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***In Vivo* Luminescent Imaging of NF- κ B Activity and NF- κ B Related Serum Cytokine Levels Predict Pain Sensitivities in a Rodent Model Of Peripheral Neuropathy**

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Abstract

Background—Methods for the detection of the temporal and spatial generation of painful symptoms are needed to improve the diagnosis and treatment of painful neuropathies and to aid preclinical screening of molecular therapeutics.

Methods—In this study, we utilized *in vivo* luminescent imaging of NF- κ B activity and serum cytokine measures to investigate relationships between the NF- κ B regulatory network and the presentation of painful symptoms in a model of neuropathy.

Results—The chronic constriction injury model led to temporal increases in NF- κ B activity that were strongly and non-linearly correlated with the presentation of pain sensitivities (i.e. mechanical allodynia and thermal hyperalgesia). The delivery of NEMO-binding domain peptide reduced pain sensitivities through the inhibition of NF- κ B activity in a manner consistent with the demonstrated non-linear relationship. Importantly, the combination of non-invasive measures of NF- κ B activity and NF- κ B regulated serum cytokines produced a highly predictive model of both mechanical ($R^2=0.86$) and thermal ($R^2=0.76$) pain centered on the NF- κ B regulatory network (NF- κ B, IL-6, CXCL1).

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Author Contributions

R.B. and L.S. were responsible for the integrity of the work as a whole, from inception to publication. R.B., L.S., I.K., D.K., V.K., G.S. made substantial contributions to conception, design and acquisition of data; and revising the article critically for important intellectual content. R.B., M.S. J.H., and K.R. contributed to analysis and interpretation of the data and drafting the article. All authors discussed the results and approved of the final version to be published.

Conclusions—Using *in vivo* luminescent imaging of NF- κ B activity and serum cytokine measures, this work establishes NF- κ B and NF- κ B regulated cytokines as novel multivariate biomarkers of pain-related sensitivity in this model of neuropathy that may be useful for the rapid screening of novel molecular therapeutics.

Introduction

Painful neuropathies can be induced following peripheral nerve compression or chemical irritation, as seen following intervertebral disc (IVD) herniation. However, the underlying molecular events that contribute to painful neuropathy remain poorly understood. Clinically, determining the tissue source and associated spinal level can make developing effective treatment plans difficult, especially for those associated with IVD disorders (Boden et al., 1990; Deyo and Weinstein, 2001). A better understanding of the underlying mechanisms involved in the generation of painful symptoms and the development of novel biomarkers of pain are needed to improve the diagnosis and treatment of painful neuropathies and aid in the rapid screening of novel therapeutics.

The transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) is involved in the regulation of over 150 genes in inflammation, immunity, apoptosis, and cell-proliferation (Bonizzi and Karin, 2004) and this NF- κ B regulatory network has been linked to neuropathy via an array of inflammatory cytokines (e.g TNF- α , IL-1 β , IL-6) (see appendix S1) through the activation of NF- κ B activity via TNF- α and IL-1 β (Kaltschmidt and Kaltschmidt, 2009; Makarov, 2000; Tak and Firestein, 2001). In models of peripheral nerve ligation or biochemical irritation, NF- κ B activity has been observed in satellite cells of the dorsal root ganglion, astrocytes of the dorsal horn and Schwann cells of the sciatic nerve (Ledeboer et al., 2005; Ma and Bisby, 1998; Miyoshi et al., 2008). Despite an incomplete understanding of a role for NF- κ B in the presentation of pain in peripheral neuropathy, inhibiting NF- κ B activity can result in the attenuation of painful symptoms (Fu et al., 2010; Meunier et al., 2007; Sun et al., 2012; Suzuki et al., 2009) and indicate a causal role for NF- κ B in the presentation of painful symptoms after peripheral injury.

Imaging of NF- κ B activity, using a transgenic luminescent NF- κ B reporter mouse model (Carlsen et al., 2004; Izmailova et al., 2007), has potential to reveal the temporal and spatial distribution of transcription factor activation following a defined injury and act as a novel imaging pain biomarker (Bowles et al., 2014). Luminescent imaging was used to study the relationships between NF- κ B activity, NF- κ B regulated cytokines, and the presentation of painful symptoms in a model of peripheral neuropathy. This is the first time luminescent imaging has been used to monitor the regional and temporal progression of inflammation in a model of peripheral neuropathy. The chronic constriction injury model was chosen due to its repeatability and ability to model the mechanical compression and biochemical irritation of peripheral nerve observed during IVD herniation induced neuropathies. The network of NF- κ B related measures (luminescent imaging and NF- κ B regulated serum cytokines) was tested for its ability to predict pain sensitivities in this model to propose a multivariate biomarker of pain. The clinically relevant NF- κ B specific inhibitor NF- κ B essential modulator (NEMO)-binding domain peptide (NBD), which inhibits NF- κ B activity by disrupting the I κ B kinase complex, was delivered to test the relationship between the

measured NF- κ B activity and pain in this model. This approach was chosen to test the usefulness of NF- κ B activity imaging in screening therapeutics.

