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Video Article

# Development of an Antigen-driven Colitis Model to Study Presentation of Antigens by Antigen Presenting Cells to T Cells

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## Abstract

Inflammatory bowel disease (IBD) is a chronic inflammation which affects the gastrointestinal tract (GIT). One of the best ways to study the immunological mechanisms involved during the disease is the T cell transfer model of colitis. In this model, immunodeficient mice (RAG<sup>-/-</sup> recipients) are reconstituted with naive CD4<sup>+</sup> T cells from healthy wild type hosts.

This model allows examination of the earliest immunological events leading to disease and chronic inflammation, when the gut inflammation perpetuates but does not depend on a defined antigen. To study the potential role of antigen presenting cells (APCs) in the disease process, it is helpful to have an antigen-driven disease model, in which a defined commensal-derived antigen leads to colitis. An antigen driven-colitis model has hence been developed. In this model OT-II CD4<sup>+</sup> T cells, that can recognize only specific epitopes in the OVA protein, are transferred into RAG<sup>-/-</sup> hosts challenged with CFP-OVA-expressing *E. coli*. This model allows the examination of interactions between APCs and T cells in the lamina propria.

## Video Link

The video component of this article can be found at <http://www.jove.com/video/54421/>

## Introduction

The intestine is the largest surface of the body that is exposed to the external environment. Vast arrays of resident microbes colonize the human intestine to form the intestinal microbiota (or microflora). This is estimated to consist of up to 100 trillion microbial cells and constitutes one of the most densely populated bacterial habitats known in biology<sup>1-3</sup>. In the GIT bacteria colonize an intestinal niche where they survive and multiply<sup>4</sup>. In return, the microbiota endows the host with additional functional features not encoded on its genome<sup>1</sup>. For example the microbiota stimulates the proliferation of epithelial cells, produces vitamins that hosts cannot produce by themselves, regulates metabolism and protects against pathogens<sup>4-6</sup>. Given this beneficial relationship, some authors have suggested that humans are "super-organisms" or "holobionts" that are a mix of bacterial and human genes<sup>7,8</sup>. Given the beneficial impact of the microbiota on the (human) host, the intestinal immune system needs to tolerate commensal microbes to enable their existence in the lumen but also kill the pathogens that invade from luminal side<sup>9-11</sup>. The intestinal immune system has developed mechanisms to distinguish between harmless and potentially harmful luminal microbes; however these mechanisms are not yet well understood<sup>12</sup>. Maintaining intestinal integrity requires a tightly regulated immune homeostasis to keep the balance between tolerance and immunity<sup>13</sup>. An imbalance in immune homeostasis contributes to the induction of intestinal diseases such as inflammatory bowel disease (IBD)<sup>3,14</sup>.

There are two major types of IBD: Crohn's disease (CD) and ulcerative colitis (UC). Patients with these diseases usually suffer from rectal bleeding, severe diarrhea and abdominal pain<sup>15,16</sup>. The single cause of IBD is still unknown, but a combination of genetic factors, environmental influences and dysregulated immune responses might be the key event for disease development<sup>15</sup>.

Animal models for IBD have been used for over 50 years. In the last few decades new IBD model systems have been developed to test the various hypotheses concerning the pathogenesis of IBD<sup>17,18</sup>. The best-characterized model of chronic colitis is the T-cell transfer model that induces disruption of T-cell homeostasis<sup>19,20</sup>. This model involves transferring naive T cells from immunocompetent mice into hosts that lack T and B-cells (such as RAG<sup>-/-</sup> and SCID mice)<sup>16,21</sup>. The development of disease in this model is monitored for 3-10 weeks by evaluating the presence of diarrhea, reduced physical activity, and loss of body weight. This is so called the wasting syndrome<sup>16</sup>. Compared to the healthy mice the colonic tissue of transplanted hosts is thicker, shorter and heavier<sup>16</sup>. Using the T cell transfer model, it is possible to understand how different T cell populations can contribute to the pathogenesis of IBD<sup>22</sup>. The T cell transfer model does not analyze the interactions between APCs and T cells in the disease process in an antigen-specific manner. It has been shown that an interaction between myeloid cells and lymphoid cells could

be responsible for the development of intestinal inflammation<sup>23</sup>. Although many aspects of IBD have been clarified, the initial events that lead to the disease development still need to be clearly understood.

It has been shown that in the absence of microbiota transfer colitis cannot be established<sup>24</sup>. Recently, several theories suggest that IBD could be a result of an immune response against commensal bacteria<sup>25</sup>. Authors have also proposed that commensal bacteria are essential to induce inflammation in the distal intestine<sup>26</sup>. In germ free (GF) animals the intestinal immune system is generally impaired<sup>27,28</sup>, but a colonization of these mice with a mixture of specific-pathogen-free bacteria results in the development of the fully-competent intestinal immune system<sup>29</sup>. Hence, the microbiota seems to be a key element in the pathogenesis of IBD, either as a mechanism that predisposes to or protects against the development of intestinal inflammation<sup>30,31</sup>. Current theories suggest that IBD is a result of microbial imbalance, called dysbiosis, in genetically predisposed patients<sup>32</sup>, but it is not clear yet if the dysbiosis is the cause or the consequence of the disease<sup>12</sup>. Considering the role of microorganisms in the development of IBD, *in vitro* experiments showed that CD4<sup>+</sup> T cells can be activated by APCs pulsed with intestinal bacteria<sup>33,34</sup>.

Moreover, it has been shown that antigens from different commensal bacterial species, such as *E. coli*, *Bacteroides*, *Eubacterium* and *Proteus*, are able to activate CD4<sup>+</sup> T cells<sup>35</sup>. This indicates that presentation of bacterial antigens to T cells is of importance for the development of IBD. To reduce the complexity of multiple antigens derived by the microflora in the disease process, an *E. coli* strain has been created that produces the OVA antigen. Transfer colitis was induced by injecting OVA-specific T cells into RAG<sup>-/-</sup> animals colonized with OVA-expressing *E. coli*.

