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In vivo imaging of mucosal CD4⁺ T cells using single photon emission computed tomography in a murine model of colitis

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Abstract

Immune responses that occur in the context of human infectious and inflammatory diseases are usually studied by sampling cells from peripheral blood, from biopsies, or by end-point harvests at necropsy. These approaches are likely to yield information that is incomplete and/or non-representative. Here, we report the development and validation of a non-invasive method to localize and to quantitate the disposition of specific subpopulations of cells in vivo. In a murine model of dextran sulfate sodium (DSS)-induced colitis, CD4⁺ T cells were visualized in the colon by single photon emission computed tomography (SPECT-CT) after injection of monoclonal, non-depleting, indium-111 (¹¹¹In) labeled anti-CD4⁺ antibodies. The SPECT-CT colon uptake ratio (CUR) was found to correlate ($p < 0.01$) with the number of total CD4⁺ T cells and with standard measures of pathology (colon length, cell counts, and histopathologic evidence of apoptosis, edema, and cellular infiltrates) as assessed by direct examination of diseased colon. Each of these parameters, including the SPECT-CT signal uptake, increased as a function of DSS dose ($p < 0.05$). We conclude that CT-SPECT imaging using an ¹¹¹In-labeled anti-CD4⁺ antibody is reflective of traditional parameters of pathology in this experimental model of murine colitis. This approach should be readily applicable to the imaging of discrete cell subpopulations in non-human primates and in humans, thus augmenting our understanding of infectious diseases and inflammation in vivo.

Keywords

CD4⁺ T cell; SPECT imaging; Murine colitis; Non-invasive imaging

1. Introduction

Physiologic and pathophysiologic events that occur at the intestinal mucosal interface are clearly important but only poorly understood. By example, inflammatory bowel diseases (IBD), such as ulcerative colitis and Crohn's disease, are disorders characterized by

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inflammation of the gastrointestinal tract (Hue et al., 2006; Xavier and Podolsky, 2007). Previous studies report that the pathogenesis of these disorders involves factors that are immune, genetic, and environmental, particularly microbial (Doganci et al., 2005; Drakes et al., 2005; Fichtner-Feigl et al., 2005; Gad, 2005; Danese et al., 2006; Hue et al., 2006; Xavier and Podolsky, 2007). Yet, even as our understanding of the molecular and genetic basis of these diseases has expanded, our knowledge about the role of the adaptive immune response in the context of IBD remains limited.

Immune responses in vivo are usually studied by periodic sampling of cells from the peripheral blood or lymphoid organs, or by end-point harvests of discrete organs at necropsy. These approaches are informative but suboptimal: only a fraction of total lymphocytes is circulating at any one time (Haase, 1999), cell yields may be non-representative (Lumadue et al., 1998), and longitudinal analyses, particularly of biopsies, are difficult to perform. Such limitations are perhaps most problematic when attempting to analyze immune responses that occur in the gastrointestinal tract, an anatomically complex organ system that is difficult to sample in a repeated and comprehensive fashion. Accordingly, our knowledge about the disposition and composition of mucosal immune responses (e.g., in the setting of inflammatory bowel disease or lentiviral infection) remains largely inferential.

To gain a more comprehensive view of events in vivo, mucosal immunopathology has been imaged by a number of non-invasive approaches, including transabdominal ultrasound, computed tomography enterography, magnetic resonance enterography, and positron emission tomography (de Lima Ramos et al., 1998; Rubini et al., 2001; Albert et al., 2002; Lemberg et al., 2005; Seiderer et al., 2005; Loffler et al., 2006). Each of these methods has its own advantages and disadvantages. Some (e.g., computed tomography) require exposure to radiation but are more readily available, while others (e.g., transabdominal ultrasound) are prone to operator error while remaining cost effective. None, however, is amenable to the localization and enumeration of specific subpopulations of lymphocytes in vivo.

Many small animal models of IBD have been investigated (Elson et al., 1995; Dohi et al., 2000; Takahashi et al., 2002; Dresner-Pollak et al., 2004; Van der Sluis et al., 2006; Elson et al., 2007). The dextran sulfate sodium (DSS) model is a chemically induced colitis that causes epithelial damage and recruitment of innate inflammatory cells (Stevceva et al., 2001). Although previous studies indicate that the acute inflammation is caused by macrophages and neutrophils, these studies have been limited to tracking and quantitating innate immune cells and have not clearly delineated the role of adaptive immune cells. Recent studies have indicated that CD4⁺ T cells become activated in the periphery in this model and home to the colon during the first 3 to 7 days of a DSS-induced colitis. Therefore, it is likely that the absolute number of mucosal CD4⁺ T cells increases during acute administration of DSS (Sund et al., 2005; Da Silva et al., 2006).

