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## **Use of Bioluminescence Imaging to Track Neutrophil Migration and its Inhibition in Experimental Colitis**

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**Short title:** Tracking neutrophil recruitment

**Abbreviations used in this paper:** DSS, dextran sodium sulphate; IBD, inflammatory bowel disease; PMN, polymorphonuclear; IL-8, Interleukin 8; MIP-2, Macrophage Inflammatory Protein-2; KC, keratinocyte-derived chemokine; mrKC, murine recombinant KC.

## Summary

Inflammatory bowel disease (IBD) is associated with neutrophil infiltration into the mucosa and crypt abscesses. The chemokine IL-8 (murine homologs KC and MIP-2) and its receptor CXCR2 are required for neutrophil recruitment; thus, blocking this engagement is a potential therapeutic strategy. In the present study we developed a pre-clinical model of neutrophil migration suitable for investigating the biology of and testing new drugs that target neutrophil migration. Peritoneal exudate neutrophils from transgenic  $\beta$ -actin-*luciferase* mice were isolated 12 hours after intraperitoneal injection with thioglycollate and were phenotypically and functionally assessed. Exudate cells were injected intravenously into recipients with dextran sodium sulphate (DSS)-induced colitis followed by bioluminescence imaging of whole-body and *ex vivo* organs at 2, 4 and 16-22 hours post-transfer. Anti-KC antibody or an isotype control were administered at 20 $\mu$ g/mouse 1 hour before transfer followed by whole-body and organ imaging 4 hours post-transfer. The peritoneal exudate consisted of 80% neutrophils, 39% of which were CXCR2<sup>+</sup>. *In vitro* migration towards KC was inhibited by anti-KC. *Ex vivo* bioluminescent imaging showed that neutrophil trafficking into the colon of DSS recipients was inhibited by anti-KC, 4 hours post cell transfer. In conclusion, this study describes a new approach for investigating neutrophil trafficking that can be used in pre-clinical studies to evaluate potential inhibitors of neutrophil recruitment.

**Keywords:** Inflammatory bowel disease; neutrophils; DSS colitis; bioluminescence imaging

## **Introduction**

Polymorphonuclear (PMN) neutrophil transmigration across the mucosa and into intestinal crypts is a major characteristic of the Inflammatory bowel diseases (IBD): Crohn's disease (CD) and Ulcerative colitis (UC). Excessive or unchecked neutrophil recruitment can lead to tissue damage mainly due to the persistent release of harmful inflammatory cytokines, reactive oxygen species and proteases by the infiltrated cells [1]. In active IBD, histological evidence of high density neutrophil accumulation in the intestinal lumen directly correlates with epithelial injury and clinical disease activity [2]. Therefore, targeting neutrophil influx is a potential therapeutic strategy for IBD.

The CXC chemokines; human Interleukin-8 (IL-8/CXCL8) and the murine functional homologs keratinocyte-derived chemokine (KC/CXCL1) and macrophage inflammatory protein-2 (MIP-2/CXCL2) are neutrophil chemoattractants, that orchestrate their activation and recruitment from the blood into sites of infection, inflammation and injury by promoting endothelial adhesion and transmigration [3]. Their biological effects are mediated by binding to two high affinity receptors; CXCR1 and CXCR2 [4]. CXCR2 has proven to be a potent mediator of PMN recruitment in pre-clinical models of arthritis [5], allergy [6], respiratory disease [7] and ulcerative colitis [8]. Increased mucosal expression of these chemokine receptors and their ligands in IBD explains the massive influx of leukocytes in active disease. The up-regulation of IL-8 in the colonic mucosa of IBD patients [9, 10] correlates well with the histological degree of inflammation and chemokine mRNA expression [11] [12]. The pivotal involvement of KC and MIP-2 in PMN infiltration into inflammatory sites is also well documented [13] [14]. Furthermore, a marked increase

in KC and MIP-2 have been reported in colons of mice with acute phase dextran sulphate sodium (DSS) -induced colitis [15].

Traditional methods used to track neutrophil recruitment such as static histological analysis of fixed tissues following adoptive transfer of dye-labeled cells, do not provide temporal or spatial information within the physiological environment of lymphoid tissues [16]. While, white cell scintigraphy has been used to study neutrophil migration in both pre-clinical and clinical IBD studies [17, 18] there are well recognized disadvantages associated with radiotracers including the adverse effect on cell viability, radioactive decay and poor resolution [19]. In this study, a novel method to track neutrophil recruitment is described; involving bioluminescence imaging of adoptively transferred luciferase-expressing peritoneal exudate cells. We also investigated the blocking effect that an anti-KC antibody may have on neutrophil homing to the inflamed intestines of mice with DSS-induced colitis. The results from these studies clearly show selective trafficking of luciferase-expressing cells to inflamed colon 4h post cell transfer with a significant reduction in neutrophil trafficking in the anti-KC treated DSS mice.

## **Materials and Methods**

### **Mice**

Male and female wild-type (wt) FVB/N mice, 8-12 wk old, were obtained from Harlan (UK). The  $\beta$ -actin/luciferase expressing (*luc*<sup>+</sup>) transgenic FVB/N mice were purchased from Caliper Life Sciences, Alameda, CA, USA. All mice were housed individually and in a conventional environment (temperature 21°C, 12h light: 12h darkness, humidity 50%) in a dedicated animal holding facility. They were fed a standard non-sterile pellet diet and tap water ad libitum. Mice were allowed  $\geq 2$  wk to acclimatize before entering the study. All animal procedures were performed according to national ethical guidelines.