This model is based on recent evidence suggesting that CX<sub>3</sub>CR1<sup>+</sup> MPs, a major cell subset in the colonic lamina propria (cLP)<sup>36</sup>, are interacting with CD4<sup>+</sup> T cells during transfer colitis<sup>37</sup>. MPs sample the intestinal lumen for particulate antigen, such as bacteria, using their dendrites<sup>36,38,39</sup>. Previous studies demonstrated that MPs can also take up soluble antigens, such as OVA, introduced into the intestinal lumen<sup>40,41</sup>. Given the abundance of CX<sub>3</sub>CR1<sup>+</sup> MPs in the cLP, it is possible that these cells can sample luminal bacteria and interact with CD4 T cells. Confocal imaging of mice transplanted with OVA-specific CD4<sup>+</sup> T cells colonized with *E. coli* CFP-OVA, show that CX<sub>3</sub>CR1<sup>+</sup> MPs are in contact with OT-II CD4<sup>+</sup> T cell during the development of antigen-driven colitis. This model enables the study of the antigen presentation process between intestinal APCs and T cells specific only for particular antigen-expressing bacteria in the gut lumen.

## Protocol

Mice were bred and kept under specific pathogen-free (SPF) conditions in the animal facility of Ulm University (Ulm, Germany). All animal experiments were performed according to the guidelines of the local animal use and care committee and the National Animal Welfare Law.

### 1. Construction of the pCFP-OVA Plasmid

1. Amplify the full size OVA gene using the primers Ova\_SpeI\_fw (3'-GACCAACTAGTATGGAATTTGTTTTGATGTATT-5') and Ova\_ClaI\_rev (3'-GACCAGATCGATTAAGGGGAAACACATCTGCC-5')<sup>37</sup> using plasmid pCI-OVA (ampicillin resistant)<sup>42</sup> as a template. Clone the PCR product (*i.e.* the OVA coding sequence) into a commercially available phage mid-vector using SpeI and ClaI restrictions sites of the primers and vector to yield the specific OVA-plasmid.
2. Amplify the CFP-coding gene together with the strong constitutive promoter *P<sub>hyper</sub>* using plasmid pAD<sub>1</sub>-cCFP (chloramphenicol resistant)<sup>43</sup> as a template and primers CFP\_SacII\_fw (3'-GATCGACCGCGTTCTTGAAGACGAAAGGGCC-5') and CFP\_SpeI\_rev (3'-GATCGAACTAGTACCACCACCACCACCTTTGTAGAGTTCATCCATGC-5')<sup>37</sup>.
3. Clone the CFP-coding gene into the specific OVA-plasmid (ampicillin resistant) using the SpeI and SacI restriction sites of the primers and vector<sup>37</sup>. This introduces a spacer of 6 glycine residues between the CFP-coding from OVA-coding sequence.

### 2. Construction of *E. coli* pCFP-OVA

1. Grow *E. coli* strain DH10B in 100 ml of Luria Bertani (LB) medium aerobically at 37 °C on a rotary shaker overnight.
2. Mix 800 µl of bacterial culture with 200 µl of glycerol to create glycerol stock and store at -80 °C.
3. Add 50 µl of *E. coli* strain DH10B from glycerol stock in 5 ml of LB medium and grow them aerobically at 37 °C on a rotary shaker overnight.
4. Transfer the culture in a baffled 1 L Erlenmeyer flask with 250 ml of LB medium at 37 °C in a rotary shake.
5. Grow bacteria to an optical density at 600 nm (OD<sub>600</sub>) of 0.5 and then chill the culture on ice for 30 min. Centrifuge the bacteria at 5,000 x g for 10 min at 4 °C.
6. Resuspend bacteria with 100 ml of ice-cold sterile H<sub>2</sub>O and centrifuge the bacteria at 5,000 x g for 10 min at 4 °C. Resuspend bacteria with 100 ml of ice-cold 10% glycerol and centrifuge the bacteria at 5,000 x g for 10 min at 4 °C. Repeat this last step twice.
7. After the final wash step, resuspend bacteria in 700 µl of 10% glycerol. Freeze 50 µl aliquots in liquid nitrogen and store at -80 °C.
8. Thaw a 50 µl aliquot on ice and transfer into a pre-chilled electroporation cuvette (2 mm electrode distance).
9. Add 5 µl of plasmid DNA (100 ng) to the 50 µl *E. coli* aliquots. Perform electroporation with a single pulse at 25 µF, 200 ohms (Ω) and 2.5 kV.
10. Resuspend bacteria in 1 ml pre-warmed SOC medium (5 g/L yeast extract, 2 g/L tryptone, 10 mM sodium chloride, 2.5 mM potassium chloride, 10 mM magnesium chloride, 10 mM magnesium sulfate) and incubate at 37 °C aerobically for 1 hr.
11. Plate bacteria on LB-agar plates supplemented with ampicillin 100 µg/ml (add 100 µl of a 100 mg/ml ampicillin stock to 100 ml of LB-agar) and incubate overnight aerobically at 37 °C.
12. Pick single colonies and re-streak them in LB-agar plates supplemented with ampicillin 100 µg/ml. Incubate overnight aerobically at 37 °C.
13. Pick single colonies and grow them overnight in 10 ml of LB media supplemented with ampicillin 100 µg/ml for plasmid isolation. Use commercially available plasmid mini prep kit and elute plasmid DNA with 30 µl dH<sub>2</sub>O (distilled water) according to manufacturer's protocol. Verify plasmids by restriction analysis and DNA sequencing<sup>37,42,43</sup>.
14. Set up a culture of positive clones of *E. coli* pCFP-OVA in 100 ml of LB medium supplemented with ampicillin 100 µg/ml. Grow aerobically at 37 °C on a rotary shaker overnight.
15. Mix 800 µl of bacterial culture with 200 µl of pure glycerol and store at -80 °C.

