Smart imaging of acute lung injury: exploration of myeloperoxidase activity using in vivo endoscopic confocal fluorescence microscopy

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1Soins Intensifs Médicaux, Département de Médecine; 2 Centre de Recherche Clinique du CHUS; 3Centre d’Imagerie Moléculaire de Sherbrooke; and 4Laboratoire de Chimie Médicale, Institut de Pharmacologie de Sherbrooke Faculté de Médecine et des Sciences de la Santé, Université de Sherbrooke, Sherbrooke, Quebec, Canada

Submitted 14 October 2014; accepted in final form 24 July 2015

Chagnon F, Bourgouin A, Lebel R, Bonin M, Marsault E, Lepage M, Lesur O. Smart imaging of acute lung injury: exploration of myeloperoxidase activity using in vivo endoscopic confocal fluorescence microscopy. Am J Physiol Lung Cell Mol Physiol 309: L543–L551, 2015. First published July 31, 2015; doi:10.1152/ajplung.00289.2014.—The pathophysiology of acute lung injury (ALI) is well characterized, but its real-time assessment at bedside remains a challenge. When patients do not improve after 1 wk despite supportive therapies, physicians have to consider open lung biopsy (OLB) to identify the process(es) at play. Sustained inflammation and inadequate repair are often observed in this context. OLB is neither easy to perform in a critical setting nor exempt from complications. Herein, we explore intravital endoscopic confocal fluorescence microscopy (ECFM) of the lung in vivo combined with the use of fluorescent smart probe(s) activated by myeloperoxidase (MPO). MPO is a granular enzyme expressed by polymorphonuclear neutrophils (PMNs) and alveolar macrophages (AMs), catalyzing the synthesis of hypochlorous acid, a by-product of hydrogen peroxide. Activation of these probes was first validated in vitro in relevant cells (i.e., AMs and PMNs) and on MPO-non-expressing cells (as negative controls) and then tested in vivo using three rat models of ALI and real-time intravital imaging with ECFM. Semiquantitative image analyses revealed that in vivo probe-related cellular/background fluorescence was associated with corresponding enhanced lung enzymatic activity and was partly prevented by specific MPO inhibition. Additional ex vivo phenotyping was performed, confirming that fluorescent cells were neutrophil elastase⁺ (PMNs) or CD68⁺ (AMs). This work is a first step toward “virtual biopsy” of ALI without OLB.

endoscopic confocal fluorescence microscopy; myeloperoxidase; polymorphonuclear neutrophils; macrophages; lung; inflammation

ACUTE LUNG INJURY (ALI)/acute respiratory distress syndrome (ARDS)-related acute respiratory failure (ARF) is a diffuse and severe lung condition of growing incidence and serious outcome (4, 13). Diffuse alveolar damage (DAD) is its pathological hallmark, in which the initial and exudative acute phase is essentially comprised of a coercive inflammation. The latter results from a massive infiltration of alveolar spaces and distal parenchyma by inflammatory cells such as alveolar macrophages (AMs) and polymorphonuclear neutrophils (PMNs) (6, 28, 34). More specifically, pneumonia is responsible for more than two-thirds of all cases of ARF and infections and is the fourth most frequent cause of hospitalizations (1). Systemic infection (also called sepsis) kills 60,000–90,000 people every year in the US only (35) despite the fact that a majority of pneumonia cases respond well to treatments. Although infection is initially causative and generally treated efficiently with antibiotics, major lung insults come mostly from consecutive processes that are self-amplified independently of the primary microbiological cause and often are neither self-resolved nor efficiently cured. Consequently, nearly 50% of those patients do not demonstrate any clear evidence of improvement after 1 wk and remain dependent on ventilation support (6, 28, 34). This “stagnation” of the clinical condition is a source of serious concern and can last more than 1 wk, during which the outcome is uncertain. If the repair process fails and inflammation persists, patient death can occur due to terminal respiratory failure. For instance, the presence and activity of myeloperoxidase (MPO) are elevated in bronchoalveolar lavage fluid and lung tissue of ALI/ARDS (23), where it can modulate lung epithelial response to inflammation (15). MPO and elastase are contained in azurophilic granules and are important members of the enzymatic arsenal of AMs and PMNs. Upon cellular activation, MPO converts hydrogen peroxide to hypochlorous acid, a powerful oxidant, and bactericidal reactive oxygen species (ROS) (36), which can also be released pericellularly, especially in extracellular traps (NETs), or concentrated in phagosomes (16, 20, 25, 27, 28).