### **Colitis Induction and Assessment: DSS model**

For the bioluminescence imaging studies, acute colitis was induced in the recipient wild-type FVB/N mice by administering 4% DSS (47 kDa, TdB Consultancy, Uppsala, Sweden) in drinking water. The mice were exposed to DSS for 5 days followed by one day on tap water. DSS was changed once during the 5 days. Disease progression was assessed by monitoring body weight loss, stool consistency (0=normal, well-formed pellets, 1=changed formed pellets, 2=loose stool, 3=diarrhoea) and fur texture/posture (0=smooth coat/ not hunched, 1=mildly scruffy/mildly hunched, 2=very scruffy/very hunched) which were recorded to generate a daily disease activity index (DDAI). Distal colonic tissue samples were collected, weighed and homogenized in 50ml PBS + 2 protease inhibitor cocktail tablets (Roche, UK) + 10% FCS (Gibco, UK). Homogenates were centrifuged for 12 minutes at 14,000 RPM at 4°C. Chemokine and cytokine levels were measured in the

supernatants using a Meso Scale Discovery (MSD) 96-well mouse pro-inflammatory 7 plex kit and the electro-chemiluminescent multiplex system Sector 2400 imager (Meso Scale Discovery, Gaithersburg, MD, USA), as per manufacturers instructions.

### **Induction of peritonitis and isolation of luc<sup>+</sup> donor neutrophils**

Peritoneal exudate cells are primed, highly chemotactic and more functionally responsive in comparison to blood PMN leukocytes [20]. Thus, we chose to isolate these cells for both the *in vitro* and *in vivo* studies. Localized inflammation was induced in the peritoneal cavity of mice by intraperitoneal (i.p) injection of 4% thioglycollate (Difco, Detroit, MI, USA) broth that had been previously autoclaved and stored at 4°C. Approximately 12 hours later, a peritoneal lavage was performed on the mice following sacrifice by decapitation. Briefly, 5ml of harvest medium {1x sterile Ca<sup>2+</sup>/ Mg<sup>2+</sup>- free PBS (pH 7.2, Gibco) supplemented with 0.02% EDTA (Sigma-Aldrich Ireland) and 0.5% heat-inactivated FCS (Gibco)}, was injected into the peritoneal cavity. The peritoneal wall was then gently massaged and the fluid withdrawn. This was repeated twice with 80-90% recovery of the lavage fluid. The lavage fluid was pooled and centrifuged at 300 x g for 10 minutes at 25°C to recover leukocytes. The lavage solution was washed twice by re-suspending in 10ml sterile PBS (Gibco) and centrifuging at 300 x g for 10 minutes. Leukocytes were counted using a haemocytometer. Approximately 5 x 10<sup>6</sup> cells per mouse were harvested.

### **Phenotypic Characterization of the peritoneal exudate using FACS analysis**

Peritoneal exudate cells from three wild-type FVB/N mice were isolated and pooled as described above and re-suspended at 1 x 10<sup>6</sup> cells/ml. To this cell suspension; 50µL of each mAb dye mix was added with incubation in the dark at 4°C for 30 minutes.

The mAbs used for flow cytometry included; anti-CD11c (IgG1) phycoerythrin cyanine dye 7 (PE-Cy7) HL3, anti-Ly6G (IgG2b) PE RB6-8C5, anti-CD4 (IgG2a) PE RM4-5, anti-CD49b (IgM) fluorescein isothiocyanate (FITC) DX5 (all from BD Pharmingen, UK), anti-F4/80 (IgG2b) TRI-COLOR BM8 (Caltag, UK), anti-CD8 (IgG1) PE, anti-CD3 (IgG2B) FITC, anti-CXCR2 (IgG2a) allophycocyanin (APC) (R&D Systems, UK), and anti-B220 (IgG2a) Alexafluor (AF) 700 RA3-6B2 (Serotec, UK). For analysis of activation marker expression the mAbs used were anti-CD11b (IgG2b) FITC MI/70 and anti-CD69 (IgG1) PE-Cy7 H1.2F3 (BD Pharmingen). Following staining, the cells were washed twice with blocking buffer {PBS + 1% bovine serum albumin (BSA, Sigma-Aldrich) + 1% rat serum (Sigma-Aldrich) + 1% hamster serum (Sigma-Aldrich) + 1% mouse serum (Dako Diagnostics, Ireland) + 0.1% sodium azide (Sigma-Aldrich)} and fixed in 3% formalin for analysis. Relative fluorescence intensities were measured using a LSRII cytometer and BD Diva software (Becton Dickinson, UK). For each sample 20,000 events were recorded. The percentage of cells labeled with each mAb was calculated in comparison with cells stained with isotype control antibody. Background staining was controlled by labeled isotype controls (BD Biosciences, Caltag and Serotec) and FMO (fluorescence minus one). The results represent the percentage of positively stained cells in the total cell population exceeding the background staining signal.

### **Transwell™ in vitro chemotaxis assay**

To analyze the functional migration activity of the peritoneal exudate cells towards recombinant KC in the presence or absence of an anti-KC antibody, a 96-well Neuroprobe ChemoTx Chemotaxis plate (Receptor Technologies, Adderbury, UK) with 5µm pore polycarbonate filters was used, as previously described [21]. Peritoneal exudates from wild-type FVB/N mice were obtained by peritoneal lavage



12 hours post 4% thioglycollate injection and re-suspended at a concentration of  $8 \times 10^6$  cells/ml in serum-free RPMI 1640 media. The chemotactic factor; murine recombinant mrKC (Peprotech, UK), diluted in serum-free RPMI-1640 to 10ng/ml and with or without 0.1  $\mu\text{g/ml}$  or 10 $\mu\text{g/ml}$  monoclonal anti-mouse CXCL1/KC antibody (R&D Systems), was added to the lower chamber. Following placement of the filter membrane over the lower wells, 25 $\mu\text{l}$  cells ( $2 \times 10^5$ ) were added to the upper chamber of each well. The plate was incubated for 4 hours at 37°C with 5% CO<sub>2</sub>. Inserts were removed and the number of neutrophils that migrated into the bottom chamber was determined by counting using a haemocytometer and trypan blue. For each experiment, the % migration after subtraction of the control (RPMI medium alone) was given for KC alone (no anti-KC) and for two concentrations of anti-KC antibody.

