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Supplemental Information

Positive Regulation of Interleukin-1β Bioactivity by Physiological ROS-Mediated Cysteine S-Glutathionylation

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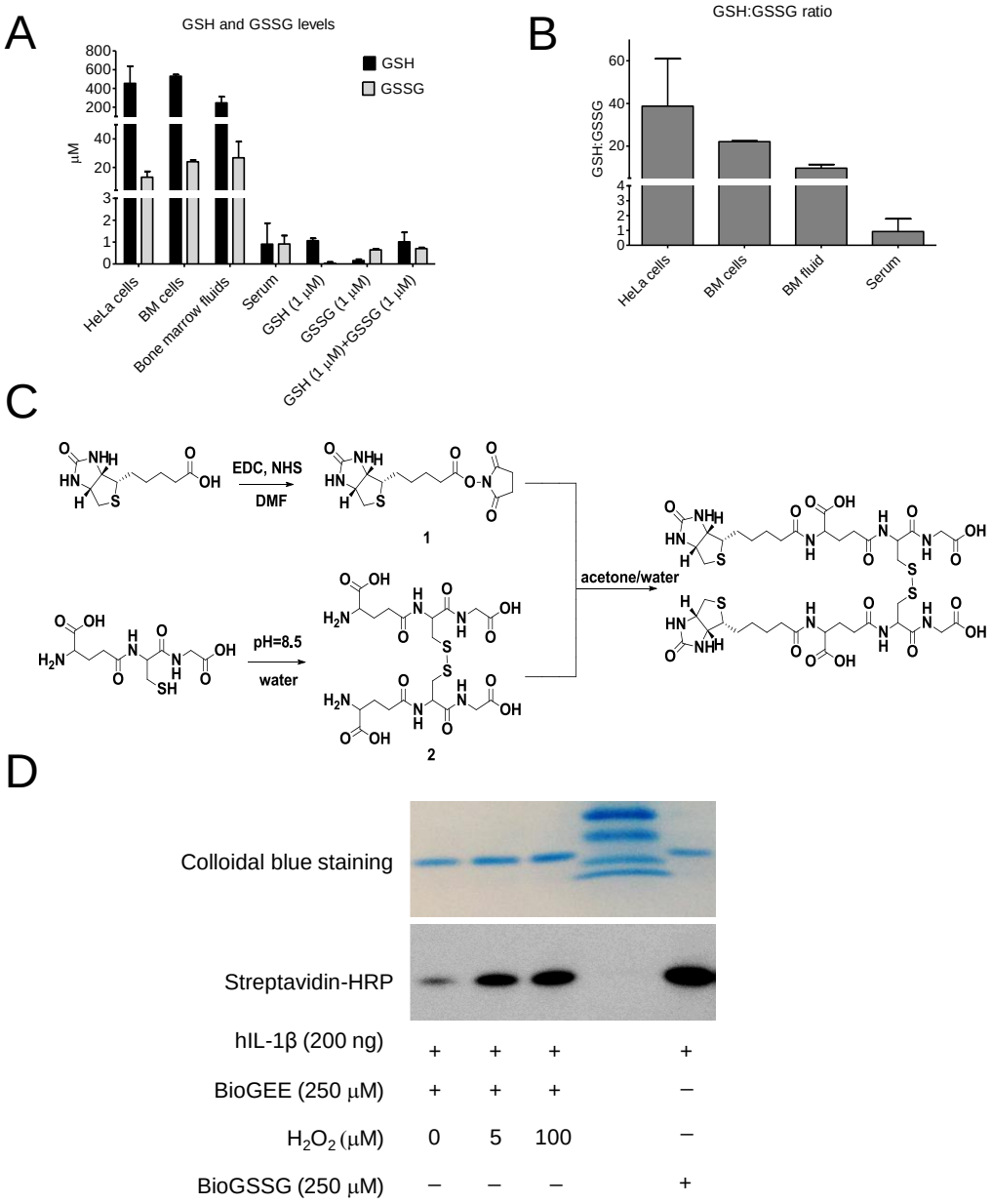

Figure S1. Related to Figure 1. **(A)** GSH and GSSG levels in HeLa and BM cells, BM fluid, and serum. Pure GSH and GSSG were used as controls. **(B)** GSH to GSSG ratios in HeLa and BM cells, bone marrow fluid, and serum. Results are the means (±SD) of three independent experiments. **(C) Synthesis of biotin-GSSG-biotin (BioGSSG)**. 1, N-hydroxysuccinimido biotin. 2, Glutathione disulfide. The final product was purified by HPLC (total yield: 83%) and confirmed by LC-MS (Waters Acquity UPLC/MICROMASS detector). **(D) Human IL-1β (hIL-1β) can be directly S-glutathionylated by GSSG in the absence of H_2O_2, or by reduced glutathione (GSH) in the presence of H_2O_2.** hIL-1β was incubated with the indicated amount of biotinylated reduced glutathione (BioGSH), H_2O_2, or BioGSSG, resolved on non-reducing 4%-12% gradient PAGE gel, and then probed for the biotinylated-glutathione modification using streptavidin-HRP. Total protein loading was evaluated by colloidal blue staining.

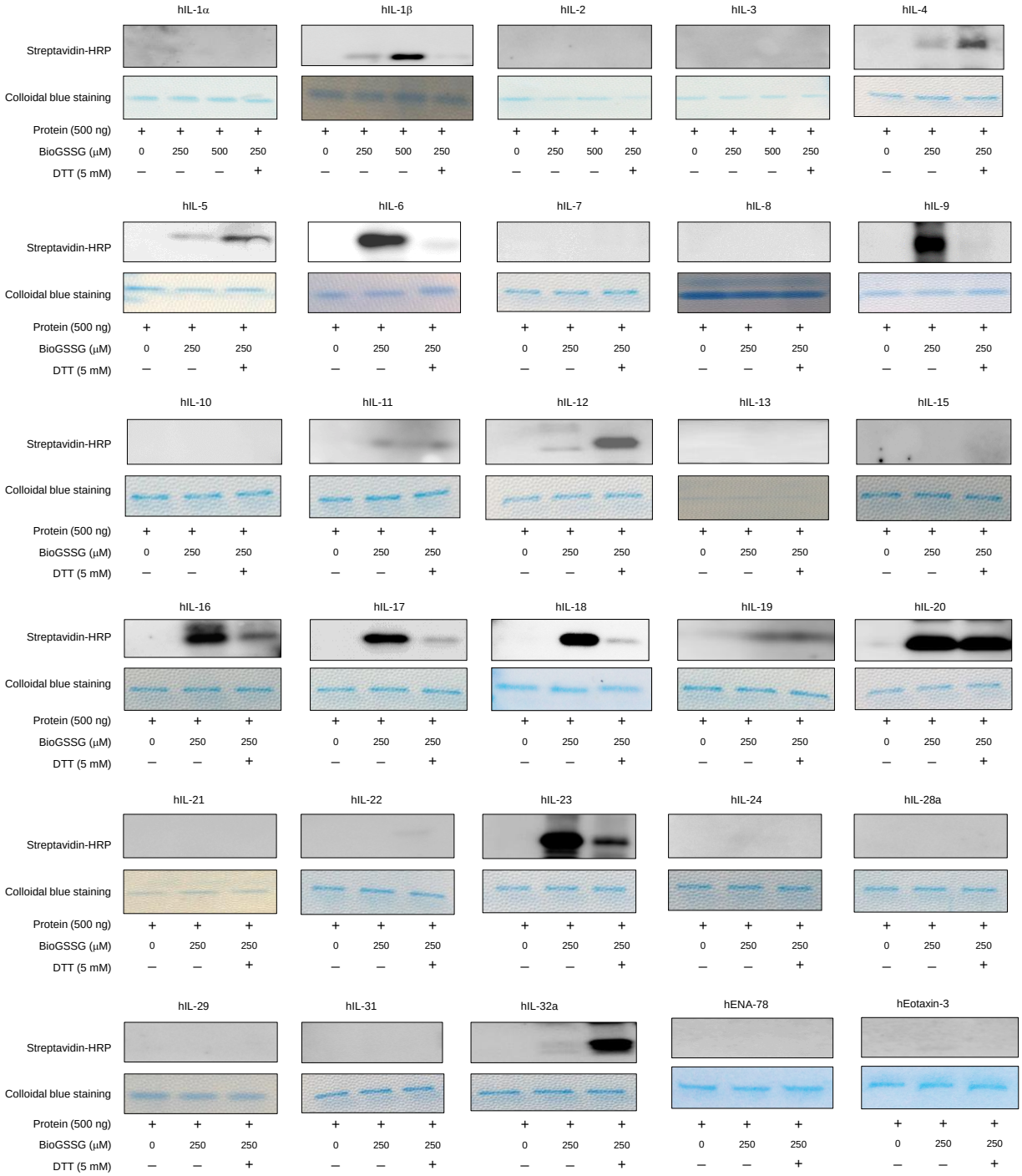

Figure S2. Related to Figure 1. S-Glutathionylation of human cytokines and chemokines. Human cytokines and chemokines were treated with the indicated amounts of BioGSSG and DTT as described in the Methods. Proteins were then resolved on non-reducing 4%-12% gradient PAGE gels and probed for the biotinylated-glutathione modification using streptavidin-HRP. Total protein loading was evaluated by colloidal blue staining.