To assess this clinical stagnation, modern imaging techniques (such as high-resolution computed or positron emission tomographies) can be helpful but have the following drawbacks: 1) heavy infrastructures requiring moving patients, which are not always reasonable for frail patients in critical condition, 2) they are potentially toxic (injection of contrast agents), and 3) insufficient resolution at the cellular and molecular levels (5). When modern imaging is not sufficiently contributive, open lung biopsy (OLB) is the “gold standard” to directly assess the nature/composition, intensity, and precise chronological “history” of DAD (26). This is the only test that allows the microscopic analysis of processes occurring in the lung. However, OLB is burdened by unacceptable risks and complications for patients in critical condition. Bronchoalveolar lavages (BAL) with distal airspace-lining fluid (BALF) recovery allow for analyses of free cell inflammatory/repair molecule profile but lack informative access to parenchymal tissues (11, 34).
Accordingly, 1) new strategies for improved diagnostic tools are required, 2) it is imperative to develop a tool that is available at bedside to allow “real-time” monitoring of lung repair without OLB to adapt treatment as needed, and 3) detecting the aforementioned MPO activity in activated AMs and PMNs would allow the characterization of sustained inflammation and unresolved lung tissue repair processes.

Endoscopic confocal fluorescence microscopy (ECFM) is emerging as a potentially powerful surrogate to white light microscopy, and confocal apparatuses have recently gained huge technical improvements, including miniaturization of optical fibers, high resolution, and very fast acquisition systems (14, 21, 35). In this work, we present the first report of an ECFM system used in conjunction with a dedicated fluorescence probe applied to ALI/ARDS preclinical models. This proof of concept will be a preliminary step toward a method for intravital “virtual biopsy” of the lung. In vitro, ex vivo, and in vivo testing of an activatable fluorescence probe targeting MPO activity in combination with ECFM are presented.

METHODS

Smart Probe Selection and Synthesis

Sulfonaphthoaminophenyl fluorescein (SNAPF) was synthesized as described by Shepherd et al. (30). SNAPF was used as a reference probe for detection of MPO activity, exhibiting strong fluorescence in the near-infrared wavelength, when activated.

4-[6-(4-Aminophenoxo)-3-oxo-3H-xanthene-9-yl]benzene-1,3-disulfonic acid (DSAPF) as well as 6-(hydroxy-3-oxo-3H-xanthene-9-yl)benzene-1,3-disulfonic acid (DSF) were produced by our probe synthesis plate form. The SNAPF probe originally described by Shepherd et al. (30) was based on a naphthofluorescein. In this work, we replaced the naphthalene moieties of SNAPF by benzenes, yielding the homologous fluorescein structure ($\lambda_{ex}$ = 502 nm and $\lambda_{em}$ = 522 nm, in PBS, pH 7.4). This new molecule, labeled DSAPF, is then suitable for use in the green channel of the CellVizio and is a smart activatable probe version that possesses very low detectable spontaneous fluorescence in the absence of specific hypochloric acid-related triggering and exhibits fluorescence in the FITC wavelength when activated. Full optical characterization was observed (unpublished observations).

DSF is the “already activated” version of DSAPF, exhibiting strong and sustained fluorescence (in the FITC wavelength) and serving as positive control in specific experiments. Briefly, DSF was synthesized as described by Hilderbrand et al. (17). DSAPF was obtained in two steps starting from crude disulfofluorescein. An ipso substitution was performed on DSF using 4-nitro-fluorobenzene in dimethylformamide at 175°C for 15 min. This step was followed by a reduction with iron m. For illustration purposes, images were contrast enhanced and normalized by the signal measured at time $t = 0$ (5 min) for the cells exposed to DSAPF without MPO inhibitor. All measurements were performed three times.

CONFOCAL MICROSCOPY. Cells processed as described above were used for confocal microscopy imaging. DSAPF or DSF was added to the cells with or without the MPO inhibitor. Cells were examined with a Plan Apo 60 oil immersion objective NA 1.42 on an inverted spectral scanning confocal microscope FV1000 (Olympus, Tokyo, Japan). Specimens were laser-excited at 488 nm (40 mW Blue Argon laser). Serial horizontal optical sections of 512 by 512 pixels with two line averaging were taken at 0.4-$\mu$m intervals through the entire thickness of the cells (optical resolution: lateral 0.2 $\mu$m, axial 0.8 $\mu$m). For illustration purposes, images were contrast enhanced and pseudocolored according to their original fluorochrome (FluoView Software; Olympus).

