Published in final edited form as:

Nat Med. 2009 April ; 15(4): 455–461. doi:10.1038/nm.1886.

Bioluminescence imaging of myeloperoxidase activity *in vivo*

Shimon Gross¹, Seth T Gammon¹, Britney L Moss¹, Daniel Rauch², John Harding², Jay W Heinecke³, Lee Ratner², and David Piwnica-Worms^{1,4}

¹Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington University School of Medicine, St. Louis, Missouri, USA

²Division of Molecular Oncology, Department of Medicine, Washington University School of Medicine, St. Louis, Missouri, USA

³Department of Medicine, University of Washington, Seattle, Washington, USA

⁴Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri, USA

Abstract

The myeloperoxidase (MPO) system of activated phagocytes is central to normal host defense mechanisms, and dysregulated MPO contributes to the pathogenesis of inflammatory disease states ranging from atherosclerosis to cancer. Here we show that upon systemic administration, the small molecule luminol enables noninvasive bioluminescence imaging (BLI) of MPO activity *in vivo*. Luminol-BLI allowed quantitative longitudinal monitoring of MPO activity in animal models of acute dermatitis, mixed allergic contact hypersensitivity, focal arthritis and spontaneous large granular lymphocytic tumors. Bioluminescence colocalized with histological sites of inflammation and was totally abolished in gene-deleted *Mpo*^{-/-} mice, despite massive tissue infiltration of neutrophils and activated eosinophils, indicating that eosinophil peroxidase did not contribute to luminol-BLI *in vivo*. Thus, luminol-BLI provides a noninvasive, specific and highly sensitive optical readout of phagocyte-mediated MPO activity *in vivo* and may enable new diagnostic applications in a wide range of acute and chronic inflammatory conditions.

The heme-containing enzyme MPO is a key component of the cytotoxic armamentarium of phagocytic white blood cells^{1,2}. MPO is by far the most abundant protein product in azurophilic granules of neutrophils (5%), constitutes approximately 1% of monocyte protein and is found in the lysosomes of other polymorphonuclear leukocytes and macrophages. The phagosomal oxidative burst is initiated by a stimulus-dependent assembly of the phagocytic NADPH oxidase (Phox), a multimeric protein complex located on the phagosomal membrane. Phox then reduces molecular oxygen to produce superoxide anion (O₂^{•-}), which further dismutates to yield the relatively unreactive hydrogen peroxide (H₂O₂)¹. Upon phagocytic activation, large quantities of active MPO are secreted into phagosomes, catalyzing the production of highly bactericidal hypochlorous acid (HOCl) with H₂O₂ and chloride ions (Cl⁻) as substrates (Fig. 1a)¹.

Correspondence should be addressed to D.P.-W. (piwnica-wormsd@mir.wustl.edu).

Note: Supplementary information is available on the Nature Medicine website.

Author Contributions: S.G. designed and performed most of the experiments, spearheaded data analysis and wrote and edited the manuscript; S.T.G. performed experiments and edited the manuscript; B.L.M. performed experiments and edited the manuscript; D.R. assisted with experiments and edited the manuscript; J.H. assisted with experiments; J.W.H. provided crucial reagents, helped design experiments and edited the manuscript; L.R. provided crucial reagents, helped design experiments and edited the manuscript; D.P.-W. guided the project, designed experiments, analyzed data and wrote and edited the manuscript.

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Aside from HOCl, MPO catalyzes the formation of other reactive molecular species such as tyrosyl radicals, aldehydes and perhaps hydroxyl radicals ($\text{OH}^\bullet$)^{1,2} and oxidizes nitric oxide ($\text{NO}^\bullet$) to produce nitrite³. Each of these products can directly or indirectly activate cellular signaling cascades or further react to induce cellular injury of both the invading species and the host tissue. It is, therefore, not surprising that MPO contributes to the microbicidal activities of phagocytes¹ and is implicated in the pathogenesis of many disorders, such as rheumatoid arthritis⁴, atherosclerosis^{2,5,6}, renal glomerular injury⁷, pulmonary fibrosis⁸, Alzheimer's disease⁹, Parkinson's disease¹⁰ and selected cancers¹¹.

Luminol (5-amino-2,3-dihydro-1,4-phthalazine-dione) is a redox-sensitive compound that emits blue luminescence ($\lambda_{\text{max}} = 425 \text{ nm}$) when exposed to an appropriate oxidizing agent. The high stability, low cost and relatively simple synthesis of luminol has rendered it useful in a variety of fields ranging from metallurgy, analytical chemistry, biochemistry, clinical diagnostics and forensic sciences for detecting reactive intermediates. Luminol-mediated luminescence enables detection of extraordinarily low concentrations of oxidizing species in biological systems, and, indeed, use of luminol to study isolated phagocytes and whole blood was reported 25–30 years ago^{12,13}. Luminol-mediated luminescence enables *ex vivo* analyses of the phagocytic oxidative burst upon stimulation with a myriad of soluble activators, opsonized particles or intact microorganisms^{14,15}. Luminol is also used clinically to screen neutrophils *ex vivo* for defects in oxidative metabolism such as chronic granulomatous disease¹⁴ and MPO deficiency¹⁶.