Methods

Chronic Constriction Injury Model and Experimental Timeline

The transgenic mice used for this study were engineered to carry cDNA for firefly luciferase downstream of NF- κ B response elements (n=39, male, BALB/c-Tg(NF κ B-RE-luc), age 12 weeks; Taconic Farms Inc., Hudson, NY). All procedures were performed with approval of the Duke University IACUC and mice were acclimated for one week prior to the start of the study.

A subset of mice (n=24) were randomly divided into two groups that received either chronic constriction injury (CCI) surgery (CCI; n=12) or a sham surgery (SHAM; n=12). The CCI surgery was performed under general anesthesia (2% isoflurane inhalation) and sterile conditions according to the methods described by Bennett and Xie and modified for mice (Bennett and Xie, 1988). Briefly, after induction of general anesthesia, the posterior thigh was prepped with chlorhexidine. A 1 cm incision was made on the posterior thigh. The sciatic nerve was exposed and loosely ligated at three sites at one mm intervals with 6-0 chromic gut suture (Syneture; Covidien, Mansfield, MA). The skin was closed with a 4-0 nylon suture. In the sham group, surgery was performed to expose the nerve without any ligation or suture placement. One set of randomly selected animals (n=6/group, CCI and SHAM) was sacrificed on postoperative day 3 and a second set of animals was sacrificed on postoperative day 28 (n=6/group, CCI and SHAM). Animals were imaged prior to sacrifice using *in vivo* luminescent imaging to quantify NF- κ B activity preoperatively and on postoperative days 3, 7, 14, 21, and 28. These time points were selected to capture the early expression of TNF- α , IL-1 β , and IL-6 and presentation of pain in the CCI model, while also capturing the sustained pain sensitivities and IL-6 expression that have been shown out to day 28. Additionally, mechanical allodynia and thermal hyperalgesia were measured at these same time points. The sciatic nerve, adjacent muscle, and spleen were harvested for *ex vivo* imaging immediately after sacrifice. Finally, serum was obtained from the maxillary vein using standard procedures preoperatively and on days 3, 14, and 28 in a subset of animals to assay for select NF- κ B regulated systemic pro- and anti- inflammatory cytokines.

NEMO-Binding Domain Peptide Delivery

A randomly selected subset of mice (n=15) underwent CCI surgery as described above, and was then randomly divided into two groups that received either NEMO-binding domain (NBD) peptide in vehicle, as described below, (NBD; n=8) or vehicle alone (VEHICLE; n=7). The NBD peptide sequence was *DRQIKIWFQNRRMKWKK*TALDWSWLQTE, which included both the NEMO binding domain and a cell permeable domain from *Drosophila antennapedia* (AnaSpec, Fremont, CA). The NBD peptide was weighed and solubilized immediately before injection at room temperature in a small volume of DMSO, and then diluted in PBS to a final concentration of 2% DMSO and 100 μ M NBD peptide. NBD peptide (3 mg/kg i.p.; ~200 μ l) was delivered in PBS with 2% DMSO every other day from day 1 to 27. *In vivo* luminescent imaging, mechanical allodynia, and thermal

hyperalgesia measures were performed preoperatively and on days 3, 7, 14, 21, and 28, as above. Animals were sacrificed on day 28.

