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(54) Title: ANTIBODIES AGAINST FLAGELLIN AND USES THEREOF

(57) Abstract: The present invention provides a novel class of monoclonal antibodies which have a high affinity, broad spectrum neutralizing reactivity to flagellin from various Gram-negative bacteria including, but not limited to, E. coli, Salmonella, Serratia, Proteus, Enterobacter, Citrobacter, Campylobacter and Pseudomonas. The present invention further provides methods of treating inflammatory bowel disease (IBD) and methods of treating enterobacterial infections using anti-flagellin antibodies in humans, other animals and birds.

## ANTIBODIES AGAINST FLAGELLIN AND USES THEREOF

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United States government has certain rights in the invention.

### Cross-Reference to Related Application

This application claims priority to U.S. Provisional Patent Application No.  
60/967,718, filed September 5, 2007, entitled, "Antibodies Against Flagellin and Uses  
10 Thereof," which is hereby incorporated by reference in its entirety.

### Background of the Invention

The human intestine is colonized by a large and diverse population of  
commensal bacteria and, on occasion, is exposed to potentially pathogenic bacteria. One  
15 particular subset of intestinal bacteria have flagella, which are whip-like organelles that  
attach to a rotatory motor embedded in the bacterial cell wall. Flagella provide bacteria  
with motility and enable these microbes to reach, adhere and eventually invade or  
colonize a particular niche in their host. An individual flagellum is composed of  
approximately 20,000 subunits of the monomeric protein flagellin. Due to physical  
20 constraints by its function, flagellin has a relatively conserved structure among widely  
diverse bacterial species (Steiner, T.S. *Infect Immun.* 2007 Feb;75(2):545-52).

Flagellin is highly antigenic and is a major immunoglobulin target in a variety of  
infectious events (Sitaraman *et al.*, *Am J Physiol Gastrointest Liver Physiol.* 2005  
Feb;288(2):G403-6). As such, it is a potent and direct activator of the innate immune  
25 system. From the perspective of the host, flagellin is a microbial-associated molecular  
pattern (MAMP) *i.e.*, a microbial-associated determinant that can be perceived by the  
innate immune system, typically by pattern recognition receptors. Flagellin therefore  
serves as a danger signal across a wide variety of eukaryotes and is a potent inducer of  
inflammatory effector responses in the mammalian gut (Neish, A.S., *Am J Physiol*  
30 *Gastrointest Liver Physiol.* 2007 Feb;292(2):G462-6). Specifically, upon detection of  
miniscule levels of the monomeric protein flagellin, the mammalian germline encoded  
cell surface receptor Toll-like receptor 5 (TRL5) can directly promote a mucosal  
inflammatory response and trigger a massive induction of host gene expression designed

to arm and protect the host against the invading microbe. The resulting inflammatory cascade triggered by flagellin can be profound, causing clinical manifestations and tissue damage (Gewirtz, A.T., *Am J Physiol Gastrointest Liver Physiol.* 2007 Mar;292(3):G706-10).

5 Inflammatory bowel disease (IBD) is characterized by inflammation of the bowel, *i.e.*, the large or small intestine, and causes abdominal pain, rectal bleeding and/or diarrhea. The most common types of IBD are Ulcerative Colitis and Crohn's Disease. While the inflammation in Ulcerative Colitis is more superficial and limited to the inner lining of the colon and rectum, the inflammation associate with Crohn's  
10 Disease extends from the mucosa through the entire thickness of the bowel wall and can affect any area of the gastrointestinal tract from the mouth to the anus.

IBD, in general, and Crohn's Disease, in particular, is thought to be driven by exaggerated mucosal immune responses to enteric microflora. For example, in a spontaneously colitic mouse model (C3H/HeJBir mice), sera screened for differential  
15 expression of bacterial protein antigens identified hundreds of antigens, with approximately 25% being bacterial flagellins (Lodes, *et al.*, *J Clin Invest.* 2004 May;113(9):1296-306). Additionally, it has been demonstrated that flagellin released by commensal *E. coli* isolates activates the expression of chemokines (*e.g.*, IL-8) that ultimately cause recruitment of activated neutrophils. Neutrophils however, are not  
20 merely an indicator of active IBD, but instead are thought to cause much of the damage and symptoms associated with active inflammation and to actually drive acute flares of IBD (Gewirtz, A.T., *Am J Physiol Gastrointest Liver Physiol.* 2007 Mar;292(3):G706-10).

Further, the identification of a dominant-negative TLR5 polymorphism, which  
25 reduces the adaptive immune response to flagellin and negatively associates with Crohn's Disease, suggests that immune responses to flagellin are not merely associated with Crohn's Disease, but instead actually promote the pathogenic response (Gewirtz, *et al.*, *Am J Physiol Gastrointest Liver Physiol.* 2006 Jun;290(6):G1157-63).

Notwithstanding, the specific organisms that drive the immune response associated with  
30 IBD are not well defined and there still remains a question as to whether host responses to flagellin are, in fact, part of the healthy, beneficial immune response or, alternatively, whether they are part of an aberrant immune response that should be therapeutically

targeted (Gewirtz, A.T., *Am J Physiol Gastrointest Liver Physiol.* 2007 Mar;292(3):G706-10).

Current treatments for IBD typically involve administration of anti-inflammatory drugs, corticosteroids such as prednisone, immune system suppressors, antibiotics, as well as anti-diarrheals, laxatives, pain relievers or other over-the-counter (OTC) drugs, and in some cases surgery. These therapies, however, have clear drawbacks in that they are associated with potentially long-term side effects and are merely palliative in nature. Accordingly, improved treatments for IBD, as well as other flagellated bacterial infections would be beneficial.

10

### **Summary of the Invention**

The present invention provides a novel class of high affinity monoclonal antibodies that bind to flagellin and neutralize a broad spectrum of bacteria including, but not limited, to gram-negative bacteria, such as *E. coli*, *Salmonella*, *Serratia*, *Proteus*, *Enterobacter*, *Citrobacter*, *Campylobacter* and *Pseudomonas*. Accordingly, the antibodies of the present invention can be used to treat, prevent and diagnose a variety of bacterial diseases, including both infectious and non-infectious diseases in humans, other animals and birds.

Antibodies of the invention generally are characterized as having one or more of the following properties: (i) neutralization (*i.e.*, inhibition) of bacterial flagellin, (including flagellin bound to bacteria or “free”, circulating flagellin in the systemic circulation); (ii) cross-reactivity with flagellin from a broad spectrum of bacteria; (iii) inhibition of bacterial invasion into susceptible epithelial cells; (iv) binding to flagellin with an affinity of at least  $10^{10} \text{ M}^{-1}$ ; (v) reduction or prevention of flagellin-induced tissue injury; (vi) reduction or prevention of flagellin-stimulated neutrophil infiltration; (vii) reduction or prevention of colonic mucosal congestion, erosion and/or hemorrhagic ulcerations associated with IBD; and (viii) reduction or prevention of cytokine production, including MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2, IL-6 and IL-8, and pro-inflammatory free radical synthesizing enzymes, such as the inducible nitric-oxide synthases; (ix) ability to opsonize bacteria; and (x) ability to promote macrophage ingestion of bacteria.

From a structural standpoint, particular representative antibodies of the invention include a heavy chain variable region comprising an amino acid sequence which is at

least 80% (e.g., 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to the heavy chain variable region amino acid sequence set forth in SEQ ID NO:1 or 2. Other particular antibodies of the present invention include a light chain variable region comprising an amino acid sequence which is at least 80% (e.g., 85%, 90%, 95%, 96%, 97%, 98% or 5 99%) identical to the light chain variable region amino acid sequence set forth in SEQ ID NO:3 or 4. The antibodies may also include both of the aforementioned heavy chain and light chain variable regions.

The variable heavy and light chain regions of the antibodies typically include one or more complementarity determining regions (CDRs). These include one or more 10 CDR1, CDR2, and CDR3 regions. Accordingly, other particular antibodies of the present invention include one or more CDR sequences selected from a heavy chain variable region CDR1 comprising SEQ ID NO:5 or 6; a heavy chain variable region CDR2 comprising SEQ ID NO:7 or 8; a heavy chain variable region CDR3 comprising SEQ ID NO:9 or 10; a light chain variable region CDR1 comprising SEQ ID NO:11 or 15 12; a light chain variable region CDR2 comprising SEQ ID NO:13 or 14; a light chain variable region CDR3 comprising SEQ ID NO:15 or 16; and combinations thereof.

The antibodies may also comprise one or more CDRs which are at least 80% (e.g., 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to any of the aforementioned 20 CDRs, or combinations of CDRs.

Also provided by the present invention are antibodies that bind to the same or overlapping epitopes bound by any of the aforementioned antibodies. In a particular embodiment, these antibodies cross-react with a variety of gram-negative bacteria, including *Proteus Vulgaris*, *non-pathogenic E. Coli*, *Citrobacter freundii*, *Serratia 25 marcenscens*, *Enterobacter cloacae*, *Campylobacter jejuni*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Salmonella muenchen*, *Proteus mirabilis* and *Enteropathogenic E. Coli*. In another particular embodiment, the antibodies bind to an epitope on flagellin of *Salmonella muenchen* located between amino acids 37-43, part of another highly conserved RINSA region (amino acids 31-52) 30 within a conserved N-terminal region.

In another aspect, the invention pertains to antibodies that cross compete for binding to flagellin with the anti-flagellin antibodies described herein. For example, the present invention provides for an antibody that cross competes for binding to flagellin

with an antibody comprising heavy and light chain variable regions comprising the amino acid sequences set forth in SEQ ID NOs: 2 and 4 or SEQ ID NOs: 1 and 3, respectively. The invention also pertains to antibodies that bind to an epitope on flagellin recognized by an antibody described herein. For example, the present  
5 invention provides for an antibody that binds to an epitope on flagellin recognized by an antibody comprising heavy and light chain variable regions comprising the amino acid sequences set forth in SEQ ID NOs: 2 and 4 or SEQ ID NOs: 1 and 3, respectively.

Antibodies of the present invention include all known immunoglobulin forms and other protein scaffolds with antibody-like properties. For example, the antibody can  
10 be a murine antibody, a human antibody, a humanized antibody, a chimeric antibody or a protein scaffold with antibody-like properties, such as fibronectin or Ankyrin repeats. The antibody also can have any of the following isotypes: IgG1, IgG2, IgG3, IgG4, IgM, IgA1, IgA2, IgAsec, IgD and IgE. Antibodies of the invention also include antibody fragments, such as an Fab, Fab'2, ScFv, SMIP, affibody, avimer, nanobody or a domain  
15 antibody.

In one embodiment, the invention provides fully human antibodies (*i.e.*, which contain human CDR and framework sequences) that bind to and neutralize flagellin. Particular human antibodies of the invention comprise a heavy chain variable region from a human VH 1-24 or VH 3-23 germline gene, and/or a light chain variable region  
20 from human VK A26 or VK V2-17 germline gene. The sequences of these and other human germline genes are publicly available and can be found, for example, in the "VBase" human germline sequence database (available on the Internet at [www.mrc-cpe.cam.ac.uk/vbase](http://www.mrc-cpe.cam.ac.uk/vbase)) and the "IMGT" database (available on the Internet at <http://imgt.cines.fr/>), and are hereby incorporated by reference.

Antibodies of the invention can be administered alone or in combination with  
25 other therapeutic agents. For example, the antibodies can be administered in combination with (*i.e.*, together with or linked to) cytotoxins, antibacterial agents, including antibiotics and/or other therapeutic antibodies. In one embodiment, the antibody is linked to a second antibody (*i.e.*, thereby forming a bispecific antibody) or  
30 other binding agent that binds to a different target (*e.g.*, an Fc receptor on an immune cell) or a different epitope on flagellin.

In yet another aspect, the present invention provides isolated nucleic acids encoding the aforementioned antibodies of the invention. In particular embodiments, the

nucleic acid encodes a heavy chain variable region comprising a nucleotide sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to, or which hybridizes under high stringency conditions to, SEQ ID NO:19 or 20; or a light chain variable region comprising a nucleotide sequence which is at least 80% (*e.g.*, 85%, 90%, 95%, 96%, 97%, 98% or 99%) identical to, or which hybridizes under high stringency conditions to, SEQ ID NO:21 or 22; or combinations of such heavy and light variable regions.

The present invention also provides hybridomas that express and/or produce the aforementioned antibodies.

Further provided by the invention are kits comprising one or more of the aforementioned antibodies, optionally, with instructions for use in treating or diagnosing bacterial diseases associated with flagellin in humans, other animals and birds.

As noted above, antibodies of the present invention can be used in a broad variety of diagnostic and therapeutic applications, or used in the manufacture of one or more medicaments for diagnostic or therapeutic applications. These applications include treatment and prevention of both infectious and non-infectious bacterial diseases. Particular non-infectious diseases include, but are not limited to, inflammatory bowel diseases (IBDs), such as Crohn's Disease and colitis. Other particular diseases include gram negative bacterial infections (*e.g.*, enterobacterial infections) sepsis and septic shock, in particular. Still other particular diseases include Anthrax, Bacterial Meningitis, Botulism, Brucellosis, Cat Scratch Disease, Cholera, Diphtheria, Epidemic Typhus, Impetigo, Legionellosis, Leprosy, Leptospirosis, Listeriosis, Lyme Disease, Melioidosis, MRSA infection, Nocardiosis, Pertussis, Plague, Pneumococcal pneumonia, Psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), Salmonellosis, Scarlet Fever, Shigellosis, Syphilis, Tetanus, Trachoma, Tuberculosis, Tularemia, Typhoid Fever, Urinary Tract Infections and Necrotizing enterocolitis.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

### **Brief Description of the Drawings**

**Figure 1** shows the nucleotide sequence (SEQ ID NO:19) and primary amino acid sequence (SEQ ID NO:1) of the heavy chain variable region of murine mAb 741. The CDR1 (SEQ ID NOs: 23 and 5), CDR2 (SEQ ID NOs: 25 and 7) and CDR3 (SEQ

ID NOs:27 and 9) nucleotide and amino acid sequences regions, respectively, are delineated.

**Figure 2** shows the nucleotide sequence (SEQ ID NO:21) and primary amino acid sequence (SEQ ID NO:3) of the light chain variable region of murine mAb 741. The CDR1 (SEQ ID NOs:29 and 11), CDR2 (SEQ ID NOs:31 and 13) and CDR3 (SEQ ID NOs:33 and 15) regions are delineated.

**Figure 3** shows the nucleotide sequence (SEQ ID NO:20) and primary amino acid sequence (SEQ ID NO:2) of the heavy chain variable region of human mAb 763. The CDR1 (SEQ ID NOs:24 and 6), CDR2 (SEQ ID NOs:26 and 8) and CDR3 (SEQ ID NOs:28 and 10) regions are delineated.

**Figure 4** shows the nucleotide sequence (SEQ ID NO:22) and primary amino acid sequence (SEQ ID NO:4) of the light chain variable region of human mAb 763. The CDR1 (SEQ ID NOs:30 and 12), CDR2 (SEQ ID NOs:32 and 14) and CDR3 (SEQ ID NOs:34 and 16) regions are delineated.

**Figure 5** shows the binding affinities of chimeric mAb 741C and murine mAb 741, as assessed by competition ELISA.

**Figure 6** depicts the macroscopic histological alterations of the colons of mice treated with either a sham-vehicle (**Figure 6a**), a control-vehicle (**Figure 6b**), DNBS and 20 mg/kg of mAb 763 (**Figures 6c, 6c1 and 6c2**), DNBS and 5 mg/kg of a positive control mAb, Infliximab (**Figures 6d and 6d1**), or DNBS and 20 mg/kg of an isotype human control mAb, CBH2 (**Figures 6e and 6e1**), as well as the macroscopic damage score for each of these experimental groups (**Figure 6f**).

**Figure 7** depicts the histopathological features of the colons of mice treated with either a sham-vehicle (**Figure 7a**), a control-vehicle (**Figures 7b and 7b1**), DNBS and 20 mg/kg of mAb 763 (**Figures 7c and 7c1**), DNBS and 5 mg/kg of a positive control mAb, Infliximab (**Figures 7d, 7d1, and 7d2**), or DNBS and 20 mg/kg of an isotype



human control mAb, CBH2 (**Figures 7e and 7e1**), as well as the histological score for each of these experimental groups (**Figure 7f**).

**Figure 8** is a graph illustrating the change in body weight in grams of mice  
5 treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of mAb 763, DNBS and 5 mg/kg of a positive control mAb, Infliximab, or DNBS and 20 mg/kg of an isotype human control mAb, CBH2.

**Figure 9** is a graph illustrating myeloperoxidase (MPO) activity in mice treated  
10 with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of mAb 763, DNBS and 5 mg/kg of a positive control mAb, Infliximab, or DNBS and 20 mg/kg of an isotype human control mAb, CBH2.

**Figure 10** depicts the macroscopic histological alterations of the colons of mice  
15 treated with either a sham-vehicle (**Figure 10a**), a control-vehicle (**Figure 10b**), DNBS and 20 mg/kg of mAb 741 (**Figures 10c, 10c1 and 10c2**), DNBS and 10 mg/kg of mAb 741 (**Figures 10d, 10d1 and 10d2**), DNBS and 5 mg/kg of mAb 741 (**Figures 10e and 10e1**), DNBS and 20 mg/kg of mAb 763 (**Figures 10f and 10f1**), and DNBS and 20 mg/kg of an isotype mouse control mAb, 18.8 (**Figures 10g and 10g1**), as well as the  
20 macroscopic damage score for each of these experimental groups (**Figure 10h**).

**Figure 11** depicts the histopathological features of the colons of mice treated  
with either a sham-vehicle (**Figure 11a**), a control-vehicle (**Figures 11b and 11b1**),  
DNBS and 20 mg/kg of mAb 741 (**Figures 11c and 11c1**), DNBS and 10 mg/kg of mAb  
25 741 (**Figures 11d and 11d1**), DNBS and 5 mg/kg of mAb 741 (**Figures 11e and 11e1**),  
DNBS and 20 mg/kg of mAb 763 (**Figure 11f**), DNBS and 20 mg/kg of an isotype  
mouse control mAb, 18.8 (**Figures 11g**), as well as the histological score for each of  
these experimental groups (**Figure 11h**).

**Figure 12** is a graph illustrating the change in body weight in grams of mice  
30 treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of mAb 741, DNBS and 10 mg/kg of mAb 741, DNBS and 5 mg/kg of mAb 741, DNBS and 20 mg/kg of mAb 763, and DNBS and 20 mg/kg of an isotype mouse control mAb, 18.8.

**Figure 13** is a graph illustrating myeloperoxidase (MPO) activity in mice treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of mAb 741, DNBS and 10 mg/kg of mAb 741, DNBS and 5 mg/kg of mAb 741, DNBS and 20 mg/kg of mAb 763, and DNBS and 20 mg/kg of an isotype mouse control mAb, 18.8.

**Figure 14** is a graph showing the effects of mAb 741 and mAb 763 on mortality in an *in vivo* murine model of colitis, wherein the mice were sham treated or treated with either DNBS and a control-vehicle, DNBS and 5 mg/kg of mAb 741, DNBS and 10 mg/kg of mAb 741, DNBS and 20 mg/kg of mAb 741, DNBS and 20 mg/kg of mAb 18.8, or DNBS and 20 mg/kg of mAb 763.

**Figure 15** depicts the macroscopic histological alterations of the colons of mice treated with either a sham-vehicle (**Figure 15a**), a control-vehicle (**Figure 15b**), DNBS and 10 mg/kg of mAb 763 (**Figures 15c, 15c1 and 15c2**), DNBS and 3 mg/kg of mAb 763 (**Figures 15d and 15d1**), DNBS and 1 mg/kg of mAb 763 (**Figures 15e and 15e1**), DNBS and 0.3 mg/kg of mAb 763 (**Figures 15f and 15f1**), and DNBS and 10 mg/kg of an isotype human control mAb, CBH2 (**Figures 15g and 15g1**), as well as the macroscopic damage score for each of these experimental groups (**Figure 15h**).

20

**Figure 16** depicts the histopathological features of the colons of mice treated with either a sham-vehicle (**Figure 16a**), a control-vehicle (**Figures 16b and 16b1**), DNBS and 10 mg/kg of mAb 763 (**Figures 16c, 16c1 and 16c2**), DNBS and 3 mg/kg of mAb 763 (**Figure 16d**), DNBS and 1 mg/kg of mAb 763 (**Figure 16e**), DNBS and 0.3 mg/kg of mAb 763 (**Figure 16f**), and DNBS and 10 mg/kg of an isotype human control mAb, CBH2 (**Figure 16g**), as well as the histological score for each of these experimental groups (**Figure 16h**).

25

**Figure 17** is a graph illustrating the change in body weight in grams of mice treated with either a sham-vehicle, a control-vehicle, DNBS and 10 mg/kg of mAb 763, DNBS and 3 mg/kg of mAb 763, DNBS and 1 mg/kg of mAb 763, DNBS and 0.3 mg/kg of mAb 763, and DNBS and 10 mg/kg of an isotype human control mAb, CBH2.

30

**Figure 18** is a graph illustrating myeloperoxidase (MPO) activity in mice treated with either a sham-vehicle, a control-vehicle, DNBS and 10 mg/kg of mAb 763, DNBS and 3 mg/kg of mAb 763, DNBS and 1 mg/kg of mAb 763, DNBS and 0.3 mg/kg of mAb 763, and DNBS and 10 mg/kg of an isotype human control mAb, CBH2.

5

**Figure 19** is a chart depicting the effects of mAb 763, a positive control mAb (Infliximab) and CBH2, a non-relevant human mAb control, on DNBS-induced mediator (*i.e.*, MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2 and IL-8) production from colon extracts.

10

**Figure 20** is a graph depicting the inhibitory effects of mAb 763, a positive control mAb (Infliximab) and CBH2, a non-relevant human mAb control, on colonic mediator (*i.e.*, MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2 and IL-8) production in a DNBS-induced colitis model.

15

**Figure 21** depicts the macroscopic histological alterations of the colons of mice treated with either a sham-vehicle (**Figure 21a**), a control-vehicle (**Figure 21b**), DNBS and 20 mg/kg of chimeric mAb 741C (**Figure 21c**), DNBS and 20 mg/kg of a murine mAb 741 (**Figure 21d**), or DNBS and 20 mg/kg of a non-specific, murine control mAb (**Figure 21e**).

20

**Figure 22** depicts the macroscopic damage score of mice treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of chimeric mAb 741C, DNBS and 20 mg/kg of a murine mAb 741, or DNBS and 20 mg/kg of a non-specific, control mAb (mAb 18.8).

25

**Figure 23** depicts the histological score of mice treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of chimeric mAb 741C, DNBS and 20 mg/kg of a murine mAb 741, or DNBS and 20 mg/kg of a non-specific, control mAb (mAb 18.8).

30

**Figure 24** is a graph illustrating the change in body weight in grams of mice treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of chimeric

mAb 741C, DNBS and 20 mg/kg of murine mAb 741, or DNBS and 20 mg/kg of a non-specific, control mAb (mAb 18.8).

**Figure 25** is a graph illustrating myeloperoxidase (MPO) activity in mice treated with either a sham-vehicle, a control-vehicle, DNBS and 20 mg/kg of chimeric mAb 741C, DNBS and 20 mg/kg of murine mAb 741, or DNBS and 20 mg/kg of a non-specific, control mAb (mAb 18.8).

**Figure 26** is a graph showing the effects of chimeric mAb 741C on mortality in an *in vivo* murine model of colitis.

**Figure 27** is a graph depicting the inhibitory effects of mAb 741 and chimeric mAb 741C on TNF $\alpha$  production in a DNBS-induced colitis model.

**Figure 28** is a graph depicting the inhibitory effects of mAb 741 and chimeric mAb 741C on IL-1- $\beta$  production in a DNBS-induced colitis model.

**Figure 29** is a graph depicting the inhibitory effects of mAb 741 and chimeric mAb 741C on MIP-1 production in a DNBS-induced colitis model.

**Figure 30** is a graph showing the specific, wide-spread, reactivity of mAb 741 to a variety of gram-negative bacteria in a live bacterial ELISA assay.

**Figure 31** is a graph showing the specific, wide-spread reactivity of mAb 763 to a variety of gram-negative bacteria in a live bacterial ELISA assay.

**Figure 32** is a graph showing that anti-flagellin mAbs 741 and 763 inhibit flagellin activity in an NO production assay.

### **Detailed Description of the Invention**

In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

**I. Definitions**

As used herein, the term “flagellin” carries its art recognized meaning as referring to a monomeric subunit of bacterial flagellum. The term “flagellin” includes the monomeric protein flagellin bound to bacteria, free circulating flagellin, and flagellin subunits of an individual flagellum or flagella. The amino acid sequences of flagellins from different bacterial strains are known in the art and are widely conserved, as discussed by Steiner, T.S. (*Infect Immun.* 2007 Feb;75(2):545-52), the teachings of which are incorporated by reference herein. Preferred antibodies of the invention cross react with flagellins of multiple bacterial species, including, but not limited to, *Proteus Vulgaris*, non-pathogenic *E. Coli*, *Citrobacter freundii*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis*, and *Enteropathogenic E. Coli*. Representative flagellin sequences, include, but are not limited to, the sequences set forth below.

15 *Proteus mirabilis* (GI:1169696) (SEQ ID NO:35)

MAQVINTNYLSLVTQNNLNKSQGTLSAIERLSSGLRINSAKDDAAGQAIANRF  
 TSNVNGLTQASRNANDGISIAQTTEGALNEINNLLQRIRELTVQAKNGTNSNSDI  
 TSIQNEVKNVLDEINRISEQTQFNGVKVLSGEKSEMVIQVGTNDNETIKFNLDKV  
 20 DNDTLGVASDKLFDTKTEKKGVTAAGAGVTDKAKKINAAATLDMMSVSLVKEFN  
 LDGKPVTDKFIVTKGGKDYVATKSDFELDATGTKLGLKASATTEFKVDAGKDV  
 KTLNVKDDALATLDKAINRIDESRSKLGAIQNRFFESTINNLNNTVNNLSASRSRIL  
 DADYATEVSNMSRGQILQQAGTSVLAQANQVPQTVLSLLR  
 (Belas, *et al.* (1994). *Gene* 148, 33-41.)