16. Centrifuge the rest of the overnight culture at 5,000 x g for 10 min at room temperature (RT) and resuspend the pellet in phosphate buffered saline (PBS). Place 10  $\mu$ l of the suspension onto glass slide and cover it with a coverslip.
17. Observe the samples in a standard fluorescence microscopy to confirm the expression fluorescence of CFP-OVA (CFP excitation at 450 nm, emission at 480 nm). Analyze the bacteria starting with magnification 400X.
18. Centrifuge 1 ml of bacteria from overnight cultures at 5,000 x g for 10 min, RT. Resuspend the pellet in 40  $\mu$ l of PBS and boil at 95 °C for 10 min.
19. Centrifuge the samples at 30,000 x g for 10 min and use the supernatants for Western blot analysis<sup>37</sup>. Use the rabbit-raised anti-OVA polyclonal antibody diluted 1:400.

### 3. Generation of OT-II/Red Mice

1. Cross a male/female OT-II mouse with a female/male mouse expressing red fluorescent protein (both strains commercially available) in order to generate the OT-II/Red mice<sup>37</sup>.

### 4. Spleen Cell Isolation

1. Euthanize the OT-II/Red mice by cervical dislocation after anesthesia by inhalation of any halogenated ether (up to 5% for induction) commercially available.
2. Use scissors to cut the left superior abdominal part of the mouse. Under this abdominal part there is the spleen (oval shape with a dark-red color). Use scissors and tweezers to remove it.
  1. Extract the spleen of the OT-II/Red mice and pass it through a 100  $\mu$ m cell strainer positioned on a 50 ml tube using the plunger of a 1 ml syringe in order to obtain a single-cell suspension. Wash the strainer twice with 10 ml of PBS supplemented with 1% heat inactivated fetal bovine serum (FBS).
3. Centrifuge the cell suspension at 390 x g for 5 min at RT. Resuspend the pellet with 5 ml of pre-warmed lysis buffer (144 mM NH<sub>4</sub>Cl, 17 mM Tris, pH= 7.2) to lyse the red blood cells. Incubate 10 min at 37 °C.
4. Wash the sample with 25 ml of PBS supplemented with 1% FBS. Centrifuge at 390 x g for 5 min at RT. Resuspend the pellet in 10 ml of PBS supplemented with 1% FBS.
5. Count the cells using a Neubauer counting chamber. Mix 10  $\mu$ l of the cells suspension with 10  $\mu$ l of a 0.4% Trypan Blue solution. Incubate for 1-2 min.
  1. Fill the hemocytometer chamber with 10  $\mu$ l of stained cells. Count cells under the microscope in four 1 x 1 mm<sup>2</sup> squares of one chamber and determine the average number of cells per square. Viable cells appear white, dead cells appear blue. To determine the number of cells/ml multiply cell number by factor of dilution. If 2-4 quadrants are being counted, divide by the number of quadrants.

### 5. CD4<sup>+</sup> CD62L<sup>+</sup> T Cell Enrichment

1. Enrich spleen cells for CD4<sup>+</sup> CD62L<sup>+</sup> T cells via magnetic isolation kit using the principles of negative or positive selection. Here, use negative selection to isolate CD4<sup>+</sup> T cells and use positive selection to further enrich for the CD62L<sup>+</sup> population within the CD4<sup>+</sup> T cell pool.  
NOTE: In the negative selection procedure, the cells, which will be not isolated (here: all CD4 negative cells) are labeled with specific antibodies coupled with magnetic microbeads. During the column washing steps the CD4<sup>+</sup> T cells will pass through the column so that they can be easily collected. In the positive selection procedure cells to be isolated are labeled with appropriate antibody coupled with magnetic microbeads. During the column washing steps the labeled cells will stay in the column and they can be collected by removing the column from the magnetic field and rinsing it with the buffer.
2. For the negative selection procedure, resuspend 10<sup>7</sup> total spleen cells in 40  $\mu$ l of cold PBS supplemented with 0.5% bovine serum albumin (BSA). Add 10  $\mu$ l of biotin labeled antibody cocktail supplied with the magnetic isolation kit (specific antibodies binding the non-CD4<sup>+</sup> T cells) and incubate 15 min on ice.
  1. Add 30  $\mu$ l of cold PBS supplemented with 0.5% BSA and 20  $\mu$ l of anti-biotin microbeads. Incubate 20 min on ice.
  2. Add to the sample 10 ml of PBS supplemented with 0.5% BSA and centrifuge at 390 x g for 5 min. Repeat this step twice. Resuspend the cells in 1 ml of cold PBS supplemented with 0.5% BSA.
  3. Place the column specific for the negative selection procedure in the magnetic field and rinse with 3 ml of cold PBS supplemented with 0.5% BSA onto the column. Add the cell suspension onto the column and wash 3 times with 3 ml of cold PBS supplemented with 0.5% BSA.
  4. Centrifuge cells of the effluent at 390 x g for 5 min. Effluent passing through the column contains unlabeled CD4<sup>+</sup> T cells. Resuspend the pellet in 1 ml of PBS supplemented with 0.5% BSA and count the cells.
  5. Count the cells using a Neubauer counting chamber. Mix 10  $\mu$ l of the cells suspension with 10  $\mu$ l of a 0.4% Trypan Blue solution. Incubate for 1-2 min.
    1. Fill the hemocytometer chamber with 10  $\mu$ l of stained cells. Count cells under the microscope in four 1 x 1 mm<sup>2</sup> squares of one chamber and determine the average number of cells per square. Viable cells appear white, dead cells appear blue. To determine the number of cells/mL multiply cell number by factor of dilution. If 2-4 quadrants are being counted, divide by the number of quadrants.
3. For the positive selection procedure, add 10  $\mu$ l of CD62L microbeads per 10<sup>7</sup> pre-enriched CD4<sup>+</sup> T cell fraction. Incubate 15 min on ice.
  1. Add to the sample 10 ml of PBS supplemented with 0.5% BSA and centrifuge at 390 x g for 5 min. Repeat this step twice. Resuspend in 500  $\mu$ l of cold PBS supplemented with 0.5% BSA.

