Glutathione S-Transferase P Influences Redox and Migration Pathways in Bone Marrow

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Abstract

To interrogate why redox homeostasis and glutathione S-transferase P (GSTP) are important in regulating bone marrow cell proliferation and migration, we isolated crude bone marrow, lineage negative and bone marrow derived-dendritic cells (BMDDCs) from both wild type (WT) and knockout (Gstpt1/p2−/−) mice. Comparison of the two strains showed distinct thiol expression patterns. WT had higher baseline and reactive oxygen species-induced levels of S-glutathionylated proteins, some of which (sarco-endoplasmic reticulum Ca2+-ATPase) regulate Ca2+ fluxes and subsequently influence proliferation and migration. Redox status is also a crucial determinant in the regulation of the chemokine system. CXCL12 chemotactic response was stronger in WT cells, with commensurate alterations in plasma membrane polarization/permeability and intracellular calcium fluxes; activities of the downstream kinases, ERK and Akt were also higher in WT. In addition, expression levels of the chemokine receptor CXCR4 and its associated phosphatase, SHP-2, were higher in WT. Inhibition of CXCR4 or SHP-2 decreased the extent of CXCL12-induced migration in WT BMDDCs. The differential surface densities of CXCR4, SHP-2 and inositol trisphosphate receptor in WT and Gstpt1/p2−/− cells correlated with the differential CXCR4 functional activities, as measured by the extent of chemokine-induced directional migration and differences in intracellular signaling. These observed differences contribute to our understanding of how genetic ablation of GSTP causes higher levels of myeloproliferation and migration.

Introduction

The bone marrow produces all the differentiated hematopoietic cells for peripheral blood. This tissue is extremely sensitive to alterations in redox homeostasis, as proliferation and differentiation are influenced by physiological changes in a number of factors that are sensitive to reactive oxygen species (ROS; [1,2]). ROS and the oxidation/reduction of thiols have key roles in cell signaling events that regulate a variety of biological functions [3–7]. Reduced glutathione (GSH) is the most abundant non-protein thiol at cellular concentrations that range from 0.1 to 10 mM [8]. Glutathione S-transferase P (GSTP) is one of a family of GST isozymes and has functions as a catalytic enzyme, protein chaperone, kinase regulator and in regulating the forward reaction of protein S-glutathionylation [1,9]. This post-translational modification occurs in certain protein clusters that have roles in events regulating cell proliferation [10]. It is reasonable to speculate that the difference in ROS levels in myeloid progenitor and quiescent hematopoietic stem cells (HSCs) may act in intracellular signaling events that drive HSC differentiation and that modulation of redox-sensitive cysteines through S-glutathionylation may have a key role in these events.

As early as 1953 a role for cysteines and thiols in bone marrow cell proliferation was established [11]. Various drugs that disrupt thiol homeostasis have been shown to exert a redox-based influence on components of bone marrow proliferation. For example, N-acetyl cysteine has been used in the management of patients with HIV, enabling more robust immune responses through T helper cells [12]; NOV-002 (a glutathione disulfide mimic) enhances marrow recovery following immunosuppressive drugs in both rodents [13] and humans [14]; TLK199, [γ-glutamyl-S-(benzyl)-cysteinyl-R(-) phenyl glycine diethyl ester] (Telitra) as a GSH peptidomimetic inhibitor of GSTP stimulates myeloproliferation in both rodents [15] and man [16,17]. In mice, Telitra increases all peripheral blood cell lineages in wild type mice as compared to GSTP-deficient mice. Even in the absence of
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**Telitra**, GSTP-null animals have increased hematopoietic progenitor cell (HPC) numbers, myeloid cell differentiation and proliferation [18]. **Telitra** has shown positive results (decreased requirements for red blood cell, platelet and growth factor support) in ongoing Phase 2 clinical trials for patients with myeloproliferative syndrome [16,17], a stem cell disorder characterized by ineffective blood cell production and an increased risk for transformation to acute leukemia.

Bone marrow is a relatively hypoxic tissue (1% to 2% O2) [19], but within the three-dimensional marrow compartment, self-renewing HSCs, HPCs and mature blood cells are able to migrate in a site/niche-specific fashion regulated by factors such as O2 and Ca2+ gradients. At any given time, approximately 75% of HSCs are in a quiescent phase of the cell cycle [20]. At the bone-marrow interface (osteoblastic niche), the microenvironment favors HSC quiescence, while closer to blood vessels (vascular niche), proliferation and differentiation is more likely [21–25]. Osteoclast and osteoblast-mediated bone remodeling results in an increased extracellular Ca2+ in the endostium and Ca2+ gradient between osteoblastic and vascular niches, enabling HSCs to sense and migrate appropriately [26]. Adhesive molecules, cytokines and chemokine signaling determine population and niche characteristics. The chemokine CXCL12 plays an essential role in retaining and maintaining HSCs in bone marrow and depletion of a related cytokine, CXCR4, increases HSCs in the peripheral blood [27,28]. The interplay between ROS and thiol balance/gradients is critical to myeloproliferation and/or migration, as the redox status can be regulated by shifts of thiol-disulfide equilibrium [2]. Since pharmaceutical inhibition of GSTP has translational applications in myeloproliferation, the present studies were designed to address how genetic ablation of GSTP impacts bone marrow cell redox parameters and influences downstream events that contribute to proliferation and migration in this tissue.

**Results**

Increased DNA synthesis in Gstp1/p2−/− bone marrow cell populations

Cell proliferation was assessed by BrdU incorporation. In the presence of stem/progenitor cell growth factors (stem cell factor, thrombopoietin and Flt3L), BrdU incorporation was ~10% higher in the knock out (Gstp1/p2−/−) lineage-negative (Lin−) cells, compared to wild type (WT) Lin− cells. Gstp1/p2−/− bone marrow derived-dendritic cells (BMDDCs) had a 63% higher DNA synthesis rate than WT cells in response to GM-CSF stimulation. For both Lin− cells and BMDDCs, significant differences were observed between WT and Gstp1/p2−/− mice (Fig. 1). These results support our previous publication that ablation of GSTP either genetically or pharmacologically results in the over-production of lymphoid, erythroid and myeloid lineage cell lineages as well as platelets [26].

