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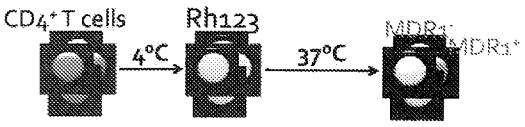
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(54) Title: DIAGNOSTIC AND THERAPEUTIC METHODS RELATED TO INFLAMMATORY BOWEL DISEASES

Figure 1



(57) Abstract: The present invention provides methods and compositions for monitoring MDR1 transporter function in human IBD patients. Also provided in the invention are methods for stratification of IBD patients for bile acid directed therapies, and therapeutic methods that integrate the patient stratification with bile acid sequestration therapies.

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Diagnostic and Therapeutic Methods Related to Inflammatory Bowel Diseases

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the priority of U.S. Provisional patent application serial number 62/353,914, filed June 23, 2016, the disclosure of which is incorporated by reference herein in its entirety.

STATEMENT OF GOVERNMENT SUPPORT

This invention was made with government support under AI119728 awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND OF THE INVENTION

The mechanisms underlying chronic intestinal inflammation in inflammatory bowel disease (IBD) patients are unknown, and likely vary depending on individual patient genetics. For example, different portions of the intestinal tract are affected by the two common forms of inflammatory bowel diseases, ulcerative colitis (UC; the colon) and Crohn's disease (CD; the ileum). Unlike *bona fide* autoimmune diseases (e.g., type 1diabetes, multiple sclerosis), IBDs are not driven by conspicuous auto-antigens. As a result, treatments for inflammatory diseases (IBDs) such as ulcerative colitis and Crohn's disease remain non-specific and ineffective. In large part, this is because the mechanisms underlying chronic intestinal inflammation in IBD patients are unknown, and likely vary depending on individual patient genetics.

Therefore, it has become increasingly evident that future advances in IBD therapy will require a more personalized approach, where therapies are aligned with individual patient genetics. The present invention is directed to this and other unmet needs in the art.

SUMMARY OF THE INVENTION

In one aspect, the invention provides methods for quantifying MDR1 activity in T cells of a human subject. The methods entail (a) assaying MDR1 function in at least one MDR1-expressing effector T cell subsets in a biological sample obtained from the subject,

and (b) comparing the assayed MDR1 function to a background MDR1 function determined with at least one control T cell subset that does not express MDR1. This allows a quantitative measurement of MDR1 activity in T cells of the human subject. In these methods, the MDR1-expressing effector T cell subset is elected from the group consisting of (i) $CD4^+$ effector/memory T cells ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve T cells ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory T cells ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory T cells ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$).

In some of the methods, the biological sample contains peripheral blood mononuclear cells from the subject. In some methods, MDR1 function is assayed via determining the frequency of $MDR1^+$ cells in each T cell subset. In some of these methods, the assayed MDR1 function is the combined $MDR1^+$ cell frequency from the at least one MDR1-expressing effector T cell subsets. In some of these methods, the assayed MDR1 function is the combined $MDR1^+$ cell frequency from effector T cell subsets (i) to (iv). In some methods of the invention, the employed control T cell subset is (i) $CD4^+$ naïve T cells (Tnaive, $CD4^+CD25^-CD45RO^-CCR7^{hi}$) or (ii) regulatory T cells (Treg, $CD4^+CD25^{hi}$) that are present in the same biological sample from the subject. In some methods, the effector T cell subsets from the biological sample are separated via fluorescence-activated cell sorting (FACS). In some of these methods, $MDR1^+$ cells are determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells of the effector T cell subsets.

Some methods of the invention further involve determining $MDR1^+$ activity in cells of the MDR1-expressing effector T cell subsets that are treated with a MDR1 inhibitor. Some methods of the invention utilize a biological sample that contain cryopreserved peripheral blood mononuclear cells. In some of these methods, the determined $MDR1^+$ cell frequency is the combined MDR1 cell frequency from all four effector T cell subsets (i) to (iv) noted above, and $MDR1^+$ cell is determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells in each of the effector T cell subsets. Some methods of the invention are directed to quantifying or monitoring $MDR1^+$ activity in a subject that is afflicted with an inflammatory bowel disease (IBD). In some of these methods, the IBD is Crohn's disease or ulcerative colitis.

In another aspect, the invention provides methods for identifying a human patient with an inflammatory bowel disease (IBD) for treatment with bile acid sequestration therapies. These methods involve (a) obtaining a biological sample containing $CD3^+$ T cells from a candidate

IBD patient and from at least one healthy control subject, (b) determining the combined $MDR1^+$ cell frequency in two or more effector T cell subsets in the biological sample consisting of (i) $CD4^+$ effector/memory T cells ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve T cells ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory T cells ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory T cells ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$), and (c) comparing the combined $MDR1^+$ cell frequency of the candidate IBD patient to the combined $MDR1^+$ cell frequency of the control subject. If there is a substantive decrease of the combined $MDR1^+$ cell frequency of the candidate IBD patient from the combined $MDR1^+$ cell frequency of the control subject, the candidate IBD patient is identified as one suitable for treatment with bile acid sequestration therapies.

In some methods, the candidate IBD subject is a Crohn's disease patient. In some of these methods, the combined $MDR1^+$ cell frequency is combined $MDR1^+$ cell frequency of all effector T cell subsets (i) to (iv). In some methods, determining the combined MDR1+ cell frequency additionally includes quantifying a baseline $MDR1^+$ cell frequency in at least one control T cell subset that does not express MDR1. For example, the control T cell subset can be (i) $CD4^+$ naïve T cells (Tnaive, $CD4^+CD25^-CD45RO^-CCR7^{hi}$) or (ii) regulatory T cells (Treg, $CD4^+CD25^{hi}$) present in the same biological sample. In some methods, the employed biological sample contains peripheral blood mononuclear cells from the patient. In some methods, the effector T cell subsets from the biological sample are separated via fluorescence-activated cell sorting (FACS). In some of these methods, $MDR1^+$ cell is determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells of the effector T cell subsets.

In another aspect, the invention provides methods for treating an inflammatory bowel disease (IBD) in a human patient. The methods entail (a) detecting MDR1 loss-of-function in a candidate IBD patient, and (b) administering to the patient a pharmaceutical composition comprising a therapeutically effective amount of a bile acid sequestrant. This allows treatment of the inflammatory bowel disease in the patient. In some of these methods, MDR1 loss-of-function is detected by (a) obtaining a biological sample containing $CD3^+$ T cells from the candidate patient, (b) determining the combined $MDR1^+$ cell frequency in two or more effector T cell subsets in the biological sample consisting of (i) $CD4^+$ effector/memory ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$), and (c) comparing the

combined MDR1$^+$ cell frequency of the candidate IBD patient to combined MDR1$^+$ cell frequency in the same biological sample from one or more healthy control subjects. Some of the therapeutic methods of the invention are directed to treating patients suffering from Crohn's disease.

A further understanding of the nature and advantages of the present invention may be realized by reference to the remaining portions of the specification and claims.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows Rh123 efflux as a method for visualizing expression/function of the xenobiotic membrane transporter, MDR1. Cells are labeled with the fluorescent MDR1 substrate, rhodamine 123 (Rh123) at 4°C, where MDR1 is inactive. Rh123 is a membrane permeant vital dye that labels mitochondria. Labeled cells are washed to remove excess dye, and moved to a 37°C incubator to active the MDR1 pump. Cells that express MDR1 efflux Rh123, and thus lose fluorescence as determined by FACS analysis.

Figure 2 shows discrimination of T cell subsets by flow cytometry. Frozen PBMC from a representative healthy adult donor was thawed and stained with FACS antibodies to discriminate T cell subsets after (1) gating on lymphocytes; (2) excluding doublets; and (3) gating on viable CD3+ T cells (exclusion of a membrane impermeant viability dye). 6 T cell subsets are identified: (1) CD4$^+$ naïve (Tnaive, CD4$^+$CD25$^-$CD45RO$^-$CCR7^{hi}); (2) regulatory T cells (Treg, CD4$^+$CD25^{hi}); (3) CD4$^+$ effector/memory (CD4$^+$ Teff, CD4$^+$CD25$^-$ CD45RO$^+$CCR7^{lo}); (4) CD8$^+$ naïve (CD4$^-$CD25$^-$CD45RO$^-$CCR7^{hi}); (5) CD8$^+$ effector/memory (CD8$^+$ Teff, CD4$^-$CD25$^-$CD45RO$^+$CCR7^{lo}); and (6) CD8$^+$ short-lived effector/memory (CD8$^+$ Temra, CD4$^-$CD25$^-$CD45RO$^-$CCR7^{lo}). Representative of more than 100 FACS staining experiments performed on healthy control and IBD patient samples.

Figure 3 shows MDR1-dependent Rh123 efflux in human CD3$^+$ T cell subsets. Rh123 efflux in the 6 T cell subsets as identified as in Figure 2 from a pooled stock of healthy adult PBMC was determined by FACS analysis. Percentage of MDR1-expressing cells are shown based on background staining in elacridar-treated cells (shaded peaks). The combined frequency of Rh123^{lo} (MDR1$^+$) cells in the latter four subsets (CD4$^+$ Teff, CD8$^+$ naïve, CD8$^+$ Teff, and CD8$^+$ Temra), calculated as the "cumulative MDR1 score," was used to indicate MDR1 function (expression and activity) in a given PMBC sample.

Figure 4 demonstrates assay stability. Cumulative MDR1 score (blue) and percentage of MDR1$^+$ (Rh123^{lo}) CD4$^+$ Teff cells (red) from 11 independent staining experiments on the same stock PBMC as in Figure 3. Coefficient of variation (CV) is indicated.

Figure 5 shows MDR1 function in human subjects. (A) MDR1 function in male (*left*) and female (*right*) human adult controls and IBD patients. (B) Correlation between MDR1 function and age within healthy human adults (*left*), UC patients (*middle*), and CD patients (*right*). Pearson correlation (*r*) values are shown for each plot.

Figure 6 shows Rh123 efflux in mice Teff cells and MDR1 function in human peripheral blood T cell subsets. (A) MDR1-dependent Rh123 efflux in Teff cells (gated as in Fig. 2) from tissues of SAMP1/YitFc, or MHC-matched wild type (WT, AKR) mice. Background Rh123 efflux shown in elacridar-treated cells (shaded peaks). Representative of 3 experiments. (B) MDR1 function (calculated as in Fig. 5B) in peripheral blood T cell subsets from healthy adult donors ($n = 49$), ulcerative colitis (UC) patients ($n = 53$), and Crohn's disease (CD) patients ($n = 56$). Solid red lines indicate means of each group; hatched red line indicates mean MDR1 function of healthy donors. 3 standard deviations below the healthy donor mean (-3x SD) is indicated by hatched grey line. Three CD patients display MDR1 function less than the -3x SD value of all healthy donors (blue highlights).

Figure 7 shows MDR1-dependent Rh123 efflux in T cell subsets from healthy donor PBMC or the indicated MDR1^{lo} CD patients identified in Figure 6B analyzed on the same day. Background Rh123 efflux in healthy control T cell subsets treated with elacridar is shown (shaded peaks).

Figure 8 shows age and medication history information of human subjects examined for MDR1 function. (A) medication history (medications) of UC and CD patients in this cohort. (B) Age of disease onset of UC and CD patients in this cohort. (C) MDR1 function in healthy adults, UC patients, and CD patients with or without ileitis that have or have not required surgery. (D-F) MDR1^{lo} CD patients identified in (B) are highlighted blue. * $P < .05$, *** $P < .001$, **** $P < .0001$, one-way ANOVA.

DETAILED DESCRIPTION OF THE INVENTION

I. Overview

Current approaches to characterize Inflammatory Bowel Disease (IBD) patients involve whole-genome/whole-exome DNA sequencing. However, information obtained from the analysis cannot be used to dictate clinical decisions in IBD therapy. The present invention is predicated in part on the discovery by the present inventors of a fundamentally new mechanism that regulates T cell-mediated inflammation in the ileum, a specialized portion of the distal small intestine that is overwhelmingly targeted in IBD patients, esp. Crohn's disease. It was found that pro-inflammatory subsets of CD4+ T cells, namely IL-17A-producing Th17 cells and

IFNγ-secreting Th1 cells, upregulate expression of a germline-encoded xenobiotic transporter, MDR1 (ABCB1; P-glycoprotein). Once expressed, MDR1 interacts with conjugated bile acids (CBAs) to enforce Th17 and Th1 cell persistence, limit inflammatory cytokine expression (TNFα, IFNγ), and suppress ileitis. CBAs are cytotoxic digestive metabolites produced in the liver that recycle through the ileal lamina propria during enterohepatic circulation; this involves active reabsorption of luminal CBAs by ileocytes, and is blocked by bile acid sequestrants, such as cholestyramine. Accordingly, bile acid sequestration restores MDR1-deficient T cell homeostasis in the small intestine and attenuates ileitis. Further, it was found that spontaneous MDR1 loss-of-function is associated with Crohn's disease-like ileitis in SAMP1/YitFc mice, which is also reduced by bile acid sequestration. Thus, immune homeostasis in the ileum involves coordinated, druggable, and tissue-specific interactions between mucosal T cells and circulating bile acids.

The inventors further developed a cell-based screen to identify IBD patients that are suitable for bile acid sequestration therapies. Specifically, the cell-based screen enables identification of subsets of IBD patients displaying loss of MDR1 expression/function. As noted above, IBD patients displaying MDR1 loss-of-function are predicted to be effectively treatable with bile acid sequestrants, e.g., cholestyramine (CME) or other similar therapeutic modalities that interfere with intestinal bile acid reabsorption. The invention accordingly provides diagnostic methods that allow stratification of IBD patients based on MDR1 function as defined in this cell-based assay. The invention additionally provides therapeutic methods that integrate bile acid sequestration therapies with the patient profiling strategy disclosed herein. A more detailed guidance for practicing methods of the invention is provided below.

II. Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which this invention pertains. The following references provide one of skill with a general definition of many of the terms used in this invention: *Academic Press Dictionary of Science and Technology*, Morris (Ed.), Academic Press (1st ed., 1992); *Oxford Dictionary of Biochemistry and Molecular Biology*, Smith et al. (Eds.), Oxford University Press (revised ed., 2000); *Encyclopaedic Dictionary of Chemistry*, Kumar (Ed.), Anmol Publications Pvt. Ltd. (2002); *Dictionary of Microbiology and Molecular Biology*, Singleton et al. (Eds.), John Wiley &

Sons (3rd ed., 2002); *Dictionary of Chemistry*, Hunt (Ed.), Routledge (1st ed., 1999); *Dictionary of Pharmaceutical Medicine*, Nahler (Ed.), Springer-Verlag Telos (1994); *Dictionary of Organic Chemistry*, Kumar and Anandand (Eds.), Anmol Publications Pvt. Ltd. (2002); and *A Dictionary of Biology (Oxford Paperback Reference)*, Martin and Hine (Eds.), Oxford University Press (4th ed., 2000). In addition, the following definitions are provided to assist the reader in the practice of the invention.

Bile acid sequestrants are a group of resins used to bind certain components of bile in the gastrointestinal tract. They bind to the major bile acids to form an insoluble complex that prevents reabsorption and leads to fecal excretion. Moreover, the depletion of bile acid stores promotes increased bile acid synthesis which reduces the pool of intracellular cholesterol, thereby helping to maintain a lower cholesterol level. Appreciation for the effects of bile acid sequestrants has led to the use of these agents for several other clinical applications, particularly diarrhea of various etiologies.

Bile acid sequestration or bile acid sequestration therapy refers to therapeutic use of bile acid sequestrants in patients to bind bile acids in the intestine and increase the excretion of bile acids in the stool. Due to the fact that bile acid sequestrants exert their pharmacologic effect almost exclusively in the GI tract with very little systemic absorption, these agents are generally well tolerated. The therapies were initially developed for the treatment of hypercholesterolemia. The most important role of bile acid sequestrants in the control of GI-related disorders has stemmed from its value in the control of diarrhea related to bile acid malabsorption. This condition has numerous etiologies, including Crohn's disease, cholecystectomy, ileal resection, vagotomy, and celiac disease.

The term "contacting" has its normal meaning and refers to combining two or more agents (e.g., polypeptides or small molecule compounds) or combining agents and cells. Contacting can occur in vitro, e.g., combining two or more agents or combining an agent and a cell or a cell lysate in a test tube or other container. Contacting can also occur in a cell or in situ, e.g., contacting two polypeptides in a cell by coexpression in the cell of recombinant polynucleotides encoding the two polypeptides, or in a cell lysate. Contacting can also occur inside the body of a subject, e.g., by administering to the subject an agent which then interacts with the intended target (e.g., a tissue or a cell).