In Vivo NF-κB Activity Luminescent Imaging

Mice were injected with D-luciferin (150 mg/kg i.p., Caliper Life Sciences Inc., Hopkinton, MA) 10 minutes prior to imaging to allow D-luciferin distribution. Under anesthesia, whole-body NF-κB activity-related luminescence was then imaged on an *in vivo* imaging system (IVIS Spectrum or IVIS Kinetic, Perkin Elmer, Waltham, MA) with a 15 second exposure. In order to quantify luminescence, an elliptical ROI (1×1.5 cm) was placed on the ipsilateral limb (Fig. 1). The ROI was selected to encompass the majority of NF-κB activity variance observed in the imaging dataset (Fig.1 and Video S1). At each time point, the ROI luminescence (LUM_{ROI}) was measured as photons/sec/cm² for all animals. Luminescence data for each animal at each time point was normalized to preoperative values from the same animal (N_{LUM}) according to,

$$N_{LUM} = \frac{LUM_{ROI}}{(LUM_{ROI})_{preoperative}} \quad [1]$$

Ex Vivo NF-κB Activity Luminescent Imaging

Ex vivo imaging was performed to determine the tissue sources of NF-κB activity related luminescence. At sacrifice, the sciatic nerve, nerve-adjacent muscle tissue, and spleen were harvested for immediate *ex vivo* luminescent imaging. Tissues were placed in a 12-well plate containing 300 µg/ml D-luciferin in Dulbecco's Phosphate-Buffered Saline (Gibco, Grand Island, NY). After 5 minutes, the tissue culture plate was imaged on the IVIS Spectrum (Perkin Elmer; 15 second exposure). Selected ROI around each tissue was measured as photons/sec/cm² ($LUM_{Sciatic\ Nerve}$: sciatic nerve luminescence, LUM_{Muscle} : muscle luminescence, LUM_{Spleen} : spleen luminescence). Immediately after *ex vivo* luminescent imaging on day 3, the ipsilateral and contralateral sciatic nerves from a randomly selected subset of CCI mice were placed in optimal cutting temperature (OCT) embedding media (Sakura Finetek, Torrance CA) and flash-frozen in liquid nitrogen for immunofluorescence experiments to identify NF-κB activity cell source (appendix S2).

Mechanical Allodynia and Thermal Hyperalgesia

Mice were acclimated to cages and testing procedures for mechanical allodynia and thermal hyperalgesia for a one-week period prior to start of experiment. Mechanical allodynia was measured in the ipsilateral hind paw using the “up-down” method (Chaplan et al., 1994) to calculate the 50% withdrawal threshold (50%WT), as previously described (Bowles et al., 2014). Data were collected preoperatively and on days 3, 7, 14, 21, and 28 with all data normalized as a percentage of preoperative values,

$$N_{50\%WT} = \frac{50\%WT}{50\%WT_{preoperative}} \times 100. \quad [2]$$

On the same days as mechanical allodynia testing, thermal hyperalgesia was measured in the ipsilateral hind paw using the Hargreaves method to calculate latency to paw withdrawal (LAT), as previously described (Bowles et al., 2014). Thermal hyperalgesia withdrawal data for each animal at each time point was normalized as a percentage of the preoperative values from the same animal (N_{LAT}),

$$N_{LAT} = \frac{LAT}{LAT_{\text{preoperative}}} \times 100. \quad [3]$$

Serum Cytokines

The serum cytokines interleukin-1 beta (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12p70 (IL-12p70), chemokine C-X-C motif ligand 1 (CXCL1), tumor necrosis factor-alpha (TNF- α), interferon-gamma (IFN- γ), and monocyte chemotactic protein-1 (MCP-1) were quantified using multiplex and single immunoassays (Mouse Pro-inflammatory 7-plex and Mouse MCP-1 single-plex, Meso Scale Discovery, Rockville, MD) in triplicate. The mean intra-assay coefficients of variation for the 7-plex pro-inflammatory and MCP-1 immunoassay were 3.3% and 2.8%, respectively. A SECTOR Imager 2400 (Meso Scale Discovery, Rockville, MD) was used for all serum measurements.

Statistics

ANOVA Analysis—*Ex vivo* imaging (LUM_{Sciatic} , LUM_{Mus} , LUM_{Spleen}) and serum cytokine data were analyzed by two-way analysis of variance (ANOVA) with Tukey post hoc test, treating day and group as factors. Significance was tested at $\alpha = 0.05$. One half of the lower limit of detection was used for any serum cytokine value below the level of detection for the purpose of performing statistical analyses

Repeated measures ANOVA was run separately for responses N_{LUM} , $N_{50\%WT}$, N_{LAT} in order to determine if the mean response differed by day, treatment, or the interaction of day and treatment. Variables were transformed to the log scale (base 10) to better meet the normality assumptions. *Post-hoc* analysis performed using Tukey post hoc test.