25 *Pseudomonas aeruginosa* (GI:3386643) (SEQ ID NO:36)

MALTVNTNIASLNTQQRNLNNSASLNTSLQRLSTGSRINSAKDDAAGLQIANRRT  
 SQVNGLVATKNANDGISLAQTAEGALQQSTNILQRMRLSLQSANGSNSDSER  
 30 TALNGEVKQLQKELDRISNTTTFGGRKLLDGSFGVASFQVGSAAANEIISVGIDEM  
 SAESLNGTYFKADGGGAVTAATASGTVDIAIGITGGSVAVNVKVDKMGNETAEQ  
 AAKIAAAVNDANVGIGAFTDGAQISYVSKASADGTTSAVSGVAITDTGSTGAG  
 TAAGTTTTFTEANDTVAKIDISTAKGAQSAVLVIDEAIKQIDAQRADLGAVQNRFD  
 NTINNLKNIGENVSAARGRIEDTDFAAETANLTKNQVLQQAGTAILAQANQLPQ  
 35 SVLSLLR  
 (Spangenberg, C. *et al.*, (1996). *FEBS Lett.* 396, 213-217)

*Escherichia coli* (GI:1655807) (SEQ ID NO:37)

40 MAQVINTNSLSLITQNNLNKNQSALSSSIERLSSGLRINSAKDDAAGQAIANRFTS  
 NIKGLTQAARNANDGISVAQTTEGALSEINNLLQRIRELTVQATTGTNSDSDLDSI  
 QDEIKSRLDEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGETITIDLKIDSST

LGLNGFNVNGKGTITNKAATVSDLTSAGAKLNTTGLYDLKTENTLLTTDAAFD  
 KLGNGDKVTVGGVDYTYNAKSGDFTTKSTAGTGVDAQAADSASKRDALA  
 ATLHADVGKSVNGSYTTKDGTVSFETDSAGNITIGGSQAYVDDAGNLTNNAGS  
 AAKADMKALLKAASEGSDGASLTFNGTEYTIKATPATTTPVAPLIPGGITYQAT  
 5 VSKDVLSETKAAAATSSITFNSGVLSKTIGFTAGESSDAAKSYVDDKGGITNVA  
 DYTYSYSVNKDNGSVTVAGYASATDTNKDYAPAIGTAVNVNSAGKITTETTS  
 GSATTNPLAALDDAISSIDKFRSSLGAIQNRLLSAVTNLNNTTTLNLSEAQSRIQDA  
 DYATEVSNMSKAQIIQQAGNSVLAKANQVPQQVLSLLQG

10 Serratia marcescens (GI:514988) (SEQ ID NO:38)

MAQVINTNSLSLMAQNNLNKSQSSLGTAIERLSSGLRINSAKDDAAGQAISDRFT  
 ANIKGLTQASRNANDGISLAQTTEGALNEVNDNLQNRRLTVQAQNGSNSTSDL  
 KSIQDEITQRMSEINRISEQTDFNGVKVLSSDQKLTIQVGANDGETIDIDLQGLTGF  
 15 DVTENGTKIGSAIADKAMVKDDTGTDAFDLGEFQTTGGALEKATLVSGKTKD  
 GKEGYIQTDDAATGAKTYATAKIDDKGVVTKGADVTDVKDPLATLDKALAQ  
 VDGLRSSLGAVQNRFDVSNLNSTVNNLSASQSRIQDADYATEVSNMSRAHILQ  
 QAGTSVLAQANQSTQNVLSLLR

(Akatsuka, H. *et al.*, (1995). *Gene* 163, 157-158)

20

Salmonella muenchen (GI:1333832) (SEQ ID NO:39)

MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFT  
 ANIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANGTNSQSDLD  
 25 SIQAEITQRLNEIDRVSGQTQFNGVKVLAQDNLTIQVGANDGETIDIDLKEISSK  
 TLGLDKLVQDAYTPKETAVTVDKTTYKNGTDTITAQSNTDIQTAIGGGATGVT  
 GADIKFKDGQYYLDVKGGASAGVYKATYDETTKKNIDTTDKTPLATAEATAI  
 RGTATITHNQIAEVTKEGVDTTVAQAALAAAGVTGADKDNLSLVKLSFEDKNG  
 KVIDGGYAVKMGDDFYAATYDEKQVQLLLNNHYTDGAGVLQTGAVKFGGAN  
 30 GKSEVVTATVVGKTYLASDLKHNFRGTGGELKEVNTDKTENPLQKIDAALAQVD  
 TLRSDLGAVQNRFNSAITNLGNTVNNLSSARSRIEDSDYATEVSNMSRAQILQQA  
 GTSVLAQANQVPQNVLSLLR

(Wei, L.N. *et al.*, (1985). *J. Mol. Biol.* 186, 791-803)

35

Salmonella typhimurium (GI:153979) (SEQ ID NO:40)

MAQVINTNSLSLLTQNNLNKSQSALGTAIERLSSGLRINSAKDDAAGQAIANRFT  
 ANIKGLTQASRNANDGISIAQTTEGALNEINNNLQRVRELAVQSANSTNSQSDL  
 40 DSIQAEITQRLNEIDRVNGQTQFSGVKVLAQDNLTIQVGANDGETIDIDLKQINS  
 QTLGLDTLVQKQYKVSDDAATVTGYADTTIALDNSTFKASATGLGGTDEKID  
 GDLKFDDTTGKYAKVTVTGGTGKDGYYEVSVDKTNGEVTAAVTPATVTTA  
 TALSGKMYSANPDSIAKAAALTAAGVTGTASVVKMSYTDNNGKTIDGGLAVK  
 VGDDYYSATQDKDGSISIDTTKYTADNGTSKTALNKLGGADGKTEVVTIDGKT  
 45 YNASKAAGHDFKAEPALAEQAAKTTENPLQKIDAALAQVDTLRSDLGAVQNR  
 NSAITNLGNTVNNLSSARSRIEDSDYATEVSNMSRAQILQQAGTSVLAQANQVP  
 QNVLSLLR

(Joys, T.M. (1985). *J. Biol. Chem.* 260, 15758-15761.)

As used herein, the term “bacteria” or “bacterium” refers to unicellular prokaryotic microorganisms, *i.e.*, organisms without a cell nucleus or any other membrane-bound organelles. Bacteria are typically a few micrometres in length and individual bacteria have a wide-range of shapes, ranging from spheres to rods to spirals.

5 Although the vast majority of bacteria are rendered harmless or beneficial by the protective effects of the immune system, a few pathogenic bacteria cause infectious diseases.

As used herein, “gram-negative bacteria” or “gram-negative bacterium” refer to bacteria having characteristic staining properties under the microscope, where they  
10 either do not stain or are decolorized by alcohol during Gram's method of staining.

Gram-negative bacteria generally have the following characteristics: (1) their cell wall only contains a few layers of peptidoglycan (which is present in much higher levels in Gram-positive bacteria); (2) the cells are surrounded by an outer membrane containing lipopolysaccharide (which consists of Lipid A, core polysaccharide, and O-  
15 polysaccharide) outside the peptidoglycan layer; (3) porins exist in the outer membrane, which act like pores for particular molecules; (4) there is a space between the layers of peptidoglycan and the secondary cell membrane called the periplasmic space; (5) the S-layer is directly attached to the outer membrane, rather than the peptidoglycan; (6) if present, flagella have four supporting rings instead of two; (7) no teichoic acids or  
20 lipoteichoic acids are present; (8) lipoproteins are attached to the polysaccharide backbone, whereas in Gram-positive bacteria no lipoproteins are present; and (9) most do not sporulate.

Examples of gram-negative bacteria include, but are not limited to, *Escherichia coli*, *Enterobacteriaceae*, *Moraxella*, *Helicobacter*, *Burkholderia cepacia*,  
25 *Stenotrophomonas*, *Bdellovibrio*, *acetic acid bacteria*, *cyanobacteria*, *spirochaetes*, *green sulfur and green non-sulfur bacteria*, *Neisseria gonorrhoeae*, *Neisseria meningitides*, *Moraxella catarrhalis*, *Hemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, *Enterobacter cloacae*, *Serratia marcescens*, *Helicobacter pylori*, *Salmonella enteritidis*, and  
30 *Salmonella typhi*.

As used herein, “a bacterial infectious disease” is a disease or infection caused by bacteria.

As used herein, “a gram negative bacterial infection” is a disease or infection caused by gram negative bacteria.

As used herein, “an enterobacterial infection” is an infection caused by *Enterobacteriaceae*.

5 As used herein, “*Enterobacteriaceae*” and “enterobacteria” refer to a large family of bacteria, including many of the more familiar pathogens, such as *Salmonella* and *Escherichia coli*. Genetic studies place them among the Proteobacteria, and they are given their own order (Enterobacteriales). Members of the *Enterobacteriaceae* are rod-shaped, and are typically 1-5 μm in length. Like other Proteobacteria, they have Gram-  
10 negative stains, and they are facultative anaerobes, fermenting sugars to produce lactic acid and various other end products. They also reduce nitrate to nitrite. Unlike most similar bacteria, enterobacteria generally lack cytochrome C oxidase, although there are exceptions (e.g., *Plesiomonas*). Most have many flagella used to move about, but a few genera are non-motile. They are non-spore forming, and except for *Shigella dysenteriae*  
15 strains they are catalase-positive. Many members of this family are a normal part of the gut flora found in the intestines of humans and other animals, while others are found in water or soil, or are parasites on a variety of different animals and plants.

Examples of *Enterobacteriaceae* include, but are not limited to, *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophonus*, *Azotivirga*, *Blochmannia*,  
20 *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia* (e.g. *Erwinia amylovora*), *Escherichia* (e.g. *Escherichia coli*), *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella* (e.g. *Klebsiella pneumoniae*), *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*,  
25 *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Candidatus Phlomobacter*, *Photorhabdus* (e.g. *Photorhabdus luminescens*), *Plesiomonas* (e.g. *Plesiomonas shigelloides*), *Pragia* *Proteus* (e.g. *Proteus vulgaris*), *Providencia*, *Rahnella*, *Raoultella*, *Salmonella*, *Samsonia*, *Serratia* (e.g. *Serratia marcescens*), *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia* (e.g. *Yersinia pestis*), and *Yokenella*.

30 Examples of enterobacterial infections include, but are not limited to, Anthrax (by the bacterium *Bacillus anthracis*), Bacterial Meningitis (caused by a variety of bacteria, including, but not limited to, *Neisseria meningitides*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus*



*aureus*, *Streptococcus agalactiae* and *Haemophilus influenzae*), Botulism (caused by bacterium *Clostridium botulinum*), Brucellosis (caused by bacteria of the genus *Brucella*), *Campylobacteriosis* (caused by bacteria of the genus *Campylobacter*), Cat Scratch Disease (caused by *Bartonella henselae* and *Bartonella clarridgeiae*), Cholera  
5 (caused by the bacterium *Vibrio cholerae*), Diphtheria (caused by *Corynebacterium diphtheriae*), Epidemic Typhus (causative organism is *Rickettsia prowazekii*), Impetigo (caused by several bacteria, including, *Staphylococcus aureus* and *Streptococcus pyogenes*), Legionellosis (caused by bacteria belonging to the genus *Legionella*), Leprosy (Hansen's Disease) (caused by the bacterium *Mycobacterium leprae*),  
10 Leptospirosis (caused by spirochaetes of the genus *Leptospira*), Listeriosis (caused by the bacterium *Listeria monocytogenes*), Lyme Disease (caused by spirochete bacteria from the genus *Borrelia*), Melioidosis (caused by the bacterium *Burkholderia pseudomallei*), MRSA infection (caused by *Staphylococcus aureus*), Nocardiosis (bacterium of the genus *Nocardia*, most commonly *Nocardia asteroides* or *Nocardia brasiliensis*), Pertussis (Whooping Cough) (caused by the bacterium *Bordetella pertussis*), Plague (caused by the enterobacteria *Yersinia pestis*), Pneumococcal pneumonia (caused by a variety of bacteria, including, but not limited to, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*), Psittacosis  
20 (caused by a bacterium called *Chlamydophila psittaci*), Q fever (caused by infection with *Coxiella burnetii*), Rocky Mountain Spotted Fever (RMSF) (by *Rickettsia rickettsii*), Salmonellosis (caused by bacteria of the genus *Salmonella*), Scarlet Fever, Shigellosis (caused by bacteria of the genus *Shigella*), Syphilis (caused by *Treponema pallidum*), Tetanus (*Clostridium tetani*), Trachoma, Tuberculosis (caused by  
25 *mycobacteria*, mainly *Mycobacterium tuberculosis*), Tularemia (by the bacterium *Francisella tularensis*), Typhoid Fever (caused by the bacterium *Salmonella typhi*), and Urinary Tract Infections (caused by bacteria such as *Escherichia coli*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter spp.*,  
30 *Pseudomonas* and *Enterococcus*).

As used herein, “gram-positive bacteria” or “gram-positive bacterium” refer to bacteria that retain the stain or that are resistant to decolourisation by alcohol during Gram's method of staining. Gram-positive bacteria generally have the following

characteristics: (1) a very thick cell wall (peptidoglycan); (2) if a flagellum is present, it contains two rings for support as opposed to four in Gram-negative bacteria because Gram-positive bacteria have only one membrane layer; and (3) teichoic acids and lipoteichoic acids are present, which serve to act as chelating agents, and also for certain types of adherence. Examples of gram-positive bacteria genera include, but are not limited to, *Bacillus*, *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*.

As used herein, “Inflammatory Bowel Disease (IBD)” refers to a group of chronic intestinal diseases characterized by inflammation of the bowel, *i.e.*, the large or small intestine. The most common types of IBD are Ulcerative Colitis and Crohn's Disease. The symptoms of IBD include abdominal pain, diarrhea, bloody diarrhea, severe urgency to have a bowel movement, fever, loss of appetite, weight loss, anemia. IBD can also cause intestinal complications including profuse bleeding from the ulcers, perforation of the bowel, strictures and obstructions, fistulae, perianal disease, toxic megacolon and cancer. The disease can be limited to the intestine or affect the skin, joints, spine, liver, eyes, and other organs.

As used herein, “Crohn's Disease” is a form of IBD that causes severe irritation in the gastrointestinal tract. It usually affects the lower small intestine (*i.e.*, the ileum) or the colon, but can affect other parts of the digestive system including the small intestine, mouth, esophagus, and stomach. The inflammation in Crohn's Disease involves the entire thickness of the bowel wall. There are five different types of Crohn's disease: (1) Ileocolitis (the most common form, which affects the ileum and the colon); (2) Ileitis (which affects the ileum); (3) Gastroduodenal Crohn's Disease (which causes inflammation in the stomach and the duodenum); (4) Jejunoileitis (which causes spotty patches of inflammation in the top half of the small intestine (*i.e.*, the jejunum)); and (5) Crohn's (Granulomatous) Colitis (which affects only affects the large intestine).

As used herein, “Ulcerative Colitis” is a form of IBD that affects the colon (the large intestine) alone and inflammation is confined to the mucosa (the inner lining) of the intestine. It can be difficult to diagnose because its symptoms are similar to other intestinal disorders and Crohn's Disease.

The term “Toll-like receptor (TLR)” as used herein, refers to an important family of innate immune receptors that recognize pathogen-associated molecular patterns, *i.e.*,

evolutionarily conserved structures that are required for microbial fitness and are not present in the host .

The term “Toll-like receptor 5 (TLR5)” as used herein, refers to the Toll-like receptor which recognizes and binds bacterial flagellin from both gram-positive and gram-negative and activates host inflammatory responses. TLR5 is specifically expressed in monocytes, immature dendritic cells and epithelial cells.

The term “neutralizes” and “inhibits” are used interchangeably herein, and refer to any statistically significant decrease in the biological activity (e.g., motility) of flagellin, including full blocking of the activity. For example, “neutralizes” or “inhibits” can refer to a decrease of about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% in flagellin activity.

In particular embodiments of the invention, neutralization or inhibition of flagellin activity results in one or more of the following effects: it prevents bacterial invasion into susceptible epithelial cells, reduces the symptoms of an enterobacterial infection or IBD in a subject, reduces the extent and severity of flagellin-induced tissue injury, reduces flagellin-stimulated neutrophil infiltration, decreases colonic mucosal congestion, erosion and hemorrhagic ulcerations associated with IBD, inhibits or decrease the production of mediators (e.g., MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2 and IL-8); and/or counteracts a reduction in body weight associated with IBD.

The term “antibody” or “immunoglobulin,” as used interchangeably herein, includes whole antibodies and any antigen binding fragment (*i.e.*, “antigen-binding portion”) or single chains thereof. An “antibody” comprises at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V<sub>H</sub>) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as V<sub>L</sub>) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The V<sub>H</sub> and V<sub>L</sub> regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each V<sub>H</sub> and V<sub>L</sub> is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding

domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system. Exemplary antibodies of the invention include mAbs 741  
5 and 763, and antigen-binding portions thereof.

The term “antigen-binding portion” of an antibody (or simply “antibody portion”), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen (*e.g.*, flagellin). It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length  
10 antibody. Examples of binding fragments encompassed within the term “antigen-binding portion” of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V<sub>L</sub>, V<sub>H</sub>, CL and CH1 domains; (ii) a F(ab')<sub>2</sub> fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V<sub>H</sub> and CH1 domains; (iv) a Fv fragment consisting  
15 of the V<sub>L</sub> and V<sub>H</sub> domains of a single arm of an antibody, (v) a dAb including V<sub>H</sub> and V<sub>L</sub> domains; (vi) a dAb fragment (Ward *et al.* (1989) *Nature* 341, 544-546), which consists of a V<sub>H</sub> domain; (vii) a dAb which consists of a V<sub>H</sub> or a V<sub>L</sub> domain; and (viii) an isolated complementarity determining region (CDR) or (ix) a combination of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore,  
20 although the two domains of the Fv fragment, V<sub>L</sub> and V<sub>H</sub>, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V<sub>L</sub> and V<sub>H</sub> regions pair to form monovalent molecules (known as single chain Fv (scFv); see *e.g.*, Bird *et al.* (1988) *Science* 242, 423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85, 5879-  
25 5883). Such single chain antibodies are also intended to be encompassed within the term “antigen-binding portion” of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies. Antigen-binding  
30 portions can be produced by recombinant DNA techniques, or by enzymatic or chemical cleavage of intact immunoglobulins.

The term “monoclonal antibody” as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, *i.e.*, the individual antibodies comprising the population are identical except for possible naturally

occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. Monoclonal antibodies can be prepared using any art recognized technique and those described herein such as, for example, a hybridoma method, as described by Kohler *et al.* (1975) *Nature*, 256:495, a transgenic animal, as described by, for example, (see *e.g.*, Lonberg, *et al.* (1994) *Nature* 368(6474): 856-859), recombinant DNA methods (see, *e.g.*, U.S. Pat. No. 4,816,567), or using phage antibody libraries using the techniques described in, for example, Clackson *et al.*, *Nature*, 352:624-628 (1991) and Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991). Monoclonal antibodies include chimeric antibodies, human antibodies and humanized antibodies and may occur naturally or be recombinantly produced.

The term "recombinant antibody," refers to antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (*e.g.*, a mouse) that is transgenic or transchromosomal for immunoglobulin genes (*e.g.*, human immunoglobulin genes) or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody, *e.g.*, from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library (*e.g.*, containing human antibody sequences) using phage display, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences (*e.g.*, human immunoglobulin genes) to other DNA sequences. Such recombinant antibodies may have variable and constant regions derived from human germline immunoglobulin sequences. In certain embodiments, however, such recombinant human antibodies can be subjected to *in vitro* mutagenesis and thus the amino acid sequences of the V<sub>H</sub> and V<sub>L</sub> regions of the recombinant antibodies are sequences that, while derived from and related to human germline V<sub>H</sub> and V<sub>L</sub> sequences, may not naturally exist within the human antibody germline repertoire *in vivo*.

The term "chimeric immunoglobulin" or antibody refers to an immunoglobulin or antibody whose variable regions derive from a first species and whose constant regions derive from a second species. Chimeric immunoglobulins or antibodies can be

constructed, for example by genetic engineering, from immunoglobulin gene segments belonging to different species.

The term "human antibody," as used herein, is intended to include antibodies having variable regions in which both the framework and CDR regions are derived from human germline immunoglobulin sequences as described, for example, by Kabat et al. (See Kabat, *et al.* (1991) *Sequences of proteins of Immunological Interest, Fifth Edition*, U.S. Department of Health and Human Services, NIH Publication No. 91-3242). Furthermore, if the antibody contains a constant region, the constant region also is derived from human germline immunoglobulin sequences. The human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (*e.g.*, mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody", as used herein, is not intended to include antibodies in which CDR sequences derived from the germline of another mammalian species, such as a mouse, have been grafted onto human framework sequences.

The human antibody can have at least one or more amino acids replaced with an amino acid residue, *e.g.*, an activity enhancing amino acid residue which is not encoded by the human germline immunoglobulin sequence. Typically, the human antibody can have up to twenty positions replaced with amino acid residues which are not part of the human germline immunoglobulin sequence. In a particular embodiment, these replacements are within the CDR regions as described in detail below.

The term "humanized immunoglobulin" or "humanized antibody" refers to an immunoglobulin or antibody that includes at least one humanized immunoglobulin or antibody chain (*i.e.*, at least one humanized light or heavy chain). The term "humanized immunoglobulin chain" or "humanized antibody chain" (*i.e.*, a "humanized immunoglobulin light chain" or "humanized immunoglobulin heavy chain") refers to an immunoglobulin or antibody chain (*i.e.*, a light or heavy chain, respectively) having a variable region that includes a variable framework region substantially from a human immunoglobulin or antibody and complementarity determining regions (CDRs) (*e.g.*, at least one CDR, preferably two CDRs, more preferably three CDRs) substantially from a non-human immunoglobulin or antibody, and further includes constant regions (*e.g.*, at least one constant region or portion thereof, in the case of a light chain, and preferably three constant regions in the case of a heavy chain). The term "humanized variable

region” (*e.g.*, “humanized light chain variable region” or “humanized heavy chain variable region”) refers to a variable region that includes a variable framework region substantially from a human immunoglobulin or antibody and complementarity determining regions (CDRs) substantially from a non-human immunoglobulin or antibody.

A “bispecific” or “bifunctional antibody” is an artificial hybrid antibody having two different heavy/light chain pairs and two different binding sites. Bispecific antibodies can be produced by a variety of methods including fusion of hybridomas or linking of Fab' fragments. See, *e.g.*, Songsivilai & Lachmann, (1990) *Clin. Exp. Immunol.* 79, 315-321; Kostelny *et al.* (1992) *J. Immunol.* 148, 1547-1553.

As used herein, a “heterologous antibody” is defined in relation to the transgenic non-human organism or plant producing such an antibody.

An “isolated antibody,” as used herein, is intended to refer to an antibody which is substantially free of other antibodies having different antigenic specificities (*e.g.*, an isolated antibody that specifically binds to flagellin is substantially free of antibodies that specifically bind antigens other than flagellin). In addition, an isolated antibody is typically substantially free of other cellular material and/or chemicals. In one embodiment of the invention, a combination of “isolated” monoclonal antibodies having different flagellin binding specificities are combined in a well defined composition.

As used herein, “isotype” refers to the antibody class (*e.g.*, IgM or IgG1) that is encoded by heavy chain constant region genes. In one embodiment, an antibody or antigen binding portion thereof is of an isotype selected from an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, an IgAsec, an IgD, or an IgE antibody isotype. In some embodiments, a monoclonal antibody of the invention is of the IgG1 isotype. In other embodiments, a monoclonal antibody of the invention is of the IgG2 isotype.

As used herein, “isotype switching” refers to the phenomenon by which the class, or isotype, of an antibody changes from one Ig class to one of the other Ig classes.

As used herein, “nonswitched isotype” refers to the isotypic class of heavy chain that is produced when no isotype switching has taken place; the CH gene encoding the nonswitched isotype is typically the first CH gene immediately downstream from the functionally rearranged VDJ gene. Isotype switching has been classified as classical or non-classical isotype switching. Classical isotype switching occurs by recombination

events which involve at least one switch sequence regions in a gene encoding an antibody. Non-classical isotype switching may occur by, for example, homologous recombination between human  $\sigma_{\mu}$  and human  $\Sigma_{\mu}$  ( $\delta$ -associated deletion). Alternative non-classical switching mechanisms, such as intertransgene and/or interchromosomal recombination, among others, may occur and effectuate isotype switching.

As used herein, the term “switch sequence” refers to those DNA sequences responsible for switch recombination. A “switch donor” sequence, typically a  $\mu$  switch region, will be 5' (*i.e.*, upstream) of the construct region to be deleted during the switch recombination. The “switch acceptor” region will be between the construct region to be deleted and the replacement constant region (*e.g.*,  $\gamma$ ,  $\epsilon$ , etc.). As there is no specific site where recombination always occurs, the final gene sequence will typically not be predictable from the construct.

An “antigen” is an entity (*e.g.*, a proteinaceous entity or peptide) to which an antibody or antigen-binding portion thereof binds. In various embodiments of the present invention, an antigen is flagellin.

The term “epitope” or “antigenic determinant” refers to a site on an antigen to which an immunoglobulin or antibody specifically binds. Epitopes can be formed both from contiguous amino acids or noncontiguous amino acids juxtaposed by tertiary folding of a protein. Epitopes formed from contiguous amino acids are typically retained on exposure to denaturing solvents, whereas epitopes formed by tertiary folding are typically lost on treatment with denaturing solvents. An epitope typically includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acids in a unique spatial conformation. Methods of determining spatial conformation of epitopes include techniques in the art and those described herein, for example, x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, *e.g.*, *Epitope Mapping Protocols in Methods in Molecular Biology*, Vol. 66, G. E. Morris, Ed. (1996).