Altered redox status in Gstp1/p2−/− bone marrow cell populations

Crude bone marrow cells (BMCs) or BMDDCs derived from WT or Gstp1/p2−/− mice were used to evaluate the dynamics of S-glutathionylation following exposure to reagents that induce either oxidative (H2O2) or nitrosative stress [PABA/NO] (O2−,[2,4-dinitro-3-[4-(N-methylamino)benzoyloxy]phenyl]1-[N,N-dimethylamino]diazene-1-tum,1,2-diolate), a diazeniumdiolate prodrg which releases NO [29]. PABA/NO induces limited levels of protein nitrosylation/nitration and high levels of S-glutathionylation [30,31]. As shown in Fig. 2, monoclonal anti-GSH antibodies directed against the GS-moiety detected S-glutathionylated proteins in BMCs and BMDDCs following either H2O2 or PABA/NO treatment. Under basal conditions, low levels of S-glutathionylated proteins were detectable in BMCs or BMDDCs (primarily actin [32]). H2O2 treatment produced S-glutathionylation of a limited number of proteins, while PABA/NO led to rapid S-glutathionylation of numerous proteins. A dose-dependent increase in total protein S-glutathionylation was observed following either H2O2 or PABA/NO in both BMCs and BMDDCs. Following drug treatments, S-glutathionylation levels of proteins in Gstp1/p2−/− cells were consistently lower than WT.

The impact of GSTP expression on the redox state of the intracellular protein thiol levels was further examined (Fig. 3A). Significantly higher levels of reduced intracellular protein thiols were observed in Gstp1/p2−/− Lin− cells and BMDDCs when compared to WT cells.

In addition, cysteine reactive Isotope-coded affinity tag (ICAT) and LC/MS specifically identified 333 thiol active proteins, of which we selected proteins where abundance ratios between Gstp1/p2−/− and WT were >1.2, or <0.8, including: vimentin, apoptotic chromatin condensation inducer 1, transition- al endoplasmic reticulum ATPase, aldokekuto-reductase family 1, peroxiredoxin 4, rho GTPase-activating protein 17, triose-phosphate isomerase 1, ras GTPase-activating-like protein IQGAP1, prolyl 4-hydroxylase, S100 calcium binding protein A9, serine/threonine kinase 16 (Table 1).

We also measured the in situ levels of reduced and oxidized glutathione (GSH and GSSG) in bone marrow populations derived from WT and Gstp1/p2−/− mice. As shown in Fig. 3B, compared to WT cells, levels of reduced GSH were higher, whereas levels of oxidized GSSG were lower in Gstp1/p2−/− crude BMCs, Lin− cells or BMDDCs. Significant differences were only observed in GSH levels in Gstp1/p2−/− crude BMCs and BMDDCs when compared to WT cells. In addition, a MALDI-MS bone imaging method was developed to visualize GSH and GSSG simultaneously in bone marrow tissues without any labeling (Fig. 3C). This method generated a multidimensional spatial expression map of the biomolecules directly from a tissue section. As seen in the differential ion intensities of GSH and GSSG, the bone marrow distribution results were consistent with the biochemical measurements showing that differences in qualitative distribution of GSH and GSSG were present between the WT and Gstp1/p2−/− samples.

Altered intracellular calcium dynamics in Gstp1/p2−/− bone marrow cell populations

Fig. 4 shows typical calcium responses of Lin− cells and BMDDCs after stimulation with either A23187 or Thapsigargin (ThG). A23187 is an ionophore that forms lipid-soluble complexes with divalent metal cations, increasing specific permeability of membranes to Ca2+. A23187 induced a rapid increase in [Ca2+]i, followed by a sustained high level of [Ca2+]i, in Lin− cells. Alternatively, only a transient increase in [Ca2+]i was observed in BMDDCs, with no obvious differences between WT and Gstp1/p2−/− cells (Fig. 4A). Inhibition of the sarco-endoplasmic reticulum Ca2+-ATPase (SERCA) pump by ThG is a commonly used method for manipulating calcium stores. ThG-induced rises in [Ca2+]i reflect the passive leak of Ca2+ from the endoplasmic reticulum (ER) following SERCA inhibition [33,34]. In the absence of extracellular Ca2+, ThG induced a rapid and transient elevation of [Ca2+]i, in both Lin− cells and BMDDCs. Interestingly, Gstp1/p2−/− Lin− cells and BMDDCs showed significantly lower Ca2+ responses to ThG compared to WT cells (Fig. 4B), implying that SERCA activity may be different between WT and Gstp1/p2−/− cells. Supporting this conclusion, S-
glutathionylation increases SERCA2 activity [35] and GSTP promotes S-glutathionylation [32]. Therefore, we investigated whether GSTP influenced the S-glutathionylation level of SERCA2 in BMDDCs. To detect S-glutathionylated SERCA2 under control conditions, immunoprecipitation with the anti-GSH antibody followed by immunoblotting with the anti-SERCA2 antibodies was used. As shown in Fig. 4C, compared with Gstp1/p2−/− BMDDCs, WT cells have significant higher levels of S-glutathionylated SERCA2 under control conditions. There were no quantitative differences in SERCA2 mRNA levels or protein expression levels between WT and Gstp1/p2−/− cells (Fig. 4D).