Correlations and Predictive Modeling—To verify the source tissues of *in vivo* NF- κ B activity luminescence (N_{LUM}), a multi-linear regression was performed to produce a linear model of N_{LUM} with the independent variables $LUM_{\text{Sciatic Nerve}}$ and LUM_{Muscle} ,

$$LUM_{ROI} = \gamma_1 LUM_{\text{sciaticNerve}} + \gamma_2 LUM_{\text{Muscle}} \quad [4]$$

To test for hypothesized relationships between pain-related measures ($N_{50\%WT}$, N_{LAT}), NF- κ B activity (N_{LUM}), and serum cytokines (IL-6, CXCL1, IL-1 β , MCP-1, IFN- γ , IL-12p70, and IL-10), pairwise correlations were calculated using data from day 3, 14, and 28 (all measures collected on these days). Repeated measures were accounted for using the within subjects correlation method of Bland and Altman (Bland and Altman, 1995). Significance of the correlations was reported with a Bonferroni correction of $\alpha = 0.001$ (0.05/45). All variables were log-transformed (base-10) prior to statistical analysis.

A predictive model of pain sensitivities was built using NF- κ B activity luminescent imaging and serum cytokine measures. Linear models for $N_{50\%WT}$ and N_{LAT} were built using partial least squares (PLS) regression from the log-transformed variables N_{LUM} , IL-6, CXCL1, IL-1 β , MCP-1, IFN- γ , and IL-10. The PLS method was selected for its ability to handle the multicollinearity in the predictor variables and repeated measures. A threshold for variable influence on projection (VIP) was set at 0.8 for inclusion in the final linear model (Eriksson et al., 2006). In order to estimate the predictive power of the models, K-fold cross-validation with 6 subsamples was used to calculate Q^2 . All regression analysis was performed using JMP Pro (JMP, Cary, NC).

Results

NF- κ B Activity in CCI

NF- κ B activity luminescence (N_{LUM}) increased in the ipsilateral limb after CCI surgery (Fig. 1) and was significantly elevated compared to sham controls ($p < 0.001$; repeated measures ANOVA). Increases in N_{LUM} were observed on the order of 20-fold through the first 2 weeks and dropped down to 8-fold after day 14. Luminescence was distributed from the midline of the animal through the distal ipsilateral limb at early time points in some animals, but subsided near the midline of the animal over time and was localized in the distal ipsilateral limb at later time points ($> \text{day } 7$) (Fig. 1A). Mean regional NF- κ B activity measurements (video S1) demonstrated luminescence was distributed from the midline to the distal region at day 3, but largely concentrated in the distal regions of the limb and with signal dissipating near the midline and increasing in the distal region from day 3 to 14. The overall signal peaks from day 3 to 14 and decreases for all regions beyond day 14, but with elevated signal being observed in the distal region observed at all time points. Increased signals in the midline region are only observed at early time points, while distal regions remain prominent throughout the study.

Behavioral Assessments

CCI resulted in the induction of significantly elevated sensitivity to mechanical stimuli (mechanical allodynia; $p < 0.0001$) and thermal stimuli (thermal hyperalgesia; $p = 0.0004$) compared to sham controls in the ipsilateral limb (Fig. 1D). Increased sensitivities were sustained in both measures over the full 28 days of this study.

Serum Cytokines

Serum levels of IL-6 were significantly elevated on day 3 in both the CCI ($p = 0.0006$) and sham ($p = 0.006$) operated groups compared to preoperative levels, but only CCI-operated values remained elevated by day 14 compared to preoperative ($p = 0.0002$) and sham levels ($p = 0.03$) (Fig. 2). Additionally, serum levels of CXCL1 were elevated in the CCI ($p < 0.0001$) and sham ($p = 0.02$) groups compared to preoperative levels at day 14, but with CCI showing significant increases ($p = 0.01$) compared to sham. There was no evidence of between-group or time-related differences in serum cytokines MCP-1, IFN- γ , IL-1 β , IL-10, IL-12p70.

Ex Vivo NF- κ B Activity Luminescent Imaging

Ex vivo imaging revealed significant elevation of NF- κ B activity in the injured sciatic nerve (Lum_{Sciatic Nerve}), as well as the muscle (Lum_{Muscle}) and spleen (Lum_{Spleen}) (Fig. 3A). Specifically, NF- κ B activity luminescence was significantly elevated in the ipsilateral sciatic nerve in the CCI group compared to sham at both day 3 ($p = 0.0009$) and 28 ($p < 0.0001$). NF- κ B activity luminescence was also significantly elevated in the muscle ($p = 0.0009$) and spleen ($p = 0.04$) at day 3 in the CCI group compared to sham. *In vivo* NF- κ B activity luminescence (N_{LUM}) was well described when modeled by the *ex vivo* sciatic nerve (Lum_{Sciatic Nerve}) and muscle (Lum_{Muscle}) NF- κ B activity luminescence using a multiple linear regression ($R^2 = 0.79$, $p < 0.0001$) (Fig. 3B).