Also encompassed by the present invention are antibodies that bind the same or an overlapping epitope as the particular antibodies described herein, *i.e.*, antibodies that compete for binding to flagellin, or bind to an epitope on flagellin recognized by the particular antibodies described herein. For example, the antibodies of the present invention may specifically bind to an epitope located between amino acids 1-55 of flagellin from *Salmonella* (Genbank Accession No.GI:1333832) (SEQ ID NO:39) or *Pseudomonas* (Genbank Accession No.GI:3386643) (SEQ ID NO:36). In one



embodiment, the antibodies of the present invention may specifically bind to an epitope located between amino acids 1-40 or 30-50 or 30-40 or 37-43 or 31-47 or 41-52 of flagellin from *Salmonella* (Genbank Accession No.GI:1333832) (SEQ ID NO:39) or *Pseudomonas* (Genbank Accession No.GI:3386643) (SEQ ID NO:36).

5           Antibodies that recognize the same or an overlapping epitope can be identified using routine techniques such as an immunoassay, for example, by showing the ability of one antibody to block the binding of another antibody to a target antigen, *i.e.*, a competitive binding assay. Competitive binding is determined in an assay in which the immunoglobulin under test inhibits specific binding of a reference antibody to an  
10           antigen, such as flagellin. Numerous types of competitive binding assays are known, for example: solid phase direct or indirect radioimmunoassay (RIA), solid phase direct or indirect enzyme immunoassay (EIA), sandwich competition assay (see Stahli *et al.*, (1983) *Methods in Enzymology* 9:242); solid phase direct biotin-avidin EIA (see Kirkland *et al.*, (1986) *J. Immunol.* 137:3614); solid phase direct labeled assay, solid  
15           phase direct labeled sandwich assay (see Harlow and Lane, (1988) *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press); solid phase direct label RIA using I-125 label (see Morel *et al.*, (1988) *Mol. Immunol.* 25(1):7); solid phase direct biotin-avidin EIA (Cheung *et al.*, (1990) *Virology* 176:546); and direct labeled RIA. (Moldenhauer *et al.*, (1990) *Scand. J. Immunol.* 32:77). Typically, such an assay involves the use of  
20           purified antigen (*e.g.*, flagellin) bound to a solid surface or cells bearing either of these, an unlabeled test immunoglobulin and a labeled reference immunoglobulin. Competitive inhibition is measured by determining the amount of label bound to the solid surface or cells in the presence of the test immunoglobulin. Usually the test immunoglobulin is present in excess. Usually, when a competing antibody is present in  
25           excess, it will inhibit specific binding of a reference antibody to a common antigen by at least 50-55%, 55-60%, 60-65%, 65-70% 70-75% or more.

          As used herein, the terms “specific binding,” “specifically binds,” “selective binding,” and “selectively binds,” mean that an antibody or antigen-binding portion thereof, exhibits appreciable affinity for a particular antigen or epitope and, generally,  
30           does not exhibit significant cross-reactivity with other antigens and epitopes.

          “Appreciable” or preferred binding includes binding with an affinity of at least  $10^6$ ,  $10^7$ ,  $10^8$ ,  $10^9$   $M^{-1}$ , or  $10^{10}$   $M^{-1}$ . Affinities greater than  $10^7$   $M^{-1}$ , preferably greater than  $10^8$   $M^{-1}$  are more preferred. Values intermediate of those set forth herein are also intended to be

within the scope of the present invention and a preferred binding affinity can be indicated as a range of affinities, for example,  $10^6$  to  $10^{10}$   $M^{-1}$ , preferably  $10^7$  to  $10^{10}$   $M^{-1}$ , more preferably  $10^8$  to  $10^{10}$   $M^{-1}$ . An antibody that “does not exhibit significant cross-reactivity” is one that will not appreciably bind to an undesirable entity (*e.g.*, an  
5 undesirable proteinaceous entity). Specific or selective binding can be determined according to any art-recognized means for determining such binding, including, for example, according to Scatchard analysis and/or competitive binding assays.

The term “ $K_D$ ,” as used herein, is intended to refer to the dissociation equilibrium constant of a particular antibody-antigen interaction or the affinity of an antibody for an  
10 antigen. In one embodiment, the antibody or antigen binding portion thereof according to the present invention binds an antigen (*e.g.*, flagellin) with an affinity ( $K_D$ ) of 50 nM or better (*i.e.*, or less) (*e.g.*, 40 nM or 30 nM or 20 nM or 10 nM or less), as measured using a surface plasmon resonance assay or a cell binding assay. In a particular  
15 embodiment, an antibody or antigen binding portion thereof according to the present invention binds flagellin with an affinity ( $K_D$ ) of 8 nM or better (*e.g.*, 7 nM, 6 nM, 5 nM, 4 nM, 2 nM, 1.5 nM, 1.4 nM, 1.3 nM, 1nM or less), as measured by a surface plasmon resonance assay or a cell binding assay. In other embodiments, an antibody or antigen  
20 binding portion thereof binds an antigen (*e.g.*, flagellin) with an affinity ( $K_D$ ) of approximately less than  $10^{-7}$  M, such as approximately less than  $10^{-8}$  M,  $10^{-9}$  M or  $10^{-10}$  M or even lower when determined by surface plasmon resonance (SPR) technology in a BIACORE 3000 instrument using recombinant flagellin as the analyte and the antibody as the ligand, and binds to the predetermined antigen with an affinity that is at least two-fold greater than its affinity for binding to a non-specific antigen (*e.g.*, BSA, casein)  
other than the predetermined antigen or a closely-related antigen.

25 The term “ $K_{off}$ ,” as used herein, is intended to refer to the off rate constant for the dissociation of an antibody from the antibody/antigen complex.

The term “EC50,” as used herein, refers to the concentration of an antibody or an antigen-binding portion thereof, which induces a response, either in an *in vitro* or an *in vivo* assay, which is 50% of the maximal response, *i.e.*, halfway between the maximal  
30 response and the baseline.

As used herein, “glycosylation pattern” is defined as the pattern of carbohydrate units that are covalently attached to a protein, more specifically to an immunoglobulin protein.

The term “naturally-occurring” as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory  
5 is naturally-occurring.

The term “rearranged” as used herein refers to a configuration of a heavy chain or light chain immunoglobulin locus wherein a V segment is positioned immediately adjacent to a D-J or J segment in a conformation encoding essentially a complete V<sub>H</sub> or V<sub>L</sub> domain, respectively. A rearranged immunoglobulin gene locus can be identified by  
10 comparison to germline DNA; a rearranged locus will have at least one recombined heptamer/nonamer homology element.

The term “unrearranged” or “germline configuration” as used herein in reference to a V segment refers to the configuration wherein the V segment is not recombined so as to be immediately adjacent to a D or J segment.

The term “nucleic acid molecule,” as used herein, is intended to include DNA molecules and RNA molecules. A nucleic acid molecule may be single-stranded or  
15 double-stranded, but preferably is double-stranded DNA.

The term “isolated nucleic acid molecule,” as used herein in reference to nucleic acids encoding antibodies (*e.g.*, V<sub>H</sub>, V<sub>L</sub>, CDR3) that bind to flagellin, is intended to refer  
20 to a nucleic acid molecule in which the nucleotide sequences encoding the antibody are free of other nucleotide sequences encoding antibodies that bind antigens other than flagellin, which other sequences may naturally flank the nucleic acid in human genomic DNA.

Alternatively, antibodies can comprise an amino acid sequence which is encoded  
25 by a nucleotide sequence which hybridizes, *e.g.*, hybridizes under stringent conditions to a nucleotide sequence disclosed herein. As used herein, the term “hybridizes under stringent conditions” is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 60% homologous to each other typically remain hybridized to each other. Preferably, the conditions are such that sequences at  
30 least about 65%, more preferably at least about 70%, and even more preferably at least about 75% or more homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those of ordinary skill in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-

6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

The term “modifying,” or “modification,” as used herein, is intended to refer to changing one or more amino acids in the antibodies. The change can be produced by adding, substituting or deleting an amino acid at one or more positions. The change can be produced using known techniques, such as PCR mutagenesis. For example, in some embodiments, an antibody identified using the methods of the invention can be modified, to thereby modify the binding affinity of the antibody to flagellin.

The present invention also encompasses “conservative amino acid substitutions” in the sequences of the antibodies of the invention, *i.e.*, nucleotide and amino acid sequence modifications which do not abrogate the binding of the antibody encoded by the nucleotide sequence or containing the amino acid sequence, to the antigen, *i.e.*, flagellin. Conservative amino acid substitutions include the substitution of an amino acid in one class by an amino acid of the same class, where a class is defined by common physicochemical amino acid side chain properties and high substitution frequencies in homologous proteins found in nature, as determined, for example, by a standard Dayhoff frequency exchange matrix or BLOSUM matrix. Six general classes of amino acid side chains have been categorized and include: Class I (Cys); Class II (Ser, Thr, Pro, Ala, Gly); Class III (Asn, Asp, Gln, Glu); Class IV (His, Arg, Lys); Class V (Ile, Leu, Val, Met); and Class VI (Phe, Tyr, Trp). For example, substitution of an Asp for another class III residue such as Asn, Gln, or Glu, is a conservative substitution. Thus, a predicted nonessential amino acid residue in an anti-flagellin antibody of the present invention is preferably replaced with another amino acid residue from the same class. Methods of identifying nucleotide and amino acid conservative substitutions which do not eliminate antigen binding are well-known in the art (see, *e.g.*, Brummell *et al.*, *Biochem.* 32:1180-1187 (1993); Kobayashi *et al.* *Protein Eng.* 12(10):879-884 (1999); and Burks *et al.* *Proc. Natl. Acad. Sci. USA* 94:412-417 (1997)).

The term “non-conservative amino acid substitution” refers to the substitution of an amino acid in one class with an amino acid from another class; for example, substitution of an Ala, a class II residue, with a class III residue such as Asp, Asn, Glu, or Gln.

Alternatively, in another embodiment, mutations (conservative or non-conservative) can be introduced randomly along all or part of an anti-flagellin antibody coding sequence, such as by saturation mutagenesis, and the resulting modified anti-flagellin antibodies can be screened for binding activity.

5 A “consensus sequence” is a sequence formed from the most frequently occurring amino acids (or nucleotides) in a family of related sequences (See *e.g.*, Winnaker, *From Genes to Clones* (Verlagsgesellschaft, Weinheim, Germany 1987). In a family of proteins, each position in the consensus sequence is occupied by the amino acid occurring most frequently at that position in the family. If two amino acids occur  
10 equally frequently, either can be included in the consensus sequence. A “consensus framework” of an immunoglobulin refers to a framework region in the consensus immunoglobulin sequence.

Similarly, the consensus sequence for the CDRs of can be derived by optimal alignment of the CDR amino acid sequences of flagellin antibodies of the present  
15 invention.

For nucleic acids, the term “substantial homology” indicates that two nucleic acids, or designated sequences thereof, when optimally aligned and compared, are identical, with appropriate nucleotide insertions or deletions, in at least about 80% of the nucleotides, usually at least about 90% to 95%, and more preferably at least about 98%  
20 to 99.5% of the nucleotides. Alternatively, substantial homology exists when the segments will hybridize under selective hybridization conditions, to the complement of the strand.

The percent identity between two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical  
25 positions/total # of positions x 100), taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, as described in the non-limiting examples below.

30 The percent identity between two nucleotide sequences can be determined using the GAP program in the GCG software, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. The percent identity between two nucleotide or amino acid sequences can also be determined using

the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4. In addition, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch  
5 (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package, using either a Blossum 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6.

The nucleic acid and protein sequences of the present invention can further be  
10 used as a "query sequence" to perform a search against public databases to, for example, identify related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid  
15 molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped  
20 BLAST programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used.

The nucleic acids may be present in whole cells, in a cell lysate, or in a partially purified or substantially pure form. A nucleic acid is "isolated" or "rendered substantially pure" when purified away from other cellular components or other  
25 contaminants, *e.g.*, other cellular nucleic acids or proteins, by standard techniques, including alkaline/SDS treatment, CsCl banding, column chromatography, agarose gel electrophoresis and others well known in the art. *See*, F. Ausubel, *et al.*, ed. *Current Protocols in Molecular Biology*, Greene Publishing and Wiley Interscience, New York (1987).

30 The nucleic acid compositions of the present invention, while often in a native sequence (except for modified restriction sites and the like), from either cDNA, genomic or mixtures thereof may be mutated, in accordance with standard techniques to provide gene sequences. For coding sequences, these mutations, may affect amino acid

sequence as desired. In particular, DNA sequences substantially homologous to or derived from native V, D, J, constant, switches and other such sequences described herein are contemplated (where “derived” indicates that a sequence is identical or modified from another sequence).

5           The term “operably linked” refers to a nucleic acid sequence placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the  
10           sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not  
15           exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. A nucleic acid is “operably linked” when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence. With respect to transcription regulatory sequences, operably linked means  
20           that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. For switch sequences, operably linked indicates that the sequences are capable of effecting switch recombination.

          The term “vector,” as used herein, is intended to refer to a nucleic acid molecule  
25           capable of transporting another nucleic acid to which it has been linked. One type of vector is a “plasmid,” which refers to a circular double stranded DNA loop into which additional DNA segments may be ligated. Another type of vector is a viral vector, wherein additional DNA segments may be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*,  
30           bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) can be integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the

expression of genes to which they are operatively linked. Such vectors are referred to herein as “recombinant expression vectors”(or simply, “expression vectors”). In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. The terms, “plasmid” and “vector” may be used interchangeably.

5 However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The term “recombinant host cell” (or simply “host cell”), as used herein, is intended to refer to a cell into which a recombinant expression vector has been  
10 introduced. It should be understood that such terms are intended to refer not only to the particular subject cell but to the progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term “host cell” as used herein.

15 The terms “treat,” “treating,” and “treatment,” as used herein, refer to therapeutic or preventative measures described herein. The methods of “treatment” employ administration to a subject, an antibody of the present invention, for example, a subject having an infection or disease associated with flagellin or predisposed to having such an infection or disease, in order to prevent, cure, delay, reduce the severity of, or ameliorate  
20 one or more symptoms of the infection or disease in order to prolong the survival of a subject beyond that expected in the absence of such treatment.

The terms “effective amount” and “therapeutically effective amount” as used herein, refers to that amount of an antibody thereof that binds flagellin, which is sufficient to effect treatment, prognosis or diagnosis of an infection or disease  
25 associated with flagellin, as described herein, when administered to a subject. A therapeutically effective amount will vary depending upon the subject and the infection or disease condition being treated, the weight and age of the subject, the severity of the infection or disease condition, the manner of administration and the like, which can readily be determined by one of ordinary skill in the art. The dosages for administration  
30 can range from, for example, about 1 ng to about 10,000 mg, about 5 ng to about 9,500 mg, about 10 ng to about 9,000 mg, about 20 ng to about 8,500 mg, about 30 ng to about 7,500 mg, about 40 ng to about 7,000 mg, about 50 ng to about 6,500 mg, about 100 ng to about 6,000 mg, about 200 ng to about 5,500 mg, about 300 ng to about 5,000 mg,



about 400 ng to about 4,500 mg, about 500 ng to about 4,000 mg, about 1 µg to about 3,500 mg, about 5 µg to about 3,000 mg, about 10 µg to about 2,600 mg, about 20 µg to about 2,575 mg, about 30 µg to about 2,550 mg, about 40 µg to about 2,500 mg, about 50 µg to about 2,475 mg, about 100 µg to about 2,450 mg, about 200 µg to about 2,425 mg, about 300 µg to about 2,000, about 400 µg to about 1,175 mg, about 500 µg to about 1,150 mg, about 0.5 mg to about 1,125 mg, about 1 mg to about 1,100 mg, about 1.25 mg to about 1,075 mg, about 1.5 mg to about 1,050 mg, about 2.0 mg to about 1,025 mg, about 2.5 mg to about 1,000 mg, about 3.0 mg to about 975 mg, about 3.5 mg to about 950 mg, about 4.0 mg to about 925 mg, about 4.5 mg to about 900 mg, about 5 mg to about 875 mg, about 10 mg to about 850 mg, about 20 mg to about 825 mg, about 30 mg to about 800 mg, about 40 mg to about 775 mg, about 50 mg to about 750 mg, about 100 mg to about 725 mg, about 200 mg to about 700 mg, about 300 mg to about 675 mg, about 400 mg to about 650 mg, about 500 mg, or about 525 mg to about 625 mg, of an antibody of the present invention. Dosage regimens may be adjusted to provide the optimum therapeutic response. An effective amount is also one in which any toxic or detrimental effects (*i.e.*, side effects) of an antibody are minimized and/or outweighed by the beneficial effects.

The term “patient” includes human and other mammalian subjects that receive either prophylactic or therapeutic treatment.

As used herein, the term “subject” includes any human or non-human animal. For example, the methods and compositions of the present invention can be used to treat a subject having a bacterial disease. In a particular embodiment, the subject is a human. The term “non-human animal” includes all vertebrates, *e.g.*, mammals and non-mammals, such as non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, *etc.*

The term “sample” refers to tissue, body fluid, or a cell from a patient or a subject. Normally, the tissue or cell will be removed from the patient, but *in vivo* diagnosis is also contemplated. Other patient samples, include urine, tear drops, serum, cerebrospinal fluid, feces, sputum, cell extracts *etc.*

The term “therapeutic agent” refers to any agent which acts in conjunction with or synergistically with the antibody to treat or prevent an infection-associated infection or disease. Therapeutic agents include, but are not limited to, chemotherapeutic agents, cytotoxic agents, anti-inflammatory agents, *e.g.*, a steroidal or nonsteroidal

inflammatory agent, or a cytotoxin antimetabolites (*e.g.*, methotrexate, 6-mercaptapurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (*e.g.*, mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclothosphamide, busulfan, dibromomannitol, streptozotocin, 5 mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (*e.g.*, daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (*e.g.*, dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (*e.g.*, vincristine and vinblastine).

The term “cytotoxin” or “cytotoxic agent” includes any agent that is detrimental 10 to (*e.g.*, kills) cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof.

15 Various aspects of the invention are described in further detail in the following subsections.

## **II. Methods for Producing Anti-Flagellin Antibodies**

### **(i) Monoclonal Antibodies**

20 Monoclonal antibodies of the invention can be produced using a variety of known techniques, such as those described in the examples, as well as the standard somatic cell hybridization technique described by Kohler and Milstein (1975) *Nature* 256: 495, viral or oncogenic transformation of B lymphocytes or phage display technique using libraries of human antibody genes. In particular embodiments, the 25 antibodies are fully human monoclonal antibodies.

Accordingly, in one embodiment, a hybridoma method is used for producing an antibody that binds flagellin. In this method, a mouse or other appropriate host animal can be immunized with flagellin protein (or a fragment of flagellin) in order to elicit lymphocytes that produce or are capable of producing antibodies that will specifically 30 bind to this antigen. Suitable flagellin protein can be obtained using a variety of methods, purified from a source, produced recombinantly or chemically synthesized. In a particular embodiment of the present invention, antibodies are raised against flagellin

from *Salmonella* (Genbank Accession No.GI:1333832) (SEQ ID NO:39) or *Pseudomonas* (Genbank Accession No.GI:3386643) (SEQ ID NO:36).

Alternatively, lymphocytes may be immunized *in vitro*. Lymphocytes can then be fused with myeloma cells using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell (Goding, *Monoclonal Antibodies: Principles and Practice*, pp.59-103 (Academic Press, 1986)). Culture medium in which hybridoma cells are growing is assayed for production of monoclonal antibodies directed against the antigen. After hybridoma cells are identified that produce antibodies of the desired specificity, affinity, and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies:Principles and Practice*, pp. 59-103 (Academic Press, 1986)). Suitable culture media for this purpose include, for example, D-MEM or RPMI-1640 medium. In addition, the hybridoma cells may be grown *in vivo* as ascites tumors in an animal. The monoclonal antibodies secreted by the subclones can be separated from the culture medium, ascites fluid, or serum by conventional immunoglobulin purification procedures such as, for example, protein A-Sepharose, hydroxylapatite chromatography, gel electrophoresis, dialysis, or affinity chromatography.

In another embodiment, antibodies (and binding fragments thereof) that bind flagellin can be isolated from antibody phage libraries generated using the techniques described in, for example, McCafferty *et al.*, *Nature*, 348:552-554 (1990). Clackson *et al.*, *Nature*, 352:624-628 (1991), Marks *et al.*, *J. Mol. Biol.*, 222:581-597 (1991) and Hoet *et al* (2005) *Nature Biotechnology* 23, 344-348 ; U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*. Additionally, production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.*, *Bio/Technology*, 10:779-783 (1992)), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.*, *Nuc. Acids. Res.*, 21:2265-2266 (1993)), may also be used.

In a particular embodiment, the antibodies of the invention are fully human antibodies. Such antibodies can be produced using a variety of known methods, for example, the phage display technique described by Hoet *et al.*, *supra*. This technique

involves the generation of a human Fab library having a unique combination of immunoglobulin sequences isolated from human donors and having synthetic diversity in the heavy-chain CDRs is generated. The library is then screened for Fabs that bind to flagellin.

5           Additionally, fully human antibodies directed against flagellin can be generated using transgenic or transchromosomic mice carrying parts of the human immune system rather than the mouse system (see *e.g.*, Lonberg, *et al.* (1994) *Nature* 368(6474): 856-859; Lonberg, N. *et al.* (1994), *supra*; reviewed in Lonberg, N. (1994) *Handbook of Experimental Pharmacology* 113:49-101; Lonberg, N. and Huszar, D. (1995) *Intern. Rev. Immunol.* 13: 65-93, and Harding, F. and Lonberg, N. (1995) *Ann. N.Y. Acad. Sci.* 10   764:536-546. See further, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016; 5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent No. 5,545,807 to Surani *et al.*; PCT Publication Nos. WO 92/03918, WO 93/12227, WO 94/25585, WO 97/13852, WO 98/24884 and WO 15   99/45962, all to Lonberg and Kay; and PCT Publication No. WO 01/14424 to Korman *et al.*).

Other techniques for generating fully human antibodies of the invention include the use of a mouse that carries human immunoglobulin sequences on transgenes and transchromosomes, such as a mouse that carries a human heavy chain transgene and a 20   human light chain transchromosome (see *e.g.*, PCT Publication WO 02/43478 to Ishida *et al.*).

Still further, alternative transgenic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-flagellin antibodies of the invention. For example, an alternative transgenic system referred to as 25   the Xenomouse (Abgenix, Inc.) can be used; such mice are described in, for example, U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6, 150,584 and 6,162,963 to Kucherlapati *et al.*

Moreover, alternative transchromosomic animal systems expressing human immunoglobulin genes are available in the art and can be used to raise anti-flagellin 30   antibodies of the invention. For example, mice carrying both a human heavy chain transchromosome and a human light chain transchromosome can be used; as described in Tomizuka *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:722-727. Furthermore, cows carrying human heavy and light chain transchromosomes have been described in the art

(Kuroiwa *et al.* (2002) *Nature Biotechnology* 20:889-894) and can be used to raise antibodies of the present invention.

In yet another embodiment, antibodies of the present invention can be prepared using a transgenic plant and/or cultured plant cells (such as, for example, tobacco, maize  
5 and duckweed) that produce such antibodies. For example, transgenic tobacco leaves expressing antibodies or antigen binding portions thereof can be used to produce such antibodies by, for example, using an inducible promoter (see, *e.g.*, Cramer *et al.*, *Curr. Top. Microbol. Immunol.* 240:95 118 (1999)). Also, transgenic maize can be used to express such antibodies and antigen binding portions thereof (see, *e.g.*, Hood *et al.*, *Adv. Exp. Med. Biol.* 464:127 147 (1999)). Antibodies can also be produced in large amounts  
10 from transgenic plant seeds including antibody portions, such as single chain antibodies (scFv's), for example, using tobacco seeds and potato tubers (see, *e.g.*, Conrad *et al.*, *Plant Mol. Biol.* 38:101 109 (1998)). Methods of producing antibodies or antigen binding portions in plants can also be found in, *e.g.*, Fischer *et al.*, *Biotechnol. Appl. Biochem.* 30:99 108 (1999), Ma *et al.*, *Trends Biotechnol.* 13:522 7 (1995); Ma *et al.*, *Plant Physiol.* 109:341 6 (1995); Whitelam *et al.*, *Biochem. Soc. Trans.* 22:940 944 (1994) and U.S. Patent Nos. 6,040,498 and 6,815,184.

The binding specificity of the antibodies of the present invention can be identified using any technique including those disclosed here, can be determined by  
20 immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). The binding affinity of a monoclonal antibody or portion thereof can be determined by the Scatchard analysis of Munson *et al.*, *Anal. Biochem.*, 107:220 (1980). Art recognized techniques can also be used to alter or optimize particular binding specificities and/or affinities (see, for  
25 example, Carter PJ, *Nature Reviews Immunology* 6: 343-357 (2006)).

In certain embodiments, partial antibody sequences derived from antibodies of the invention may be used for producing structurally and functionally related antibodies. For example, antibodies interact with target antigens predominantly through amino acid residues that are located in the six heavy and light chain complementarity determining  
30 regions (CDRs). For this reason, the amino acid sequences within CDRs are more diverse between individual antibodies than sequences outside of CDRs. Because CDR sequences are responsible for most antibody-antigen interactions, it is possible to express recombinant antibodies that mimic the properties of specific naturally occurring

antibodies by constructing expression vectors that include CDR sequences from the specific naturally occurring antibody grafted onto framework sequences from a different antibody with different properties (see, *e.g.*, Riechmann, L. *et al.*, 1998, *Nature* 332:323-327; Jones, P. *et al.*, 1986, *Nature* 321:522-525; Tamura *et al.*, *J Immunol.*, 2000 Feb 5 1;164(3):1432-41; and Queen, C. *et al.*, 1989, *Proc. Natl. Acad. Sci. U.S.A.* 86:10029-10033). Such framework sequences can be obtained from public DNA databases that include germline antibody gene sequences.

Thus, in one embodiment, one or more structural features of the particular anti-flagellin antibodies of the invention are used to create structurally related anti-flagellin antibodies that retain the functional properties of the parent antibodies of the invention, 10 such as binding to the same epitope or overlapping epitopes bound by the anti-flagellin antibodies exemplified herein, as well as cross-competing for antigen-binding with the anti-flagellin antibodies exemplified herein.