In Vivo Studies

ALI models and imaging procedures and analyses. Pathogen-free Sprague-Dawley adult rats (250–350 g) received care in compliance with the Guide for the Care and Use of Experimental Animals from the Canadian Council of Animal Care (CCAC; 1993, 2nd ed.). Protocols were approved by the Université de Sherbrooke Ethics Committee for Animal Care and Experimentation. ALI was induced by 1) intratracheal (I/T) instillation of Bleomycin sulfate (5 U/kg), 2) I/T instillation of LPS (Escherichia coli 055:B5 LPS; 3 mg/kg) (Sigma, St-Constant, QC, Canada), or 3) hyperoxia (85%) exposure in a plexiglass chamber, as described previously (10, 19). All models induced fast inflammatory cell recruitment/infiltration, with PMN dominance for the first two models and AM dominance for the latter
(22). All animals were imaged between 24 and 72 h after ALI initiation. For this purpose, rats were anesthetized with 1 ml/kg ketamine-xylazine (87 and 13 mg/ml, respectively), tracheostomized with a 14-G catheter (inner diameter 2.2 mm), and the right jugular vein was cannulated using an angiocatheter (P50; VWR, Mississauga, ON, Canada). They were then positioned upright, and 0.06 mg/ml of DSAPF or 0.3 mg/ml SNAPF in PBS was nebulized in the right main bronchus through a microdose nebulizer (Penn-Century). Animals were left to recover with oxygen support on a warmed pad. After 3 h of incubation, a 1.5-mm miniprobe (ProFlex S-1500) connected to a Cellvizio Dual Band system (Mauna-Kea Tech) was introduced through the I/T catheter to explore distal airways and spaces of the right lung with oxygen supplementation and with or without supportive bag ventilation when needed. This ECFM apparatus allowed simultaneous dual-wavelength illumination and detection of fluorescence (488-nm green and 660-nm red channels, respectively). Occasionally, SNAPF-expressing cell imaging was recorded in an area of interest within the near-infrared channel, and dual labeling was secured by FITC-dextran 500-kDa (500 µl, 5 mg/ml; Sigma) intraveneously (iv) injection to obtain a microcirculatory contrast. Dedicated in vivo experiments were performed using DSAPF and SNAPF (or vehicle-PBS alone) I/T instillation at the same concentrations as above to assess smart probe’s tolerance and innocuity. After 3 h, left lungs were ligated and snap-frozen, and the right lungs underwent a BALF (PBS; 5 ml) both for cytotoxicity and cell death assays. Lung tissues and BALF-free cells served for caspase-3 activity assays, whereas cell-free BALF was used for LDH activity assays (see details below).

In vivo semiquantitative assessment of MPO activity was performed in specific experiments selecting the LPS model as the strongest provider of PMN-driven MPO enzymatic activation. MPO inhibitor ABAH or vehicle alone (80 mg/kg i/P in DMSO) was injected intraperitoneally 1 h before DSAPF nebulization (37); 5 × 2-min consecutive videos (including one baseline sequence prior probe spraying) were recorded. Each video consists of a descent of the endoscope through the airways of the right lung at a random position with respect to the flexibility of the Proflex S-1500 and to the relative smallness of rat airways. CellVizio video acquisitions were analyzed in MATLAB (MathWorks, Natick, MA). Briefly, 1) video frames were selected manually to sample the video acquisition without analyzing the same object/cell multiple times, 2) these frames were smoothed using a Gaussian filter (3 × 3), and 3) a background image was calculated using a second Gaussian filter (containing an empty ROI, contrast of the MPO probes were calculated based on images acquired with DAPI, as described previously (10).)

Signal-to-noise ratio (SNR) and Webber contrast were calculated based on images acquired from the green (DSAPF) or red (SNAPF) channels. Regions of interest (ROIs) were drawn over the cells being tracked. Here, the SNR is defined as the ratio of the mean intensity of the ROI, \( I_{ROI} \), over the standard deviation of the background signal, \( \sigma_{bg} \).