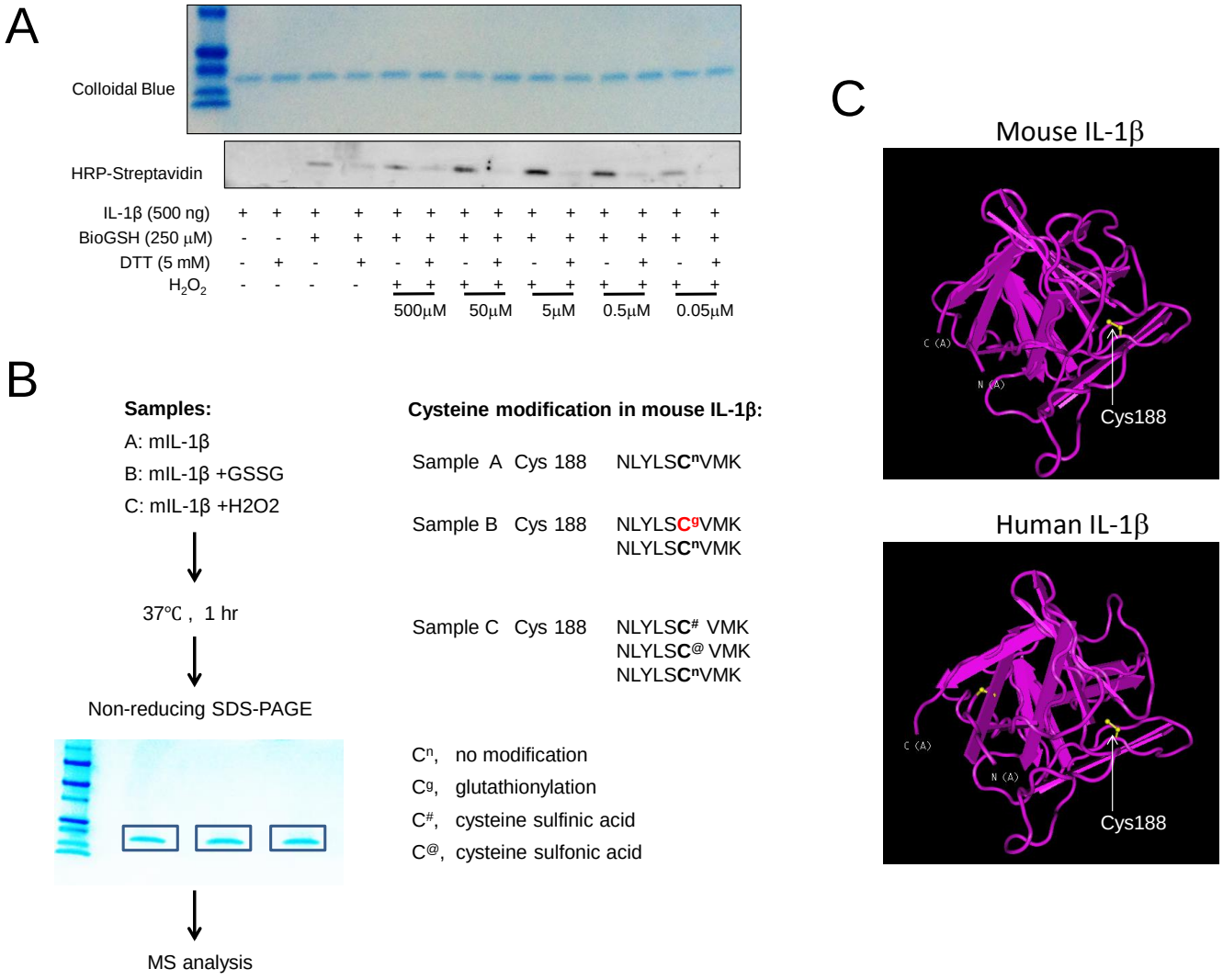

Figure S3. Related to Figure 1. (A) S-glutathionylation of mouse IL-1β (mIL-1β). The experiment was conducted essentially as described in **Figure 1**. mIL-1β was incubated with the indicated amounts of biotinylated reduced glutathione (BioGSH), H_2O_2, or DTT, resolved on a non-reducing 4%-12% gradient PAGE gel, and then probed for the biotinylated-glutathione modification using streptavidin-HRP. Total protein loading was evaluated by colloidal blue staining. **(B) Cysteine modifications of mouse IL-1β were analyzed by mass spectrometry.** The experiment was essentially conducted as described in **Figure 1**. Cysteine S-glutathionylation was only detected when mIL-1β was incubated with oxidized glutathione (GSSG). **(C) Three-dimensional structures of mature mouse and human IL-1β.** C(A) and N(A) represent the C- and N-terminals, respectively. Side chains of cysteine residues are highlighted in yellow. The conserved cysteine (Cys188), on which S-glutathionylation occurs, is indicated. Three-dimensional protein structures of mature human and mouse IL-1β were obtained from the Protein Data Bank (PDB) and annotated using Cn3D software (NCBI).