"Inflammation" or "inflammatory response" refers to an innate immune response that occurs when tissues are injured by bacteria, trauma, toxins, heat, or any other cause. The damaged tissue releases compounds including histamine, bradykinin, and serotonin. Inflammation refers to both acute responses (i.e., responses in which the inflammatory

processes are active) and chronic responses (i.e., responses marked by slow progression and formation of new connective tissue). Acute and chronic inflammation can be distinguished by the cell types involved. Acute inflammation often involves polymorphonuclear neutrophils; whereas chronic inflammation is normally characterized by a lymphohistiocytic and/or granulomatous response. Inflammation includes reactions of both the specific and non-specific defense systems. A specific defense system reaction is a specific immune system reaction response to an antigen (possibly including an autoantigen). A non-specific defense system reaction is an inflammatory response mediated by leukocytes incapable of immunological memory. Such cells include granulocytes, macrophages, neutrophils and eosinophils.

Inflammatory bowel disease (IBD) refers to a group of inflammatory conditions of the colon and small intestine, including Crohn's disease, ulcerative colitis, Behcet's disease, collagenous colitis, diversion colitis, ischemic colitis, and lymphocytic colitis. Crohn's disease (CD) and ulcerative colitis (UC) are the principal types of inflammatory bowel disease. The first clue in the diagnosis of IBD is the symptom such as unrelenting diarrhea, blood or mucus in the stool (more common with ulcerative colitis than Crohn's disease), fever and abdominal pain. However, some of these symptoms may also be present with a parasitic infection, diverticulitis, celiac disease, colon cancer, or other less common conditions. Therefore, often IBDs are diagnosed only after ruling out other possible causes for the symptoms, including ischemic colitis, infection, irritable bowel syndrome (IBS), diverticulitis and colon cancer. To help confirm a diagnosis of IBD, a combination of tests and procedures can be used. These include blood tests for anemia or infection, fecal occult blood test, endoscopic procedures (such as colonoscopy, flexible sigmoidoscopy, upper endoscopy, capsule endoscopy, and double-balloon endoscopy) , and imaging procedures (such as X-ray, CT scan, magnetic resonance imaging (MRI) and small bowel imaging.

Crohn disease (CD) is idiopathic chronic enteritis of unknown origin, and is a disease with non-specific inflammatory symptoms extended from the small intestine to the large intestine. Signs and symptoms often include abdominal pain, diarrhea (which may be bloody if inflammation is severe), fever, and weight loss. Other complications may occur outside the gastrointestinal tract and include anemia, skin rashes, arthritis, inflammation of the eye, and feeling tired. The skin rashes may be due to infections as well as pyoderma gangrenosum or erythema nodosum. Bowel obstruction also commonly occurs and those with the disease are at greater risk of bowel cancer. Diagnosis is based on a number of findings including biopsy and appearance of the bowel wall, medical imaging and description of the disease. There are

no medications or surgical procedures that can cure Crohn's disease. Treatment options can only help with symptoms, maintain remission, and prevent relapse. For example, high calorie fluid or enteral nutrition therapy has been used as a long-term treatment in improving nutritional status. For treatment based on medications, salazosulfapyridine, metronidazole, adrenocortical steroids, immunosuppressants, 5-aminosalicylic acid (5-ASA) and the like are typically used.

Ulcerative colitis is a diffuse non-specific inflammatory disease that mainly invades the mucosa or submucosa of large intestine, frequently forming erosion or ulcer therein. The main symptom of active disease is diarrhea mixed with blood. Ulcerative colitis has much in common with Crohn's disease, but what sets it apart from Crohn's disease is that ulcerative colitis only affects the colon and rectum, rather than the whole GI tract. Ulcerative colitis is an intermittent disease, with periods of exacerbated symptoms, and periods that are relatively symptom-free. Although the symptoms of ulcerative colitis can sometimes diminish on their own, the disease usually requires treatment to go into remission. Although UC has no known cause, there is a presumed genetic risk. The disease may be triggered in a susceptible person by environmental factors. Dietary modification may reduce the discomfort of a person with the disease. Treatment of UC is with anti-inflammatory drugs, immunosuppression, and biological therapy targeting specific components of the immune response. Examples include salazosulfapyridine, adrenocortical steroids, immunosuppressants, and 5-aminosalicyclic acid (5-ASA). Colectomy (partial or total removal of the large bowel through surgery) is occasionally necessary if the disease is severe, does not respond to treatment, or if significant complications develop. A total proctocolectomy (removal of the entirety of the large bowel and rectum) can cure ulcerative colitis as the disease only affects the large bowel and rectum and does not recur after removal of the latter.

Multidrug resistance protein 1 (MDR1), aka P-glycoprotein (permeability glycoprotein 1; P-gp), ATP-binding cassette sub-family B member 1 (ABCB1) or cluster of differentiation 243 (CD243), is an important protein of the cell membrane that pumps many foreign substances out of cells. More formally, it is an ATP-dependent efflux pump with broad substrate specificity. It exists in animals, fungi and bacteria and likely evolved as a defense mechanism against harmful substances. MDR1 is extensively distributed and expressed in the intestinal epithelium where it pumps xenobiotics (such as toxins or drugs) back into the intestinal lumen, in liver cells where it pumps them into bile ducts, in the cells of the proximal tubule of the kidney where it pumps them into urine-conducting ducts, and in the capillary endothelial cells composing the blood--brain barrier and blood-testis barrier,

where it pumps them back into the capillaries. MDR1 is a glycoprotein that in humans is encoded by the *ABCB1* gene.

T lymphocytes recognize antigens as peptides bound to major histocompatibility complex (MHC) molecules. The fine specificity of the T cell is defined by the T-cell receptor (TCR). Most T lymphocytes express the TCR αß and either CD4 or CD8 molecules. These molecules stabilize the TCR-peptide/MHC interaction, are essential for intrathymic selection, and contribute to transmembrane signaling, with important roles in the development and activation of helper and cytotoxic T cells. All T cells originate from haematopoietic stem cells in the bone marrow. Haematopoietic progenitors derived from haematopoietic stem cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8. As they progress through their development they become double-positive thymocytes ($CD4^+CD8^+$), and finally mature to single-positive ($CD4^+CD8^-$ or $CD4^-CD8^+$) thymocytes that are then released from the thymus to peripheral tissues. These precursor cells then differentiate into various type of mature or effector T cells which can be $CD4^+$ T cells, $CD8^+$ T cells, or $CD4^-CD8^-$ double negative (DN) T cells.

T helper (Th) cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages, among other functions. These cells are also known as $CD4^+$ T cells because they express the CD4 protein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of Antigen Presenting Cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response. These cells can differentiate into one of several subtypes, including TH1, TH2, TH3, TH17, or TFH, which secrete different cytokines to facilitate a different type of immune response. The mechanism by which T cells are directed into a particular subtype is poorly understood, though signaling patterns from the APC are thought to play an important role.

Cytotoxic T cells (TC cells, or CTLs) destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as $CD8^+$ T cells since they express the CD8 glycoprotein at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of nearly every cell of the body. Through IL-10, adenosine and other molecules secreted by regulatory T cells, the $CD8^+$ cells can be inactivated to an anergic state, which prevent autoimmune diseases such as experimental autoimmune encephalomyelitis.

Memory T cells are a subset of antigen-specific T cells that persist long-term after an infection has resolved. They quickly expand to large numbers of effector T cells upon re-exposure to their cognate antigen, thus providing the immune system with "memory" against past infections. Memory T cells comprise two subtypes: central memory T cells (TCM cells) and effector memory T cells (TEM cells). Memory cells may be either $CD4^+$ or $CD8^+$. Memory T cells typically express the cell surface protein CD45RO.

The term "subject" for purposes of diagnosis or treatment refers to any animal classified as a mammal, e.g., human and non-human mammals. Examples of non-human animals include dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, and etc. Unless otherwise noted, the terms "patient" or "subject" are used herein interchangeably. Preferably, the subject is human. Unless otherwise noted, human patient and human subject are used interchangeably herein.

The term "treating" or "alleviating" includes the administration of compounds or agents to a subject to prevent or delay the onset of the symptoms, complications, or biochemical indicia of a disease (e.g., an inflammatory disorder such as IBD), alleviating the symptoms or arresting or inhibiting further development of the disease, condition, or disorder. Subjects in need of treatment include those already suffering from the disease or disorder as well as those being at risk of developing the disorder. Treatment may be prophylactic (to prevent or delay the onset of the disease, or to prevent the manifestation of clinical or subclinical symptoms thereof) or therapeutic suppression or alleviation of symptoms after the manifestation of the disease. In the treatment of a disease or disorder associated with or mediated by systemic autoimmunity or neuroinflammation, a therapeutic agent may directly decrease the pathology of the disease, or render the disease more susceptible to treatment by other therapeutic agents.

III. Assays for monitoring MDR1 activity

The invention provides methods for monitoring or quantifying MDR1 activity (or MDR1 expression/function) in human subjects. In some embodiments, the methods can be used to monitor MDR1 activity in human IBD patients such as ulcerative colitis (UC) patients and Crohn's disease (CD) patients. Unless otherwise noted, the MDR1 activity to be monitored in the methods of the invention refers to the net efflux pump activity of the MDR1 protein in a target cell or a population of cells (e.g., a population of an effector T cell subset). Thus, a modified MDR1 activity can be the result of altered expression or cellular level of the protein and/or the biochemical function of the protein. As detailed below, quantifying MDR1

activity in IBD patients can allow the clinician to identify patients with MDR1 loss-of-function that are suitable for bile acid directed therapies. In some embodiments, monitoring MDR1 activity in IBD patients undergoing treatment can enable assessment of efficacy of the treatment.

IBD patients can be clinically diagnosed with standard procedures described herein which are routinely employed in the art, e.g., endoscopy and histology examination. To assess or monitor the MDR1 activity (i.e., net outcome from MDR1 expression and pump function) in an IBD patient, the frequency of MDR1+ cells in one or more MDR1-expressing effector T cell subsets is determined. MDR1+ cells are determined via contacting or labeling the cells with a probe or MDR1 substrate which can pumped out of the cell by a functional MDR1 protein. The effector T cells are typically isolated from a biological sample obtained from the patient. For example, the biological sample can be mononuclear cells from a peripheral blood sample from the patient as exemplified herein. The MDR1-expressing effector T cell subsets for assessing frequency of MDR1+ cells include (1) CD4+ effector/memory T cells (Teff, CD4+CD25-CD45RO+CCR7lo), (2) CD8+ naïve T cells (CD8+CD25-CD45RO-CCR7hi), (3) CD8+ effector/memory T cells (Teff, CD8+CD25-CD45RO+CCR7lo), and (4) CD8+ short-lived effector T cells (Temra, CD8+CD25-CD45RO-CCR7lo). As exemplified herein, the different T cell subsets can be readily separated with appropriate antibodies against the various surface markers and flow cytometric analysis. In some embodiments, the frequency of $MDR1^+$ cells in at least 2 of these MDR1-expressing effector T cell subsets is determined. In some other embodiments, the frequency of $MDR1^+$ cells in at least 3 of the MDR1-expressing effector T cell subsets is determined. In some preferred embodiments, the frequency of $MDR1^+$ cells in all 4 of the MDR1-expressing effector T cell subsets is determined. In various embodiments, the determined MDR1 activity in the biological sample (e.g., PMBC sample) is based on the combined $MDR1^+$ cell frequency (or "cumulative MDR1 score") in the examined effector T cell subsets.

MDR1 is an ATP-dependent efflux pump with broad substrate specificity. Any xenobiotic compounds or agents that are transported by MDR1 across the cell membrane may be used in the methods of the invention for detecting $MDR1^+$ cells. Typically, the substrate used for monitoring MDR1 activity is a labeled probe or detectable agent. For example, the cells can be labeled with fluorescent dyes such as Rhodamine123 (Rh123) or MitoTracker under conditions (e.g., low temperature such as 4°C) that MDR1 is inactive. After washing the cells to remove excess dye, MDR1 activity can then be determined by quantifying

MDR1$^+$ cells under conditions (e.g., high temperature such as 37°C) that MDR1 is active. Using Rh123 as an example, MDR1$^+$ cells will display little or significantly diminished fluorescence signal (Rh123$^-$ or Rh123^{lo}) as the substrate is pumped out by active MDR1 protein. Similar to the sorting of the different T cell subsets from the biological sample obtained from the IBD patients, MDR1$^+$ (Rh123$^-$) cells in the T cell subsets can also be readily determined and quantified via fluorescence-activated cell sorting (FACS) as exemplified herein.

To control for background signal, the methods can also include one or more T cell subsets that do not express MDR1. For example, in addition to the 4 MDR1-expressing effector T cell subsets noted above, the methods of the invention can also include determination of MDR1$^+$ cell frequency in CD4+ naïve T cells (CD4+CD25-CD45RO-CCR7hi) and/or CD4+ regulatory T cells (Treg, CD4+CD25hi). Typically, these control T cell subsets are obtained from the same subject. In some preferred embodiments, the control cells are isolated from the same biological sample (e.g., PMBC sample) from which the MDR1-expressing effector T cell subsets are isolated. In some other embodiments, baseline or background MDR1 activity may be additionally controlled with the same MDR1-expressing effector T cells in the presence of a MDR1 inhibitor. For example, as exemplified herein, background efflux of a fluorescent MDR1 substrate can be quantified using MDR1-expressing cells treated with MDR1 antagonist elacridar.

IV. Diagnostic and therapeutic applications

Some methods of the invention are intended for use in identifying human subjects with MDR1 loss-of-function in the MDR1-expressing T cells. Some of these methods are directed to screening IBD patients for bile acid sequestration therapies. To determine whether there is MDR1 loss-of-function in a subject (e.g., an IBD patient), MDR1 activity (cumulative MDR1 score) determined with MDR1-expressing cells from the subject is compared to that of a positive control value. Typically, the positive control value is the determined value with the sample biological sample (e.g., PMBC cells) from one or more healthy subjects via the same assay. In some embodiments, the mean MDR1 activity determined from a group of healthy subjects is used as the positive control value. As noted above, one or more MDR1$^-$ T cell subsets from the candidate IBD patient and the healthy control can be included in the assay to determine baseline MDR1 activity. As a further control for background signal, the methods can further include assaying MDR1 activity in the same MDR1-expressing T cell subsets that are treated with a MDR1 antagonist.

Once the cumulative MDR1 score of the MDR1-expressing T cell subsets from the candidate patient is determined, it is compared to the control cumulative MDR1 score determined with the MDR1-expressing T cell subsets from the healthy control subjects. If the MDR1 activity of the candidate subject (e.g., an IBD patient) substantially departs, or deviates with statistical significance, from the control value, the candidate subject is identified as one who has MDR1 loss-of-function. Thus, some methods of the invention also include determination of the mean value and standard deviation ("s.d.") of the cumulative MDR1 scores of the healthy control subjects. A substantial departure or statistically significant deviation means at least 1.5 x s.d. below the mean value of the healthy control subjects. In some embodiments, a substantial departure means at least 2 x s.d. or 2.5 x s.d. below the mean control value. In some embodiments, a substantial departure means at least 3 x s.d. or 3.5 x s.d. below the mean control value. In some other embodiments, a substantial departure means at least 4 x s.d. or more below the control value. If the MDR1 activity (cumulative MDR1 score) in an IBD patient (e.g., a CD patient) substantially departs from that of the control subjects, the patient is identified as having MDR1 loss-of-function relative to that of the healthy control subjects.

In some embodiments, the invention provides therapeutic methods for treating a subset of IBD patients that have impaired or diminished MDR1 activity (or MDR1 loss-of-function). These therapeutic applications combine the diagnostic methods described herein and bile acid sequestration therapies. Thus, IBD patients can be first screened for MDR1 activity in effector T cell subsets as described herein. Patients who are determined to have MDR1 loss-of-function are then subject to treatment with a bile acid sequestration therapy. The patients are typically administered with a pharmaceutical composition that contains a therapeutically effective amount of a bile acid sequestrant. Several well-known bile acid sequestrants can be readily employed in the therapeutic regimen for the subject. Examples of suitable bile acid sequestrants include cholestyramine (CME) (Questran, Prevalite), colestipol (Colestid, Flavored Colestid), and colesevelam (Welchol). Clinical protocols for using bile acid sequestrants have been developed in the treatment of a number of gastrointestinal diseases or disorders. See, e.g., Taylor et al., J. Infect. Dis. 1980;141:92-7, 1980; Fromm and Malavolti, Clin. Gastroenterol. 15:567-82, 1986; Pusl et al., Clin. Rev. Allergy Immunol. 28:147-57, 2005; Puleston et al., Gut. 54:441-2, 2005; Westergaard et al., Curr. Treat. Options Gastroenterol. 10:28-33, 2007; Wedlake et al., Gut 58:A117–A117, 2009; Odunsi-Shiyanbade et al., Clin. Gastroenterol. Hepatol. 8:159-65, 2010; and Reiner et al., Fundam.

Clin. Pharmacol. 24:19-28, 2010. These protocols can be readied modified in the therapeutic methods of the invention for treating IBD patients.