In this work, the Webber contrast method was selected because the feature (ROI) was relatively small against a mostly uniform background, and the objective was to establish how well individual cells could be monitored by ECFM. The Webber contrast was defined as follows:

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W_c = \frac{I_{ROI} - I_b}{I_b}
\]

where \( I_b \) is the mean intensity of the background. For both SNR and contrast, a high value indicates that the cells are clearly visible in the image.

Calculations were performed over the first 150 frames (images) of video using MatLab (MathWorks). Eighteen videos were analyzed, nm using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

Cytotoxicity and cell death assays: lactate dehydrogenase and caspase-3 activities. One hundred milligrams of lung tissue was homogenized in corresponding ice-cold buffers. Lung samples and BALFs were centrifuged at 10,000 g for 15 min. Extracellular (released) enzymatic activity in cell-free BALF samples were processed according to the lactate dehydrogenase (LDH) activity assay kit (Sigma), whereas intracellular enzymatic activity in BALF cell pellets and lung tissue was prepared according to the caspase-3 colorimetric assay kit (BioVision, Milpitas, CA). Absorbances were measured at 450 and 405 nm using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). LDH activity was calculated in mU/ml according to the NADH standard curve and caspase-3 activity in \( \delta \)-optical density before/after addition of the specific labeled substrate DEVD-pNA.

Cryopreserved lung slice immunofluorescence microscopy. At the end of in vivo ECFM procedures, a contralateral left-side BAL (5–10 ml of PBS) was performed for free cell differential profile assessment using Wright-Giemsa staining. Then, in vivo tissue fixation was performed with cold 2% paraformaldehyde, followed by cold 20% sucrose iv infusions (30 min slow injection each) and overnight incubation of whole lungs in a 20% sucrose bath at 4°C. The lungs were then preserved in Clear Frozen Section Compound (OCT; VWR), and the right previously imaged lung was sampled for ex vivo studies. Cryosections 10 µm thick were harvested from this OCT-embedded block and placed on positively charged slides (VWR). Nonspecific binding was neutralized by incubating slides in 10% donkey serum (Life Technologies, Burlington, ON, Canada) for 1 h, and slices were covered with rabbit polyclonal anti-aquaporin 5 (AQP-5), anti-neutrophil elastase (NE; 1:200 and 1:100, respectively; Abcam, Toronto, ON, Canada), or mouse anti-CD68 (1:100; Novus Biologicals, Littleton, CO). Staining was revealed with a donkey anti-rabbit or anti-mouse rhodamine conjugated secondary antibody (1:100; Santa Cruz Biotechnology, Dallas, TX), and slides were mounted with Vectashield Hardset Mounting Medium containing DAPI (Vector Laboratories, Burlingame, CA) for nuclear counterstaining. Ex vivo observations were performed using an Axiostkop 2 fluorescence microscope (Carl Zeiss, Thornwood, NY). Photomicrographs were captured using Retiga SRV cooled color digital camera (Qimaging, Burnaby, BC, Canada). Images were processed using Image Pro software (Media Cybernetics, Silver Spring, MD). Randomized selected pieces of ALI lungs were processed as above but paraffin embedded, cut, and either stained with hematoxylin and eosin or recovered with FITC-rhodammin communis agglutinins (RCA-1, 0.015 mg/ml; Vector Laboratories) and mounted with Vectashield medium containing DAPI, as described previously (10).

Video quality analysis. Signal-to-noise ratio (SNR) and Webber contrast of the MPO probes were calculated based on images acquired from the green (DSAPF) or red (SNAPF) channels. Regions of interest (ROIs) were drawn over the cells being tracked. Here, the SNR is defined as the ratio of the mean intensity of the ROI, \( I_{ROI} \), over the standard deviation of the background signal, \( \sigma_{bg} \).

Ex Vivo Validation

MPO activity in lung tissues. Frozen lung samples were homogenized in 50 mM potassium phosphate, pH 6.0. Homogenates were centrifuged at 10,000 rpm for 15 min, and the pellets were resuspended in 50 mM potassium phosphate with 50 mM hexadecyltrimethylammonium bromide. Then, samples were sonicated and snap-frozen in liquid nitrogen. The samples were centrifuged at 10,000 rpm for 10 min. Supernatants were mixed with 50 mM potassium phosphate containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. The absorbance was measured at 450 nm using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