Altered CXCL12/CXCR4 signaling in Gstp1/p2−/− bone marrow cell populations

CXCL12 binds primarily to CXCR4 and initiates divergent signaling pathways downstream of ligand binding, resulting in a number of important cellular responses including chemotaxis, mobilization of intracellular calcium and activation of ERK1/2 and Akt kinases. CXCL12-mediated chemotaxis is mediated at least in part by activation of PI3 kinase/Akt and ERK1/2. Calcium mobilization by CXCL12 is achieved via phospholipase C beta activation and formation of inositol trisphosphate (IP3) and diacylglycerol. IP3/IP3 receptor (IP3R) signaling triggers the opening of the Ca2+ channel on the surface of ER, and thus release of Ca2+ into the cytoplasm [36,37]. We compared the results of GStP ablation in Lin(−) cells (Fig. S1) and BMDDCs (Fig. 5) on chemotaxis, calcium mobilization, plasma membrane potential and intracellular signaling (Akt and ERK activation) after CXCL12 treatment. Both Lin(−) cells and BMDDCs migrated toward CXCL12. The number of Lin(−) cells and BMDDCs that migrated in response to CXCL12 was significantly higher than those not exposed to CXCL12 as a chemotactant (control). This CXCL12-dependent migration was stronger in WT Lin(−) cells and BMDDCs than in Gstp1/p2−/− cells (Fig. S1A and Fig. 5A).

Figs. S1B and Fig. 5B show the dynamics of intracellular calcium in Lin(−) cells and BMDDCs after stimulation with CXCL12. CXCL12 induced a rapid and transient elevation of [Ca2+]i in both Lin(−) cells and BMDDCs through IP3/IP3R signaling. Interestingly, similar to the results obtained with ThG-induced calcium release from ER stores through SERCA inhibition, Gstp1/p2−/− Lin(−) cells and BMDDCs showed significantly different Ca2+ responses to CXCL12 as compared to WT cells. Stronger intracellular calcium oscillations were observed in WT Lin(−) cells and BMDDCs than in Gstp1/p2−/− cells following CXCL12. The response in WT cells was characterized by a relatively rapid rate of rise and robust peak change in [Ca2+]i, whereas the response in Gstp1/p2−/− cells was characterized by a relatively slow rate of rise and small peak change in [Ca2+]i.

[Ca2+]i, affects ion permeability and membrane potential and so we determined whether different Ca2+ responses between WT and Gstp1/p2−/− cells were also reflected by different potential responses under CXCL12 stimulation. Plasma membrane potential was determined using the slow-response potential-sensitive dye, bis-1,3-diethyliothiobarbituric acid (trimetine oxonol) (DiS-BAC2/3). The anionic dye can enter depolarized cells where it binds to intracellular proteins or membranes and exhibits enhanced fluorescence. Increased depolarization results in additional influx of the anionic dye and thus, an increase in fluorescence. Conversely, hyperpolarization is indicated by a decrease in fluorescence. Fig. S1C and Fig. 5C show the dynamics of plasma membrane potential in Lin(−) cells and BMDDCs following stimulation with CXCL12. Following CXCL12 stimulation, depolarization of plasma membranes was detected in both WT and Gstp1/p2−/− Lin(−) cells, whereas delayed, amplified and reversible plasma membrane depolarization was observed in Gstp1/p2−/− cells (Fig. S1C). For BMDDCs, CXCL12 caused membrane hyperpolarization in both WT and Gstp1/p2−/− cells, and this hyperpolarization was stable during incubation. The effects of CXCL12 on membrane potential were slightly more pronounced in WT BMDDCs (Fig. 5C).

Next we assessed whether the different chemotactic responses of WT and Gstp1/p2−/− cells following CXCL12 stimulation were...
also reflected at other steps of intracellular signaling. As shown in Fig. 5D, treatment of BMDDCs with CXCL12 induced a rapid phosphorylation and activation of Akt and ERK in both WT and Gstp1/p2−/− cells. Compared to Gstp1/p2−/− cells, more intense phosphorylation of both Akt and ERK was observed in WT cells after CXCL12 stimulation. At 15 mins, pERK levels did drop compared to pAkt, perhaps reflecting the slightly different roles that these kinases play in regulating proliferative pathways.

To find a possible explanation for the differential chemotactic responsiveness and intracellular signaling capacity between WT and Gstp1/p2−/− cells, we determined chemokine CXCL12 receptor CXCR4, IP3 receptor IP3R mRNA levels as well as protein expression (Fig. 5E and F). Both WT and Gstp1/p2−/− BMDDCs expressed CXCR4 and IP3R, with significantly higher mRNA and protein expression levels of CXCR4 and IP3R3 in WT than in Gstp1/p2−/− cells, likely linked to the different redox status of those cells. In addition, existing evidence implies that expression of the thiol active phosphatase SHP-2 is important in regulating CXCR4 signaling [38,39] and our data indicate that SHP-2 expression was lower in Gstp1/p2−/− than in WT cells (Fig. 5E). Furthermore, we analyzed the effect of SHP-2 specific inhibitor PHPS1 [40] and CXCR4 specific antagonist AMD3100 on CXCL12-induced migration of BMDDCs. PHPS1 or AMD3100 treatment significantly reduced the CXCL12-induced migration in both WT and Gstp1/p2−/− BMDDCs, although it had a greater effect on the WT cells (Fig. 5G). Taken together, the differential surface density of CXCR4 and SHP-2 in WT and Gstp1/p2−/− cells may correlate with the differential CXCR4 functional activity measured by the extent of CXCL12-induced cellular responses in hematopoietic cells.

Discussion

The present studies were undertaken to determine why either genetic ablation or pharmacological inhibition of GSTP enhances myeloproliferation and migration, producing increased numbers of all committed cell lineages [15,18]. Existing literature implies that ROS can regulate certain bone marrow niches and influence proliferative status [1]. Consequently, we undertook a series of studies to determine how various bone marrow cell types from Gstp1/p2−/− mice differed from their WT counterparts. Because ROS conditions have direct influence on cellular redox homeostasis, a number of thiol-dependent pathways are implicated, and these downstream targets are influenced by the presence/absence of GSTP.