Subjects with various clinical subtypes of IBD may be suitable for the methods of the invention. Some methods of the invention are directed to patients with Crohn's disease (CD). Crohn's disease is a disease of chronic inflammation that can involve any part of the gastrointestinal tract. Commonly, the distal portion of the small intestine, i.e., the ileum, and the cecum are affected. In other cases, the disease is confined to the small intestine, colon, or anorectal region. CD occasionally involves the duodenum and stomach, and more rarely the esophagus and oral cavity. Several features are characteristic of the pathology of CD. The inflammation associated with CD, known as transmural inflammation, involves all layers of the bowel wall. Thickening and edema, for example, typically also appear throughout the bowel wall, with fibrosis present in long-standing forms of the disease. The inflammation characteristic of CD is discontinuous in that segments of inflamed tissue, known as "skip lesions," are separated by apparently normal intestine. Furthermore, linear ulcerations, edema, and inflammation of the intervening tissue lead to a "cobblestone" appearance of the intestinal mucosa, which is distinctive of CD.

A hallmark of CD is the presence of discrete aggregations of inflammatory cells, known as granulomas, which are generally found in the submucosa. Some CD cases display typical discrete granulomas, while others show a diffuse granulomatous reaction or a nonspecific transmural inflammation. As a result, the presence of discrete granulomas is indicative of CD, although the absence of granulomas is also consistent with the disease. Thus, transmural or discontinuous inflammation, rather than the presence of granulomas, is a preferred diagnostic indicator of CD. Quantifying MDR1 activity or function in CD patients would help the clinician to devise appropriate treatment and/or to monitor progresses of an ongoing treatment.

In some embodiments, the diagnostic and/or therapeutic methods of the invention are directed to patients with ulcerative colitis (UC). Ulcerative colitis is a disease of the large intestine characterized by chronic diarrhea with cramping, abdominal pain, rectal bleeding, loose discharges of blood, pus, and mucus. The manifestations of UC vary widely. A pattern of exacerbations and remissions typifies the clinical course for about 70% of UC patients, although continuous symptoms without remission are present in some patients with UC. Local and systemic complications of UC include arthritis, eye inflammation such as uveitis, skin ulcers, and liver disease. In addition, UC, and especially the long-standing, extensive form of the disease is associated with an increased risk of colon carcinoma.

UC is a diffuse disease that usually extends from the most distal part of the rectum for a variable distance proximally. The term "left-sided colitis" describes an inflammation that involves the distal portion of the colon, extending as far as the splenic flexure. Sparing of the rectum or involvement of the right side (proximal portion) of the colon alone is unusual in UC. The inflammatory process of UC is limited to the colon and does not involve, for example, the small intestine, stomach, or esophagus. In addition, UC is distinguished by a superficial inflammation of the mucosa that generally spares the deeper layers of the bowel wall. Crypt abscesses, in which degenerated intestinal crypts are filled with neutrophils, are also typical of UC.

In comparison with CD, which is a patchy disease with frequent sparing of the rectum, UC is characterized by a continuous inflammation of the colon that usually is more severe distally than proximally. The inflammation in UC is superficial in that it is usually limited to the mucosal layer and is characterized by an acute inflammatory infiltrate with neutrophils and crypt abscesses. Monitoring and quantifying MDR1 function in UC patients can also provide valuable information that can be helpful to determining a suitable course of therapeutic intervention.

Indeterminate colitis (IC) is another clinical subtype of IBD that includes both features of CD and UC. Such an overlap in the symptoms of both diseases can occur temporarily (e.g., in the early stages of the disease) or persistently (e.g., throughout the progression of the disease) in patients with IC. Clinically, IC is characterized by abdominal pain and diarrhea with or without rectal bleeding. For example, colitis with intermittent multiple ulcerations separated by normal mucosa is found in patients with the disease. Histologically, there is a pattern of severe ulceration with transmural inflammation. The rectum is typically free of the disease and the lymphoid inflammatory cells do not show aggregation. Although deep slit-like fissures are observed with foci of myocytolysis, the intervening mucosa is typically minimally congested with the preservation of goblet cells in patients with IC. Like CD and UC patients, subjects with IC can also be examined for MDR1 function via the methods of the invention.

In addition to the diagnostic and therapeutic methods, the invention further provides kits or pharmaceutical combinations for carrying out the methods described herein. The kits can be readily used in profiling IBD patients for those that are suitable for bile acid sequestration therapies. The kits of the invention typically contain reagents for determining MDR1 activity in a biological sample, e.g., via Rh123 efflux assay as described herein. In various embodiments, the kits can include reagents for collecting a biological sample (e.g.,

PMBC cells) from patients, an agent for labeling the cells in the biological sample for determining MDR1 function via a suitable assay (e.g., fluorescent label Rh123 for Rh123 efflux assay), and monoclonal antibodies for gating the different T cell subsets in flow cytometry analysis. In some embodiments, the kits can optionally include control biological samples (e.g., PMBC cells) that are obtained from healthy subjects. The diagnostic kits can further include packaging material for packaging the reagents and a notification in or on the packaging material. The kits can additionally include appropriate instructions for use and labels indicating the intended use of the contents of the kits. The instructions can be present on any written material or recorded material supplied on or with the kit or which otherwise accompanies the kit.

Mdr1-dependent dye efflux and a novel CRISPR-generated reporter mouse show that Mdr1 is also expressed in mouse Th17 and Th1 cells in vivo, where it is increased in the small intestine lamina propria, highest in the ileum, and induced by CD103$^+$ dendritic cells (DCs)

Immune competency requires naïve CD4$^+$ T_H cell differentiation into functionally distinct subsets of cytokine-secreting effector (Teff) cells (Nakayamada et al., 2012). These subsets, which include IFNγ-producing Th1 cells and IL-17-secreting Th17 cells, also down-regulate lymphoid homing receptors and up-regulate tissue homing chemokine receptors and integrins, which drives egress out of secondary lymphoid organs and migration into inflamed peripheral tissues. Unlike most non-lymphoid tissues, mucosal tissues such as the intestine are continuously exposed to potentially pathogenic stimuli. As such, Teff cells circulate in and out of the intestinal lamina propria at steady state (Morton et al., 2014).

Presence of pro-inflammatory Teff cells in the intestinal mucosa is potentially deleterious, and exaggerated cytokine expression by Th17 and Th1 cells is associated with pathogenesis of inflammatory bowel diseases (IBDs) (Kaser et al., 2010). Accordingly, numerous mechanisms are deployed to actively suppress Teff cell function in the gut, the vast majority of which involve symbiotic interactions with commensal bacteria (Huttenhower et al., 2014; Longman et al., 2013). In addition to restricting colonization by intestinal pathogens (Lee et al., 2013), commensal microbes restrict trafficking of intestinal CX_3CR1^+ phagocytes to mesenteric lymph nodes to prevent T_H cell priming against enteric antigens (Diehl et al., 2013). In addition, several commensal species produce short-chain fatty acids (e.g., butyrate) or polysaccharide A to stimulate peripheral development of Foxp3$^+$ T regulatory (pTreg) cells and IL-10-expression (Arpaia et al., 2013; Mazmanian et al., 2008; Round and Mazmanian, 2010). Microbiota also direct conversion of intestinal pTregs into

$CD4^+CD8\alpha\alpha^+$ intraepithelial lymphocytes ($CD4_{IEL}$) for additional control over mucosal immune homeostasis (Sujino et al., 2016). Still other enteric bacteria have pro-inflammatory functions. Segmented filamentous bacteria (SFB), for example, promote Th17 cell development and induce IL-17 expression by these cells in the ileum (Atarashi et al., 2015; Ivanov et al., 2009; Sano et al., 2015). Accordingly, SFB colonization in mice is associated with pathogenesis of both intestinal and systemic inflammatory diseases (Kriegel et al., 2011; Stepankova et al., 2007; Teng et al., 2016; Wang et al., 2015). Based on these and many other findings, major translational initiatives are now underway to characterize the human microbiome in both healthy individuals and patient cohorts, and to test the efficacy of microbiota-directed therapies (*e.g.*, fecal transplantation) in human inflammatory diseases.

Far less is known about how the mucosal immune system interacts with non-microbial intestinal products. For example, bile acids (BAs) are a fundamental class of amphipathic intestinal detergents synthesized from cholesterol in the liver (Hofmann and Hagey, 2014). BAs are stored in the gall bladder and deposited into the duodenum in response to food intake, where they aid in digestion by emulsifying cholesterol and other insoluble dietary lipids (Hofmann and Hagey, 2014). Intriguingly, BAs, and in particular those conjugated to the amino acid glycine or taurine, are actively reabsorbed in the terminal ileum by specialized enterocytes expressing the apical bile acid transporter, Asbt (gene symbol *Slc10a2*) (Dawson et al., 2003; Dawson et al., 2009). Actively recycled BAs accumulate in the ileal mucosa and ultimately transit into portal veins for re-circulation back to the liver. This enterohepatic circulation of BAs – first described in the 1960's – establishes that the ileal mucosa is the only mammalian tissue exposed to high extracellular concentrations of conjugated BAs (Hofmann and Hagey, 2014). Yet still, the immunologic consequences of mucosa-associated BAs in the ileum remain unknown.

We previously identified the ATP-binding cassette (ABC)-containing xenobiotic transporter, MDR1 (ABCB1, P-glycoprotein) as preferentially expressed in intestinal *vs.* circulating human Th17 and Th1 cells (Ramesh et al., 2014). MDR1 is a plasma membrane-associated ATP-dependent efflux pump best known for expression in drug-resistant tumor cells, where it effluxes structurally unrelated chemotherapeutic compounds (Gottesman et al., 2002). Intriguingly, mice lacking Mdr1a (*Abcb1a*) are prone to spontaneous colitis (Panwala et al., 1998), which has been ascribed to defects in both epithelial and hematopoietic compartments (Maxwell et al., 2015; Staley et al., 2011). Further, polymorphisms in the gene encoding human MDR1 (*ABCB1*) have been sporadically associated with IBDs (Annese et al., 2006). Yet despite these data suggesting important physiologic functions, MDR1 remains

widely regarded as a dedicated "drug handler" (Borst and Schinkel, 2013; Schinkel, 1997). However, orthologs of MDR1 are present in bacteria, suggesting that this transporter has broader and more fundamental functions in cell biology beyond interfacing with 20th century pharmaceuticals.

Here we use both Mdr1-dependent dye efflux and a novel CRISPR-generated reporter mouse to show that Mdr1 is also expressed in mouse Th17 and Th1 cells *in vivo*, where it is increased in the small intestine lamina propria, highest in the ileum, and induced by $CD103^+$ dendritic cells (DCs). Consistent with this pattern of expression, Mdr1 regulates Th17 and Th1 cell function selectively in the ileum; T_H cells lacking Mdr1 – due to germline ablation (*Abcb1a/1b*$^{-/-}$) or shRNAmir-mediated knockdown – transfer Crohn's disease-like ileitis in *Rag1*$^{-/-}$ hosts and overexpress several pro-inflammatory cytokines in the ileum, most notably TNFα and IFNγ. Importantly, both increased expression and selective function of Mdr1 in the ileum is independent of microbiota, as judged by experiments in germ free wild type mice and antibiotic-treated *Rag1*$^{-/-}$ mice. Mdr1 acts specifically in the presence of conjugated BAs to suppress Th17 and Th1 cell cytokine expression and enforce survival. Accordingly, mucosal homeostasis of Mdr1-deficient Th17 and Th1 cells *in vivo* is rescued by either genetic ablation of the ileal BA transporter, Asbt, or dietary administration of the BA sequestrant, cholestryramine, both of which prevent active reabsorption of luminal BAs into the ileal mucosa. Finally, we show overt MDR1 loss-of-function in both ileitis-prone (SAMP1/YitFc) mice and a subset of patients with ileal Crohn's disease. Thus, in addition to interacting with local microflora, lymphocytes employ specialized functions in the ileum to prevent potentially pathogenic interaction with mucosa-associated BAs.

Mdr1 is preferentially expressed by Th17 and Th1 cells in the ileum

Appreciating that MDR1 function *in vivo* involves complex interaction with unknown metabolites, we sought to interrogate the immunologic function of this transporter using *in vivo* mouse models rather than human cell culture systems. However, Mdr1 expression in mouse lymphocyte is both poorly characterized and important to understand when considering function. Using efflux of the fluorescent Mdr1 substrate rhodamine 123 (Rh123) (Ludescher et al., 1992) as a surrogate for expression, we found that – as in humans (Ramesh et al., 2014) – Mdr1 is expressed in a portion of endogenous mouse Teff cells at steady state. Rh123 efflux in Teff cells is due to Mdr1, and not related transporters, as: (*i*) Rh123 efflux is abolished by treatment with the Mdr1 antagonist, elacridar (Hyafil et al., 1993), (*ii*) Rh123 efflux is absent in Teff cells from Mdr1-deficient (FVB.*Abcb1a/1b*$^{-/-}$) mice (Schinkel et al., 1997), and (*iii*) wild type Teff cells FACS-sorted based on Rh123 efflux are

enriched for expression of *Abcb1a* – the mouse ortholog of human *ABCB1* – but not other multidrug transporter mRNAs (*e.g.*, *Abcc1*, *Abcc3*, *Abcg2*). Also as in humans, Mdr1 is absent in mouse naïve T_H cells, higher in Teff cells *vs.* Tregs, and expressed in both Th17 and non-Th17 (mostly Th1) Teff cells, the latter judged by Rh123 efflux experiments in cells from Th17 fate-mapping reporter mice.

However useful, Rh123 efflux is still an indirect measure of Mdr1 expression. Moreover, commercial antibodies are neither suitable for flow cytometry nor specific for mouse Mdr1 over related transporters. Therefore, to enable direct and specific tracking of Mdr1 expression in mouse lymphocytes, we employed CRISPR/Cas9 genome editing in wild type C57Bl/6 (B6) zygotes (Wang et al., 2013; Yang et al., 2013) to generate an Mdr1 fluorescent reporter mouse. We replaced the stop codon of *Abcb1a* with a reporter cassette containing a self-cleaving P2A peptide and the fluorescent reporter, ametrine (, to facilitate stoichiometric translation of independent Mdr1 and ametrine proteins from a single bi-cistronic mRNA. Although our founder reporter mouse carried a small (9-nucleotide) in-frame deletion near the 3' terminal end of *Abcb1a* in the reporter allele, this has no bearing on Mdr1 transport function. Further, ametrine expression in splenic T_H cells from reporter mice is largely restricted to $CD44^{hi}$ Teff cells, and faithfully discriminates those with high-level Mdr1 transport (Rh123 efflux) activity and *Abcb1a* gene expression.

Whether judged by Rh123 efflux in wild type T_H cells or ametrine expression in Mdr1-reporter T_H cells, we found that Mdr1 is increased markedly in Teff cells from small intestine lamina propria (siLP) compared with counterparts from colon lamina propria (cLP) or other tissues, including spleen and mesenteric lymph nodes (MLN), or bone marrow, lung, liver, and skin (data not shown). Increased Mdr1 expression in small intestinal Teff cells is evident in wild type mice of several genetic backgrounds (*e.g.*, B6, FVB, BALB/c) (data not shown), and in $Rag1^{-/-}$ mice injected with wild type or Mdr1-reporter naïve T_H cells. Within siLP, Teff cell expression of Mdr1 increases in a gradient fashion from proximal to distal, with highest expression observed in the ileum.

Increased Mdr1 expression in small intestinal Teff cells could be due to selective survival or local upregulation. In support of the latter, we found that Mdr1 expression in Teff cells is transient, and induced locally by intestinal dendritic cells (DCs). First, Teff cells (*e.g.*, Th1, Th2, Th17) generated *in vitro* from naïve T_H cells do not express Mdr1. Second, *ex vivo*-sorted $Mdr1^+$ Teff cells lose Mdr1 expression rapidly upon *in vitro* culture. Third, *ex vivo*-sorted $Mdr1^-$ Teff cells acquire Mdr1 expression upon transfer into $Rag1^{-/-}$ mice, and Mdr1 expression here again is highest in siLP. Fourth, Mdr1 expression in *ex vivo*-sorted $Mdr1^-$

Teff cells is induced *in vitro* upon co-culture with small intestinal $CD11c^+$ DCs, but not small intestinal $CD11b^+CD11c^-$ macrophages or $CD11c^+$ DCs from spleen. Intestinal DCs that induce Mdr1 expression in Teff cells *in vitro* are heterogeneous for expression of CD103 and CX_3CR1. However, only purified $CD103^+$ DCs, and not CX_3CR1^+ phagocytes, induce Mdr1 in co-cultured Teff cells. Finally, Mdr1 expression in Teff cells *in vivo* is independent of microbiota; high-level Teff cell expression of Mdr1 is evident in siLP from germ free (GF) mice, as well as mice lacking toll-like receptor (*Myd88*$^{-/-}$) or inflammasome (*Asc*$^{-/-}$) signaling components. Accordingly, intestinal DCs from both specific pathogen-free (SPF) and GF mice induce Mdr1 expression in co-cultured Teff cells. Thus, Mdr1 expression is a locally acquired and prominent trait of Th17 and Th1 cells infiltrating the ileum.

