AD_____

Award Number:
W81XWH-17-1-0128

TITLE:
Noninvasive optical imaging of glomerulonephritis

PRINCIPAL INVESTIGATOR:
Alfred Hyoungju Kim

CONTRACTING ORGANIZATION: Washington University
Saint Louis, MO 63130

REPORT DATE:
March 2019

TYPE OF REPORT:
Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command

Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: (Check one)

♦ Approved for public release; distribution unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE March 2019	**2. REPORT TYPE** Final	**3. DATES COVERED** 15 May 2017 - 15 Nov 2018

4. TITLE AND SUBTITLE
Noninvasive optical imaging of glomerulonephritis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-17-1-0128

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Alfred Hyoungju Kim

E-Mail: akim@wustl.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The Washington University
One Brookings Drive
Saint Louis, MO 63130-4862

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Material Command
Fort Detrick, MD 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

We have identified new near-infrared fluorescence (NIRF) probes that specifically detect the presence of glomerulonephritis. This was achieved using two innovative technologies: 1) fluorescence molecular tomography (FMT), an optical imaging approach that noninvasively monitors biologic processes quantitatively and in three-dimensions utilizing probes that fluoresce in the near-infrared spectrum of light; and 2) activatable NIRF probes, which leverage fluorescence in the near-infrared range (650-900 nm) excited by nonionizing radiation, is minimally absorbed by water and hemoglobin, and activates in the presence of an enzyme that is in high concentrations in a tissue of interest.

We found that NIRF probes activated by either cathepsin B or elastase, which derive from inflammatory macrophages and neutrophils respectively, correlate with the onset of proteinuria induced by experimental glomerulonephritis (GN) in mice. Two models of GN were used: nephrotoxic nephritis and the MRL/lpr lupus model. Importantly, this finding was specific for GN, as another model of renal injury, the CD2AP knockout mouse which models focal segmental glomerulonephritis yielded poor cathepsin B and elastase induced signals. These data support their use as a novel tool to detect GN, and possible application in humans for the detection of inflammatory kidney disease.

15. SUBJECT TERMS
Lupus nephritis, glomerulonephritis, inflammation, non-invasive imaging, near-infrared fluorescence

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER** *(include area code)*
Unclassified	Unclassified	Unclassified	Unclassified	11	USAMRMC

Table of Contents

Introduction
The gold standard for diagnosing lupus nephritis (LN) is the renal biopsy. While it has several significant benefits, this procedure is not without risk (i.e. bleeding) and only interrogates a minute portion of the renal parenchyma. Thus, missed diagnoses or misdiagnoses occur leading to repeat biopsies and inadequate treatment. Recently, near-infrared fluorescent (NIRF) probes have been utilized to image protein activity noninvasively in animals and humans. We propose that specific inflammatory processes observed in nephritis such as intrarenal macrophage activation can be noninvasively detected and monitored using NIRF-based optical imaging approaches. We aim to initiate these studies in experimental models of glomerulonephritis (GN), so we can track longitudinally the progression of disease with noninvasive imaging in comparison to conventional surrogates of disease activity such as proteinuria. We anticipate these data will warrant the need to further examine NIR-based imaging approaches as a novel tool for monitoring experimental glomerulonephritis and as an innovative diagnostic for detecting LN in patients with SLE.

Keywords
Systemic lupus erythematosus
Lupus nephritis
Glomerulonephritis
Non-invasive imaging
Near-infrared fluorescence
Inflammation
Cathepsin B
Elastase
Animal models