2. Place the specific column for the negative selection procedure in the magnetic field and rinse with 500  $\mu$ l of cold PBS supplemented with 0.5% BSA onto the column. Add cell suspension onto the column and wash 3 times with 500  $\mu$ l of cold PBS supplemented with 0.5% BSA.
3. Remove the column from the magnetic field and place it on a suitable collection tube. Add 1 ml of PBS supplemented with 0.5% BSA onto the column and flush out CD4<sup>+</sup> CD62L<sup>+</sup> T cells retained in the column by firmly pushing the plunger into the column.  
NOTE: The isolated CD4<sup>+</sup> CD62L<sup>+</sup> T cells are now ready for downstream applications, such as *in vivo* experiments. According to the manufacturer's instruction from the CD4<sup>+</sup> CD62L<sup>+</sup> T cells isolation kit, the microbeads are not toxic. Due to the small size (around 50 nm), they do not activate cells and they will not saturate cell surface epitopes. Therefore the isolated cells can be transferred into immunodeficient mice to induce colitis<sup>37,44,45</sup>.
4. Count the cells using a Neubauer counting chamber. Mix 10  $\mu$ l of the cells suspension with 10  $\mu$ l of a 0.4% Trypan Blue solution. Incubate for 1-2 min.
  1. Fill the hemocytometer chamber with 10  $\mu$ l of stained cells. Count cells under the microscope in four 1 x 1 mm<sup>2</sup> squares of one chamber and determine the average number of cells per square. Viable cells appear white, dead cells appear blue. To determine the number of cells/ml multiply cell number by factor of dilution. If 2 - 4 quadrants are being counted, divide by the number of quadrants.

## 6. Induction of Antigen-driven Colitis

1. Inject intraperitoneally 3 x 10<sup>5</sup> OT-II/Red CD4<sup>+</sup> CD62L<sup>+</sup> T cells into CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice. For induction of antigen-specific colitis, administer *E. coli* pCFP-OVA every second day at a dose of 1 x 10<sup>8</sup> colony-forming unit (CFU) in 100  $\mu$ l of PBS per animal by gavage or oral instillation behind the incisors.
2. Monitor twice weekly the body weight of transplanted mice and their clinical condition (presence of blood in the stool, stool consistency and activity of mice).

## 7. Tissue Samples for Histopathological Examination

1. Take tissue samples from the large intestine of mice, fix in neutral-buffered formalin (10%) and embed in paraffin as previously described<sup>36,46</sup>.
2. Section the paraffin samples on a microtome, mount on slides, and perform the H&E stain<sup>47,48</sup>.
3. Categorize the histology of the large intestine as published previously<sup>47,48</sup>: normal (score 0); mild colitis (score 1), moderate colitis (score 2) and severe colitis (score 3).

## 8. Isolation of Colonic Lamina Propria Cells

1. Euthanize transplanted mice by cervical dislocation after anesthesia by inhalation of any halogenated ether (up to 5% for induction) commercially available.
2. Place the animal down with the belly facing up. Use forceps to grab the skin anteriorly to the urethral opening. Use scissors to cut the skin along the ventral midline from the groin to the chest. Pull the skin back on the sides.
3. Cut through the transparent peritoneal muscle wall and opening up the body cavity. Identify the colon that goes from the anus to the caecum<sup>49</sup>. Cut the 2 extremities and free the tissue from any fat.
4. Open the colon longitudinally using a dissection scissors. Use a tweezers to shake the colon tissue in a petri dish containing 25 ml of PBS supplemented with 1% FBS to remove debris and mucous. Cut the colon into 5 - 8 mm pieces.
5. Place the colon segments in a 50 ml tube containing 20 ml of the Wash Buffer. The Wash buffer contains 500 ml DPBS, 10mM Hepes and 5mM ethylenediaminetetraacetic acid (EDTA).
6. Vortex the tube for 30 sec at maximal speed.
7. Incubate the tube with the colon segments in a water bath at 37 °C with 200 rpm shaking for 10 min.
8. After the incubation, vortex the tube for 30 sec at maximal speed.
9. Discard the supernatants and place the tissue samples in a new 50 ml tube containing 20 ml of the Wash buffer. Repeat steps 6-9 three times.
10. Discard the supernatants and place the tissue samples in a petri dish containing 25 ml of PBS. Use a tweezers to shake gently the tissue samples in the petri dish for few seconds to remove residual epithelial cells. Repeat this step three times.
11. Cut colonic tissues into 2 x 2 mm<sup>2</sup> pieces and digest them by incubation in 10 ml of Roswell Park Memorial Institute (RPMI) medium with 0.5 mg/ml collagenase type VIII from *Clostridium histolyticum* and 10 U/ml of DNaseI.
12. Vortex the tube for 30 sec at maximal speed.
13. Incubate the colon fragments in a water bath at 37 °C with 200 rpm shaking for 35 min and vortex the tube for 30 sec at maximal speed every 5 min.
14. At the end of the incubation vortex the tube for 30 sec at maximal speed.
15. Collect the digested fragments by passing them through a 70  $\mu$ m cell strainer positioned on a new 50 ml tube.
16. Wash the sample by adding 20 ml of DPBS and centrifuge at 400 g for 5 min. Then resuspend the cells in 1 ml of DPBS.
17. Count the cells using a Neubauer counting chamber. Mix 10  $\mu$ l of the cells suspension with 10  $\mu$ l of a 0.4% Trypan Blue solution. Incubate for 1-2 min.
  1. Fill the hemocytometer chamber with 10  $\mu$ l of stained cells. Count cells under the microscope in four 1 x 1 mm<sup>2</sup> squares of one chamber and determine the average number of cells per square. Viable cells appear white, dead cells appear blue. To determine the number of cells/ml multiply cell number by factor of dilution. If 2-4 quadrants are being counted, divide by the number of quadrants.