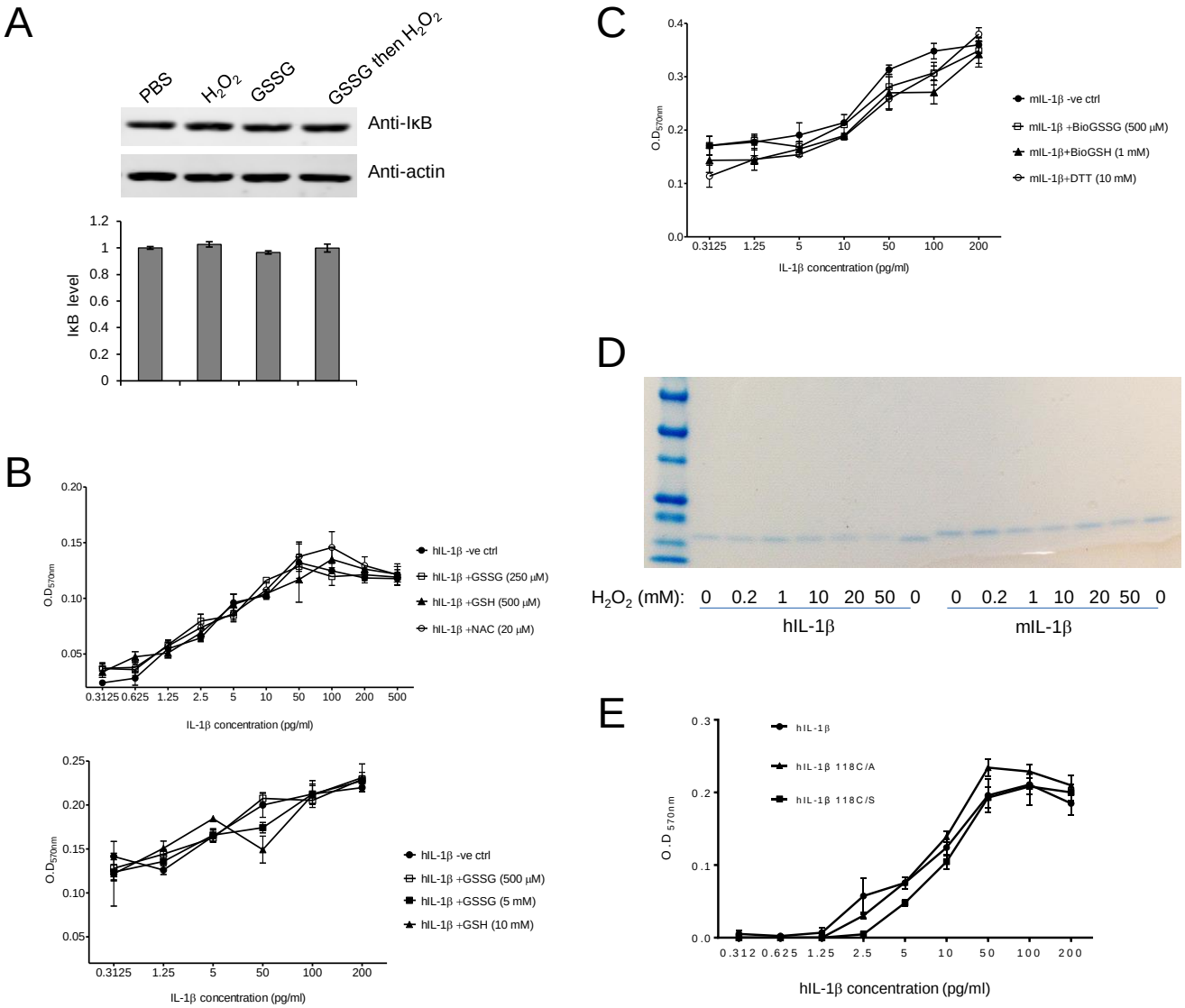

Figure S4. Related to Figure 2. (A) Treatment with GSSG or H2O2 alone does not trigger IκB degradation. HeLa cells were incubated with GSSG (final concentration 1 μM which is equivalent to the amount in the 200pg/μl IL-1 sample in Figure 2) or H_2O_2 (final concentration 0.4 μM which is equivalent to the amount in the 200pg/μl IL-1 sample in Figure 2) for 30 min. IκB degradation was measured as described in **Figure 2**. Shown are the means (±SD) of three independent experiments. **(B-C) Cysteine S-glutathionylation does not affect IL-1β bioactivity directly. (B)** Bioactivity of human IL-1β was measured using a D10 cell proliferation assay. D10.G4.1 cells were incubated with the indicated concentrations of IL-1β for 3 days. Cell viability was determined by the MTT assay and absorbance was determined at 570 nm. **(C)** Bioactivity of mouse IL-1β. Data are expressed as mean OD±SD of triplicate measurements. **(D) H_2O_2-treatment does not cause degradation of IL-1β.** hIL-1β (500 ng) and mIL-1β (500 ng) were incubated with the indicated amounts of H_2O_2 overnight, separated on SDS-PAGE, and then stained with colloidal blue. **(E) Cys188 is dispensable for IL-1β bioactivity.** The biological activity of wild-type and indicated mutant forms of human IL-1β was assessed using a D10 cell proliferation assay as described in **Figure S4B.** Data are expressed as mean OD±SD of triplicate measurements.

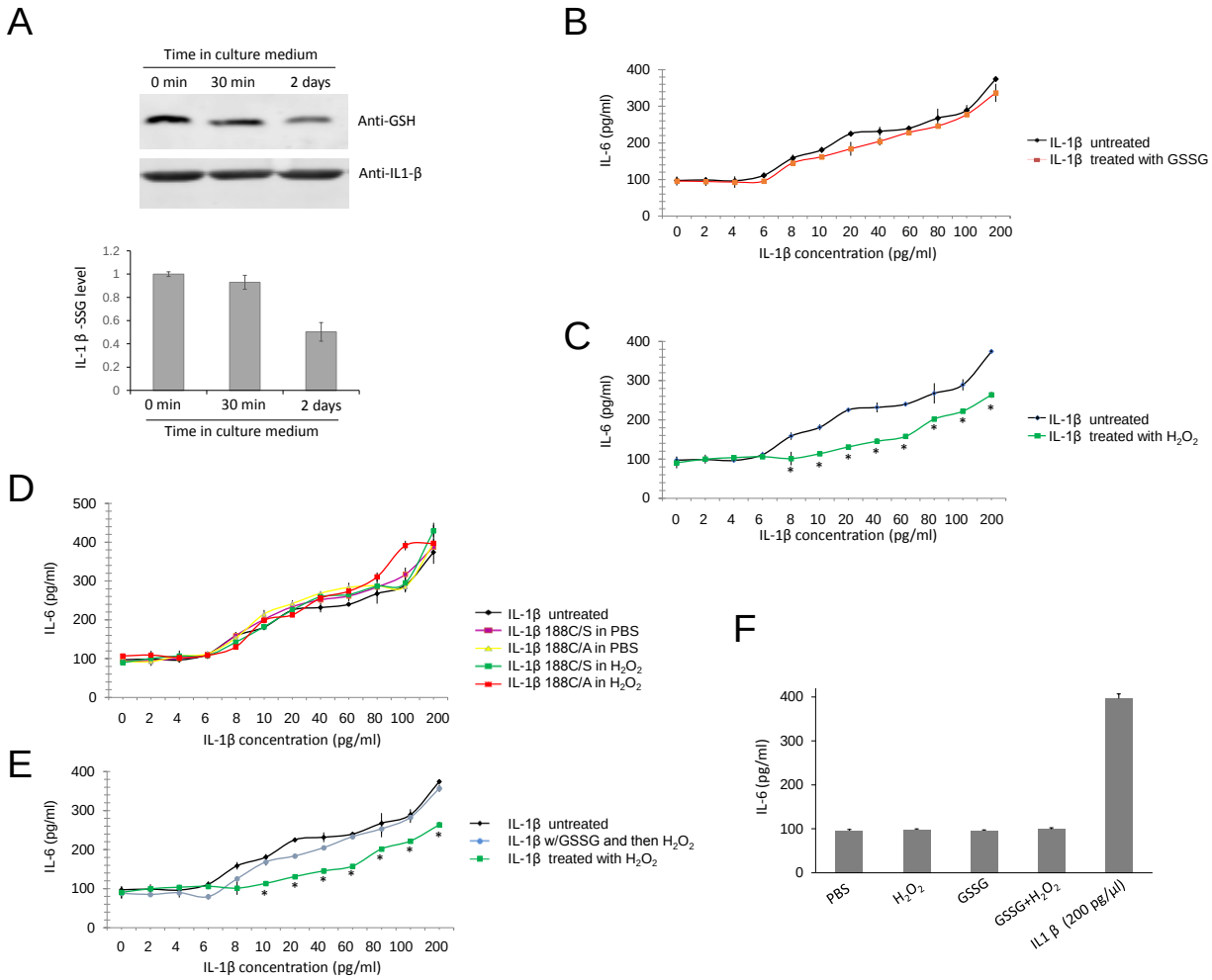

Figure S5. Related to Figure 2. (A) IL-1β glutathionylation is relatively stable in culture medium with cells. Glutathionylated IL-1β was incubated in culture medium (DMEM/Nut.Mix.F-12/10% FBS) for indicated time. The level of IL-1beta glutathionylation was assessed as described in Figure 1. **(B-F) Bioactivity of IL-1β was measured by monitoring IL-1β-induced IL-6 production by mouse embryonic fibroblasts (MEF) cells.** MEF cells were incubated with the indicated concentrations of IL-1β for 2 days. The amount of secreted IL-6 was measured using a Mouse IL6 ELISA kit (eBioscience). **(B) The effect of glutathionylation on IL-1β bioactivity**. IL-1β was pretreated with or without GSSG (250 µM) for one hour at 37°C before adding to MEF cell cultures. **(C) The effect of H_2O_2-induced oxidation on IL-1β bioactivity**. IL-1β was pretreated with H_2O_2 (100 µM) for one hour at 37°C before adding to MEF cell cultures. **(D) Cys188 is dispensable for IL-1β bioactivity.** The wild-type and indicated mutant forms of IL-1β were pretreated with or without H_2O_2 (100 µM) for one hour at 37°C before adding to MEF cell cultures. **(E) The effect of glutathionylation on H_2O_2-induced deactivation of IL-1β**. IL-1β was pretreated overnight with GSSG (250 µM) and then with H_2O_2 (100 µM) for another 30 min at 37°C before adding to MEF cell cultures. Shown are the means (±SD) of three independent experiments. * p<0.01versus control (untreated). **(F) Treatment with GSSG or H2O2 alone does not trigger IL-6 secretion by MEF cells**. MEF cells were incubated with GSSG (final concentration 1 µM which is equivalent to the amount in the 200pg/µl IL-1 sample) or H_2O_2 (final concentration 0.4 µM which is equivalent to the amount in the 200pg/µl IL-1 sample) for 2 days. Shown are the means (±SD) of three independent experiments.

