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Induction of Intestinal Graft-Versus-Host Disease and its Mini-Endoscopic Assessment in Live Mice

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To the

editorial board of Journal of Visualized Experiments (JoVE)

10/26/2018

Dear Dr. Bajaj
Dear members of the editorial board,

we are pleased to re-submit our revised manuscript (JoVE58871R1) entitled

"Induction of Intestinal Graft-Versus-Host Disease and its Mini-Endoscopic Assessment in Live Mice"

by Vera Buchele et al. to JoVE (section: Immunology and Infection) for publication.

In addition to the comments and suggestions of the reviewers that we addressed as indicated in our previous point-by-point response letter from October 17th, here we submit a second revision of our manuscript attempting to additionally accommodate the editorial comments that we received October 18th 2018. Please appreciate that we adapted as well the title of the manuscript to meet the editorial request.

Overall we feel confident that we submit a significantly improved manuscript to JoVE due to and complying with the reviewers' and editorial recommendations.

Thank you very much for your continuous consideration to publish our manuscript in JoVE.

Yours faithfully, on behalf of the authors

Prof. Dr. med. Kai Hildner
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TITLE:

Induction of Intestinal Graft-versus-host Disease and Its Mini-endoscopic Assessment in Live Mice

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KEYWORDS:

Hematology, gastroenterology, immunology, allogeneic hematopoietic stem cell transplantation, colitis, intestinal GvHD, endoscopy, mucosal inflammation, gastrointestinal tract histopathology

SUMMARY:

Here, we present a protocol that describes allogeneic hematopoietic stem cell transplantation and allows repetitive mini-endoscopic evaluations of the distal colon in situ for the presence, characteristics, and severity of colonic inflammation within live mice suffering from intestinal graft-versus-host disease.

ABSTRACT:

Acute graft-versus-host disease (GvHD) represents the most severe complication that patients previously undergoing allogeneic hematopoietic stem cell transplantation (allo-HCT) face and is frequently associated with a poor clinical outcome. While, for instance, GvHD manifestations of the skin are usually responsive to established immune-suppressive therapies and are, hence, not taking a fatal course, the presence and the intensity of intestinal GvHD, especially of the mid-to-lower parts of the gut, strongly influence the outcome and overall survival of patients with acute GvHD. Therapeutic options are essentially limited to the classic immune-suppressive agents yielding only moderate disease-mitigating effects. Hence, detailed knowledge about the tissue-resident immune cascade, changes in the intestinal microbiota, and the stromal response prior, upon, and after intestinal GvHD onset are urgently needed to understand the events and mechanisms underlying its pathogenesis and to develop innovative therapeutic options. Murine models of GvHD are frequently employed to identify and functionally assess molecules and

pathways putatively driving intestinal GvHD. However, means to specifically monitor and evaluate intestinal inflammation over time are essentially lacking since established scores to assess and grade acute GvHD are routinely comprised of various parameters rather than reflecting systemic GvHD manifestations. The detailed evaluation of intestinal GvHD has been restricted to studies using euthanized mice, thereby essentially excluding longitudinal (i.e., kinetic) analyses of the colonic compartment under a given experimental condition (e.g., antibody-mediated blockade of a proinflammatory cytokine) in live mice (i.e., in vivo). The mini-endoscopic in situ assessment of the distal colon of allo-HCT-treated mice described here allows a) a detailed macroscopic evaluation of different aspects of intestinal inflammation and b) the option to collect tissue samples for downstream analyses at various time points over the course of the observation period. Overall, the mini-endoscopic approach provides a major advance in preclinical noninvasive monitoring and assessment of intestinal GvHD.

INTRODUCTION:

Hematopoietic malignancies directly arising from the hematopoietic stem cell compartment and uncontrolled, rapidly progressing, and severe immune-mediated disorders are often indications to perform allo-HCT[1,2]. However, although accounting for the occurrence of the prognostic beneficial graft-versus-tumor response, donor lymphocytes are frequently inducing and promoting an unwanted immune-mediated attack of healthy tissue components within the allo-HCT recipient, a process that is called graft-versus-host disease[3]. Manifestations in the gut, the so-called intestinal GvHD, represent the most dreaded complication of acute GvHD, severe forms of which are routinely associated with a high mortality[1,2,4].

Overall, murine models of allo-HCT have emerged as invaluable tools to identify and study immune-mediated mechanisms underlying the pathogenesis of GvHD[5]. However, kinetic assessment of, for instance, beneficial effects of novel therapeutic interventions over time in live mice is routinely based on the determination of clinical GvHD scores[6]. While these scores are suitable to reflect, for instance, the overall disease burden (i.e., the systemic GvHD), clinical scores lack the sensitivity to reliably mirror organ-specific manifestations (e.g., in the gut). Hence, conclusions, for instance with respect to gut-protective effects of a given therapeutic intervention, that are based on these scoring systems usually fall short.

Despite major advances through the invention of the novel whole-body imaging modalities in combination with the usage of either bioluminescent or fluorescent genetic mouse models[7,8], methodologies to directly and specifically assess the intestinal manifestation of GvHD in live mice are lacking. Hence, the rationale behind the protocol of the endoscopic assessment of the intestinal GvHD phenotype described in the next section is to overcome this obstacle. Furthermore, the motivation is also to reduce experimental mice numbers since, so far, a detailed assessment of the cellular, morphological, and molecular characteristics (e.g., by histopathology or molecular biology) of intestinal GvHD manifestation has ultimately required the sacrifice of the experimental mouse.

Our institution has previously reported on the methodology of a mini-endoscopic assessment of colonic manifestations in the course of syngeneic colitis models[9]. In the protocol presented here,

we have refined and adapted the colonoscopic scoring matrix for alloresponse-driven colitis in live mice with intestinal GvHD upon transplantation of alloreactive HCT and donor lymphocytes in an MHC class I fully mismatched setting. We identified four parameters suitable to reflect intestinal GvHD-related colonic lesions. Furthermore, we established a system that allows a fine-tuned grading of any single determinant, resulting in a new score that readily informs the reader about the severity of intestinal GvHD present in a given mouse at a given time point. Histopathological analyses confirmed that an endoscopic score above a certain threshold is reliably predicting moderate-to-high grade tissue inflammation. Hence, mini-endoscopic evaluation appears to represent a working substitute for the gold standard histopathology that routinely requires the sacrifice of the experimental mice. Importantly, this protocol can be applied at virtually any given time point and can be repeatedly used during the course of the disease[10,11]. Furthermore, in contrast to the usage of bioluminescence-dependent approaches, no labor-intense and time-consuming measures like intercrossing genetically modified mice are required and, hence, the methodology can be applied on virtually any mouse line of interest.

Taken together, given the detrimental clinical perspective of allo-HCT patients with severe intestinal GvHD, rapid scientific progress and more insight into the molecular mechanisms underlying the immune pathogenesis are urgently needed. Similarly, important, ethical considerations demand that the gain of knowledge should be achieved with the usage of the minimal number of experimental mice. Hence, both recognized claims on the research community exploring intestinal GvHD can be advanced by implementing serial mini-endoscopic evaluations of the colon in the experimental work chain to monitor and grade intestinal GvHD in live experimental mouse models, as described and validated in the protocol presented here.

PROTOCOL:

The experimental methods described here have been approved by the government of Mittelfranken, Bavaria, Germany.

1. GvHD induction

1.1. Day 0: Total body irradiation of the recipient mice

1.1.1. Use female CD45.2^{+} H2kd^{+} BALB/c mice that are at least 10 weeks old as recipients.

1.1.2. Weigh and record the weight of the recipient mice prior to GvHD induction.

NOTE: Ensure that the recipient has a minimal body weight of 20 g. The starting body weight will serve as the reference value to calculate the weight loss of the individual mouse over the course of GvHD induction and progression.

1.1.3. Place up to five mice in the container of the X-ray irradiator.

1.1.4. Irradiate the mice by total body irradiation with a single dose of 8 Gy of X-ray. Use Cs^{137} as a radiation source.

1.2. Day 1: Reconstitution of the irradiated mice with T-cell-depleted bone marrow

NOTE: The transplantation of allogeneic bone marrow cells, as outlined and detailed under section 1.2, should take place within 24 h after irradiation. Perform the procedures described below in a sterile tissue culture hood and use filtered reagents. Use allogeneic CD45.1/Ly5.1 B6.SJL-*Ptprca Pepcb*/BoyCrl mice as donors for T-cell-depleted bone marrow.

1.2.1. Euthanize CD45.1/Ly5.1 B6.SJL mice that will donate allogeneic bone marrow cells in accordance with the institutional guidelines, 12–24 h after the irradiation of the recipient BALB/c mice. For this, anesthetize the CD45.1/Ly5.1 B6.SJL mice by inhalation of 5% isoflurane. Continue with the isoflurane exposure over 1 min after breathing arrest and confirm euthanasia by cervical dislocation.

1.2.2. Disinfect the fur and skin of the mouse thoroughly with 70% ethanol.

1.2.3. Position the mouse onto a clean working sheath so that the mouse is in a prone position, with its hindquarters facing the experimenter. Lift the fur at the Achilles' heel with the tip of a Semken forceps with a length of 13 cm and serrated curved tips. Incise the skin between the forceps and the heel with hardened 85 cm fine scissors with straight tips.

1.2.4. Elongate the incision cranially (i.e., from the heel over the lower leg and thigh to the hip region). Remove the skin and fur from the hind limb with the help of the forceps.