## 9. Extracellular Staining for FCM Analysis

1. Centrifuge the cLP cells 5 min at 390 x g. Resuspend the cells in 1 ml of FACS A (PBS supplemented with 1% BSA and 0.1% sodium azide) NOTE: Sodium azide is highly toxic. It can cause hypotension, hypothermia, headache convulsions or death. Dilute solutions of sodium azide (0.1 to 1.0%) usually do not present extraordinary dangers to the user, but they are eye and skin irritants. Therefore, use sodium azide only in the chemical hood while wearing a laboratory coat and a double pair of disposable nitrile gloves.
2. Incubate the cells for 15 min at RT with the monoclonal antibody (mAb) 2.4G2 directed against the FcγRIII/II CD16/CD32 (0.5-1 μg mAb/10<sup>6</sup> cells) diluted in FACS A.
3. Add 200 μl of FACS A to the cells and centrifuge 5 min at 390 x g. Repeat this step twice. Incubate the cells with 0.5 ng/10<sup>6</sup> cells of the relevant mAb for 20 min at 4 °C. Dilute the antibodies in FACS A.
4. Add 200 μl of FACS A to the cells and centrifuge 5 min at 390 x g. Repeat this step twice. Resuspend the samples in 100 μl of FACS A.
5. Analyze T cells from CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice reconstituted with OT-II/Red CD4 T cells at the flow cytometer<sup>37</sup>.
6. Analyze data using commercially available software.

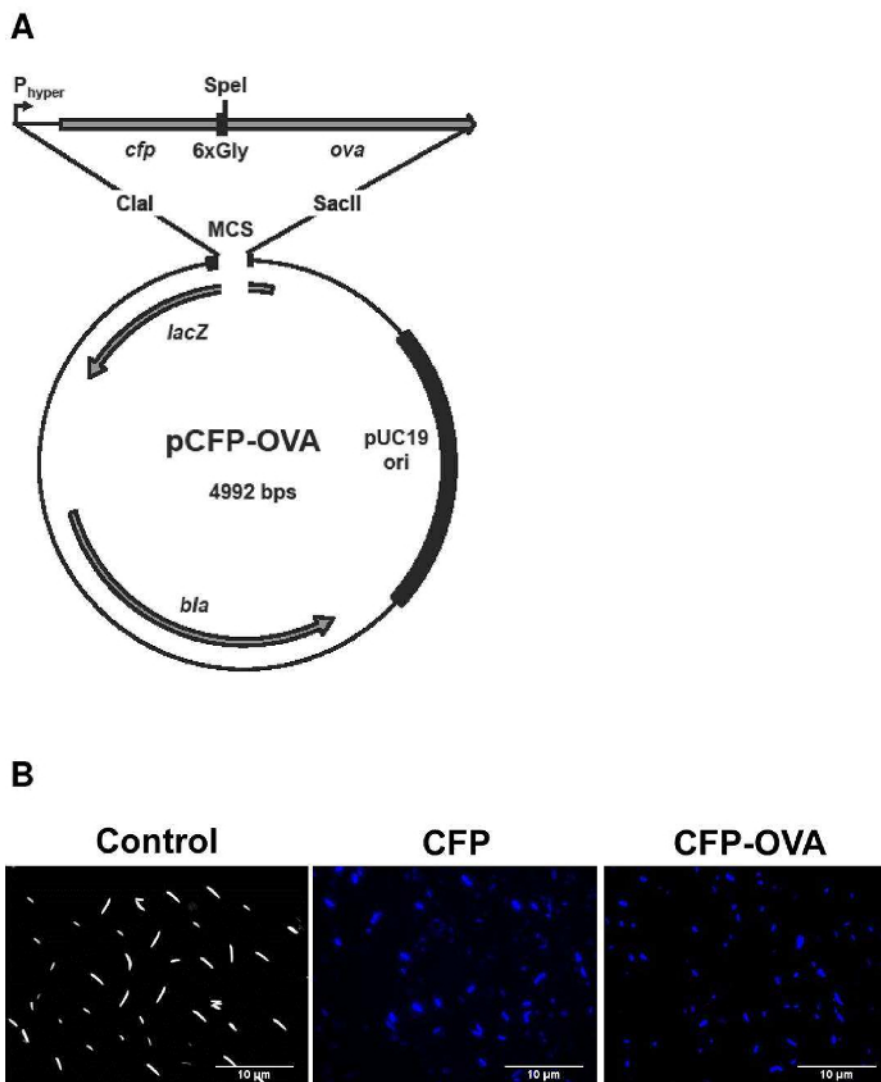
## 10. Confocal Microscopy Analysis

1. Analyze the colon of CX<sub>3</sub>CR1<sup>GFP/+</sup> mice reconstituted or not with OT-II/Red CD4<sup>+</sup>CD62L<sup>+</sup> T cell and fed orally every second day with 1 x 10<sup>8</sup> CFU of *E. coli* strain DH10B pCFP-OVA.
2. Remove the colon from the mice using scissors and tweezers. Open it longitudinally using a dissection scissors.
3. Cut a 5-8 mm piece of colon sample using scissors, put onto glass slides and cover with coverslips.
4. Analyze the colon samples with a confocal microscope at a magnification of 40/1.30.
5. Detect the CX<sub>3</sub>CR1<sup>+</sup> MPs, labeled with green fluorescein protein (GFP), using a filter set with excitation at 488 nm and emission at 509 nm.
6. Detect the CD4<sup>+</sup> T cell expressing red protein using a filter set with excitation at 554 nm and emission at 586 nm.
7. Detect *E. coli* pCFP-OVA using a filter set with excitation at 450 nm and emission at 480 nm.
8. Define an area of interest and generate scatter diagrams as previously described<sup>50</sup> to carry out the co-localization analysis. Use 2 different formulas: the Overlap Coefficient according to Manders and the Pearson's Coefficient.