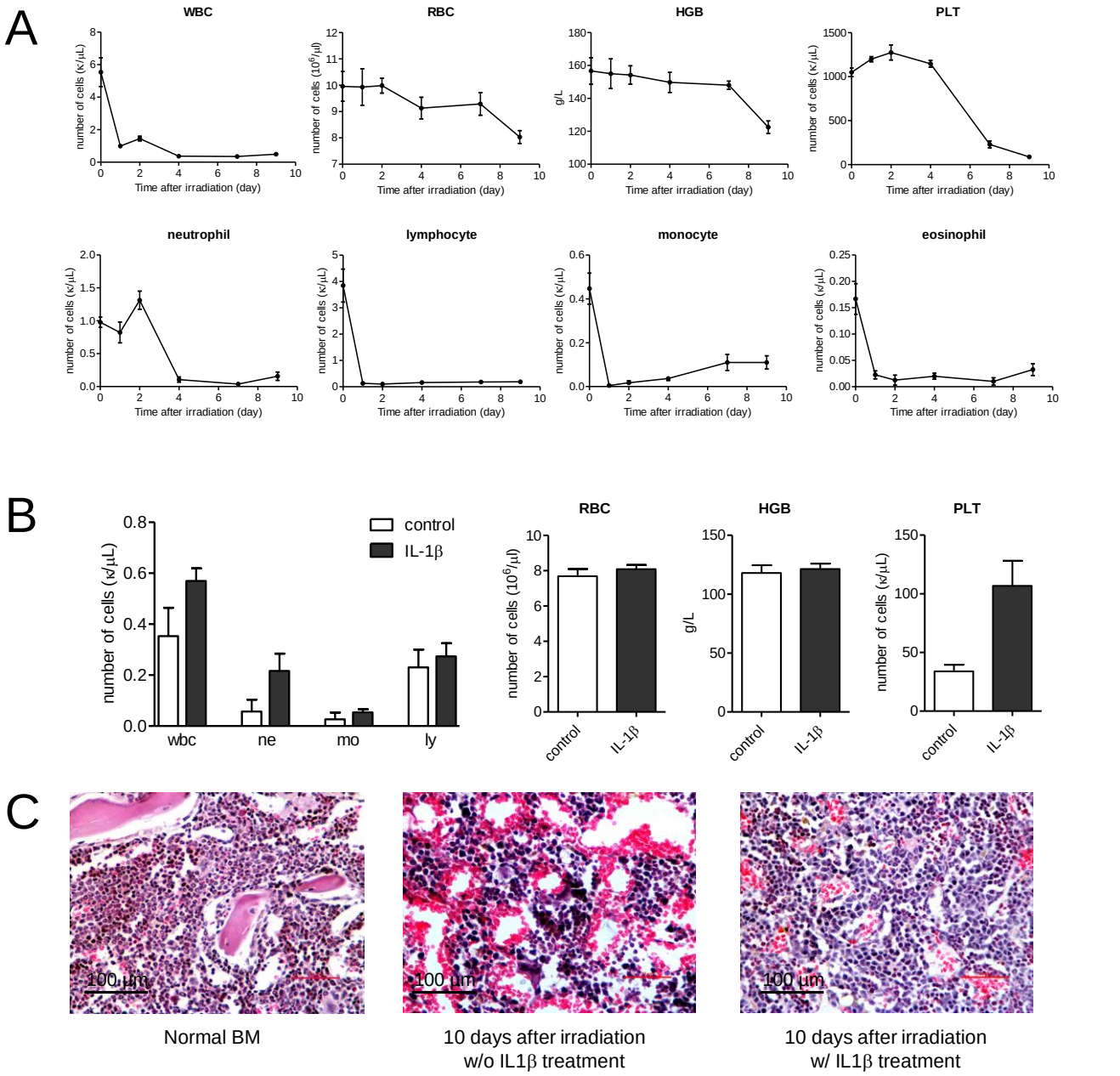

Figure S6. Related to Figure 4. IL1β accelerates bone marrow recovery in irradiated mice. (A) WT C57BL/6 male mice (8-week-old) were treated with 6Gy γ-irradiation (0.64Gy/min×9.4 min). Mice were sacrificed and the peripheral blood was collected by retro-orbital bleeding at indicated time points. Peripheral blood cell counts were evaluated using the Hemavet automated cell counter (Sysmex, XT2000i) (n=3-4). **(B)** WT C57BL/6 mice (male, 10-week-old) were pretreated with 1.0 μg/mouse IL-1β (administered i.p.) 20 h before irradiation. The mice were then given a dose of 5.5Gy γ-irradiation (whole body irradiation, 1.20Gy/min). On day 10 following irradiation, mice were sacrificed and their peripheral blood cell counts and hemoglobin levels (HGB) were evaluated. Results are the means (±SD) of three independent experiments. * p<0.01versus control. **(C)** The femur was fixed and hematoxylin and eosin (H&E) staining performed on femur sections. Representative images of three independent experiments are shown.

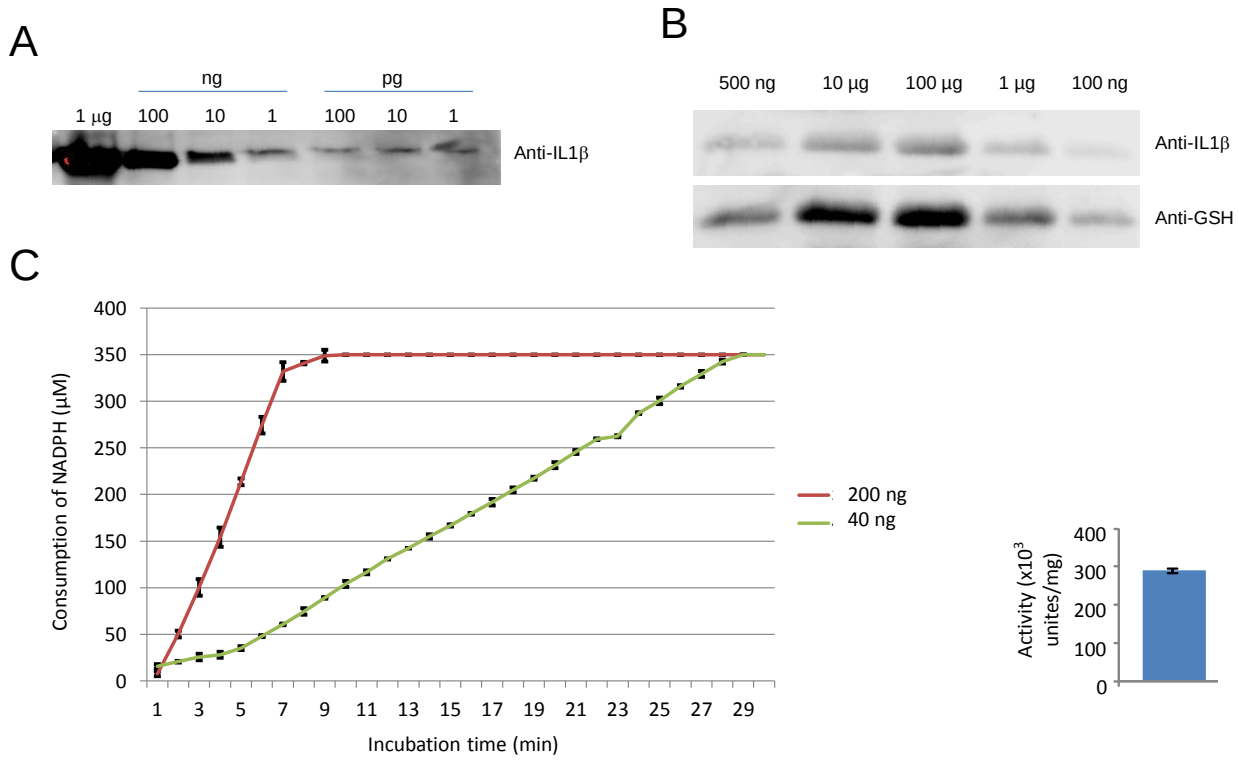

Figure S7. (A-B) Related to Figure 4. Detection of intraperitoneally injected recombinant mouse IL-1β and its S-glutathionylation by western blotting. A) Indicated amounts of His-tagged recombinant mouse IL-1β were injected intraperitoneally into wild-type mice (8-12 week old). The recombinant IL-1β was pulled down from the serum using Ni-NTA agarose beads 24 h after the injection. Precipitated IL-1β was assessed by western blotting using anti-mouse IL-1β antibodies. **B)** S-glutathionylation of injected recombinant IL-1β. Indicated amounts of His-tagged recombinant mouse IL-1β were injected i.p. Recombinant IL-1β was pulled down from the serum using Ni-NTA agarose beads 72 h after injection. S-glutathionylation was detected using an anti-GSH antibody. **C) Related to Figure 5. Glutaredoxin activity of recombinant mouse Grx1.** Purified recombinant mouse Grx1 protein was mixed with a reaction buffer consisting of 137 mM Tris-HCl buffer (pH 8.0), 0.5 mM GSH, 1.2 units of GSSG reductase, 2.5 mM 2-hydroxyethyl disulfide (HED), 0.35 mM NADPH, and 1.5 mM EDTA (pH 8.0). The reaction proceeded at 30°C, and Grx activity was measured spectrophotometrically at 340 nm. One unit was defined as consumption of 1 µmol of NADPH per min. Shown in the lower panel is the number of units per mg protein, calculated based on data collected from 40 ng Grx1 protein. Shown are means ± SD of three experiments.