Our characterization of bone marrow cells identified a number of consistent differences between WT and Gstp1/p2−/− cells with respect to thiol balance. For example, since GSTP can catalyze the...
forward reaction of S-glutathionylation [32,41], its absence diminished the capacity of cells to establish high levels of either general or specific protein S-glutathionylation (Fig. 2 and Fig. 4C). Presumably as a consequence of this, free protein thiols and GSH levels were higher in resting BMCs, BMDDCs and Lin(−) cells from Gstp1/p2−/− mice (Fig. 3A and B). Moreover, in this report, we demonstrate for the first time that MALDI-MS bone imaging can be used for the simultaneous in situ visualization of both GSH and GSSG in sectioned bones with an intact bone marrow compartment (Fig. 3C). These results, while predominantly qualitative in nature, confirm the biochemical analyses that detail differences between GSH/GSSG in WT and Gstp1/p2−/− mice. From left to right: scanned image of matrix sprayed MALDI slide of mouse femur with bone marrow; corresponding images of: GSH ions at m/z = 306.08 and GSSG ions at m/z = 611.14. Color heat map of the data points in the GSH and GSSG images represent averaged individual ion signal intensities of the spots. doi:10.1371/journal.pone.0107478.g003

Figure 3. Protein thiols, GSH and GSSG levels in bone marrow cells. (A, B) Intracellular reduced protein thiols (A), and GSH/GSSG levels (B) in crude BMCs, Lin(−) cells and BMDDCs. Intracellular reduced thiol and GSH levels were measured by means of a sulfhydryl-specific fluorescent probe; intracellular GSSG levels were determined based on the reduction of GSSG in the presence of glutathione reductase and NADPH and on measurement of NADPH fluorescence decrease. Values are means (±SD) from at least three independent experiments, with asterisks (*) indicating statistical significant differences between (p<0.05). (C) Representative MALDI-MS images of GSH and GSSG in sectioned femur showing bone marrow distribution in WT and Gstp1/p2−/− mice. From left to right: scanned image of matrix sprayed MALDI slide of mouse femur with bone marrow; corresponding images of: GSH ions at m/z = 306.08 and GSSG ions at m/z = 611.14. Color heat map of the data points in the GSH and GSSG images represent averaged individual ion signal intensities of the spots.

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hydroxyase, triose-phosphate isomerase 1, transitional endoplasmic reticulum ATPase; cytoskeletal proteins (vimentin); apoptotic proteins (apoptotic chromatin condensation inducer 1); calcium binding protein (S100 calcium binding protein A9); regulatory proteins (rho GTPase-activating protein 17); scaffold proteins (ras GTPase-activating-like protein IQGAP1) and signaling proteins (serine/threonine kinase 16). Quantitative expression differences could indicate a number of other ways that GSTP genotype could influence bone marrow cell function. In particular, although their specific role in marrow cell proliferation has yet to be addressed, the relevance of S100 and kinases seems self-evident. In addition, the altered expression of IQGAP1 is interesting in light of our recent data showing that sulfiredoxin (a redox active protein with roles in de glutathionylation) has sequence homology to IQGAP proteins and involved in controlling cell migration [42,43].

The redox status in cells is crucial in the regulation of the chemokine system, exemplified by the observations that antioxidants decrease chemokine receptor expression and chemotaxis, while H2O2 or general GSH-depletion, increases chemokine receptor expression and chemotactic responses [44]. Accordingly, while both WT and Gstp1/p2−/− BMDDCs expressed CXCR4, the former had significantly higher miRNA and protein expression.
levels (Fig. 5E and F), implying a link to the different redox status of the cells. The thiol-dependent phosphatase SHP-2 regulates CXCR4 signaling [38,39]. Previous studies have shown that SHP-2 functions as an adaptor molecule, which can bind to several proteins and then transduce various proliferation/migration signals. CXCR4, SHP-2 and cbl collectively participate in the formation of a multimeric signaling complex and over-expression of SHP-2 increases CXCL12-induced chemotaxis, whereas phosphatase inhibitors significantly inhibit CXCL12-induced migration [38].

The SHP-2 specific inhibitor PHPS1 or CXCR4 phosphatase inhibitors significantly inhibit CXCL12-induced chemotaxis, whereas the SHP-2 specific inhibitor PHPS1 or CXCR4 inhibitors could prove to be physiologically significant and pertinent to the observed differences between WT and Gsp1/p2−/− bone marrow cells (where SHP-2 expression was lower). Taken together, the differential surface density of CXCR4 and SHP-2 in WT and Gsp1/p2−/− cells could be correlated with the differential CXCR4 functional activity measured by the extent of chemokine-induced directional migration and the intracellular signaling capacity (e.g. PI3K, MAPKs, calcium oscillation, etc.) (Fig. S1 and Fig. 5A-D). It should be noted that CXCR4 is expressed in multiple cell types in the immune and central nervous systems, hematopoietic stem/progenitor cells, endothelial and epithelial cells and cancer cells. Its ligand, CXCL12, is expressed/secreted in various tissues and organs (bone marrow, liver, lung, skin, skeletal muscle, brain, kidney and heart). CXCL12/CXCR4 signaling plays an important and unique role in the regulation of stem/progenitor cell trafficking, inflammation, embryo/organogenesis, tissue/organ regeneration, and tumor progression, angiogenesis, metastasis, and survival [37,46,47]. Thus, our observed link between GSTP and different levels/activities of CXCR4 and genesis, tissue/organ regeneration, and tumor progression, angiogenesis, metastasis, and survival [37,46,47]. Thus, our observed link between GSTP and different levels/activities of CXCR4 and genesis, tissue/organ regeneration, and tumor progression,