Cytotoxicity and cell death assays: lactate dehydrogenase and caspase-3 activities. One hundred milligrams of lung tissue was homogenized in corresponding ice-cold buffers. Lung samples and BALFs were centrifuged at 10,000 g for 15 min. Extracellular (released) enzymatic activity in cell-free BALF samples were processed according to the lactate dehydrogenase (LDH) activity assay kit (Sigma), whereas intracellular enzymatic activity in BALF cell pellets and lung tissue was prepared according to the caspase-3 colorimetric assay kit (BioVision, Milpitas, CA). Absorbances were measured at 450 and 405 nm using a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA). LDH activity was calculated in mU/ml according to the NADH standard curve and caspase-3 activity in \( \delta \)-optical density before/after addition of the specific labeled substrate DEVD-pNA.
two for the SNAPF probe and 16 for the DSAPF probe. The selection of these videos was based on the presence of at least one cell in the first 150 frames. ROIs were automatically drawn using an absolute threshold. A single ROI corresponding to the largest cell was selected.

Statistical Analysis

Results were expressed as means ± SD for at least three experiments. Differences in FACS data were analyzed with GraphPad Prism 6 Software (GraphPad Software, La Jolla, CA), using two-way ANOVA for repeated measurements or Student’s t-tests vs. Mann-Whitney U-tests when relevant, and with statistical significance set at \( P < 0.05 \). Differences in MPO enzymatic activity in the presence of an inhibitor were studied using a one-way ANOVA with statistical significance at \( P < 0.05 \) based on the hypothesis that the inhibitor should lower the activity.

RESULTS

In Vitro Validation

Probe cell cross-reactivity assays. LPS-stimulated AMs were incubated with DSAPF or DSF (Fig. 1A). Fluorescence intensity of DSAPF increased over time, and a final 2.5-fold increase was noted at 150 min. This increase was significantly reduced by coinoculation with the ABAH (specific MPO inhibitor) \( (P < 0.0001; \text{ Fig. 1A}) \). DSF incubated cells exhibited neither obvious change in intracellular fluorescence \( (P = 0.93) \) nor significant effect with ABAH \( (P = 0.19) \). Image examples of intracellular fluorescence obtained in unstimulated and stimulated AMs after 1 h of incubation with DSAPF are shown in Fig. 1B, images a and b, respectively.

Blood-isolated PMNs activated with LPS are known to exhibit high MPO activity. Those were incubated with DSAPF or DSF (Fig. 2A). A 2.8-fold fluorescence increase was noted 150 min after exposure to DSAPF. The level of intracellular fluorescence of DSAPF in the presence of MPO inhibitor plateaued after 90 min. At 150 min, the fluorescence of DSAPF was found to be halved by the presence of an MPO inhibitor \( (P < 0.0001) \). Image examples of intracellular fluorescence obtained in unstimulated and stimulated PMNs after 2 h of incubation with DSAPF are shown in Fig. 2B, images a and b, respectively (with focus presented in images c and d, respectively).

None of the other cell lines tested from epithelial, endothelial, lymphocytic, or fibroblastic origins exhibited any significant intracellular fluorescence when incubated with DSAPF, even after LPS stimulation. Measurements by FACS (Fig. 3A) confirmed that no significant intracellular fluorescence was present in these cell lines. Extracellular fluorescence could be detected with DSF (Fig. 3B).

DSF probe signal was found to be roughly stable in all cells, with the exception of PMNs and Beas2B cells: 75/38% increase in intracellular fluorescence without/with MPO inhibitor for the former and 17/32% decrease in intracellular fluorescence without/with MPO inhibitor for the latter. However, DSF signal was not related to MPO activity but rather to cell penetration or phagocytosis or membrane sticking.

In Vivo Imaging

Generally, video recordings using the Proflex S-1500 were restricted to easily accessible rat airways, with some possible limitation inherent to the size of this probe. More specifically, the intermediate, cardiac, and diaphragmatic lobes should have been explored most of the time and contained minor airways exhibiting diameters ≤1.5 mm (24). However, sometimes, as observed in human reports, this probe can reach alveolar spaces (acini) and sacs by randomly progressing toward distal airways and either by moving through occasional buds or by penetrating through a bronchiolar wall, with consecutive change in the viewing profile (32).