In another embodiment, one or more structural features of the particular antibodies of the invention are used to create structurally related anti-flagellin antibodies 15 that retain functional properties of the parent antibodies of the invention, such as (i) neutralizing flagellin; (ii) inhibiting the activity of flagellin; (iii) cross-reacting with a broad spectrum of gram-negative bacteria; (iv) inhibiting bacterial invasion into susceptible epithelial cells; (v) binding to flagellin with an affinity of at least  $10^6 \text{ M}^{-1}$ ; 20 (vi) reducing the symptoms of an enterobacterial infection or IBD in a subject; (vii) reducing the extent and severity of flagellin-induced tissue injury; (viii) reducing flagellin-stimulated neutrophil infiltration; (ix) decreasing colonic mucosal congestion, erosion and hemorrhagic ulcerations associated with IBD; (x) inhibiting or decreasing the production of mediators (*e.g.*, MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2 and IL-8); and (xi) 25 counteracting a reduction in body weight associated with IBD.

Methods known in the art for creating such structural and functional related antibodies include, for example, Marks *et al.* (*Biotechnology* (N Y).1992 Jul;10(7):779-83) (monoclonal antibodies diversification by shuffling light chain variable regions, then heavy chain variable regions with fixed CDR3 sequence changes), Jespers *et al.*, 30 (*Biotechnology* (N Y).1994 Sep;12(9):899-903) (selection of human antibodies from phage display repertoires to a single epitope of an antigen), Sharon *et al.*, (*Proc Natl Acad Sci U S A.* 1986 Apr;83(8):2628-31) (site-directed mutagenesis of an invariant amino acid residue at the variable-diversity segments junction of an antibody); Casson *et*

*al.*, (*J Immunol.* 1995 Dec 15;155(12):5647-54) (evolution of loss and change of specificity resulting from random mutagenesis of an antibody heavy chain variable region).

In one embodiment, one or more CDR regions of antibodies of the invention can  
5 be combined recombinantly with known human framework regions and CDRs to create additional, recombinantly-engineered, anti-flagellin antibodies of the invention. The heavy and light chain variable framework regions can be derived from the same or different antibody sequences.

It is well known in the art that antibody heavy and light chain CDR3 domains  
10 play a particularly important role in the binding specificity/affinity of an antibody for an antigen. See, for example, Brummel et al. (*Biochemistry.* 1993 Feb 2;32(4):1180-7), which showed that binding activity is retained in a wide range of CDR3 mutants for each of the four residues that directly hydrogen bond to the antigen. Only Gly<sup>102</sup> could not be replaced without significant loss of affinity (*see also*, Hall et al., *J. Immunol.*, 149:1605-  
15 1612 (1992); Polymenis et al., *J. Immunol.*, 152:5318-5329 (1994); Jahn et al., *Immunobiol.*, 193:400-419 (1995); Klimka et al., *Brit. J. Cancer*, 83:252-260 (2000); Beiboer et al., *J. Mol. Biol.*, 296:833-849 (2000); Rader et al., *Proc. Natl. Acad. Sci. USA*, 95:8910-8915 (1998); Barbas et al., *J. Am. Chem. Soc.*, 116:2161-2162 (1994); Ditzel et al., *J. Immunol.*, 157:739-749 (1996)). Accordingly, in certain embodiments,  
20 antibodies can be prepared to include the heavy and/or light chain CDR3s of the antibodies of the present invention (*e.g.*, SEQ ID NOs:9 and 10 and/or SEQ ID NOs:15 and 16). The antibodies can further include the heavy and/or light chain CDR2s of the antibodies of the present invention (*e.g.*, SEQ ID NOs:7 and 8 and/or SEQ ID NOs:13 and 14). The antibodies can further include the heavy and/or light chain CDR1s of the  
25 antibodies of the present invention (*e.g.*, SEQ ID NOs:5 and 6 and/or SEQ ID NOs:11 and 12).

The CDR1, 2, and/or 3 regions of the engineered antibodies described above can comprise the exact amino acid sequence(s) as those disclosed herein (*e.g.*, CDRs of monoclonal antibody 741 ("mAb 741") and monoclonal antibody 763 ("mAb 763"), set  
30 forth in SEQ ID NOs:5, 7, 9, 11, 13, 15 and 6, 8, 10, 12, 14 and 16, respectively). However, the ordinarily skilled artisan will appreciate that some deviation from the exact CDR sequences may be possible while still retaining the ability of the antibody to bind flagellin effectively (*e.g.*, conservative amino acid substitutions). Accordingly, in

another embodiment, the engineered antibody may be composed of one or more CDRs that are, for example, 90%, 95%, 98%, 99% or 99.5% identical to one or more CDRs of mAbs 741 and 763.

In another embodiment, one or more residues of a CDR may be altered to modify  
5 binding to achieve a more favored on-rate of binding. Using this strategy, an antibody having ultra high binding affinity of, for example,  $10^{10} \text{ M}^{-1}$  or more, can be achieved. Affinity maturation techniques, well known in the art and those described herein, can be used to alter the CDR region(s) followed by screening of the resultant binding molecules for the desired change in binding. Accordingly, as CDR(s) are altered, changes in  
10 binding affinity as well as immunogenicity can be monitored and scored such that an antibody optimized for the best combined binding and low immunogenicity are achieved.

In addition to, or instead of, modifications within the CDRs, modifications can also be made within one or more of the framework regions, FR1, FR2, FR3 and FR4, of  
15 the heavy and/or the light chain variable regions of an antibody, so long as these modifications do not eliminate the binding affinity of the antibody.

In another embodiment, it may be desirable to modify the antibody of the invention with respect to effector function, so as to enhance the effectiveness of the antibody in treating an inflammatory bowel disease in a subject, for example. For  
20 example cysteine residue(s) may be introduced in the Fc region, thereby allowing interchain disulfide bond formation in this region. The homodimeric antibody thus generated may have improved internalization capability and/or increased complement-mediated cell killing and antibody-dependent cellular cytotoxicity (ADCC). See Caron *et al.*, *J. Exp Med.* 176:1191-1195 (1992) and Shopes, B. *J. Immunol.* 148:2918-2922  
25 (1992). Homodimeric antibodies with enhanced anti-tumor activity may also be prepared using heterobifunctional cross-linkers as described in Wolff *et al. Cancer Research* 53:2560-2565 (1993). Alternatively, an antibody can be engineered which has dual Fc regions and may thereby have enhanced complement lysis and ADCC capabilities. See Stevenson *et al. Anti-Cancer Drug Design* 3:219-230 (1989).

30 Also encompassed by the present invention are bispecific antibodies and immunoconjugates, as discussed below.



(ii) Bispecific Antibodies

Bispecific antibodies of the present invention include at least one binding specificity for flagellin and at least one binding specificity for another target (such as an immune cell (*e.g.*, an Fc receptor on an immune cell) or a second epitope on flagellin).

5 Bispecific antibodies can be prepared as full length antibodies or antibody fragments (*e.g.* F(ab')<sub>2</sub> bispecific antibodies).

Methods for making bispecific antibodies are well known in the art. For example, production of full length bispecific antibodies can be based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two  
10 chains have different specificities (see, *e.g.*, Millstein *et al.*, *Nature*, 305:537-539 (1983)). Further details of generating bispecific antibodies can be found, for example, in Suresh *et al.*, *Methods in Enzymology*, 121:210 (1986) and in Brennan *et al.*, *Science*, 229: 81 (1985), which describes a chemical linkage process for making bispecific antibodies. Various techniques for making and isolating bispecific antibody fragments  
15 directly from recombinant cell culture have also been described. For example, bispecific antibodies have been produced using leucine zippers (see, *e.g.*, Kostelny *et al.*, *J. Immunol.*, 148(5):1547-1553 (1992)). Another strategy for making bispecific antibody fragments by the use of single-chain Fv (sFv) dimers has also been reported (see, *e.g.*, Gruber *et al.*, *J. Immunol.*, 152:5368 (1994)).

20

(iii) Immunoconjugates

In another aspect, the present invention provides immunoconjugates that bind to flagellin and target therapeutic agents (*e.g.*, a toxin) to particular classes of bacteria. Immunoconjugates can be formed by conjugating (*e.g.*, chemically linking or  
25 recombinantly expressing) antibodies of the invention to suitable therapeutic agents. Suitable agents include, for example, a cytotoxic agent, a toxin (*e.g.* an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof), and/or a radioactive isotope (*i.e.*, a radioconjugate). Enzymatically active toxins and fragments thereof which can be used include diphtheria A chain, nonbinding active fragments of  
30 diphtheria toxin, exotoxin A chain (from *Pseudomonas aeruginosa*), ricin A chain, abrin A chain, modeccin A chain, alpha-sarcin, Aleurites fordii proteins, dianthin proteins, Phytolaca americana proteins (PAPI, PAPII, and PAP-S), momordica charantia inhibitor, curcin, crotin, sapaonaria officinalis inhibitor, gelonin, mitogellin, restrictocin,

phenomycin, enomycin and the tricothecenes. A variety of radionuclides are available for the production of radioconjugated anti-flagellin antibodies. Examples include  $^{212}\text{Bi}$ ,  $^{131}\text{I}$ ,  $^{131}\text{In}$ ,  $^{90}\text{Y}$  and  $^{186}\text{Re}$ .

Immunoconjugates of the invention can be made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaredehyde), bis-azido compounds (such as bis (p-azidobenzoyl) hexanediamine), bis-diazonium derivatives (such as bis-(p-diazoniumbenzoyl)-ethylenediamine), diisocyanates (such as tolyene 2,6-diisocyanate), and bis-active fluorine compounds (such as 1,5-difluoro-2,4-dinitrobenzene). For example, a ricin immunotoxin can be prepared as described in Vitetta *et al.*, *Science* 238: 1098 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody (see, *e.g.*, WO94/11026).

### **III. Methods for Screening Anti-Flagellin Antibodies**

Subsequent to producing antibodies that bind to flagellin, the antibodies can be screened and selected for various properties, such as (i) their effect on bacterial invasion into susceptible epithelial cells, (ii) inhibition of flagellin-stimulated NO or IL-8 production from epithelial cells, (iii) bacterial opsonophagocytosis, (iv) macrophage ingestion of bacteria, (v) superoxide production, (vi) ability to neutralize flagellin, (vii) ability to inhibit the activity of flagellin, (viii) cross-reactivity with a broad spectrum of gram-negative bacteria, (ix) ability to inhibit bacterial invasion into susceptible epithelial cells, (x) ability to bind to flagellin with an affinity of at least  $10^6\text{ M}^{-1}$ , (xi) capability of reducing the symptoms of an enterobacterial infection or IBD in a subject, (xii) capability of reducing the extent and severity of flagellin-induced tissue injury, (xiii) capability of reducing flagellin-stimulated neutrophil infiltration; (xiv) capability of decreasing colonic mucosal congestion, erosion and hemorrhagic ulcerations associated with IBD; (xv) capability of inhibiting or decreasing the production of mediators (*e.g.*, MDA, IL-1 $\beta$ , TNF $\alpha$ , MIP-1, MIP-2 and IL-8); and (xvi) capability of counteracting a reduction in body weight associated with IBD, using a variety of assays that are well known in the art. Assays for screening for such properties include the assays

exemplified and described herein, as well as those well known in the art, such as binding to immobilized recombinant or bacterial flagellin on ELISA, binding to recombinant or bacterial extracts on SDS-PAGE, affinity binding determinations to purified antigens by BIACore analysis.

5           Antibodies or antigen binding portions thereof that bind to the same or overlapping epitopes as one or more antibodies of the present invention can also be identified using standard techniques known in the art and described herein. For example, in order to screen for antibodies which bind to the same or an overlapping epitope on flagellin bound by an antibody of interest, a cross-blocking assay, such as  
10           that described in *Antibodies*, A Laboratory Manual, Cold Spring Harbor Laboratory, Ed Harlow and David Lane (1988), can be performed.

#### **IV. Pharmaceutical Compositions**

          In another aspect, the present invention provides compositions, *e.g.*, a  
15           pharmaceutical composition, containing one or a combination of antibodies of the invention thereof, of the present invention, formulated together with a pharmaceutically acceptable carrier. In one embodiment, the compositions include a combination of multiple (*e.g.*, two or more) isolated antibodies of the invention, which bind different epitopes on flagellin.

20           As used herein, “pharmaceutically acceptable carrier” includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Preferably, the carrier is suitable for intravenous, intramuscular, subcutaneous, parenteral, spinal or epidermal administration (*e.g.*, by injection or infusion). Depending on the route of  
25           administration, the active compound, *i.e.*, antibody, bispecific and multispecific molecule, may be coated in a material to protect the compound from the action of acids and other natural conditions that may inactivate the compound.

          A “pharmaceutically acceptable salt” refers to a salt that retains the desired biological activity of the parent compound and does not impart any undesired  
30           toxicological effects (see *e.g.*, Berge, S.M., *et al.* (1977) *J. Pharm. Sci.* 66:1-19). Examples of such salts include acid addition salts and base addition salts. Acid addition salts include those derived from nontoxic inorganic acids, such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydroiodic, phosphorous and the like, as well as from

nontoxic organic acids such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, aromatic acids, aliphatic and aromatic sulfonic acids and the like. Base addition salts include those derived from alkaline earth metals, such as sodium, potassium, magnesium, calcium and the like, as well as from nontoxic organic amines, such as N,N'-dibenzylethylenediamine, N-methylglucamine, chlorprocaine, choline, diethanolamine, ethylenediamine, procaine and the like.

Pharmaceutical compositions of the invention can be administered alone or in combination therapy, *i.e.*, combined with other agents. For example, the combination therapy can include a composition of the present invention with at least one or more additional therapeutic agents, such as chemotherapeutic agents. The pharmaceutical compositions of the invention can also be administered in conjunction with radiation therapy.

Pharmaceutical compositions of the invention can administered by a variety of methods known in the art. As will be appreciated by the skilled artisan, the route and/or mode of administration will vary depending upon the desired results. The active compounds can be prepared with carriers that will protect the compound against rapid release, such as a controlled release formulation, including implants, transdermal patches, and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Many methods for the preparation of such formulations are patented or generally known to those skilled in the art. *See, e.g., Sustained and Controlled Release Drug Delivery Systems*, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978.

To administer a compound of the invention by certain routes of administration, it may be necessary to coat the compound with, or co-administer the compound with, a material to prevent its inactivation. For example, the compound may be administered to a subject in an appropriate carrier, for example, liposomes, or a diluent.

Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Liposomes include water-in-oil-in-water CGF emulsions as well as conventional liposomes (Strejan *et al.* (1984) *J. Neuroimmunol.* 7:27).

Pharmaceutically acceptable carriers include sterile aqueous solutions or dispersions and sterile powders for the extemporaneous preparation of sterile injectable

solutions or dispersion. The use of such media and agents for pharmaceutically active substances is known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the pharmaceutical compositions of the invention is contemplated. Supplementary active compounds can also be  
5 incorporated into the compositions.

Therapeutic compositions typically must be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other ordered structure suitable to high drug concentration. The carrier can be a solvent or dispersion medium containing, for  
10 example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. In many cases, it will be preferable to include isotonic agents, for example, sugars,  
15 polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, monostearate salts and gelatin.

Sterile injectable solutions can be prepared by incorporating the active  
20 compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by sterilization microfiltration. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile  
25 injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying (lyophilization) that yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Dosage regimens are adjusted to provide the optimum desired response (*e.g.*, a therapeutic response). For example, a single bolus may be administered, several divided  
30 doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. For example, the human antibodies of the invention may be administered once or twice weekly by subcutaneous injection or once or twice monthly by subcutaneous injection.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subjects to be treated; each unit contains a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Therapeutic compositions of the present invention include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods known in the art of pharmacy. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated, and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the composition which produces a therapeutic effect. Generally, out of one hundred per cent, this amount will range from about 0.001 per cent to about ninety percent of active ingredient, preferably from about 0.005 per cent to about 70 per cent, most preferably from about 0.01 per cent to about 30 per cent.

The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal,

intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, epidural and intrasternal injection and infusion.

Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the invention include water, ethanol, polyols  
5 (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of surfactants.

10 These compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of presence of microorganisms may be ensured both by sterilization procedures, supra, and by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include  
15 isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

When the antibodies of the present invention are administered as  
20 pharmaceuticals, to humans and animals, they can be given alone or as a pharmaceutical composition containing, for example, 0.001 to 90% (more preferably, 0.005 to 70%, such as 0.01 to 30%) of active ingredient in combination with a pharmaceutically acceptable carrier.

Regardless of the route of administration selected, antibodies of the present  
25 invention and/or the pharmaceutical compositions thereof, are formulated into pharmaceutically acceptable dosage forms by conventional methods known to those of skill in the art.

Actual dosage levels of the active ingredients in the pharmaceutical compositions of the present invention may be varied so as to obtain an amount of the active ingredient  
30 which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being toxic to the patient. The selected dosage level will depend upon a variety of pharmacokinetic factors including the activity of the particular compositions of the present invention employed, or the

ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion of the particular compound being employed, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular compositions employed, the age, sex, weight, condition, general health and  
5 prior medical history of the patient being treated, and like factors well known in the medical arts. A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the compounds of the invention employed in the pharmaceutical composition at levels lower than that  
10 required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of a composition of the invention will be that amount of the compound which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described above. It is preferred that administration be  
15 intravenous, intramuscular, intraperitoneal, or subcutaneous, preferably administered proximal to the site of the target. If desired, the effective daily dose of a therapeutic composition may be administered as two, three, four, five, six or more sub-doses administered separately at appropriate intervals throughout the day, optionally, in unit dosage forms. While it is possible for a compound of the present invention to be  
20 administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition).

Therapeutic compositions can be administered with medical devices known in the art. For example, in a preferred embodiment, a therapeutic composition of the invention can be administered with a needleless hypodermic injection device, such as the  
25 devices disclosed in U.S. Patent Nos. 5,399,163, 5,383,851, 5,312,335, 5,064,413, 4,941,880, 4,790,824, or 4,596,556. Examples of well-known implants and modules useful in the present invention include: U.S. Patent No. 4,487,603, which discloses an implantable micro-infusion pump for dispensing medication at a controlled rate; U.S. Patent No. 4,486,194, which discloses a therapeutic device for administering  
30 medications through the skin; U.S. Patent No. 4,447,233, which discloses a medication infusion pump for delivering medication at a precise infusion rate; U.S. Patent No. 4,447,224, which discloses a variable flow implantable infusion apparatus for continuous drug delivery; U.S. Patent No. 4,439,196, which discloses an osmotic drug



delivery system having multi-chamber compartments; and U.S. Patent No. 4,475,196, which discloses an osmotic drug delivery system. Many other such implants, delivery systems, and modules are known to those skilled in the art.

In certain embodiments, antibodies of the invention can be formulated to ensure proper distribution *in vivo*. For example, the blood-brain barrier (BBB) excludes many highly hydrophilic compounds. To ensure that the therapeutic compounds of the invention cross the BBB (if desired), they can be formulated, for example, in liposomes. For methods of manufacturing liposomes, see, *e.g.*, U.S. Patents 4,522,811; 5,374,548; and 5,399,331. The liposomes may comprise one or more moieties which are selectively transported into specific cells or organs, thus enhance targeted drug delivery (*see, e.g.*, V.V. Ranade (1989) *J. Clin. Pharmacol.* 29:685). Exemplary targeting moieties include folate or biotin (*see, e.g.*, U.S. Patent 5,416,016 to Low *et al.*); mannosides (Umezawa *et al.*, (1988) *Biochem. Biophys. Res. Commun.* 153:1038); antibodies (P.G. Bloeman *et al.* (1995) *FEBS Lett.* 357:140; M. Owais *et al.* (1995) *Antimicrob. Agents Chemother.* 39:180); surfactant protein A receptor (Briscoe *et al.* (1995) *Am. J. Physiol.* 1233:134), different species of which may comprise the formulations of the inventions, as well as components of the invented molecules; p120 (Schreier *et al.* (1994) *J. Biol. Chem.* 269:9090); *see also* K. Keinanen; M.L. Laukkanen (1994) *FEBS Lett.* 346:123; J.J. Killion; I.J. Fidler (1994) *Immunomethods* 4:273.

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#### V. Methods of Using Anti-Flagellin Antibodies

The present invention provides methods of using antibodies that bind to and neutralize bacterial flagellin in a variety of therapeutic and diagnostic applications.

Suitable diseases that can be treated and/or diagnosed using the antibodies provided herein include, for example, IBD, Ulcerative Colitis and Crohn's Disease, as well as infectious diseases, including, but not limited to, gram negative bacterial infections (*e.g.*, enterobacterial infections), sepsis, septic shock, Anthrax (by the bacterium *Bacillus anthracis*), Bacterial Meningitis (caused by a variety of bacteria, including, but not limited to, *Neisseria meningitides*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae* and *Haemophilus influenzae*), Botulism (caused by bacterium *Clostridium botulinum*), Brucellosis (caused by bacteria of the genus *Brucella*), *Campylobacteriosis* (caused by bacteria of the genus *Campylobacter*), Cat Scratch

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Disease (caused by *Bartonella henselae* and *Bartonella clarridgeiae*), Cholera (caused by the bacterium *Vibrio cholerae*), Diphtheria (caused by *Corynebacterium diphtheriae*), Epidemic Typhus (causative organism is *Rickettsia prowazekii*), Impetigo (caused by several bacteria, including, *Staphylococcus aureus* and *Streptococcus pyogenes*),  
5 Legionellosis (caused by bacteria belonging to the genus *Legionella*), Leprosy (Hansen's Disease) (caused by the bacterium *Mycobacterium leprae*), Leptospirosis (caused by spirochaetes of the genus *Leptospira*), Listeriosis (caused by the bacterium *Listeria monocytogenes*), Lyme Disease (caused by spirochete bacteria from the genus *Borrelia*), Melioidosis (caused by the bacterium *Burkholderia pseudomallei*), MRSA infection  
10 (caused by *Staphylococcus aureus*), Nocardiosis (bacterium of the genus *Nocardia*, most commonly *Nocardia asteroides* or *Nocardia brasiliensis*), Pertussis (Whooping Cough) (caused by the bacterium *Bordetella pertussis*), Plague (caused by the enterobacteria *Yersinia pestis*), Pneumococcal pneumonia (caused by a variety of bacteria, including, but not limited to, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Haemophilus*  
15 *influenzae*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*, and *Legionella pneumophila*), Psittacosis (caused by a bacterium called *Chlamydophila psittaci*), Q fever (caused by infection with *Coxiella burnetii*), Rocky Mountain Spotted Fever (RMSF) (by *Rickettsia rickettsii*), Salmonellosis (caused by bacteria of the genus  
20 *Salmonella*), Scarlet Fever, Shigellosis (caused by bacteria of the genus *Shigella*), Syphilis (caused by *Treponema pallidum*), Tetanus (*Clostridium tetani*), Trachoma, Tuberculosis (caused by *mycobacteria*, mainly *Mycobacterium tuberculosis*), Tularemia (by the bacterium *Francisella tularensis*), Typhoid Fever (caused by the bacterium *Salmonella typhi*), and Urinary Tract Infections (caused by bacteria such as *Escherichia*  
25 *coli*, *Staphylococcus saprophyticus*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Enterobacter* spp., *Pseudomonas* and *Enterococcus*).

Antibodies of the present invention are particularly useful for treating enterobacterial infections, and can be selected for broad reactivity with multiple entobacterial strains, such *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*,  
30 *Arsenophonus*, *Azotivirga*, *Blochmannia*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, *Morganella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Candidatus*

*Phlomobacter, Photorhabdus, Plesiomonas, Pragia Proteus, Providencia, Rahnella, Raoultella, Salmonella, Samsonia, Serratia, Shigella, Sodalis, Tatumella, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia and Yokenella.*

The antibodies can be administered alone or with other therapeutic agents, which  
 5 act in conjunction with or synergistically with the antibodies, to treat diseases. Such  
 therapeutic agents include, for example, toxins, chemotherapeutic agents, small  
 molecules and radiation

Also within the scope of the present invention are kits comprising antibodies and  
 antigen binding portions thereof of the invention which optionally include instructions  
 10 for use in treating a disease associated with flagellin. The kits may include a label  
 indicating the intended use of the contents of the kit. The term label includes any  
 writing, marketing materials or recorded material supplied on or with the kit, or which  
 otherwise accompanies the kit.

Other embodiments of the present invention are described in the following  
 15 Examples.

The present invention is further illustrated by the following examples which  
 should not be construed as further limiting. The contents of Sequence Listing, figures  
 and all references, patents and published patent applications cited throughout this  
 application are expressly incorporated herein by reference.

20

## EXAMPLES

### **Example 1: Generation of Anti-Flagellin Antibodies**

#### **Antigen Construction**

25 The gene fragment corresponding to amino acids 1–156 of the flagellin gene of  
*Salmonella muenchen* was used as an antigen (Genbank Accession No. GI:47233) (SEQ  
 ID NO:41):

30 aaggaaaagatcatggcacaagtcattaatacaaacagcctgtcgctgttgaccagaataacctgaacaaatcccagtcgct  
 ctgggcaccgctatcgagcgtctgtcttccggctgcgtatcaacagcgcgaaagacgatgcggcaggtcaggcgattgctaa  
 ccgtttcaccgcgaacatcaaaggctgactcaggcttcccgtaacgtaacgacggatctccattgcgagaccactgaagg  
 cgcgctgaacgaaatcaacaacaacctgcagcgtgtgcgtgaactggcggttcagctgtaacgggtactaactcccagctg  
 acctgactctatccaggctgaaatcaccagcgtctgaacgaaatcgaccgtgtatccggtcagactcagttcaacggcgtga  
 aagtctctggcgcaggacaacaccctgacctccaggttggtgccaacgac

35

The antigen was prepared by expression of a cDNA clone obtained by PCR amplification of DNA from *S. muenchen* using a sense primer designated 1S (5'-CGCGGATCCCAATGGCACAAGTCATTAATAACA) (SEQ ID NO:17) and an antisense primer designated 468A (5'-

5 TCCGCTCGAGTTAAATAGTTTCACCGTCGTTGGCACC) (SEQ ID NO:18). Underlined nucleotides represent adaptor sequences added to the ends of primers to maintain proper reading frame and facilitate cloning (BamHI recognition sites on sense primers and XhoI sites on antisense primers). The template DNA for PCR was plasmid CL402, a clone of pBR322 containing a 3.8-kb EcoRI fragment of *S. muenchen*

10 chromosomal DNA that harbors the 1.5-kb flagellin gene. PCR-generated flagellin DNA were digested with BamHI plus XhoI, gel purified, and subcloned into the BamHI/XhoI sites at the 3' end of the His tag in expression vector pET 30C (Novagen, San Diego, CA). The correct reading frame and integrity of subcloned DNA was verified by DNA sequence analysis. The recombinant plasmids were then introduced

15 into *Escherichia coli* BL21 (DE3) (Novagen) by transformation and selected in the presence of kanamycin (50 ug/ml).