## Table 1. List of selected proteins and their abundance ratios between WT and Gsp1/p2−/− BMDDCs.

| Accession | Gene names | Protein names | Abundance ratio (GSP1/P2−/−/WT) |
|-----------|------------|--------------|---------------------------------|
| 31902755  | Vim        | Vimentin     | 1.6                             |
| 9625006   | Acr1       | Apoptotic chromatin condensation inducer 1 | 1.6 |
| 94408013  | Vcp        | Transitional endoplasmic reticulum ATPase | 1.4 |
| 10946870  | Akr1A4     | Aldo-keto reductase family 1, member A4 (aldehyde reductase) | 1.4 |
| 7948999   | Prdx4      | Peroxiredoxin 4 | 1.2 |
| 169790947 | Arhgap17   | Rho GTPase-activating protein 17 | 1.2 |
| 6678413   | Tpi1       | Triose-phosphate isomerase 1 | 1.2 |
| 242332572 | Iqgap1     | Ras GTPase-activating-like protein IQGAP1 | 1.2 |
| 42415475  | P4hb       | Poly 4-hydroxylase, beta polypeptide | 1.2 |
| 6677837   | S100a9     | S100 calcium binding protein A9 (calgranulin B) | 0.7 |
| 31534784  | Stk16      | Serine/threonine protein kinase 16 | 0.6 |

Amplitude of a CXCL12-mediated oscillation of intracellular Ca2+ through its release from and influx into intracellular stores (E/S)R, myochondria, etc) is proportional to GSTP level and is similar in Lin(−) cells (Fig. S1B). Both the IP3/R channel and the plasmaemmal Ca2+-ATPase (PMCA) pump can be reversibly S-glutathionylated. IP3R channel activity is enhanced by glutathiolation, whereas PMCA pump activity is inhibited [48]. Our data are consistent with the hypothesis that GSTP can promote S-glutathionylation of the IP3R and PMCA causing Ca2+ release from IP3-sensitive internal Ca2+ stores and elevation of basal intracellular Ca2+ levels - in the absence of extracellular Ca2+ [49]. In Lin(−) cells, these effects cause an initial plasma membrane depolarization and GSTP-dependent additional depolarization/ hyperpolarization in the presence of extracellular Ca2+ (Fig. S1C), most likely indicating a Ca2+-induced Ca2+-release effect. The amplitude of CXCL12 effects on mobilizing intracellular Ca2+ in BMDDC is also GSTP dependent and similar to that in Lin(−) cells, but with minimal oscillations in the absence of extracellular Ca2+ (Fig. 5B). CXCL12 can also cause a continuous, GSTP-independent hyperpolarization of plasma membranes in

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BMDDCs (in the presence of extracellular Ca\(^{2+}\), Fig. 5C). This conclusion is compatible with differences of plasma membrane-mediated Ca\(^{2+}\) fluxes in Lin(−) cells and BMDDCs and would fit the model where CXCL12-mediated intracellular Ca\(^{2+}\) dynamics and plasma membrane depolarization is linked with differences in GSTP mediated protein S-glutathionylation.

Overall, genetic ablation of GSTP is causally linked with multiple events that contribute to the regulation of bone marrow cell proliferation and migration. A common link is the perturbation of redox homeostasis and those documented in this paper help to explain why Telintra, as an inhibitor of GSTP, has clinical activity as a small molecule myeloproliferative drug [16,17].

**Materials and Methods**

**Mice**

C57BL/6 wild type mice were purchased from Jackson Laboratory (Bar Harbor, ME). Gstp1/p2\(^{-/-}\) mice were generated as described earlier [50]. The mice were bred and kept in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-certified animal facility of the Medical University of South Carolina (MUSC). All of the mice were used at approximately 8–12 weeks of age. The Institutional Animal Care and Use Committee of Medical University of South Carolina approved all of the experimental procedures used in this study.

**Primary cells and culture conditions**

The femurs and tibias were harvested from WT or Gstp1/p2\(^{-/-}\) mice immediately after cervical dislocation. Crude bone marrow cells were flushed from the bones into RPMI-1640 culture medium (HyClone, Logan, UT) using 26-gauge needles and 10-ml syringes, and filtered through a 40\(\mu\)m nylon cell strainer (BD, Franklin Lakes, NJ) to prepare single-cell suspensions. Red blood cells were then lysed with ACK (Ammonium-Chloride-Potassium) lysis buffer (Gibco, Life Technologies, Carlsbad, CA).

Bone marrow derived-dendritic cells were generated according to previous reported procedures [51,52] with minor modifications. Murine BMCs (4–5\(\times\)10\(^5\)/ml, 10 ml/plate) were plated into RPMI-1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin (all from Mediatech, Manassas, VA), and 20 ng/ml recombinant mouse GM-CSF (BioAb-Chem, Ladson, SC) (DC medium) into 100-mm culture dishes (Sarstedt, Newton, NC). Fresh DC medium was added on day 4 and was gently replaced by fresh DC medium containing 10 ng/
ml recombinant mouse GM-CSF on day 7. Immature BMDDCs (non-adherent and loosely adherent cells) were used in experiments on day 8.

For BM (Lin(−)) cells, BMCs were centrifuged through Lympholyte-M (Cedarlane, Burlington, NC) to isolate BM-mononuclear cells (MNCs). BM-MNCs were incubated on ice for 30 min with biotin-conjugated rat antibodies specific for murine CD4, CD8A, CD45R/B220, Gr-1 and Ter-119 (BD Pharmingen, BD Biosciences, San Jose, CA). The labeled mature lymphoid and myeloid cells were depleted by incubation on ice for 30 min with sheep anti-rat IgG Dynabeads (Invitrogen, Life Technologies, Carlsbad, CA) at a bead: cell ratio of 3:1 with gentle rotation. Cells binding the Dynabeads were removed with a DynaMag-15 magnet (Invitrogen, Life Technologies). The negatively isolated Lin(−) cells were washed twice with PBS containing 0.1% BSA and resuspended in StemSpan™ Serum-Free Expan-
Cell treatment with H2O2 or PABA/NO

Wild type and Gstp1/p2−/− BMCs or BMDDCs (1 × 10^6 cells/ml) suspended in complete medium (RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin) were exposed to different concentrations of H2O2 (1, 5 or 10 mM) or PABA/NO (0.5, 1 or 2 μM) at 37°C for 15 min as indicated in individual experiments. The reaction was terminated by immediate centrifugation at 500 g, 4°C for 5 min. Supernatants were removed and pellets washed once with ice-cold PBS and then solubilized by ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 5 mM NEM, plus a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)]. Cell lysates/supernatants were collected from the cells after spinning at 16,000 g for 10 min at 4°C and used for immunoblotting.