Although luminol can react with many reactive oxygen species (ROS) produced during the phagocytic oxidative burst, studies with isolated phagocytes from normal volunteers and subjects with MPO deficiency indicate that the luminol reaction is dependent on MPO activity^{15,17}. However, the identity of the actual oxidizing agent and the location of luminol oxidation (that is, intra- or extracellular compartment) remain a matter of continuous debate (Fig. 1a)^{15,17–20}. Whereas enzyme-catalyzed luminescence (bioluminescence) of luminol in isolated cell systems has proven useful, extrapolation to a specific readout of MPO activity *in vivo* is not obvious, as numerous competing redox reactions and compartments concurrently exist (for example, heme-mediated oxidation, eosinophil peroxidase-catalyzed generation of ROS, and so on).

Luminol is relatively nontoxic, well absorbed and rapidly excreted upon systemic administration²¹, and it was used to treat humans with alopecia areata in the 1960s²². Thus, we hypothesized that systemic administration of luminol in concert with BLI could specifically probe MPO activity in live animals.

Results

Use of luminol to monitor MPO activity *in vitro*, *ex vivo* and *in vivo*

Luminol-dependent bioluminescence of stimulated phagocytes and whole blood has been extensively studied with photo-multiplier tube (PMT)-based luminometers. To determine whether BLI can be similarly used to detect oxidant production in biological material, we used a charge-coupled device-based BLI system to monitor phagocyte activation in 1 μl of heparinized whole blood (Fig. 1). Blood incubated with luminol (100 μM) was treated with the potent protein kinase C activator phorbol 12-myristate 13-acetate (PMA) or vehicle control (DMSO) (Fig. 1b). PMA induced a time-dependent increase in bioluminescence, peaking approximately 20–25 min after stimulation at approximately 30–40-fold over background values (Fig. 1b,d). No significant change in bioluminescence was observed in unstimulated cells (Fig. 1b). The timing and magnitude of the BLI responses were in excellent agreement with previous reports of phagocytic activation performed with PMT-based luminometers, and BLI also provided the spatial resolution lacking in luminometers^{15,17}. Furthermore, whereas

others have reported using 5×10^5 to 1×10^7 purified phagocytes to achieve a detectable signal with PMT-based readouts, in our studies, $<5 \times 10^3$ phagocytes in 1 μ l of whole blood were sufficient to produce BLI signal-to-background ratios of > 30 . Thus, charge-coupled device–based luminol-BLI offers both spatial resolution and 100–1,000-fold increased sensitivity over PMT-based photon counting.

Previous studies with *isolated* neutrophils suggested that luminol-dependent bioluminescence during a phagocytic oxidative burst is dependent on MPO activity^{15,17}. However, because *in vitro* studies have shown that other oxidants can cause luminol-dependent bioluminescence, a recent study examining the applicability of luminol as an *in vivo* probe for imaging ROS production concluded that luminol bioluminescence *in vivo* resulted from direct interactions with H_2O_2 ²³. Hypothetically, uncharged and relatively unreactive H_2O_2 could readily diffuse across biological membranes, generating highly reactive $OH^\bullet$ upon catalysis by free or possibly heme-bound metals, for example, hemoglobin and cytochromes. Thus, $OH^\bullet$ may react directly with luminol to produce light independently of MPO activity. Therefore, we performed a series of experiments to establish the mechanism of luminol bioluminescence and the requirement of luminol bioluminescence for MPO *in vitro* (with purified MPO), *ex vivo* (in whole blood) and *in vivo* (with animal imaging).

First, we analyzed the impact of pharmacological inhibition of MPO on luminol bioluminescence *in vitro*. Light production was measured from a solution containing purified human MPO, H_2O_2 , luminol and increasing concentrations of a relatively selective MPO inhibitor (4-aminobenzoic acid hydrazide (4-ABAH)²⁴, 0–500 μ M; Fig. 1c). The bioluminescence output was inversely proportional to 4-ABAH concentration (apparent half-maximal inhibitory concentration (IC_{50}) of 1.0 ± 0.7 μ M), suggesting that it is dependent on MPO activity.