#### Expression and Purification of Recombinant Antigen

A single colony of *E. coli* containing the recombinant plasmid was grown at 37

20 °C in Luria broth containing 50 ug/ml kanamycin to an A600 of 0.5 and then induced for 3 h with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Following induction, bacteria were harvested and washed with phosphate-buffered saline (PBS, pH 7.2). Cell-free lysates were prepared in 6 M guanidine chloride containing 5 mM imidazole and 0.1% Nonidet P-40 (binding buffer). After removing the insoluble material by centrifugation,

25 the lysate was applied to a nickel-nitrilotriacetic acid-agarose (Qiagen, Valencia, CA) column, washed extensively with binding buffer, and then eluted with binding buffer containing 200 mM imidazole. The purified proteins were extensively dialyzed against PBS, and protein concentrations were determined by the Bradford method. The final

30 proteins were analyzed by 10% SDS-PAGE and visualized with Coomassie Blue staining to assess protein purity, integrity, and concentration.

### Immunization

Female BALB/c mice of 12 week old were immunized with 50 ug of fusion protein in complete adjuvant. On day 14, 28 and 42 mice were boosted with 50 ug of protein in incomplete adjuvant. Three days after final boost, spleen cells will be prepared for fusion with SP2/O myeloma cells (ATCC). Antibody titer in serum was measured by ELISA and using recombinant *S. muenchen* flagellin as antigen.

### Preparation of Monoclonal Antibodies

Anti-flagellin monoclonal antibodies were produced using previously described methods (Harlow and Lane, Antibodies, CSH laboratories). Splens from immunized mice were broken apart with sterile forceps and passed through a sterile stainless-steel strainer by pressing the spleen tissue with the glass plunger. Splenocytes were collected, washed once in serum-free DMEM medium and then fused with SP2/O myeloma cells using sterile PEG (polyethylene glycol) solution. After fusion cells were seeded in 96-well microtiter plates and selected against HAT medium. Hybridoma culture supernatants were screened by ELISA using 96 well plates coated with recombinant flagellin and amplified the positive ones and tested for Ig-subclass (Southern Biotechnology Associates). The candidate hybridoma lines producing IgG subclass were selected and cloned by limited dilution.

### Purification of Monoclonal Antibodies

Murine monoclonal antibodies to flagellin were purified using standard immunology techniques. In brief, hybridoma cells were grown in roller bottles for 14 days in BD medium and tissue culture supernatants were collected. Antibodies were further purified by passing over a 10 ml protein G-sepharose affinity column, washed extensively with PBS, and eluted with 0.1 M glycine (pH 2.5). After dialysis of the eluate against PBS, antibodies titers were tested by ELISA and tested the purity by SDS-polyacrylamide gels.

### Example 2: Generation of Human Monoclonal Antibodies

Human peripheral blood lymphocytes (PBL's) were obtained by leukophoresis from donors positive for anti-Salmonella muenchen IgG. PBL's were separated on a Ficoll-Hypaque gradient, washed twice, counted and resuspended in PBS.

BALB/c mice were exposed to split dose total body irradiation (4 Gy followed 3 days later by 10-11 Gy) from a gamma beam <sup>60</sup>Co source with a focal skin distance of 75 cm and a dose rate of 0.7 Gy/min. Following irradiation, each recipient mouse was immediately injected i.v. with 4-6 x 10<sup>6</sup> of SCID/NOD bone marrow cells and i.p. with 100 x 10<sup>6</sup> human PBL's. These reconstituted mice were thereafter immunized once i.p. with 2.5 mcg recombinant SM flagellin protein (a/a 1-156) in 0.15% Aluminum hydroxide gel. Fourteen days later, animals were bled from the retro-orbital vein and screened for IgG reactivity to coated SM flagellin protein on ELISA plates and determination of human IgG. Spleen cells from mice that exhibited positive screening of plasma were harvested and taken for fusion with the human-mouse heteromyeloma HMMA2.11TG/020 at a ratio of 3:1. Fusion was performed with 50% w/v PEG 1500 and fused cells seeded at a concentration of 30,000 cells per well in a 96 well U-bottom, microtiter plate in complete RPMI medium containing hypoxanthine, aminopterin and thymidine (HAT) supplement. Cells were fed with fresh HAT medium 1 week later. Two weeks after fusion, supernatants were harvested for ELISA of human IgG and anti-SM flagellin reactivity. Medium was replaced with fresh hypoxanthine, thymidine (HT)-containing medium. Hybridoma cultures secreting specific anti-SM flagellin IgG were cloned by limiting dilution at 0.5 cells per well in a 96 well U bottom microtiter plate. One clone, INO-763, repeatedly demonstrated the highest binding as well as being stable.

### **Example 3: Hybridoma Sequencing**

mRNA was extracted from two hybridoma cell lines, the first (mAb 741) expressing a murine antibody, and the second (mAb 763) expressing a human antibody. The mRNA was reverse transcribed and antibody specific transcripts were PCR amplified. PCR products were cloned for determination of the nucleotide and amino acid sequences of the heavy and light chain variable regions of both antibodies. Heavy chain variable region mRNA was amplified using a set of six degenerate primer pools (HA to HF) and light chain variable region mRNA was amplified using a set of eight degenerate primer pools (LA to LH).

#### **mAb 741 Heavy Chain**

Strong DNA bands of approximately the expected size were observed in primer pools HB and HE. DNA from each band was purified and cloned, and four clones were

sequenced. All four clones aligned to give a functional, rearranged heavy chain (Table 1, Figure 1).

mAb 741 Light Chain

5 Strong DNA bands of the expected size were observed in primer pools LB, LC and LG. DNA from each band was purified and cloned, and a total of nine clones were sequenced. Six of the clones were found to align with the well described aberrant kappa transcript found in some hybridomas and three clones aligned to give a functional, rearranged light chain (Table 1, Figure 2).

10 **TABLE 1: Antibody Sequence Analysis<sup>1</sup>**

	<b>VH Chain</b>	<b>VK Chain</b>
CDR 1 Length	5 amino acids	10 amino acids
CDR 2 Length	17 amino acids	7 amino acids
CDR 3 Length	4 amino acids	9 amino acids
Mouse Germline <sup>2</sup>	J558.51 (93%)	Kn4 (96%)
Closest Human Germline <sup>2</sup>	VH1-24 (65%)	A26 (64%)

<sup>1</sup>CDR definitions and sequence numbering according to Kabat.

<sup>2</sup>Germline ID(s) indicated followed by % homology.

mAb 763 Heavy Chain

15 A weak DNA band of the expected size was observed in primer pool HA. DNA from this band was purified and cloned, and three clones were sequenced. All clones aligned to give a functional, rearranged heavy chain (Table 2, Figure 3).

mAb 763 Light Chain

20 Strong DNA bands of the expected size were observed in primer pools LB, LC and LG, and a weak band was observed in pool LH. DNA from each band was purified and cloned and a total of eighteen clones were sequenced. Twelve of the clones (from pools LB, LC and LG) aligned with the well described aberrant kappa transcript found in some hybridomas. Five clones (from pool LH) aligned to give a functional, rearranged light chain  
 25 (Table 2, Figure 4). Pool LH primers are specific for lambda light chains and this was confirmed by the sequence analysis.

**TABLE 2: Antibody Sequence Analysis<sup>1</sup>**

	<b>VH Chain</b>	<b>Vλ Chain</b>
CDR 1 Length	5 amino acids	11 amino acids
CDR 2 Length	17 amino acids	7 amino acids
CDR 3 Length	10 amino acids	11 amino acids
Mouse Germline <sup>2</sup>	N/A	N/A
Closest Human Germline <sup>2</sup>	VH3-23 (87%)	V2-17 (92%)

<sup>1</sup>CDR definitions and sequence numbering according to Kabat.

<sup>2</sup>Germline ID(s) indicated followed by % homology.

#### 5 **Example 4: Generation of Chimeric Antibodies**

As described above in Example 3, variable regions from the murine 741 anti-flagellin antibody were successfully cloned and sequenced. These murine variable region genes were then combined with human IgG1 heavy chain and kappa light chain constant regions and expressed in NS0 cells to produce a chimeric anti-flagellin antibody (referred to herein as mAb 741C or chimeric mAb 741C) as follows.

##### A. Expression of Chimeric Antibody

The variable regions of mAb 741 were transferred to appropriate expression vector (*e.g.*, an Antitope expression vector) system for IgG1 heavy chains. NS0 cells were transfected via electroporation and selected using methotrexate (Sigma Catalogue No. M8407-500MG). A number of methotrexate resistant colonies were identified and cell lines positive for IgG expression were expanded. After several passages, the cell line with the highest level of IgG expression (mAb 741-4G4) was selected and expanded. Chimeric IgG1 from the mAb 741-4G4 stable cell line was purified from cell culture supernatants on a Protein A sepharose column (GE Healthcare Catalogue No. 110034-93). The concentration of the antibody was calculated by UV absorption based upon a calculated molar extinction coefficient such that  $E_{c(0.1\%)} \text{ at } 280\text{nm} = 1.41$ . 20mg of the expressed chimeric antibody was purified. The purified chimeric antibody was analyzed by SDS-PAGE.

25



### B. Binding of the Chimeric Antibody to Flagellin

The binding of chimeric mAb 741C to flagellin was assessed by competition ELISA. A dilution series of NS0-derived chimeric IgG1 or mouse control antibody from 20µg/ml to 0.01µg/ml was premixed with a constant concentration of

5 biotinylated control antibody (0.6µg/ml) before incubating for 1 hour at room temperature on a Nunc Immulo MaxiSorp 96 well flat bottom microlitre plate (Fisher Catalogue No. DIS-971-030J) pre-coated with 100µl 1µg/ml Salmonella muenchen Flagellin (supplied by Inotek) per well. The binding of the biotinylated mAb was determined by detection with streptavidin-HRP (Sigma Catalogue No. S5512) and

10 OPD substrate (Sigma Catalogue No. P9187-50SET) and absorbance at 490nm was measured on a Dynex Technologies MRX TC II plate reader. The results obtained (Figure 5) show that the chimeric mAb 741C and mouse control antibodies have similar binding profiles, with IC50 values of 1.59µg/ml and 1.90µg/ml respectively.

### 15 Example 5: Generation of Humanized Antibodies

Antibodies of the invention can also be humanized using a variety of known techniques known in the art, such as those taught in U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen

*et al.*, the substance of which is incorporated herein by reference. Additionally, the

20 antibodies of the invention can be humanized using composite human antibody technologies, as described below.

#### A. Design of Humanized Heavy and Light Chains

The sequences of mAb 741 (or other anti-flagellin antibodies) heavy and light

25 chain variable regions can be analyzed to identify CDRs, unusual amino acids and residues critical to binding as follows.

First, protein models of the murine antibody variable regions can be generated using existing antibody structures as templates. Structural information from the protein model can then be used to identify and compare residues critical for antibody

30 conformation and binding with structurally equivalent residues from existing antibody structures and sequence databases. These amino acids can then be candidates for inclusion in one or more variants of the final humanized sequences.

Segments of monoclonal antibody heavy and light chain variable region amino acid sequences can then be compared with corresponding segments of human variable region sequences in order to identify potential heavy and light chain human sequences for possible inclusion in the humanized sequences.

5 A series of at least ten of each humanized heavy and light chain variable regions can then be designed entirely from segments of human variable region sequences. Alternative variants will differ in the inclusion of residues which might be critical to restoration of the original monoclonal antibody binding efficiency with the objective that the number of alterations in the frameworks needed to restore binding efficiency will be  
10 kept to the minimum and generation of T-cell epitopes avoided. Potential T cell epitopes as determined by *in silico* methods can be considered in the selection of alternative variants.

#### B. Construction of Humanized Heavy and Light Chains

15 Humanized variable regions can be constructed by PCR-ligation of long synthetic oligonucleotides. The initial heavy and light chain variable region genes can be used as templates for construction of additional sequences by mutagenesis using overlapping PCR with mutagenic oligonucleotide primers. Restriction enzyme sites can then be engineered upstream and downstream of each of the variable heavy and light  
20 chains for cloning into the appropriate expression vector (*e.g.*, an Antitope expression vector). The entire DNA sequence can be confirmed to be correct for each modified variable region cassette.

#### C. Construction of Expression Plasmids Encoding Humanized Antibodies with Human Constant Regions

25 At least ten humanized variable regions can be transferred into mammalian expression vectors as follows. First, the DNA sequences for each variable region can be inserted into mammalian expression vectors between an upstream cytomegalovirus immediate/early promoter/enhancer (CMV-ie) plus the immunoglobulin signal sequence and a downstream the immunoglobulin constant region. The heavy chain vector  
30 includes a genomic human IgG constant region of choice (IgG1, or IgG4) and the dhfr gene for selection in mammalian cells. The light chain vector includes the genomic human  $\kappa$  constant region.

DNA samples can then be prepared for transfection into mammalian cells. The humanized antibody heavy and light chain-encoding plasmids can be co-transfected into mammalian cells by electroporation.

5           D.     Generation of Humanized Antibody-Producing Cell Lines and Selection of Lead Humanized Antibody

Individual heavy and light chain plasmids can be paired in order to produce a final series of antibodies combining variant humanized variable region sequences. These combinations may also include chimeric heavy and light chains in order to  
10 determine the effects of individual modified humanized chains on binding efficiency.

Heavy and light chain plasmid DNA pairs can then be transfected into mammalian cells by electroporation, stable cell lines can be selected and tested for antibody production. Cell lines producing humanized antibodies comprising combinations of heavy and light chains can then be expanded and antibody samples  
15 (typically 100ug) purified.

Antibodies can then be tested in a binding assay in order to determine the humanized best antibody lead. The entire coding sequence of the lead humanized antibody can then be subcloned into an appropriate mammalian expression vector.

20     **Example 6: Anti-Flagellin Monoclonal Antibodies Are Protective in a Murine DNBS-Induced Colitis Model**

The following materials and methods were utilized in each of the following studies presented within this example:

25           *Animals*

The study was carried out in 6-8 weeks old (25-30 g) CD1 male mice (Harlan Nossan Udine, Italy). The animals were housed in a controlled environment and provided with standard rodent chow and water. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific  
30 purposes (D.M. 116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

### Administration of Antibodies

All monoclonal antibodies were administered by intra-peritoneal injection one time only at 60 min prior to the DNBS challenge. In all the groups of animals, no evidence for lethargy or irritation was observed during the experimental time  
5 frame.

### Induction of Experimental Colitis

Colitis was induced with a very low dose of DNBS (4 mg per mouse) by using a modification of the method first described in mice. In preliminary experiments, this dose of DNBS was found to induce reproducible colitis without mortality. Mice were  
10 anesthetized by Enflurane. DNBS (4 mg in 100  $\mu$ l of 50% ethanol) was injected into the rectum through a catheter inserted 4.5cm proximally to the anus. Carrier alone (100  $\mu$ l of 50% ethanol) was administered in control experiments. Thereafter, the animals were kept for 15 minutes in a Trendelenburg position to avoid reflux. After colitis and sham-  
15 colitis induction, the animals were observed for 3 days. On Day 4, the animals were weighed and anaesthetized with chloral hydrate, and the abdomen was opened by a midline incision. The colon was removed, freed from surrounding tissues, opened along the antimesenteric border and processed for histology.

### Evaluation of colon damage

After its removal, the colon was gently rinsed with saline solution, opened  
20 by a longitudinal incision, and immediately examined under a microscope. The visible colonic damage was assessed by a scoring system as previously described (Wallace *et al.*, 1992).

### Optical Microscopy

After fixation for 1 week at room temperature in Dietrich solution (14.25%  
25 ethanol, 1.85% formaldehyde, 1% acetic acid), samples were dehydrated in graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, New Jersey). Thereafter, 7- $\mu$ m sections were deparaffinized with xylene, stained with hematoxylin-eosin and observed in a Dialux 22 Leitz (Wetzlar, Germany) microscope. In order to have a quantitative estimation of colon damage, section (n = 6 for each animals) was  
30 scored by an independent observer blinded to the experimental protocol. The following morphological criteria were considered: score 0, no damage; score 1 (mild), focal epithelial edema and necrosis; score 2 (moderate), diffuse swelling and necrosis of the villi; score 3 (severe), necrosis with presence of neutrophil infiltrate in the submucosa;

score 4 (highly severe), widespread necrosis with massive neutrophil infiltrate and hemorrhage.

#### Myeloperoxidase activity

Myeloperoxidase activity, an indicator of polymorphonuclear leukocyte  
5 accumulation, was determined. At the specified time following the intracolonic  
injection of DNBS, colon tissues were obtained and weighed. Each piece of tissue  
was homogenised in a solution containing 0.5% hexa-decyl-trimethyl-ammonium  
bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and centrifuged  
for 30 min at 20,000 x g at 4° C. An aliquot of the supernatant was then allowed to  
10 react with a solution of tetra-methyl-benzidine (1.6 mM) and 0.1 mM H<sub>2</sub>O<sub>2</sub>. The  
rate of change in absorbance was measured spectrophotometrically at 650 nm.  
Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol  
of peroxide min<sup>-1</sup> at 37° C and and was expressed in U per gram weight of wet  
tissue.

#### 15 Reagents

All other reagents used were purchased from Sigma Chemical Company  
(Milan, Italy).

#### Data analysis

All values in the figures and text are expressed as mean ± standard error  
20 (S.E.M.) of the mean of *n* observations. For the *in vivo* studies *n* represents the  
number of animals studied. In the experiments involving histology, the figures  
shown are representative of at least three experiments performed on different  
experimental days. The results were analyzed by one-way analysis of variance  
followed by a Bonferroni *post-hoc* test for multiple comparisons. A *P*-value less  
25 than 0.05 was considered significant.



induced by DNBS-administration in mice (Figure 8). On the contrary the treatment with CBH2 (20 mg/kg) did not reduced the loss of body weight induced by DNBS-administration in mice (Figure 8). The colitis caused by DNBS was also characterized by an increase in myeloperoxidase activity, an indicator of the neutrophils accumulation in the colon (Figure 9). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DBNS-mice contained a large number of neutrophils. On the contrary, mAb 763 (20 mg/kg) treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Figure 9). Similarly, the treatment with Infliximab reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 9). On the contrary the treatment with CBH2 (20 mg/kg) did not reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 9).

**B. Effects of Full-Dose Response Curve of mAb 741 (0.3-10 mg.kg) Versus DNBS-Induced Colitis**

1. Experimental Groups: Animals were randomly divided into 7 groups (n=15 for each group):

Group	DNBS	Treatment	Dosing
1	No	Sham-vehicle	• 0.2 ml vehicle i.p. (saline solution)
2	Yes	Control-Vehicle	• 0.2 ml vehicle i.p. (saline solution)
3	Yes	mAb 763	• 20 mg/kg i.p.
4	Yes	mAb 741	• 5 mg/kg i.p.
5	Yes	mAb 741	• 10 mg/kg i.p.
6	Yes	mAb 741	• 20 mg/kg i.p.
7	Yes	mAb 18.8	• 20 mg/kg i.p.

2. Effects of mAb 763 and mAb 741 Treatment on the Degree of Colitis

No histological alteration was observed in the colon tissue from sham-treated mice (see Figures 10a; see macroscopic score 10h, Figure 11a see histological score

11h). Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon and rectum showed presence of mucosal congestion, erosion and hemorrhagic ulcerations (see Figure 10b; see macroscopic score 10h). The histopathological features included a transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-treated mice (see Figures 11b and 11b1; see histological score 11h). The treatment with mAb 763 (20 mg/kg) significantly reduced the extent and severity of the macroscopic (see Figure 10f and 10f1; see macroscopic score 9h) and histological signs of colon injury (see Figure 11f; see histological score 11h). Similarly, the treatment with mAb 741 (20 and 10 mg/kg) reduced in a dose dependent manner the macroscopic and histological signs of colon injury (see Figures 10c, 10c1, 10c2, 10d, 10d1, and 10d2; see macroscopic score 10h; Figs. 11c, 11c1, 11d, and 11d1; see histological score 11h respectively. On the contrary the treatment with mAb 741 at 5 mg/kg did not reduced the extent and severity of the macroscopic (see Figure 10e, 10e1; see macroscopic score 10h) and histological signs of colon injury (Figs. 11e and 11e1 respectively, see histological score 11h). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a significant reduction in body weight (compared with the sham groups of mice) (Figure 12).

mAb 763 (20 mg/kg) treatment resulted in a significant reduction of lost of body weight induced by DNBS-administration in mice (Figure 12). Similarly, the treatment with mAb 741 (20 and 10 mg/kg) reduced in a dose dependent manner the lost of body weight induced by DNBS-administration in mice (Figure 12). On the contrary the treatment with mAb 741 at 5 mg/kg did not reduced the lost of body weight induced by DNBS-administration in mice (Figure 12). The colitis caused by DNBS was also characterized by an increase in myeloperoxidase activity, an indicator of the neutrophils accumulation in the colon (Figure 13). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DNBS-mice contained a large number of neutrophils. mAb 763 (20 mg/kg) treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Figure 13). Similarly, the treatment with mAb 741 (20 and 10 mg/kg) reduced in a dose dependent manner the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 13). On the contrary the treatment with mAb 741 at 5



mg/kg did not reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 13).

Treatment with mAb 18.8 (20 mg/kg) did not reduced the macroscopic (Figures 10g and 10g1; see macroscopic score 10h) and histological signs of colon injury (see 5 Figure 11g, see histological score 11h), the degree of PMN infiltration (Figure 12) and the lost of body weight induced by DNBS-administration in mice (Figure 13).

3. Effects of mAb 763 and mAb 741 Treatment on the Mortality

DNBS-mice, which had received vehicle, developed severe hemorrhagic 10 diarrhoea, and 46% of these animals died within 4 days (Figure 14). In contrast, no DNBS-treated mice which had been treated with mAb 763 (20 mg/kg) died. Similarly, only 6.6% of the DNBS-treated mice, which had been treated with mAb 741 (20, 10 and 5 mg/kg) had hemorrhagic diarrhoea and died. Please note that DNBS-mice, which had received monoclonal antibody 18.8 (20 mg/kg) developed severe hemorrhagic diarrhoea, 15 and 40% of these animals died within 4 days.

C. Effects of Full-Dose Response Curve of mAb 763 (5-20 mg/kg) Versus DNBS-Induced Colitis

1. Experimental Groups: Animals were randomly divided into 7 20 groups (n=15 for each group):

Group	DNBS	Treatment	Dosing
1	No	Sham-vehicle	• 0.2 ml vehicle i.p. (saline solution)
2	Yes	Control-Vehicle	• 0.2 ml vehicle i.p. (saline solution)
3	Yes	mAb 763	• 10 mg/kg i.p.
4	Yes	mAb 763	• 3 mg/kg i.p.
5	Yes	mAb 763	• 1 mg/kg i.p.
6	Yes	mAb 763	• 0.3 mg/kg i.p.
7	Yes	mAb CBH2	• 10 mg/kg i.p.

## 2. Effects of mAb 763 Treatment on the Degree of Colitis

No histological alteration was observed in the colon tissue from sham-treated mice (see Figure 15a; see macroscopic score 15h; Figure 16a; see histological score 5 16h). Four days after intra-colonic administration of DNBS, the colon appeared flaccid and filled with liquid stool. The macroscopic inspection of cecum, colon and rectum showed presence of mucosal congestion, erosion and hemorrhagic ulcerations (Figures 15b and 15b1; see macroscopic score 15h). The histopathological features included a 10 transmural necrosis and edema and a diffuse leukocyte cellular infiltrate in the submucosa of colon section from DNBS-treated mice (Figures 16b and 16b1; see histological score 16h). The treatment with mAb 763 (10 mg/kg) significantly reduced the extent and severity of the macroscopic (Figures 15c and 15c1; see macroscopic score 15h) and histological signs of colon injury (Figures 16c, 16c1, 16c2; see histological 15 score 15h). On the contrary the treatment with mAb 763 at 3, 1, 0.3 mg/kg did not reduced the extent and severity of the macroscopic (Figures 15d, 15d1, 15e, 15e1, 15f, and 154f1, respectively; see macroscopic score 15h) and histological signs of colon injury (Figures 16d, 16e and 16f, respectively; see histological score 16h). Four days after colitis induced by DNBS treatment, all mice had diarrhea and a significant 20 reduction in body weight (compared with the sham groups of rats) (Figure 17). mAb 763 (10 mg/kg) treatment resulted in a significant reduction of lost of body weight induced by DNBS-administration in mice (Figure 17). On the contrary the treatment with mAb 763 at 3, 1, 0.5 mg/kg did not reduced the lost of body weight induced by DNBS-administration in mice (Figure 17). The colitis caused by DNBS was also characterized 25 by an increase in myeloperoxidase activity, an indicator of the neutrophils accumulation in the colon (Figure 18). This finding is consistent with the observation made with light microscopy that the colon of vehicle-treated DNBS-mice contained a large number of neutrophils. On the contrary, mAb 763 (10 mg/kg) treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon 30 (Figure 18). On the contrary the treatment with mAb 763 at 3, 1, 0.5 mg/kg did not reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 18).

Please note that the treatment with CBH2 (10 mg/kg) did not reduce the macroscopic (Figures 15g and 15g1; see macroscopic score 13h) and histological signs of colon injury (Figures 16g, see histological score 16h), the degree of PMN infiltration (Figure 17) and the loss of body weight induced by DNBS-administration in mice  
5 (Figure 18).