1.2.5. Perform the same procedure on the other hind limb.

1.2.6. Remove the hind limbs by cutting through the adjacent hip joints.

1.2.7. Cut away the rear paws. Cut through the knee joint. Store the thigh and shank of every hind limb together in a 100 mm Petri dish filled with phosphate-buffered saline (PBS) solution on ice.

1.2.8. Clean the femur and tibia bones carefully by removing as much muscle tissue as possible from every thigh and shank. Transfer the femur and tibia bones to a 50 mL tube filled with sterile RPMI 1640 medium and place it on wet ice until all tibia and femur bones collected in step 1.2.7 are cleaned from muscle tissue.

1.2.9. To isolate the bone marrow, transfer one femur or tibia bone at a time (which has been cleaned as described in step 1.2.8) from the 50 mL tube to a Petri dish (with a diameter of 92 mm) filled with RPMI 1640 medium. Cut off the ends of the femur or tibia with a scalpel to get access to the cavity of the bone containing the bone marrow.

1.2.10. Prepare a 50 mL collection tube with 5 mL of RPMI 1640 medium. Insert a 26 G needle attached to a 1 mL syringe filled with 1 mL of RPMI 1640 medium into the bone cavity and flush

the bone marrow out of the cavity into the collection tube by pushing the plunger.

1.2.11. Repeat step 1.2.10 until the bone appears light and shiny (i.e., the bone cavity is visibly devoid of reddish bone marrow). Discard the empty bone.

1.2.12. Repeat steps 1.2.9 - 1.2.11, using all femur and tibia bones from step 1.2.8, and collect all recovered bone marrow pieces in the same 50 mL collection tube from step 1.2.10. Keep the collection tube on wet ice until all bones from step 1.2.8 have been processed accordingly.

1.2.13. Create a single-cell suspension by softly pipetting the flushed, crumbly bone marrow pieces up and down. Filter the bone marrow single-cell suspension through a 40 µm mesh screen cell strainer placed on a new 50 mL collection tube.

1.2.14. Centrifuge the 50 mL collection tube containing the bone marrow single-cell suspension at 450 x *g* for 5 min at 4 °C. Discard the supernatant.

1.2.15. Resuspend the pellet in 5 mL of ACK lysing buffer (1 mM Na_2EDTA; 10 mM $KHCO_3$; 144 mM NH_4Cl; pH 7.2) for red blood cell lysis. Incubate the cell suspension for 3 min at room temperature. Afterward, immediately add 10 mL of PBS solution to the cell suspension.

1.2.16. Centrifuge the suspension at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the cells in 2 mL of PBS solution.

1.2.17. Count the bone marrow cells, using a hemocytometer. Preserve an aliquot of 6 x 10^6 cells for flow cytometry analysis to check the purity of the cells after cell purification (see step 1.2.20).

1.2.18. For the depletion of T cells from the bone marrow single-cell suspension, use a commercially available cell purification kit. Magnetically deplete $CD90.2^+$ cells (i.e., lymphocytes) from the total bone marrow cells, following the manufacturer's protocol.

1.2.19. Count the T-cell-depleted bone marrow cells that have been isolated as described in step 1.2.18, using a hemocytometer. Preserve an aliquot of 1 x 10^6 cells for flow cytometry analysis to be performed later on, as described in step 1.2.20.

NOTE: The on-average cell yield derived from one donor mouse is usually from 2 x 10^7 to 3.4 x 10^7 T-cell-depleted bone marrow cells.

1.2.20. Confirm a successful T-cell depletion by flow cytometry. Stain 1 x 10^6 cells, preserved from steps 1.2.17 (bone marrow cells before magnetic cell separation) and 1.2.19 (T-cell-depleted bone marrow cells after magnetic cell separation), with the following antibodies: α-CD45.1 (A20), α-CD3 (17A2), α-CD4 (GK1.5), and α-CD8α (53-6.7). Use the remaining cells from step 1.2.17 as an unstained control and for single-staining controls, to set up the flow cytometer.

1.2.20.1. Transfer 1 x 10^6 cells from each sample to a well of a 96-well plate with a V-bottom to

perform the flow cytometric staining procedure.

1.2.20.2. Spin down the cells within the 96-well plate (step 1.2.20.1) at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the cells in 100 µL of FACS buffer (PBS supplemented with 3% filtered bovine serum).

1.2.20.3. Centrifuge the cells within the 96-well plate at 450 x *g* for 5 min at 4 °C. Discard the supernatant.

1.2.20.4. Add an appropriate amount of the antibody of choice (compare with step 1.2.20) to the samples for single staining in 100 µL of FACS buffer.

1.2.20.5. Prepare a mixture of all antibodies (master mix) containing the appropriate amounts sufficient to separately stain the bone marrow cell aliquots preserved from prior (see step 1.2.17) and after (see step 1.2.19) magnetic T-cell depletion. Add 100 µL of the master mix to both aliquots.

1.2.20.6. Add only 100 µL of FACS buffer to the unstained sample.

1.2.20.7. Incubate the cells within the 96-well plate for 20 min at 4 °C in the dark.

1.2.20.8. Add 100 µL of FACS buffer and centrifuge the cells within the 96-well plate at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the cells in 100 µL of FACS buffer.

1.2.20.9. Centrifuge the cells at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the cells in 250 µL of FACS buffer.

1.2.20.10. Transfer the samples to a 5 mL polystyrene round-bottom tube and analyze them with a flow cytometer instrument that is able to detect the fluorescent molecules that were used to label the antibodies employed to characterize the cell samples (see step 1.2.20).

1.2.20.11. Compare the cell composition from before and after the magnetic cell separation.

NOTE: A purity of approximately 95% $CD45.1^{+}CD3^{-}$ (i.e., T-cell-depleted bone marrow) cells within the live gate is usually achieved.

1.2.21. Wash the T-cell-depleted bone marrow cell suspensions 2x with PBS solution (450 x *g* for 5 min at 4 °C) and, finally, resuspend the cells in PBS solution, adjusting the cell concentration to 5 x 10^7 cells/mL. Keep the cells on wet ice until the injection.

1.2.22. Inject T-cell-depleted bone marrow cells into the recipient mice, which were irradiated the day before (see section 1.1).

1.2.22.1. Place the experimental mouse in a leak-proof chamber and induce anesthesia by

inhalation of up to 4% isoflurane, until the mouse is unconscious. Confirm analgesia and loss of consciousness by testing the loss of the righting reflex and the subsequent loss of the pedal withdraw reflex.

1.2.22.2. Inject 5 x 10^6 T-cell-depleted bone marrow cells (i.e., 100 µL of 5x10^7 cells/mL) containing PBS solution with a 30 G syringe intravenously into the retrobulbar space containing the venous sinus.

1.3. Day 2: Transfer of T cells

NOTE: Perform the described procedures below in a sterile tissue culture hood and use filtered reagents. Use CD45.2 C57Bl/6 (wild-type; WT) mice as donors for alloreactive T cells. The usage of congenic marker systems allows the distinguishability between recipient (CD45.2$^+$ H2kd$^+$ Balb/c mice) and donor hematopoietic cell types (bone marrow cells: CD45.1$^+$ H2kb$^+$ B6.SJL mice; allogeneic T cells: CD45.2$^+$ H2kb$^+$ C57/Bl6 mice).

1.3.1. Euthanize C57Bl/6 mice in accordance with the applying institutional guidelines and authorities. Therefore, anesthetize mice by inhalation with a concentration of 5% isoflurane. Continue the isoflurane exposure until 1 min after breathing arrest and confirm euthanasia by cervical dislocation. Disinfect the fur and skin of the mouse thoroughly with 70% ethanol.

1.3.2. Place a strainer with a 40 µm mesh screen on a 50 mL collection tube. Remove the spleen and place it onto the strainer.

1.3.3. With a syringe plunger, comminute the spleen in the strainer. Wash the strainer and syringe plunger with PBS to collect all splenocytes.

1.3.4. Centrifuge the cells within the 50 mL collection tube at 450 x *g* for 5 min at 4 °C. Discard the supernatant.

1.3.5. Resuspend the splenocytes in 3 mL of ACK lysing buffer to lyse red blood cells. Incubate the cell suspension for 3 min. Afterward, add 10 mL of PBS and centrifuge the cells at 450 x *g* for 5 min at 4 °C. Discard the supernatant and resuspend the splenocytes in PBS.

1.3.6. Count the spleen cells, using a hemocytometer. Divert an aliquot of 6 x 10^6 cells for flow cytometry analysis, to evaluate the magnitude of purification in step 1.3.9.

1.3.7. For a total CD3$^+$ T-cell isolation from total splenocytes, use a commercially available cell purification kit. Isolate the splenic T cells, following the manufacturer's protocol.

1.3.8. Count the splenic CD3$^+$ T cells isolated as described in step 1.3.7, using a hemocytometer. Preserve an aliquot of 1 x 10^6 T cells for flow cytometry analysis.

NOTE: The on-average yield of splenic T cells per donor mouse isolated by magnetic cell

separation ranges from 1.3 x 10^7 to 2 x 10^7 cells.

1.3.9. Confirm the success of the T-cell isolation by flow cytometry. For a detailed description of the staining protocol, confer and follow steps 1.2.20.1. - 1.2.20.10.