4-ABAH also inhibits MPO activity in cultured cells²⁴, and we therefore used this compound to analyze MPO-dependent luminol bioluminescence in PMA-stimulated whole blood. Metals such as iron are highly abundant in whole blood but are confined to specific cellular compartments, that is, hemoglobin within erythrocytes or cytochromes in mitochondrial membranes. To our surprise, although H_2O_2 generated in response to PMA stimulation could theoretically diffuse and react with hemoglobin, cytochromes or free iron, production of bioluminescence by luminol (Fig. 1d,e) was almost completely inhibited by 4-ABAH (apparent IC_{50} of 50 ± 15 μ M; Fig. 1e). These observations strongly suggest that even in the presence of other physiologic redox catalysts, luminol bioluminescence requires MPO.

To validate the role of MPO, we analyzed whole-blood samples from $Mpo^{+/+}$ or $Mpo^{-/-}$ mice for luminol bioluminescence upon stimulation with PMA (Fig. 1f). As previously shown with neutrophils isolated from MPO-deficient individuals¹⁶, luminol bioluminescence was undetectable in whole blood of $Mpo^{-/-}$ mice (Fig. 1f). Collectively, these data indicate that in whole blood *ex vivo*, luminol bioluminescence depends on both the presence of MPO and activation of phagocytes. Moreover, we now show that the concurrent presence of intact erythrocytes, a rich source of heme iron, does not affect MPO-specific luminol bioluminescence activity.

To determine the relative contribution of other ROS to the bioluminescence signal induced by luminol in stimulated blood *ex vivo*, we focused on two enzymatically catalyzed ROS products, $O_2^{\bullet-}$ and $NO^\bullet$, generated by the Phox complex and nitric oxide synthase (NOS), respectively, as well as their reaction product, peroxynitrite ($ONOO^-$). As expected, pretreatment of whole blood *ex vivo* with the potent Phox inhibitor diphenyleneiodonium culminated in complete abrogation of PMA-induced bioluminescence (Fig. 1g). This was not unexpected, as Phox is biochemically directly upstream of MPO (Fig. 1a). In contrast, and in agreement with a

previous report²⁵, inhibition of NOS by L-N^G-monomethyl arginine citrate (L-NMMA) did not cause a significant reduction in luminol-dependent bioluminescence (Fig. 1g). These data suggest that peroxynitrite-dependent bioluminescence, a result of NO[•] oxidation by O₂^{•-}, did not contribute substantially to luminol bioluminescence *ex vivo*.

We next determined the biochemical requirements and pharmacokinetics of luminol bioluminescence in intact mice and whether MPO activity could be imaged *in vivo*. Glucose oxidase catalyzes the oxidation of β-D-glucose with the concomitant production of peroxide, releasing 1 mole of H₂O₂ for each mole of glucose consumed. Thus, one can use immobilized glucose oxidase *in vivo* as an H₂O₂ generator²⁶. We embedded glucose oxidase, MPO, MPO plus glucose oxidase or vehicle (PBS) in Matrigel solution and established subcutaneous implants of these mixtures on the backs of *nu/nu* mice (*n* = 3, Fig. 2a). Upon systemic intraperitoneal (i.p.) administration of luminol, intense bioluminescence was emitted only from the MPO plus glucose oxidase implants (Fig. 2b), reaching maximum values 10 min after injection of luminol and then decaying to baseline at between 65 and 90 min after injection in a biphasic manner (Fig. 2c). Bioluminescence from all other implants was essentially equal to background values (Fig. 2c).

Imaging MPO activity in inflammatory foci *in vivo*

To analyze whether luminol could be used to noninvasively monitor physiological MPO activity *in vivo*, we tested two mouse models of acute inflammation.

Topical application of PMA onto the earlobes of mice induces acute dermatitis, manifested by local swelling, erythema and infiltration of neutrophils²⁷. We used this model for assessment of MPO activity by luminol-BLI in *Mpo*^{+/+} and *Mpo*^{-/-} mice 24 h after topical application of PMA (Fig. 3a). Bioluminescence was locally emitted from PMA-treated earlobes of *Mpo*^{+/+} mice, reaching background-subtracted levels of 13.1 ± 0.2-fold over vehicle-treated ears. Serial BLI after luminol injection showed a gradual decay to background levels within 4 d after PMA application (data not shown). Bioluminescence from PMA-treated ears of *Mpo*^{-/-} mice was 1.9 ± 0.5-fold over vehicle-treated ears, that is, essentially at background levels (Fig. 3a).