Relationships Between NF- κ B Activity, Serum Cytokines, and Pain-Related Sensitivities

Significant correlations were observed between NF- κ B activity, serum cytokines, and pain-related sensitivities in the CCI model (Table S1, Fig. 4). Strong relationships were observed between NF- κ B activity (N_{LUM}) and both pain sensitivity measures, mechanical allodynia ($R = -0.79$, $p < 0.0001$) and thermal hyperalgesia ($R = -0.72$, $p < 0.0001$). Relationships between NF- κ B activity (N_{LUM}), mechanical allodynia ($N_{50\%WT}$), and thermal hyperalgesia (N_{LAT}) were non-linear before taking log-transform (Fig. 4a). For both mechanical allodynia and thermal hyperalgesia, the dynamic range of the relationship occurs in the 0 to ~10 fold range of N_{LUM} . Additionally, serum IL-6 showed significant relationships with mechanical allodynia ($R = -0.70$, $p = 0.0002$) and thermal hyperalgesia ($R = -0.64$, $p = 0.0008$). NF- κ B activity (N_{LUM}) was positively correlated to serum IL-6 ($R = 0.71$, $p < 0.0001$). No evidence of additional significant correlations was detected between NF- κ B activity (N_{LUM}), mechanical allodynia ($N_{50\%WT}$), thermal hyperalgesia (N_{LAT}), and serum levels of IL-6, CXCL1, IL-1 β , MCP-1, IFN- γ , IL-12p70, and IL-10.

Predictive Model of Pain Sensitivities

The non-invasive measures of NF- κ B activity (N_{LUM}) and serum cytokines were used to build models of mechanical allodynia ($N_{50\%WT}$) and thermal hyperalgesia (N_{LAT}) using partial least squares regression (Fig. 4B). Both mechanical allodynia and thermal hyperalgesia were well predicted with NF- κ B activity (N_{LUM}), serum IL-6, and serum CXCL1 as independent variables, with NF- κ B activity being the most influential variable in the models (highest VIP and magnitude of standardized model coefficients). Specifically, the mechanical allodynia model was able to describe ~86% (R^2) of the variability using NF- κ B activity (VIP = 1.7), serum IL-6 (VIP = 1.4), and serum CXCL1 (VIP = 1.1). Using K-fold cross validation, this same model was able to predict ~79% (Q^2) of the variability in the data. The thermal hyperalgesia model was able to describe ~76% (R^2) of the variability using NF- κ B activity (VIP = 1.7), serum IL-6 (VIP = 1.3), and serum CXCL1 (VIP = 1.1) as independent variables. Using K-fold cross validation this same model was able to predict ~65% (Q^2) of the variability in the data.

Treatment of Pain Sensitivities with NBD

Systemic delivery of NBD decreased both NF- κ B activity and pain sensitivities following CCI, and in a manner that preserved the non-linear relationships between these variables

(Fig. 4C and 5). NBD delivery significantly reduced NF- κ B activity (N_{LUM}) compared to CCI ($p < 0.0001$) and vehicle ($p = 0.01$) groups and was not significantly different from sham levels ($p = 0.9$) (Fig. 5A, B). A modest effect of the vehicle (2% DMSO) was observed with a significant decrease in NF- κ B activity (N_{LUM}) in the vehicle group compared to the CCI group ($p = 0.01$). However, N_{LUM} in the vehicle group was significantly elevated compared to NBD ($p = 0.01$) and sham groups ($p = 0.007$). Delivery of NBD reduced pain sensitivities to sham levels for mechanical allodynia ($N_{50\%WT}$) and thermal hyperalgesia (N_{Lat}) (Fig. 5C). Specifically, mechanical allodynia after NBD delivery was significantly decreased compared to both CCI ($p < 0.0001$) and vehicle groups ($p < 0.0001$), and not significantly elevated compared to sham ($p = 0.4$). Thermal hyperalgesia after NBD delivery was significantly decreased compared to both CCI ($p = 0.002$) and vehicle groups ($p = 0.003$), and not significantly elevated compared to sham ($p = 0.8$).