1.3.9.1. Stain 1 x 10^6 splenocytes of steps 1.3.6 (before the magnetic cell separation) and 1.3.8 (after magnetic cell separation) with the following antibodies: α-CD45.2 (104), α-CD3 (17A2), α-CD4 (GK1.5), and α-CD8α (53-6.7).

1.3.9.2. Use the remaining cells from step 1.3.6 as an unstained control and for single staining with the respective antibodies, to set up the flow cytometer.

1.3.9.3. Compare the cell composition before and after magnetic cell separation.

NOTE: A purity of ≥95% of $CD45.2^+CD3^+$ T cells within the lymphocyte live gate should be accomplished.

1.3.10. Wash the T cells 2x with PBS (450 x *g* for 5 min at 4 °C) and, finally, resuspend the cells in PBS solution, adjusting the cell concentration to 7 x 10^6 cells/mL. Keep the cells on ice until the injection.

1.3.11. Inject alloreactive, splenic T cells (see step 1.3.10) into irradiated BALB/c mice who have previously received $CD45.1^+$ T-cell-depleted bone marrow cells (see step 1.2.22).

1.3.11.1. Induce anesthesia by placing the experimental mouse in a leak-proof chamber in which it is exposed to up to 4% isoflurane until it loses its consciousness. Confirm analgesia and loss of consciousness by testing the loss of the righting reflex and the subsequent loss of the pedal withdraw reflex.

1.3.11.2. Inject 0.7 x 10^6 alloreactive $CD3^+$ T cells with a 30 G syringe (i.e., 100 µL of 7 x 10^6 cells/mL) containing PBS solution (see step 1.3.10), intravenously into the retrobulbar space of the mice, to induce GvHD. Denominate these mice as the experimental group.

1.3.11.3. Inject a different group of mice with 100 µL of PBS alone, intravenously into the retrobulbar space, and denominate this group as the control group, subsequently called “no-T-cell-transplanted (noT) mice”.

2. Assessment of systemic GvHD

NOTE: Perform this procedure in a semi-sterile surrounding in an experimental room licensed and equipped for handling live mice within the animal facility.

2.1. Follow the clinical course of GvHD in individual mice; specifically, assess and score the experimental mice 3x a week for the presence of clinical GvHD symptoms, based on a scoring

system described below and adapted from a scoring system established previously by Cook et al.[6].

2.1.1. Weigh each mouse individually, record the weight, and calculate the specific body weight loss in reference to the body weight that was determined prior to the start of the experiment (corresponding to 100%) on day 0 (step 1.1.2). Score a weight loss of less than 10% of the starting weight with grade 0, a weight loss between 10% and 25% with grade 1, and a weight loss of more than 25% with grade 3.

2.1.2. Score the activity level of each mouse, discriminating mice with a normal activity level (score = 0) from mice with a moderately decreased activity level (score = 1) and mice with stationary behavior unless they are externally stimulated (score = 2).

2.1.3. Score the fur texture, thereby discriminating a normal, shiny, and kempt fur (score = 0) from a moderately ruffled fur (score = 1) and the presence of bald spots (score = 2).

2.1.4. Score the skin texture, discriminating normal skin (score = 0) from sporadic spots of scaling skin (e.g., at the tail and the paws) (score = 1) and obvious scaling and sore skin areas (score = 2).

2.1.5. Analyze the consistency of secreted stool fractions. Score stool with a normal (i.e., hard consistency) with grade 0, stool with deformable and soft stool with grade 1, and stool without any consistency and, hence, with severe diarrhea with grade 2.

2.1.6. Sum up all separately scored parameters to a maximal clinical score of 12 per mouse.

2.2. Consider and evaluate the criteria of ceasing the experiment due to the current disease severity level of the individual mouse, in accordance with the applying institutional guidelines and authorized animal protocol.

3. Assessment of intestinal GvHD

3.1. Scoring of GvHD-related lesions by mini-endoscopy of the distal colorectal region

NOTE: Perform this in a semisterile surrounding in an experimental room licensed and equipped for handling live mice within the animal facility. Use a mini-endoscopic workstation that is approved for the use of small animals.

3.1.1. Prepare the endoscopic device by covering the telescope (with a diameter of 1.9 mm and a length of 10 cm) with the endoscopic examination sheath. Clean and sterilize the endoscope with water and ethanol.

3.1.2. Switch on the computer, the adjustable xenon light source, and the camera module, and set the focus in a way that objects close to the lens give a clear image.

3.1.3. Connect the air pump to the endoscopic sheath. To visualize the air stream, dip the tip of the endoscope into a beaker glass filled with water. Regulate the air stream by adjusting the valve between the air pump and the endoscopic sheath. Adjust it to a slow, constant air stream, reflected by a few continuously ascending bubbles.

3.1.4. Place a mouse into a leak-proof chamber. Induce anesthesia by inhalation of up to 4% isoflurane until the mouse is unconscious. Confirm analgesia and an absent state of the reflexes by testing the loss of the righting reflex and the subsequent loss of the pedal withdraw reflex.

3.1.5. Use an appropriate veterinary anesthesia machine in accordance with the animal protocol used. Transfer the mouse from the chamber to the colonoscopy workspace. Here, position the anesthetized mouse, with its nose in the nosecone (which is connected to the anesthesia instrument), onto a clean working sheath so that the mouse is in a prone position, facing the experimenter with its hindquarters.

3.1.6. To maintain anesthesia during the colonoscopy procedure, reduce the isoflurane concentration to approximately 2%. Monitor the mouse's respiration and response to stimulation during the colonoscopy and adjust the isoflurane concentration at the vaporizer if needed. Cover the mouse's eyes with salve to prevent them from getting dry.

3.1.7. Control the efficacy of the anesthesia by pinching between the toes of the mouse. In the case of absent reactivity, proceed to step 3.1.8.

3.1.8. Lift the tail just above the tail root with one hand and carefully insert the endoscope, positioned in the other hand, via the anus into the rectum.

3.1.9. While the air stream inflates the colorectal lumen, slowly and carefully move the endoscope forward in aboral direction.

3.1.10. Place the endoscope in the middle of the colonic lumen and maintain this position in order to minimize contact with the colonic wall and to avoid making scratches, deeper wall-related injuries (e.g., resulting in bleeding), or even wall perforation (see step 3.1.17). Control this step by permanently watching the video stream (i.e., under constant visualization of the colonic compartment).

3.1.11. If feces constrict the view, remove the endoscope and perform an enema by rectally flushing with up to 2 mL of saline, using a soft Pasteur pipette. Use as little liquid as possible, to prevent an unintended dilution of the feces and other secondary effects negatively affecting the characteristics of the mucosal surface and, ultimately, the scoring results.

3.1.12. Try to regularly position the endoscope at a defined (i.e., distinct) anatomic site within the distal colon to standardize the scoring of colonic inflammation and to optimize the comparability of scoring results between mice and across experiments.

NOTE: Normally, with the rigid endoscope, a maximal depth of 4 cm can be reached via the rectal route. Between 2 and 4 cm aborally, the colon regularly turns into a flexure that cannot be passed with the rigid colonoscope. Use the colon region distally adjacent to the flexure as the default scoring spot.

3.1.13. Start to record the video stream and begin with the scoring of inflammation.

3.1.14. Evaluate the GvHD-related inflammation of the colon by scoring the parameters explained and depicted in **Table 1** and **Figure 3**.

3.1.14.1. Move the endoscope at the scoring spot gently and slightly back- and forward to assess the different parameters.

3.1.14.2. Assess the parameter “translucency”, as well as the “stool consistency”, by positioning the endoscope in a wider distance in relation to the colonic wall.

3.1.14.3. Assess the parameters “granularity” and “vascularity” by positioning the endoscope in close proximity to the colonic wall. For this task, carefully apply well-dosed tension to the colonic wall with the tip of the endoscope.

3.1.15. Upon completion of the scoring process, stop recording.

NOTE: Afterward, the recorded video clip can be used in total or used to generate representative images (i.e., screenshots) of the mini-endoscopically assessed criteria overall contributing to colonic inflammation.

3.1.16. Carefully remove the endoscope.

3.1.17. Discontinue isoflurane exposition by transferring the mouse to a separate cage in which it is exposed to ambient air. Warm the mouse with a red-light lamp and observe the mouse until it recovers from the anesthetic and regains full consciousness.

NOTE: Due to the air inflation of the colon during the endoscopy, the abdominal region of the mouse may appear puffed up directly afterward. While this is normal and of transient nature, prolonged signs of massive and even progressive inflation of the abdomen and failure of recovery of the mouse might indicate an unintended perforation of the colon (in this case, the mouse needs to be euthanized immediately).

3.1.18. Upon complete recovery, place the mouse back into its respective cage.

3.1.19. Optional approach: It is also possible to take view-guided tissue biopsies. Take the following steps.

NOTE: The colonoscopy will be performed in the same way as described in steps 3.1.1 - 3.1.18,

with the following modifications.

3.1.19.1. In step 3.1.1, use an endoscopic examination sheath with a built-in working channel, instead of a simple examination sheath without working channels, for covering the telescope. Introduce a biopsy forceps into the working channel until its tip is just visible in front of the endoscope at the video screen because this largely prevents unintended injury to the intestine.

3.1.19.2. Proceed with steps 3.1.2 - 3.1.11. Insert the endoscope into the colon and push it carefully forward to the spot of interest.