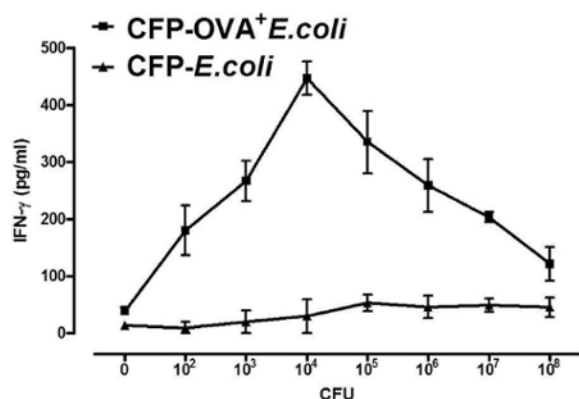
## Representative Results

To establish an antigen-driven colitis model an *E. coli* strain has been constructed that contains a plasmid in which the gene for the CFP is fused to the coding sequence for the chicken ovalbumin protein and the fusion construct is expressed under control of the strong constitutive promoter *P<sub>hyper</sub>* (**Figure 1A**). Fluorescent microscopy shows that the recombinant *E. coli* pCFP-OVA, but not the parental *E. coli* DH10B, expresses CFP (**Figure 1B**). CFP-OVA- producing *E. coli* is able to activate OT-II cells *in vitro* and induce IFN-γ production in contrast to an *E. coli* strain expressing CFP only (**Figure 2**). **Figure 3A** shows *ex vivo* 3D confocal imaging of the colonic tissue of CX<sub>3</sub>CR1<sup>GFP/+</sup> mice fed with *E. coli* pCFP-OVA. *E. coli* pCFP-OVA can be detected within colonic crypts located close to intestinal epithelial cells and co-localizes with CX<sub>3</sub>CR1<sup>+</sup> phagocytes. To determine the relative proportion of *E. coli* pCFP-OVA internalized by defined CX<sub>3</sub>CR1<sup>+</sup> phagocytes, the blue and green fluorescence intensity were determined by scatter blots. The analysis revealed that CX<sub>3</sub>CR1<sup>+</sup> cells sampled 11.9 ± 1.5% of *E. coli* pCFP-OVA detected in the colon (**Figure 3B**). In OT-II/Red animals, CD4<sup>+</sup> T cells are characterized by Red expression and Vβ 5.1 expression shown by flow cytometry (**Figure 4**). **Figure 5A** shows a general overview of the antigen driven colitis model. OT-II/Red<sup>+</sup> CD4<sup>+</sup> T CD62L<sup>+</sup> cells were adoptively transferred into CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> recipients that were then gavaged every second day with *E. coli* pCFP-OVA. When challenged with *E. coli* pCFP-OVA, transferred CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice lose body weight and develop clinical signs of colitis (**Figure 5B**). **Figure 6** shows *ex vivo* 3D confocal imaging of colonic tissue 7, 14 and 21 days after reconstitution of heterozygous CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> recipients with OT-II/Red<sup>+</sup> cells challenged with *E. coli* pCFP-OVA. Seven days after cell transfer only a few CX<sub>3</sub>CR1<sup>+</sup> phagocytes have sampled *E. coli* pCFP-OVA and OT-II/Red<sup>+</sup> cells were not detected. Fourteen days after cell transfer, CX<sub>3</sub>CR1<sup>+</sup> phagocytes that sampled *E. coli* pCFP-OVA were located close to OT-II/Red<sup>+</sup> cells. Twenty-one days after cell transfer a high number of CX<sub>3</sub>CR1<sup>+</sup> cells that have sampled *E. coli* pCFP-OVA and OT-II/Red<sup>+</sup> cells, dispersed throughout the cLP in close proximity to CX<sub>3</sub>CR1<sup>+</sup> phagocytes.



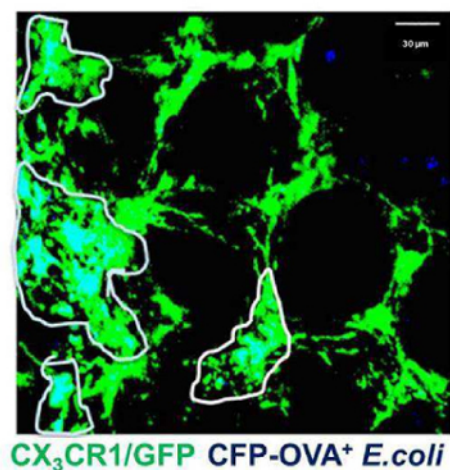


**Figure 1: CFP-OVA Producing *E. coli*.** (A) Map of pCFP-OVA with relevant restriction sites, the gene encoding OVA and the bright blue fluorescent protein CFP. (B) Bright field and fluorescent microscopic images of wild type *E. coli* DH10B, *E. coli* DH10B pCFP and *E. coli* DH10B pCFP-OVA. Scale bar: 10  $\mu$ m. [Please click here to view a larger version of this figure.](#)