Discussion and Conclusions

The chronic-constriction injury (CCI) model has been widely used to model neuropathy based on the loose ligation of the sciatic nerve with chromic gut sutures that provides both a mechanical compression and a chemical irritation of the peripheral nerve (Jaggi et al., 2011). Ligation of the peripheral nerve induces Wallerian degeneration with immune cell recruitment to the injury site, glial cell activity, and neuroinflammation that leads to painful symptoms. The model produces a rapid and sustained pain response, which includes mechanical allodynia, thermal hyperalgesia, and changes in gait and posture (Bennett and Xie, 1988; De Vry et al., 2004; Vrinten and Hamers, 2003). Given its extended study for pain presentation, the CCI model is of great interest for studying the relationship between NF- κ B activation and the temporal presentation of pain using non-invasive imaging of luciferase in the NF κ B-RE-luc mouse.

Sustained NF- κ B activity was observed in the ipsilateral limb after chronic constriction injury of the sciatic nerve, which showed strong relationships to the presentation of painful symptoms (i.e. mechanical allodynia and thermal hyperalgesia) and elevations in serum levels of IL-6/CXCL1. Both IL-6 and CXCL1 are regulated by NF- κ B (Bachmeier et al., 2008; Takeshita et al., 1996) and IL-6 was correlated with local NF- κ B activity measures in the ipsilateral limb. Both mechanical compression and chemical irritation may lead to increased mRNA or protein expression of an array of inflammatory cytokines including IL-6 in the affected nerve (Ellis and Bennett, 2013; Moalem and Tracey, 2006; Rothman et al., 2011). Importantly, NF- κ B is a key mediator of signaling pathways for the expression of IL-6, consistent with the relationship between NF- κ B activity and IL-6 levels observed here. The direct or indirect interaction of IL-6 with nociceptors has been suggested as a possible contributor to nociceptor sensitivity in both peripheral neuropathy and discogenic back pain (Risbud and Shapiro, 2013; Sommer and Kress, 2004). Specifically, in relation to the findings of this study, IL-6 is capable of directly sensitizing nociceptors through trans-signaling and the subsequent activation of protein kinase C and regulation of TRPV1 (Obreja et al., 2005; Quarta et al., 2011; Rose-John, 2012). In addition, IL-6 can also modulate spinal cord synaptic transmission to induce central sensitization (Kawasaki et al., 2008). For this reason, the relationships observed in this study are consistent with observed underlying mechanisms of IL-6 regulation and nociceptor sensitization by IL-6. It is

important to note that plasma IL-6 levels have been associated with psychological stress (LeMay et al., 1990) and may not directly be related to NF- κ B levels in the ipsilateral limb. However, regardless of source, this observation suggests that a set of non-invasive measures of the NF- κ B regulatory network, such as NF- κ B-regulated cytokines studied here, may be useful surrogate measures of pain. Together, this work establishes NF- κ B and NF- κ B regulated cytokines as novel multivariate biomarkers of pain-related sensitivity in this model of neuropathy. The relationship between pain sensitivities and NF- κ B activity was non-linear and plateaued at NF- κ B activity (N_{LUM}) fold changes of ~ 10 and greater. This plateau may suggest that therapeutics targeted at the NF- κ B pathway would need to decrease NF- κ B activity below a threshold before seeing therapeutic efficacy. Therapies that fail to reach this threshold, could in theory, successfully reduce NF- κ B activity, but fail to see a therapeutic response.

Surprisingly, despite many papers implicating a central mechanism for NF- κ B activity, luminescence imaging demonstrated strong and persistent levels of NF- κ B activity distal to the injury site with evidence of central NF- κ B activity dissipating by day 7. For this reason, this work focused on the peripheral NF- κ B activity. NF- κ B activity detected in the ipsilateral limb via luminescence was produced at the tissue level by a sustained increase in NF- κ B activity in the sciatic nerve and a transient increase in NF- κ B activity in the adjacent muscle tissue that was unrelated to the surgery. These data indicate an NF- κ B activation specific to the injured sciatic nerve. Importantly, ipsilateral limb luminescence at late time points largely represents NF- κ B activity at the sciatic nerve. Immunofluorescence indicated luciferase was closely colocalized with GFAP positive cells (e.g. Schwann cells) as a primary source of NF- κ B activity luminescence in the sciatic nerve after CCI (Figure S1 and appendix S2), which is consistent with previous literature demonstrating a reduction of pain behavior after transgenic inhibition of NF- κ B activity in GFAP expressing cells (Fu et al., 2010). NF- κ B activity became localized to the peripheral regions of the ipsilateral limb around day 7, with NF- κ B activity near the midline dissipating at these time points. This may elucidate why NF- κ B inhibition targeted at the central nervous system is effective at time points before day 7 but loses efficacy when delivered at day 7 in a spinal nerve ligation model (Zang et al., 2010).