3.1.19.3. Employ the help of a second experimenter for the task of taking colonic tissue biopsies. Ask the second experimenter to navigate the tip of the biopsy forceps to the colonic region of choice by pushing the forceps within the working channel slowly forward until the jaws of the forceps can be opened. Constantly watch the video stream during this procedure to minimize the risk of injuring the colonic wall.

3.1.19.4. Recover a colonic wall tissue sample by carefully opening and closing the jaws of the biopsy forceps.

NOTE: Do this with caution to prevent the perforation of the colonic wall. In the rare case of a colonic perforation, immediately sacrifice the affected mouse by applying measurements in accordance with the institutional guidelines and under the continuous administration of anesthetics.

3.1.19.5. Pull back and remove the closed forceps out of the working channel. Remove the specimen from the jaws of the biopsy forceps by dipping and carefully shaking the opened forceps in sterile PBS solution. Afterward, choose proper storage conditions for the tissue sample, in accordance with the planned downstream analyses. After disinfection with 70% ethanol, the forceps might be reused to take further biopsies.

3.1.19.6. After taking biopsies, remove the endoscope and finish the colonoscopy as described in steps 3.1.16 - 3.1.18.

3.2. Histopathological analysis

3.2.1. Between days 26 and 30 after the X-ray irradiation, euthanize the allo-HCT recipient mice in accordance with applying institutional guidelines and authorities. For this, anesthetize the mice by inhalation of 5% isoflurane. Continue with the isoflurane exposure for 1 min after breathing arrest and confirm euthanasia by cervical dislocation. Thoroughly disinfect the fur and skin of the mouse with 70% ethanol.

3.2.2. Open the abdominal cavity and remove the colon. Transfer it to a Petri dish (with a diameter of 92 mm) with PBS.

3.2.3. Fill a 5 mL dispenser tip with PBS. With the help of a Semken forceps with a length of 13 cm and serrated curved tips, slip the distal part of the colon (approximately 5 mm) over the tip of the dispenser tip. Carefully fix the colon with the forceps at the dispenser tip and flush the colon with PBS by pressing down the plunger of the dispenser tip to remove feces from the intestine.

3.2.4. Cut out a colonic segment located about 5 mm from the anus, with the help of a scalpel.

3.2.5. Fix the resected gut specimen in 4.5% formaldehyde overnight. Before embedding it in paraffin, place the tissue in an upright position.

3.2.6. Cut 3 µm cross sections of paraffin-embedded colon tissue and stain them with hematoxylin and eosin (HE), using standard staining protocols.

3.2.7. Prepare HE-stained cross sections of colon tissue samples.

3.2.8. Adapted from the scoring matrix reported by Kaplan et al.[12], histopathologically score the inflammatory activity semiquantitatively, with scores from 0–3: no inflammation (score = 0), mild inflammation (score = 1), moderate inflammation (score = 2), and severe inflammation (score = 3).

NOTE: Ideally, employ for this task the expertise of a pathologist who is experienced in the evaluation of murine intestinal GvHD and blinded to the nature of the experimental and control groups.

REPRESENTATIVE RESULTS:

The current protocol, describing the mini-endoscopic evaluation of intestinal GvHD-associated lesions of the distal colon, has been established and validated in mice previously subjected to the systemic induction of a severe acute GvHD model. In this study, we used an MHC class I fully mismatched model system in which BALB/c mice were lethally irradiated, followed by the transplantation of T-cell-depleted allogeneic bone marrow and by the administration of GvHD-inducing alloreactive C57BL/6 $CD3^+$ T lymphocytes. T-cell-depleted bone marrow cells and splenic T cells for GvHD induction were purified by magnetic separation. The purity of these cell populations was determined by flow cytometry, and representative results of the purification process are displayed in **Figure 1**, showing a sufficient depletion of T cells from total bone marrow cells and a consistent enrichment of spleen-derived $CD3^+$ T cells prior to their transfer to previously irradiated mice. The clinical course displayed in **Figure 2** demonstrates the reproducibly robust induction of the clinical systemic GvHD phenotype upon allo-HCT in the presence (WT, in black) vs. absence (noT, grey) of alloreactive donor T lymphocytes. The scoring system previously reported by Cooke et al. represents a sum core in which six parameters are assessed and graded: body weight, posture, activity, skin and fur texture, and stool consistency[6]. The control mice experienced only one, early occurring peak of the clinical score, between days 5 and 10, that was similarly observed in T-cell-receiving mice and is, therefore, largely due to the irradiation-associated systemic inflammatory response. Regardless, clinical scores of donor T-

cell-receiving mice started to rise earlier, showed a higher total peak, only transiently decreased after initially peaking, and then, essentially increased again, continuously, over the remaining period of the experiment. Overall, these results are in agreement with the interpretation that T-cell-receiving mice show stronger and more progressive signs of systemic GvHD compared to noT mice.

The donor T-lymphocytes-receiving allo-HCT mice showed various signs of acute organ-related and systemic GvHD manifestations, overall resulting in high sum scores, while the control mice lacked moderate to severe affections, especially at later time points. However, signs of intestinal GvHD are underrepresented in the systemic GvHD scoring system, where the only assessed gut-related parameter is stool consistency. As shown in **Table 1,** mini-endoscopically assessable criteria were defined, specifically intended for the precise description, scoring, and grading of intestinal GvHD-associated lesions, by adapting criteria previously reported for the evaluation of syngeneic colitis to the context of alloresponse-driven colitis[9]. **Figure 3** displays typical examples for each individual criterion, illustrating the type and extent of intestinal GvHD-associated lesions, thereby visualizing the grading matrix applied during the mini-endoscopic evaluation of the distal colon of GvHD-prone mice upon transplantation of MHC class I fully mismatched donor lymphocytes.

Figure 4A shows that intestinal GvHD sum score results that are based on criteria defined in **Table 1** and displayed in **Figure 2** easily enable the experimenter to discriminate donor lymphocyte-receiving mice with severe signs of intestinal inflammation from control mice that are essentially devoid of GvHD. To validate the mini-endoscopically based grading system, histopathological studies were performed by using a previously reported microscopic grading system[12]. The data in **Figure 4B** confirm that the colon of mice severely affected by GvHD-related inflammation, as evidenced by high colonoscopic sum scores, display similarly strong histopathological signs of inflammation, given a histopathological sum score of ≥2 postmortem. In contrast, colon tissues of control mice display no (score 0) or, at most, mild (score 1) histopathological signs of inflammation, in line with the virtual absence of mini-endoscopically detectable signs of colitis. Furthermore, as shown in **Figure 4C, D**, correlation studies between mini-endoscopically and histopathologically assessed colitis activity and systemic GvHD scores were performed. Importantly, these studies demonstrated that mini-endoscopically determined sum scores of ≤3 reliably predict the absence of mid-to-higher grade (i.e., ≥2) intestinal GvHD-associated colonic inflammation scores obtained from histopathological grading. Finally, the severity of endoscopically assessed intestinal GvHD shows a correlation with the systemic GvHD activity.

FIGURE AND TABLE LEGENDS:

Figure 1: Flow cytometric quality assessment of magnetically purified T-cell-depleted bone marrow cells and allogeneic splenic CD3$^+$ T cells. (**A**) Depletion of CD90.2$^+$ bone marrow cells achieved by magnetic separation, using a commercially available purification kit. The purity of T-cell-depleted bone marrow cells was determined by flow cytometry, by staining cells prior to and after T-cell depletion with anti-CD45.1 and anti-CD3. Results from one representative experiment are shown. (**B**) Splenic T cells were magnetically purified, employing a commercially available

purification kit. The purity was determined by flow cytometry, comparing the frequencies of CD45.2 and CD3 costaining of samples derived from before and after T-cell isolation. Results from one representative experiment are shown.

Figure 2: Clinical course of GvHD, employing an MHC class I fully mismatched model. To induce acute GvHD, BALB/c mice were whole-body irradiated on day 0. The mice received T-cell-depleted bone marrow cells 24 h later (d1). On day 2, the mice were injected with allogeneic splenic $CD3^+$ T cells (wild-type [WT]; $n = 9$). As a control, some mice received T-cell-depleted bone marrow alone (noT; $n = 9$). The mice were specifically assessed three times a week for the presence and severity of clinical symptoms of GvHD. The displayed data represent mean values ± SEM of the clinical scores obtained from individual mice of the indicated treatment group from two representative experiments. The data were analyzed by two-way ANOVA, followed by Bonferroni's multiple comparisons posttest. ****$p < 0.0001$ was considered significant.

Figure 3: Representative images illustrating grading examples underlying the scoring matrix of the intestinal GvHD-related colon alterations assessed by a mini-endoscopic evaluation of the colons of live, allo-HCT-treated mice. To complement and illustrate the detailed description of the used scoring grading and system in **Table 1**, representative mini-endoscopic images of the evaluated severity levels (grading) for all parameters individually in the colon of anesthetized, live mice undergoing allo-HCT between days 26 and 30 before as described in **Figure 1** are displayed here.