Single photon emission computed tomography (SPECT) technology has been utilized to assess the degree of colitis in animal models of IBD as well as clinically in human patients with intestinal inflammation (Bennink et al., 2004; van Montfrans et al., 2004; Bennink et al., 2005). SPECT is a useful modality to assess the degree of inflammation because of its non-invasive nature. Small animal SPECT can image radioactive distribution in vivo with a resolution that is on the order of millimeters. With the advent of combined SPECT and computed tomography (SPECT-CT) technology, overlay images have added anatomic correlations to the functional images produced by SPECT. Past experiments have indicated that SPECT-CT imaging may be a useful modality in a DSS model of colitis. In these studies, however, radiolabeled neutrophils or WBCs were administered, precluding the ability to localize and enumerate a specific subpopulation of cells (i.e., CD4⁺ T cells) (Weldon et al., 1996; Charron et al., 1998; Biancone et al., 2005).

We have chosen to address this problem with a non invasive radiographic method that can be used to label, localize, and quantitate cell subpopulations of interest in vivo. We show here that it is possible to use SPECT-CT to visualize mucosal CD4⁺ T cells in mice after infusion of a monoclonal, non-depleting, anti-CD4⁺ antibody radiolabeled with indium-111 (¹¹¹I). When a chemical colitis is induced by dextran sodium sulfate (DSS), the signal provided by SPECT-CT correlates well with traditional parameters of disease severity, including histopathology, cell count, and colon length. This approach should be readily transferable to other murine models of inflammation and infection. We anticipate that it will also find applications in the analysis of IBD and lentiviral infections in non-human primates and in humans.

2. Results and discussion

2.1. Dose-dependent colitis induced by DSS

Oral administration of dextran sulfate sodium results in colonic mucosal epithelial damage, leading to an influx of macrophages and neutrophils with consequential bacterial invasion and ultimate lymphocyte activation and cytokine secretion (Elson et al., 1995; Da Silva et al., 2006). We chose this murine model of human inflammatory bowel disease (over, for instance, murine models such as the IL-10^{-/-} mouse) because it was more amenable to a dose-response analysis of SPECT-CT imaging (Tsuchiya et al., 2003). Fig. 1 shows the results of a pilot experiment in which groups of mice ($n = 3$ each) were provided 1–9% DSS in their drinking water for varying periods of time. The number of lymphocytes and of CD4⁺ T cells in the colon of treated mice reached a plateau at a dose of 5% DSS (Fig. 1A). When mice were treated with this dose of DSS for 3–15 days, a 9-day treatment period was associated with a tolerable degree of weight loss (7.3%) (Fig. 1B). In subsequent experiments, 0–5% DSS was accordingly administered for 9 days, so that SPECT-CT imaging could be evaluated across a spectrum of disease severity.

2.2. 5% DSS mice develop severe colitis

Established parameters of colitis were evaluated in groups of mice ($n=4-7$ each) treated with 0%, 3%, or 5% DSS for 9 days. As shown in Fig. 2A, colon lengths were shorter in mice treated with 5% DSS mice compared to those treated with either 3% or 0% DSS ($p<0.01$). At the same time, significant differences were also observed in the total lymphocyte counts (Fig. 2B) and in the absolute CD4⁺ T cell counts (Fig. 2C) between the 3% and 5% DSS groups and between the control and the 5% DSS groups ($p<0.01$ for all differences except those between 0–3% and 3–5% DSS in the yields of CD4⁺ T cells, where instead $p<0.05$). Histologically, and as expected, the DSS-induced colitis in these animals was associated with edema, inflammatory cell infiltration, and mucosal thickening with destruction of epithelial cells, and a significant difference ($p<0.05$) in total histopathologic score was observed between all three groups. Representative examples of edema, cellular infiltration, and apoptosis (observed in the 5% DSS group) are shown in Fig. 3A. These three parameters scored higher in each of the 7 mice in the 5% DSS group and contributed to their overall higher histopathologic score (Fig. 3B) ($p<0.01$, comparing the 5% DSS to the control group not treated with DSS).