Cytokines that can not be glutathionylated:			
IL-1α			
IL-2	IL-8	IL-21	IL-29
IL-3	IL-10	IL-22	IL-31
IL-7	IL-13	IL-24	ENA 78 (CXCL15)
	IL-15	IL-28A	Eotaxin-3 (CCL26)
Cytokines that can be glutathionylated - glutathionylation is enhanced by DTT treatment:			
IL-4	IL-11	IL-19	IL-32a
IL-5	IL-12	IL-20	
Cytokines that can be glutathionylated - glutathionylation is suppressed by DTT treatment:			
IL-1β			
IL-6	IL-9	IL-17	IL-23
	IL-16	IL-18	

Table S1. Related to Figure 1. S-glutathionylation of human cytokines and chemokines.

Patient ID	Treatment	Date	Case	Glutaredoxin	Increased
Normal BM	n/a	n/a	n/a	<10%	n/a
	n/a	n/a	n/a	negative	n/a
	n/a	n/a	n/a	negative	n/a
	n/a	n/a	n/a	<10%	n/a
	n/a	n/a	n/a	negative	n/a
#1	Dx AML	12/30/2013	13:S46507	2%	
	day 11 post induction	1/9/2014	14:S841	30%	
	day 31 post induction	1/29/2014	14:S3124	5%	**negative**
#2	Dx AML	3/16/2013	13:S11217	2%	
	day 10 post induction	4/5/2013	13:S12920	50%	
	day 35 post induction	4/30/2013	13:S15999	30%	positive
#3	Dx AML	2/18/2010	10:S7769	2%	
	day 15 post induction	3/5/2010	10:S10159	40%	
	day 25 post induction	3/15/2010	10:S11638	30%	positive
#4	Dx AML	3/18/2010	10:S12196	2%	
	day 12 post induction	3/29/2010	10:S13868	40%	
	day 28 post induction	4/14/2010	10:S16783	30%	positive
#5	Dx AML	5/6/2010	10:S20489	20%	
	day 14 post induction	5/24/2010	10:S23321	30%	
	day 24 post induction	6/3/2010	10:S25121	10%	**negative**
#6	Dx AML	5/28/2010	10:S24389	10%	
	day 14 post induction	6/11/2010	10:S26456	20%	
	day 32 post induction	6/29/2010	10:S29353	5%	**negative**
#7	Dx AML	8/11/2010	10:S36130	2%	
	day 14 post induction	8/25/2010	10:S38256	30%	
	day 30 post induction	9/8/2010	10:S40250	40%	positive
#8	Dx AML	8/13/2010	10:S36366	2%	
	day 13 post induction	8/26/2010	10:S38455	40%	
	day 27 post induction	9/9/2010	10:S40489	20%	positive
#9	Dx AML	8/5/2010	10:S35177	2%	
	day 14 post induction	8/19/2010	10:S37255	30%	
	day 34 post induction	9/9/2010	10:S40491	30%	positive
#10	Dx AML	9/7/2010	10:S40129	2%	
	day 10 post induction	9/17/2010	10:S41982	30%	
	day 27 post induction	10/4/2010	10:S44660	30%	positive
#11	Dx AML	12/6/2010	10:S55578	10%	
	day 10 post induction	12/20/2010	10:S57949	30%	
	day 30 post induction	1/13/2011	11:S2151	50%	positive
#12	Dx AML	12/17/2010	10:S57678	10%	
	day 10 post induction	12/27/2010	10:S58726	30%	
	day 34 post induction	1/20/2011	11:S3091	30%	positive
#13	Dx AML	2/7/2011	11:S5610	2%	
	day 14 post induction	2/23/2011	11:S8351	40%	
	day 30 post induction	3/14/2011	11:S11287	10%	positive
#14	Dx AML	3/17/2011	11:S11991	20%	
	day 12 post induction	3/30/2011	11:S14152	30%	
	day 31 post induction	4/18/2011	11:S17451	50%	positive

#15	Dx AML	4/13/2011	11:S16588	60%	
	day 14 post induction	4/27/2011	11:S18926	30%	
	day 28 post induction	5/11/2011	11:S21358	30%	**negative**
#16	Dx AML	5/6/2011	11:S20711	20%	
	day 12 post induction	5/18/2011	11:S22686	30%	
	day 19 post induction	5/26/2011	11:S24225	10%	**negative**
#17	Dx AML	5/11/2011	11:S21512	2%	
	day 13 post induction	5/25/2011	11:S24002	20%	
	day 29 post induction	6/13/2011	11:S27050	40%	positive
#18	Dx AML	12/27/2012	S12-56771	20%	
	day 10 post induction	1/7/2013	S13-655	10%	
	day 25 post induction	1/22/2013	S13-2709	70%	positive
#19	Dx AML	6/3/2008	S08-23722	80%	
	day 14 post induction	6/17/2008	S08-26189	10%	
	day 41 post induction	7/14/2008	S08-30145	50%	**negative**
#20	Dx AML	7/21/2009	S09-32810	2%	
	day 14 post induction	8/4/2009	S09-35142	70%	
	day 30 post induction	8/20/2009	S09-37751	80%	positive
#21	Dx AML	12/30/2009	S09-59127	2%	
	day 13 post induction	1/12/2010	S10-1587	80%	
	day 33 post induction	2/2/2010	S10-5145	70%	positive
#22	Dx AML	1/16/2010	S10-2503	50%	
	day 13 post induction	1/29/2010	S10-4662	80%	
	day 27 post induction	2/12/2010	S10-6983	20%	**negative**
#23	Dx AML	4/18/2013	13:S14527	20%	
	day 14 post induction	5/6/2013	13:S16881	20%	
	day 29 post induction	5/21/2013	13:S19143	70%	positive
#24	Dx AML	2/18/2012	12:S8513	negative	
	day 10 post induction	3/9/2012	12:S11860	5%	
	day 27 post induction	3/26/2012	12:S14580	80%	positive
#25	Dx AML	4/20/2012	12:S19251	5%	
	day 12 post induction	5/8/2012	12:S22330	negative	
	day 28 post induction	5/24/2012	12:S25454	50%	positive
#26	Dx AML	2/1/2013	13:S4351	2%	
	day 10 post induction	2/14/2013	13:S6116	50%	
	day 31 post induction	3/7/2013	13:S8881	30%	positive
#27	Dx AML	11/16/2011	11:S53343	2%	
	day 12 post induction	11/28/2011	11:S55012	5%	
	day 21 post induction	12/8/2011	11:S57091	50%	positive

Table S2. Related to Figure 6. Glutaredoxin staining in bone marrow biopsies of acute myeloid leukemia (AML) patients. In this study, all 27 patients were diagnosed with AML (Dx AML). Standard chemotherapy induction was usually initiated shortly after diagnosis. Expression of glutaredoxin (Grx1) was assessed by immunostaining using a specific human Grx1 antibody. The percentage of BM cells expressing Grx1 was counted. Patients that satisfied the following criteria were scored as "positive": a) the percentage of Grx1-expressing cells in the BM was greater than 10% one month after induction chemotherapy; and b) one month after induction, the percentage of Grx1-expressing cells in the BM was at least two-fold increased compared to at the time of diagnosis.