Accomplishments
- What were the major goals of the project?
  - 1) Determine sensitivity of cathepsin B (CatB) and elastase probes for detecting early changes of GN using the fluorescence molecular tomography.
  - 2) Develop and test NIRF probes to C3d, CD20, and test their ability to detect early (C3d) and late (CD20) GN
  - 3) Assess if improvement of glomerulonephritis can be detected after treatment with cyclophosphamide
- What was accomplished under these goals?
  - *Major activities*
    - 1) We have demonstrated that both inducible NIRF probes to CatB and elastase are capable of specifically detecting GN in mice administered nephrotoxic nephritis.
    - 2) The CatB-activated probes are specific for GN and distinguishes from another glomerular injury animal model.
  - *Specific objectives*
    - 1) Examine whether CatB and elastase-activated probes can detect early GN changes using the nephrotoxic nephritis (NTN) model of GN.
    - 2) Examine whether CatB and elastase-activated probes can detect early GN changes using the MRL/lpr mouse model of LN.
    - 3) Develop NIRF probes specific to C3d and CD20
    - 4) Examine whether NIRF probes to C3d and CD20 detect early (C3d) or late (CD20) changes of GN using the NTN model.
    - 5) Examine whether all of these probes can detect renal improvement following treatment of NTN mice with cyclophosphamide.
  - *Significant results/key outcomes*
    - Experimental approach: Two mouse models of GN are used to establish the feasibility of FMT: 1) Nephrotoxic nephritis (NTN) induced by the injection of sheep-derived nephrotoxic serum (NTS), and 2) *MRL/Mp-Fas^{lpr}/Fas^{lpr}* model for LN. NTN is a well-described model for GN with a rapid onset of proteinuria in 129x1/SvJ mice (1 day), and development of TIN (tubulointerstitial nephritis) (~14 days) with ~50% mortality (~day 28). MRL/Mp-*Fas^{lpr}*/*Fas^{lpr}* (referenced as MRL/lpr from here on) is an established mouse model of SLE that develop robust immune-complex GN at 12 weeks of age

and die between 17 (females) to 22 (males) weeks of age. Control mice (wild-type 129x1/SvJ or MRL/Mp) are age- and sex-matched.

At defined time points (NTN: early GN=day 0, 1, 4, 7, 10, late GN=14, 21, 28; MRL/lpr: weeks 6, 10, 12, 14, 16, 18, 20, 22), urine was collected, kidneys were harvested to histologically confirm the presence of GN, TIN, and complement deposits, and flow cytometry was performed on single-cell suspensions of proteolytically digested kidneys to confirm intrarenal macrophages ($Ly6G^-$ $F4/80^+CD11b^+Ly6C^+$), neutrophils ($Ly6G^+CD11b^+Ly6C^-Gr1^+$), and B cells ($B220^+$). Proteinuria was evaluated using both SDS-PAGE and urinary albumin/creatinine ratios (albumin detection: Bethyl Laboratories mouse albumin ELISA, creatinine detection: BioAssay QuantiChrom Assay Kit).

Also, at these time points, NIR probes specific to enzymes (cathepsin B: Perkin Elmer CatB 750 FAST, elastase: Perkin Elmer neutrophil elastase 680 FAST), complement split products (labeled whole antibody or $F(ab)_2$ to C3d) or immune cells ($F(ab)_2$ to CD20) was injected 6 hours prior to imaging. Isofluorane-anesthetized mice were imaged using FMT (Perkin Elmer FMT 4000). FMT uses a transillumination mode combined with computer algorithms to generate three-dimensional maps of fluorophores inside living animals Images will also be obtained prior to probe delivery to evaluate baseline fluorescence. Fluorescence measurements were normalized then reconstructed. Fluorescence measurements from the kidney and non-kidney regions was quantified using TrueQuant imaging software (PerkinElmer).

Results: In Figure 1, representative images for CatB-activated probes obtained from the FMT-4000 imaging system are shown. In Figure 2. quantification of CatB-activated probe (A and B), proteinuria (C), and renal macrophages (D) are shown. Each parameter was assessed several times during the early GN phase (days 0, 1, 3, 5, 7, and 10). Mice were reused for longitudinal imaging: one set were imagined on days 0, 3, and 7, and a second set on days 1, 5, and 10.