Examples of ECFM performed with DSAPF in distal airways and spaces of hyperoxic lungs are shown first. Fluorescent positive cells were found in liquid edema (Fig. 4, A and B). These MPO+ cells were mobile, crawling with time and sometimes exhibiting granular staining patterns (Fig. 4, B and C). A representative video of these imaging sequences was supplied in Supplemental Video S1: Supplemental Material for this article may be found on the American Journal of Physiology: Lung Cellular and Molecular Physiology website. The estimated size of

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**Fig. 1.** In vitro detection of myeloperoxidase (MPO) activity in LPS-stimulated bronchoalveolar macrophages (AMs). Mixed human cell populations (mainly AMs) coming from bronchoalveolar lavages with distal airspace-lining fluid (BALFs) were sampled, exposed to LPS, and studied (\( n = 6 \)). A: time course fluorescence-activated cell sorting (FACS) analysis of 4-(6-(4-aminophenoxo)-3-oxo-3H-xanthen-9-yl)benzene-1,3-disulfonic acid (DSAPF) or 6-(hydroxy-3-oxo-3H-xanthen-9-yl)benzene-1,3-disulfonic acid (DSF) intracellular signal in LPS-stimulated BALF cells with or without MPO inhibitor. Results are presented as means ± SD. ■ DSF; black and white horizontal squares, DSF with MPO inhibitor; □ DSAPF; black and white vertical squares, DSAPF with MPO inhibitor. B: confocal microscopy imaging of bronchoalveolar cells after 1-h incubation with DSAPF. Image a: unstimulated cells with marginal intracellular fluorescence; image b: several stimulated large cells with round nucleus and macrophage (AM)-like phenotype exhibiting significant granular fluorescence (white arrows). **P ≤ 0.003 (t = 90, 120, and 150) vs. t = 0; †††P < 0.0001, DSAPF vs. DSAPF with MPO inhibitor.**
these positive cells (11.4 ± 4.9 μm) suggests that AMs accounted for most of the labeled cells.

Examples of ECFM performed with SNAPF, which was neutubelized in a rat model of Bleomycin-injured lungs, are also presented. Animals were also injected iv with FITC-Dextran when a ROI with MPO⁺ cells was detected for dual-band imaging aim (Supplemental Video S2, A and B). Various large and smaller fluorescent positive cells ranging in size between 8 and 15 μm (presumably AMs and PMNs) were observed within the countercontrast of FITC-dextran pulmonary circulation exhibiting various sizes of microvessels with networking characteristic of a major-to-minor airway location.

Both videos were representative of in vivo experimentations completed in 12 rats/model.

Video quality was assessed using the SNR and contrast with either DSAPF or SNAPF probes. More ECFM experiments were carried out using the DSAPF, and SNAPF was used for comparison purposes. Using DSAPF and green channel of the CellVizio, SNR and contrast were 7.9 ± 1.7 and 0.8 ± 0.2, respectively (n = 16). For contrast average calculations, three outliers were found using a ROUT test and discarded. For SNAPF, SNR and contrast were 9.0 ± 1.1 and 1.4 ± 0.5, respectively (n = 2). These values were not significantly different between probes, although additional work using the SNAPF would be required to confirm the data. SNR and contrast values of Supplemental Videos S1 and S2, A and B, were 9.4 and 7.2, 8.2 and 1.1, and 9.8 and 1.8, respectively.

Quantification of the fluorescence detected during the CellVizio acquisitions was performed. Figure 5 shows the average cell intensity, average background across the cells, and contrast between these values for the three cohorts of animals (n = 6): control (no LPS challenge), LPS (I/T)-challenged, and LPS (I/T)-challenged that have received MPO inhibitor. A significant contrast increase (+60%) was observed in LPS lungs (P < 0.05 vs. control) and was reversed by MPO inhibitor pretreatment (P < 0.01 vs. LPS) (Fig. 5). MPO inhibitor pretreatment with I/T vehicle alone did not differ from the control (data not shown).

