A novel NIR dye for in vivo temporal tracking of labeled macrophages to sites of acute inflammation

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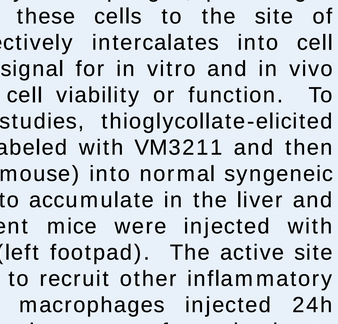
Abstract

Understanding the pathophysiology of inflammatory disease processes at the cellular and biochemical level is a major challenge in basic research and drug development. The difficulty in recapitulating complex biological processes in vitro, in particular, raises the importance of robust and quantitative in vivo techniques to assess progressive inflammation and therapeutic intervention. For example, the recruitment of monocytes and macrophages from the blood to sites of tissue infection or trauma is crucial for the induction of acute or chronic inflammation. To assess the biological changes that occur in acute inflammation, we used a simple model of acute neutrophil-driven inflammation, carrageenan-induced paw edema (CPE), generated in mice. A carrageenan solution (1% in PBS) was injected in the right footpad of each mouse to cause overt swelling and inflammation, and PBS was injected in each left footpad as a non-inflamed control site. We used non-invasive fluorescent imaging (Fluorescence Molecular Tomography™ [FMT]) to assess these changes, validating the model by measuring edema with a near infrared (NIR) vascular leak agent and inflammatory cell cathepsin and neutrophil elastase activity with NIR protease activatable agents. These agents all provided robust and statistically significant signal increases in inflamed versus control paws and correlated well with changes in paw thickness. In addition, a novel lipophilic cell-labeling agent was developed (VM3211) as a means to label primary macrophages, providing a tool to monitor the recruitment of these cells to the site of inflammation. This NIR agent effectively intercalates into cell membranes to provide a long-lasting signal for in vitro and in vivo studies with imperceptible effects on cell viability or function. To perform these macrophage-tracking studies, thioglycollate-elicited macrophages from donor mice were labeled with VM3211 and then transferred intravenously ($5x10^6$ cells/mouse) into normal syngeneic mice. The labeled cells were allowed to accumulate in the liver and lungs for 24h, and then the recipient mice were injected with carrageenan (right footpad) and PBS (left footpad). The active site of inflammation was used as a means to recruit other inflammatory cells, including the VM3211-labeled macrophages injected 24h earlier. Paw swelling and NIR FMT imaging was performed prior to carrageenan injections and at 3, 24, 48, 72, and 168 h thereafter. Macrophages selectively trafficked into inflamed paws and accumulated there, showing statistically significant elevations in fluorescence from 3-72h. A single prophylactic dose of dexamethasone (10 mg/kg IP) was sufficient to delay macrophage influx for at least 24h and decrease the peak levels of macrophage influx by approximately 40%. As expected, the peak macrophage influx accounted for ~1% of the total injected cells (50,000 cells/mouse), and control experiments characterized the linearity of cell quantification for VM3211-labeled macrophages.

1 VM3211 Characterization

Property	Specification
MW	1173 g mol^{-1}
Fluorescence[1] ▪ Peak excitation ▪ Peak emission	676 nm 696 nm
Purity[2]	> 95%
Appearance	Blue solid

1. Absorbance, excitation, and fluorescence in PBS
2. As determined by SE-HPLC and measuring absorbance at 675 nm

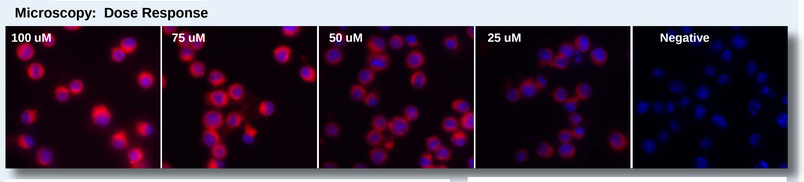


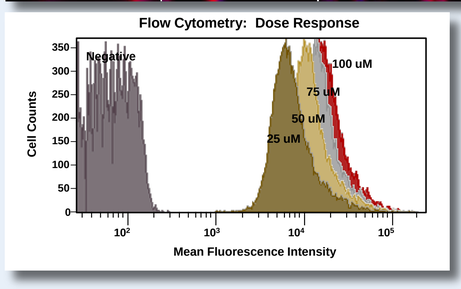
The cell-labeling agent VM3211 is a modified fluorophore designed to intercalate into cellular membranes. This agent has absorption and emission peaks at 676 nm and 696 peak nm in 1x PBS, respectively (ε=260,000 M/cm).