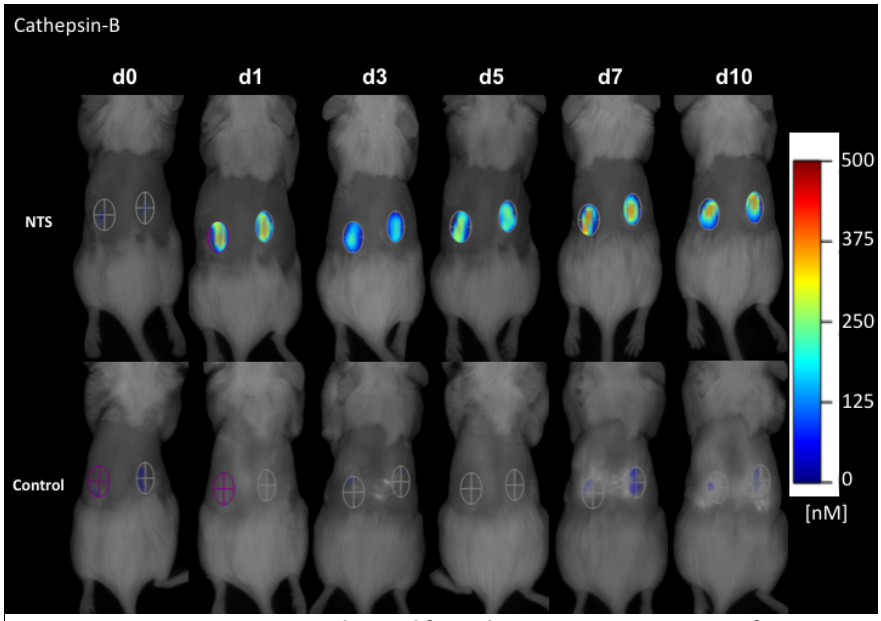

Figure 1. Representative images obtained from the FMT-4000 instrument of CatB-activated dye within the kidneys of mice with early GN. Quantification of dye is color-coded with the scale on the right.

A substantial accumulation of CatB-activated probe was observed as early as day 1 (Fig 1, Fig 2A), which correlated with a rise in proteinuria (Fig 2C). The increased CatB-activated signal and proteinuria was elevated for at least 7 days. Importantly, since mice were reused for longitudinal imaging, no spillover fluorescence was observed in-between time points (Fig 2B).

We originally hypothesized that the source of renal CatB would be from inflammatory macrophages, however the number of renal macrophages did not increase in concordance with the CatB signal (Fig 2D). In fact, substantial macrophage infiltration did not occur until day 7. While we do not know the explicit reason for this discordance between macrophage numbers and CatB probe detection, we speculate that either an increase of renal macrophage CatB expression on a per cell basis occurred during the early time period, or expression of CatB within the renal parenchyma itself increased during this injury phase. Indeed, CatB upregulation was observed in the proximal tubules

of proteinuric rats (Olbricht CJ, *Eur J Clin Chem Clin Biochem*, **30**:675, 1992). To determine the source of CatB, we plan to use flow cytometry to examine of macrophage expression of CatB increases during the early GN phase. In addition, we will perform immunofluorescence on frozen kidney sections from mice with early GN and stain for CatB to confirm the source of CatB.