**D. Effects of mAb 763 on DNBS-Induced Mediator Production from Colon Extracts**

The effect of mAb 763 (10, 20 mg/kg), Infliximab (5 mg/kg) and non-relevant  
10 human mAb (20 mg/kg) on the production of DNBS-induced mediators was assessed (Figures 19 and 20).

**1. Measurement of Cytokines and Chemokines**

TNF- $\alpha$ , IL-10, IL-8, MIP-1, MIP-2 and IL-1 $\beta$  colonic levels were evaluated  
15 from colon tissues collected at 4 days after DNBS administration following preparation of colonic extracts. Quantitative determination of the levels of inflammatory mediators in the extracts were measured using commercially-available, ELISA kits.

**2. Malondialdehyde (MDA) Measurement**

Malondialdehyde levels in the colon tissue were determined as an indicator of  
20 lipid peroxidation. Colon tissue, collected at the specified time, were homogenised in 1.15% KCl solution. An aliquot (100  $\mu$ l) of the homogenate was added to a reaction mixture containing 200  $\mu$ l of 8.1% superoxide dismutaseium dodecyl sulphate, 1500  $\mu$ l of 20% acetic acid (pH 3.5), 1500  $\mu$ l of 0.8% thiobarbituric acid and 700  $\mu$ l distilled  
25 water. Samples were then boiled for 1 h at 95° C and centrifuged at 3,000 x g for 10 min. The absorbance of the supernatant was measured by spectrophotometry at 650 nm.

**3. Data Analysis**

All values in the figures and text are expressed as mean  $\pm$  standard error (S.E.M.)  
30 of the mean of  $n$  observations. For the *in vivo* studies  $n$  represents the number of animals studied. In the experiments involving histology, the figures shown are representative of at least three experiments performed on different experimental days. The results were

analysed by one-way analysis of variance followed by a Bonferroni *post-hoc* test for multiple comparisons. A *P*-value less than 0.05 was considered significant.

4. Effects of mAb 763 Treatment on Inflammatory Mediator  
Production in Colon Tissues

The colitis caused by DNBS was also characterized by an increase in all inflammatory mediators examined (Figures 19 and 20). MDA levels were elevated ~ 6 fold in DNBS-treated animals, which were significantly attenuated by mAb 763 and infliximab. However, the non-relevant human mAb failed to affect DNBS-stimulated MDA production. Additionally, for all other colonic inflammatory mediators, DNBS caused a significant increase in IL-1 $\beta$ , TNF, MIP-1, MIP-2 and IL-8 levels, whose production were significantly attenuated with INO-763 but not by a human, non-relevant mAb (Figures 19 and 20).

E. Effects of Chimeric mAb at 20 (mg/kg) on DNBS-Induced Colitis

1. Experimental Groups: Animals were randomly divided into 5 groups (n=15 for each group):

Group	DNBS	Treatment	Dosing
1	No	Sham-vehicle	• 0.2 ml vehicle i.p. (saline solution)
2	Yes	Control-Vehicle	• 0.2 ml vehicle i.p. (saline solution)
3	Yes	mAb 741	• 20 mg/kg i.p.
4	Yes	Chimeric mAb 741C	• 20 mg/kg i.p.
5	Yes	Control mAb	• 20 mg/kg i.p.

2. Effects of Chimeric mAb Treatment on the Degree of Colitis

No histological alteration was observed in the colon tissue from sham-treated mice (see Figure 210; see macroscopic score Figure 22; see histological score Figure 23). Treatment with chimeric mAb 741C (20 mg/kg) significantly reduced the extent and severity of the macroscopic (see Figure 22) and histological signs of colon injury (see Figure 21c). Similarly, the treatment with murine mAb 741 significantly reduced the macroscopic and histological signs of colon injury. On the contrary, treatment with

a non-specific control murine mAb (mAb 18.8) (20 mg/kg) did not reduced the extent and severity of the macroscopic (see Figure 22) and histological signs of colon injury (see Figure 21). Four days after colitis induced by DNBS treatment, all mice except for those treated with the chimeric mAb, had diarrhea and a reduction in body weight  
5 (compared with the sham groups of mice) (Figure 24). Chimeric mAb 741C (20 mg/kg) treatment resulted in a significant reduction of loss of body weight induced by DNBS-administration in mice (Figure 24).

The colitis caused by DNBS was also characterized by an increase in myeloperoxidase activity, an indicator of the neutrophils accumulation in the colon  
10 (Figure 25). On the contrary, chimeric mAb 741C (20 mg/kg) treatment significantly reduced the degree of PMN infiltration (determined as increase in MPO activity) in inflamed colon (Figure 25). Similarly, the treatment with mAb 741 reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 25). On the contrary the treatment with a non-specific control murine mAb  
15 (mAb 18.8) (20 mg/kg) did not reduced the neutrophils infiltration in the colon tissues induced by DNBS-administration in mice (Figure 25).

### 3. Effect of Chimeric mAb Treatment on Mortality

DNBS-mice, which had received vehicle, developed severe hemorrhagic  
20 diarrhoea, and greater than 50% of these animals died within 4 days (Figure 26). In contrast, no DNBS-treated mice which had been treated with chimeric mAb 741C (20 mg/kg) died.

### F. Effects of Chimeric mAb 741C on DNBS-Induced Mediator Production from Colon Extracts

  
25

The effect of chimeric mAb 741C (20 mg/kg), murine mAb 741, and an isotype mouse control mAb, 18.8 (20 mg/kg) on the production of DNBS-induced mediators was assessed (Figures 27-29).

#### 30 1. Measurement of Cytokines and Chemokines

TNF- $\alpha$ , IL-1 $\beta$  and MIP-1 colonic levels were evaluated from colon tissues collected at 4 days after DNBS administration following preparation of colonic extracts.

Quantitative determination of the levels of inflammatory mediators in the extracts were measured using commercially-available, ELISA kits.

## 2. Data Analysis

5 All values in the figures and text are expressed as mean  $\pm$  standard error (S.E.M.) of the mean of  $n$  observations. For the *in vivo* studies  $n$  represents the number of animals studied. In the experiments involving histology, the figures shown are representative of at least three experiments performed on different experimental days. The results were analysed by one-way analysis of variance followed by a Bonferroni *post-hoc* test for  
10 multiple comparisons. A  $P$ -value less than 0.05 was considered significant.

## 3. Effects of Chimeric mAb 741C Treatment on Inflammatory Mediator Production in Colon Tissues

The colitis caused by DNBS was characterized by an increase in all three  
15 inflammatory mediators examined (Figures 27, 28 and 29). TNF- $\alpha$ , IL-1 $\beta$  and MIP-1 levels were significantly elevated in DNBS-treated animals and were significantly attenuated by both mAb 741 and chimeric mAb 741C. However, the isotype mouse control mAb, 18.8, failed to affect the production of DNBS-stimulated inflammatory  
20 mediators.

### **Example 7: Anti-Flagellin Monoclonal Antibodies Cross-React with a Broad Spectrum of Gram-Negative Bacteria**

Dose-response binding relative to mAb 763 and mAb 741 was assessed towards a panel of different gram-negative bacteria (*i.e.*, *Proteus Vulgaris*, *non-pathogenic E. Coli*, *Citrobacter*,  
25 *Serratia marcenscens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis*, *Enteropathogenic E. Coli*) in a live bacterial ELISA assay. Both mAb 763 and mAb 741 bound cross-reactively to a variety of different bacteria with highest binding observed towards *Proteus mirabilis*, *Salmonella typhimurium*, *Serratia marcenscens*, and *Enteropathogenic E. Coli* (Figures 30 and 31).

30

**Example 8: Binding Affinities**

The binding affinity of mAb 763 was determined towards Salmonella and Pseudomonas flagellin using the BIACore system. The affinity of mAb 763 towards Salmonella flagellin was  $\sim 10^{-10}$  M and the affinity of mAb 763 towards Pseudomonas flagellin was  $\sim 10^{-6}$  M.

5

**Example 9: *In Vivo* Treatment of Colitis**

The effects of the anti-flagellin antibodies of the present invention (*e.g.*, mAb 741 and mAb 763) can be tested in an IL-10 k/o mouse model of colitis. For example, a 10 week model can be employed wherein the antibodies are administered at either week 6 (*i.e.*, a prophylactic regimen) or week 9 (*i.e.*, a treatment paradigm wherein colitis has been established). At week 16, animals can be sacrificed and markers of colonic injury assessed.

10

**Example 10: *In Vitro* Nitric Oxide Production Assay**

An *in vitro* nitric oxide (NO) production assay was used to assess the functional characteristics of murine mAb 741 and human mAb 763. DLD-1 cells (ATCC) were grown at 37 °C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) and were supplemented with 10% FBS, 4 mM glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, 1 mM sodium pyruvate, and antibiotics. Cells, between passages 5 and 15, were seeded at a density of 50,000 cells/cm<sup>2</sup> in 96-well plates and allowed to grow 72–96 hours to confluence before use. Growth medium was changed the day before use. Cells were washed once with DMEM without FBS (but containing antibiotics) before the addition of flagellin proteins. Flagellin to be tested were added to 100  $\mu$ l growth medium containing 0.5% FBS and 100U/ml IFN- $\gamma$  in each well. To neutralize NO production activity, the recombinant flagellin was incubated with antibodies for 1 hour at room temperature and then added to DLD-1 cells in 96 well plate. After 20 hours of incubation at 37°C, the culture medium was removed and tested for NO<sub>2</sub>-/NO<sub>3</sub>- concentration by Greiss assay (Salzman *et al. Am J Physiol.* 268, 361-73 (1995)).

15

20

25

As depicted in Figure 32, both mAb 741 and mAb 763 inhibited flagellin activity in the NO production assay.

30

**Example 11: Additional *In Vitro* Functional Assays**

A number of *in-vitro* functional assays can be performed to assess the functional characteristics of the anti-flagellin antibodies of the present invention (e.g., murine mAb 741, chimeric mAb 741 and 763). Such assays can include, but are not limited to, assays  
 5 designed to assess the effect of anti-flagellin antibodies on (1) bacterial invasion into susceptible epithelial cells, (2) inhibition of flagellin-stimulated NO or IL-8 production from epithelial cells, (3) bacterial opsonophagocytosis, (3) macrophage ingestion of bacteria, (4) bacterial “killing” and (5) superoxide production.

10 **Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Any combination of the embodiments disclosed in the dependent claims are  
 15 contemplated to be within the scope of the invention.

**Incorporation by Reference**

All publications, patents, and pending patent applications referred to herein are hereby incorporated by reference in their entirety.

20

**SUMMARY OF SEQUENCE LISTING**

<b>SEQ ID NO:</b>	<b>SEQUENCE</b>	<b>SEQ ID NO:</b>	<b>SEQUENCE</b>
1	VH a.a. 741	19	VH n.t. 741
2	VH a.a. 763	20	VH n.t. 763
3	VK a.a. 741	21	VK n.t. 741
4	VK a.a. 763	22	VK n.t. 763
5	VH CDR1 a.a. 741	23	VH CDR1 n.t. 741
6	VH CDR1 a.a. 763	24	VH CDR1 n.t. 763
7	VH CDR2 a.a. 741	25	VH CDR2 n.t. 741
8	VH CDR2 a.a. 763	26	VH CDR2 n.t. 763
9	VH CDR3 a.a. 741	27	VH CDR3 n.t. 741
10	VH CDR3 a.a. 763	28	VH CDR3 n.t. 763
11	VK CDR1 a.a. 741	29	VK CDR1 n.t. 741
12	VK CDR1 a.a. 763	30	VK CDR1 n.t. 763
13	VK CDR2 a.a. 741	31	VK CDR2 n.t. 741
14	VK CDR2 a.a. 763	32	VK CDR2 n.t. 763
15	VK CDR3 a.a. 741	33	VK CDR3 n.t. 741
16	VK CDR3 a.a. 763	34	VK CDR3 n.t. 763



17	Sense Primer Designated 1S	35	Proteus mirabilis (GI:1169696)
18	Antisense Primer Designated 468A	36	Pseudomonas aeruginosa (GI:3386643)
		37	Escherichia coli (GI:1655807)
		38	Serratia marcescens (GI:514988)
		39	Salmonella muenchen (GI:1333832)
		40	Salmonella typhimurium (GI:153979)
		41	Amino acids 1–156 of flagellin gene of Salmonella muenchen (GI:47233)

## CLAIMS

1. A method of treating inflammatory bowel disease (IBD) in a subject, comprising administering to the subject a therapeutically effective amount of an isolated monoclonal antibody which binds to and neutralizes flagellin.  
5
2. A method of treating a gram negative bacterial infection in a subject, comprising administering to the subject a therapeutically effective amount of an isolated monoclonal antibody which binds to and neutralizes flagellin.  
10
3. A method of neutralizing enterobacteria comprising contacting the enterobacteria with an antibody that binds to and inhibits the activity of flagellin.
4. The method of any of claims 1-3, wherein the antibody comprises  
15 (a) a heavy chain variable region comprising an amino acid sequence set forth in SEQ ID NO:1 or an amino acid sequence at least 80% identical thereto, or  
(b) a light chain variable region comprising an amino acid sequence set forth in SEQ ID NO:3 or an amino acid sequence at least 80% identical thereto.
- 20 5. The method of any of claims 1-3, wherein the antibody comprises a heavy chain variable region comprising an amino acid sequence set forth in SEQ ID NO:1 and a light chain variable region comprising an amino acid sequence set forth in SEQ ID NO:3.
6. The method of any of claims 1-3, wherein the antibody cross competes for  
25 binding to flagellin with an antibody comprising heavy and light chain variable regions comprising the amino acid sequences set forth in SEQ ID NOs: 1 and 3, respectively.
7. The method of any of claims 1-3, wherein the antibody binds to an epitope on flagellin recognized by an antibody comprising heavy and light chain variable regions  
30 comprising the amino acid sequences set forth in SEQ ID NOs: 1 and 3, respectively.
8. The method of claim 1, wherein the IBD is Crohn's Disease or colitis.

9. The method of claim 1, wherein the IBD is caused by an enterobacteria.
10. The method of claim 2, wherein the gram negative bacterial infection is an enterobacterial infection.
- 5
11. The method of claim 10, wherein the enterobacterial infection is selected from the group consisting of Anthrax, Bacterial Meningitis, Botulism, Brucellosis, Cat Scratch Disease, Cholera, Diphtheria, Epidemic Typhus, Impetigo, Legionellosis, Leprosy, Leptospirosis, Listeriosis, Lyme Disease, Melioidosis, MRSA infection, 10 Nocardiosis, Pertussis, Plague, Pneumococcal pneumonia, Psittacosis, Q fever, Rocky Mountain Spotted Fever (RMSF), Salmonellosis, Scarlet Fever, Shigellosis, Syphilis, Tetanus, Trachoma, Tuberculosis, Tularemia, Typhoid Fever, sepsis, septic shock and Urinary Tract Infections.
- 15
12. The method of claim 3 or 9, wherein the enterobacteria is selected from the group consisting of *Alishewanella*, *Alterococcus*, *Aquamonas*, *Aranicola*, *Arsenophonus*, *Azotivirga*, *Blochmannia*, *Brenneria*, *Buchnera*, *Budvicia*, *Buttiauxella*, *Cedecea*, *Citrobacter*, *Dickeya*, *Edwardsiella*, *Enterobacter*, *Erwinia*, *Escherichia*, *Ewingella*, *Grimontella*, *Hafnia*, *Klebsiella*, *Kluyvera*, *Leclercia*, *Leminorella*, *Moellerella*, 20 *Morganella*, *Obesumbacterium*, *Pantoea*, *Pectobacterium*, *Candidatus Phlomobacter*, *Photorhabdus*, *Plesiomonas*, *Pragia Proteus*, *Providencia*, *Rahnella*, *Raoultella*, *Salmonella*, *Samsonia*, *Serratia*, *Shigella*, *Sodalis*, *Tatumella*, *Trabulsiella*, *Wigglesworthia*, *Xenorhabdus*, *Yersinia* and *Yokenella*.
- 25
13. The method of any of claims 1-5, wherein the antibody cross reacts with a gram-negative bacteria selected from the group consisting of *Proteus Vulgaris*, non-pathogenic *E. Coli*, *Citrobacter*, *Serratia marcenscens*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis*, and *Enteropathogenic E. Coli*.
- 30
14. The method of any of claims 1-5, wherein the antibody specifically binds to an epitope located between amino acids 1-55 of flagellin from *Salmonella* (SEQ ID NO:39) or *Pseudomonas* (SEQ ID NO:36).

15. The method of claim 14, wherein the antibody specifically binds to an epitope located between amino acids 1-40 of flagellin from *Salmonella* (SEQ ID NO:39) or *Pseudomonas* (SEQ ID NO:36).
- 5 16. The method of any of the preceding claims, wherein the subject is human.
17. The method of any of the preceding claims, wherein the antibody is administered intravenously, intramuscularly, or subcutaneously to the subject.
- 10 18. The method of any of the preceding claims, wherein the antibody is administered in combination with a second therapeutic agent.
19. The method of claim 18, wherein the second therapeutic agent is a second antibody.
- 15 20. The method of claim 18, wherein the second therapeutic agent is an antibiotic.
21. The method of any of the preceding claims, wherein the antibody is selected from the group consisting of a human antibody, a humanized antibody, a bispecific antibody and a chimeric antibody.
- 20 22. The method of any of the preceding claims, wherein the antibody is selected from the group consisting of a Fab, Fab'2, ScFv, SMIP, affibody, avimer, nanobody, and a domain antibody.
- 25 23. The method of any of the preceding claims, wherein the antibody is selected from the group consisting of an IgG1, an IgG2, an IgG3, an IgG4, an IgM, an IgA1, an IgA2, an IgAsec, an IgD, and an IgE antibody.
- 30 24. A chimeric monoclonal antibody that binds to flagellin comprising a heavy chain variable region comprising an amino acid sequence set forth in SEQ ID NO:1.

25. A chimeric monoclonal antibody that binds to flagellin comprising a light chain variable region comprising an amino acid sequence set forth in SEQ ID NO:3.
26. A chimeric antibody that binds to flagellin comprising a heavy and light chain variable region comprising the amino acid sequences set forth in SEQ ID NOs:1 and 3.
27. A chimeric monoclonal antibody that binds to flagellin and comprises heavy and light chain variable region CDR1, CDR2 and CDR3, wherein the
- heavy chain variable region CDR1 comprises SEQ ID NO:5;
  - a heavy chain variable region CDR2 comprises SEQ ID NO:7;
  - a heavy chain variable region CDR3 comprises SEQ ID NO:9;
  - a light chain variable region CDR1 comprises SEQ ID NO:11;
  - a light chain variable region CDR2 comprises SEQ ID NO: 13; and
  - a light chain variable region CDR3 comprises SEQ ID NO: 15.

15

*Fig. 1*

**741 Heavy Chain Sequences**

10 20 30 40 50 60 70 80 90 100  
 CAGGTTCAACTGCAGCAGTCTGGGGCTGAGCTGGTGGCCCTGGGGCTTCAGTGACGTGCTCCCTGCAAGGCTTCGGGGCCACACATTACT**GACTATGAGA**  
 Q V Q L Q Q S G A E L V G P G A S V T L S C K A S G H T F T D Y E  
 10 20 30  
 CDR 1

110 120 130 140 150 160 170 180 190 200  
**TGCACTGGGTGAAGCAGACACCCTGTGCATGGCCCTGGAATGGATTGGAGGTATTGATCCCTGAAACTGGGTGGTACTGGCCTACAAATCAGAACTCAAGGGCGA**  
 M H W V K Q T P V H G L E W I G G I D P E T G G T A Y N Q K L K G E  
 40 50 52 A 60  
 CDR 2

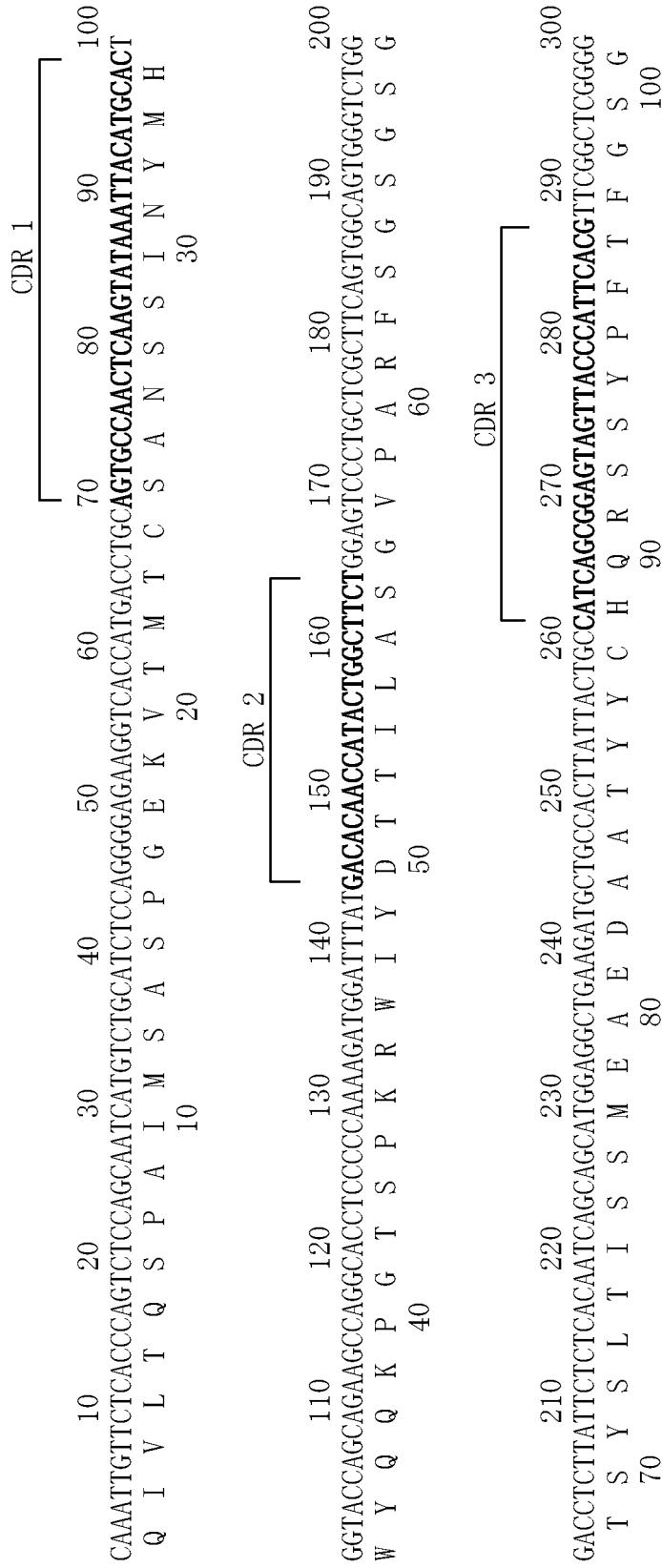
210 220 230 240 250 260 270 280 290 300  
 GGCCACACTGACTGCAGACAAATCCTCCACACAGCCCTACATGGAGCTCCGCGAGCCTGACATCTGAGGACTCTGGCGTCTATTACTGTAGCATTT**ACCTT**  
 A T L T A D K S S N T A Y M E L R S L T S E D S A V Y Y C T I Y L  
 70 80 82 A B C 90 96  
 CDR 3

310 320 330 340  
**GACTACTGGGGCCGAGGCACCCTCTCACAGTCTCCTCA**  
 D Y W G R G T T L T V S S  
 102 110

CDR definitions and protein sequence numbering according to Kabat. CDR nucleotide and protein sequences are highlighted in bold typeface.

*Fig. 2*

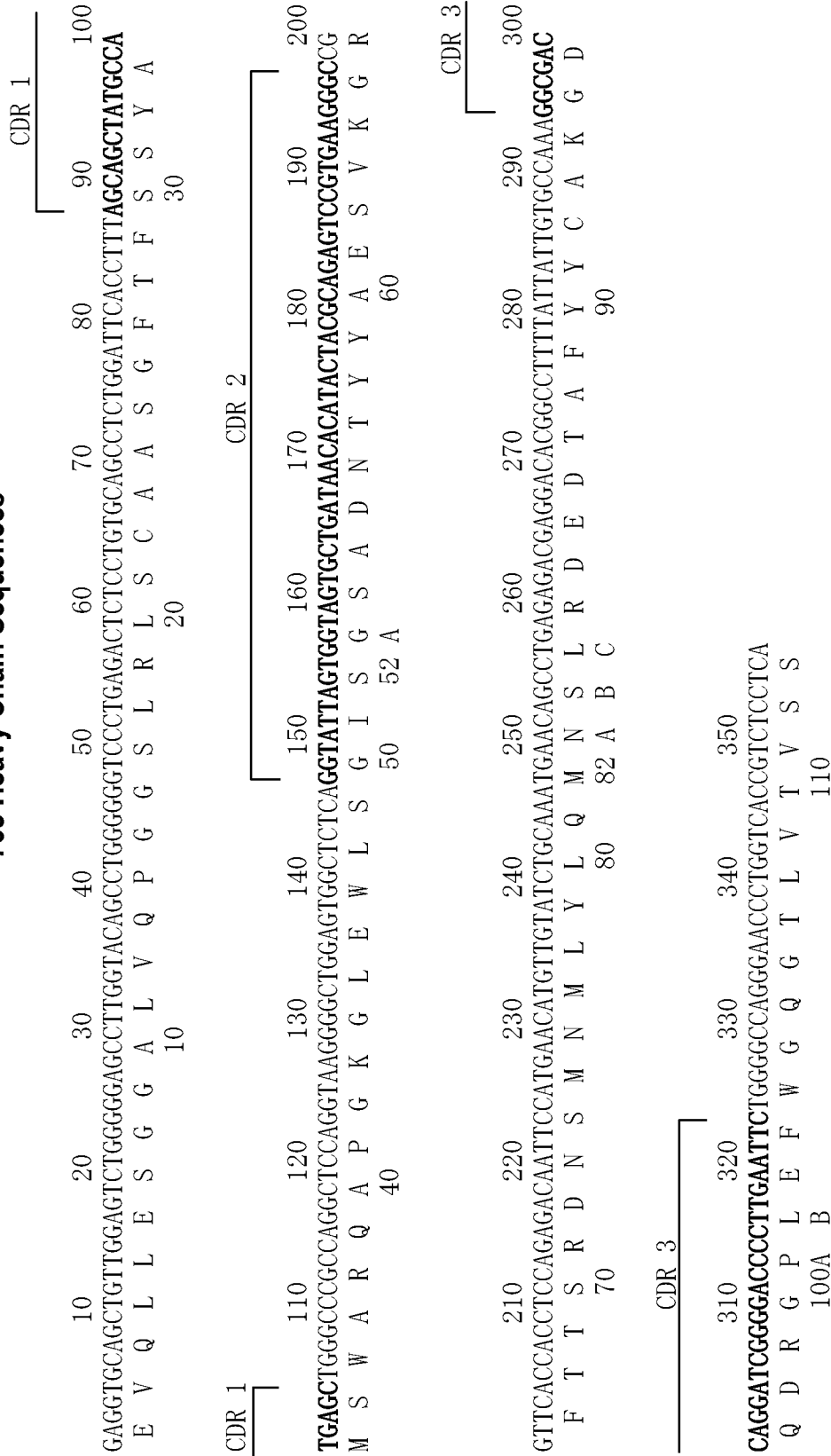
**741 Light Chain Sequences**



CDR definitions and protein sequence numbering according to Kabat. CDR nucleotide and protein sequences are highlighted in **bold** typeface.