### **Bioluminescence Neutrophil Trafficking Model**

To establish an efficient model to track and quantify neutrophil migration, we developed a neutrophil trafficking model using a *luc*<sup>+</sup> transgenic donor mouse line in conjunction with bioluminescent imaging (Fig. 1). Expression of the luciferase reporter gene is detectable in all tissues including white blood cells of the transgenic  $\beta$ -actin-*luc*<sup>+</sup> mice. It has been demonstrated that *luc*<sup>+</sup> cells emit visible light photons that penetrate tissues and are externally and quantitatively detectable with high sensitivity [22]. Thus,  $4 \times 10^6$  *luc*<sup>+</sup> donor neutrophils were adoptively transferred intravenously (i.v.) via tail-vein of wild-type FVB/N recipients with DSS-induced colitis. Naïve wild-type FVB/N mice with or without transferred *luc*<sup>+</sup> donor neutrophils were included as appropriate control groups. Bioluminescence imaging was performed, as previously described [23], using an *IVIS* 100 charge-coupled

device (CCD) imaging system (Xenogen, Alameda, CA.) at 2, 4, 16-22 h post adoptive cell transfer. Briefly, the recipient mice were injected i.p. with the exogenous substrate D-Luciferin (120mg/kg bodyweight) (BioThema AB, Sweden), following gaseous anesthesia with isoflurane and transferred to the imaging chamber. Emission images were collected with 2 minute integration times. Following the whole-body bioluminescent imaging, the mice were injected with an additional dose of D-luciferin. Five minutes later, the mice were sacrificed and the organs were removed and imaged for 2 minutes. Bioluminescent signal was quantified by creation of regions of interest (ROIs). To standardize the data, light emission was quantified from the same surface area (ROI) for each organ type. In addition, background light emission, taken from ROIs created on organs of non-recipient non-DSS control animals, was subtracted from test organs. Imaging data was analyzed and quantified with Living Image Software (Xenogen) and expressed as photons/second/cm<sup>2</sup>.

### **Use of the model to show inhibition of neutrophil infiltration into inflamed tissue by anti-KC**

DSS recipient mice (3 and 5 respectively) received purified isotype control rat IgG2ak (BD Pharmingen) or a monoclonal rat anti-mouse CXCL1/KC antibody (R&D Systems) at a concentration of 20µg / mouse i.p., 1 hour pre-adoptive transfer of the *luc*<sup>+</sup> peritoneal neutrophils. Four hours, post adoptive cell transfer, whole-body and *ex vivo* bioluminescent organ imaging was carried out in the same manner as described above.

## **Statistics**

Data are expressed as means  $\pm$  SEM unless otherwise stated. Statistical significance was determined with the unpaired Student's t-test using commercially available statistic software (GraphPad Software, San Diego, CA.). P values  $<0.05$  were considered as statistically significant (\* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ ).

## Results

### Phenotypic characterization of the peritoneal exudate

To determine neutrophil purity and the overall phenotype profile of the peritoneal exudate cells 12 h post thioglycollate-induction of peritonitis, immunofluorescence flow cytometry was performed. The data revealed a neutrophil purity of 80%, i.e. LY6G<sup>+</sup> cells (Fig. 1a), with clear expression of the activation molecule CD69 on these neutrophils as shown by mean fluorescence intensity (Fig. 1b). CXCR2, the major receptor for human IL-8 and the murine homologs KC and MIP-2, was expressed on 39% of the neutrophils (Fig. 1c). The remaining 20% of the exudate consisted of 10% T lymphocytes (CD3<sup>+</sup>) and the rest being macrophages (F4/80<sup>+</sup>), NK cells (DX5<sup>+</sup>), dendritic cells (CD11c<sup>+</sup>) and B cells (B220<sup>+</sup>) (data not shown). Thus, the high percentage of activated neutrophils in the peritoneal exudate population demonstrates that these are suitable for adoptive transfer and neutrophil trafficking studies.

### Recombinant KC-stimulated neutrophil chemotaxis *in vitro* is reduced in the presence of anti-KC

The chemotactic capability of the peritoneal exudate cells towards mrKC and the ability of an anti-KC antibody to inhibit this chemotaxis was examined *in vitro* using a Transwell system. Addition of mrKC to the bottom chamber of a 96-well Neuroprobe Chemotx plate induced mobilization of peritoneal exudate neutrophils from the upper chamber. This migration was reduced by two different concentrations of anti-KC. In the presence of mrKC, there was an 8% increase in % neutrophil transmigration compared to the RPMI medium control and this value was decreased to 2.8% and 1.5% by 0.1µg/ml and 10µg/ml anti-KC, respectively (Fig. 1d). This chemotaxis assay confirmed the suitability of the peritoneal exudate cells for adoptive

transfer. Neutrophil migration towards recombinant Macrophage Inflammatory Protein-2 (MIP-2) instead of mrKC was also tested with similar results (data not shown).

### **Kinetic analysis of neutrophil trafficking in DSS-induced colitis**

In the absence of inflammation, neutrophils (activated and responsive to KC) did not migrate to the colons of naïve mice indicating the necessity for localized gastrointestinal inflammation (Figures 4 and 5). Acute DSS colitis was, therefore, induced in recipient mice. Inflammation was confirmed by assessing body weight change and total daily disease activity index (DDAI) (Fig. 2a-b) and by the significant increases in levels of KC and the pro-inflammatory cytokines IL-1 $\beta$  and IL-6 in the distal colons of the DSS mice as previously reported [15, 24] (Fig. 2c). In addition, we and others have provided both histological and MPO data confirming the colonic tissue damage caused by DSS administration [25-29]. Following induction of colitis, the temporal recruitment of neutrophils in living animals was analyzed by performing whole-body and *ex vivo* organ bioluminescence imaging at 2, 4, and 16-22 h following adoptive transfer of *luc*<sup>+</sup> peritoneal exudate cells. Whole-body imaging confirmed presence of transferred viable neutrophils in recipient mice at all time points (data not shown). At the early time points of 2 and 4 h post adoptive cell transfer, *ex vivo* imaging of organs revealed high neutrophil infiltration, as measured by bioluminescent signal, in the lungs, spleens and livers of recipient DSS mice (Fig. 3 c, d and e). The neutrophil signal in the colon was increased by 93% at 4 h compared to 2 h (Fig. 4a). At the later time point of 16-22 h, neutrophil presence in the colon remained high (Fig. 3a) but had decreased in the spleen, liver and lungs (Fig. 3c, d and e). Thus, the data show a robust signal in the inflamed colon at all time

points post cell transfer. There was no evidence of neutrophil recruitment to the small intestines of DSS recipient mice at any of the time points studied (data not shown).