Luminescence imaging of NF- κ B activity and NF- κ B regulated serum cytokine measures were used to generate a multivariate biomarker of pain sensitivities (mechanical allodynia and thermal hyperalgesia) with PLS regression. PLS regression provided a method that could provide accurate reports on the relative importance of each factor in the model despite the colinearity in the data that is naturally present when measuring multiple components from the NF- κ B regulated network. The strong relationship between pain and NF- κ B activity was demonstrated in the multivariate model, as NF- κ B activity was the most predictive variable in the model according to both VIP and standardized model coefficients. Despite the strong relationship between NF- κ B activity and pain sensitivities, combining it with other measures from the NF- κ B regulatory network (IL-6 and CXCL1) produced a more predictive model ($R^2 = 0.86$) than NF- κ B activity alone ($R^2 = 0.64$). Cytokines act in a complex network of signaling pathways such that utilizing multiple and temporal measures from a regulatory network (e.g. NF- κ B), instead of individual cytokines, can provide more powerful biomarkers.

Luminescent imaging of NF- κ B activity provided both a minimally invasive system to monitor disease progression and therapeutic intervention. We delivered NBD peptide, an NF- κ B specific inhibitor, to test the screening utility of the NF- κ B activity reporter system, while simultaneously testing the relationships of NF- κ B activity to pain sensitivities. NBD peptide decreased pain sensitivities to sham levels in the same non-linear fashion as demonstrated previously. No animals demonstrated any deleterious effects due to peptide delivery. The vehicle (2% DMSO) also inhibited NF- κ B activity, consistent with previous work demonstrating DMSO's ability to affect NF- κ B activity (Essani et al., 1997; Vlahopoulos et al., 1999). The preservation of the non-linear relationship between NF- κ B activity and pain sensitivities in the presence of two independent sources of NF- κ B inhibition (i.e. NBD and DMSO) verify this strong non-linear relationship, while also demonstrating the therapeutic ability of NBD peptide to reduce pain sensitivities.

The relationships between NF- κ B activity and painful sensitivity presented within this manuscript is quite consistent with prior findings for a model of inflammatory osteoarthritis (Bowles et al., 2014). Despite very distinct disease models, both models showed strong relationships between pain sensitivities, NF- κ B activity, and serum IL-6. This relationship was evident in the inflammatory driven phase of the osteoarthritis model, and is consistent with the strong inflammatory component in the CCI model of neuropathy. Nevertheless, there were differences in the dependence of NF- κ B activity on other cytokines (IL-10 and IL-1 β) in the osteoarthritis model that do not emerge as contributing variables in the CCI model. The ability for consistent predicted relationships between NF- κ B activity and inflammatory pain components suggests a common molecular mechanism for pain in both models, and suggests a utility for these non-invasive measures to predict this type of pain sensitivity in a broad spectrum of disease models. It is important to note that this model addresses the inflammatory components of pain sensitivities and does not account for the non-inflammatory contributors of neuropathic pain, such as abnormal collections of sodium channels in damaged peripheral nerve fibers (Waxman et al., 1999), decreased inhibition of neuronal activity via loss of inhibitory neurons (Taylor, 2009), and altered central processing (Latremoliere and Woolf, 2009). Future work will focus on imaging techniques to incorporate some of these parameters (e.g. alterations in sodium channel gene expression) in to the modeling.

This study utilized luminescent imaging of NF- κ B activity and serum measures of NF- κ B-regulated cytokines to investigate relationships between the NF- κ B regulatory network and the presentation of painful symptoms after chronic constriction injury of peripheral nerve. The CCI model induced increases in NF- κ B activity that were strongly and non-linearly correlated with the developing pain sensitivities in this model and the delivery of NBD peptide was able to reduce these pain sensitivities. Importantly, the combination of the non-invasive measures of NF- κ B activity and NF- κ B regulated serum cytokines produced a highly predictive multivariate biomarker of pain centered on the NF- κ B regulatory network. This work demonstrates a non-invasive set of biomarkers that are predictive of pain in this model of neuropathy and can aid in the rapid screening of novel therapeutics by reducing the need for laborious behavioral pain measures. Future work will be necessary to test how these results translate to human neuropathies with the hopes of developing novel NF- κ B pathway targeted treatments and novel multivariate biomarkers for pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What's Known About this Topic?