Mdr1 selectively regulates T cell function and inflammation in the ileum

Naïve T_H cells lacking Mdr1 (FVB.*Abcb1a/1b*$^{-/-}$) induce more aggressive weight loss upon transfer into *Rag1*$^{-/-}$ mice than wild type counterparts. Inflammation in this transfer model is generally restricted to the colon (Ostanin et al., 2009), and indeed, both wild type and Mdr1-deficient T_H cells induce severe colitis. However, Mdr1-deficient T_H cells also induce marked ileitis, whereas wild type T_H cells do not. Like Mdr1 expression, ileitogenic function of Mdr1-deficient T_H cells is independent of microbiota; depletion of enteric bacteria with broad-spectrum antibiotics has little impact on weight loss in *Rag1*$^{-/-}$ mice transplanted with Mdr1-deficient T_H cells, and these mice still display ileitis. Accordingly, ileitis transferred by Mdr1-deficient T_H cells in untreated *Rag1*$^{-/-}$ mice is not transmissible by co-housing, and is not associated with dysbiosis, as judged by 16S rDNA sequencing. By contrast, antibiotics strongly attenuate weight loss and colitis in *Rag1*$^{-/-}$ mice injected with wild type T_H cells. Importantly, shRNAmir-mediated knockdown (Fellmann et al., 2013) of Mdr1 in wild type T_H cells also leads to increased weight loss and ileitis in transferred *Rag1*$^{-/-}$ mice.

Consistent with small bowel pathology, Mdr1-deficient $ROR\gamma t^+$ (Th17) and $ROR\gamma t^-$ (mostly Th1) Teff cells recovered from siLP of transferred *Rag1*$^{-/-}$ mice display pronounced over-expression of TNFα and IFNγ *vs.* wild type counterparts. Mdr1-deficient Th17 cells in siLP also display increased IL-17A production compared with wild type Th17 cells. Importantly, elevated cytokine expression in Mdr1-deficient *vs.* wild type Th17 and Th1 cells is specific to the small intestine and occurs irrespective of antibiotic treatment. As with Mdr1 expression, TNFα over-expression in Mdr1-deficient *vs.* wild type Th17 and Th1 cells is most conspicuous in the distal small intestine (i.e., ileum). shRNAmir-mediated Mdr1 knockdown in wild type Teff cells also triggers TNFα and IFNγ over-expression in siLP of transferred

Rag1$^{-/-}$ mice, compared with those expressing a control shRNAmir against *Cd8a* (shCD8). Further, Mdr1 intrinsically regulates Teff cell homeostasis in siLP, as judged by congenic transfer experiments where *Rag1*$^{-/-}$ mice are transplanted with mixtures of untransduced, shCD8-expressing, and shMdr1-expressing cells discriminated by different retroviral reporters (*e.g.*, GFP, ametrine). Compared with bystander control cells, Mdr1 knockdown drives increased Teff cell expression of TNFα and IFNγ, but only in siLP. Other T_H cell functional parameters are not impacted by Mdr1-deficiency *in vivo*, including development of RORγt$^+$ Th17 cells and Foxp3$^+$ pTregs, expression of tissue-homing chemokine receptors (CCR6, CXCR3, CCR9), and integrins (α4β7), and expression of IL-10 and IL-22, cytokines involved in suppressing intestinal inflammation and enforcing epithelial barrier function, respectively (Davidson et al., 1996; Dudakov et al., 2015). Thus, locally acquired Mdr1 expression enforces mucosal Th17 and Th1 cell homeostasis in the ileum and suppresses inflammation.

Mdr1 interacts with conjugated bile acids

Preferential expression and selective function of Mdr1 in the ileum suggests local interaction with tissue-restricted metabolites. Indeed, soluble small intestine luminal content (siLC), but not colon luminal content (cLC), interacts with Mdr1, as judged by competitive, dose-dependent inhibition of Rh123 efflux in wild type Teff cells. Small intestinal metabolites that interact with Mdr1 could be of host or bacterial origin. However, given high-level expression of Mdr1 in Teff cells from GF mice and mucosal dysfunction of Mdr1-deficient Teff cells in antibiotic-treated *Rag1*$^{-/-}$ mice, we hypothesized that Mdr1 interacts principally with host metabolites. As detailed above, bile acids (BAs; *a.k.a.*, bile salts) are an abundant class of lipophilic detergents synthesized by hepatocytes in the liver and restricted to the small intestine due to active reabsorption in the ileum (Dawson et al., 2009). Because of their detergent-like properties, BAs are also cytotoxic to cells at high extracellular concentrations, as in the case of cholestatic liver diseases where BA accumulation in the liver leads to hepatocellular necrosis, inflammation, and liver failure (Poupon et al., 2000). Indeed, siLC from wild type mice – but not cLC – is cytotoxic to cultured T_H cells. Two results confirm that BAs are the principal Mdr1-interacting metabolites within siLC. First, BA levels are equivalent in siLC from GF and SPF mice, and both GF and SPF siLC interact with Mdr1. Second, depleting BAs from either GF or SPF siLC with the BA sequestering resin, cholestyramine (Arnold et al., 2014), abolishes both cytotoxicity and Mdr1 interaction. BA levels in siLC from T_H cell-transferred *Rag1*$^{-/-}$ mice are also unaffected by antibiotic

treatment, and siLC from both control and antibiotic-treated *Rag1*$^{-/-}$ mice is cytotoxic to cultured T_H cells and interacts with Mdr1.

Five major BA species are found in mammals: cholic acid (CA), chenodeoxycholic acid (CDCA), and β-muricholic acid are primary BAs synthesized in the liver by hepatocytes; deoxycholic acid (DCA) and lithocholic acid (LCA) are secondary BAs generated by enteric bacteria (Hofmann and Hagey, 2014). Each BA species can exist in a free/unconjugated form, or as conjugated to the amino acids taurine or (less frequently) glycine (Hofmann and Hagey, 2014). BA conjugation decreases cytotoxicity and membrane permeability, whilst enabling Asbt-dependent reabsorption in the ileum (Dawson et al., 2003; Dawson et al., 2009; Hofmann and Hagey, 2014). Whereas LCA is largely insoluble in aqueous solution, CA, DCA, CDCA, and β-muricholic acid are all readily soluble; all are cytotoxic to cultured wild type T_H cells at high concentrations (2.5-10 mM), and all modulate Mdr1 transport activity in a dose-dependent manner at lower concentrations (0.5-2 mM). For each BA species, however, only glycine- or taurine-conjugated BAs (CBAs) interact with Mdr1. As only CBAs are actively recycled through the ileal mucosa, specific interaction between Mdr1 and CBAs thus predicts – as observed above – that Mdr1-dependent phenotypes are amplified in the ileum.

CBAs modulate Mdr1 function reversibly, without affecting mRNA (*Abcb1a*, *Abcb1b*) or protein expression (data not shown). In addition, both mouse siLC and synthetic CBAs interact with human MDR1 – whether ectopically expressed in Mdr1^{-} mouse embryonic fibroblasts, or endogenously expressed in primary human CD4^{+} Teff cells. Finally, CBAs interact with Mdr1 indirectly; they are neither direct transport substrates nor inhibitors. CBAs do not compete for Mdr1 binding with established chemotherapeutic substrates (e.g., vinblastine, verapamil), and do not stimulate Mdr1 ATPase activity *in vitro* (Swartz et al., 2013). At the same time, Mdr1 transport activity is required for Teff cell homeostasis in the presence of CBAs (see below), indicating that CBAs are not Mdr1 antagonists.

Mdr1 regulates T cell homeostasis in the presence of conjugated bile acids

Given the interaction between Mdr1 and CBAs, and the fact that loss or knockdown of Mdr1 prompts increased cytokine expression in mucosal Teff cells, we hypothesized that CBAs suppress pro-inflammatory cytokine expression in Teff cells via Mdr1. Indeed, both glycine- and taurine-conjugated CBAs (e.g., CDCA) acutely inhibit TNFγ expression in *ex vivo*-stimulated Teff cells in dose- and Mdr1-dependent manners. By contrast, unconjugated

BAs suppress TNFα expression in both wild type and Mdr1-deficient Teff cells consistent with the fact that only CBAs interact with Mdr1.

Several results indicate that Mdr1 transport function is important for TNF□ regulation in Teff cells exposed to CBAs. First, inhibiting Mdr1 transport activity with elacridar in wild type Teff cells increases TNFα expression in the presence – but not absence – of CBAs. Second, CBA-mediated inhibition of TNFα expression in the Mdr1^{-} mouse T cell lymphoma line EL4 requires ectopic expression of transport-competent human MDR1 (hMDR1); CBAs do not repress TNFα expression in parental EL4 cells, EL4 cells expressing an empty retrovirus, or EL4 cells expressing a transport-deficient human MDR1 mutant (ΔMDR1; Y401A/Y1044A) (Kim et al., 2006). EL4 cells were used for these experiments because ectopic expression of mouse or human MDR1 is not tolerated in primary T_H cells. Finally, Teff cells do not express the known cell surface CBA receptor, Tgr5 (Pols et al., 2011), and unlike Mdr1, Teff cell expression of TNFα in the presence of CBAs *in vitro* or in the small intestine *in vivo* is not influenced by Tgr5-deficiency.

To confirm that actively recycled CBAs are responsible for the over-expression of pro-inflammatory cytokines by mucosal Mdr1-deficient Teff cells *in vivo*, we transferred congenic mixtures of untransduced, shCD8-expressing, and shMdr1-expressing cells into co-housed *Rag1*$^{-/-}$ mice that have or lack the ileal bile acid transporter, Asbt. Indeed, whereas shMdr1-expressing Teff cells display marked over-expression of TNFα relative to bystander controls in siLP from Asbt-sufficient *Rag1*$^{-/-}$ mice, there is no difference in TNFα expression between control and shMdr1-expressing Teff cells in siLP from *Rag1*$^{-/-}$ mice lacking Asbt.

MDR1 transport activity also enforces Teff cell survival in the face of more chronic CBA exposure. In our *Rag1*$^{-/-}$ transfer experiments, we observed that absolute numbers of Mdr1-deficient T_H cells recovered from tissues of transferred *Rag1*$^{-/-}$ mice were modestly, but consistently, reduced *vs.* wild type T_H cells, both in the presence and absence of antibiotics. To more carefully assess the role of Mdr1 in Teff cell persistence *in vivo*, we again transferred congenic mixtures of untransduced, shCD8-expressing and shMdr1-expressing T_H cells into *Rag1*$^{-/-}$ hosts. Compared to bystander controls expressing shCD8, Teff cells expressing shMdr1 show reduced persistence in all tissues. Given extensive Teff cell circulation through the intestinal lamina propria (Morton et al., 2014), this phenotype might also reflect local dysfunction of Mdr1-deficient Teff cells in the presence of ileal CBAs. Indeed, tCDCA kills 80-90% of splenic wild type T_H cells after 18-24 hours, and those that survive are uniformly Mdr1^{+} Teff cells, as judged by either Rh123 efflux in wild type cells, or ametrine expression in Mdr1-reporter cells. Accordingly, neither elacridar-treated wild

type Teff cells nor Teff cells lacking Mdr1 (*Abcb1a/1b*$^{-/-}$) survive long-term tCDCA exposure. In addition, survival of Mdr1^{-} EL4 cells treated overnight with tCDCA also requires ectopic expression of transport-competent hMDR1. Together, these results demonstrate that Mdr1 is required for Teff cell homeostasis in the presence of locally recycled CBAs in the ileum.

EXAMPLES

The following examples are provided to further illustrate the invention but not to limit its scope. Other variants of the invention will be readily apparent to one of ordinary skill in the art and are encompassed by the appended claims.

Example 1 Cell-based screening of MDR1 loss-of-function via Rh123 efflux assay

The basic Rh123 efflux assay is schematically depicted in Figure 1. To identify IBD patients displaying MDR1 loss-of-function, a robust and quantitative cell-based screening platform was developed. Specifically, the screen uses cryopreserved (frozen) peripheral blood mononuclear cells (PBMCs) from healthy donors or IBD patients. It was found that the process of freezing and thawing PBMC does not affect the assay (data not shown). In addition, the screen measures the combined frequency of MDR1^{+} cells within all MDR1-expressing human CD4^{+} and CD8^{+} T cell subsets to better reflect germline-encoded function. To this end, a panel of FACS antibodies were used in the assay to allow for visualization of MDR1-dependent Rh123 efflux in 6 different viable CD3+ T cell subsets: (1) CD4+ naïve T cells (CD4+CD25-CD45RO-CCR7hi); (2) CD4+ regulatory T cells (Treg, CD4+CD25hi); (3) CD4+ effector/memory T cells (Teff, CD4+CD25-CD45RO+CCR7lo); (4) CD8+ naïve T cells (CD8+CD25-CD45RO-CCR7hi); (5) CD8+ effector/memory T cells (Teff, CD8+CD25-CD45RO+CCR7lo); and (6) CD8+ short-lived effector T cells (Temra, CD8+CD25-CD45RO-CCR7lo) (Figure 2). Whereas the first 2 subsets do not express MDR1, subsets 3-6 display MDR1 expression/function, the combined percentage of Rh123lo (MDR1+) cells in these 4 subsets enables calculation of a "cumulative MDR1 score" (Figure 3). This measurement is used to identify IBD patients displaying statistical MDR1 loss-of-function (see below).

The ability to simultaneously visualize MDR1-dependent Rh123 efflux in multiple subsets is important for monitoring assay specificity, and for increasing the likelihood that significantly reduced MDR1 function in multiple MDR1-expressing T cell lineages is due to genetic perturbation (as opposed to environmental effects). To enable quantitative analysis, each healthy control or IBD patient sample is split into 2 equal parts after Rh123 labeling and

prior to Rh123 efflux at 37°C. The first half of cells have nothing added (i.e., media alone), whereas the second half of cells are treated with the selective MDR1 antagonist, elacridar (Hyafil et al., *Cancer Res.* 1993 Oct 1;53(19):4595-602). Inclusion of elacridar enables us to quantify the percentage of Rh123lo (MDR1+) cells within each subset taking into account background Rh123 fluorescence in inhibitor (elacridar)-treated cells (Figure 3).

Finally, to ensure that fluctuation in MDR1 activity between healthy control and IBD patient samples is due to biological but not technical variation, we thaw and analyze a control stock of frozen healthy adult donor PBMC along with up to 10 individual healthy control/IBD patient samples. Analyses of this control stock PBMC over 11 independent Rh123 efflux experiments shows an excellent coefficient of variation (CV) of 8.3% for the cumulative MDR1 score in our stock PBMC (Figure 4); current best practice for cell-based screens recommends CV's < 10%, and tolerates CV's < 20%.

Example 2 Examining MDR1 activity in ileitis-prone mice and human IBD patients

SAMP1/YitFc (SAMP) mice are one of the only pre-clinical models of spontaneous Crohn's disease-like ileitis. We first assayed MDR1-dependent Rh123 efflux from tissues of SAMP1/YitFc, or MHC-matched wild type (WT, AKR) mice. It was found that SAMP Teff cells are MDR1-null (Fig. 6A). By quantifying the cumulative MDR1 scores, we then analyzed MDR1 activities in CD4+ and CD8+ effector T cells from PMBC samples from healthy human adults, as well as human UC patients and CD patients with ileitis. As determined by the D'Agostino-Pearson omnibus normality test, normal distribution of MDR1 function scores were observed with all healthy adults ($n = 49$), ulcerative colitis (UC) patients ($n = 53$), and Crohn's disease (CD) patients ($n = 56$). The data indicate that MDR1 function is reduced in both male and female CD patients (Fig. 5A), and decreases with age in CD and UC patients, but not in healthy adults (Fig. 5B). Notably, MDR1 function is not influenced by IBD-related surgeries (e.g., bowel resections), or medication history.

Importantly, we found that three CD patients (1 male, 2 females) display statistical MDR1 loss-of-function (*i.e.*, cumulative MDR1 function scores less than 3 standard deviations below the healthy donor mean) (Fig. 6B). Each of these $MDR1^{lo}$ CD patients display marked impairment in MDR1 transport activity in all T cell subsets analyzed (Fig. 7). They also show atypically low MDR1 function for their age (Fig. 5B), and have unique medication histories (Fig. 8A). Clinically, all three $MDR1^{lo}$ CD patients were diagnosed at an earlier than average age (Fig. 8B), and have a history of aggressive small bowel disease. Indeed, MDR1 function is lowest among CD patients with treatment-refractory ileitis (*i.e.*, have required surgical intervention) (Fig. 8C), including two of the three $MDR1^{lo}$ CD

patients; the third was only recently diagnosed. Together, these data suggest that MDR1 function influences CD susceptibility and disease severity.

Example 3 MDR1 regulates T cell homeostasis in the presence of conjugated bile acids

Given that MDR1 interacts with small intestinal CBAs, and loss or knockdown of MDR1 in Teff cells leads to increased TNFα and IFNγ expression in the small intestine, we hypothesized that CBAs suppress pro-inflammatory cytokine expression via MDR1. Indeed, it was found that both glycine- and taurine-conjugated CDCA acutely inhibit TNFα expression in *ex vivo*-stimulated Teff cells in dose- and MDR1-dependent manners. By contrast, unconjugated CDCA suppresses TNFα expression in both wild type and MDR1-deficient Teff cells, consistent with the fact that only CBAs interact with MDR1. Elevated TNFα expression in CBA-treated MDR1-deficient Teff cells is not recapitulated in the presence of other detergents (e.g., tween 20, NP-40, etc.), indicating that MDR1 does not generically regulate Teff cell function in the face of membrane damage. Further, unlike MDR1, TNFα regulation by CBAs does not involve the cell surface BA receptor, Tgr5 (*Gpbar1*). Indeed, Teff cells do not express Tgr5 *in vivo*, and neither germline ablation nor shRNA-mediated knockdown of Tgr5 affects Teff cell TNFα expression in the presence of CBAs *in vitro* or in the small intestine *in vivo*.