To directly localize the injected rat anti-mouse CD4 antibodies, sections were counterstained with a rabbit anti-rat Ig antibody and an Alexa Fluor 555-conjugated anti-rabbit Ig antibody. As shown in Fig. 3C, sections from rat intestine (endowed with a rich representation of surface Ig⁺ B cells) stained positively with the rabbit anti-rat Ig antibody (C-1), but such staining was not observed when the same antibody was applied to sections of untreated mouse intestine (C-2). Intestinal sections from a mouse given 5% DSS and then injected with the ¹¹¹In-labeled DOTA rat anti-mouse CD4 conjugate showed cell-surface staining (reflective of mouse CD4 expression) with the anti-rat antibody (C-3 and C-4, at low and high power, respectively).

2.3. CD4⁺ T cells can be visualized by SPECT-CT

To determine whether the above changes could be detected by SPECT-CT, ¹¹¹In-labeled DOTA rat anti-mouse CD4 conjugates were injected in vivo into mice with varying degrees of DSS-induced colitis. A representative example of the overlay produced by CT followed by SPECT scanning is shown in Fig. 4A. In this example, a mouse provided with 5% DSS was imaged. Sagittal images from this animal are shown and regions of specific antibody uptake are graded by color with red indicating maximum uptake. To determine which organs harbored radiolabeled antibody, portions of liver, colon, kidney, and bladder were isolated and imaged ex vivo; all but the latter organ showed uptake of the labeled antibody (Fig. 4B). Since retroperitoneal structures such as kidneys were excluded from the region of interest visualized by SPECT-CT, this analysis indicated that the intraperitoneal activity was localized to bowel and not affected by bladder uptake. Fig. 4C compares coronal, axial, and sagittal SPECT-CT images from a control and a 5% DSS mouse. In both animals, the far superior region of red activity in the coronal and sagittal images corresponds with liver uptake. A 3-D composite rendering (Fig. 4D) was performed to assist in visualization of the colon. In these images, colons are displayed as the green structures in the three dimensional reconstruction. Uptake of radiolabeled anti-CD4 antibody is not evident in the bowels loops of control mice (top) but clearly evident in the bowel loops of mice treated with 5% DSS (bottom).

2.4. ROI quantitative image analysis

The amount of labeled antibody (measured in terms of microcuries/mg) found in the colons of each group is compared in Fig. 5A (left). As expected from the images of Fig. 4, colonic activity was greater with increasing doses of DSS ($p < 0.05$ between the 5% and the control groups). Fig. 5A (right) shows the results of the AMIDE software analysis of reconstructed SPECT-CT images for the colon uptake ratio (CUR), normalized as mean colon activity/mean muscle activity. In this case, statistically significant differences were observed between all groups ($p < 0.01$ between the 0 and 5% dose; $p < 0.05$ between the 0–3% and 3–5% doses). To correlate the degree and extent of colitis with SPECT-CT imaging, regression analyses were performed to relate the CUR to the colon length, total lymphocytes, histopathologic scores, and total CD4⁺ T cells (Fig. 5B). Except in the case of colon length ($p < 0.05$), all of these analyses revealed highly significant differences ($p < 0.01$ for total lymphocytes and $p < 0.001$ for histopathologic scores and CD4⁺ T cells).

These data demonstrate that SPECT-CT imaging can be used to localize and to quantitatively assess inflammation in a murine model of DSS-induced colitis. ¹¹¹In-labeled anti-CD4 antibodies localized to areas of colonic inflammation and correlated with the degree of pathology, as assessed by total cell counts, CD4⁺ T cell counts, and histopathology. Upon 3-D rendering, CD4⁺ T cell infiltrates could be observed and quantitated in the colon of the DSS-treated mice. This method now makes it possible to define and to quantify immune responses within this important organ on a non-invasive basis, an opportunity that we believe will complement and expand upon our knowledge of the physiology and pathophysiology of the immune system.