### **Effects of KC blockade on neutrophil influx during DSS-induced colitis**

To illustrate the potential of the bioluminescence neutrophil trafficking model, we assessed the effect of a chemokine blocking antibody, anti-KC. Four hours post adoptive transfer of *luc*<sup>+</sup> neutrophils from transgenic donors, a clear bioluminescent signal was apparent in the whole-body images of all the recipient DSS mice and of the naïve control mice in contrast to the non-recipient non-DSS control, specifically in the upper part of the body and in the inguinal lymph nodes (Fig. 4a). These images confirm that the recipient mice received viable luciferase-expressing cells that can be detected *in vivo*. However, since some attenuation of optical signal is expected to occur with tissue depth, *ex vivo* imaging of the organs is necessary for accurate visualization and quantitation of neutrophil localization.

*Ex vivo* imaging of the organs revealed high neutrophil presence (i.e. bioluminescent signal) in the spleens and lungs of the IgG control-treated and anti-KC-treated DSS recipients, confirming our observations from the whole-body imaging. There was no significant increase or decrease in neutrophil recruitment to liver, spleen or lungs in the anti-KC treated group compared to the IgG control-treated group [Fig. 5b (i) and (ii)]. However, a significant reduction in the signal from the colons of the DSS-recipients that were treated with anti-KC compared to the IgG control-treated recipients was observed (Fig. 4b and Fig. 5a). Similar to the kinetic study, no bioluminescence signal was evident in the small intestines of both IgG control-treated and anti-KC treated groups (data not shown). In the naïve recipients, i.e. the control group, there was significantly higher localization of neutrophils in the

liver, spleen and lungs compared to the DSS recipient mice [Fig. 5b (i) and (ii)]. However, in contrast to the DSS recipients, there was no bioluminescence signal evident in the naïve colons (Fig. 5a).

## Discussion

In both human and experimental IBD, PMN invasion of the intestinal lamina propria and crypts correlates with tissue damage and clinical symptoms, suggesting that targeting neutrophil recruitment is a viable therapeutic strategy for IBD. This study presents a robust model to analyze the biology of neutrophil trafficking that can also be used in pre-clinical studies to evaluate new therapeutic compounds specifically aimed at blocking neutrophil recruitment. The first step in developing the model was to characterize the purity and functional properties of the neutrophil population from thioglycollate -induced peritonitis. Phenotypic analysis of the peritoneal exudate isolated 12 hours post i.p. administration of thioglycollate, revealed 80% neutrophil purity. In addition, the cells were activated and functionally responsive to recombinant KC *in vitro* and their chemotaxis was inhibited by the presence of an anti-KC antibody. These results showed that the post-thioglycollate peritoneal exudate population of neutrophils was appropriate for the adoptive transfer model.

Bioluminescence imaging of whole-body and *ex vivo* organs was used to track and quantify neutrophil trafficking following adoptive transfer of *luc*<sup>+</sup> peritoneal exudate cells from transgenic donors. This is a non-invasive technology allowing real-time detection of tagged cells *in vivo* using CCD cameras due to the detection of visible light produced by luciferase-catalyzed reactions [30]. In contrast to other imaging modalities such as Positron Emission Tomography (PET), Single photon emission computed tomography (SPECT) and Magnetic resonance imaging (MRI); bioluminescence imaging is less complicated, less labor intensive and relatively low cost while still providing quantitative, spatial and temporal data. In addition, bioluminescence overcomes the problems commonly encountered with using



fluorescent labels such as CFSE and GFP, namely the exponentially decreasing light intensity with tissue depth and the limited sensitivity and specificity as a result of endogenous tissue autofluorescence [31, 32]. So far, bioluminescence has been used to monitor infection progression, transgene expression, tumor growth and metastasis, transplantation, toxicology and gene therapy [30]. In the context of cell tracking, Sheikh et al., successfully used bioluminescence imaging to track bone marrow mononuclear cell homing in ischemic myocardium [33], while Costa et al., used a retroviral vector containing luciferase and GFP to illuminate the migratory patterns of CD4<sup>+</sup> T cells in a mouse model of multiple sclerosis [34]. These and other studies have shown robust correlation between cell numbers and bioluminescence signals [23]. The results of the present study demonstrated that the adoptively transferred neutrophils preferentially migrated to the diseased sites in the recipient animals with DSS-induced colitis, with high infiltration of the colon at all time points investigated. In contrast, high transit through the lungs and spleen was evident at early time points following cell transfer but declined at the later time point. This is probably due to re-direction of the transferred neutrophils to the inflamed colon with return to basal conditions in these organs. Since the route of administration of the donor cells was intravenous (i.v.), neutrophil localization to the lungs, liver and spleen of the recipient mice reflects the natural route of circulation. In fact, it is possible that the higher neutrophil presence in the inflamed colon at the later time points of 4h and 16-22h compared to 2h post adoptive transfer of cells is due to the fact that a recovery time of at least 2 hours is necessary to allow transferred cells to equilibrate in the circulation following i.v. administration. There was significantly higher neutrophil presence in the lungs, liver and spleen of the naïve recipients compared to in the DSS recipients which was most likely due to the absence of gut inflammation. Similar findings have

been noted in previous studies where neutrophil presence in the spleen declined in patients with severe inflammatory disease compared to normal subjects. The explanation for this being that the pooled cells had been re-directed to inflammatory foci [35, 36]. In addition, we investigated the utility of the bioluminescence model as a tool to dissect the biology of and test new drugs that target neutrophil migration using a blocking antibody against KC. Significant inhibition of neutrophil recruitment to the inflamed colons of the anti-KC treated mice compared to IgG control-treated was clearly evident using this system. Interestingly, it has been reported that treatment of mice with TNBS-induced colitis with anti-KC ameliorated disease by reducing neutrophil migration and MPO [37].