MDR1 transport activity is important for CBA-dependent TNFα suppression. First, we observed that inhibition of MDR1 function in wild type Teff cells via elacridar increases TNFα expression in the presence, but not absence, of tauro-chenodeoxycholic acid (tCDCA). As expected, elacridar has no impact on TNFα expression in MDR1-deficient Teff cells. Second, tCDCA-mediated inhibition of TNFα expression in the MDR1$^-$ mouse T cell lymphoma line EL4 requires ectopic expression of transport-competent human MDR1 (hMDR1); tCDCA does not repress TNFα expression in parental EL4 cells, EL4 cells expressing an empty retrovirus, or EL4 cells expressing a transport-deficient MDR1 mutant. EL4 cells were used for these experiments because constitutive MDR1 expression is not tolerated in primary T_H cells. Finally, tCDCA down-regulates *Tnf* mRNA expression in an MDR1-dependent manner, indicating regulation of gene expression.

MDR1 transport activity also enforces T cell survival in the face of more chronic CBA exposure, consistent with the fact that loss or knockdown of MDR1 results in impaired Teff cell persistence *in vivo*. First, we observed that tCDCA kills 80-90% of *ex vivo*-isolated wild type T_H cells after 18-24 hours. Second, the surviving 10-20% of tCDCA-treated T_H cells are uniformly MDR1$^+$ Teff cells, judged either by Rh123 efflux in wild type cells, or ametrine expression in MDR1-reporter cells. Third, inhibition of MDR1 transport activity by

elacridar abolishes wild type Teff cell survival in the presence of long-term tCDCA treatment, but has no effect on survival in the absence of tCDCA. Fourth, MDR1-deficient T_H cells do not survive chronic tCDCA exposure, even in the absence of elacridar. Prolonged survival of EL4 cells in the presence of tCDCA also requires ectopic expression of transport-competent hMDR1. In conclusion, these data indicate that MDR1 directly and specifically regulates Teff cell homeostasis in the presence of CBAs.

Example 4 Bile acid sequestration remedies MDR1-deficient T_H cell dysfunction in *vivo*

Our studies revealed that MDR1 directly and specifically regulates Teff cell homeostasis in the presence of conjugated bile acids (CBAs). We therefore hypothesized that BA sequestrants such as cholestyramine (CME), which bind CBAs in the lumen of the small intestine and prevent reabsorption through the ileal lamina propria, will correct MDR1-deficient T_H cell dysfunction *in vivo*. After confirming CME treatment increases fecal bile acid excretion in *Rag1*$^{-/-}$ mice transplanted with MDR1-deficient T_H cells, indicative of reduced intestinal reabsorption, we found that CME treatment: (*i*) suppresses ileitis; (*ii*) increases MDR1-deficient Teff cell persistence in small intestine; and (*iii*) reduces TNFα and IFNγ expression by MDR1-deficient Th1 and Th17 cells in the small intestine.

Bile acid sequestration can have systemic effects on metabolism, confounding analysis of weight loss; reduced BA recirculation to the liver stimulates *de novo* BA biosynthesis by hepatocytes, reduces cholesterol, and shows metabolic benefit in animal models of obesity and diabetes. However, we observed that CME does not impact T_H cell-induced colitis, and has no effect on Teff cell function outside of the small intestine. Therefore, these data suggest that locally recycled CBAs interact with mucosal Teff cells in the ileum *in vivo*, where they promote pro-inflammatory Th17 and Th1 cell dysfunction in the absence of MDR1.

If Teff cells infiltrating the ileum upregulate Mdr1 expression to prevent potentially pathogenic interaction with locally recycled CBAs, then bile acid sequestrants such as cholestyramine (CME) (Arnold et al., 2014) – that bind CBAs in the intestinal lumen and prevent their reabsorption into the ileal lamina propria – could be pharmacologically useful to limit mucosal dysfunction of Mdr1-deficient Teff cells and reduce associated ileitis without inducing systemic immune suppression. To test this, we treated Rag1$^{-/-}$ mice transplanted with wild type or Mdr1-deficient T_H cells with or without CME upon onset of weight loss to assess therapeutic effects. We confirmed that CME-treated Rag1$^{-/-}$ recipients display increased fecal BA excretion, indicative of reduced ileal BA reabsorption (Bhat et al., 2003; Dawson et al., 2003), and we found that CME selectively attenuates Mdr1-defcient T_H cell-

induced ileitis; CME has no impact on normal small bowel histology in $Rag1^{-/-}$ mice receiving wild type T_H cells, and does not affect colitis induced by either wild type or Mdr1-deficient T_H cells. In line with the fact that CME does not attenuate colitis in this model, CME has only modest effects on weight loss, albeit more so in $Rag1^{-/-}$ mice transplanted with Mdr1-deficient T_H cells where ileitis is reduced. CME also normalizes TNFα and IFNγ expression between wild type and Mdr1-deficient Th17 and Th1 cells in siLP without influencing Teff cell cytokine expression outside of the small intestine. Although BA sequestration can have broader effects on host metabolism in the liver (Bhat et al., 2003; Fan et al., 2014; Hofmann and Hagey, 2014), these results are consistent with the notion that BA sequestration also acts locally in the ileum to prevent pathogenic interactions between mucosa-associated CBAs and Mdr1-deficient Teff cells.

Given these results, we hypothesized that MDR1 loss-of-function might be evident in other clinical or pre-clinical instances of ileitis. The SAMP1/YitFc (SAMP) mouse is one of the only pre-clinical models of spontaneous Crohn's disease-like ileitis (Pizarro et al., 2003). SAMP ileitis occurs with 100% penetrance by 30 weeks of age, and displays histologic similarity to CD (Rodriguez-Palacios et al., 2015). Like Mdr1-deficient cells, SAMP Teff cells display increased TNFα and IFNγ expression in siLP, and transfer ileitis in SCID recipients (Kosiewicz et al., 2001; Marini et al., 2003). SAMP ileitis also develops under germ free conditions, indicating disease pathogenesis does not require enteric bacteria (Barnes et al., 2007). Using Rh123 efflux as a measure of Mdr1 function, we found that SAMP Teff cells are indeed Mdr1-null. Mdr1 loss-of-function in SAMP mice parallels expression of an altered Abcb1a transcript that is present at reduced levels compared to MHC-matched wild type (AKR) mice and that lacks exon 23 by sequence alignment (Figure 7B). Exclusion of exon 23 from SAMP Abcb1a mRNA is not due to a genomic deletion (Rodriguez-Palacios et al., 2014), which is consistent with a previously reported retroviral insertion found near the 5' boundary of Abcb1a exon 23 in other SAMP-related strains (Zhang et al., 2008).

MDR1 function is also reduced in circulating T cells from Crohn's disease (CD) patients compared to either healthy adults or ulcerative colitis (UC) patients. This measure of MDR1 function incorporates the combined frequency of $Rh123^{lo}$ ($MDR1^+$) cells within all MDR1-expressing human $CD4^+$ and $CD8^+$ T cell subsets to better reflect germline-encoded function ($CD4^+$ Teff cells, $CD8^+$ naïve T cells, $CD45RO^+$ and $CD45RO^-$ $CD8^+$ Teff cells). We ensured assay reliability over time by analyzing MDR1 function in a control stock of healthy donor PBMC in parallel with each independent batch of patient samples, and we

confirmed that all data are normally distributed. MDR1 function is reduced in both male and female CD patients, and decreases with age in CD and UC patients, but not in healthy adults. Notably, MDR1 function in IBD patient PBMC is not generically influenced by the number of bowel surgeries or medication history.

Three CD patients in particular (1 male, 2 females) display marked MDR1 loss-of-function (i.e., cumulative MDR1 function scores < 3 standard deviations below the healthy donor mean). Each of these $MDR1^{lo}$ CD patients show impaired transport activity in all MDR1-expressing T cell lineages; they also show atypically low MDR1 function relative to other patients of similar age, and have unique medication histories. Clinically, all three $MDR1^{lo}$ CD patients were diagnosed at an earlier than average age, and two of these patients have a history of aggressive small bowel disease requiring surgical intervention; the third $MDR1^{lo}$ patient was only recently diagnosed upon presenting with ileitis. That deficiencies in MDR1 function are observed in humans and enriched for within ileal CD patients is both predicted by our animal studies, and supportive of the notion that MDR1 has unique functions that safeguard immune homeostasis in the ileum.

Here we show that Th17 and Th1 cells "adapt" upon migration into the lamina propria of the ileum. Ileal adaptation involves upregulation of the xenobiotic membrane transporter, Mdr1, and permits homeostasis in the presence of locally recycled bile acids. $CD4^+$ effector/memory (Teff) lineages, particularly Th17 cells, are recognized for displaying functional plasticity following initial differentiation; plasticity referring to instances where Teff cells of one lineage (e.g., $IL\text{-}17A^+$ Th17 cells) modify effector cytokine expression to take on characteristics of another lineage (*e.g.*, IFNg expression) (Nakayamada et al., 2012).

By contrast, adaptation occurs when Teff lineages acquire novel functions within discrete tissue microenvironments. Adaptation of Th17 and Th1 cells shown here in the ileum is consistent with previous reports showing tissue-specific functions in $Foxp3^+$ Tregs. In visceral adipose tissue, for example, Tregs upregulate expression of the nuclear receptor, PPARg, to suppress diabetogenic inflammation (Cipolletta et al., 2012). Tregs in skeletal muscle and lung upregulate amphiregulin (Areg) to facilitate tissue-repair following injury (Arpaia et al., 2015; Burzyn et al., 2013). And in colon lamina propria, Tregs upregulate expression of ST2, a component of the IL-33 receptor, to suppress colitogenic Teff cell function (Schiering et al., 2014). Most recently, Mucida and colleagues showed that Tregs adapt to the intestinal epithelium by converting to $CD4^+CD8aa^+$ intraepithelial lymphocytes (Sujino et al., 2016). Our results suggest that adaptation within non-lymphoid tissues is a common feature of circulating lymphocytes, which highlights the impact of the tissue

microenvironment on lymphocyte function, raises important new concepts in the pathogenesis of tissue-specific inflammatory diseases, and predicts that tissue-specific immune modulation is possible in patients.

Our results also show that steady-state circulation of conjugated bile acids (CBAs) through the ileal mucosa has direct and important consequences on mucosal Teff cell function; Teff cells lacking Mdr1 display marked and specific mucosal dysfunction in the ileum that is rescued by either genetic ablation of the ileal bile acid transporter, Asbt, or dietary administration of the bile acid sequestrant, cholestyramine. Enterohepatic CBA circulation allows for maintenance of the intestinal CBA pool *in vivo* without the need for continual replenishment through biosynthesis. A defining feature of enterohepatic CBA circulation is active reabsorption in the ileum by specialized enterocytes expressing the CBA transporters Asbt – that mediates transport of luminal CBAs across the apical membrane of enterocytes – and OSTa/b, which drives basolateral CBA transport from the enterocyte cytoplasm into the lamina propria (Dawson et al., 2009). Because of their fundamental activities in digestion and nutrient absorption, CBAs have evolved to be synthesized in the liver and reabsorbed in the ileum independent of microbiota (Hofmann and Hagey, 2014). Indeed, we show that Mdr1 is both highly expressed in small intestinal Teff cells from germ free mice, and required for mucosal Teff cell homeostasis in antibiotic-treated *Rag1*$^{-/-}$ mice. Thus, in addition to interacting with microbiota, mucosal Teff cells have evolved mechanisms to interface directly with host-derived intestinal metabolites.

Surprisingly little is known about the concentration and composition of the mucosa-associated CBA pool in the ileum, though it is reasonable to expect that mucosa-associated CBAs are present in the ileum at micro-molar concentrations; between the well established concentrations of CBAs in the ileal lumen (1-10 mM) and those observed in the portal vein (~ 50 mM) (Dawson et al., 2009; Hofmann and Hagey, 2014). This estimate also assumes that CBA concentrations within the ileal lamina propria fluctuate throughout the day, increasing in response to food intake and decreasing during periods of fasting. In line with these estimated concentrations, we find that individual CBAs (*e.g.*, taurine- or glycine-conjugated chenodeoxycholic acid), modulate Mdr1 function, TNFa expression, and survival in cultured Teff cells starting at ~ 300 mM. Endogenous bile acids present in the small intestine lumen – whether from wild type, germ free, or antibiotic-treated mice – interact with Mdr1 at even lower concentrations (25-100 mM). We used taurine-conjugated chenodeoxycholic acid (tCDCA) in our *in vitro* experiments because it is both a hydrophobic primary BA synthesized by hepatocytes independent of microbiota and a high-affinity Asbt transport

substrate (Craddock et al., 1998). Indeed, treating splenic wild type and Mdr1-deficient Teff cells with tCDCA recapitulates several aspects of Mdr1-deficient Teff cell mucosal dysfunction observed in small intestines of transferred *Rag1*$^{-/-}$ mice, namely increased TNFa expression and decreased persistence; these effects of tCDCA on cultured Teff cells are both time- and dose-dependent. Thus, a full appreciation of the complex interplay between mucosal Teff cells and mucosa-associated CBAs *in vivo* requires further insight into the size and composition of the intestinal CBA pool *in vivo*, the duration of Teff cell persistence in the ileum, and how these parameters change in the context of disease.

Much remains to be learned about how CBAs interact with Mdr1, and how these interactions in turn dictate Teff cell survival and function. We show that CBAs acutely modulate Mdr1 function without acting as either direct transport substrates or inhibitors. Rather, several lines of evidence suggest a model wherein Mdr1 transports endogenous lipids upon CBA exposure to maintain plasma membrane integrity. First, CBAs are membrane impermeant detergents that influence cell physiology primarily by disrupting plasma membrane architecture (Jean-Louis et al., 2006; Zhou et al., 2009; Zhou et al., 2013). Second, Mdr1 is a broad-specificity "lipid flippase", capable of transporting diverse phospholipid species from the inner to outer membrane leaflet (Sharom et al., 2005). Third, the closest homologue of human MDR1 is ABCB4, a dedicated phosphatidylcholine (PC) transporter that secretes PC into bile in a manner that is indirectly stimulated by CBAs (Morita and Terada, 2014; Vasiliou et al., 2009). This CBA-stimulated lipid transport model is consistent with our findings that CBA exposure leads to competitive inhibition Mdr1-dependent Rh123 efflux without binding directly to Mdr1 (Figure S6H), while at the same time modulating Teff cell survival and function in a manner that requires Mdr1 transport activity. Membrane disorganization induced by CBAs in the absence of Mdr1 could result in overt membrane damage, which we have not observed (data not shown), or lead to altered signaling due to disruption of spatially-organized plasma membrane microdomains, such as cholesterol-rich lipid rafts (Jean-Louis et al., 2006; Zhou et al., 2013). Indeed, our study opens new avenues for interrogating the endogenous substrates and functions of Mdr1, which our results suggest are critical for mucosal immune homeostasis in the ileum.

Our results also have important implications for the understanding and treatment of inflammatory diseases targeting the ileum, in particular Crohn's disease (CD). Ileitis is the most common and distinctive feature of CD, yet specific pathophysiologies underlying ileitis remain unclear and likely vary from patient-to-patient as a result of both individual genetic and environmental risk factors (Kaser et al., 2010). Indeed, we show that ileitis-prone

(SAMP1/YitFc) mice harbor a null mutation in the mouse ortholog of human MDR1 (*Abcb1a*). In addition, we show overt MDR1 loss-of-function in a small group (~ 5%; 3 of 56) of ileal CD patients. Whether MDR1 loss-of-function in these patients is due to genetic mutations is not yet clear, though the fact that MDR1 function is markedly reduced in all major MDR1-expressing T cell lineages is consistent with impaired germline-encoded function. Thus, whereas overt MDR1 loss-of-function is not a feature of all or even most ileal CD patients, these data suggest that impaired MDR1 function could contribute to ileitis in a subset of CD patients in a manner that involves interaction with additional susceptibility loci.

That Mdr1 regulates ileitis in a manner that depends on genetic context is also evident in mice. In our experiments, for example, Mdr1-deficiency (in T_R cells) interacts with Rag1-deficiency to promote ileitis on the FVB background. By contrast, spontaneous ileitis has not been reported in FVB.*Abcb1a*$^{-/-}$ mice that instead are prone to colitis (Panwala et al., 1998; Staley et al., 2011). Further, whereas the *Abcb1a* null mutation in SAMP1/YitFc mice described here is associated with spontaneous ileitis, similar *Abcb1a* mutations in other senescence-accelerated mouse (SAM) strains are only sporadically associated with ileitis (Zhang et al., 2008). Of course, Mdr1 is widely expressed *in vivo*, and deficiency in Mdr1 is thus likely to impact many cell types and tissues, most prominently intestinal epithelial cells (Maxwell et al., 2015). By contrast, our *Rag1*$^{-/-}$ transfer data demonstrate direct, cell-intrinsic regulation of mucosal Teff cell function by Mdr1, which is evident by both genetic ablation and shRNAmir-mediated knockdown on multiple genetic backgrounds (*e.g.*, FVB, B6). Thus, we postulate that bile acid-driven dysfunction of Mdr1-deficient Teff cells in the ileum lays a pathogenic foundation for progression to overt ileitis following additional genetic lesions.