The SPECT-CT imaging procedure described here offers a novel and non-invasive method that may aid in the diagnosis of disease and in the quantitative analysis of disease progression, e.g., as a function of therapy or the lack thereof. In the research setting, a non-invasive quantification of CD4⁺ T cell infiltration could be used as a tool to select animals for therapeutic trials, since traditional parameters of inflammation (i.e., weight loss and bloody stools) may not accurately predict disease severity and pathologic involvement (Ohman et al., 2000). Ultimate translation into clinical studies may serve to provide guidance for directed endoscopic biopsy to regions of localized inflammation. It could also be applied to other clinical situations in which quantification and visualization of selected lymphocyte subpopulations in the

intestinal mucosa might be diagnostic, such as in the setting of HIV disease, or indicative of response to new medical therapies.

With further refinement and with the development of additional probes, we anticipate that this technique may be more generally applicable to the detection of other cell subpopulations and of additional markers of mucosal inflammation. If so, it might then be used in conjunction with clinical parameters and necropsy tissue to longitudinally follow colitis progression in small animals and non-invasively determine disease severity. Furthermore, we anticipate that this modality could be readily applied to the analysis of lymphocyte subpopulations within non-human primates and humans. In this manner, it may be possible to more clearly define changes that occur in the disposition, composition, and number of such cells in the context of inflammation and/or infection in vivo.

3. Materials and methods

3.1. Animals

Female BALB/c mice (14–20 weeks of age and weighing 18–20 g; Charles River) were housed and maintained by the UCSF Lab Animal Resource Center 1–3 weeks before the experiments began. Food and water were given as per standard protocol. Protocols for animal experiments were approved by of the Committee on Animal Research at the University of California, San Francisco.

3.2. Induction of colitis by DSS

In the first set of experiments, groups of mice ($n = 3$) were provided 1, 3, 5, 7, or 9% DSS in their drinking water for 10 days. Lymphocytes were isolated and counted per the protocol listed below at the end of 10 days. Once it was determined that the optimal dose of DSS for colitis was 5%, a second experiment was performed in which mice were divided into groups ($n = 3$) and given 5% DSS in drinking water for 3, 6, 9, 12, or 15 days. Colons were harvested and lymphocytes counted per the protocol below, and a treatment duration of 9 days was deemed ideal for the imaging experiments.

For the imaging study, mice were divided into 3 groups: a control group receiving no DSS, a mild colitis group given 3% DSS, and a moderate colitis group given 5% DSS. A total of 17 mice were included in the study and 16 were included in the final data analysis (one mouse died during antibody injection). For induction of DSS colitis, mice were continuously fed either control drinking water, 3% DSS in water, or 5% DSS in water for 7 days. At day seven, 350 μg of ^{111}In -labeled anti-CD4 antibody was injected via the tail vein. Imaging was conducted 48 h thereafter. The total duration of DSS administration or sterile water control was 9 days.

3.3. Preparation of indium-111 labeled antibody

Rat monoclonal antibodies against murine CD4 (clone YTS 177) were kindly provided by Hermann Waldmann, Sir William Dunn School of Pathology, Oxford, England. These antibodies were covalently conjugated at the ϵ amino group of lysine residues with the commercially available *N*-hydroxysuccinimide ester of 1,4,7,10-tetra-azacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA) (Macrocyclics Inc., Dallas, Texas), per standard methods (Liu et al., 2003). Antibodies were conjugated with an average of 4 DOTA per CD4 antibody. The final concentration of prepared DOTA/CD4 conjugate was 8.12 mg/ml in 0.3 M ammonium acetate buffer, pH 7. DOTA-CD4 (0.7 mg) was diluted with equal volume of 1 M ammonium acetate buffer, pH 6. 57.8 + 11.9 Mbq ^{111}In (Perkin Elmar, Shelton, CT) and was pretreated by incubation with half the volume of the buffer solution in room temperature for 20 min. The ^{111}In DOTA-CD4 reaction mixtures were heated at 40 °C for 60 min. The reaction was terminated by adding neutralized diethyle-netriamene pentaacetate (DTPA) to a final

concentration of 2 mM. Following labeling, the radioimmunoconjugates were filtered using a molecular weight cut-off centrifugal filter (Microcon YM3, Millipore Billerica, MA). Thin-layer chromatography (TLC) was performed and imaged with TLC Image Scanner (Bioscan, Washington DC) to determine the labeling efficiency. The specific activity of labeling was 82.6 ± 16.9 MBq/mg.