Cell treatment with CXCL12

Wild type and Gstp1/p2−/− BMDDCs were harvested, resuspended at a concentration of 1 × 10^6 cells/ml in RPMI-1640 and kept in the incubator at 37°C for another 2 hours to reduce the basal activity of intracellular signaling pathways. Then BMDDCs were stimulated with chemokine, 200 ng/mL CXCL12 (BioAbChem) for various times (2–15 min). Stimulation was terminated by centrifugation at 500 g, 4°C for 5 min. Supernatants were removed and pellets washed once with ice-cold PBS and then solubilized by ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 40 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 5 mM sodium fluoride, 2 mM sodium orthovanadate, plus a protease inhibitor cocktail]. Cell lysates/supernatants were collected from the cells after spinning at 16,000 g for 10 min at 4°C and used for immunoblotting.

Immunoblotting

Total soluble protein was quantitated by bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL). Cell lysates were resolved in SDS-loading buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, (±) 5 mM tris(2-carboxyethyl)phosphine (TCEP)) and heated to 95°C for 5 min. Equal amounts of protein were electrophoretically separated on 7.5%, 10% or 4–20% SDS-PAGE (BioRad, Hercules, CA) and transferred to Low Fluorescent PVDF membranes (Millipore, Billerica, MA) or nitrocellulose membranes (BioRad) by Trans-Blot Turbo Transfer System (BioRad). PVDF or nitrocellulose membranes were incubated in Odyssey blocking buffer (LI-COR, Lincoln, NE) for 1 hour to reduce non-specific binding and then probed with appropriate primary antibodies (diluted in Odyssey blocking buffer) at 4°C overnight. Immunoblots were then developed with infrared (IR) fluorescence IRDye secondary antibodies (LI-COR) at a dilution of 1:15,000, imaged with a two-channel (red and green) IR fluorescence Odyssey CLx imaging system (LI-COR) and quantified with Image Studio 3.0 software (LI-COR). The following antibodies were used for immunoblot: rabbit polyclonal GSTP1 (MBL, Woburn, MA), mouse monoclonal anti-GSH (Vironen, Watertown, MA), rabbit polyclonal anti-Cdk2, mouse monoclonal anti-Phospho-ERK (Tyr204), rabbit polyclonal anti-SHP-2, mouse monoclonal anti-SERCA2 (all from Santa Cruz Biotechnology, Dallas, Texas), rabbit polyclonal anti-beta actin, rabbit polyclonal anti-CXCR4, rabbit polyclonal anti-ERK (both from Abcam, Cambridge, MA), rabbit polyclonal anti-Akt, rabbit polyclonal anti-Phospho-Akt (Ser473), rabbit monoclonal anti-IP3R (all from Cell Signaling Technology), IRDye 800CW Goat anti-Mouse IgG, IRDye 800CW Goat anti-rabbit IgG, IRDye 680RD Goat anti-mouse IgG, and IRDye 680RD Goat anti-Rabbit IgG (all from LI-COR).

Flow cytometry

Cell surface CXCR4 expression was determined by fluorescence-activated cell sorter (FACS) analysis using FITC-conjugated rat anti-mouse CXCR4 mAb or FITC-conjugated rat IgG2b (both from BD Pharmingen) as isotype control. A total of 0.5 × 10^6 cells were incubated with primary antibodies in FACS buffer (2% FBS in PBS) for 30 min at room temperature, washed once in FACS buffer, resuspended in 250 μL of FACS buffer and analyzed on a BD FACS Calibur analytical flow cytometer and CellQuest Pro software (BD Pharmingen). After gating on CD11c (APC-conjugated hamster anti-mouse CD11c mAb, Affymetrix, eBioscience, San Diego, CA) positive cells, 20,000 events per sample were analyzed.

Immunoprecipitation of S-glutathionylated SERCA2

Five hundred micrograms of protein from WT or Gstp1/p2−/− BMDDCs lysates were pre-cleared by incubation with protein A/G-agarose beads (Santa Cruz Biotechnology) for 1 hour at 4°C. After the removal of protein A/G-agarose beads by brief centrifugation, the samples were incubated with monoclonal anti-GSH antibody for 2 hours at 4°C. The antibody-antigen complexes were immunoprecipitated by incubating with protein A/G-agarose beads overnight at 4°C. Non-specific bound proteins were removed by washing protein A/G-agarose beads once with ice-cold lysis buffer and twice with PBS. Bound immunocomplexes were solubilized in SDS-loading buffer and analyzed by subsequent SDS-PAGE, probing of the immunoblots with goat polyclonal anti-SERCA2 antibody (Santa Cruz Biotechnology). As a reagent control, cell lysates were incubated with isotype control IgG and subjected to the same procedures.

RNA isolation and Real-Time RT-PCR analysis

Total RNA was prepared using the Isolate II RNA mini kit (Bioline, Taunton, MA) and cDNA was then generated with the iScript cDNA synthesis kit (Bio-Rad) according to the manufacturers’ protocols. Subsequently, quantification of gene expression was performed in duplicates using iQSYBR Green supermix (Bio-Rad) with detection on a MyiQ Real-Time PCR System (Bio-Rad). The reaction cycles used were 95°C for 5 min, and then 40 cycles at 95°C for 15 s and 58°C for 1 min followed by melt curve analysis. The following primers were used: CXCR4, forward 5′-TCAGTGGCTGACGCTCTTT-3′, reverse 5′-CTTGGCCCTTTGACTGTTGGT-3′; GAPDH, forward, 5′-CCCAGCAAGGACACTGAGCAA-3′, reverse 5′-CCCAGCAAGGACACTGAGCAA-3′.
AGGCCCCCTCTGTTATTAGG-3'; IP3R1, forward 5'-AGAAGGAGGAGGCTGTTAGT-3'; IP3R2, forward 5'-CAACCGAAGC-CTTGGAGACCTTGAG-3'; reverse 5'-TTGCGCAGAGGGT-TGATGACTCT-3'; IP3R3, forward 5'-AGACCCGGCCTG-CTCAGTACTGAAAG-3', reverse 5'-GTGCACTGTCGCA-GATGGCAGGT-3'; SERCA2, forward 5'-GATTCCCCGTCGTTTTGAC-3', reverse 5'-CCACAGGGAGGAGGAT-3'. Relative gene expression quantification was based on the comparative threshold cycle (CT) method (2−△△CT) with normalization of the raw data to the included housekeeping gene (β-actin).