**Ex Vivo Validation**

All experimental models generated ALI with macroscopic and microscopic evidence of congestion/edema, inflammation, or hepatisation, together with cellular insult and tissue remodelling [Fig. 6, A, image a; B, image a; and C, image a]. Inflammation remodelling was dominant in LPS and Bleomycin lungs, whereas edema was obvious in hyperoxic lungs (Fig. 6, A–C). ALI models exhibited incremental tissular MPO activity, which culminated within LPS lungs (P < 0.05 vs. control) and was significantly reduced by pretreatment with MPO inhibitor (P < 0.01 vs. LPS without MPO inhibitor; Fig. 6D). BALF revealed short-term recruitment/activation of inflammatory cells in distal airways and spaces, with AM predominance for the hyperoxic lungs (85 ± 2%, n = 6; Fig. 7A, image a), PMN predominance for the LPS lungs (96 ± 0.4%, n = 6; Fig. 7A, image c), and a PMN/AM mix in the Bleomycin lungs (58 ± 6%, n = 6; Fig. 7A, image b). CD68⁺ cells (a selective granular marker of AMs with no significant expression on other cells) and NE⁺ cells (a selective marker of PMNs with no significant expression on AMs and no expression on other cells), with or without colocalization with activated DSAPF (MPO⁺ cells), were observed mainly in BALF cells coming from hyperoxic lungs for the former and Bleomycin or LPS lungs from the latter (Fig. 7A, images d–f).
Further identification of PMNs and AMs was performed in lung tissues using AQP-5 (mainly epithelial and endothelial selective with no significant expression on phagocytes) (6), NE, and CD68 labeling. AQP5 staining did not overlap with MPO cells (Fig. 7B, images a and b), confirming MPO activation is not epithelial driven. On the other hand, several MPO cells were also NE or CD68, indicating that MPO activation was detected in PMNs and AMs, respectively (Fig. 7B, images c and d). Overall, this supports ECFM results, where both types of cells were observed, suggesting that the density and MPO activation state could be monitored in PMNs and AMs individually. Further MPO cells were detected in distal airspaces, some with a granular pattern and some with a more uniform intracellular pattern (presumably PMNs) (Fig. 7B, images a–d).

Toxicity and cell death assays revealed no evidence of superimposed level of cell injury/apoptosis after smart probe instillation, with similar LDH contents in cell-free BALFs (vehicle: 26.7 ± 2.3 mU/ml vs. MPO probes: 23.2 ± 1 mU/ml, n = 3), 2) 1.21 ± 0.01- and 1.2 ± 0.01-fold increases of caspase-3 activities in vehicle- and MPO probe-exposed lungs, respectively (vs. no substrate-added optical density; n = 3), and 3) marginal and proportionate caspase-3 activities in BALF-free cells (1.03 ± 0.01- and 1.04 ± 0.01-fold increases with vehicle- vs. MPO probe-exposed airspaces, n = 3).

**DISCUSSION**

Detection of intracellular MPO activity was selected as a biomarker of cellular ROS synthesis and inflammatory processes in ALI/ARDS models. Corresponding converting enzyme activity is expected to be present mainly inside MPO-expressing inflammatory cells (i.e., AMs and PMNs) and NETs and sometimes detectable in the extracellular space. AMs and PMNs actively convert hydrogen peroxide to hypochlorous acid through this specific MPO-dependent pathway that is essentially concentrated into subcellular (phago)lysosomal structures, as already detected intravitally in vivo (25, 36).

The present work was aimed at detecting real-time converting enzyme activity through an increased signal-to-background fluorescence emission in MPO-expressing cells in vivo. A first MPO probe (called SNAPF) has been developed by Shepherd et al. (30) and validated in inflammatory cells. However, to maximize the versatility of a probe catalogue, a probe that emits at a lower wavelength, close to that of fluorescein (520 nm), was mandatory. Toward this end, the SNAPF molecule was modified to generate the DSAPF molecule. In addition, SNAPF has been validated in vitro (in inflammatory cells), ex vivo (in arterial vessels), and in vivo using experimental whole body fluorescence reflectance imaging (30). The ultimate objective was to assess the ability of DSAPF to specifically detect...
cells with high MPO activity profiles in vitro, ex vivo, and by in vivo high-resolution microimaging of the lung and to semiquantify MPO activity in vivo with paralleled MPO activity in lung extracts.

The DSAPF probe was first tested in vitro in cells whose type is endogenous to the healthy and sick lung. In vitro data indicated that stimulated AMs and PMNs cells activated DSAPF intracellularly, whereas other cell lines never did, even after internalization (i.e., as demonstrated in DSF assays). Adding a specific MPO inhibitor reduced this activation, without any significant impact on the fluorescence level of the positive control (DSF), suggesting that the internalization of the probe was not affected by the inhibitor. Activation occurred over several hours. The contrast measured in these data could be improved by further cell incubation with DSAPF or by delaying ECFM up to 3 h. Resulting data demonstrated the potential of DSAPF and SNAPF for in vivo lung cell monitoring of MPO activity.