Figure 4: Mini-endoscopic scoring and grading of intestinal GvHD-related inflammation of the colon, reliably predicting the presence of higher-grade colitis assessed by histopathological scoring. (**A**) Between days 26 and 29 after the induction of GvHD as described in **Figure 1**, the manifestation of intestinal GvHD was evaluated by mini-endoscopy in live, anesthetized mice. Colonic alterations were scored and graded according to the scoring and grading system depicted in **Table 1** and **Figure 2**. Representative endoscopic images of control (no T cell; noT) and wild-type (WT), T-cell-receiving mice and the mini-endoscopically assessed score (top) are shown. (**B**) The mice were sacrificed 12 h after the mini-endoscopic evaluation, and the distal part of the colon was processed and histopathologically assessed. The inflammatory activity of hematoxylin- and eosin-stained cross sections of the distal colon was graded. Representative hematoxylin- and eosin-stained cross sections of the distal colon of no T-cell- and WT T-cell-receiving allo-HCT-treated mice and the corresponding histology scores are shown. The data in panels **A** and **B** were analyzed by Student's *t*-test and are shown as mean ± SEM. ****$p < 0.0001$ was considered significant. WT: $n = 15$; noT: $n = 15$. The data represent pooled data from four individual experiments. (**C**) Interdependence of colonoscopy scoring and histological scoring. The data are shown as box plots. Each blot displays the median (black line within each box), the range of the data (min to max), and quartiles (areas of each box). WT: $n = 15$; noT: $n = 15$. The data represent pooled data from four individual experiments. (**D**) Correlation between colonoscopy scoring and clinical scoring. Both control and wild-type T-cell-receiving mice are included in the correlation analysis. The solid line represents the linear regression line. Spearman correlation coefficient (*r*-value) and *p*-value are shown. WT: $n = 12$; noT: $n = 13$. The data represent pooled data from three

individual experiments.

Table 1: Mini-endoscopic scoring and grading matrix of intestinal GvHD-related lesions in the colon of live allo-HCT-treated mice. Alterations of the endoluminal and transmural colon morphology in allo-HCT pretreated mice were assessed by a colonoscopy of the rectum and the distal colon of anesthetized, live mice, using a murine mini-endoscopy system. Colonic lesions were classified with the help of the endoscopic scoring system (modified murine endoscopic index of colitis severity [MEICS]) that is deduced from the original MEICS scoring system reported by Becker et al.[9] and adapted to the context of allo-HCT. The distal colon is visually assessed for the presence and magnitude of the following four parameters: 1. the transmittance of endoscopic light (translucency) through the colonic bowel wall as thickening of the wall; 2. the mucosal surface displaying a cobblestone appearance (granularity) of the endoluminal-oriented mucosal surface; 3. an altered vascularization pattern (vascularity); 4. endoscopically assessed stool appearance in situ (stool consistency). Each parameter is scored from 0 (no signs) to 3 (most severe phenotype), adding up to a maximum sum score of 12 per mouse.

DISCUSSION:

The protocol describes the methodology of induction and mini-endoscopic assessment of the colonic phenotype observed in the course of intestinal GvHD. It serves the wider purpose of enabling scientists to study intestinal GvHD longitudinally and noninvasively over the entire disease course (i.e., from the onset of colonic manifestation and progression until maximal disease activity).

However, there are some critical steps and important limitations inherent to the presented methodologies the experimenter needs to be aware of prior to applying the technologies in the respective scientific context. First, although we have successfully used the mini-endoscopic assessment of intestinal GvHD-related colonic lesions in a minor histocompatibility-mismatched model system[11], the provided protocol here is currently exclusively applicable to MHC class I fully mismatched acute GvHD models. While the MHC class I fully mismatched GvHD model is common and frequently used[5], it is well established that the characteristics of GvHD manifestation are highly dependent on the used mouse substrains since this strongly impacts, for instance, the sensitivity to irradiation. Furthermore, the experimental mouse source with, for instance, a varying composition of intestinal microbiota and used numbers of transferred allogeneic T cells may critically impact the kinetics and dynamics of the intestinal GvHD manifestation[13]. Hence, a critical step is to carefully test and validate the indicated measures for their ability to successfully induce intestinal GvHD as shown.

Regarding the successful implementation of the live mini-endoscopic assessment of acute intestinal GvHD, we want to stress that, while handling the endoscope is straightforward, it may require some training, to minimize the risk of experiencing the most dreaded complication (i.e., to perforate the intestinal wall with the rigid instrument in combination with the need to air-inflate the colon). Due to an increased inflammation-induced vulnerability and decreased flexibility of the colonic tissue, the gut wall integrity might be more at risk during the postradiation recovery phase (i.e., until day 10) and upon the full manifestation of intestinal

GvHD-related colitis (i.e., after approximately day 25). Importantly, however, we have not observed significantly increased complication rates dependent on the time point of endoscopy, as long as the endoscope is gently advanced under sufficient visibility. In contrast, we identified suboptimal drug dosing to be a risk factor since an insufficient depth of anesthesia can result in the occurrence of unwanted body movements of the experimental mouse and, consecutively, colonic wall perforation events. Second, the most critical step during the scoring process represents restrictions in this matter due to the unwanted presence of solid or liquid (e.g., diarrhea) feces in the gut lumen as the experimenter moves the endoscope into the scoring position. In this situation, flushing the colorectal region with saline solution is often required. However, since this procedure might compromise the scoring accuracy and specificity of several parameters (e.g., stool consistency), this caveat carefully needs to be taken into consideration upon scoring.

There are several limitations restricting the interpretation and conclusions drawn from this endoscopic approach. First, intrinsic to a methodology using an inflexible endoscope via the rectal route, evaluations are limited to the last 3–4 cm of the gastrointestinal tract (i.e., the rectum and the distal colon). Therefore, GvHD-associated affections of the proximal colon and even the small intestine are by default excluded from the evaluation, indicating that scientific questions focusing on the small intestinal phenotype of GvHD will not benefit from this approach. Second, although inflammation is one of the hallmark morphologic features of intestinal GvHD, additional characteristics, like an increased apoptosis rate of the intestinal epithelial cells and a loss of the intestinal crypt architecture, are often found and included in the histopathological workflow underlying intestinal GvHD scoring and grading by pathologists in allo-HCT patients. Here, we restrictedly correlated the mini-endoscopically assessed intestinal GvHD scores with histopathologically assessed signs of inflammation. Taking this approach, we came to the conclusion that colonic inflammation above a certain threshold in the colonoscopic scoring is reliably predicting the presence of moderate-to-high grade inflammation, as assessed by histopathological postmortem analyses. However, future studies need to investigate how, for instance, histopathologically assessed apoptosis rates relate to the mini-endoscopic total sum scores or to, for instance, one of the four individual parameters embedded in the sum score. Third, while mice suffering from histopathologically moderate-to-high-grade GvHD can be readily identified by the endoscopic scoring approach, mice with more subtle signs of intestinal inflammation may be missed by the described approach, given the fact that histopathological scoring revealed the presence of mild signs of inflammation in the group of noT mice undergoing allo-HCT alone, without the transplantation of alloreactive donor lymphocytes. Biologically, this finding might be feasible, since noT mice have been allotransplanted, and minimal colitis may, indeed, reflect the presence of low levels of GvHD due to an incomplete removal of T cells from the bone marrow cell fraction or the reconstitution of alloreactive T cells from the bone marrow over time. Future studies need to address these questions in more detail. Fourth, formally, this protocol focuses on fully established intestinal GvHD-associated colitis. However, as shown and published previously, the onset of colitis around day 15 can be endoscopically detected and quantitated[11]. Here, GvHD-prone mice could be clearly distinguished from noT mice in which the colonic phenotype might be solely due to residual signs of the irradiation-induced tissue damage or, rather, mirror the mucosal recovery response after irradiation[11]. However, future studies

need to correlate endoscopic evaluations and aim at detailed histopathological analyses of GvHD-prone mice compared to control mice at earlier time points.

Finally, an interesting, helpful add-on application of the endoscopic scoring of intestinal GvHD is the usage of the endoscopic working channel to direct a minimized biopsy forceps to the anatomic structure of choice in the colon. This feature provides, as a sort of built-in-option, the possibility to take view-guided tissue biopsies that can be further analyzed by a series of downstream techniques like histopathology, immunofluorescence staining, and real-time PCR analyses of genes of interest. However, future studies need to formally assess whether, for instance, histopathological scoring and grading of endoscopically taken biopsies are equivalent to histopathological results based on conventionally taken samples (i.e., from euthanized mice).

Overall, with this protocol describing the induction and its mini-endoscopic evaluation of intestinal GvHD, we provide a methodology to assess, score, and grade intestinal GvHD in a relevant murine GvHD model system of allo-HCT. The endoscopic assessment can be repeated in the same mouse at multiple time points over time, eliminating the need to euthanize mice to sequentially assess intestinal GvHD-associated tissue pathology (e.g., in a therapeutic setting). The mini-endoscopic scoring results proved to be comparable to results obtained by the histopathological assessment of intestinal inflammation in the colon and correlated well with systemic GvHD activity. Additionally, this procedure can be combined with view-guided biopsies via the working channel of the endoscope. Ultimately, however, future studies have to assess how far histopathologically determined morphological features other than inflammation, that are frequently found in intestinal GvHD-affected lesions, relate to the results obtained with this mini-endoscopic assessment and scoring approach.

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DISCLOSURES:
The authors have nothing to disclose.