Whether rare or more common, our findings suggest that pharmacologic strategies targeting ileal bile acid reabsorption may be therapeutic in clinical settings where ileal CD is associated with overt MDR1 loss-of-function. Although BA sequestrants, such as cholestyramine, colestipol, and colesevelam, have yet to be formally tested for efficacy in CD, a recent study found that cholestyramine induced marked endoscopic improvement and mucosal healing in a small cohort of IBD patients with associated primary sclerosing cholangitis (PSC) (Pavlidis et al., 2015). Our data suggest a mechanistic basis for this clinical result, and encourage expanded clinical efforts to test BA sequestrants in Crohn's disease.

Example 5 Methods and materials

Mice: Wild type C57Bl/6 (B6; stock no. 000664), B6.*Rag1*$^{-/-}$ (stock no. 002216), *Il17a*^{Cre} (stock no. 016879), SAMP1/YitFc (stock no. 009355), and AKR/J (stock no. 000648) mice were purchased from The Jackson Laboratory. *ROSA26*^{hCD2} transgenic mice

were generously provided by Dr. Klaus Rajewsky (Max Delbruck Center for Molecular Medicine, Berlin, Germany). *Il17a*^{Cre} mice were crossed with *ROSA26*^{hCD2} transgenic animals to produce *Il17a*$^{+/Cre}$*ROSA26*$^{+/hCD2}$ Th17 fate-mapping reporter mice. Wild type FVB/N (FVB; model no. FVB) and FVB.*Abcb1a/1b*$^{-/-}$ (model no. 1487) mice were purchased from Taconic. FVB.*Rag1*$^{-/-}$ mice were provided by Dr. Allan Bieber (Mayo Clinic, Rochester, MN). BALB/c mice were from CLEA (Japan) and maintained under specific pathogen-free (SPF) or germ-free (GF) conditions at Osaka University. *Myd88*$^{-/-}$, *Asc*$^{-/-}$, and *Cx3cr1*$^{+/EGFP}$ knock-in (heterozygous) mice were generated as described in Takeuchi et al., Immunity 11, 443-451, 1999; and Yoshida et al., Immunity 15, 569-578, 2001. The mice were maintained at Osaka University. Tgr5 knockout (*Gpbar1*$^{-/-}$) mice were generously provided by Dr. Kristina Schoonjans (EPFL, Lausanne, Switzerland). MDR1-reporter (*Abcb1a*^{ametrine}) mice were generated at Rodent Genetic Engineering Core (RGEC) at New York University (NYU) (see below). All mice were housed, bred, used in experiments, and sacrificed in accordance with protocols approved by the Institutional Animal Care and Use Committees of Scripps Florida, Osaka University, or NYU Medical Center.

CRISPR/Cas9-mediated generation of Abcb1a^{ametrine} reporter mice: Mdr1-reporter (*Abcb1a*^{ametrine}) mice were generated using the Cas9-cleavase system in wild type C57Bl/6 zygotes. Briefly, two guide RNAs (gRNAs) were identified using a publically available CRISPR design tool (http://crispr.mit.edu). These gRNAs are predicted to introduce single-stranded nicks on opposite DNA strands 22 bp upstream and 10 bp downstream of the *Abcb1a* stop codon; synthetic gRNAs were purchased (PNA Bio) and Cas9-dependent cleavage at the *Abcb1a* locus was confirmed *in vitro* prior to zygote microinjection as previously described (Kim et al., 2014). gRNA sequences are shown below:

5' gRNA (upstream of *Abcb1a* stop codon, ***protospacer in bold italics***):

5'-

G***CUUCUCAAUGGUCAGUGUGC***UGUUUUAGAGCUAGAAAUAGCAAGUUAAAAU
AAGGCUAGUC
CGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3'

3' gRNA (upstream of *Abcb1a* stop codon, ***protospacer in bold italics***):

5'-

G***AAAAUACUUAACAUCUUACA***UGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUA
AGGCUAGUC
CGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3'

To facilitate reporter transgene insertion, we constructed a homology-directed DNA repair (HDR) template. 500 bp arms of homology (AOH; 5' and 3') were purchased (IDT); the 5' AOH contains sequences corresponding to all of *Abcb1a* exon 28 (204 bp) – minus the stop codon – plus 204 bp of intron 27-28; the 3' AOH contains the first 500 bp of the 3' untranslated region (UTR) downstream of the stop codon. Synonymous point mutations were introduced into both 5' and 3' AOH at the protospacer-adjacent motifs (PAMs) to remove Cas9-cleavase activity towards the HDR template. AOHs were then assembled into an ampicillin-resistant cloning vector (pSTBlue-1, Novagen) together with bicistronic reporter elements in the following order: 5' AOH (no *Abcb1a* stop codon) – glycine-serine-glycine (GSG) linker (in-frame, 5'- GGATCCCGA-3') –P2A peptide (in-frame, 5'-GCCACGAACTTCTCTCTGTTAAGCAAGCAGGAGACGTGGAAGAAAACCCCGGTCCT-3') – ametrine open reading frame (in-frame, with stop codon) – 3' AOH. Correct assembly was confirmed by sequencing, and the linear HDR template was excised from pSTBlue-1 (PmeI and NotI), and gel purified for zygote injections.

Approximately 150 C57Bl/6 zygotes were generated by *in vitro* fertilization (IVF), and micro-injected with gRNAs (10 μg each), linearized HDR template (10 □g), and Cas9-cleavase mRNA (10 μg). 76 zygotes were gown to the 400-cell blastocyst stage and implanted into 8-week old female surrogate mothers; live pups were analyzed for transgene insertion using 5' (5'-AGTTTAACGTGTCTGCAGCTGG-3') and 3' (5'-AGCCTGCAGGATCTGTCTG-3') genotyping primers that anneal outside of the targeted region. 19 live pups were recovered from the targeting; one female founder pup showed targeted transgene insertion by genotyping.

Human blood: All experiments using human blood were conducted in accordance with an IRB protocol (IRB-14-6374) approved by the Scripps institutional review board. Buffy coats from healthy adult volunteers were purchased from OneBlood (Fort Lauderdale, FL), and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation. For profiling MDR1 function in healthy adult donors and IBD patients, frozen PBMC samples collected, prepared and stored at the UCLA Center for Inflammatory Bowel Diseases. Frozen PBMC vials, along with de-identified demographic and medical history information were sent to Scripps Florida for FACS analysis. 11 additional healthy donor frozen PBMC samples were graciously provided by Dr. Derya Unutmaz (The Jackson Laboratory, Farmington, CT).

Cell isolation: Mouse single mononuclear cell suspensions were prepared from spleen, peripheral lymph nodes, or mesenteric lymph nodes (MLN) as described in Uhlig et

al. J. Immunol. 177, 5852-5860, 2006. For some experiments, $CD4^+CD25^-$ T cells were magnetically isolated total splenocytes using an EasySep T cell negative isolation kit (Stem Cell Technologies, Inc.). For T cell transfer experiments into *Rag1*$^{-/-}$ mice (see below), $CD4^+CD25^-$ T cells from spleens of donor mice were magnetically enriched as above, and then FACS-sorted to obtain pure naïve T cells ($CD3^+CD4^+CD25^-CD62L^{hi}CD44^{lo}$). For isolation of mononuclear cells from intestinal tissues, whole small intestines (duodenum to terminal ileum) or colons (cecum to anus) were removed, flushed with PBS to remove the fecal contents, and opened longitudinally to expose the epithelium. Peyers patches were excised from small intestines. Tissues were cut into small segments (~ 3 cm) and incubated for 30 min at room temperature in DMEM media (without phenol red; Life Technologies) plus 0.15% DTT (Sigma-Aldrich) to remove mucus. After washing with media, intestines were incubated for 30 min at room temperature in media containing 1 mM EDTA (Amresco) to remove the epithelium. After washing again with media, lamina propria was digested in media containing 0.25 mg/mL liberase TL and 10 U/mL RNase-free DNaseI (both from Roche), with shaking in a bacterial incubator (Environ Shaker; Labline) for 15-25 min at 37°C. Single cell suspensions were passed through 70 μm nylon filters (BD) and mononuclear cells were isolated by 70/30% percoll gradient centrifugation (Sigma-Aldrich). Mononuclear cells were washed twice in complete T cell medium (see cell culture below), counted, and resuspended for FACS analysis or sorting. For isolation and analysis of antigen-presenting cells, mononuclear cells from spleen or small intestinal lamina propria were recovered as previously described[811], incubated with blocking antibodies (anti-CD16/32) for 5 min at 4°C, stained with antibodies against CD45, CD11b, and CD11c, and sorted based on differential CD11b/CD11c expression with the $CD45^+$ gate. Splenic dendritic cells were sorted as $CD45^+CD11c^+CD11b^-$. $Rh123^{hi}$ ($MDR1^-$) or $Rh123^{lo}$ ($MDR1^+$) Teff cells ($CD45^+CD3^+CD4^+CD25^-CD62L^{hi}CD44^{lo}$) from mesenteric lymph nodes or small intestinal lamina propria as described in Ramesh et al. (J. Exp. Med. 211: 89-104, 2014), following *ex vivo* Rh123 efflux assays. Background Rh123 efflux in cells treated with elacridar (0.1 μM) was used to set sorting gates.

T cell transfer colitis: For T cell transfer experiments using wild type FVB or FVB.*Abcb1a/1b*$^{-/-}$ mice, 0.5-1 x 10^6 naïve $CD4^+$ T cells (FACS-sorted as above) were injected (i.p.) into syngeneic FVB.*Rag1*$^{-/-}$ mice. For transfers of shCD8- or shMdr1-expressing T cells, magnetically isolated FVB wild type $CD4^+CD25^-$ T cells were transduced *in vitro* (see below), FACS-sorted as live (viability dye$^-$) ametrine$^+$ cells on day 5 post-activation, and 0.5-1 x 10^6 cells were injected (i.p.) into syngeneic FVB.*Rag1*$^{-/-}$ mice. For

shRNA mixture experiments, transduced T cells were FACS-sorted on day 5 as live (viability dye$^-$) ametrine$^-$GFP$^-$ cells (for untransduced cells), or ametrine$^+$ or GFP$^+$ cells (for transduced cells). Sorted cells were counted, mixed at 1:1:1 ratios (untransduced:GFP$^+$:ametrine$^+$), and checked by FACS to confirm equimolar ratios. 0.3-0.6 x 10^6 total mixed cells were injected (i.p.) into syngeneic FVB.*Rag1*$^{-/-}$ mice. For all experiments, *Rag1*$^{-/-}$ mice were weighed immediately prior to T cell transfer to determine baseline weight; mice were weighed twice weekly for the duration of the experiments, and euthanized if $\geq$ 20% baseline weight loss was reached. Morbidity indexes, shown throughout, are calculated by dividing the maximum percentage of body weight loss by the number of days weight loss occurred in (*e.g.*, 20% body weight loss/45 days). *Rag1*$^{-/-}$ mice injected with different donor T cells (*i.e.*, wild type *vs.* Mdr1-deficient T cells; shCD8- *vs.* shMdr1-expressing T cells) were co-housed. For antibiotic treatment experiments, mice were separated into groups receiving water alone, or water containing antibiotics: ampicillin (1 M), vancomycin (0.5 M), metronidazole (1 M), neomycin (1 M), streptomycin (2 M) (all from Sigma-Aldrich). Antibiotic treatment began ~ 3-4 weeks post-transfer (upon onset of weight loss); water (+/-antibiotics) was replaced twice weekly during assessment of weights. For cholestyramine (CME) treatment experiments, mice were separated into groups receiving standard chow, or chow supplemented with 2% CME (Sigma-Aldrich), again ~ 3-4 weeks post-transfer. Standard chow pellets were ground to a fine powder using a Vitamix Professional Series 200 food processor (VitaMix), and separated, weighed, and supplemented with 2% CME resin (w:w) as necessary. During treatment, solid food was removed and replaced with covered feeding containers (Ancare Corporation) containing powder food (+/- CME), which were placed inside the cages and exchanged for cleaning at least weekly.

Histology: Colon (proximal, distal) or small intestine (proximal, mid, distal/ileum) sections (~ 1 cm) were cut from euthanized *Rag1*$^{-/-}$ mice ~ 6-8 weeks post-T cell transfer. In some experiments, larger, ~ 10 cm segments of distal small intestine and whole colon were dissected from mice and fixed intact. All tissues were fixed in 10% neutral buffered formalin, embedded into paraffin blocks, cut for slides, and stained with hematoxylin and eosin (H&E). H&E-stained sections were analyzed and scored blindly; colons as in (Wirtz et al., 2007), and small intestines/ilea as in (Kosiewicz et al., 2001).

Flow Cytometry: With the following exceptions (see below) surface and intracellular FACS staining was performed as described in Ramesh et al., J. Exp. Med. 211: 89-104, 2014; and Atarashi et al., Nature 455: 808-812, 2008. FACS analysis of Rh123 efflux was performed as in Ramesh et al., J. Exp. Med. 211: 89-104, 2014.

Intracellular FACS analyses: For intracellular stains including transcription factors (e.g., RORγt, Foxp3), cells were fixed and permeabilized using a Foxp3 intracellular staining kit (eBioscience). For all intracellular stains, cells were stimulated for 3-4 hr with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A (all from Sigma-Aldrich) prior to antibody staining. Simultaneous intracellular staining of TNFα protein and Tnf mRNA in PMA/ionomycin-stimulated cells was achieved using a mouse PrimeFlow™ RNA Assay kit (Affymetrix, Cat.#: 88-18001). The 96-well PrimeFlow™ RNA Assay protocol was followed per manufacturer's instructions without deviation; following fixation/permeabilization, cells were stained with alexa fluor 647-conjugated (type 1) and alexa fluor 488-conjugated (type 4) target probes against mouse Tnf (NM_013693) and beta actin (Actb, NM_007393), respectively. Following target probe signal amplification and detection, cells were stained with a PE-Cy7-conjugated anti-TNFα antibody (Biolegend), a fixable viability dye (e506, eBioscience), and a cocktail of antibodies against cell-surface antigens allowing for discrimination of effector/memory T_H cells (see below for full list of antibodies).

FACS analyses of Rh123 efflux: To determine background Rh123 efflux, the MDR1 antagonist, elacridar (0.1 μM), was added to Rh123-labelled cells immediately prior to efflux at 37°C. For experiments using intestinal content – small intestine luminal content (SILC), colon luminal content (CLC) – or synthetic bile acids, Rh123-labelled cells were treated with additives prior to efflux in place of elacridar.

FACS antibodies: Anti-mouse antibodies used for FACS analysis include: Alexa700-CD45, BV650-CD3, BV711-CD4, BV605-CD25, Percp-Cy5.5-CD44, BV605-CD62L, APC-IFNγ, APC-F4/80, APC-CD4, FITC-IL-17A, Percp-Cy5.5-IL-17A, PE-Cy7-TNFα, PE-IL-4, PE-CD45, PE-CD63, PE-CX_3CR1, Pacific Blue-CD11b (all from Biolegend); mouse Fc Block (purified anti-CD16/32), PE-CF594-CD25 and PE-CF594-RORγt, PE-Siglec-F, PE-Ly-6G, FITC-Ly-6C, FITC-CD103, APC-CD11c, APC-CD45 (from BD); and e450-Foxp3, FITC-I-A(b), and FITC-CD80 (from eBioscience).

Anti-human antibodies used for FACS analysis were: BV605-CD3, BV421-CD4, PE-Cy7-CD45RO, Percp-Cy5.5-CCR7 (from Biolegend); and PE-CF594-CD25 (from BD). Vital dyes include: fixable viability dyes eFluor® 450, eFluor® 506, and eFluor® 660 (eBioscience) and 7-AAD (Biolegend). Rh123 and elacridar were purchased from Sigma-Aldrich.

FACS instruments and analyses: All FACS data was acquired on LSRII or FACSCantoII instruments (BD), and analyzed using FlowJo software (TreeStar, Inc.). For

cell sorting, cells were filtered after staining, resuspended in DMEM media (see below) without serum, and sorted on a FACS AriaII machine (BD). For quantifying percentages of $MDR1^+$ T cells, gates were set based on background Rh123 efflux in elacridar-treated cells. In some experiments, MDR1 transport activity is quantified as the change (Δ) in Rh123 mean fluorescence intensity (MFI) in elacridar- *vs.* medium-, SILC-, or synthetic bile acid-treated samples. Cytokine expression in T cells isolated from tissues of transferred *Rag1*$^{-/-}$ mice was analyzed between 5-8 weeks post-T cell transfer, depending on animal morbidity.