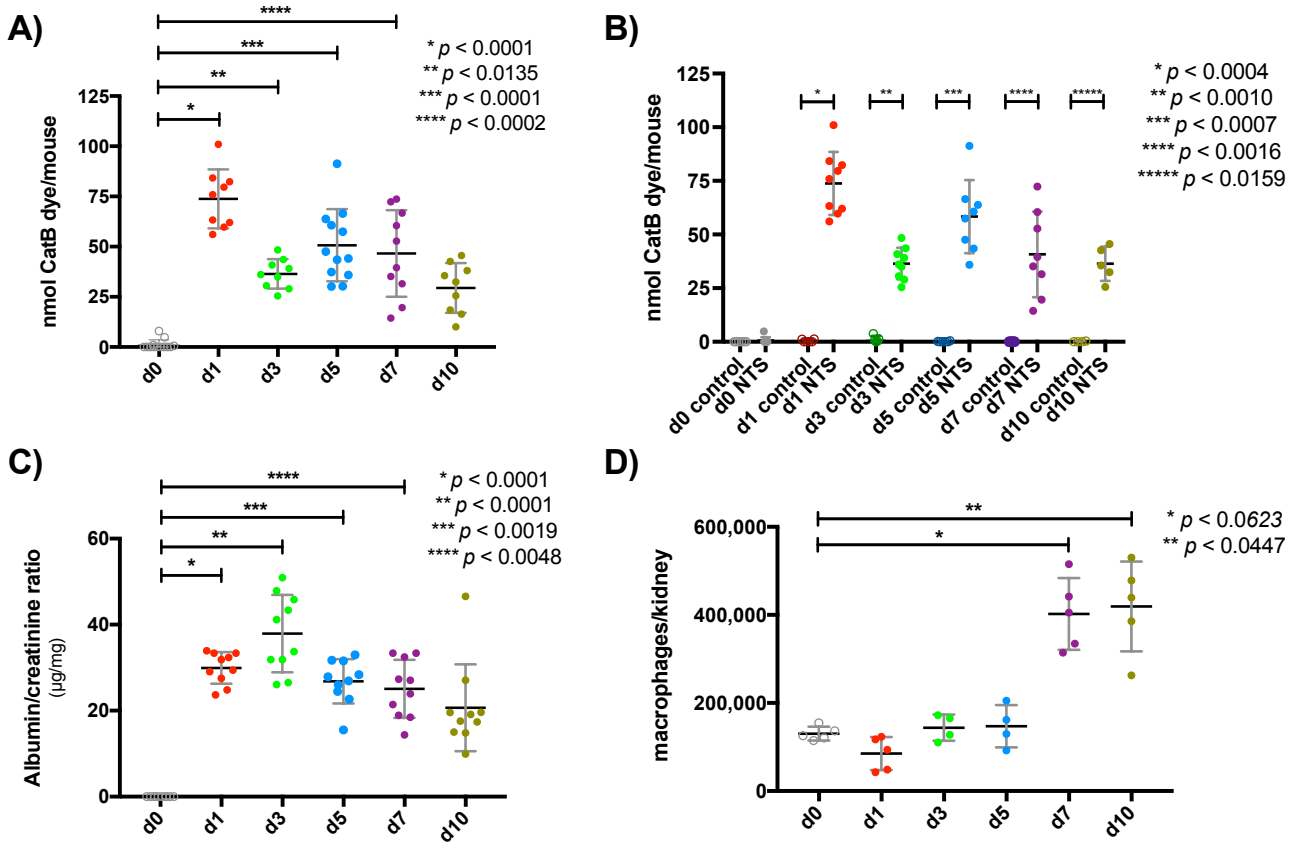

Figure 2. NIRF probes activated by CatB detect glomerulonephritis. A) Quantitative CatB signal observed per mouse over a period of 10 days post-nephrotoxic serum administration. B) Control mice imaged for CatB signal at identical time points as NTS mice. C) Urinary albumin/creatinine ratios assessed by ELISA (albumin) or a chromogenic assay (creatinine) during the same time period. D) Number of macrophages per kidney assessed by flow cytometry during the same time period. Statistical significance in A, C, and D were determined using the Kruskal-Wallis test, penalizing for multiple comparisons to day zero. Statistical significance in B was determined using the Mann-Whitney test.

Since we speculated that the source of CatB may be derived from the renal parenchyma itself, we hypothesized that renal injury of any sort may be detectable with our activatable probes. Thus, we used another well-established mouse model of glomerular injury, the CD2AP knockout mouse (Shih *et al.*, *Science*, **286**:312, 1999). These mice experience severe nephrotic syndrome and die at 6-7 weeks of age. We examined 3-week old CD2AP KO mice, which are nephrotic at this age (*urinary albumin/creatinine ratio=3.98 and 17.8 for the two CD2AP KO mice*), for the assessment of activated CatB-specific probes. A very small increase was noted compared to control mice (Fig 3) but is substantially lower than what is induced by GN despite substantial proteinuria. **These data suggest that GN specifically induces a state where CatB-activated probes can be detected by FMT.**