Acute arthritis can be chemically generated by intra-articular injection of lipopolysaccharide (LPS)²³. This focal arthritis model is well suited for imaging studies, as it allows for generation of unilateral arthritis, thus allowing each mouse to serve as its own control. We administered luminol before and up to 120 h after intra-articular injection of LPS (left joint) or vehicle (PBS, right joint) into *Mpo*^{+/+} (*n* = 5) or *Mpo*^{-/-} (*n* = 5) mice. Bioluminescence emission was readily detected from the LPS-treated joints of *Mpo*^{+/+} mice (Fig. 3b), peaking 48 h after administration of LPS at approximately 25–30-fold the background value (Fig. 3c). Serial BLI showed a gradual decay in emissions, but signal was still evident 5 d after treatment. No bioluminescence was observed in vehicle-treated joints at any time (Fig. 3a). In agreement with the dermatitis model, negligible luminol bioluminescence could be detected from either LPS- or vehicle-treated joints of *Mpo*^{-/-} mice (Fig. 3b,c) at all times.

MPO affects vascular tone and permeability at sites of inflammation in a rodent model of LPS-induced acute endotoxemia³ and inhibits migration and recruitment of neutrophils in an ultraviolet B ray–induced sunburn model²⁸. To exclude the possibility that the lack of detectable signal in the LPS-treated joints of *Mpo*^{-/-} mice was attributable to a decrease in migration, recruitment or extravasation of neutrophils, we undertook histological analyses of tissue specimens (Fig. 3d) at 48 h after treatment, the point at which we observed maximal luminol bioluminescence. In response to LPS, similar numbers of neutrophils were recruited to the intra-articular spaces of *Mpo*^{+/+} and *Mpo*^{-/-} mice (Fig. 3d,e). In both genotypes, vehicle treatment failed to induce neutrophil infiltration (Fig. 3d).

Eosinophil peroxidase does not produce BLI signal *in vivo*

Eosinophil peroxidase is known to produce bioluminescence *in vitro* when luminol is used as a substrate²⁹. Therefore, to determine the potential contribution of eosinophil peroxidase to the luminol bioluminescence signal *in vivo*, we characterized a model of allergic contact hypersensitivity. Eosinophil-dominant recruitment and activation was induced by passive immunization against dinitrophenol followed by topical challenge with dinitrofluorobenzene³⁰ in both *Mpo*^{+/+} and *Mpo*^{-/-} mice. Because the only peroxidase present at the site of allergic inflammation in *Mpo*^{-/-} mice would be eosinophil peroxidase, comparing the two *Mpo* genetic backgrounds should reveal the fraction of the bioluminescence signal originating from eosinophil peroxidase *in vivo*. Notably, induction of acute contact hypersensitivity in *Mpo*^{-/-} mice resulted in no significant luminol bioluminescence signal from the inflamed ears, whereas in *Mpo*^{+/+} mice substantial luminol bioluminescence was observed (Fig. 4a,b). Control mice showed no significant luminol-BLI signal (Fig. 4a,b). To confirm that eosinophils had indeed infiltrated the inflamed ears, we performed histological analysis of the ears 24 h after dinitrofluorobenzene treatment, which showed prominent eosinophilia within a mixed cell infiltrate (Fig. 4c). Similar numbers of eosinophils were recruited to the inflamed ears of both *Mpo*^{+/+} and *Mpo*^{-/-} mice, whereas in both genotypes vehicle treatment failed to induce eosinophilic infiltration (Fig. 4c,d). Furthermore, a monoclonal antibody to dibromotyrosine directly identified the presence of bromotyrosine adducts in the inflamed ears of both *Mpo*^{+/+} and *Mpo*^{-/-} mice, but not in vehicle-treated ears (Fig. 4c). These data directly confirm the presence of active eosinophil peroxidase in *Mpo*^{-/-} mice despite the absence of luminol-BLI signals. Thus, we conclude that the contribution of eosinophil peroxidase to luminol-BLI *in vivo* is negligible.

Luminol-BLI detects MPO activity in neutrophil-rich tumors

In *Gzmb:Tax* transgenic mice, the human T-lymphotropic virus-1– associated oncogene *Tax* is expressed under control of the *granzyme B* promoter in activated T and natural killer cells³¹. *Gzmb:Tax* mice spontaneously develop large granular lymphocytic (LGL) tumors at 200–300 d of age, and these tumors are massively infiltrated with neutrophils³¹ (Fig. 5a). To noninvasively investigate whether these tumor-residing neutrophils might be constitutively stimulated, we first analyzed tumor specimens for the presence of MPO by immunohistochemical staining (Fig. 5b). Immunoreactive MPO colocalized with neutrophils, whereas tumor LGL cells (T and natural killer cells characterized by rounded nuclei) were unreactive with antibody to MPO. We next used BLI to examine tumor-bearing *Gzmb:Tax* mice after intraperitoneal administration of luminol (Fig. 5c–g). In all cases, luminol bioluminescence colocalized with foci of tumorigenesis, although some heterogeneity was observed (for example, a lack of luminol bioluminescence emitted from the second tail tumor in Fig. 5d or bioluminescence emitted from only a part of the ear tumor in Fig. 5e). This variability was expected because these spontaneously generated tumors are histologically heterogeneous and show areas with low infiltration of neutrophils and ‘hot spots’ with a neutrophil content of $\geq 90\%$ (data not shown). To our surprise, imaging revealed confined foci of bioluminescence at regions where no evident tumors could be visualized or palpated (for example, the two foci in Fig. 5g). Histological examination of such regions revealed prevascularized, early tumor foci (approximately 200 μm diameter) comprising only a few hundred cells (Fig. 5c). These foci showed neutrophil infiltration located at the center of the tumor and not a mixed population with LGL cells, as seen with larger tumors (Fig. 5a,b), suggesting an unexpected role for MPO activity in early tumorigenesis.

