mouse hematopoietic stem cells from the bone marrow was evaluated in aliquots of murine peripheral blood by flow cytometry after costaining with anti–Sca-1 mAb, anti-ckit mAb, and lineage-specific antibody panel. The percentage of sca-1$^+$/c-kit$^+$/lin$^-$ is elevated in peripheral blood of mice injected with G-CSF in comparison with mice injected with saline buffer control. (*B*) Cell surface expression of MPO was determined in different cell populations from peripheral blood of mice treated with saline buffer or with G-CSF, (n = 3) by staining with anti-MPO polyclonal Ab (clone H-300). Flow cytometry results show absence of MPO from cell surface of murine lymphocytes and no significant variation in MPO expression on surface of monocytes or granulocytes obtained from mice treated with buffer control or with G-CSF.

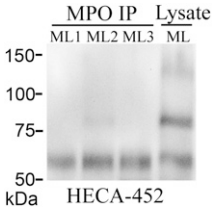

Fig. S3. The ~65-kDa MPO heavy chain is recognized by HECA-452 mAb. MPO immunoprecipitated from three different donors of hematopoietic stem and progenitor cells (HSPCs) treated with G-CSF (ML1, ML2, and ML3) and total lysate from one donor (ML) were stained in Western blot with mAb HECA-452. As shown, MPO heavy chain is reactive with mAb HECA-452.

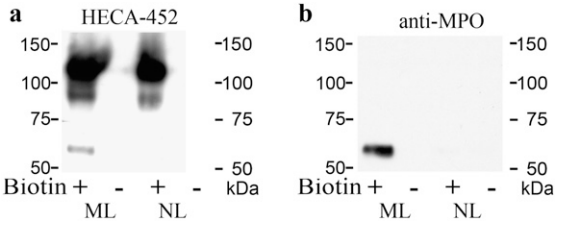

Fig. S4. The ~65-kDa molecule is expressed on the cell surface of ML and is recognized by HECA-452 and by anti-MPO mAb. MLs and NLs were biotinylated on the surface (+) or treated with DMSO control (–), lysed, and membrane proteins were precipitated with streptavidin coupled to agarose beads. Membrane proteins were released from agarose beads by boiling, resolved by gel electrophoresis, and stained in Western blots with HECA-452 (*A*) or with anti-MPO mAb 3D3, which specifically detects the MPO heavy chain (*B*). HECA-452 and anti-MPO mAb each stain the biotinylated ~65-kDa band, indicating that MPO-EL is a cell surface glycoprotein.

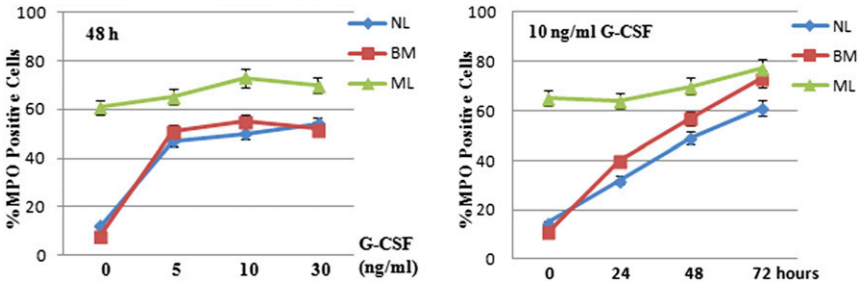

Fig. S5. Induction of surface expression of MPO on NLs, BMs, and MLs with G-CSF treatment in vitro. (*A*) Dose–response curves of cell surface MPO expression in NLs, BMs, and MLs with G-CSF treatment for 48 h in vitro (n = 5). (*B*) MPO cell surface presentation during a 72-h time course of G-CSF treatment at 10 ng/mL (n = 5). G-CSF treatment in vitro induces MPO cell surface expression in both NL and BM cells, but does not induce further MPO membrane expression in cells exposed to G-CSF in vivo.

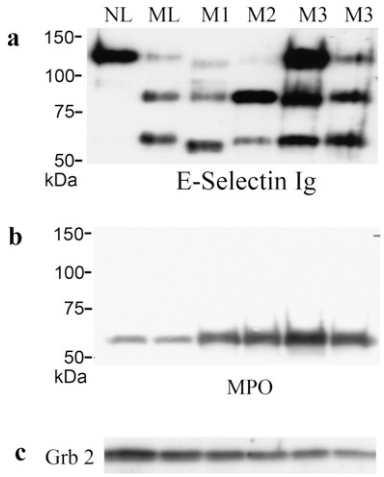

Fig. S6. Expression of MPO-EL and MPO in NLs and MLs and in blasts of patients with acute myeloid leukemia (AML). Lysates of blasts obtained from patients with AML M1 and M2 (lanes M1 and M2) and from two patients with AML M3 (acute promyelocytic leukemia, APL; lanes designated as "M3") were coelectrophoresed in parallel with NLs and MLs. All samples were normalized for protein quantity and stained in Western blots with E-selectin Ig chimera (*A*) and with anti-MPO mAb 3D3 (*B*). As an internal loading control, staining was performed with anti-Grb2 mAb (*C*). All AML samples and MLs show prominent E–Ig reactivity at ~80–90 kDa (hematopoetic cell E-/L-selectin ligand (HCELL) and at ~65 kDa (MPO heavy chain), with variable intensity of staining at ~130 kDa (CLA). NL shows prominent staining at ~130 kDa (CLA). Staining with mAb 3D3 shows an increased concentration of MPO in AML blasts in comparison with NL and ML.