Using ECFM combined with DSAPF airway nebulization, MPO activity was explored in the lungs of live animals in two models of ALI. Imaging sequences acquired in vivo were
found to contain several highly fluorescent cells normalized to a darker background. The nature of these MPO-positive cells (AMs and PMNs) was confirmed using ex vivo immunohistochemistry. Overall, this contrast was sufficient to single out and identify these cells during ECFM. Cell tracking with measurement of fluorescence intensity can be performed in videos recording image analysis of ROIs and real-time assessment of this activity in process. Actual in vivo quantification demonstrated that cell/background signal ratios can be used to differentiate between ALI with high or low levels of MPO activity, although they are variable. Future work will attempt to further improve this contrast by incubating the probe for alternative time frames and to improve DSAPF sensitivity and intracellular activation/retention with new generation probes.

The results obtained were compared by ECFM with the DSAPF and SNAPF probes. It was discussed by others that reflectance in vivo optical imaging or longer wavelengths (i.e., red or near infrared) resulted in less background (30). Herein, probes were nebulized directly into the lung, and ECFM measured the surface fluorescence. The average contrast of DSAPF and SNAPF probe imaging was found to be similar such that both probes could be used for in vivo ECFM.

The background of videos shown herein was probably produced by free extracellular probe molecules found in the lung milieu (presumably activated and released by cells physiologically or after death or MPO activated extracellularly). For instance, the activated MPO enzyme is known to be secreted by PMNs, which would lead to the observation of some residual extracellular probe activation. Indeed, flow cytometry assays revealed that the activated probe could freely penetrate cells independently from an active process (data not shown). Overall, these two phenomena could explain the background observed during lung microimaging. The presence of endogenous fluorescent molecules (e.g., elastin) could also be involved but never exhibited a similar pattern, and this was ruled out in preliminary experiments where lung autofluorescence was very barely detectable in the green channel (data not shown).

With the forthcoming addition of other concomitant specific and smart probes, ECFM is then proposed as an innovative strategy toward a “virtual biopsy” of the injured lung to assess “real-time” molecular processes and tentatively surrogating OLB. ECFM has the ability to monitor several processes simultaneously and could represent a viable alternative to OLB for critically ill patients because it is much less invasive, although sophisticated, essentially retrieve a “picture” limited to cellular processes at bedside and “virtual biopsies” will likely be feasible and translatable to critically ill and supported patients and will allow tailoring treatment through a minimally invasive image-assisted pathology monitoring. Further developments to expand the current MPO probe performance and validated in vitro and in vivo/ex vivo with whole animals or organ imaging (e.g., chemi- or bioluminescence) (3, 9, 12, 18). The ability to detect and measure MPO activity in vivo in ROIs of ALI with such precision is definitively a novel powerful tool for decision-making physicians, as it brings a smart, minimally invasive microscopy to bedside and potentially provides precious information on molecular processes at play. Molecular microimaging such as ECFM would benefit from integration or combination with other upstream imaging systems to enable it to concentrate on a particular area or region of interest to explore. This will allow for localization or “mapping” of MPO-positive cell-containing portions of the lung.

Conclusions and Perspectives

As a proof of concept, it was first demonstrated that ECFM combined with smart probe microimaging for MPO activity can detect lung cellular events at play in real time. Multiplex color smart fluorescent imaging of the distal lung with screening of cellular processes at bedside and “virtual biopsies” will likely be feasible and translatable to critically ill and supported patients and will allow tailoring treatment through a minimally invasive image-assisted pathology monitoring. Further developments to expand the current MPO probe performance and delivery and targeting of alternative processes toward more comprehensive diagnostics are currently underway. Image analyses and quantification are also a “work in progress” needing fine-tuning adjustments. Finally, followup of the natural history of ALI and the impact of targeted treatments on MPO activity assessment are also planned in the near future.

ACKNOWLEDGMENTS

We thank Drs. E. Dumoulin, E. Gaudette, Y. Poulin, and P. Larivée for their help in accessing the BALF samples and the Plate-Forme d’Analyse et de Visualisation d’Images for assistance in image analysis.

GRANTS

Support from the Quebec Bio Imaging Network strategic initiative grant, the subvention à l’innovation IPS-CRCEL of the Faculté de Médecine et des