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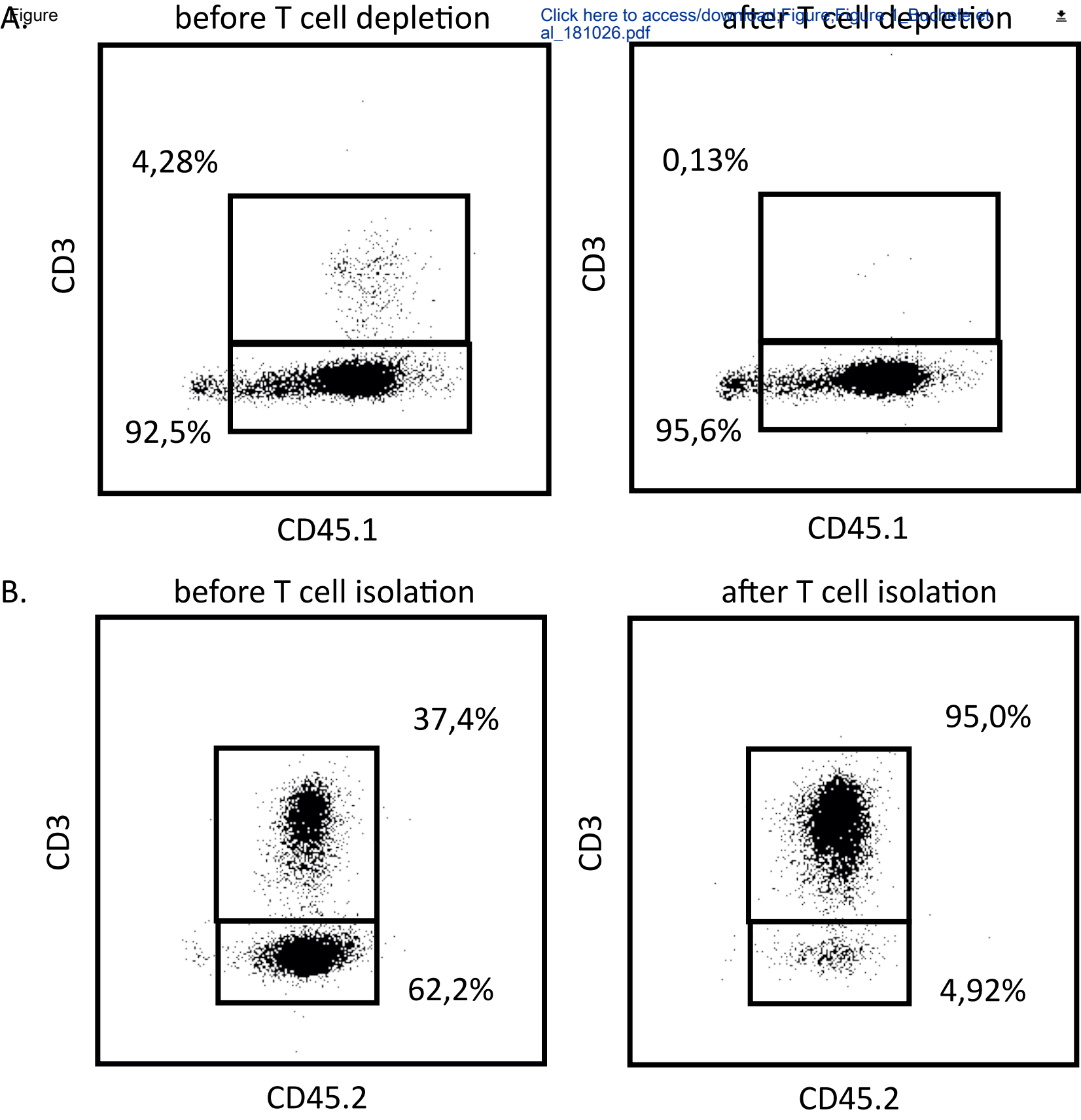

Figure

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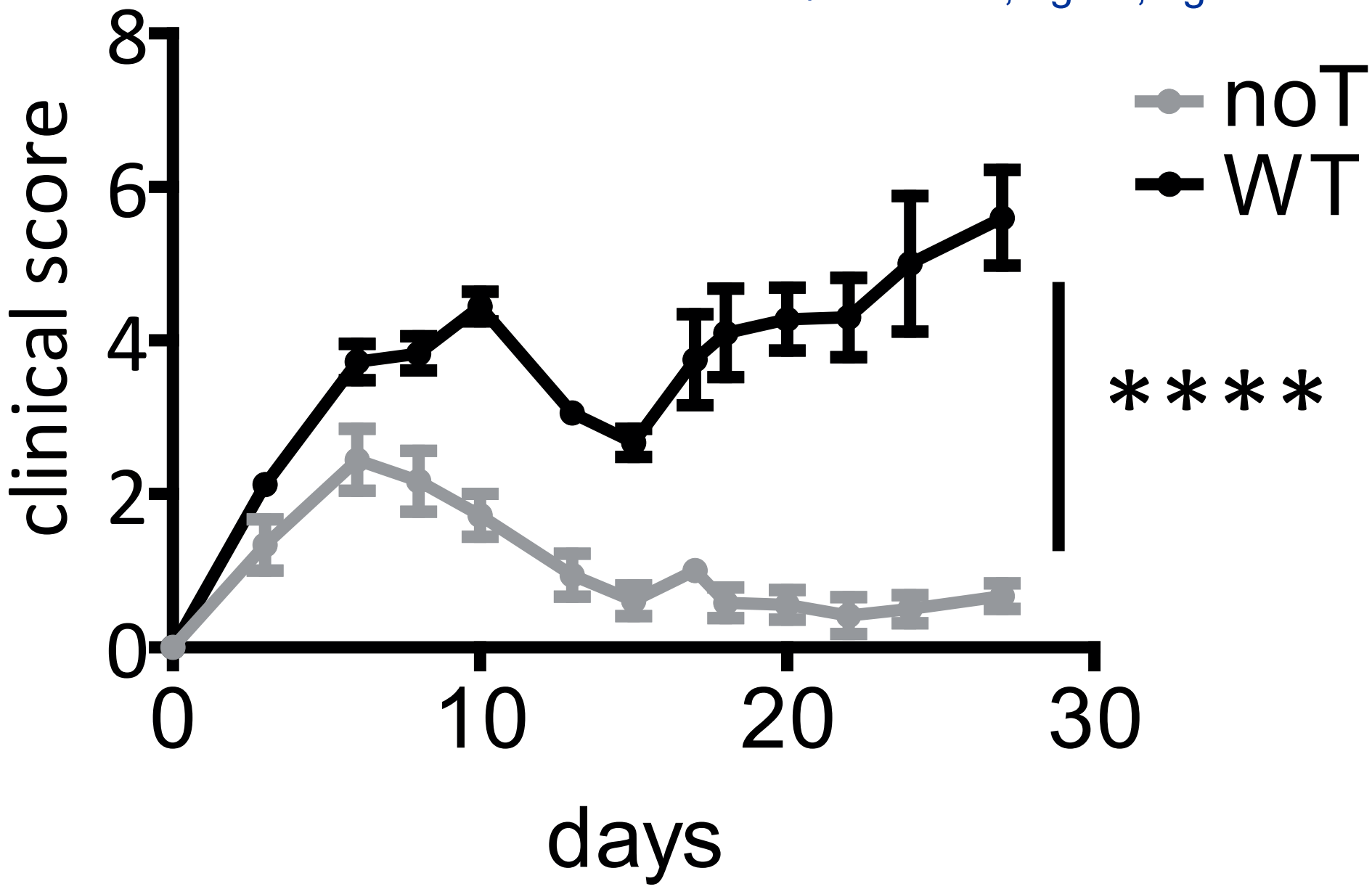