Supplemental Experimental Procedures

Mice

Grx1$^{-/-}$ mice were kindly provided by Dr. Y.S. Ho and were backcrossed for more than 16 generations onto a C57BL/6 background (Ho et al., 2007). In all experiments, age-matched C57BL/6 mice (Jackson Laboratories) were used as wild-type controls. The numbers of mice analyzed per group in each experiment are indicated in the figure legends. All experiments involving equal treatments in wild-type and mutant samples and animals were conducted by experimenters blind to conditions. All animal manipulations were conducted in accordance with the Animal Welfare Guidelines of the Children's Hospital Boston. All procedures were approved and monitored by the Children's Hospital Animal Care and Use Committee.

Biotin-GSSG-biotin (BioGSSG) synthesis

Biotin-GSSG-biotin was synthesized as described in **Supplementary Figure S1C.** All reagents including (+)-biotin and glutathione (GSH) were obtained from Sigma-Aldrich (St. Louis, MO), except for the solvents (DMF) which were from Fisher Scientific (Thermo Fisher Scientific, Waltham, MA). N-hydroxysuccinimido biotin was synthesized by strictly following the previously reported procedure and was used in the follow step without further purification(He et al., 2009). Glutathione disulfide **(2)** was prepared by dissolving GSSH (92.0 mg) into an aqueous solution with a final pH of 8.5 (3 mL) at room temperature overnight (Krezel et al., 2011; Stevens et al., 1983). N-hydroxysuccinimido biotin (100.0 mg) was dissolved in acetone (5 ml), which was added to the prepared glutathione disulfide solution. The reaction was stirred overnight at room temperature, and acetone was removed by rotary evaporation. The solution was acidified with 1.0 M HCl solution, and the crude product was filtered and collected for purification by Waters Delta 600 RP C18 HPLC (total yield: 83%) and confirmed by LC-MS (Waters Acquity UPLC/MICROMASS detector; Waters Corp., Milford, MA).

S-glutathionylation of human cytokines and chemokines

Human cytokines and chemokines were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Proteins were prepared by 1:16 dilution with distilled water (32 µl for every 2 µg of protein). 10 µl of protein solution was divided between three test tubes: DMSO (1 µl) was added to the first tube,

and BioGSSG (biotinylated oxidized glutathione, 1 µl) to the second and third tubes prior to incubation at 37°C for 30 min. 1 µl of DTT (dithiothreitol, 60 mM stock) was then added to the third test tube and 1 µl of distilled water to the first and second tubes. Samples were incubated at RT for another 30 min before being mixed with 12 µl 2x LDS loading buffer (without reducing agents, Bio-Rad Laboratories Inc. (Hercules, CA); Catalog Number 161-0737) and boiled for 7 min. Proteins were resolved on non-reducing 4%-12% gradient gels and probed by western blotting using an α-GSH antibody (Virogen, Watertown, MA; 1:1000). Total protein loading was evaluated by colloidal blue staining (Life Technologies, Carlsbad, CA).

S-glutathionylation of IL-1β *in vitro*

S-glutathionylation of IL-1β was analyzed using multiple methods. Glutathionylation of human IL-1β was identified using BioGSSG in the screening study described above (**Supplementary Figure S2**). S-glutathionylation of human and mouse IL-1β was also assessed using biotinylated reduced glutathione (BioGSH, Molecular Probes, Eugene, OR) in the presence of H_2O_2 (**Figure 1A and Supplementary Figure S1D**). Recombinant human and mouse IL-1β were incubated with the indicated amounts of BioGSH and H_2O_2 for 15 min at RT and then with or without DTT (final concentration 5 mM) for another 15 min. Protein was resolved on non-reducing 4%-12% gradient gel and probed for the biotinylated-glutathione modification using streptavidin-HRP (**Figure 1A and Supplementary Figure S1D**). Alternatively, glutathionylation of human IL-1β was induced by treatment with oxidized glutathione (GSSG) in the absence of H_2O_2. The protein was then resolved on non-reducing 4%-12% gradient gel and probed for the glutathione modification using a specific GSH antibody (**Figure 1B**). In some experiments, biotinylated-glutathionylated IL-1β was pulled down using streptavidin-agarose beads as previously described (Sakai et al., 2012). The beads were washed 3x in PBS, and bound protein was eluted by boiling in 2x LDS buffer containing 5% β-mercaptoethanol. The precipitated protein was resolved on a reducing 4%-12% gradient gel and probed with specific anti-IL-1β antibodies. Total protein loading was evaluated by colloidal blue staining or western blotting using specific human or mouse IL-1β antibodies.

Western blotting

Protein samples were separated on a 4–15% precast polyacrylamide gel (Bio-Rad) and subsequently transferred to a PVDF membrane (Amersham Biosciences; GE Healthcare Life Sciences, Little Chalfont, Bucks., UK). After blocking the membranes in TBS containing 5% BSA (Sigma) and 0.02%

Triton X-100 for 1 h, primary antibodies or streptavidin-HRP were added in blocking solution at the following dilutions: GSH (Virogen, 1:1,000), streptavidin-HRP (Sigma, 1:1,000), IL-1β (R&D Systems, Minneapolis, MN, 1:2,000), IκB (Cell Signaling Technology, Beverly, MA, 1:1,000), and β-actin (GeneTex, Irvine, CA, 1:5,000). After washing, the appropriate HRP-conjugated secondary goat antibodies (Santa Cruz Biotechnology Inc., Dallas, TX) were added (1:10,000) in block solution and incubated for 1 h. Antibody binding was detected using ECL Western Blotting Substrate (Pierce Biotechnology, Rockford, IL). Primary antibody incubations were carried out at 4°C overnight. Remaining incubations were carried out at RT. Densitometry was performed using ImageJ software Gel Analyzer plug-in (National Institutes of Health)(Luo et al., 2002).

Mass spectrometry analysis

Samples were digested with trypsin at 37 °C for 2 h in buffer containing 2 M urea and 50 mM ammonium bicarbonate, acidified with glacial acetic acid to a final concentration of 2% and desalted by ZipTip (Millipore Corp., Billerica, MA). Tryptic peptides were analyzed by highly sensitive nanospray liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) using an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific). The reversed-phase LC column was slurry-packed in-house with 5 μm, 200 Å pore size C_{18} resin (Michrom BioResources Inc., Auburn, CA) in a 100 μm i.d. × 10 cm long piece of fused silica capillary (Polymicro Technologies, Phoenix, AZ) with a laser-pulled tip. After sample injection, the column was washed for 5 min with mobile phase A (0.1% formic acid), and peptides were eluted using a linear gradient of 0% mobile phase B (0.1% formic acid, 80% acetonitrile) to 50% B in 30 min at 200 nL/minute, then to 100% B in an additional 5 min. The LTQ-Orbitrap was operated in a data-dependent mode in which each full MS scan (60,000 resolving power) was followed by eight MS/MS scans, where the eight most abundant molecular ions were dynamically selected and fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. "FT master scan preview mode", "Charge state screening", "Monoisotopic precursor selection", and "Charge state rejection" were enabled so that only the 1+, 2+, and 3+ ions were selected and fragmented by CID. Tandem mass spectra collected by Xcalibur (version 2.0.2) were searched against the pro-IL1β protein database using SEQUEST (Bioworks software from ThermoFisher, version 3.3.1) with full tryptic cleavage constraints, variable methionine oxidation, variable glutathionylation of cysteine (mass add-up for monoisotopic peptide: 305.06808), variable sulfinic acid modification of cysteine (mass add-up for monoisotopic peptide: 31.98983), and variable sulfonic acid modification of cysteine (mass add-up for monoisotopic peptide: 47.98475). Mass tolerance for precursor ions was 10 ppm and mass tolerance for fragment ions was 0.5 Da. The SEQUEST search results were filtered by the criteria "Xcorr versus

charge 1.9, 2.0, 3.0 for 1+, 2+, 3+ ions; ΔCn > 0.1; probability of randomized identification of peptide < 0.01". Confident peptide identifications were determined using these stringent filter criteria for database match scoring followed by manual evaluation of the results.

MS1 quantification

Quantification of each cysteine modification in IL-1β was conducted using Skyline software (SCIEX, Framingham, MA). Skyline MS1 filtering is a label-free quantitative software application that processes full-scan mass spectral data from proteomic experiments by extracting MS1 ion intensity chromatograms across multiple experiments (Jaffe et al., 2008; Schilling et al., 2012). Molecular weight calculation, MS1 peak picking, visualization of underlying MS/MS spectra, and automated peak integration with manual editing capabilities were all performed using Skyline following the related instructions.