Measurement of intracellular reduced protein thiol levels
ThioGlo-1 (TG-1, [3H-Naphthol][2,1-b]pyran-s-carboxylic Acid, 10-[2,5-Dihydro-2,5-dioxo-1H-pyrrol-1-yl]-9-methoxy-3-oxo, methyl ester) (Calbiochem, San Diego, CA), a maleimide sulfhydryl-specific fluorescent probe, was used to monitor intracellular reduced protein thiols as reported earlier [31]. BMCS, Lin(−) cells or BMDDCs were harvested, washed twice with PBS and then solubilized by ice-cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, plus a protease inhibitor cocktail]. Cell lysates/supernatants were collected from the cells after spinning at 16,000 g for 10 min at 4°C, and low molecular weight (<6 kDa) compounds were eliminated from the cell lysates by using size-exclusion chromatography with a micro Bio-Spin 6 column (BioRad). After measuring protein concentrations by BCA protein assay, 20 μl (~20–50 ug) cell lysate was diluted with 2 ml 20 mM sodium phosphate buffer, pH 7.4. The total intracellular reduced protein thiol levels were then determined by an immediate fluorescence increase upon the addition of 5 μM (final concentration) of TG-1. Briefly, fluorescence was measured by a QM-4 spectrofluorometer (PTI, Birmingham, NJ) in a 10 x 10 mm quartz cuvette under constant stirring at 37°C, controlled by TC 125 Temperature Controller (Quantum Northwest, Shoreline, WA), with excitation (Ex) at 379 nm and emission (Em) at 513 nm using standard kinetic mode with resolution of 0.1 sec. The emission of each sample was recorded for 100 seconds (background) before and until 500 seconds after the addition of TG-1. Saturated TG-1 kinetic mode with resolution of 0.1 sec. The concentrations of GSSG in cell lysates are normalized for protein content, and averaged using SigmaPlot 10.0 software (Systat Software, San Jose, CA).

Measurement of GSH and GSSG levels
BMCS, Lin(−) cells or BMDDCs were harvested and cell lysates were prepared in the same way as described above. Reduced GSH was determined by a fluorometric method (Ex = 379 nm, Em = 513 nm), using Cayman Thiol Detection Assay Kits (Ann Arbor, Michigan) according to the manufacturer's protocol with few modifications. Proteins were precipitated from cell lysates by adding 5% trichloroacetic acid (Sigma-Aldrich, St. Louis, MO). After 10 min at −20°C, supernatants were collected after spinning at 16,000 g for 10 min at 4°C. GSH standards (0.0625 to 5 μM) were prepared by dilution in dH₂O. The concentrations of GSH in cell lysates are expressed in nmol/mg protein. The determination of oxidized GSSG is based on its reduction by glutathione reductase using NADPH as a source of electrons and on measurement of NADPH fluorescence decrease at 37°C. The final concentrations of reagents were 2.66 μg/ml glutathione reductase and 50 μM NADPH in 1.5 ml 50 mM Tris-HCl pH 8.0 containing 1 mM EDTA and 10 μM sodium azide. The enzymatic reaction was monitored for 50 seconds before and until 300 seconds after the addition of each sample or GSSG standard with excitation at 340 nm and emission at 460 nm (resolution 0.1 sec). The concentrations of GSSG in cell lysates are expressed in nmol/mg protein. In both cases, the fluorescence was recorded by a QM-4 spectrofluorometer in standard kinetic mode as described above. GSH, GSSG, NADPH and glutathione reductase were all purchased from Sigma-Aldrich (St. Louis, MO).

ICAT labeling and nanoHPLC-Orbitrap Elite mass spectrometry
BMDDCs were harvested and the pellet was dissolved in ICAT denaturation buffer [100 mM Tris-HCl (pH 8.3), 4 M urea, 0.05% SDS, plus a protease inhibitor cocktail]. Protein (100 μg) was treated with 1.25 mM TCEP to reduce the cysteine residues and labeled with cleavable, cysteine reactive ICAT reagents according to the manufacturer’s instructions (AbSciex, Foster city, CA). Protein from WT and Gstp1/p2−/− mouse was labeled with ‘light’ (L) and ‘heavy’ (H) ICAT reagents, respectively. ICAT labeling and nano-HPLC-Orbitrap Elite MS were performed at the Proteomics Core Facility of the Medical University of South Carolina. Protein identification was performed using Mascot and Sequest nodes within the Proteome Discoverer 1.3 platform (Thermo Scientific) against a RefSeq murine database (28925 entries, downloaded 03/05/12). Parameters for peptide identification were as follows: precursor mass tolerance of 100 ppm, fragment mass tolerance of 0.8 Da, one missed cleavage, dynamic modifications of cysteine with light or heavy labeled ICAT reagent, deamidation of asparagine and glutamine, and methionine oxidation. Only high confidence peptides with a false discovery rate (FDR) <1% were included. The ICAT quantitation was performed using Proteome Discoverer 1.3 by calculating the relative intensities of the light and heavy ICAT labeled pairs. All ratios were normalized using the median ratio from all peptides identified.