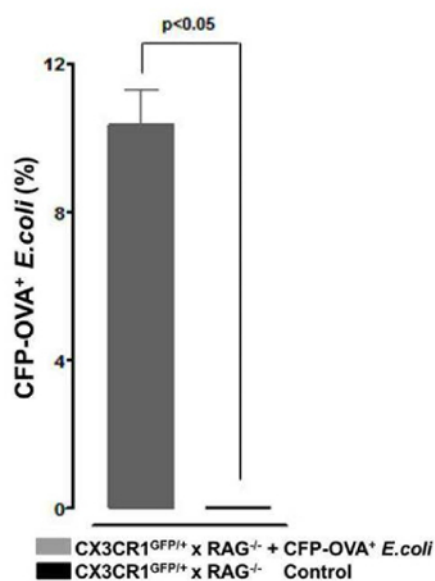


**Figure 2: OT-II Cells Stimulated with CFP-OVA Produce IFN- $\gamma$ .** After isolation from spleen, OT-II cells were stimulated with the indicated numbers of *E. coli* DH10B pCFP-OVA. Bacterial cells were inactivated following 2 hr incubation with 5  $\mu$ g/ml gentamicin. After 72 hr of culture, supernatants were collected and IFN- $\gamma$  concentrations determined by ELISA. Experiments were carried out in triplicates and data presented as mean  $\pm$  SEM. [Please click here to view a larger version of this figure.](#)

A

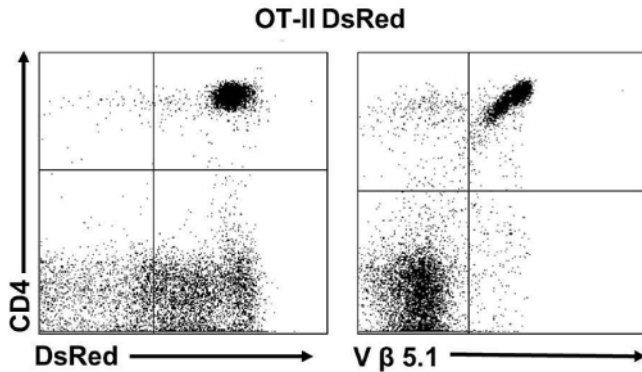


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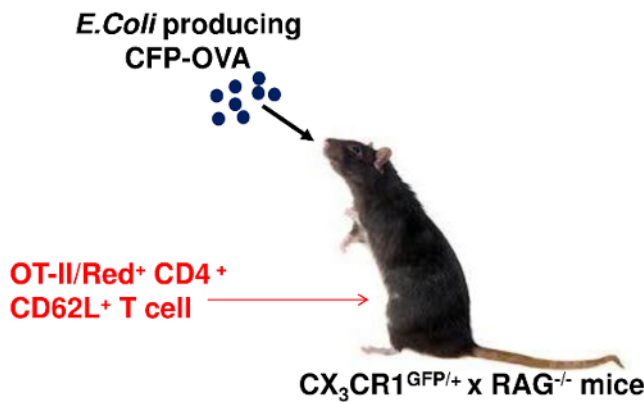
**Figure 3: CX<sub>3</sub>CR1<sup>+</sup> Phagocytes Sample *E. coli* pCFP-OVA.** (A) cLP tissue of CX<sub>3</sub>CR1<sup>GFP/+</sup> mice gavaged with  $1 \times 10^8$  *E. coli* pCFP-OVA for 7 days and analyzed by ex vivo confocal microscopy. Magnification 40X/1.30. Scale bar = 30 μm. (B) The percentage of internalized *E. coli* pCFP-OVA quantitated and data presented as mean ± SEM. In the Mann-Whitney test  $p < 0.05$  was considered statistically significant. [Please click here to view a larger version of this figure.](#)



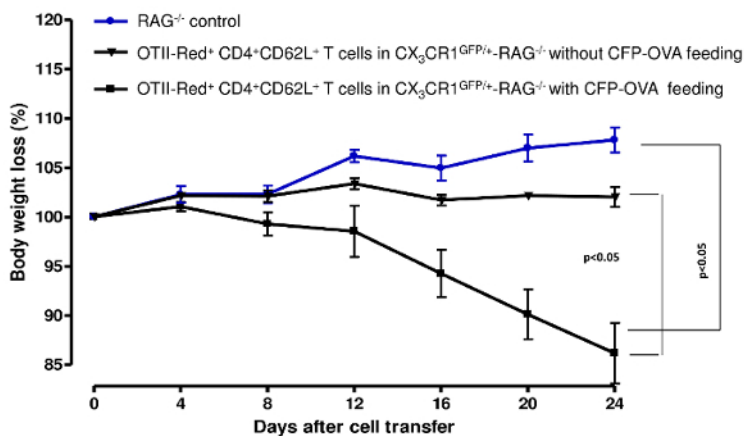


**Figure 4: Characterization of OT-II/Red<sup>+</sup>CD4<sup>+</sup> T Cells.** (A) OT-II transgenic animals were crossed with animals expressing the red fluorescent protein to obtain OT-II/Red<sup>+</sup> cells. OT-II/Red<sup>+</sup> cells were isolated from spleens of OT-II/Red transgenic animals, stained for CD4 and Vβ5.1 and analyzed by flow cytometry. [Please click here to view a larger version of this figure.](#)