Cell culture: Mononuclear cells or purified $CD4^+$ T cells were cultured in complete T cell media described in Carlson et al., J. Immunol. 192, 2167-2176, 2014. For *in vitro* T cell activation/polarization, magnetically isolated $CD4^+CD25^-$ T cells from spleen were activated by anti-CD3/anti-CD28 antibodies (non-polarizing conditions; ThN) or polarized to Th1, Th2, iTreg, or non-pathogenic Th17 cells as described in Sundrud et al., Science 324, 1334-1338, 2009. Pathogenic Th17 cells were polarized as in Lee et al., Nat. Immunol.13, 991-999, 2012. For *in vitro* induction of MDR1 expression in $CD4^+$ T cells by antigen-presenting cells (APCs), FACS-sorted $Rh123^{hi}$ ($MDR1^-$) Teff cells from small intestinal lamina propria (see above) were co-cultured in U-bottom 96-well plates with APC subsets from small intestine or spleen at a 1:2 ratio and stimulated with 1 μg/ml soluble anti-CD3 (BioLegend). MDR1 expression was analyzed on day 4 post-stimulation by staining cultures with 7-AAD and anti-CD4 antibodies following Rh123 efflux assay.

Retroviral plasmids and transductions: shRNAs against mouse CD8 (Cd8a), Mdr1 (Abcb1a, Abcb1b), and Tgr5 (Gpbar1) were purchased from TransOMIC Technologies. shRNAs were PCR amplified and cloned into GFP- or ametrine-expressing murine retroviral vectors (LMPd) containing a modified/enhanced miR-30 expression cassette. HA-tagged human MDR1 (ABCB1) was purchased from Addgene (plasmid 10957). Full-length ABCB1 was PCR-amplified without the HA-tag, and cloned into an ametrine-expressing LMPd vector without the miR-30 expression cassette. An empty LMPd.ametrine vector was generated by cutting out ABCB1 from the modified vector, blunting the restriction sites, and re-ligating. Transport-defective mutant MDR1 (ΔMDR1; Y401A/Y1044A) was generated using a QuickChange site-directed mutagenesis kit (Agilent Technologies). All retroviral constructs were confirmed by sequencing prior to use in cell culture experiments. Retroviral particles were produced in Platinum E cells (PlatE cells; gift of Dr. Matthew Pipkin, Scripps Florida), and supernatants were used to transduce anti-CD3/anti-CD28-stimualted $CD4^+CD25^-$ T cells (at 24 hr post-activation) (Sundrud et al., 2009), or NIH/3T3 fibroblasts. Transduced T cells were removed from anti-CD3/anti-CD28-coated wells at 48 hr post-

activation, expanded in T cell media containing recombinant human IL-2, and analyzed or sorted via flow cytometry on day 5.

Mouse intestinal contents: For isolation of sterile, soluble intestinal contents – small intestine luminal content (SILC), colon luminal content (CLC) - fecal contents from colon or small intestine of mice were collected into 15 mL conical tubes, weighed, and diluted in T cell media (2 parts media:1 part fecal content). Contents were mixed thoroughly by vortexing for 2 min, and solid fecal matter was pelleted by centrifugation (1,000 x g; 10 min; room temperature). Supernatants transferred to fresh eppindorf tubes were further cleared of debris by another round of high-speed centrifugation (13,000 x g; 20 min; room temperature). The resulting supernatants were collected, sterile-filtered through 0.2 µm syringe filters (VWR), aliquoted, and stored at -80°C. SILC or CLC was added to Rh123-labelled splenocytes or purified $CD4^+CD25^-$ T cells at dilutions ranging from 1-16% (2-fold dilutions) of total culture volume (v:v). Unless otherwise stated, results show T cells treated with 4% or 8% SILC or CLC. In some experiments, SILC was depleted of bile acids by pre-treatment with cholestyramine (CME; from Sigma-Aldrich). Briefly, SILC was treated for 1 hr with PBS, or a slurry of PBS containing 50 µg/mL cholestyramine (CME). Following incubation, CME-bound material was pelleted by centrifugation (1 min; 13,000 x g; room temperature), and unbound SILC supernatant was collected and used in Rh123 efflux experiments as above.

Bile acids: Synthetic bile acids: cholic acid (CA), glyco-cholic acid (gCA), tauro-cholic acid (tCA), deoxycholic acid (DCA), glyco-deoxycholic acid (gDCA), tauro-deoxycholic acid (tDCA), chenodeoxycholic acid (CDCA), glyco-chenodeoxycholic acid (gCDCA), or tauro-chenodeoxycholic acid (tCDCA) (all from Sigma-Aldrich) were reconstituted in PBS, aliquoted, and stored at 4°C. Bile acid concentrations used in cell culture experiments are indicated throughout. To test acute effects of bile acids on MDR1 transport (Rh123 efflux) activity, titrating concentrations of bile acids were added to Rh123-labelled mouse $CD4^+$ T cells, human $CD4^+$ T cells, or MDR1-expressing NIH/3T3 fibroblasts immediately prior to Rh123 efflux. For determining the effect of synthetic bile acids on cytokine expression, wild type or MDR1-deficient splenocytes, or mouse EL4 cells were pre-treated +/- glyco-CDCA or tauro-CDCA (tCDCA) for 4 hr prior to PMA/ionomycin stimulation, without washing. In some experiments, tCDCA was added to wild type splenocytes alone, or together with elacridar (0.1 µM) for 4 hr prior to stimulation and staining. For long-term survival assays, wild type or MDR1-deficient splenocytes, or EL4 cells, were treated +/- titrating concentrations of tCDCA in the presence or absence of elacridar (0.1 µM) for 20-24 hr, prior to viability dye and anti-CD4 staining. To analyze

survival of MDR1$^+$ Teff cells after tCDCA treatment, wild type splenocytes were treated +/- tCDCA (1.2 mM) for 20-24 hr as above, and then washed twice with media to remove tCDCA. After washing, splenocytes were cultured in T cell media alone for another 6 hr, and then subjected to FACS-staining and Rh123 efflux. Bile acid levels in feces (for CME treatment experiments) or small intestine luminal content (SILC) were quantified using a Total Bile Acids Assay Kit (Diazyme Laboratories). For fecal bile acid analysis, bile acids were extracted from feces with t-Butanol (Amresco) as described in Bhat et al., J. Lipid Res. 44, 1614-1621, 2003.

Mdr1a binding assays: Recombinant C-terminal hexa-histidine (his)-tagged mouse Mdr1a used for in vitro binding experiments was expressed in and purified from P. pastoris as previously described (Aller et al., 2009). Briefly, 10 µg of purified His-tagged Mdr1a in binding buffer (20 mM Hepes pH 8.0, 100 mM NaCl, 0.065% DDM and 0.2 mM TCEP) was incubated with 20-fold molar excess of BODIPY-FL vinblastine (BFV, Thermo Fisher) for 1 hr at 4 °C in presence or absence of 10-fold molar excess of verapamil (Sigma-Aldrich) or tCDCA. After binding, his-tagged Mdr1a was immobilized to 25 µl of Ni-NTA resin at 4 °C for 30 min with gentle end-over-end mixing followed by washing three times with binding buffer to remove unbound or loosely bound fluorescent inhibitor. Bound Mdr1a was then eluted with 200 µl of binding buffer containing 250 mM imidazole, and Mdr1a-bound BFV was measured. All fluorescence measurements were carried out at least in duplicates, each representing 4 independent measurements, on an Infinite M200pro (Tecan), reading emission at 520 nm after excitation at 480 nm.

RNA-sequencing: Next-generation RNA-sequencing (RNA-seq.) was performed on FACS-sorted Rh123^{hi} (Mdr1$^-$) and Rh123^{lo} (Mdr1$^+$) effector/memory T cells (Teff cells: viability dye$^-$CD45$^+$CD3$^+$CD4$^+$CD25$^-$CD44^{hi}) from mesenteric lymph nodes of FVB.Rag1$^{-/-}$ mice injected 6-8 weeks prior with wild type naïve T cells as above. Total RNA was isolated from triplicate Mdr1+/- Teff samples – in 3 independent transfer experiments – using RNeasy columns (Qiagen), with on-column DNase treatment. 500 ng of total RNA was used for analysis. Library preparation was performed using TrueSeq total RNA-Seq (Illumina), according to manufacturer's instructions. Resulting libraries were quantified using Bioanalyzer (Agilent) and qPCR (Kappa Biosystems). Barcoded libraries from each sample were combined at equimolar amounts and the mixture was loaded and sequenced on a NextSeq500 instrument (Illumina). The sequencing reads (fastq files) were mapped to the mm9 genome using TopHat (Trapnell et al., 2009). The number of reads falling into each gene defined by RefSeq gene annotations were quantified using HTSeq-count. DESeq

software (Anders and Huber, 2010) was used to detect differentially expressed genes between samples. Normalized gene expression was analyzed using the GenePattern software suite (http://genepattern.broadinstitute.org), and visualized using MultiPlot; only annotated genes displaying normalized expression (RPKM) values $\geq$ 0.5 in all replicate samples were analyzed (n = 10,019).

Nanostring: Cell pellets (10-30k cells/condition) were lysed in 5 □L RLT buffer containing fresh 2-mercaptoethanol (Qiagen). Frozen lysates were sent to Nanostring Technologies, Inc. (Seattle, WA) for analysis on the nCounter platform using a custom codeset. Raw data were normalized using nSolver software (Nanostring Technologies) and exported as raw transcript counts (normalized to housekeeper control genes) for presentation.

qPCR: RNA was isolated from cultured or ex vivo-isolated cells using RNeasy columns with on-column DNase treatment (Qiagen); this was used to synthesize cDNA via a high capacity cDNA reverse transcription kit (Life Technologies/Applied Biosystems). Taqman qPCR was performed on a StepOnePlus real time PCR instrument (Life Technologies/Applied Biosystems) as previously described (Ramesh et al., 2014). The following taqman primer/probe sets (all from Life Technologies/Applied Biosystems) were used: Abcb1a (assay ID: Mm00607939_s1); Abcb1b (assay ID: Mm00440736_m1); Gapdh (assay ID: mm99999915_g1).

16S rDNA metagenomics (microbiome analyses): Genomic DNA from mouse (cecal) stool was purified by QIAamp DNA Stool Mini Kit Stool (Qiagen) according to manufacturer's protocol. Bacterial species were identified by 16S ribosomal DNA sequencing using the Ion 16S Metagenomics kit (Life Technologies). Briefly, DNA was amplified utilizing two primer sets that selectively amplify the hypervariable regions against 16S rRNA gene V2-4-8 and V3-6, 7-9, respectively. Amplicons for each sample were pooled and adaptor ligated with barcoded adaptors specific for Ion Torrent platforms. Samples were then pooled at equimolar ratios and sequenced using 400 bp chemistry on Ion Torrent Personal Genome Machine using 316 and 318 chips. Data was analyzed with Ion Reporter software Ion 16S Metagenomics kit analysis module (Life Technologies).

cDNA sequence analysis: Cloned cDNA sequences from AKR and SAMP1/YitFc RT-PCR reactions were aligned using MegAlign Pro (DNASTAR).

Statistical analyses: Statistical analyses were performed using Prism (GraphPad). Paired or unpaired student's t tests, and one-way ANOVA were used as appropriate and are indicated throughout. Significance levels are specified in legends.

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Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

All publications, databases, GenBank sequences, patents, and patent applications cited in this specification are herein incorporated by reference as if each was specifically and individually indicated to be incorporated by reference.

WHAT IS CLAIMED IS:

1. A method for quantifying MDR1 activity in T cells of a human subject, comprising (a) assaying MDR1 function in at least one MDR1-expressing effector T cell subsets in a biological sample obtained from the subject, and (b) comparing the assayed MDR1 function to a background MDR1 function determined with at least one control T cell subset that does not express MDR1, thereby quantifying MDR1 activity in T cells of the human subject; wherein the MDR1-expressing effector T cell subset is elected from the group consisting of (i) $CD4^+$ effector/memory ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$).

2. The method of claim 1, wherein the biological sample comprises peripheral blood mononuclear cells from the subject.

3. The method of claim 1, wherein MDR1 function is assayed via determining the frequency of $MDR1^+$ cells in each T cell subset.

4. The method of claim 3, wherein the assayed MDR1 function is the combined $MDR1^+$ cell frequency from the at least one MDR1-expressing effector T cell subsets.

5. The method of claim 3, wherein the assayed MDR1 function is the combined $MDR1^+$ cell frequency from effector T cell subsets (i) to (iv).

6. The method of claim 1, wherein the control T cell subset is (i) $CD4^+$ naïve T cells (Tnaive, $CD4^+CD25^-CD45RO^-CCR7^{hi}$) or (ii) regulatory T cells (Treg, $CD4^+CD25^{hi}$) present in the same biological sample from the subject.

7. The method of claim 1, wherein the effector T cell subsets from the biological sample are separated via fluorescence-activated cell sorting (FACS).

8. The method of claim 3, wherein $MDR1^+$ cell is determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells of the effector T cell subsets.

9. The method of claim 8, further comprising determining $MDR1^+$ activity in cells of the MDR1-expressing effector T cell subsets that are treated with a MDR1 inhibitor.

10. The method of claim 1, wherein the biological sample comprises cryopreserved peripheral blood mononuclear cells, wherein the determined $MDR1^+$ cell frequency is the combined MDR1 cell frequency from effector T cell subsets (i) to (iv), and wherein $MDR1^+$ cell is determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells in each of the effector T cell subsets.

11. The method of claim 1, wherein the subject is afflicted with an inflammatory bowel disease (IBD).

12. The method of claim 11, wherein the IBD is Crohn's disease or ulcerative colitis.

13. A method for identifying a human patient with an inflammatory bowel disease (IBD) for treatment with bile acid sequestration therapies, comprising (a) obtaining a biological sample containing $CD3^+$ T cells from a candidate IBD patient and from at least one healthy control subject, (b) determining the combined $MDR1^+$ cell frequency in two or more effector T cell subsets in the biological sample consisting of (i) $CD4^+$ effector/memory ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$), and (c) comparing the combined $MDR1^+$ cell frequency of the candidate IBD patient to the combined $MDR1^+$ cell frequency of the control subject; wherein a substantive decrease of the combined $MDR1^+$ cell frequency of the candidate IBD patient from the combined $MDR1^+$ cell frequency of the control subject identifies the candidate IBD patient as one suitable for treatment with bile acid sequestration therapies.

14. The method of claim 13, wherein the candidate IBD subject is a Crohn's disease patient.

15. The method of claim 13, wherein the combined $MDR1^+$ cell frequency is combined $MDR1^+$ cell frequency of all effector T cell subsets (i) to (iv).

16. The method of claim 13, wherein determining the combined MDR1+ cell frequency further includes quantifying a baseline $MDR1^+$ cell frequency in at least one control T cell subset that does not express MDR1.

17. The method of claim 16, wherein the control T cell subset is (i) $CD4^+$ naïve T cells (Tnaive, $CD4^+CD25^-CD45RO^-CCR7^{hi}$) or (ii) regulatory T cells (Treg, $CD4^+CD25^{hi}$) present in the same biological sample.

18. The method of claim 13, wherein the biological sample comprises peripheral blood mononuclear cells from the patient.

19. The method of claim 13, wherein the effector T cell subsets from the biological sample are separated via fluorescence-activated cell sorting (FACS).

20. The method of claim 13, wherein $MDR1^+$ cell is determined via labeling cells in the biological sample with rhodamine 123 (Rh123) and flow cytometry analysis of Rh123 efflux from cells of the effector T cell subsets.

21. A method for treating an inflammatory bowel disease (IBD) in a human patient, comprising (a) detecting MDR1 loss-of-function in a candidate IBD patient, and (b) administering to the patient a pharmaceutical composition comprising a therapeutically effective amount of a bile acid sequestrant; thereby treating the inflammatory bowel disease in the patient.

22. The method of claim 21, wherein MDR1 loss-of-function is detected by (a) obtaining a biological sample containing $CD3^+$ T cells from the candidate patient, (b) determining the combined $MDR1^+$ cell frequency in two or more effector T cell subsets in the biological sample consisting of (i) $CD4^+$ effector/memory ($CD4^+$ Teff, $CD4^+CD25^-CD45RO^+CCR7^{lo}$); (ii) $CD8^+$ naïve ($CD4^-CD25^-CD45RO^-CCR7^{hi}$); (iii) $CD8^+$ effector/memory ($CD8^+$ Teff, $CD4^-CD25^-CD45RO^+CCR7^{lo}$); and (iv) $CD8^+$ short-lived effector/memory ($CD8^+$ Temra, $CD4^-CD25^-CD45RO^-CCR7^{lo}$), and (c) comparing the combined $MDR1^+$ cell frequency of the candidate IBD patient to combined $MDR1^+$ cell frequency in the same biological sample from one or more healthy control subjects.