3.4. Small animal scintigraphy

48 h prior to radionuclide imaging, 34.26 ± 0.26 MBq of ^{111}In -labeled DOTA-CD4 antibodies were injected via the tail vein. Mice were anesthetized with 1.2% isoflurane and placed prone on the animal bed with core temperature maintained at 37 °C with warm air under feedback control. Radionuclide imaging was performed with a small animal combined modality single photon emission computed tomography (SPECT-CT) imaging gamma camera (X-SPECT; Gamma Medica, Northridge, CA) using a 2 mm pinhole collimator (360° of rotation, 64 projection, 15 s/projection, and an 82×82 imaging matrix). Data were reconstructed using 10 iterations (8 subsets) of an ordered subsets-expectation maximization (OS-EM) reconstruction algorithm implemented in ANSI C (Lange and Carson, 1984), incorporating a realistic model of the pinhole collimator (i.e., depth-dependent resolution recovery).

The data were reconstructed into a $72 \times 72 \times 72$ matrix format with an isotropic voxel size of 0.65 mm. An elliptical region of interest (ROI) was defined manually over the lower half of the abdomen and image analyses were performed on reconstructed SPECT images using AMIDE Software (version 0.8.15). The voxel values obtained from the reconstructed images were initially expressed in arbitrary units. A calibration factor derived from a phantom study with ^{111}In was applied, and the results obtained from the SPECT images were expressed quantitatively as the percentage of the injection dose (%ID).

A colon uptake ratio (CUR) was derived to normalize colonic activity of the labeled anti-CD4⁺ antibody. The CUR was normalized to muscle uptake as an estimate of activity in the vascular compartment. A region of interest was produced on the right hind quarter of each mouse measuring between 16 and 23 voxel units. The CUR was calculated as:

$$\frac{\text{MBq/voxel in colon ROI}}{\text{MBq/voxel in muscle ROI}}$$

3.5. Biodistribution of indium-111 labeled antibody

Following imaging, mice were anesthetized with 3–5% isoflurane and euthanized following cervical dislocation. The mice were weighed and total body radioactivity was measured with a gamma counter (Wizard, Perkin Elmer, Milwaukee, WI). Individual organs (livers, kidneys, spleens, colons, and bladders) were isolated, weighed, and measured for radioactivity. Mouse colons were measured, cleaned, and flushed with phosphate buffered saline (PBS)(Cellgro, Herndon, CA) prior to the digestion procedure described below.

3.6. Lymphocyte isolation

Following measurements of the colons, a proximal portion (5 cm distal to cecum) and a distal portion (5 cm proximal to anal verge) were sectioned and fixed in formalin for histopathologic analysis. Mouse colons were digested with collagenase type II (Sigma-Aldrich, St. Louis, MO) using a previously described protocol (Shacklett et al., 2003). Lymphocytes were isolated by a Percoll (Sigma-Aldrich, St. Louis, MO) gradient, as previously described (Shacklett et al., 2003). Isolated lymphocytes were stained with trypan blue solution [0.4% (w/v) in normal saline (Cellgro, Herndon, VA)] with an equal volume (10 μl) of the cellular suspension and placed in the hemocytometer for counting and determination of cell viability.

The CD4⁺ T cells were enriched using Miltenyi MiniMACS Separator Kit with a Miltenyi Mouse CD4⁺ T cell isolation kit, per the manufacturers instructions (Miltenyi Biotech Inc., Auburn, CA). Isolated cells were resuspended in 1 ml of PBS and recounted, as described above.

3.7. Immunofluorescence

A biotinylated polyclonal rabbit antibody specific for rat immunoglobulin (Ig) (DakoCytomation, Glostrup, Denmark) was used to detect the presence of the injected rat anti-mouse CD4 monoclonal antibody and a goat anti-rabbit antibody conjugated with Alexa Fluor 555 (Invitrogen, Eugene, Oregon, USA) was used to detect this rabbit antibody. A standard staining protocol was performed, as previously described (Vasdev and Nayak, 2004). Five sections of colon were stained from each mouse. Negative controls for the anti-mouse CD4 antibody included sections from mice undergoing the same 9-day sterile water drinking protocol and injected with equal amounts of indium not bound with rat anti-mouse CD4⁺ antibody. Positive controls for the rabbit anti-rat Ig antibody included sections from paraffin embedded rat colons (BioChain Institute, Inc., Hayward, CA, USA). All slides were viewed and analyzed on a Leica DM 6000 microscope using Image Pro MC Version 5.1 software (MediaCybernetics, Silver Spring, Maryland, USA).