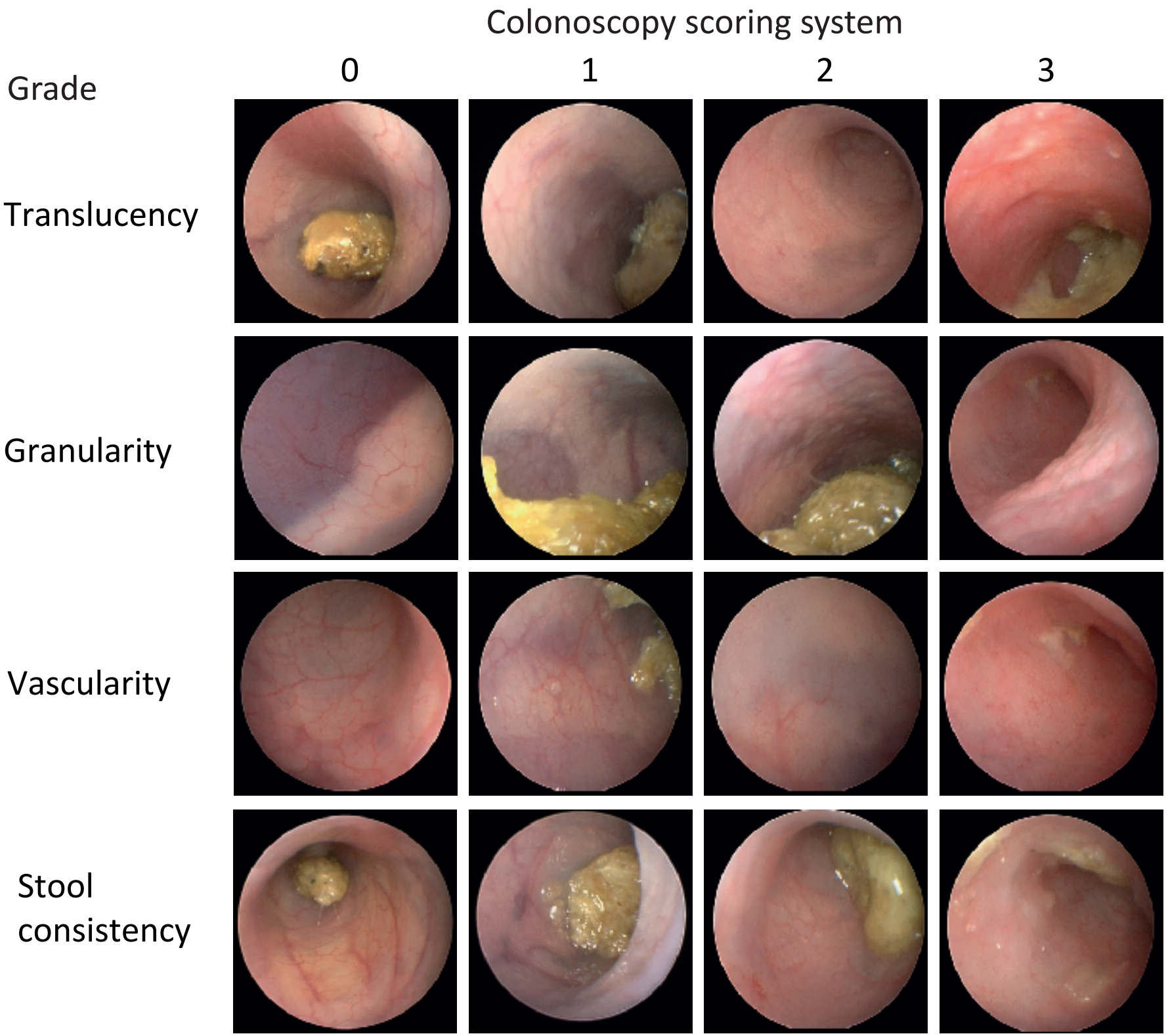

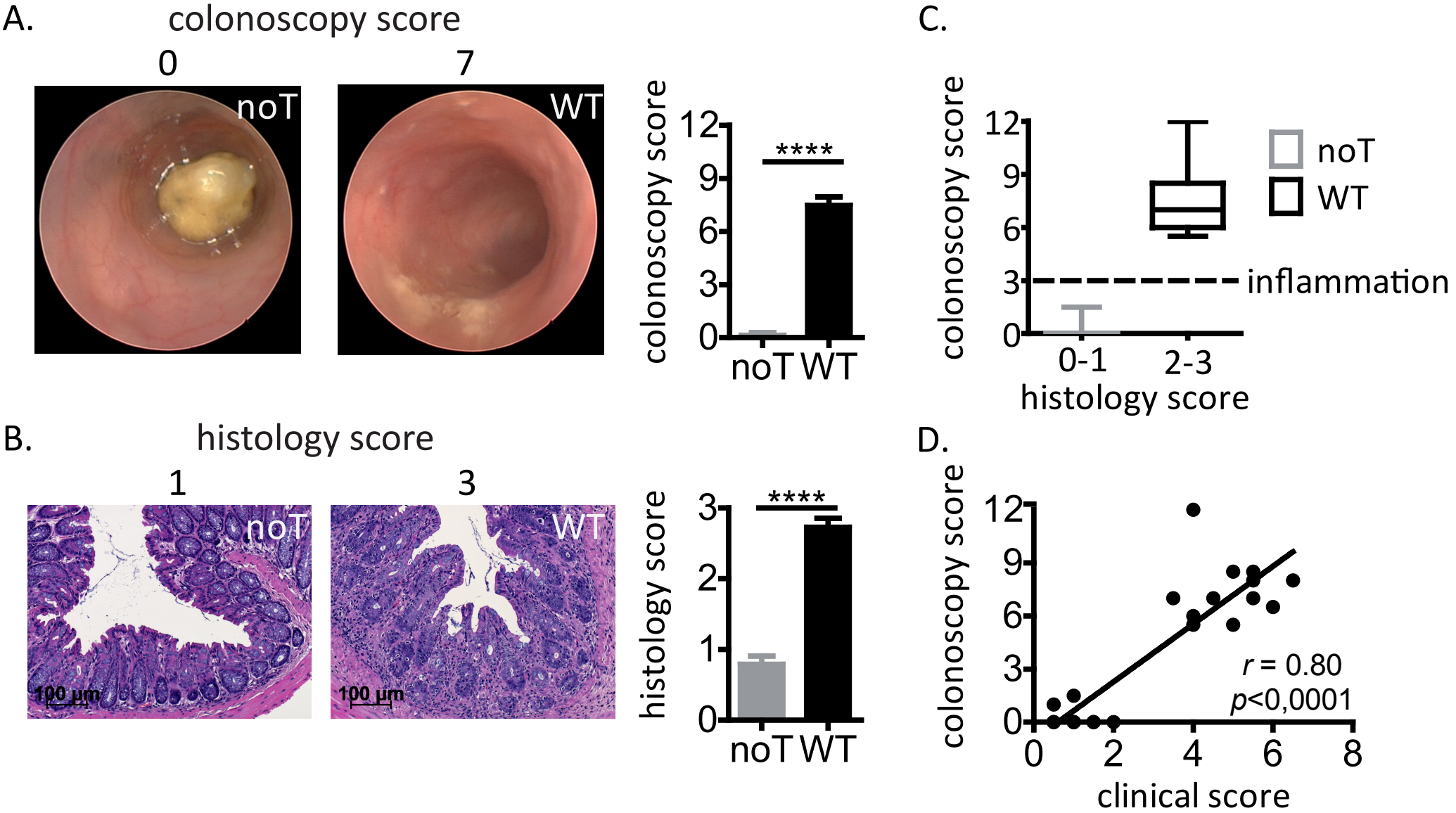

Table

Score	0	1	2	3
Translucency of the colonic wall	Extra-intestinal, inner organs (e.g. spleen) thoroughly visible	Discrete reduction of the visibility of extra-intestinal organs due to mild opacity of the colonic wall	Moderate reduction of the visibility of extra-intestinal organs due to significant opacity of the colonic wall	Lack of the visibility of extra-intestinal organs, i.e. non-transparent colonic wall
Granularity	Smooth, unaffected mucosal surface appearance; colonic crypt pattern visible	Discrete roughening and cobblestone appearance of the mucosal surface	Moderate roughening and cobblestone appearance of the mucosal surface	Severe roughening and cobblestone appearance of the mucosal surface; cushion-like appearance of the mucosa
Vascularity	Unaltered vascular pattern displaying the communicating network of large and small vessels	Discrete alterations of the vascular pattern; vessel pattern seems to fray	Some vessels are invisible; discontinuous vessel network	Contact induced bleeding; dot-like pattern of the vessels
Stool consistency	Normal; stool is hard; fine threads of mucus between feces and colonic wall can be observed	Stool is still shaped but may contain ragged edges; deformable with the tip of the endoscope	Stool is soft and unshaped stool is visibly more shining (higher water content)	Stool is loose, liquid; watery diarrhea. Stool may be randomly spread over the mucosal surface of the colon

Table of Materials

Click here to access/download;Table of Materials;Material_Buchele et al_10172018.xls

Name of Material/ Equipment	Company	Catalog Number
BIOBEAM 2000 Gamma Irradiator	Gamma-Service Meical GmbH	
Phosphate-buffered saline (PBS)	Sigma-Aldrich Co. LLC.	D8662-6x500ML
Semken Forceps (lenght: 13 cm; serrated, curved tips)	Fine Science Tools	11009-13
Hardened Fine Scissors (lenght: 8,5 cm; straight tips; cutting edge: 24 mm)	Fine Science Tools	14090-09
RPMI-1640 Medium	Sigma-Aldrich Co. LLC.	R8758-500ML
Hypodermic needle (26G)	B. Braun Melsungen AG	4657683
1 mL syring	B. Braun Melsungen AG	9166017V
50 mL tube	Sarstedt Ag & Co.KG	62,547,254
Cell strainer with a 40 µm mesh screen	BD Falcon	352340
Ammonium chloride (NH_4Cl)	Sigma-Aldrich Co. LLC.	11209
Ethylenediaminetetraacetic acid disodium salt dihydrate (Na_2EDTA)	Carl Roth GmbH & Co.KG	8043.2
Potassium bicarbonate ($KHCO_3$)	Merck KGaA	1,048,540,500
CD90.2 MicroBeads, mouse	Miltenyi Biotec GmbH	130-049-101
Pan T Cell Isolation Kit II, mouse	Miltenyi Biotec GmbH	130-095-130
Alexa Fluor 700 anti-mouse CD45.2 Antibody (clone: 104; lot: B252126; RRID: AB_493731)	Biolegend	109822
Pacific Blue anti-mouse CD3 Antibody (clone: 17A2; lot: B227246; RRID: AB_493645)	Biolegend	100214
FITC anti-mouse CD4 Antibody (clone: GK1.5; lot: B225057; RRID: AB_312691)	Biolegend	100406
PE/Cy7 anti-mouse CD45.1 Antibody (clone: A20; lot: B217246; RRID: AB_1134168)	Biolegend	110730
APC/Cy7 anti-mouse CD8a Antibody (clone: 53-6.7, lot: B247008; RRID: AB_312753)	Biolegend	100714
Filtrated bovine serum	Pan Biotec	P40-47500
96-well polystyrene V-bottom plates	Greiner Bio-One	651201
Polystyrene Round-Bottom Tube (5 mL)	Falcon	352052
BD LSRFortessa II flow cytometer	BD Bioscience Co.	
Insulin syringe with sterile interior (30G)	BD	324826
Oxy Vet Oxymat 3	Eickemeyer	
NarkoVet	Eickemeyer	213062
Plexiglass chamber	Eickemeyer	214620
Straight Forward Telescope	KARL STORZ SE &Co KG	64301 AA
Protection and Examination Sheath	KARL STORZ SE &Co KG	61029 C
Examination Sheath with working channel	KARL STORZ SE &Co KG	61029 D
Biopsy Forceps	KARL STORZ SE &Co KG	61071 ZJ
175 Watt SCB XenonLight Source	KARL STORZ SE &Co KG	20132120
Fiber Optic Light Cable	KARL STORZ SE &Co KG	495 NL
Image 1 S3 Camera Head	KARL STORZ SE &Co KG	22220030
Image 1 SCB Camera Control Unit	KARL STORZ SE &Co KG	22200020
LCD monitor	Olympus	OEV181H
Forane / Isofluran	AbbVie Inc.	B506
Formaldehyde solution 37 %	Carl Roth GmbH & Co.KG	7398.1
5,0 mL Dispenser tip	Eppendorf AG	30089456