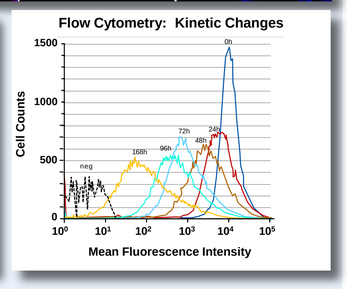
2 Cell Labeling Methods & Validation

A. RAW 264.7 cells

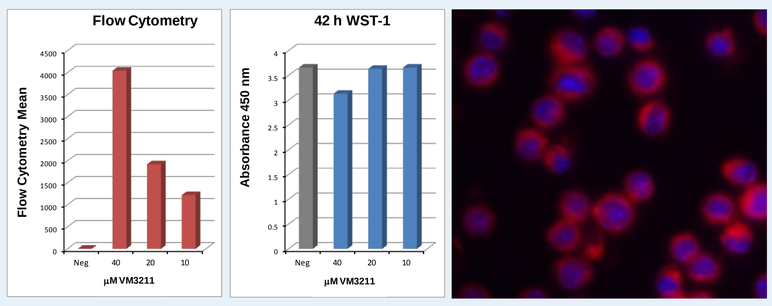
Microscopy: Dose Response







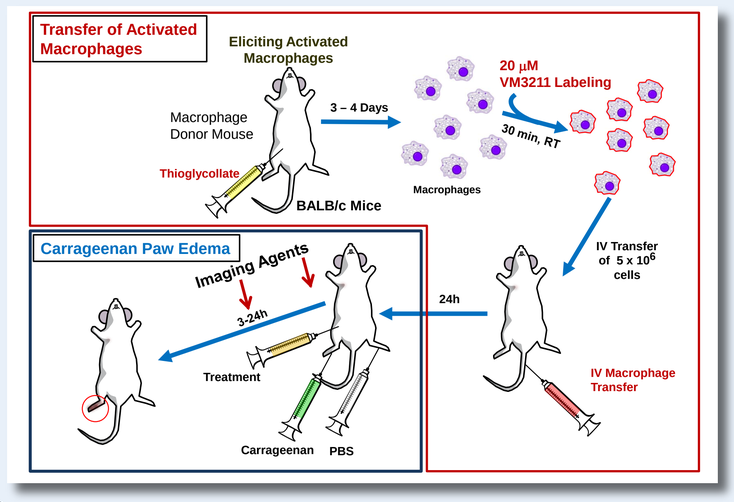
B. Primary mouse macrophages



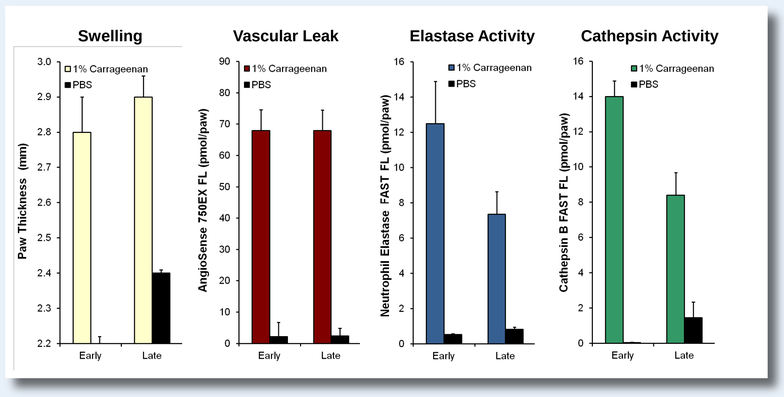
A. RAW 264.7 cells (mouse monocyte/macrophage origin) were incubated with different doses of VM3211 (15 min, RT) and assessed by microscopy and flow cytometry, showing dose-dependent labeling of cells. An additional study characterized the expected label loss over time (bottom right panel) associated with each cell division. Significant signal remains even in dividing cells over a seven day period. **B.** Primary peritoneal macrophages (thioglycollate-elicited) were labeled at three concentrations and assessed for viability (WST-1 assay), showing excellent viability at the recommended dose of 20 μM.