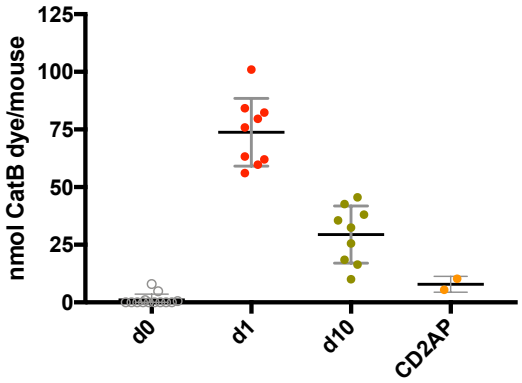

Figure 3. CatB-activated probe detection is specific to GN. Nephrotic CD2AP KO mice had minimal CatB signal compared to NTN mice.

We next examined for the ability of elastase-activated NIRF probes to detect early GN. In Figure 3, representative images of elastase-activated probes obtained from the FMT-4000 instrument are shown. In Figure 4, quantification of elastase-activated probe (A), proteinuria (B), and renal macrophages (C) are shown.

Similar to CatB, **an accumulation of elastase-activated probe was observed early (day 1) in disease course (Fig 4, Fig 5A),** which also correlated with a rise in proteinuria (Fig 5B). The

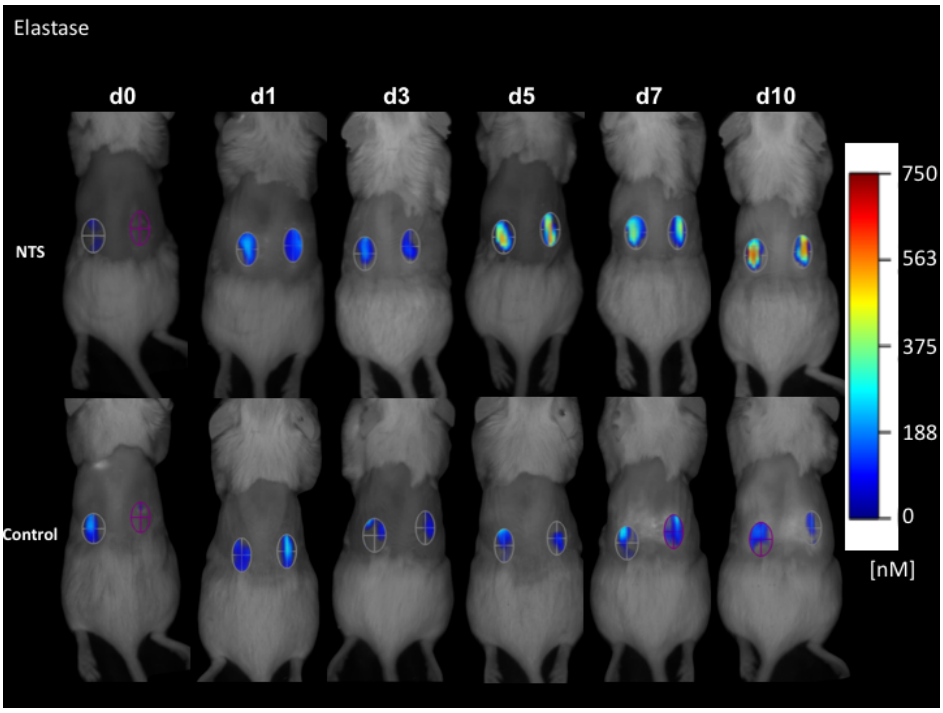

Figure 4. Representative images obtained from the FMT-4000 instrument of elastase-activated dye within the kidneys of mice with early GN. Quantification of dye is color-coded with the scale on the right.