Discussion

The central role of MPO in acute and chronic inflammation¹, as well as its pathophysiological² and prognostic value⁵ in atherosclerosis and oncogenesis, have piqued

interest in imaging MPO activity *in vivo*. To date, several attempts have been made to image MPO activity with activatable agents by means of magnetic resonance imaging^{26,32}, single photon emission computed tomography³³ and fluorescence imaging³⁴. Many of these represented proof of principle, nonphysiological studies, limited to either imaging *ex vivo*, MPO and glucose oxidase implants or LPS muscle injections *in vivo*. However, an activatable magnetic resonance imaging agent, which polymerizes and cross-links to proteins at sites of MPO activity, has recently shown promise in reporting physiological activity in healing infarcts in a mouse model with a signal-to-noise ratio of 40 (ref. 35). For comparative purposes, imaging MPO activity in MPO plus glucose oxidase Matrigel implants *in vivo* yielded signal-to-background ratios of approximately 1.7 for the magnetic resonance imaging agent and 2.6 for the single photon emission computed tomography agent. In contrast, our MPO plus glucose oxidase Matrigel data indicated that the signal-to-background ratio exceeded 3,000 for luminol-BLI (Fig. 2).

Notably, eosinophil peroxidase did not contribute to luminol-BLI *in vivo*. These data do not contradict decades of careful *in vitro* eosinophil peroxidase biochemistry, but, rather, the demonstrated mechanisms of luminol oxidation by neutrophils and eosinophils *in vitro* are consistent with our observations *in vivo*. In particular, stimulated neutrophils canonically discharge granular contents into the phagosome, wherein H_2O_2 and Cl^- react in the presence of MPO to produce concentrated HOCl^1 . In contrast, upon eosinophil activation, eosinophil peroxidase is secreted into extracellular spaces³⁶. Crucially, in purified cells *in vitro*, luminol bioluminescence has been observed within the intracellular compartment of neutrophils versus the surrounding extracellular compartment of eosinophils³⁷. Moreover, pharmacodynamics are complex *in vivo*. As systemic luminol is delivered to sites of inflammation and diffuses into phagocytic compartments of activated neutrophils, a highly concentrated stable reservoir of MPO-generated HOCl and other oxidative species will be present throughout the oxidative burst for reaction with luminol. By contrast, once eosinophil peroxidase has been secreted *in vivo*, the enzyme, reactants and products will be diluted by diffusion and bulk flow or consumed by endogenous catalase, thereby reducing local concentrations of oxidizing species necessary for bioluminescence in the presence of luminol. Furthermore, in most extravascular tissues, the extracellular milieu will contain not only Cl^- but also thiocyanate (SCN^-). SCN^- , the preferred substrate for eosinophil peroxidase³⁸, and the product, HOSCN , a much weaker oxidant than HOCl , would presumably oxidize luminol less rapidly, if at all. We, therefore, propose that the unexpected selectivity of luminol-BLI for activated MPO *in vivo* arises from differences in physiological compartmentalization and local concentration of activated MPO versus eosinophil peroxidase and their oxidative products. *In vivo*, only the phagocytic respiratory burst simultaneously provides an active enzyme (MPO), a compartment accessible to luminol (the phagosome) and sufficiently concentrated oxidative products for productive BLI.

Thus, luminol-BLI could provide a promising new tool for *in vivo* MPO research. Of note, although the side effects of luminol in humans have not been fully characterized, the metabolic and pharmacological profile of luminol reported in rodents and humans^{21,22} suggests that luminol could be further evaluated for use as an MPO activity-specific probe in humans. However, as with any optical technique, the blue spectral output of luminol-BLI potentially limits depth resolution, and development of red-shifted luminol derivatives might enhance *in vivo* applications. Luminol-BLI monitors MPO activity, not MPO protein content, and future research will require comparison of luminol-BLI signals to serum markers of MPO in clinical disease. For example, although serum concentrations of MPO correlate with the presence and severity of CAD and are regarded as a meaningful prognostic indicator⁵, it may be advantageous to colocalize the activity of a given biomarker to the actual site of disease through imaging, representing synergistic approaches to management of disease. Indeed, new diagnostic applications, such as noninvasive optical imaging of MPO-mediated pathology in

relatively superficial structures (atherosclerotic plaques located within carotid arteries, invasive psoriatic lesions or inflammatory eye diseases), as well as open surgical fields and minimally invasive endoscopic or catheter-based evaluations, can be envisioned in the future.