D10 cell proliferation assay

Recombinant human or mouse IL-1β was treated with GSSG, H_2O_2, or GSH at the indicated concentrations or in PBS alone (-ve ctrl) for 30 min at 37°C. Recombinant proteins were serially diluted in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 mM HEPES, 1 mM sodium pyruvate, 50 μM 2-ME, 2.5 μg/ml ConA, 100U/ml penicillin, 100μg/ml streptomycin, and 2 mM L-glutamine. A 100 μl sample was added per well of 96-well plates containing 2×10^4 D10.G4.1 cells (ATCC). Each sample dilution was run in triplicate. Cells were cultured with IL-1β samples at 37°C, 5% CO_2 for 72 h. Cell viability was determined using the MTT assay and absorbance was determined at 570 nm.

Determination of IL-1β biologic activity by IL-1β-induced IκB degradation

In most human cells, the nuclear NF-κB transcription factor is sequestered in the cytosol by the inhibitory IκB protein, whose ubiquitin-mediated degradation is initiated by inflammatory cytokines such as IL-1β. Agonists such as IL-1β result in phosphorylation of IκBα at serine 32 and 36. These phosphorylation events lead to ubiquitination of IκBα, followed by its rapid degradation by the 26 S proteasome. To test the biologic activity of IL-1β, HeLa cells were grown to confluence in 6-well plates. IL-1β was diluted at the indicated concentrations in 500 μl DMEM/Nut.Mix.F-12 (Gibco, Life Technologies) 10% FBS and added to the wells after being washed once with the same medium. Cells

were collected after 30 min and lysed. 100 µg of cellular extract was applied to 4-15% (wt/vol) SDS-PAGE. Western blotting for IκB was conducted using a specific anti-IκB antibody and standardized to the actin housekeeping protein. Relative amounts of IκB were quantified by densitometry using NIH ImageJ software as previously described(Luo et al., 2002).

Glutathione (GSH) and oxidized glutathione (GSSG) measurements

GSH and GSSG levels in HeLa cells, BM fluids, BM cells, and mouse sera were measured using the GSH/GSSG-Glo^{TM} assay Kit (Promega Corp., Madison, WI). The assay can detect and quantify total glutathione (GSH+GSSG), GSSG, and GSH/GSSG ratios based on a luminescence system. The BM from one femur of a C57BL/6 mouse was flushed with 200 µl PBS. The suspension was spun down at 1,000 x g for 10 min and the supernatant (BM fluid) collected. The cell pellet was re-suspended in PBS and used at a final concentration of 1 x 10^5 cells per reaction. Mouse sera were collected from fresh whole blood samples from C57BL/6 mice by centrifugation at 500 x g for 10 min. BM fluids, BM cells re-suspended in PBS, or mouse sera (35 µl) were added to a 96-well luminometer-compatible tissue culture plate. For HeLa cells, 5 x 10^3 cells were plated per well and allowed to grow overnight (20-24 h) at 37°C in a 5% CO_2 incubator. Cell media were removed and the cultured cells rinsed in 100 µl PBS. Total or oxidized glutathione lysis reagents were added to each well to obtain a final volume of 50 µl per reaction, followed by 5 min incubation. Next, 50 µl of Luciferin generation reagent was added to each well and slowly mixed by shaking for 30 min. Luciferin detection reagent was added and, after 15 min incubation, luminescence was read using the TriStar LB 941 Multimode microplate reader. A glutathione standard curve was generated by twofold serial dilutions according to the manufacturer's instructions. The total glutathione (GSH+GSSG), GSSG, and GSH/GSSG ratios were calculated using the glutathione standard curve as a reference. The GSH value was obtained by subtracting the GSSG value from the total glutathione (GSH+GSSG). For BM fluids, the BM from one mouse femur was flushed with 200 µl PBS. The volume of a mouse femur cavity is approximately 5 µl (Vogel, 1961). Therefore, the dilution factor for the BM fluids was 5:200 = 1:40. For BM cells, the cell pellet (~3 x10^6 cells) was around 5 µl (after the PBS was removed), and the pellet was re-suspended in 1 ml of PBS. Therefore, the dilution factor for BM cells was about 5:1000 = 1:200.

Plasmid construction and recombinant protein expression

Mouse Grx1 and mature human and mouse IL1β DNA were cloned and expressed using Champion pET SUMO Protein Expression Kits (Invitrogen) following the manufacturer's instructions. PCR products

were generated from corresponding cDNAs (OriGene Technologies, Rockville, MD) and then ligated into the pET SUMO vector. The resulting plasmids were transformed into One Shot®Mach1™-T1R competent cells. Positive clones were subsequently transformed into BL21(DE3) cells for overexpression. The fusion proteins were overexpressed in BL21(DE3) cells grown in LB kanamycin medium by induction with 1 mM IPTG for 4 h after the cultures had obtained an OD600 = 0.4-0.6. Preliminary purification experiments indicated that the overexpressed proteins were almost entirely in the cell lysate supernatants, so subsequent purifications were carried out under native conditions as described in the Ni-NTA Fast Start kit (Qiagen, Limburg, NL). Following elution from the Ni-NTA affinity column with Native Elution Buffer, the protein preparations were dialyzed 3 times against 100 volumes of IL1β reaction buffer (10 mM NaH_2PO_4, 150 mM NaCl, pH 7.2). SDS-PAGE of these samples showed them to be >80% pure by colloidal blue staining (Invitrogen). One liter of initial culture yielded about 20 mg of purified protein. Native proteins were generated using SUMO protease to cleave the N-terminal peptide containing the 6xHis tag and SUMO. Ni-NTA affinity columns (Qiagen) were again used to remove uncleaved proteins and cleaved 6xHis-SUMO.

Extracellular glutathionylation immunostaining.

In situ analysis of protein S-glutathionylation in mouse BM was determined as described previously (Aesif et al., 2011) with slight modifications. In brief, 4% paraformaldehyde solution (Santa Cruz)-fixed, paraffin-embedded bone sections were deparaffinized and rehydrated by passing through a series of xylene and graded alcohol solutions. Free thiol groups were blocked using blocking buffer [25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma), pH 7.4, 0.1 mM EDTA, pH 8.0, 0.01 mM neocuproine (Sigma), and 40 mM N-ethylmaleimide (Sigma)] for 30 min. S-glutathionylated cysteine groups were reduced by incubation with 13.5 μg/ml human Grx1 (American Research Products, Belmont, MA), 35 μg/ml GSSG reductase (Roche, Indianapolis, IN), 1 mM GSH, 1 mM NADPH, 18 μM EDTA, and 137 mM Tris·HCl, pH 8.0, for 20 min. After being washed with PBS, newly reduced cysteine residues were labeled with 1 mM N-(3-maleimidylpropionyl) biocytin (MPB) (Invitrogen) for 1 h before incubation with 1 μg/ml streptavidin-conjugated Alexa Fluor 488 (green, Invitrogen) for 30 min. Tissue sections were then mounted with anti-fade DAPI fluoromount (blue) and viewed under a fluorescence microscope. The mean fluorescence intensities in each imagine were measured with NIH ImageJ software as previously described (Sakai et al., 2012). Randomly selected BM fields that were largely free of non-capillary blood vessels were examined. At least three fields were counted from each slide. The fluorescence intensities for protein glutathionylation (green channel) were normalized to the nuclear DAPI staining (blue channel).

γ-irradiation and mouse survival analysis

WT C57BL/6 mice (male, 10-week-old) were pretreated with or without the indicated amount of WT or mutant forms of mouse IL-1β (administered i.p.) 20 h before irradiation. The mice were then dosed with 8Gy γ-irradiation (whole body irradiation) using a cesium-137 gamma animal irradiator (Atonomic Energy) at rate of 65.0-66.8cGy/min. Mice were monitored daily. The following criteria were utilized to evaluate death: a) cessation of vital signs such as the absence of a heartbeat and/or respiration; b) hypothermia - mice with a ventral surface temperature below 27°C were counted as dead. Hypothermia provides an earlier and more humane experimental endpoint (Kort et al., 1998; Nemzek et al., 2004; Vlach et al., 2000); we determined the fatal hypothermia temperature (FHT, the temperature at which mice will inevitably die) under our experimental conditions and found that when the ventral surface temperature was ≤27 °C, 100% (n=13) of mice died. Therefore, mice with a ventral surface temperature below 27°C were counted as dead mice and were euthanized immediately by asphyxiation by inhalation of CO_2. To compare the survival rate of different groups, mice were checked every 12 h. It is noteworthy that, since more frequent body temperature monitoring was not feasible, some mice died in the 12 h interval between temperature checks. Survival rates were analyzed using Kaplan-Meier survival curves and the log-rank test.