3.8. Assessment of inflammation in DSS-induced colitis

Two sections of colon were fixed in 10% buffered formalin and embedded in paraffin. The sections were hematoxylin- and eosin-stained and then two slides per mouse were graded blindly by an experienced pathologist (JP Grenert). The slides were evaluated and graded from 0 to 14 with points for apoptosis (0–2), edema (0–1), serosal inflammation (0–2), mucosal inflammation (0–2), submucosal inflammation (0–2), epithelial damage (0–3), and neutrophil infiltration (0–2). This grading scheme follows previously validated protocols and is representative of changes seen during the acute stage of DSS-induced colitis (Obermeier et al., 2006; Sakuraba et al., 2007).

3.9. Statistical analysis

All statistical analyses and corresponding figures were generated using GraphPad Prism Version 4® (GraphPad Software, Inc., San Diego, CA). Mann Whitney and regression *p* values are reported with significance defined as *p* < 0.05.

Abbreviations

SPECT, single photon emission computed tomography; IBD, inflammatory bowel disease; ROI, region of interest; DSS, dextran sulfate sodium; Ig, immunoglobulin..

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Fig. 1. Dose-response analysis of DSS-induced colitis. (A) Number of total lymphocytes (white bars) and CD4⁺ T cells (black bars) extracted from the colons of mice consuming varying percentages (1–9%) of DSS in their drinking water for 10 days. Each group was comprised of 3 mice and the average number of lymphocytes and CD4⁺ T cells are displayed. In the 9% group, one mouse had to be sacrificed at day 8 secondary to weight loss > 20%. (B) Percentage increase of colonic lymphocytes (black bars) and weight loss (white bars) in mice receiving 5% DSS over varying treatment intervals (3–15 days). Each group consisted of 3 mice. The baseline colonic lymphocyte count was based upon age and weight matched controls ($n=3$ mice between 18 and 20 g and 14 and 20 weeks of age).

Fig. 2. Clinical and cellular indicators of DSS-induced colitis. Results of (A) colon length, (B) total lymphocytes, and (C) CD4⁺ T cells of mice in 3 groups of DSS. All analyses were performed immediately after SPECT-CT imaging. The number of mice in each group is shown in parentheses. Bars represent average values and * $p < 0.05$, ** $p < 0.01$.

Fig. 3. Histopathologic analysis of DSS-induced colitis. (A) Representative photomicrographs showing edema, cellular infiltrates, and apoptosis induced by DSS, relative to normal colon. The bar represents 250 μ m. (B) Are the results of the histopathologic score. The number of mice in each group is in parentheses and $*p < 0.05$ and $**p < 0.01$. (C) Immunofluorescence to confirm the presence of rat anti-mouse CD4⁺ antibody in imaged tissue, with staining of control rat intestine shown in C-1 and sections from a mouse not treated (C-2) or treated with 5% DSS and stained with anti-mouse CD4 at low (C-3) and high (C-4) power. The bars in B-1, 2, 3 represent 250 μ m that in B-4 is 50 μ m. The yellow arrow in B-4 shows CD4-specific staining with the labeled rat Ig on mouse tissue.

Fig. 4. SPECT-CT images. (A) An example of the overlay strategy with a CT, SPECT, and overlay SPECT-CT image of a 5% DSS mouse. (B) Ex vivo static image of liver (L), kidney (K), colon (C), and bladder (B) from a 5% DSS-treated mouse. (C) Overlay images of a control (no DSS) and a mouse treated with 5% DSS. (D) Reconstructed 3-D images of the control mouse (top) and the colitis mouse (bottom). Green regions correlate with uptake of radiolabeled antibody.

Fig. 5. Correlation of SPECT-CT to traditional parameters of colitis. (A) Colonic activity (in microcuries/mg tissue) of radiolabeled antibody (left) and colon uptake ratio (right) between the 3 DSS groups. * $p<0.05$ and ** $p<0.01$. (B) Regression analyses to correlate uptake ratio with colon length, total lymphocytes, histology, and CD4⁺ T cells. Each point represents one of the 16 animals in these experiments.