**Fig. 3**

**763 Heavy Chain Sequences**



CDR definitions and protein sequence numbering according to Kabat. CDR nucleotide and protein sequences are highlighted **bold** typeface



**Fig. 4**

**763 Light Chain Sequences**

10 20 30 40 50 60 70 80 90 100  
 TCCTATGAGCTGACACAGCCACCCTCGGTGTCAGTGTCCCCAGGACAGACAGCCGAGCAATCACCTGCTCTGGAGATGCATTGCCAAAGCAGTATGCTTATT  
 S Y E L T Q P P S V S V S P G Q T A R I T C S G D A L P K Q Y A Y  
 11 20 30  
 CDR 1

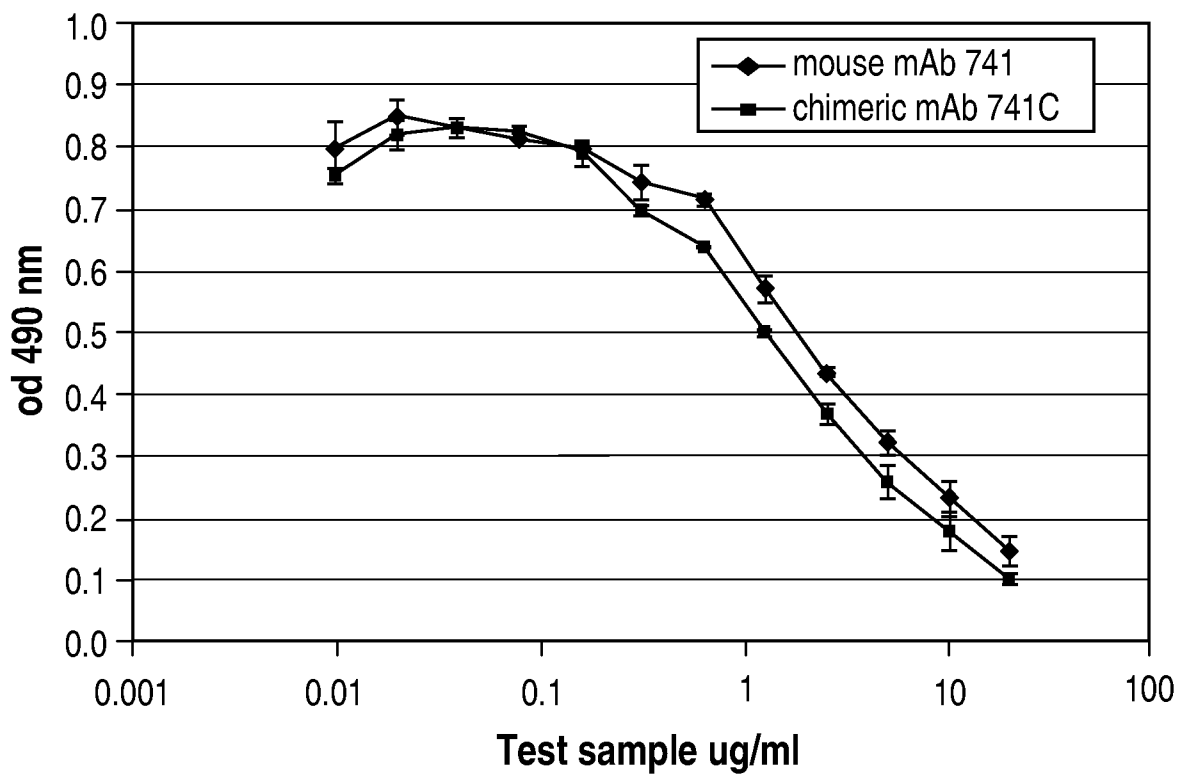
110 120 130 140 150 160 170 180 190 200  
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 W Y Q Q K P G Q A P V L L I Y **Q D T K R P S G I P E R F S S S S G**  
 40 50 60  
 CDR 2

210 220 230 240 250 260 270 280 290 300  
 GACAAACAGTCGCGTTGACCATCAGTGGAGTCCAGGACAGGACGAGGCTGACTATTACTGTCAATCAGCAGACAGCAATGATAGTATTATGCTTCGGA  
 T T V A L T I S G V Q A E D E A D Y Y C **Q S A D S N D S Y Y V F F G**  
 70 80 90 95 A B  
 CDR 3

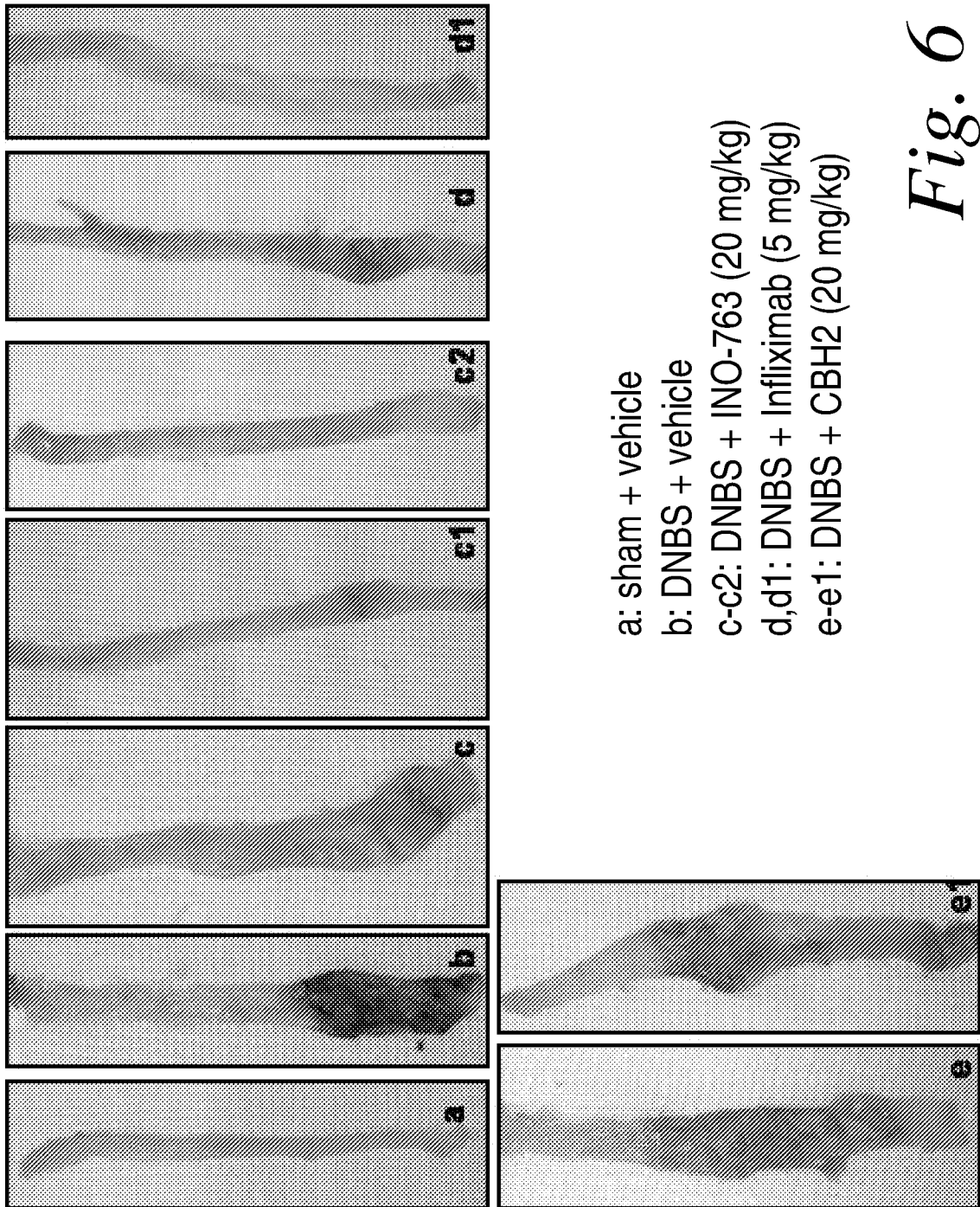
310  
 ACTGGGACCAAGGTCACCGTCCTG  
 T G T K V T V L  
 100 106A

CDR definitions and protein sequence numbering according to Kabat. CDR nucleotide and protein sequences are highlighted in **bold** typeface

### Competition Assay

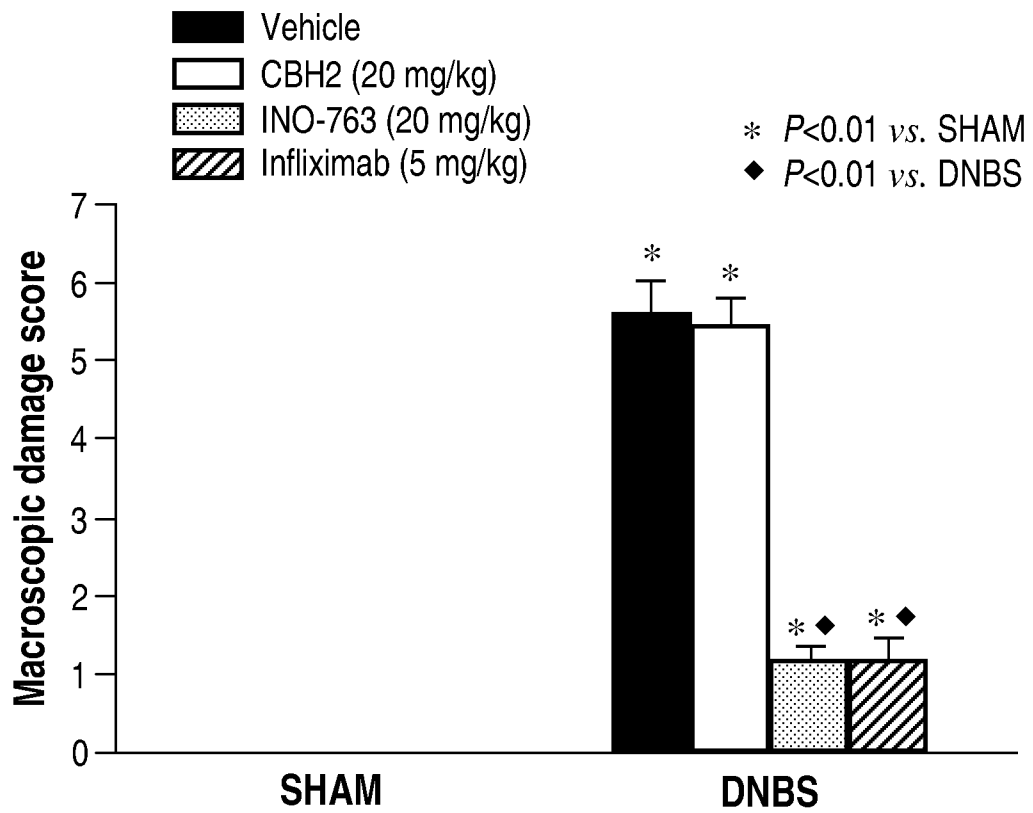


*Fig. 5*



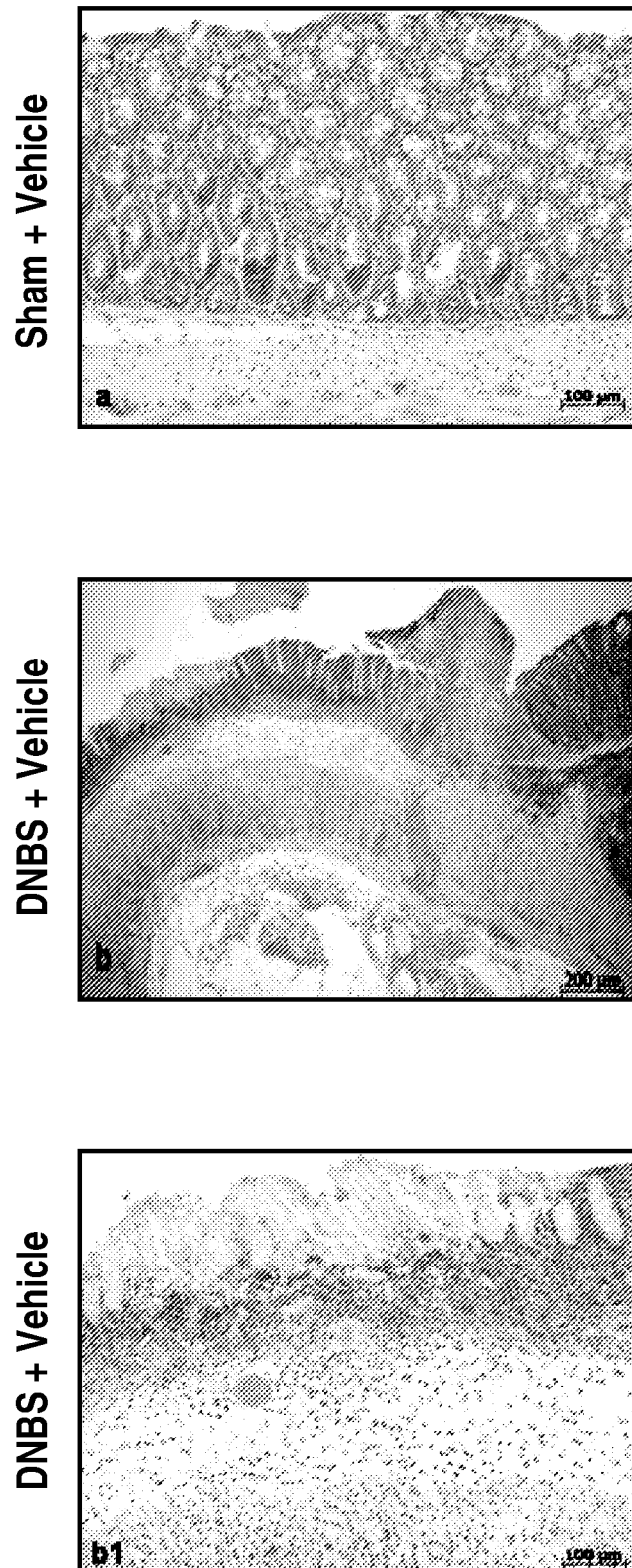
*Fig. 6*

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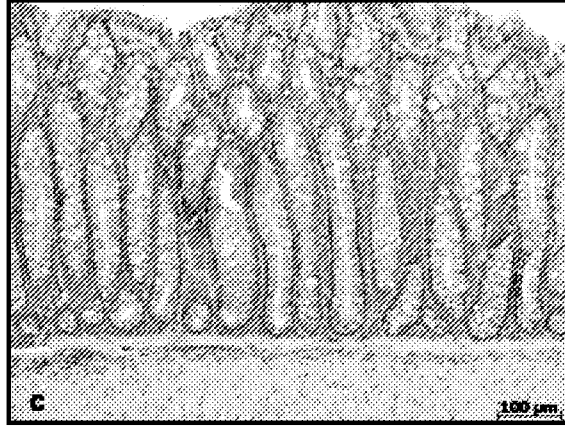
*Fig. 6F*

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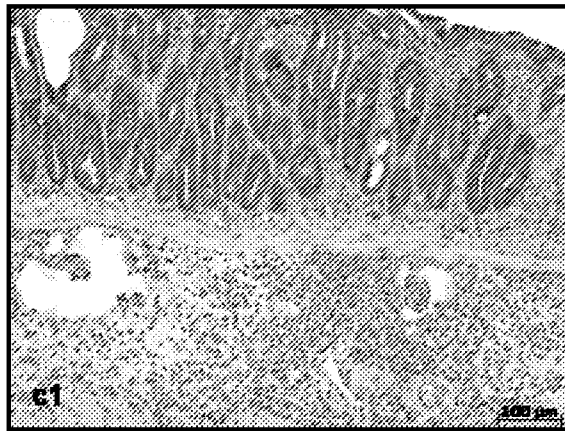


*Fig. 7*

DNBS + INO-763 (20 mg/kg)



DNBS + INO-763 (20 mg/kg)



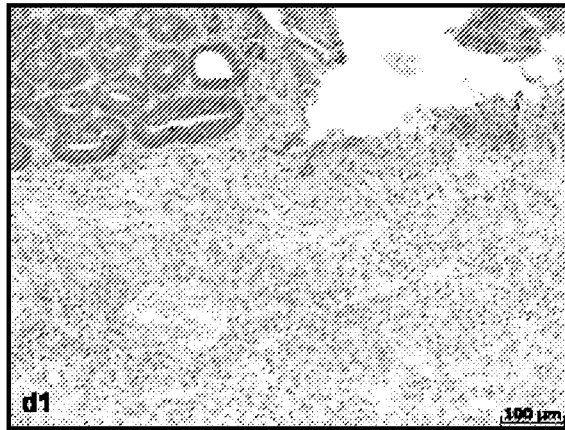
*Fig. 7*

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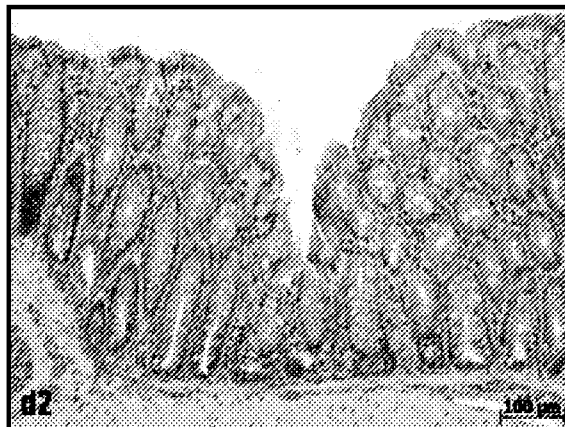
DNBS + Infliximab  
(5 mg/kg)



DNBS + Infliximab  
(5 mg/kg)

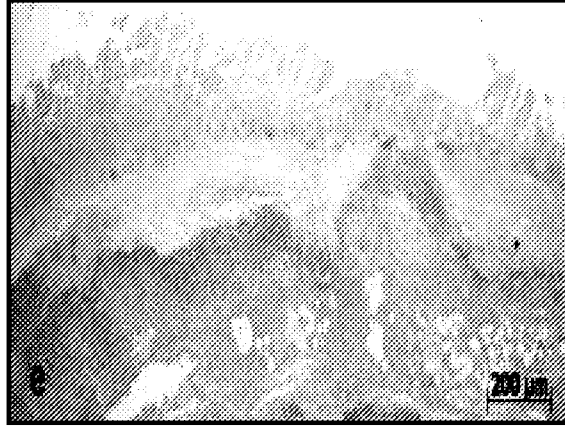


DNBS + Infliximab  
(5 mg/kg)

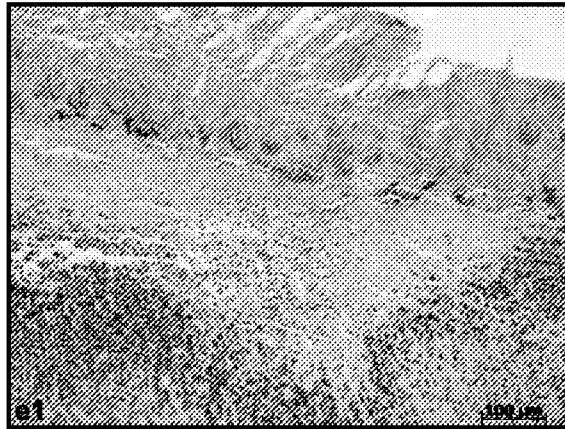


*Fig. 7*

DNBS + CBH2  
(20 mg/kg)



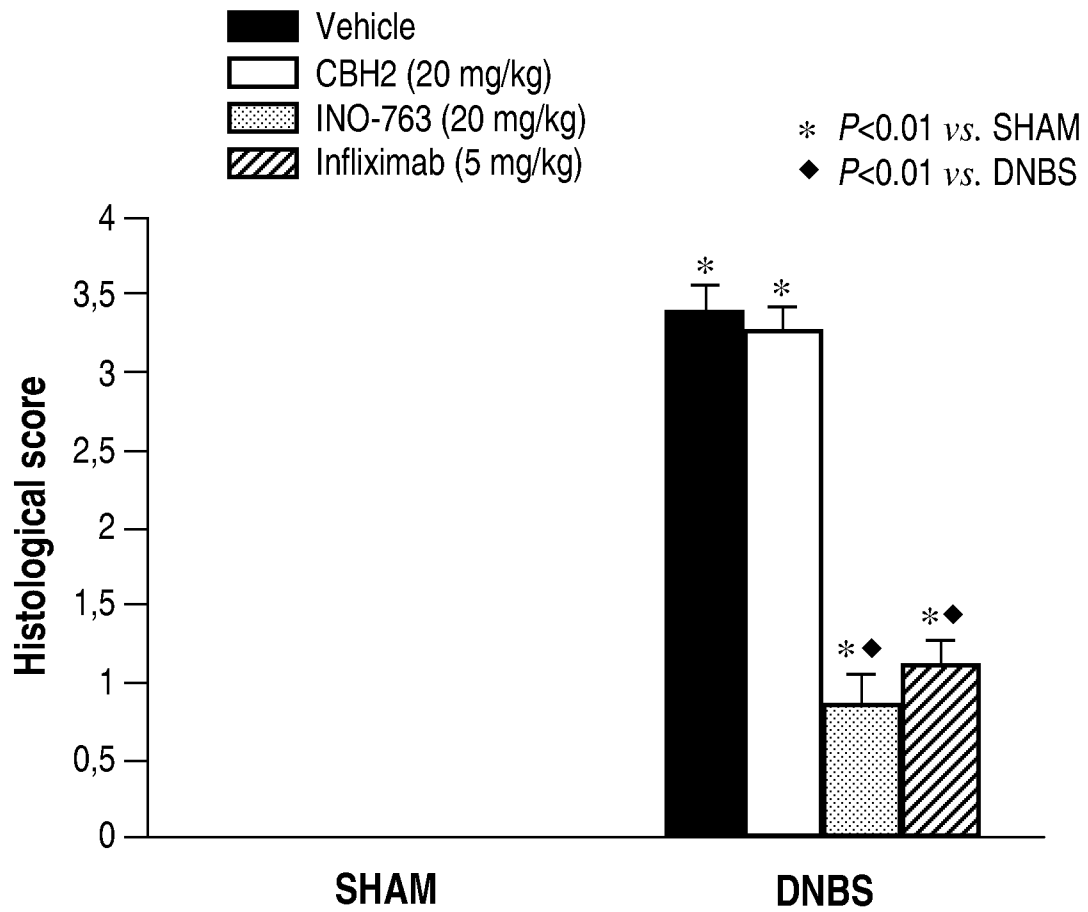
DNBS + CBH2  
(20 mg/kg)