Comments/Description
ingredient of ACK lysing buffer
ingredient of ACK lysing buffer
ingredient of ACK lysing buffer
magnet cell separation to isolate T cell-depleted bone marrow cells
magnet cell separation to isolate splenic T cells
ingredient of FACS buffer
oxygen concentrator for anesthesia
part of the experimental setup for colonoscopy
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Point-by-point letter

"Live mini-endoscopic assessment of acute intestinal Graft-versus-Host Disease in mice" by Vera Buchele et al., submitted together with the revised version from 10/18/2018

Reviewer #1:

Manuscript Summary:

The study by Buchele et al. provides the detailed description of a frequently used preclinical model for graft-versus-host disease (GVHD) followed by endoscopic evaluation of intestinal inflammation. Mouse models are key for our understanding of the complex pathophysiology of GVHD and to further investigate therapeutic approaches in a preclinical setting. However, with the large number of mouse models currently available and the methodological variations amongst the GVHD community, it might be difficult to determine and successfully perform a respective approach, especially for groups that are new in the field. Furthermore, disease severity is mainly assessed by clinical scoring systems with few indicators for intestinal inflammation during disease progress, followed by histological analysis post mortem. This study describes one of the most commonly used major-mismatch mouse models, which is easy to follow and thus likely to enhance reproducibility towards the community. Further, live-assessment of colonic inflammation displays an attractive approach for groups investigating intestinal GVHD.

Major Concerns:

The methodological procedures of this study are well described. The authors further highlight the benefits of a kinetic assessment of intestinal GVHD using endoscopy. However, in this study, only endpoint endoscopic evaluations have been performed around day 30. Have the authors also tried to evaluate earlier time points during disease progression? When are the first endoscopic lesions visible? Can the authors give any recommendations about when to perform the first endoscopic evaluation after allo-HSCT? Could irradiation-induced enteritis/colitis increase the risk of perforation?

Response to the reviewer:

We thank the reviewer for raising this important point. We sought to address these points in the revised discussion section, especially addressing the aspect of validity and sensitivity of the endoscopic approach when employed at earlier time points. According to our own published (Ullrich et al., JCI 2018) and unpublished observations, colonoscopy is suited to reproducibly and reliably detect allo-response driven colitis-induced lesions around day 15 after irradiation, i.e. 13 days after transfer of allogeneic T lymphocytes compared to noT mice receiving T cell depleted bone marrow only. At this time point routinely clinical onset of colitis can be observed. Here, endoscopic sum scores have usually returned to base line in control mice while GvHD-prone mice routinely display scores >3 with a sum score of above 3 in our experience represents a virtual threshold value indicative for the presence of colitis. However, formally we have not performed detailed correlation studies comparing endoscopic results with the gold standard histopathology. Therefore, at this point we have to refer to future studies to solidly investigate this matter to formally prove that endoscopic results are correctly staging colonic GvHD related inflammation early-on (i.e. around day 15) whereas we have shown that convincingly for the case of established colitis (i.e. around day 30) according to the data provided with this protocol.

We agree to the speculation of the reviewer that time points earlier than day 15 may be critical in respect to the specificity of the detected alterations by the endoscopic evaluation approach since irradiation induced lesions may be even more prevalent at that point and hence might dominate over the allo-T cell response-mediated GvHD-specific tissue damage. Also, as included in the revised discussion section, despite fully established intestinal GvHD perforation of the colon does not appear to represent a major adverse event in the case of proper anesthesia and careful execution of the endoscopic procedure. Hence and despite the fact that the risk of perforation during earlier time points with an assumed higher prevalence of irradiation-induced

enteritis/colitis has not been in the focus of this study and has not been formally investigated, we believe that proper execution of the endoscopy as outlined in the protocol is crucial but also effective to prevent an increased occurrence of perforation events even at earlier time points. However, as pointe out above, before around day15, the added value of such an analysis is questionable at that point.

Minor Concerns:

The introduction and the discussion contain the main and adequate aspects of clinical and molecular features of GVHD, that are also discussed properly. However, the authors might want to edit certain parts of the manuscript as the phrasing is sometimes redundant or sentences too lengthy (e.g. line 74-78).

Response to the reviewer:

We thank the reviewer for his request to increase readability of our manuscript. Accordingly, we shortened and streamlined the introductions section and revised the discussion section also taking editorial comments into consideration.

Reviewer #2:

Manuscript Summary:

An interesting detailed manuscript on mini endoscopy for assessment of murine GVHD

Major Concerns:

Please provide a more detailed analysis of endoscopic picture, histopathology and "clinical" GVHD scoring. This would significantly improve the manuscript to show how clinical grade, endoscopic grade and histopathology correlate.

Response to the reviewer:

We thank the reviewer for raising this important point. To accommodate the reviewer`s reasonable request, we added an analysis of the relationship between endoscopic scoring and systemic GvHD levels, as depicted in the new Figure 3D. Importantly, both colonic and systemic GvHD show a high grade of accordance in line with the clinical observation that severe intestinal GvHD is often associated with a profound systemic pathology and decreased quality of life due to systemic signs of inflammation.

Minor Concerns:

Introduction line 68: chronic GVHD is also mediated by donor lymphoctes

Introduction line 69: please add: "of the acute form of GVHD".....

Response to the reviewer:

We thank the reviewer for pointing out these important aspects. We rephrased the indicated passages accordingly.

Protocol: I am not sure, whether the whole transplant procedure is required in detail since most parts are published multiple times. Citation of the standard parts would be suffficient from my side. If it is the policy of the journal to provide all informations in detail I am fine with the text.

Response to the reviewer:

As we grasped the editorial policy, the detailed description of the model is favored to accordingly follow the steps leading to the core of this manuscript, i.e. the protocol how to endoscopically assess the intestinal GvHD phenotype in live mice. Hence, we currently resisted to shorten that part of the manuscript.

Line 388: delete "regardless"

Response to the reviewer:

We deleted the word “regardless”.

noT control mice: has the abbreviation been introduced?

Response to the reviewer:

We thank the reviewer for his question. We added a more detailed explanation of this abbreviation in line 336-338 to clarify this point.

Point-by point to the editorial comments _ JoVE58871R1

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-/-.

2. Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission.

We thoroughly reviewed the language and grammar of the manuscript including the revised passages to the best of our knowledge and under the given editorial time constraints.

3. The protocol section should almost entirely contain discrete steps, without large paragraphs of text between sections. Please break up the paragraphs into simple steps (with 2-3 actions per step) as much as possible throughout the protocol. Please be crisp.

We marked all changes we made in our manuscript to facilitate its tracking. Furthermore, as advised, we broke up the text of the protocol in several paragraphs trying to be in line with the journal's policy as indicated.

4. Please ensure that you describe the procedure step by step in order with all specific details required to do your experiment.

As *requested, we vigorously revised our manuscript by introducing additional subitems and indicating cross references where necessary.*

5. Once done please ensure that the protocol is no more than 10 pages long and the highlighted section is no more than 2.75 pages including heading and spacings. The highlighted section should form the most cohesive story of the protocol. Presently this is not the case. Also, notes cannot be filmed.

After editing our manuscript, the protocol section is in compliance with the editorial policy in respect to protocol length and length of the highlighted protocol section. Most importantly and as discussed in our email conversation, we have now included the core aspects of the GvHD induction protocol to make it more complete and comprehensive as requested. Accordingly, we adapted the title of the manuscript to reflect the current content of the highlighted narrative and the video recording.

6. Please ensure that there is a representative result for every section of the protocol.

Due to the inclusion of the GvHD induction in the highlighted protocol section, we added new figure (Figure1) to our manuscript displaying flow cytometry data to document representative results for the immune cell isolation procedure. The quality and quantity of transferred immune cell subpopulations is crucial in this context since it critically impacts the kinetics and dynamics of intestinal GvHD development. Therefore, the documentation of the successful isolation of the indicated cell types represents a crucial milestone assuring reproducible induction of intestinal GvHD.

7. Please ensure that the title is in alignment with the protocol described.

As discussed in our email conversation, we have now included the core aspects of the GvHD induction protocol as you suggested to make it a more comprehensive study. Accordingly, we adapted the title of the manuscript to "Induction of Intestinal Graft-Versus-Host Disease and its Mini-Endoscopic Assessment in Live Mice" thereby reflecting the current content of the highlighted narrative and the video recording.

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