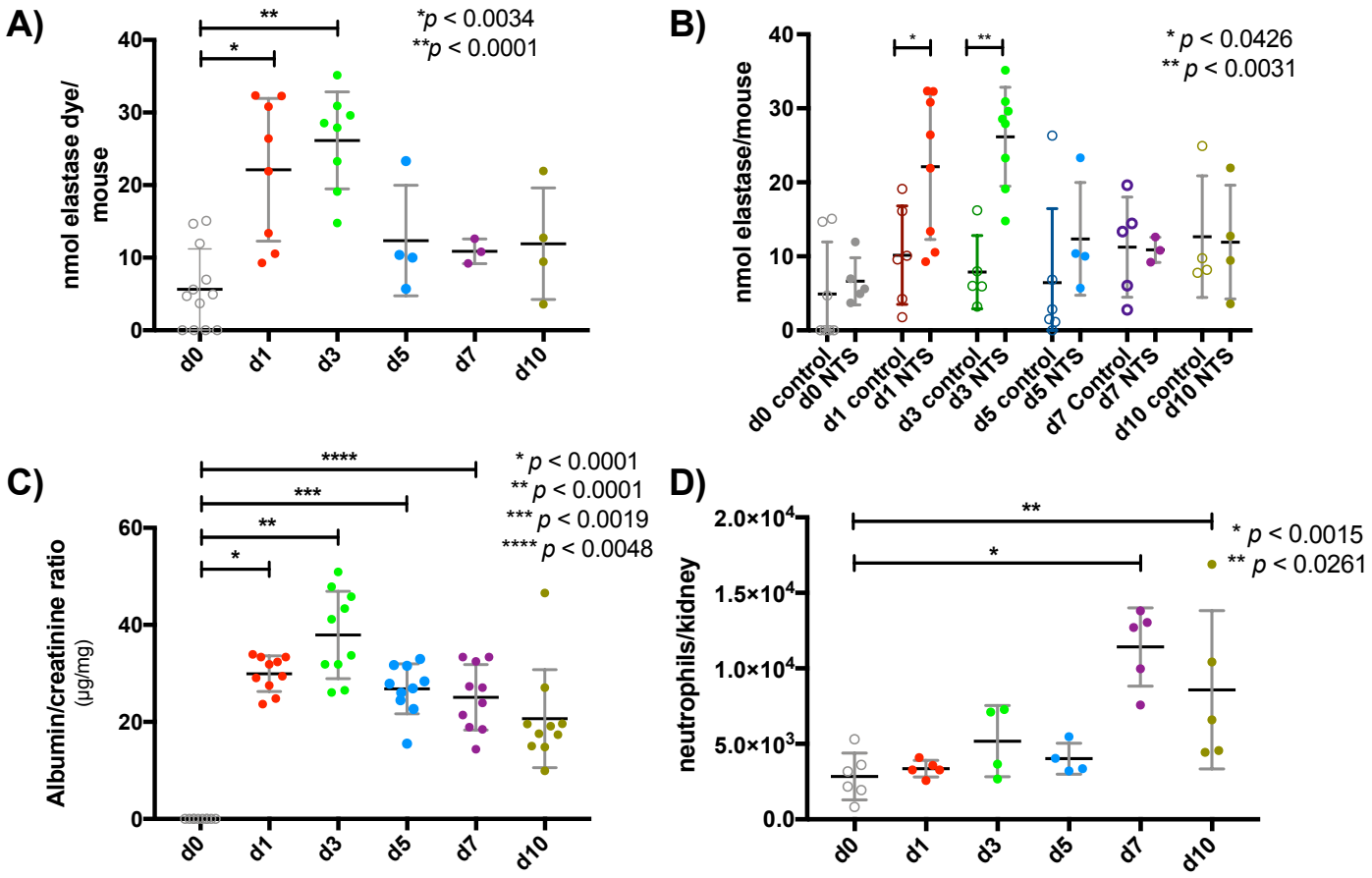

Figure 4. NIRF probes activated by elastase also detect glomerulonephritis. A) Quantitative elastase signal observed per mouse over a period of 10 days post-nephrotoxic serum administration. B) Control mice imaged for elastase signal at identical time points as NTS mice. C) Urinary albumin/creatinine ratios assessed by ELISA (albumin) or a chromogenic assay (creatinine) during the same time period. D) Number of neutrophils per kidney assessed by flow cytometry during the same time period. Statistical significance in A, C, and D were determined using the Kruskal-Wallis test, penalizing for multiple comparisons to day zero. Statistical significance in B was determined using the Mann-Whitney test.

increased elastase-generated signal and proteinuria was elevated for 3 days before extinguishing back to near baseline levels. Spillover fluorescence from prior probe administration was an issue at the later timepoints, as fluorescence started to rise at the days 7 and 10 timepoints (Fig 5B). We are repeating these experiments using new mice at each timepoint to improve signal-to-noise ratio issues and better understand the kinetics of dye elimination in the kidney. It remains unclear why the elatase-activated probe possesses spillover while the CatB-activated probe does not.