Methods

Reagents

We purchased luminol (sodium salt), PMA, LPS (B-055 type), L-NMMA, diphenyliodonium, dinitrofluorobenzene, glucose oxidase (from *Aspergillus niger*) and mouse dinitrophenol-specific IgE from Sigma-Aldrich. We obtained human MPO (EC 1.11.1.7) from Calbiochem. We prepared hydrogen peroxide (Fisher) from a 30% stock solution. We obtained 4-aminobenzoic acid hydrazide (4-ABAH) from Calbiochem. We obtained Matrigel from Becton-Dickinson.

Bioluminescence imaging

Mouse protocols were approved by the Animal Studies Committee at Washington University School of Medicine. For all BLI experiments (*in vitro*, *ex vivo* and *in vivo*), we used the IVIS 100 bioluminescence imaging system (Caliper Life Sciences) (f/stop, 1; no optical filter)³⁹. We have detailed the exposure time, binning and field of view for each experimental setup in the Supplementary Methods online.

Bioluminescence imaging assays *in vitro*

We performed assays in colorless Modified Earle's Balanced Salt Solution (MEBSS, 145 mM Na⁺, 5.4 mM K⁺, 1.2 mM Ca²⁺, 0.8 mM Mg²⁺, 152 mM Cl⁻, 0.8 mM H₂PO₄⁻, 0.8 mM SO₄²⁻, 5.6 dextrose, 4.0 HEPES, and 1% heat-inactivated FBS (vol/vol), pH 7.4 at 37 °C) in black-coated 96-well plates. To analyze the effect of 4-ABAH on luminol bioluminescence *in vitro*, we mixed 10 μM luminol with MPO (5 mU) and vehicle (MEBSS) or 4-ABAH (0–500 μM). We then added H₂O₂ to a final concentration of 50 μM and imaged the plate 10 s later.

Bioluminescence imaging of whole blood *ex vivo*

We drew fresh blood from *Mpo*^{-/-} mice⁴⁰ or control C57/BL6 *Mpo*^{+/+} mice. We performed *ex vivo* whole blood experiments in black-coated 96-well plates. Briefly, we diluted 1 μl of fresh heparinized blood in 200 μl of MEBSS supplemented with luminol (100 μM) in the absence or presence of increasing concentrations of 4-ABAH (0–6.6 mM), L-NMMA (1 mM) or diphenyliodonium (10 μM). We imaged the samples before (*t* = 0) and at the indicated time points (Fig. 1b,d) after stimulation with PMA (5 μM) or vehicle (DMSO, 1 μl).

Bioluminescence imaging of myeloperoxidase and glucose oxidase embedded in Matrigel implants *in vivo*

We added glucose oxidase (30 U), MPO (20 U), vehicle (PBS) or glucose oxidase and MPO (30 U and 20 U, respectively) to Matrigel (200 μl) at 4 °C. We injected the four different mixtures subcutaneously into different locations on the backs of NCr *nu/nu* mice²⁶. Thirty minutes later, we anesthetized the mice (isoflurane inhalation), administered luminol (200 mg per kg body weight, i.p.) and imaged them sequentially every 5 min up to 1 h.

Bioluminescence of phorbol 12-myristate 13-acetate-induced dermatitis

We applied PMA (100 μM, 20 μl) or vehicle (ethanol) topically (to the left and right ears, respectively) on the inner and outer surface of the earlobe of *Mpo*^{-/-} mice (*n* = 5) or *Mpo*^{+/+} (*n* = 5) mice²⁷. We administered luminol (i.p.) 24 h and 48 h later, and we imaged the mice 10 min later under anesthesia⁴¹.

Bioluminescence imaging of lipopolysaccharide-induced acute arthritis

We anesthetized *Mpo*^{-/-} (*n* = 5) or *Mpo*^{+/+} (*n* = 5) mice and introduced LPS (20 µg in 20 µl PBS) or vehicle (PBS, 20 µl) by intraarticular injection into the left or right ankle, respectively²³. We gave the mice luminol (i.p.) and imaged them immediately after LPS administration under anesthesia, as well as 1 d, 2 d, 3 d, 4 d and 5 d later. We prepared histological specimens from both left and right ankles of similar cohorts of mice 48 h after intra-articular injections. We fixed the specimens with paraformaldehyde, decalcified them, embedded them in paraffin, sectioned them (5-µm sections) and stained them with H&E. For each condition, we counted neutrophils in five high-power fields (magnification ×400).