Measurement of glutaredoxin enzymatic activity

Grx1 activity was assayed as described previously (Gan and Wells, 1986; Mieyal et al., 1991b; Raghavachari and Lou, 1996). In brief, cell lysate or purified mouse Grx protein was mixed with a reaction buffer consisting of 137 mM Tris-HCl buffer (pH 8.0), 0.5 mM GSH, 1.2 units of GSSG reductase, 2.5 mM 2-hydroxyethyl disulfide (HED), 0.35 mM NADPH, and 1.5 mM EDTA (pH 8.0). The reaction proceeded at 30°C, and Grx activity was measured spectrophotometrically at 340 nm. One unit was defined as consumption of 1 μmol of NADPH per min. The numbers of units per mg protein (1 mg total protein for cell lysates) under standard conditions are shown.

Amino acid sequence alignments

Primary sequence alignment of pro-IL-1β from different species was rendered with PROMALS3D(Pei et al., 2008). Human (accession number AAA74137), mouse (AAH11437), rat (AAA41426), frog

(AAI70521) and zebrafish (AAQ16563) sequence information were obtained from the NCBI protein database. The proportion cleaved from mature IL-1β by caspase-1 is in grey shade in **Figure 1**.

Hematologic analysis

Mice were anesthetized and immediately bled retro-orbitally into an EDTA-coated tube. Complete blood counts were performed using an automated hematology analyzer (Hemavet 850; Drew Scientific, Oxford, CT, or Sysmex, XT2000i). For BM cells, total cell counts were determined using a hemocytometer, and differential cell counts were conducted by microscopic analysis of Wright-Giemsa-stained cytospins or FACS analysis using a CANTOII flow cytometer with FACSDiva software (BD Biosciences, Franklin Lakes, NJ). The absolute number of neutrophils was then determined based on the cytospin or FACS analysis.

Detection of hydrogen peroxide using Amplex Red

ROS accumulation in the BM after irradiation or IL-1β treatment was measured in freshly isolated BM using an Amplex Red Hydrogen Peroxide Assay Kit (Life Technologies). Amplex Red, an H_2O_2-sensitive fluorescent probe, was prepared according to the manufacturer's instructions. WT C57BL/6 mice (male, 8-10-week-old) were treated with 1.0 μg/mouse IL-1β (administered i.p.) or with 6.0 Gy γ-irradiation (whole body irradiation, 1.20Gy/min). At the indicated times, mice were euthanized and BM samples were prepared by spinning femurs and tibias with 100 μl Krebs-Ringer phosphate buffer containing 5.5 mM glucose (pH 7.35). After further centrifugation (180 x g for 5 min) of the collected BM samples, the BM supernatant (extracellular ROS) was harvested, and 50 μl was assayed (in duplicate) in 96-well fluorescent assay plates (Thermo Fisher Scientific) containing 50 μl/well Amplex Red solution with 0.2 U HRP. Fluorescence was recorded using a fluorometer (excitation 540 nm; emission 590 nm). The H_2O_2 concentration was determined using a standard curve. The volume of the mouse femur cavity is approximately 5 μl(Vogel, 1961). Therefore, the dilution factor for the BM fluids was 5:100 = 1:20. The concentration of H_2O_2 was calculated accordingly.

Hematoxylin and eosin staining

Mice were perfused with 10% neutral buffered formalin (Sigma) immediately following euthanasia by carbon dioxide asphyxiation. Bones were decalcified with formic acid/sodium formate mix. Specimens

were then embedded in paraffin, and 6 μm thick sections were stained with hematoxylin and eosin and examined by light microscopy.

γ-irradiation and mouse survival analysis

WT C57BL/6 mice (male, 10-week-old) were pretreated with or without the indicated amount of WT or mutant forms of mouse IL-1β (administered i.p.) 20 h before irradiation. The mice were then dosed with 6Gy γ-irradiation (whole body irradiation) using a cesium-137 gamma animal irradiator (Atonomic Energy) at rate of 65.0-66.8cGy/min. Mice were monitored daily. The following criteria were utilized to evaluate death: a) cessation of vital signs such as the absence of a heartbeat and/or respiration; b) hypothermia - mice with a ventral surface temperature below 27°C were counted as dead. Hypothermia provides an earlier and more humane experimental endpoint (Kort et al., 1998; Nemzek et al., 2004; Vlach et al., 2000); we determined the fatal hypothermia temperature (FHT, the temperature at which mice will inevitably die) under our experimental conditions and found that when the ventral surface temperature was ≤27 °C, 100% (n=13) of mice died. Therefore, mice with a ventral surface temperature below 27°C were counted as dead mice and were euthanized immediately by asphyxiation by inhalation of CO_2. To compare the survival rate of different groups, mice were checked every 12 h. It is noteworthy that, since more frequent body temperature monitoring was not feasible, some mice died in the 12 h interval between temperature checks. Survival rates were analyzed using Kaplan-Meier survival curves and the log-rank test.

Detection of IL-1β S-glutathionylation *in vivo*

Purified His-tagged recombinant mouse IL-1β (1 μg/mouse) was injected i.p. into wild-type mice (8-12-week-old). Recombinant IL-1β was pulled down from the serum (400 μl serum per mouse) or BM lavage (200 μl per mouse) using Ni-NTA agarose beads at the times indicated after the injection. The amounts of precipitated IL-1β were assessed by western blotting using anti-mouse IL-1β antibodies. S-glutathionylation of injected recombinant IL-1β was detected using an anti-GSH antibody. BM lavage was prepared by spinning femurs and tibias with 200 μl Krebs-Ringer phosphate buffer containing 5.5 mM glucose (pH 7.35). For MS analysis of cysteine modification of i.p.-injected recombinant mouse IL-1β, His-tagged IL-1β alone (1 μg) or IL-1β (1 μg) plus recombinant Grx1 (1 μg) were injected i.p. into WT mice 24 h before whole body γ-irradiation (4Gy). Recombinant IL-1β was pulled down from the serum using Ni-NTA agarose beads 3 days after irradiation, separated on SDS-PAGE, and then processed for MS.

Measurement of glutaredoxin enzymatic activity

Grx1 activity was assayed as described previously (Gan and Wells, 1986; Mieyal et al., 1991a; Mieyal et al., 1991b; Raghavachari and Lou, 1996). In brief, cell lysate or purified mouse Grx protein was mixed with a reaction buffer consisting of 137 mM Tris-HCl buffer (pH 8.0), 0.5 mM GSH, 1.2 units of GSSG reductase, 2.5 mM 2-hydroxyethyl disulfide (HED), 0.35 mM NADPH, and 1.5 mM EDTA (pH 8.0). The reaction proceeded at 30°C, and Grx activity was measured spectrophotometrically at 340 nm. One unit was defined as consumption of 1 μmol of NADPH per min. The numbers of units per mg protein (1 mg total protein for cell lysates) under standard conditions are shown.

Glutaredoxin staining in BM biopsies of acute myeloid leukemia (AML) patients

Twenty-seven patients were diagnosed with AML (Dx AML). Standard chemotherapy induction was usually initiated shortly after diagnosis. Expression of glutaredoxin (Grx1) was assessed by immunostaining using a specific human Grx1 antibody. The percentage of BM cells expressing Grx1 was counted. Patients that satisfied the following criteria were scored as "positive": a) the percentage of Grx1-expressing cells in the BM was greater than 10% one month after induction chemotherapy; and b) one month after induction, the percentage of Grx1-expressing cells in the BM was at least two-fold increased compared to at the time of diagnosis.

Statistical analysis

For most experiments, the 2-tailed, unpaired, Student's t-test was used to compare groups (Microsoft Excel or Prism software). Data were presented as means (±SD). A p-value≤0.05 was considered statistically significant. Statistical power analysis was used to justify the sample size. We assumed that data were normally distributed, since most outcome values were symmetrically distributed around the mean value within each group. The variance was similar between groups as determined by the *F* test. No samples or animals subjected to successful procedures and/or treatments were excluded from the analysis. No randomization was used for animal studies, since it was not applicable in this case. For survival analysis, Kaplan-Meier survival curves were generated using survival data and groups were compared by log-rank analysis using Prism software (Graph Pad Software, Inc.).

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