Similar to CatB, we originally hypothesized that the source of renal elastase would be from neutrophils, but the number of renal neutrophils did not increase in concordance with the elastase-generated signal (Fig 5D). Again, similar to macrophage kinetics, substantial neutrophil infiltration did not occur until day 7. While the source of elastase is exclusively neutrophil-derived, inhibitors to elastase function such as α1-proteinase inhibitor, have been described to be upregulated in kidney diseases (specifically, pediatric chronic kidney disease; Polanska *et al*., *Arch Immunol Ther Exp (Warsz)*, **62**:239, 2014). These inhibitors may attenuate probe activation, thus reducing signal. We have decided not to further pursue identification of elastase inhibitors.

We are currently testing this strategy on lupus-prone mice (MRL/lpr) pre-GN onset and during GN to confirm the findings with NTS model.

Finally, with our collaborator Samuel Achilefu, we have generated probes with NIRF dyes attached to $F(ab')_2$ fragments specific to either CD20 or C3d. We have confirmed the probes have maintained their specificity to their antigen, and we will examine the ability to detect and differentiate early (Cd3) and late (CD20) GN using the NTS model.

- *Other achievements*
  - Nothing to report

- What opportunities for training and professional development has the project provided?
  - Nothing to report
- How were the results disseminated to communities of interest?
  - 2019 LUPUS meeting (Oral Presentation)
  - Data presented to patients of the Lupus Clinic at WashU at annual research symposium (April 2018)
- What do you plan to do during the next reporting period to accomplish the goals?
  - Nothing to report

Impact

- What was the impact on the development of the principal discipline of the project?
  - These data represent the first to apply noninvasive, NIRF-based, topographic imaging for the detection of GN. This extends on prior observations, which used a NIRF-based planar approach (Hao *et al*, *PLoS One*, **8**:e57418, https://doi.org/10.1371/journal.pone.0057418, 2013). The advantages of FMT (the ability to quantify signal to the picomole level, while significantly improving fluorescence detection from deep within tissue by virtue of a tomographic approach) were fully realized especially with the CatB-activated probe. Our data provides additional proof-of-principle of using noninvasive approaches to detect GN and has potential to become a novel human diagnostic approach for recognizing GN.
- What was the impact on other disciplines?
  - Nothing to report
- What was the impact on technology transfer?
  - Nothing to report
- What was the impact on society beyond science and technology?
  - Nothing to report

Changes/Problems

- Changes in approach and reasons for change
  - Nothing to report
- Actual or anticipated problems or delays and actions or plans to resolve them
  - We have had some delays generating the C3d and CD20 probes due to unanticipated chemistry issues conjugating the probe away from the antigen-binding sites. Nevertheless, we have overcome this hurdle and will test this in the NTN model.

- Additionally, as noted in above, we had spillover signal from prior injections of the elastase-activated probe in the later timepoints of the early GN assessments. We are repeating these experiments with new mice for each timepoint to reduce any noise generated from incomplete clearance of probe in the kidney.

- Changes that had a significant impact on expenditures
  - Nothing to report
- Significant changes in use or care of human subjects
  - N/A
- Significant changes in use or care of vertebrate animals
  - Nothing to report
- Significant changes in use or care of biohazards/select agents
  - N/A