Bioluminescence of allergic contact hypersensitivity

We induced allergic dermatitis by passive immunization with dinitrophenol-specific IgE and topical challenge with dinitrofluorobenzene³⁰. Briefly, we intravenously injected the mouse antibody to dinitrophenol into *Mpo*^{+/+} (*n* = 13) and *Mpo*^{-/-} (*n* = 9) mice. We injected one control mouse from each *Mpo* genetic background with vehicle (saline) only. Twenty-four hours later, we painted all left ears with dinitrofluorobenzene and all right ears with vehicle. On the next day, we imaged all mice after luminol administration (i.p.). We killed the mice immediately after imaging and prepared histological specimens from both left and right ears as described for the acute arthritis models. For each condition, we counted eosinophils and neutrophils in five high-power fields (magnification ×400).

Bioluminescence imaging of myeloperoxidase activity in spontaneous, Tax-induced large granular lymphocytic tumors

We monitored *Gzmb:Tax* mice³¹ for tumorigenesis on ears, limbs and tails by visual examination and palpation, weekly, for 200–300 d after birth. We gave ten mice with visible tumors at various sizes (1–8 mm in diameter) luminol (i.p.) and imaged them under anesthesia. We excised the tumors, prepared them for histology with H&E or immunostained them for MPO.

Immunohistochemistry for myeloperoxidase and bromotyrosine

We probed sectioned specimens for MPO with rabbit polyclonal antibody cross-reactive to both human and mouse MPO (AbCam) and visualized them with rabbit-specific antibody conjugated to horseradish peroxidase (Amersham Bioscience) and diaminobenzidine (Vector Labs). We observed negative immunostaining in the absence of primary or secondary antibodies (data not shown). We used hematoxylin as a counterstain.

We detected bromotyrosine adducts in sectioned mouse ears with dibromotyrosine-specific monoclonal antibody (Cosmo Bio) and the Vector M.O.M. Immunodetection Kit (Vector Laboratories) according to the manufacturer's instructions, with minor modifications. We visualized a horseradish peroxidase-conjugated secondary antibody with diaminobenzidine and counterstained tissue with hematoxylin. We did not observe much staining in specimens of treated ears in the absence of primary antibody (data not shown).

Statistical analyses

Data are reported as mean values ± s.e.m. We compared pairs by the Student's *t* test and considered values of *P* ≤ 0.05 significant.

Acknowledgments

We thank V. Sharma for insightful discussions. This research was funded by US National Institutes of Health grants P50 CA94056, CA105218, CA10073, CA63417 and PO1 HL030086.

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Figure 1.

Luminol bioluminescence is dependent on MPO *in vitro* and *ex vivo*. **(a)** A schematic representation of the biochemical basis of luminol bioluminescence. HOCl produced by MPO can directly or indirectly oxidize luminol to produce light. Alternatively, MPO can also use the superoxide anion (dashed line) or other ROS (for example, NO[•])³ as substrates for peroxidase-catalyzed oxidation of luminol. **(b)** Dynamic luminol-BLI of PMA-stimulated whole blood. At $t = 0$, PMA (5 μM; bottom) or vehicle (top) were added, and bioluminescence was monitored for 90 min. Images show sequential color-coded maps of photon flux superimposed on black and white photographs of the assay plates. **(c)** 4-ABAH inhibits luminol bioluminescence *in vitro*. Peak bioluminescence was determined for solutions containing luminol, purified MPO, H₂O₂ and increasing concentrations of 4-ABAH (0–500 μM). **(d,e)** 4-ABAH inhibits luminol bioluminescence in PMA-stimulated whole blood *ex vivo*. Fresh heparinized whole blood was incubated with increasing concentrations of 4-ABAH and luminol in MEBSS. At $t = 0$, PMA (5 μM) or vehicle were added, and bioluminescence was monitored for 90 min. Luminol bioluminescence (fold increase over initial) was plotted as a function of time **(d)** and concentration of 4-ABAH **(e)**, as derived from the 22.5-min time point. **(f)** Fresh heparinized whole blood from *Mpo*^{+/+} or *Mpo*^{-/-} mice was incubated with luminol in MEBSS. BLI (0–55 min) was initiated instantly upon stimulation with PMA and plotted as a function of time after PMA treatment. **(g)** Contribution of NO[•] radicals and Phox-generated O₂^{•-} to luminol bioluminescence *in cellulo*. Fresh blood from an *Mpo*^{+/+} mouse was incubated with the NOS inhibitor l-NMMA (1 mM) or the Phox inhibitor diphenyleneiodonium (DPI, 10 μM), respectively, and imaged 20 min after stimulation with PMA (5 μM). Error bars represent ± s.e.m.