3 Paw inflammation Transfer Model

A. Carrageenan-induced inflammation & macrophage transfer model



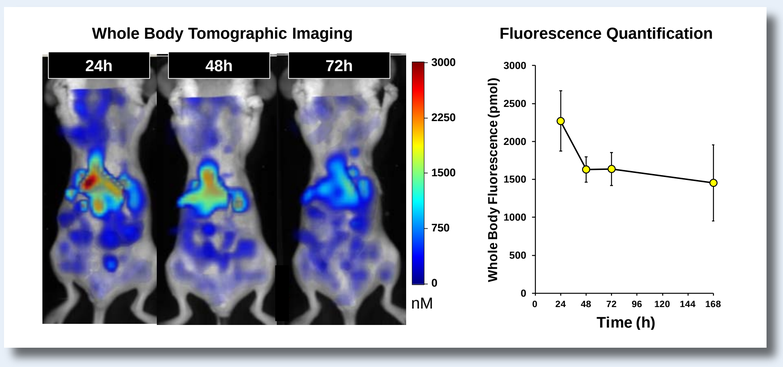
B. Paw edema model validation



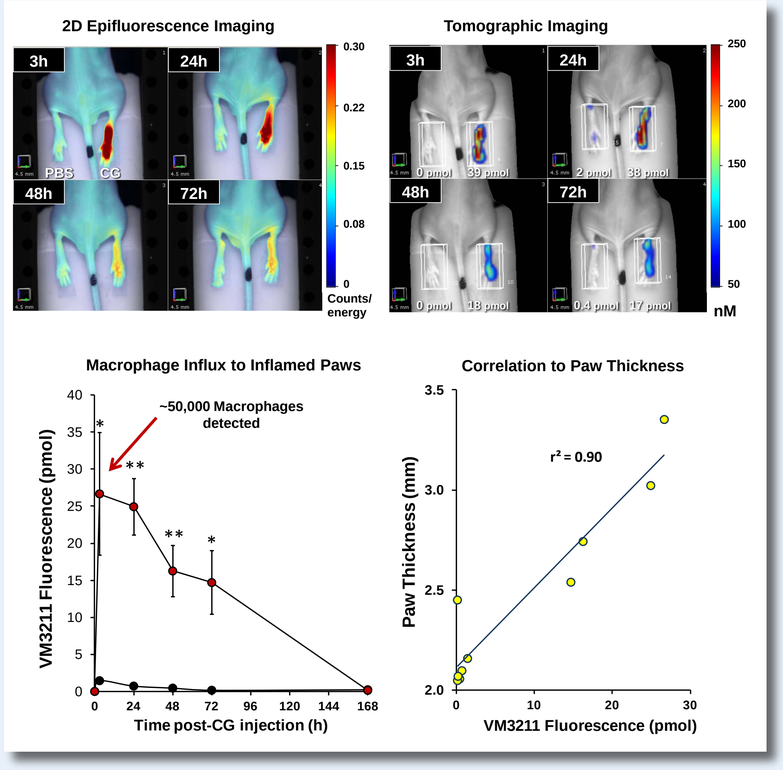
A. Protocols are illustrated for carageenan (CG)-induced paw inflammation and isolation and preparation of VM3211-labeled primary macrophages. **B.** CG-injected mice were measured for changes in paw thickness, vascular leak (AngioSense® 750EX), neutrophil elastase activity (Neutrophil Elastase 680 FAST™), and cathepsin activity (Cat B 680 FAST™) by injecting these agents at two time points, 2h (early) and 24h (late), post-CG injection. Tomographic (3D) paw imaging was performed on the FMT2500 5h after agent injection. All CG-induced responses were statistically significant at both time points ($p < 0.01$), revealing significant recruitment of inflammatory cells.

4 Timecourse of Macrophage Trafficking

A. Reconstitution of BALB/c mice with VM3211-labeled macrophages



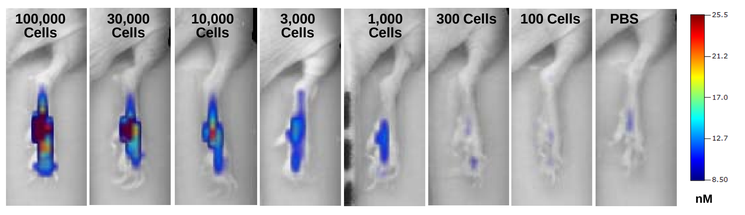
B. Tracking VM3211-labeled macrophages



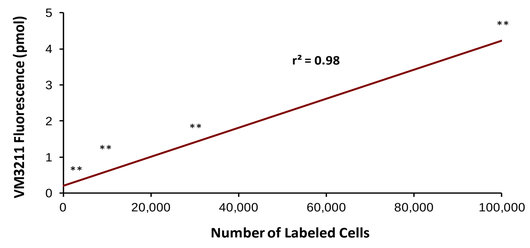
BALB/c mice received an intravenous injection of 5×10^6 thioglycollate-elicited peritoneal macrophages to provide a reservoir of fluorescent-labeled cells available for recruitment in response to an inflammatory stimulus. All mice were depilated completely to allow FMT 2500 tomographic imaging of whole body biodistribution of labeled macrophages over time. **A.** Tomographic images of mice receiving VM3211-labeled macrophages, showing widespread distribution, predominantly in the liver and lung regions. Quantification reveals retention of the majority of VM3211 signal for at least 168h. **B.** At 24h, each mouse received injections of PBS (left paw, control) and carrageenan (right paw) as an inflammatory stimulus. Imaging (both 2D and 3D) reveals high macrophage recruitment/accumulation in inflamed paws at 3-24h, with signal loss over the next 2 days. The magnitude of the fluorescence indicates ~50,000 macrophages were recruited by 3h. Fluorescence results correlated well with paw thickness measurements.