MALDI-MS bone imaging
The femurs of mice were harvested and snap frozen in the vapor phase of liquid nitrogen for at least 10 min, and then stored at −80°C until analysis. A cryostat (Microm HM550, Thermo) was used to slice the frozen bones to a thickness of 10 μm. The bone slice was attached to double sided carbon tape (SPI supplies, West Chester, PA), then mounted onto a conductive indium tin oxide coated (ITO) slide (Bruker Daltonics, Billerica, MA). Slides were desiccated at room temperature for at least 15 min before matrix application. MALDI matrix (9-aminoacridine, 5 mg/ml in 70% ethanol) was applied to the bone slices using an ImagePrep spray station (Bruker Daltonics).

MALDI-MS analysis was performed using a Solarix 7T dual source ESI/MALDI Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Bruker Daltonics). Acquisition was set up using FlexControl 3.0 and FlexImaging 4.0 software (Bruker Daltonics). Glutathione metabolites were detected in negative ion mode analyzing the mass range m/z = 50–800 with a SmartBeam II laser operating at 1000 Hz, a laser spot size of 25 μm, and a raster width of 200 μm. For each laser spot, 500 spectra were averaged and all data was normalized using root means square. Images were generated using FlexImaging 4.0 software (Bruker Daltonics). Structural confirmation of GSH and GSSG was done by collision-induced fragmentation of the indicated ions.

Chemotaxis assay
Chemotaxis assays were performed in 24-well transwell chambers with polycarbonate membranes (3 μm pore size; Corning, NY) as described by Brannah-O'Connor et al [52]. In
brief, 600 μl of either chemotaxis buffer (RPMI-1640 with 200 ng/ml CXCL12) or as a control RPMI-1640 alone were placed in the lower chambers. Upper chambers were loaded with 100 μl cell suspensions of Lin(−) cells or BMDDCs at a concentration of 3 x 10^5 cell/ml in RPMI 1640. Where indicated, BMDDCs were preincubated with 10 μM PHPS1 or 1 μM AMD3100 (both from Calbiochem) in serum-free RPMI for 2 h at 37°C prior to measuring chemotaxis. The complete chamber was kept at 37°C in the incubator for 6 or 16 h. After that, cells remaining in the upper chambers and cells that had migrated through the membrane to the lower chambers were counted with a Z1 Coulter Particle Counter (Beckman Coulter, Hialeah, FL). The percentage of migration was determined as migrated/total cells. To calculate the percentage of specific migration induced by chemokine CXCL12, the percentage of cells migrating to medium alone (control) was subtracted from the percentage of cells migrating to medium with CXCL12.

Intracellular calcium oscillations

Lin(−) cells or BMDDCs were harvested, re-suspended at a concentration of 1 x 10^6 cell/ml and incubated with 5 μM Fluo-3-AM (Invitrogen, Life Technologies, Carlsbad, CA) at 37°C for 45 min in RPMI-1640 at 37°C in the dark. All subsequent manipulations were performed with the Fluo-3-AM labeled cells protected from light. The cells were washed three times with PBS to remove extracellular dye and then re-suspended at a concentration of 1 x 10^6 cell/ml in PBS, containing 100 μM CaCl2 (Ca++)-extracellular solution) or 2 mM EGTA (zero Ca++-extracellular solution) immediately prior to use. The kinetics of intracellular free ionized calcium ([Ca2+]i) changes were measured using a QM-4 spectrofluorometer and standard kinetic mode with excitation at 506 nm and emission at 526 nm at 37°C using the Fluo-3-AM labeled cells. The emission of each sample was recorded for 100 seconds before and until 500 seconds after the addition of 100 ng/ml CXCL12. Representative traces of three independent experiments were averaged and smoothed using standard Sigma-Plot 10.0 software (Systat Software, San Jose, CA).

Plasma membrane potential measurements

Plasma membrane potential was determined using the slow-response potential-sensitive dye DiSBAC2(3) (Invitrogen, Life Technologies, Carlsbad, CA). Lin(−) cells or BMDDCs were harvested, washed three times with PBS, and re-suspended at a concentration of 0.5 x 10^6 cell/ml in PBS with 100 μM CaCl2. Cell suspensions were incubated with 5 μM (final concentration) DiSBAC2(3) in a quartz cuvette under constant stirring at 37°C for 3 min in the dark. Then the kinetics of the emission at 560 nm (Ex = 530 nm) were monitored by a QM-4 spectrofluorometer. The emission of each sample was recorded for 100 seconds before and until 500 seconds after the addition of 100 ng/ml CXCL12. Representative traces of three independent experiments were averaged and smoothed using standard Sigma-Plot 10.0 software (Systat Software, San Jose, CA).

Statistical analysis

Student’s t tests were used where P values<0.05 were regarded as statistically significant. Data were expressed as means ± SD with n equal to the number of animals/group examined under each condition.

Supporting Information

Figure S1 In Figure S1, Lin(−) cells responses to CXCL12. (A) Chemotaxis of Lin(−) cells to CXCL12. Wild type and Gstp1/p2−/− (Lin−) cells were either untreated (control) or stimulated with 200 ng/ml CXCL12 for 6 h. Values are average percentages of migration (±SD) from three independent experiments, with asterisks (*) indicating statistical significant differences between WT and Gstp1/p2−/− (Lin−) cells (p<0.05). To calculate the specific chemotaxis induced by CXCL12, the percentage of cells migrating to medium alone (control) was subtracted from the percentage of cells migrating to medium with CXCL12. (B, C) Intracellular calcium (B) and plasma membrane potential (C) dynamics in WT and Gstp1/p2−/− (Lin−) cells in response to CXCL12. The arrows indicate the addition of CXCL12. Data are representative traces of three independent experiments. (TIF)

Author Contributions

Conceived and designed the experiments: JZ ZY DMT KDT. Performed the experiments: JZ ZY PG LR EEJ YM. Analyzed the data: JZ ZY PG YM. Contributed reagents/materials/analysis tools: JZ ZY PG LR EEJ YM. Contributed to the writing of the manuscript: JZ ZY JBB YM DMT KDT.

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