Figure 2. Luminol-BLI of MPO and glucose oxidase (GOX) implants *in vivo*. **(a)** Vehicle (V; PBS), GOX, MPO or MPO plus GOX were embedded in Matrigel, and subcutaneous implants were established in *nu/nu* mice ($n = 3$). Shown is a bioluminescence image taken at $t = 0$ (before luminol administration). Scale bar, 1 cm. **(b)** BLI (5–60 min at 5-min intervals) was initiated after i.p. injection of luminol (200 mg per kg body weight). Scale bar, 1 cm. **(c)** Representative time-course of photon fluxes from the various implants in one mouse.

Figure 3.

Lack of luminol bioluminescence *in vivo* in $Mpo^{-/-}$ mice during acute inflammatory insults. (a) Luminol bioluminescence was dependent on MPO in an acute dermatitis model. Dermatitis was generated by topical application of PMA on the left ear lobe of $Mpo^{+/+}$ or $Mpo^{-/-}$ mice (shown are three representative mice for each genetic background, $n = 5$ for each group). The right ears served as vehicle (ethanol) controls. Twenty-four hours after application of PMA, luminol was administered (i.p.) and mice were imaged. Scale bar, 1 cm. (b) LPS was injected into the ankle joints of the left lower limbs of wild-type $Mpo^{+/+}$ ($n = 5$) or $Mpo^{-/-}$ mice ($n = 5$). Vehicle (PBS) was injected into the joints of the right lower limbs. At $t = 0$ h (before LPS injection) and 24 h, 48 h, 72 h, 96 h and 120 h after LPS injection, luminol was administered (i.p.), and mice were imaged. Representative images taken at $t = 48$ h are shown. Scale bars, 1 cm. (c) Background-subtracted LPS-induced luminol bioluminescence ($\pm$ s.e.m.) quantified as fold increase over vehicle-treated foot for each Mpo genotype at the indicated time points. (d) Histological analysis of the joint region showing massive infiltration of neutrophils 48 h after LPS injection in both $Mpo^{+/+}$ and $Mpo^{-/-}$ mice. Inset, high-resolution images. Scale bars, 10 μm . (e) Mean number of neutrophils per high power field ($\pm$ s.e.m.).

Figure 4.

Luminol-BLI of allergic contact hypersensitivity. **(a,b)** Acute allergic dermatitis was induced by passive immunization with dinitrophenol-specific IgE (anti-DNP IgE) and topical challenge with dinitrofluorobenzene on the left ear lobes of *Mpo*^{+/+} and *Mpo*^{-/-} mice. Right ears served as vehicle controls and mice challenged with dinitrofluorobenzene (DNFB), but not anti-DNP IgE, served as negative controls. Twenty-four hours after application of DNFB, luminol was administered, and mice were imaged. **(a)** Shown are one *Mpo*^{+/+} and one *Mpo*^{-/-} mouse. Scale bar, 1 cm. **(b)** Background (vehicle on right ear)-subtracted DNFB-induced luminol bioluminescence ($\pm$ s.e.m.) for each *Mpo* genotype. n = number of mice. **(c)** Histological analysis of proximal ear lobes, showing massive edema and eosinophilia within a mixed cell infiltrate 24 h after DNFB treatment of both *Mpo*^{+/+} and *Mpo*^{-/-} mice, but not vehicle-treated mice; lower right panel shows eosinophils in a treated *Mpo*^{-/-} mouse. Insets show immunostaining with monoclonal antibody to dibromotyrosine of the same specimens as the main panel, documenting the presence of bromotyrosine adducts in treated ears of both *Mpo*^{+/+} and *Mpo*^{-/-} mice (brown staining), but not vehicle-treated controls. Scale bars, 50 μ m. **(d)** Mean number of eosinophils or neutrophils per HPF $\pm$ s.e.m.

Figure 5.

Luminol-BLI of spontaneous LGL tumors in *Gzmb:Tax* mice. **(a)** H&E staining of a large (~1-cm) tumor from the tail of a *Gzmb:Tax* mouse, showing that the majority of the cell mass is made of neutrophils (polymorphic nuclei; magnification $\times 630$). **(b)** MPO immunostaining of the same specimen as in **a** showing localization of MPO within neutrophils (brown staining). **(c)** H&E staining of a very small (~200- μ m) tumor from the earlobe of a *Gzmb:Tax* mouse (arrow). Scale bars in **a–c**, 50 μ m. **(d–g)** Luminol-BLI of tumor-bearing *Gzmb:Tax* mice showing colocalization of luminol bioluminescence with small tumor foci (yellow arrows). Scale bars in **d** and **e**, 1 cm; scale bars in **f** and **g**, 1 mm.