5 Linearity of VM3211-Labeled Cell Detection

A. FMT Images: Direct paw implantation of VM3211-labeled macrophages



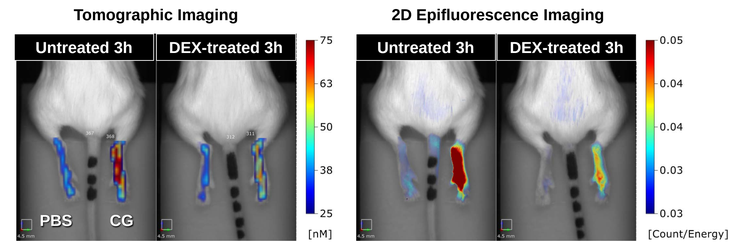
B. FMT quantification of paw fluorescence



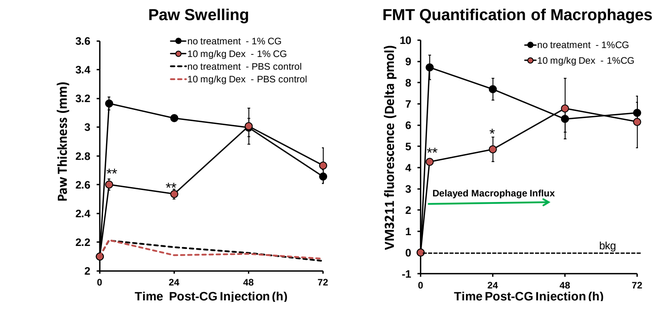
VM3211-labeled macrophages were injected at different cell numbers into the paws of hairless SKH-1E mice and imaged immediately by FMT 2500. **A.** Representative fluorescence tomographic images of paws at different cell doses are shown, and **B.** the correlation between fluorescence quantification and the cell numbers injected was determined. Asterisks indicate values with $p < 0.001$.

6 Dexamethasone Inhibition of Macrophage Recruitment

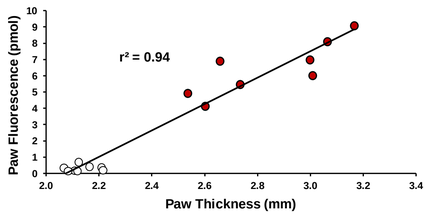
A. Imaging results



B. Paw swelling and fluorescence quantification



C. Correlating swelling and macrophage influx



BALB/c mice received an intravenous injection of 5×10^6 thioglycollate-elicited peritoneal macrophages as in the study represented in Figure 4. At the time of carrageenan injection, mice received either a single IP dose of 10 mg/kg dexamethasone or no treatment. **A.** Fluorescence tomography and 2D images of representative mice imaged at 3h post-CG are shown. **B.** Quantification of paw swelling and macrophage influx over time shows that the single treatment dose of dexamethasone delayed maximal macrophage influx by ~48h. **C.** The graph of paw thickness versus paw fluorescence across the different experimental groups reveals an excellent correlation.

Summary

We have developed a near infrared fluorescent cell labeling agent, VM3211, that can generate brightly-labeled and highly viable cells suitable for detection and longitudinal tracking in vivo. Populating normal BALB/c mice with NIR-labeled, activated primary mouse macrophages provides large numbers of cells that can be used as sensors for leukocyte recruitment to sites of inflammation. Noninvasive fluorescence tomography by FMT 2500 allowed the kinetic tracking and quantification of these cells as they migrated to a site of carrageenan (CG)-induced inflammation. As cell migration is critical for many important in vivo biological functions, this labeling approach has the potential to provide a useful tool for research in inflammation, immunology, and stem cells.

7 References

1. Eisenblatter M *et al*. In vivo optical imaging of cellular inflammatory response in granuloma formation using fluorescence-labeled macrophages. J Nucl Med. 2009; 50: 1676-1682
2. Thorek DLJ *et al.* In vivo, multimodal imaging of B cell distribution and response to antibody immunotherapy in mice. PLoS ONE. 2010; e10655.

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