- NF- κ B and inflammatory cytokines are known to play a role in peripheral neuropathy.
- Non-invasive imaging of NF- κ B activity has been demonstrated to act as a surrogate biomarker of pain sensitivities in osteoarthritis.

What Does This Study Add?

- Non-invasive imaging of NF- κ B activity can serve as a surrogate biomarker of pain sensitivities in peripheral neuropathy, with demonstrated ability to monitor therapeutic intervention.
- Serum levels of inflammatory cytokines together with NF- κ B activity are strong predictors of pain sensitivities in neuropathy.
- The relationship between NF- κ B activity and pain sensitivities is distinctly non-linear.
- NEMO binding domain peptide provides therapeutic decrease in pain sensitivities

Figure 1. Persistent NF-κB Activity Observed in Distal Limb After Chronic Constriction Injury
 (A) *In vivo* luminescent imaging of NF-κB activity in CCI and sham animals over 28 days.
 (B) Region of interest selection on ipsilateral limb used for (C) quantification of fold-change in NF-κB activity luminescence (N_{LUM}: luminescence normalized to baseline, equation 1) compared to preoperative levels over 28 days. (D) Normalized measures of mechanical allodynia (N_{50%WT}: 50% withdrawal threshold measures normalized to baseline, equation 2) and thermal hyperalgesia (N_{LAT}: latency measure normalized to baseline, equation 3) in the

ipsilateral limb for CCI and sham groups over 28 days. ($p < 0.05$, significance noted compared to sham (*))

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Figure 2. Serum IL-6 and CXCL1 Elevated After Chronic Constriction Injury

Serum cytokine levels for IL-6 and CXCL1 at preop, day 3, day 14, and day 28. ($p < 0.05$, significance noted compared to preop (#) or sham (*))

Figure 3. Elevated Sciatic Nerve and Muscle Tissue NF- κ B Activity Correlated with Whole Animal Imaging

(A) *Ex vivo* luminescent imaging of NF- κ B activity in CCI and sham sciatic nerve ($LUM_{Sciatic\ Nerve}$: non-normalized sciatic nerve luminescence), adjacent muscle (LUM_{Muscle} : non-normalized muscle luminescence), and spleen (LUM_{Spleen} : non-normalized spleen luminescence) at day 3 and 28 with representative images from day 28 ($p < 0.05$, significance noted compared to preop (#) or sham (*)). (B) Multiple linear regression describing *in vivo* luminescence (LUM_{ROI} : non-normalized region of interest luminescence) by *ex vivo* sciatic nerve ($LUM_{Sciatic\ Nerve}$: non-normalized sciatic nerve luminescence) and muscle tissue (LUM_{Muscle} : non-normalized muscle luminescence) luminescence.

Figure 4. Non-invasive NF- κ B Activity and Serum Cytokine Measures Predictive of Pain Sensitivities in Chronic Constriction Injury
(A.) Mechanical allodynia ($N_{50\%WT}$: 50% withdrawal threshold measures normalized to baseline, equation 2) and thermal hyperalgesia (N_{LAT} : latency measure normalized to baseline, equation 3) data plotted as a moving average against NF- κ B activity luminescence (N_{LUM} : luminescence normalized to baseline, equation 1) from CCI and sham groups at all time points. (B.) Partial least squares regression models of mechanical allodynia ($N_{50\%WT}$) and thermal hyperalgesia (N_{LAT}). (C) Scatter plots of mechanical allodynia ($N_{50\%WT}$) and

thermal hyperalgesia (N_{LAT}) plotted against NF- κ B Activity luminescence (N_{LUM}) for CCI, Sham, NBD, and Vehicle groups from all animals at all time points.

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Figure 5. NEMO-Binding Domain Peptide Reduces NF-κB Activity and Pain Sensitivities in Chronic Constriction Injury

(A.) Quantified NF-κB Activity (N_{LUM} : luminescence normalized to baseline, equation 1) compared between treatment groups over 28 days with (B) representative luminescent images from day 3. (C) Mechanical allodynia ($N_{50\%WT}$: 50% withdrawal threshold measures normalized to baseline, equation 2) and thermal hyperalgesia (N_{LAT} : latency measure normalized to baseline, equation 3) compared amongst treatment groups over 28 days. (Groups marked with different letters are significantly different ($p < 0.05$)).