The bioluminescence model presented here has definite and distinct advantages over other *ex vivo* techniques used to track neutrophil recruitment. First and foremost, the necessity for pre-labeling of cells is removed as the donor cells used constitutively express luciferase. This eliminates the common problems associated with cell-labeling such as alteration of cellular functions, variable labeling efficiency and elution of label from the cells of interest. In addition, our technique allows direct *ex vivo* visualization without any need for further processing of the tissues, in contrast to immunohistochemistry and MPO analysis. Histology is labor intensive and tedious while MPO assays can be problematic and do not distinguish between neutrophils and macrophages.

In conclusion, this study presents a robust model to track neutrophil recruitment which can be used to complement other available methods traditionally used for tracking neutrophils. In addition to experimental models of IBD, this versatile

technique will be useful for monitoring neutrophil trafficking during inflammatory responses in a range of disease settings and constitutes a novel approach for the assessment of potential therapeutics that aim to reduce neutrophil infiltration. Thus it can be used as an informative and specific tool for both the pharmaceutical industry and the basic research community.

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## Figure Legend

**Figure 1.** Phenotypic and functional characterization of the peritoneal exudate population. Cells were isolated from the peritoneum of FVB/N mice 12 h after i.p. injection with thioglycollate. For the phenotypic analysis, single cell suspensions were stained with flurochrome-labeled mAbs and analyzed by flow cytometry in which 20,000 events were recorded. (a) Percentage of neutrophils (i.e. Ly-6G<sup>+</sup> cells, grey area) present in the peritoneal exudate compared to isotype control antibody (black area). (b) Activation status of the neutrophils using anti-CD69 mAb (grey area) or irrelevant control antibody (black area). (c) Percentage of the LY-6G<sup>+</sup> neutrophils that were CXCR2<sup>+</sup>. Plots shown are representative of five separate experiments, and the mean values are indicated. (d) *In vitro* chemotaxis assay in which migrated cells from triplicate wells of the lower chambers were pooled and counted in trypan blue. For each experiment, the % migration after subtraction of the control (medium alone) was given for KC (alone, no anti-KC) and for two concentrations of anti-KC antibody (0.1 μg/ml and 10 μg/ml), 4 hr post incubation at 37°C. Plots shown are representative of three separate experiments using pools of 3-6 mice; \*p ≤ 0.05, \*\*p < 0.01.

**Figure 2.** Inflammation markers in DSS-induced colitis in FVB/N mice. (a) Mean % body weight changes in wild type FVB/N mice from day 0 of treatment with 4% (w/v) DSS in drinking water for 5 days followed by 1 day of water. (b) Stool consistency and fur texture/posture were used to generate a daily disease activity index (DDAI). DSS-treated groups n = 4 and non-DSS control n = 1. (c) Treatment with DSS significantly increased KC levels and levels of the pro-inflammatory cytokines IL-1β and IL-6; in the distal colons of the mice. n = 5-6 per group; \*\*p < 0.01, \*\*\*p < 0.001.



**Figure 3.** Kinetic analysis of neutrophil trafficking *in vivo*. Bioluminescent signal in organs dissected from DSS recipient mice at 2, 4 and 16- 22 h post adoptive transfer with *luc*<sup>+</sup> exudate cells. Neutrophil trafficking kinetics to (a) colon, (b) mesenteric lymph nodes (MLNs), (c) spleen, (d) liver and (e) lungs are shown. DSS-treated groups n = 5-8.

**Figure 4.** Effect of anti-mouse KC on neutrophil trafficking. (a). Representative whole body bioluminescence images of wt FVB/N recipients with DSS-induced colitis treated with isotype control rat IgG2a or anti-KC antibody, 4 hours post adoptive transfer with *luc*<sup>+</sup> cells. Non-DSS/naïve recipients were also included as a control group.

(b) *Ex vivo* images of colon of (i) non-DSS non-recipient negative control (ii) non-DSS/naïve recipient mouse and DSS recipient mice treated with (iii) IgG control or (iv) anti-KC (20µg/mouse). Non-DSS/naïve recipient, non-DSS- treated and DSS-treated groups n = 3-5.

**Figure 5.** Anti-KC administration markedly reduces neutrophil trafficking to the colons of mice with DSS-induced colitis. Summary of bioluminescent signal in (a) colons and mesenteric lymph nodes (MLNs) and b (i) liver and spleen and b (ii) lungs dissected from naïve controls and DSS mice treated with IgG control or anti-KC antibody, 4 hours post adoptive transfer with *luc*<sup>+</sup> cells. **NB:** Mean signal from the colons of naïve recipients was below the background light emission from the non-recipient control colons.  $p \leq 0.05$ ; n = 3-5 per group.