Products

- Publications, conference papers, and presentations
  - Journal publications
    - Nothing to report
  - Books or other non-periodical, one-time publications
    - Nothing to report
  - Other publications, conference papers, and presentations
    - Cheung MD, Brähler S, Huang D, Schriefer R, Renfroe SC, Black KC, Achilefu S, Akers WJ, and Kim AHJ. Noninvasive detection of experimental glomerulonephritis using near-infrared fluorescent probes activated by cathepsin B and elastase. Status of publication: In preparation.
    - Cheung MD, Brähler S, Huang D, Schriefer R, Renfroe SC, Black KC, Achilefu S, Akers WJ, and Kim AHJ. Noninvasive detection of experimental glomerulonephritis using fluorescence molecular tomography. Oral abstract presentation, LUPUS 2019, April 2019.
- Website(s) or other Internet site(s)
  - Nothing to report
- Technologies or techniques
  - We have pioneered a protocol to detect early changes of experimental GN with NIRF probes using the FMT imaging platform. We will distribute the protocol within the manuscript, initially through preprint servers (i.e bioRxiv) then through a peer-reviewed journal.
- Inventions, patent applications, and/or licenses
  - Nothing to report
- Other products
  - Nothing to report

Participants & Other Collaborating Organizations

- What individuals have worked on the project?
  - #1
    - Name: Matthew Cheung
    - Project Role: Research Assistant
    - Research Identifier: 0000-0003-4182-5220 (ORCID)
    - Nearest person month worked: 3
    - Contribution to Project: Performed the experimentation for this proposal, including animal work, urinary proteinuria assessments, mouse optical imaging, flow cytometry, and managing data.
    - Funding Support: N/A

  - #2
    - Name: Dongyue Huang
    - Project Role: Research Assistant
    - Research Identifier: 0000-0002-9017-3727 (ORCID)
    - Nearest person month worked: 7
    - Contribution to Project: Performed the experimentation for this proposal, including animal work, urinary proteinuria assessments, mouse optical imaging, flow cytometry, and managing data.

        - Funding Support: N/A
    - #3
        - Name: Kvar Black
        - Project Role: Co-Investigator
        - Research Identifier: N/A
        - Nearest person month worked: 1
        - Contribution to Project: Assisted with experimentation for this proposal, specifically mouse optical imaging and managing data.
        - Funding Support: N/A
    - #4
        - Name: Samuel Achilefu
        - Project Role: Co-Investigator
        - Research Identifier: 0000-0002-3133-6717 (ORCID)
        - Nearest person month worked: 0.5
        - Contribution to Project: Assisted with work supervision, specifically mouse optical imaging.
        - Funding Support: N/A
    - #5
        - Name: Alfred Kim
        - Project Role: Principal Investigator
        - Research Identifier: 0000-0003-4074-0516 (ORCID)
        - Nearest person month worked: 1.5
        - Contribution to Project: Supervised work, including experimental design and execution, data analysis and interpretation, and oversaw staff, as well as presentations and publications arising from the work.
        - Funding Support: N/A
- Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since last reporting period?
    - Nothing to report
- What other organizations were involved as partners?
    - Nothing to report

Special Reporting Requirements

- Collaborative Awards
    - N/A
- Quad Charts
    - See following page

Appendices

Quad chart on next page

PR162095 : Noninvasive Optical Imaging of Glomerulonephritis

PI: Alfred Kim, Washington University, MO **Budget:** $305,000.00

Autoimmune: Peer Reviewed Medical Research Program **Mechanism:** Discovery Award

Research Area(s): 505; 713 **Award Status:** 05/15/2017-11/15/2018

Study Goals:

1) Determine sensitivity of cathepsin B (CatB) and elastase probes for detecting early changes of experimental glomerunephritis using the fluorescence molecular tomography.

2) Develop and test NIRF probes to C3d, CD20, and test their ability to detect early (C3d) and late (CD20) GN

3) Assess if improvement of glomerulonephritis can be detected after treatment with cyclophosphamide

Specific Aims:

Aim 1. To evaluate the performance of FMT in monitoring experimental GN.

Subaim 1a. To determine if FMT detects GN inflammatory changes earlier that the onset of proteinuria.

Subaim 1b. To determine the sensitivity of FMT to detect TIN.

Aim 2. To determine if FMT can detect improvement in active experimental GN.

Key Accomplishments and Outcomes:

Publications: none to date

Patents: none to date

Funding Obtained: none to date