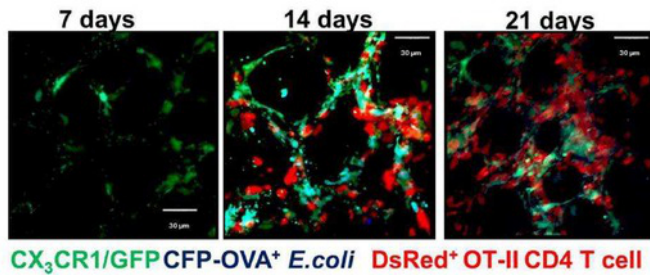
A



B



**Figure 5: OVA-driven Transfer Colitis.** (A) Schematic representation of the antigen driven colitis model. OT-II/Red<sup>+</sup> CD4<sup>+</sup> T CD62L<sup>+</sup> cells were adoptively transferred into CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice and hosts were gavaged every second day with *E. coli* pCFP-OVA. (B) Body weight of the indicated groups was measured twice a week. Mean  $\pm$  SEM body weight loss (%) is presented. In the Mann-Whitney test  $p < 0.05$  was considered statistically significant. [Please click here to view a larger version of this figure.](#)



**Figure 6: CX<sub>3</sub>CR1<sup>+</sup> MPs Communicate with OT-II Cells During OVA-driven Transfer Colitis.** (A) Colonic tissue samples were examined by *ex vivo* 3D confocal imaging at day 7, 14 and 21 after transfer of OT-II/Red<sup>+</sup> CD4<sup>+</sup> T CD62L<sup>+</sup> cells in CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> recipients. Magnification 40X/1.30. Scale bar = 30 μm.

## Discussion

As with every other model, the antigen-driven colitis model described above may present few issues that the investigator performing the technique must be aware of. When injecting the OT-II/Red<sup>+</sup> CD4<sup>+</sup> T CD62L<sup>+</sup> cells in the hosts, the investigator must be very gentle and careful to insert the needle into the peritoneal cavity. Failure to do so may result in the tearing of the intestine of the mouse which could lead to death, or a subcutaneous administration of cells which will not induce any disease.

Typically, mice will gain weight during the first week after cell transfer. The investigator should weigh the mice at the same time at each weight time point and use the same weighing balance throughout the whole procedure. Sometimes the experimental mice, especially when their weight at the beginning of the experiment is below 18 g, do not develop any signs of the wasting syndrome. However, these animals might still develop significant intestinal inflammation under macroscopic evaluation.

When working with genetically modified bacteria, contaminations can occur. To avoid these issues it is highly recommended to check the *E. coli* expressing CFP-OVA using the fluorescent microscope to analyze if the bacteria are fluorescent and are rod-shaped (the typical *E. coli* shape).

The proposed antigen driven-colitis relies on the observation that CX<sub>3</sub>CR1<sup>+</sup> MPs sample commensal luminal bacteria in the steady state and during inflammatory conditions<sup>36,37</sup>. The induction of T cells responses in the antigen-driven colitis model has been demonstrated by the highly activated phenotype of OT-II CD4<sup>+</sup> T cells from the colons of CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice challenged with the antigen, as compared to the T cells isolated from the CX<sub>3</sub>CR1<sup>GFP/+</sup> x RAG<sup>-/-</sup> mice that were not challenged with antigen<sup>37</sup>. This is consistent with previous studies in which, after adoptive transfer of OT-II T cells in RAG<sup>-/-</sup> mice, colitis was only induced when the hosts were challenged with OVA-expressing *E. coli*<sup>51</sup>.

Confocal imaging suggests that CX<sub>3</sub>CR1<sup>+</sup> phagocytes are located close to the intestinal epithelium so that they can sample *E. coli* pCFP-OVA and interact with OT-II/Red<sup>+</sup> CD4<sup>+</sup> T cells. After CX<sub>3</sub>CR1<sup>+</sup> phagocytes have sampled *E. coli* pCFP-OVA the luminal antigen is delivered to the CD103<sup>+</sup> dendritic cells (DCs) by CX<sub>3</sub>CR1<sup>+</sup> phagocytes. CD103<sup>+</sup> DCs are able to migrate to the mesenteric lymph node (MLN) to prime CD4<sup>+</sup> T cells<sup>37,52</sup>. Primed T cells assemble in the cLP where CX<sub>3</sub>CR1<sup>+</sup> phagocytes show the OVA antigen to effector T cells to induce inflammation. The delivery and presentation of the antigen by CX<sub>3</sub>CR1<sup>+</sup> phagocytes could be a key event in the development of colitis.

The ability to induce colitis with a defined bacterium expressing a specific antigen provides the opportunity to study the necessary conditions for the development of colitis<sup>24</sup>. This model shows that a specific response to an antigenic peptide (OVA expressed by *E. coli*) is sufficient to induce intestinal inflammation in RAG<sup>-/-</sup> hosts, suggesting that T cells may react to specific antigen from the intestinal microflora. A single antigen can induce intestinal inflammation in the animal models but it cannot be assumed that single antigens are the cause of human IBD. Moreover, in the transfer colitis model there are naive CD4<sup>+</sup> T cells that have unknown specificity and therefore may be reactive to multiple, as of yet unidentified antigens, from the microbiota. Major analysis and studies are needed to better clarify the role of a specific antigen in the pathogenesis of mucosal inflammation.

In recent years the usage of different animal model of colitis has led to an expansion in the knowledge of the pathogenesis of IBD<sup>53</sup>. However, due to the complexity and to the pathogenesis of the disease, there is not a definitive cure<sup>54</sup>. Using the described model, it is possible to address several aspects of IBD that are not well clarified yet, such as (i) interactions between monocytes and dendritic cells, (ii) migration of intestinal DCs to the MLN, (iii) antigen presentation, (iv) homing of effector T cells from MLN to the lamina propria and (v) activation of effector T cells in the lamina propria by CX<sub>3</sub>CR1<sup>+</sup> phagocytes. However, further studies are needed to confirm if the CX<sub>3</sub>CR1<sup>+</sup> phagocyte/CD4<sup>+</sup> T cell interaction plays a key role in the IBD pathogenesis. Mice cannot be truly representative of humans and this must be kept in mind when mouse models are used to study human IBD<sup>2</sup>.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

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