Figure 1

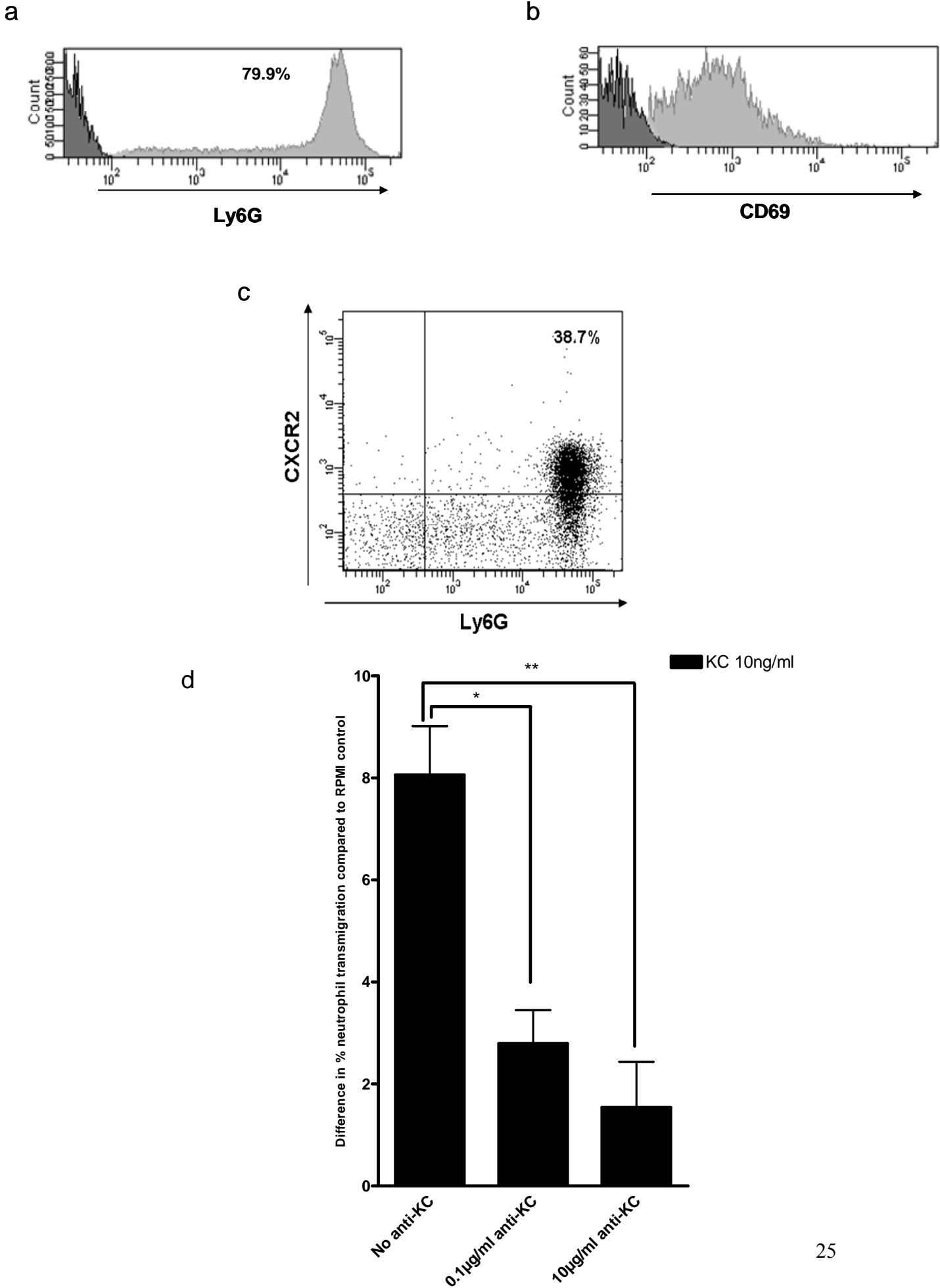
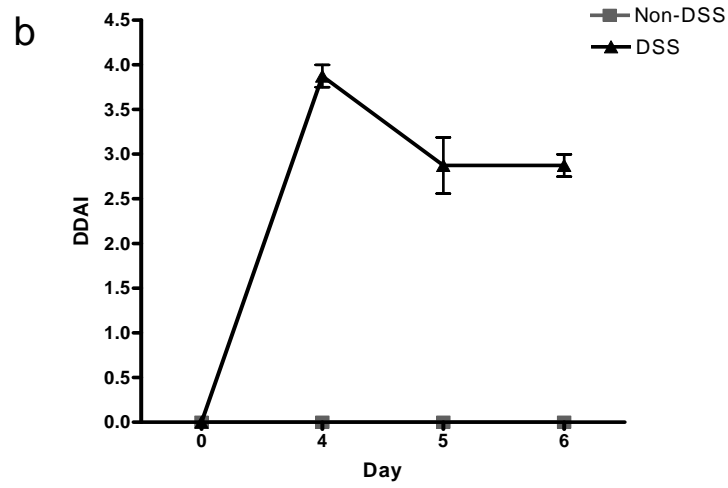
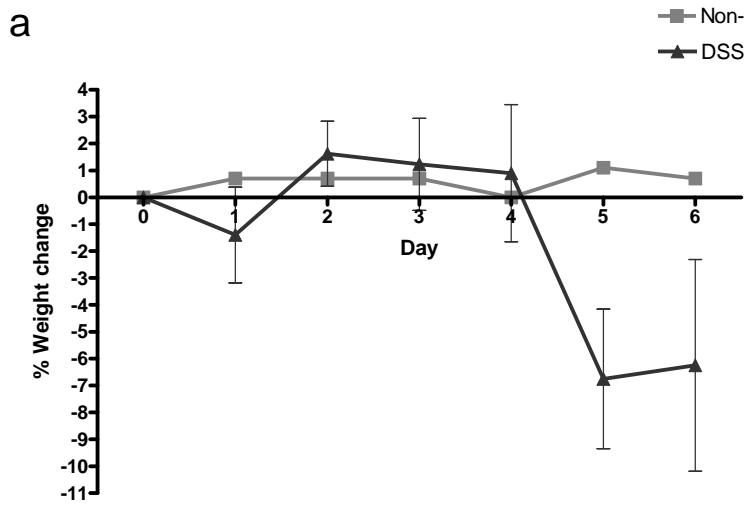