23. The method of claim 21, wherein the IBD is Crohn's disease.

Figure 1

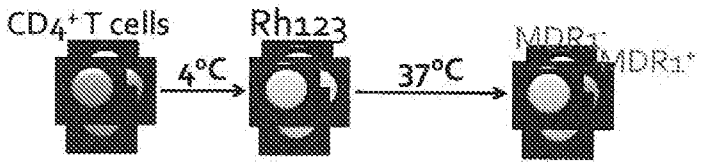


Figure 2

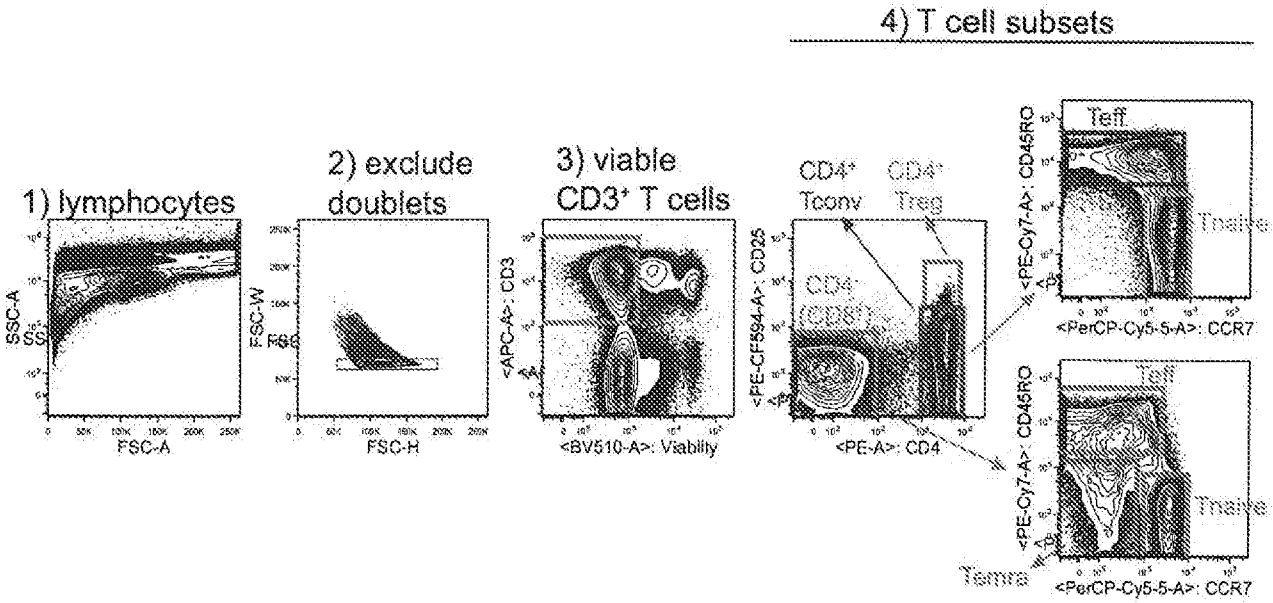


Figure 3

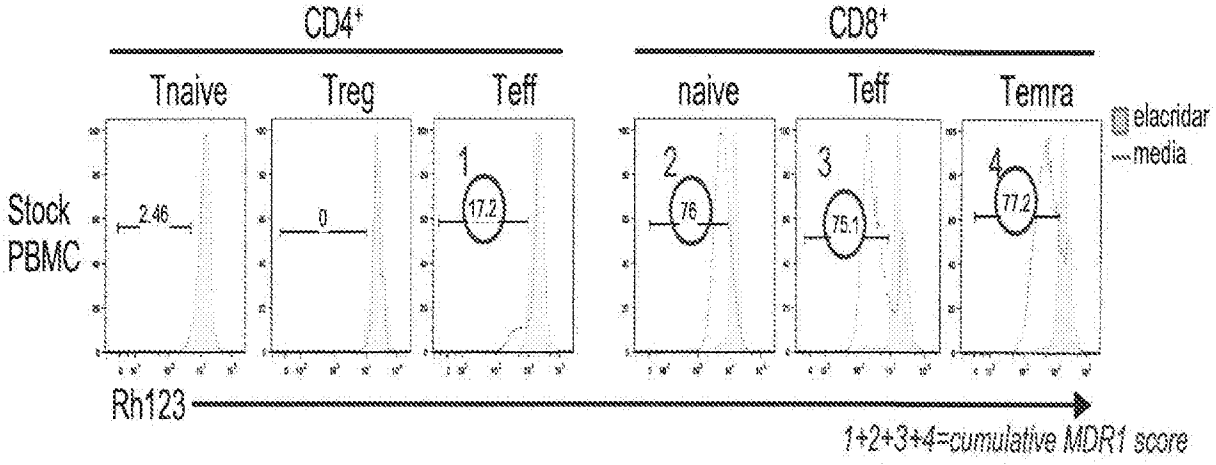


Figure 4

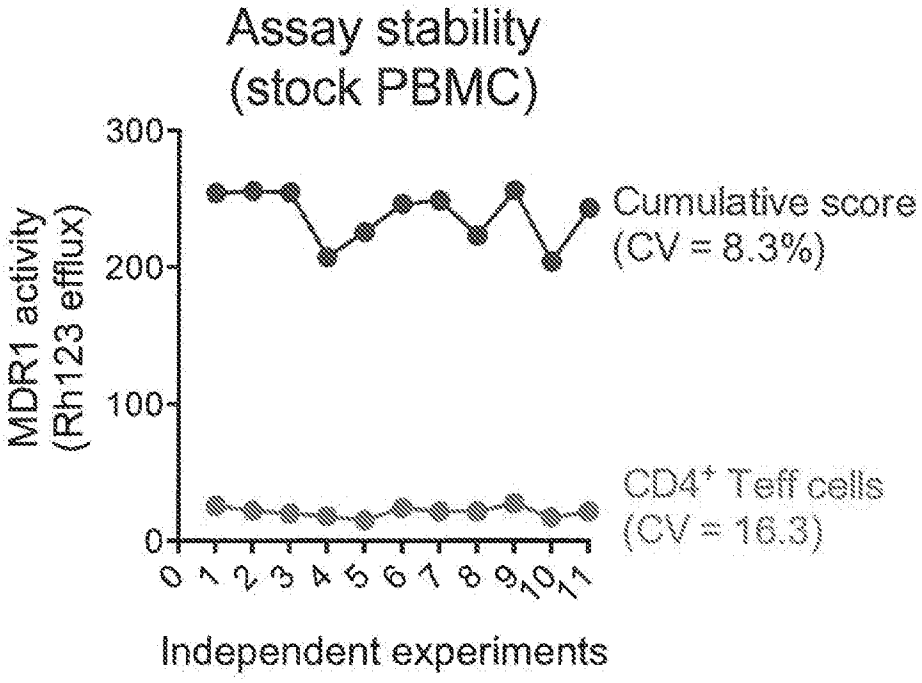


Figure 5

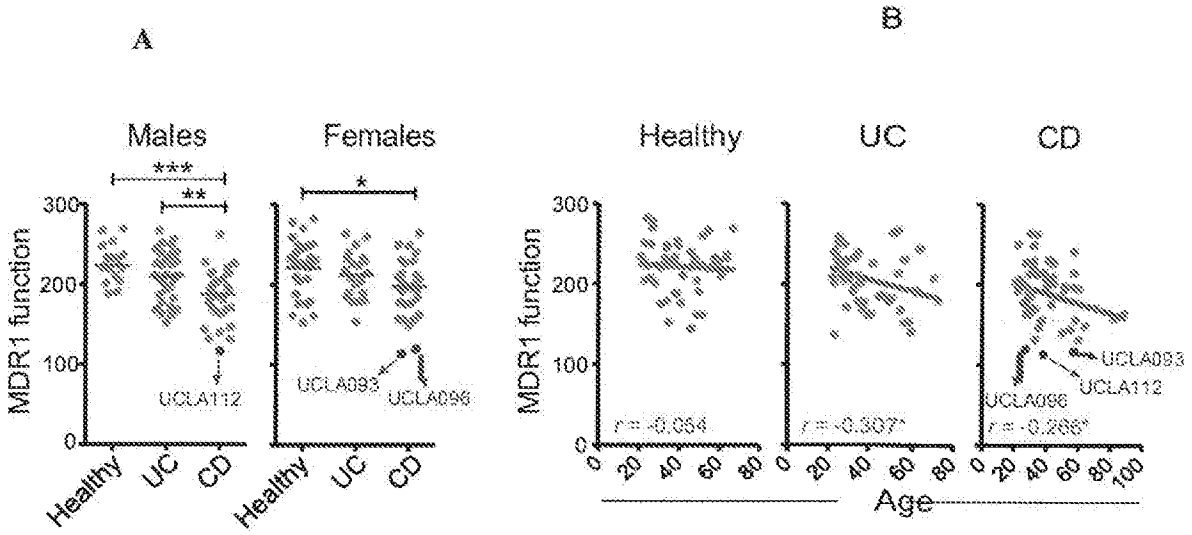


Figure 6

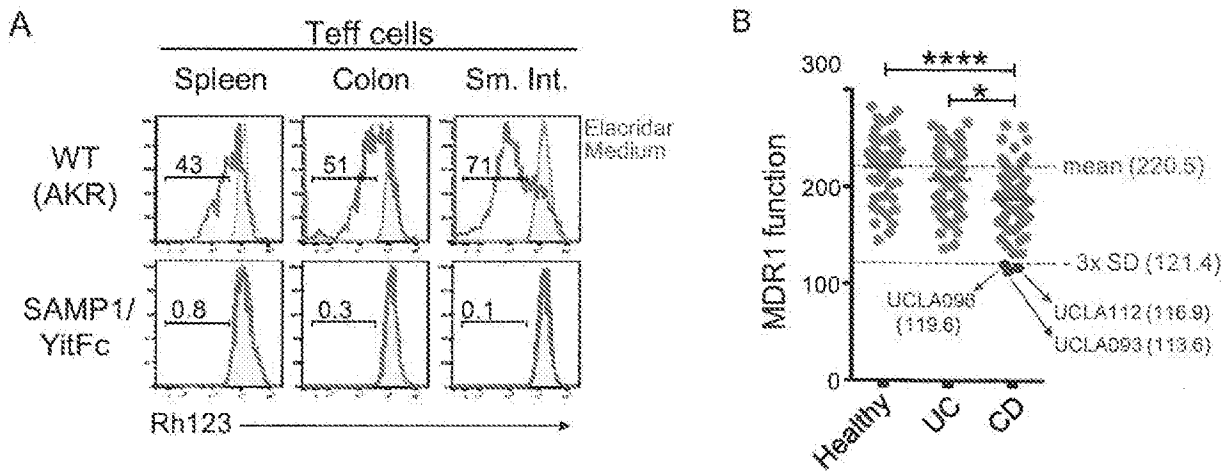


Figure 7

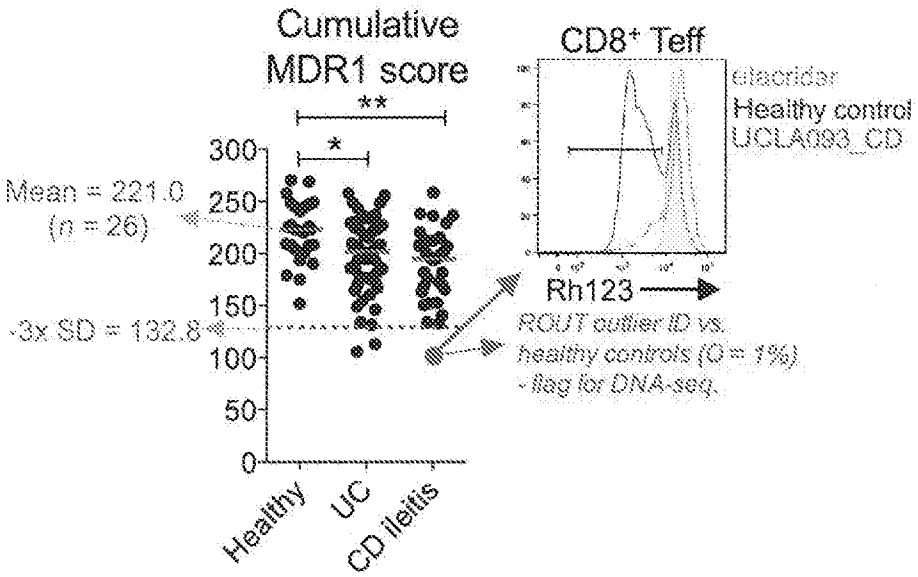
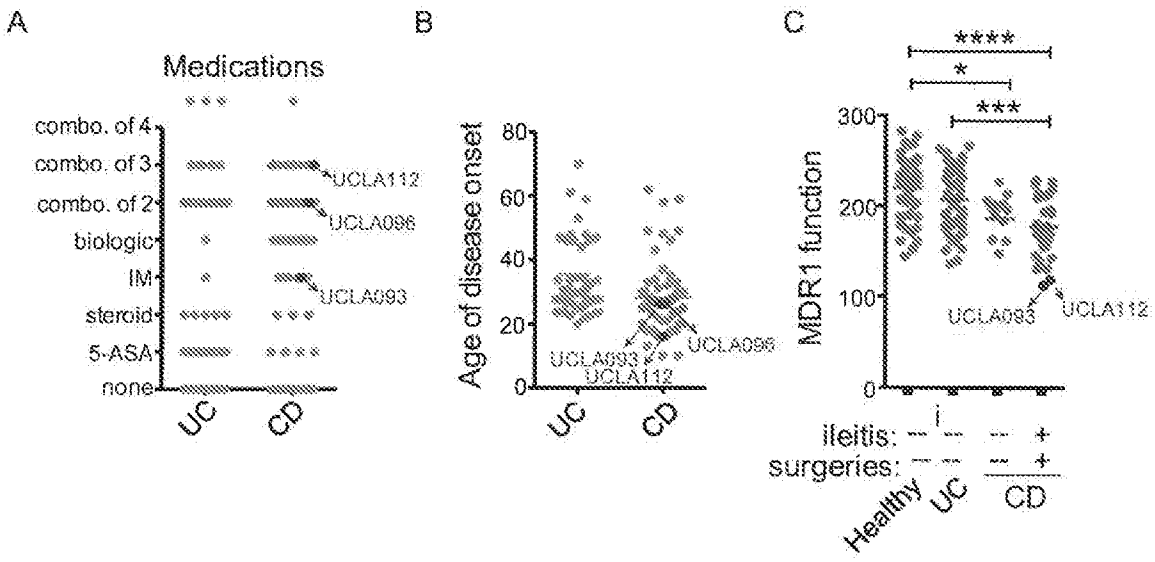


Figure 8



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/38950

A. CLASSIFICATION OF SUBJECT MATTER

IPC - G01N 33/48; C07K 14/725, 14/73, 16/00; C12Q 1/68 (2017.01)

CPC - A61B 5/4255; G01N 33/4915, 33/505, 33/6893; C12Q 1/6883

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	(ZHANG, YJ et al.) Multidrug resistance gene and its relationship to ulcerative colitis and immune status of ulcerative colitis. Genetics and Molecular Research. 19 December 2015, Vol. 13, No. 4; pages 10837-10851; abstract; page 10838, 1st paragraph; page 10839, 2nd paragraph; page 10841, 3rd paragraph; page 10845, 1st paragraph; figure 6; page 10849, 4th paragraph; DOI: 10.4238/2014.December.19.5	1-5, 7-15, 18-20
Y	(DONNENBERG, VS et al.) P-glycoprotein (P-gp) is upregulated in peripheral T-cell subsets from solid organ transplant recipients. Journal of Clinical Pharmacology. December 2001, Vol. 41, No. 12; pages 1271-1279; page 1272, 1st column, 1st paragraph and 2nd column, 1st to 3rd paragraphs; page 1273, 1st column, 2nd paragraph; figure 2; page 1278, 1st column, 1st paragraph; DOI: 10.1177/00912700122012850	1-5, 7-12, 20
Y	(DUBOC, H et al.) Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-predominant irritable bowel syndrome. Neurogastroenterology and Motility. 24 June 2012, Epub 22 February 2012, Vol. 24, No. 6; pages 513-520; page 514, 1st column, 3rd paragraph; DOI: 10.1111/j.1365-2982.2012.01893.x	13-15, 18-21, 23
Y	(JAHNEL, J et al.) Inflammatory Bowel Disease Alters Intestinal Bile Acid Transporter Expression. 1 September 2014, Epub 25 June 2014, Vol. 42, No. 9; pages 1423-1431; page 1426, 2nd column, 2nd paragraph; page 1430, 1st column, 2nd paragraph; DOI: 10.1124/dmd.114.058065	21, 23
P, X	(SUNDRUD, MS et al.) The xenobiotic transporter Mdr1 permits T cell adaptation to mucosa-associated bile acids in the ileum. Journal of Immunology. 1 May 2017, Vol. 198, No.1; entire document	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 October 2017 (12.10.2017)	02 NOV 2017
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US17/38950

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

An invitation to Furnish Nucleotide and/or Amino Acid Sequence Listing and to Pay, Where Applicable, Late Furnishing Fee ("ISA/225") was mailed on 28 July 2017 (28.07.2017). No approved electronic sequence listing was received in response to the ISA/225. Therefore, the international search was carried out only to the extent possible.