Figure 2



**c**

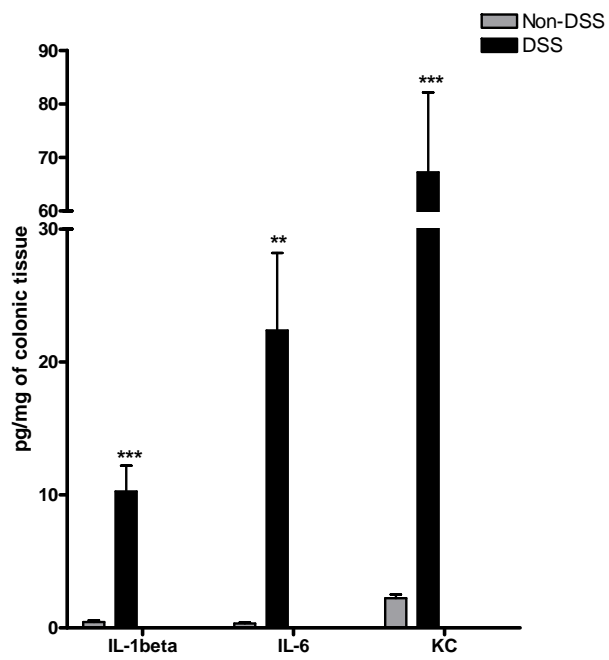


Figure 3

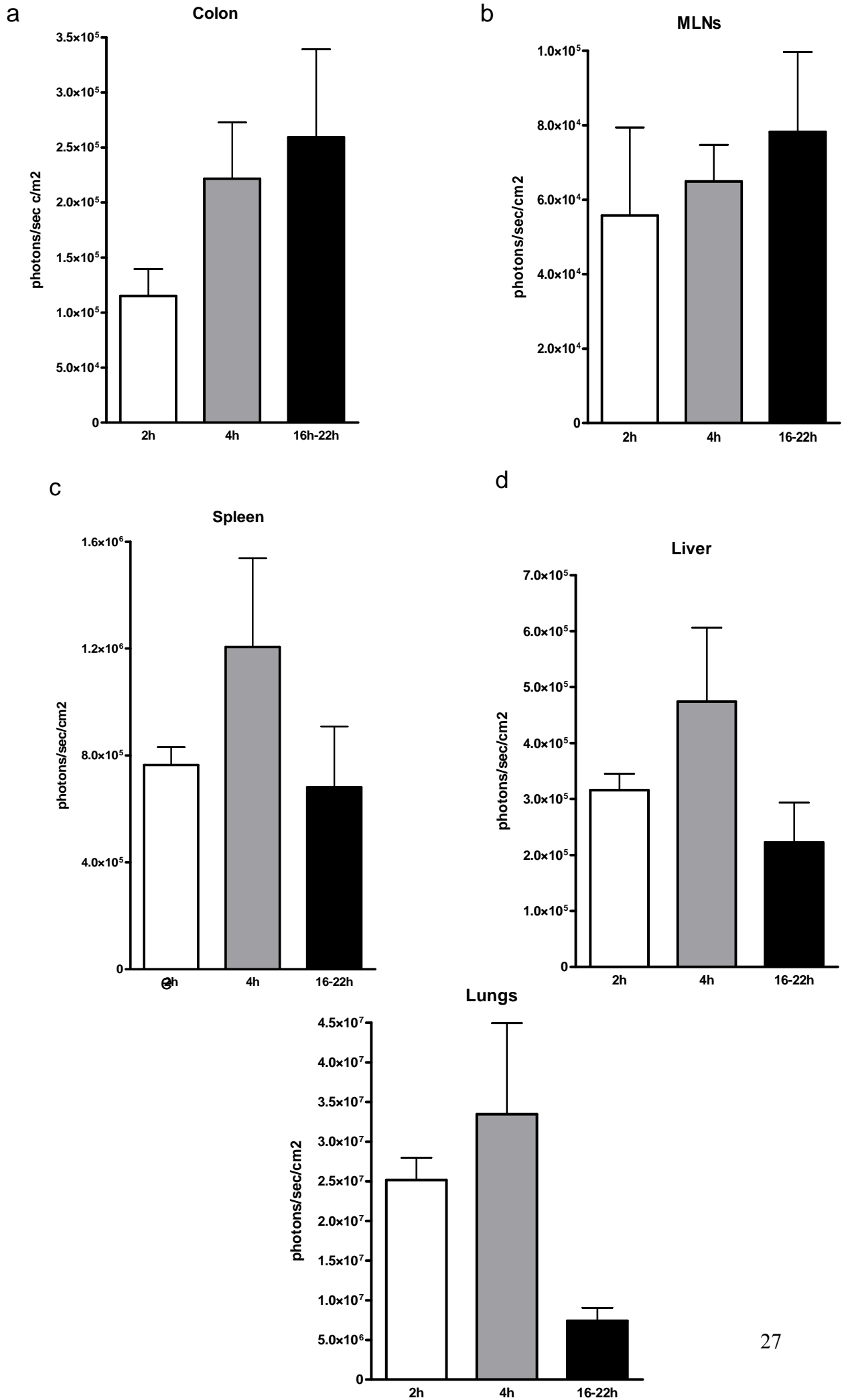
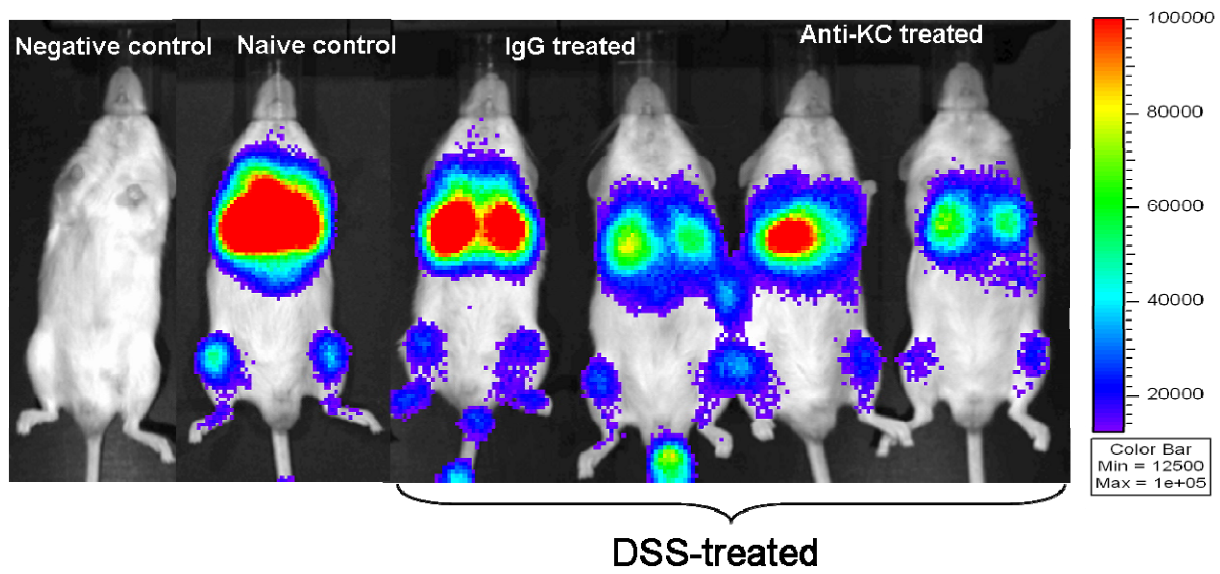


Figure 4

a



b

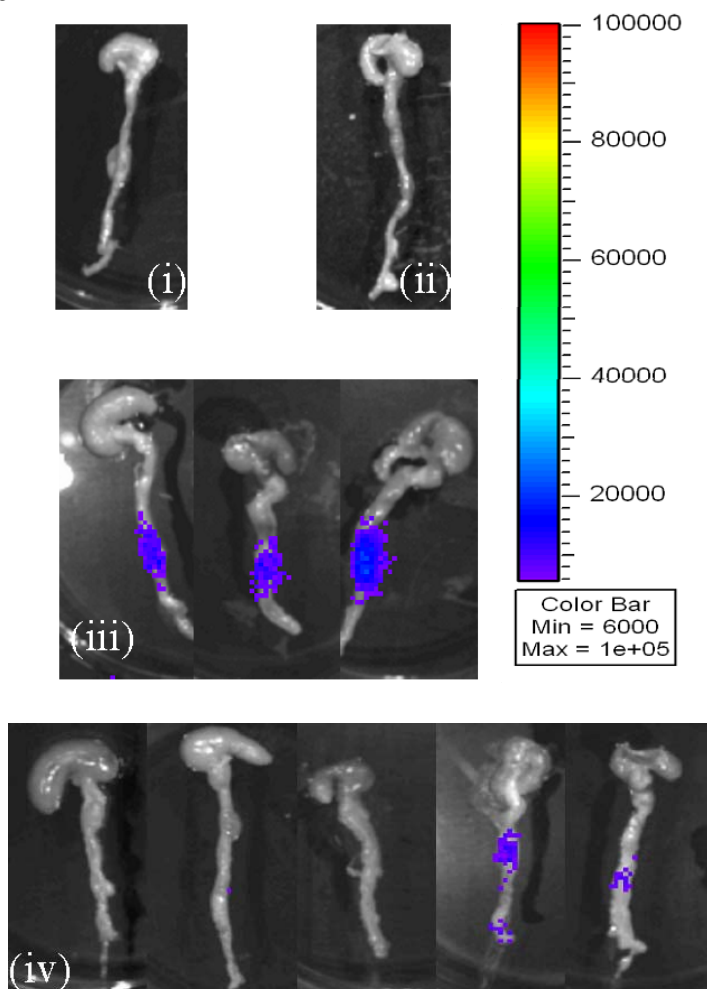
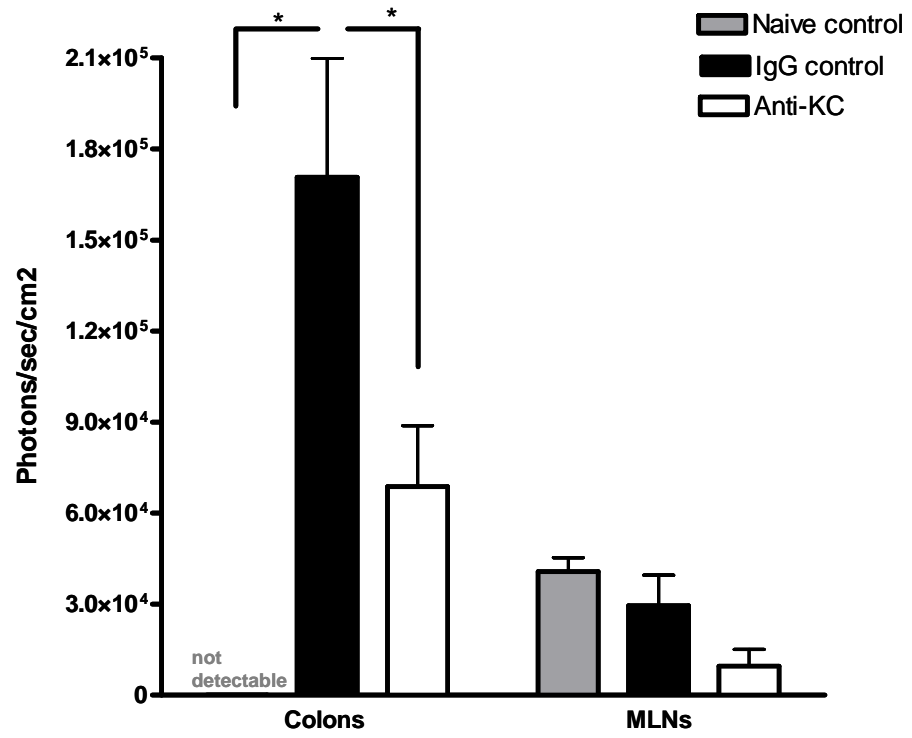


Figure 5

a



b