*Fig. 7*

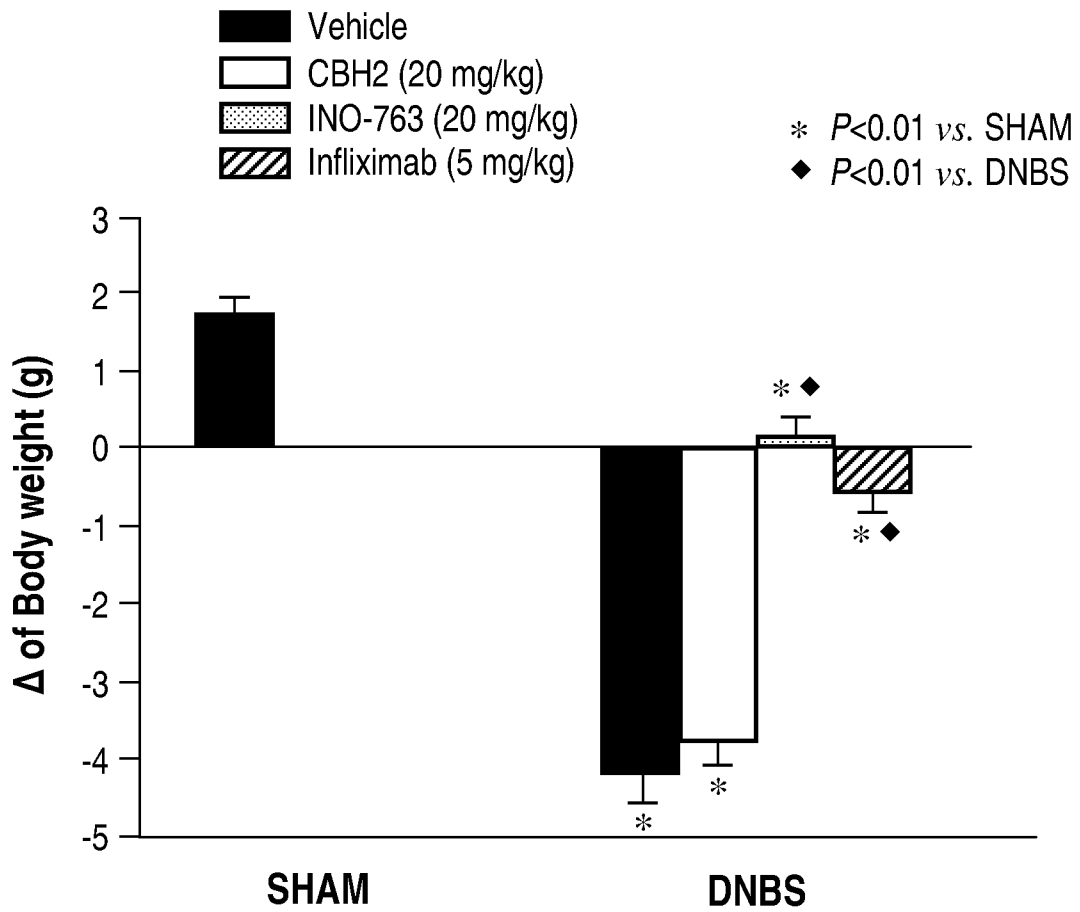


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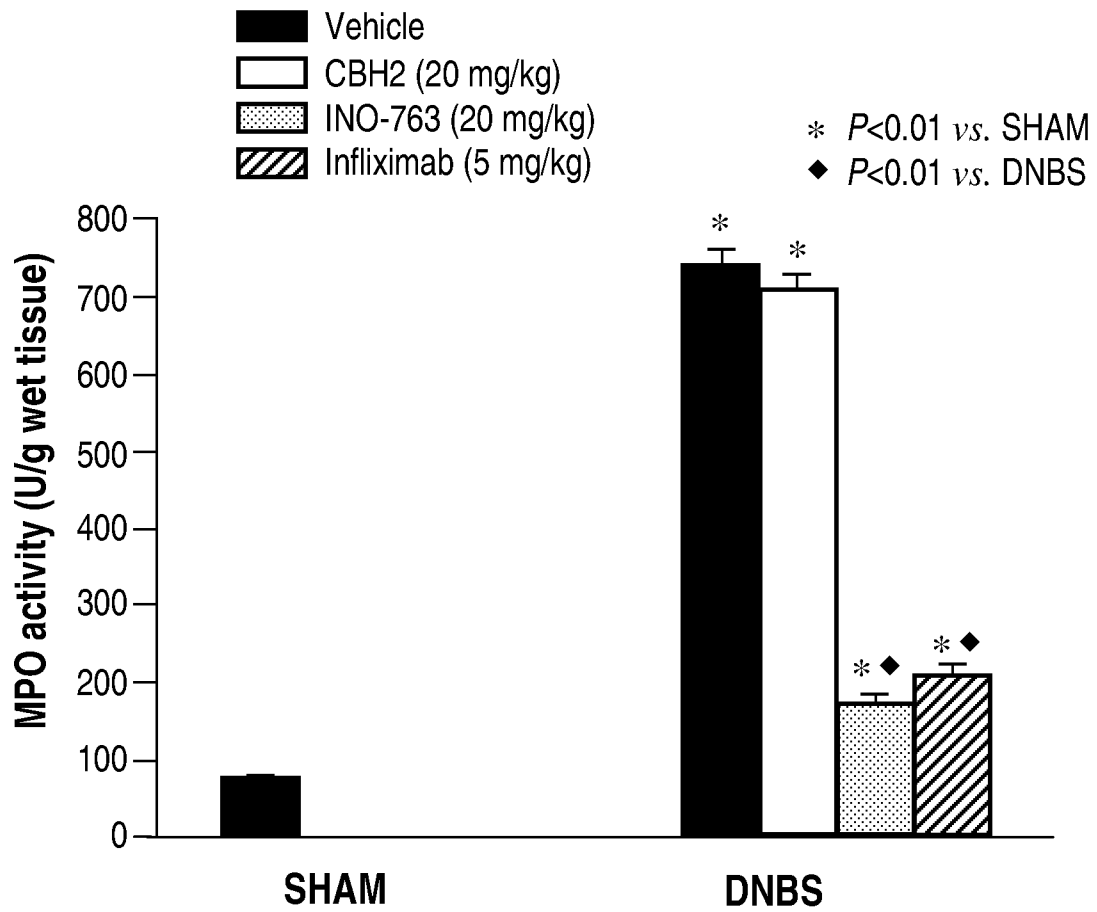
*Fig. 7F*

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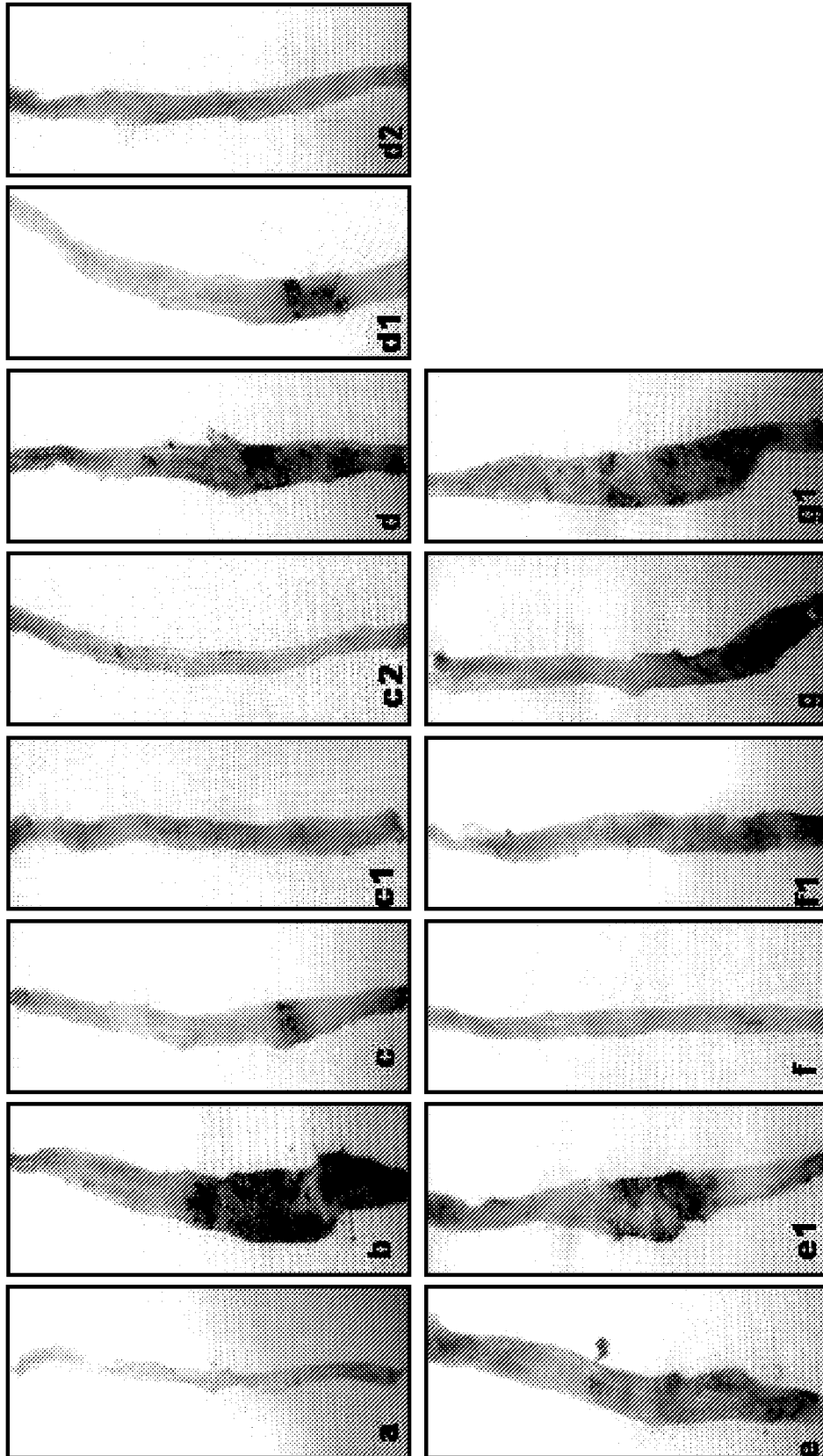


*Fig. 8*

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*Fig. 9*

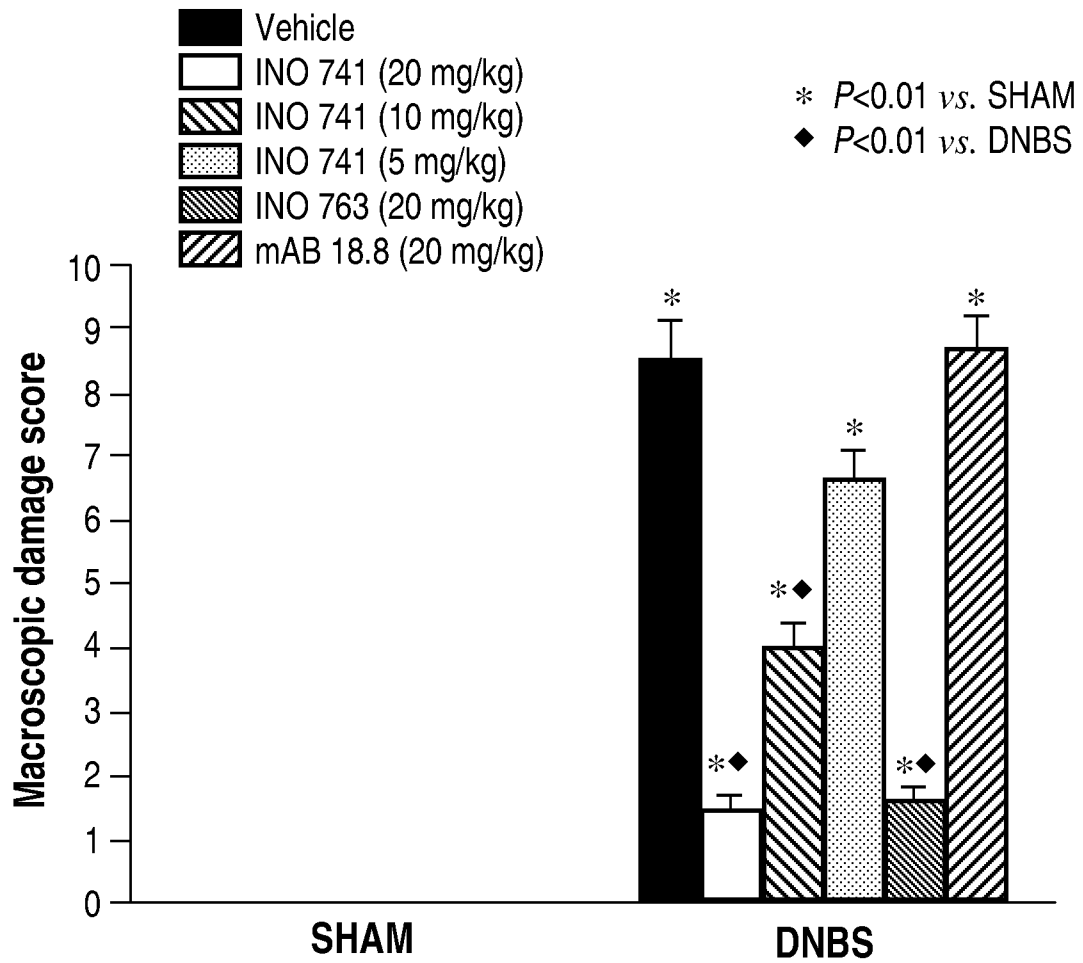


**Fig. 10**

**a:** Sham + Vehicle  
**c-c2:** DNBS + INO-741 (20 mg/kg)  
**e-e1:** DNBS + INO-741 (5 mg/kg)  
**g-g1:** DNBS + mAb 18.8 (20 mg/kg)

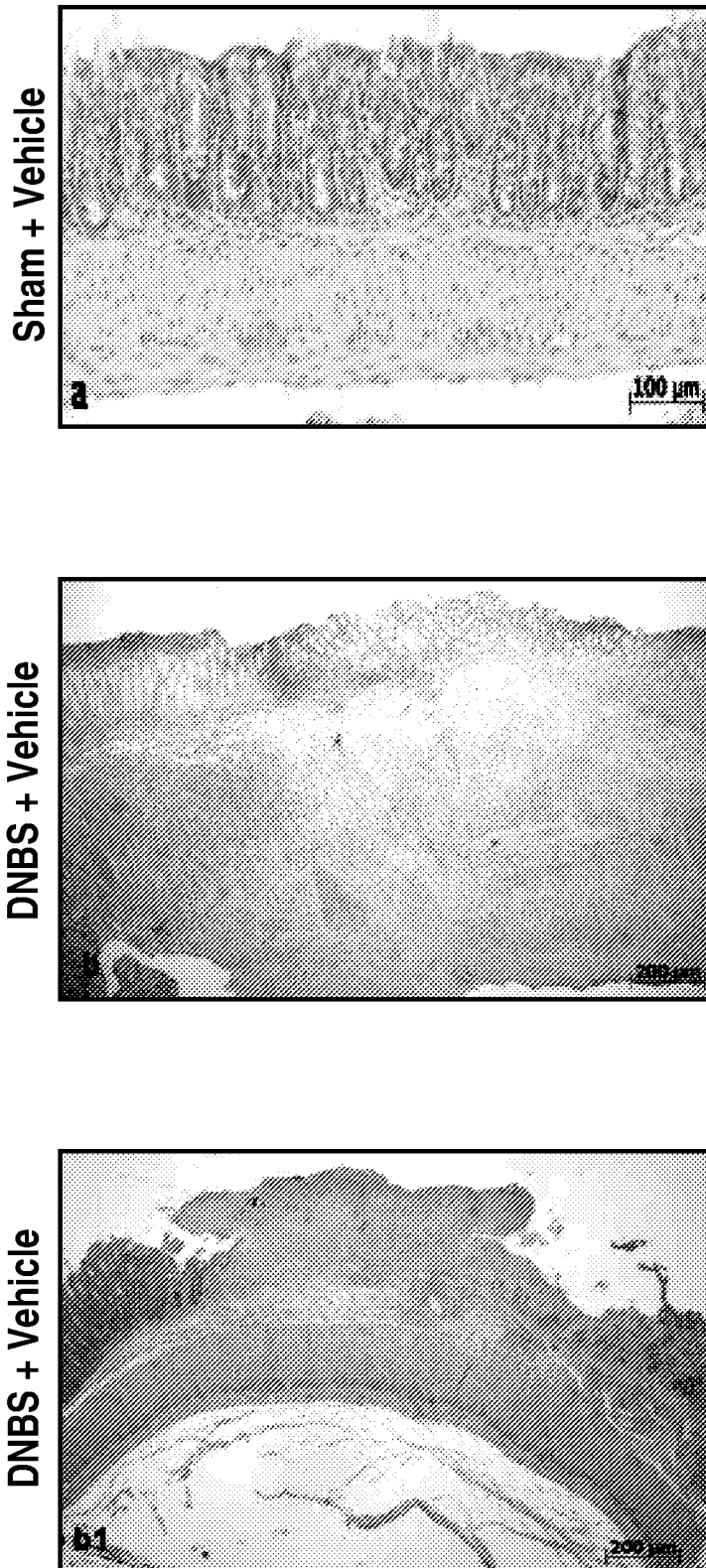
**b:** DNBS + Vehicle  
**d-d2:** DNBS + INO-741 (10 mg/kg)  
**f-f1:** DNBS + INO-763 (20 mg/kg)

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*Fig. 10h*

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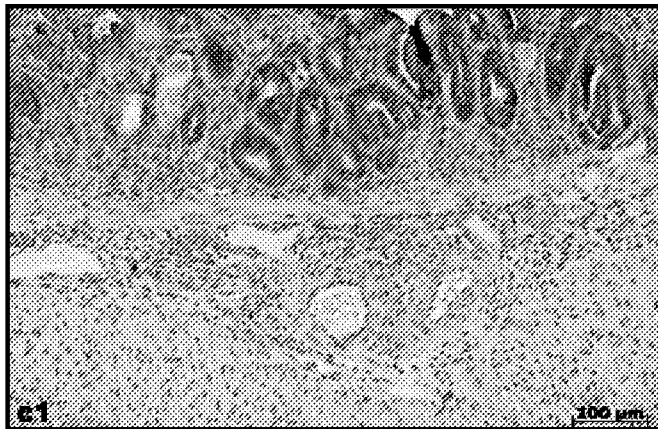
*Fig. 11*

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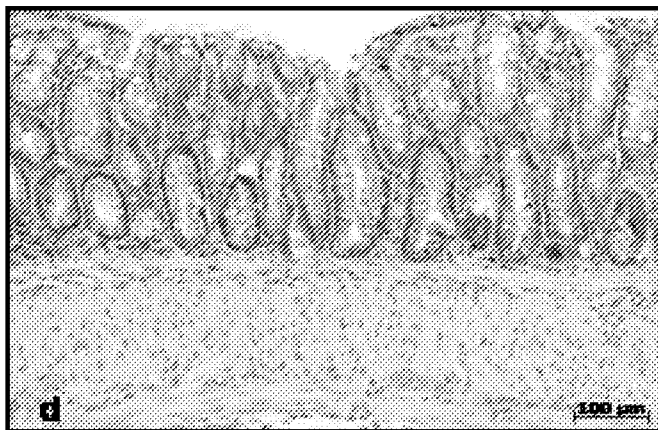
DNBS + INO 741  
(20 mg/kg)



DNBS + INO 741  
(20 mg/kg)



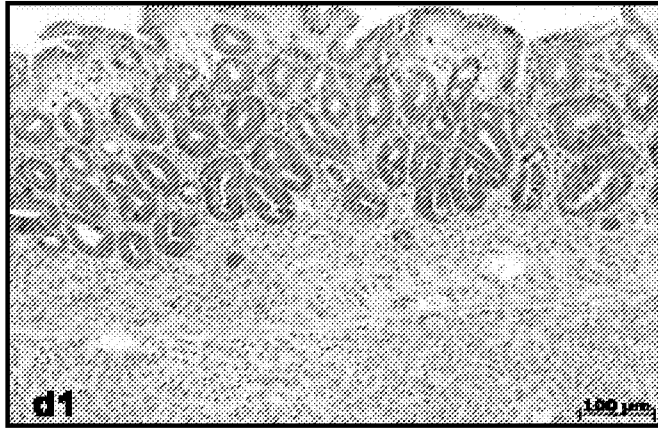
DNBS + INO 741  
(10 mg/kg)



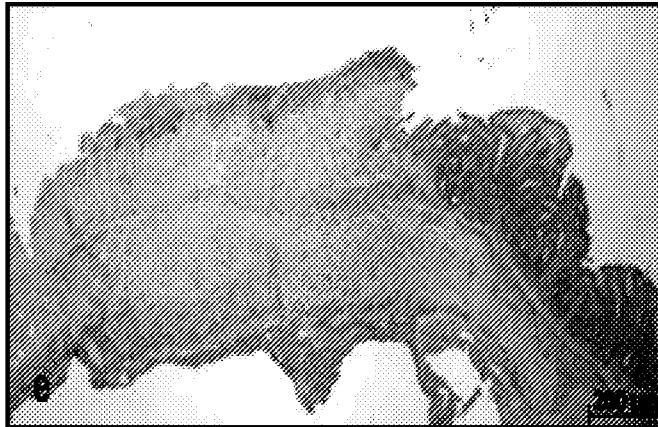
*Fig. 11*

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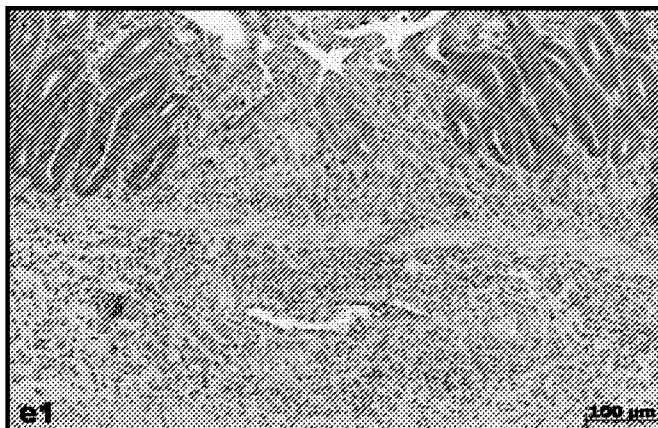
DNBS + INO 741  
(10 mg/kg)



DNBS + INO 741  
(5 mg/kg)



DNBS + INO 741  
(5 mg/kg)

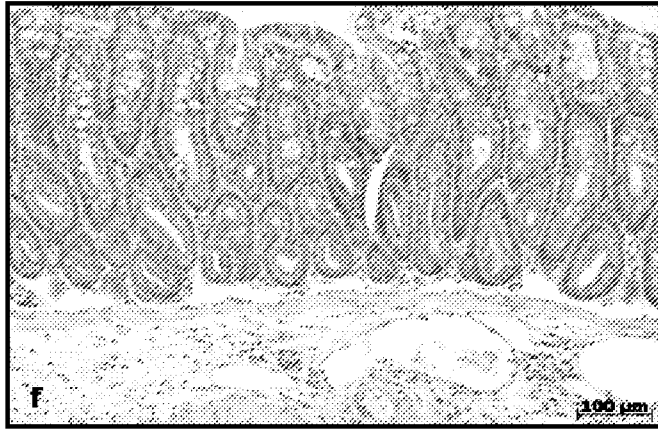


*Fig. 11*

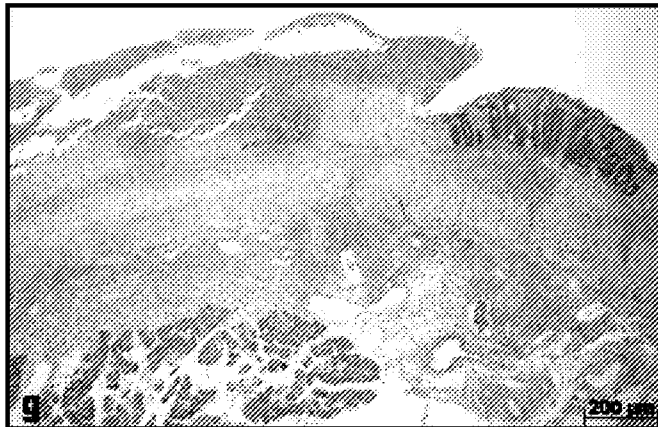


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DNBS + INO 763  
(20 mg/kg)

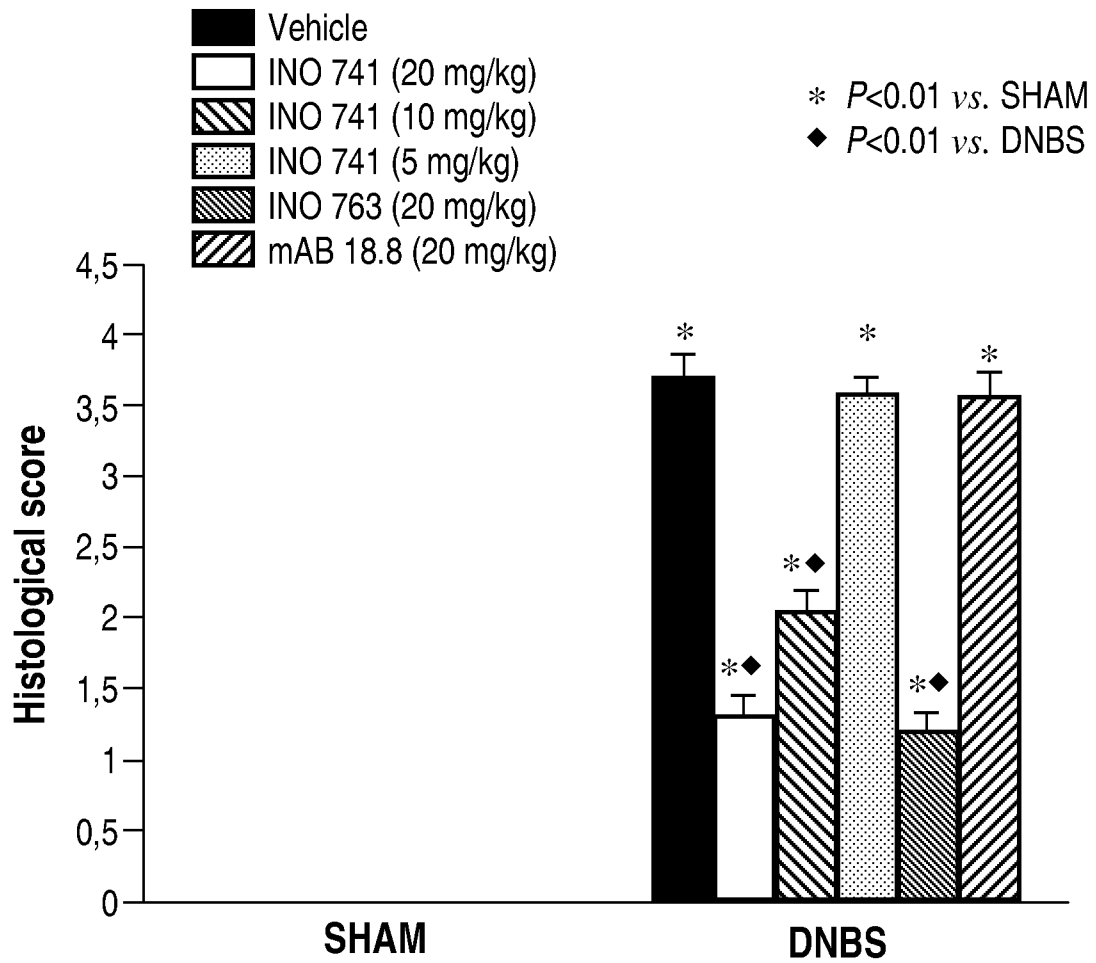


DNBS + mAb 18.8  
(20 mg/kg)



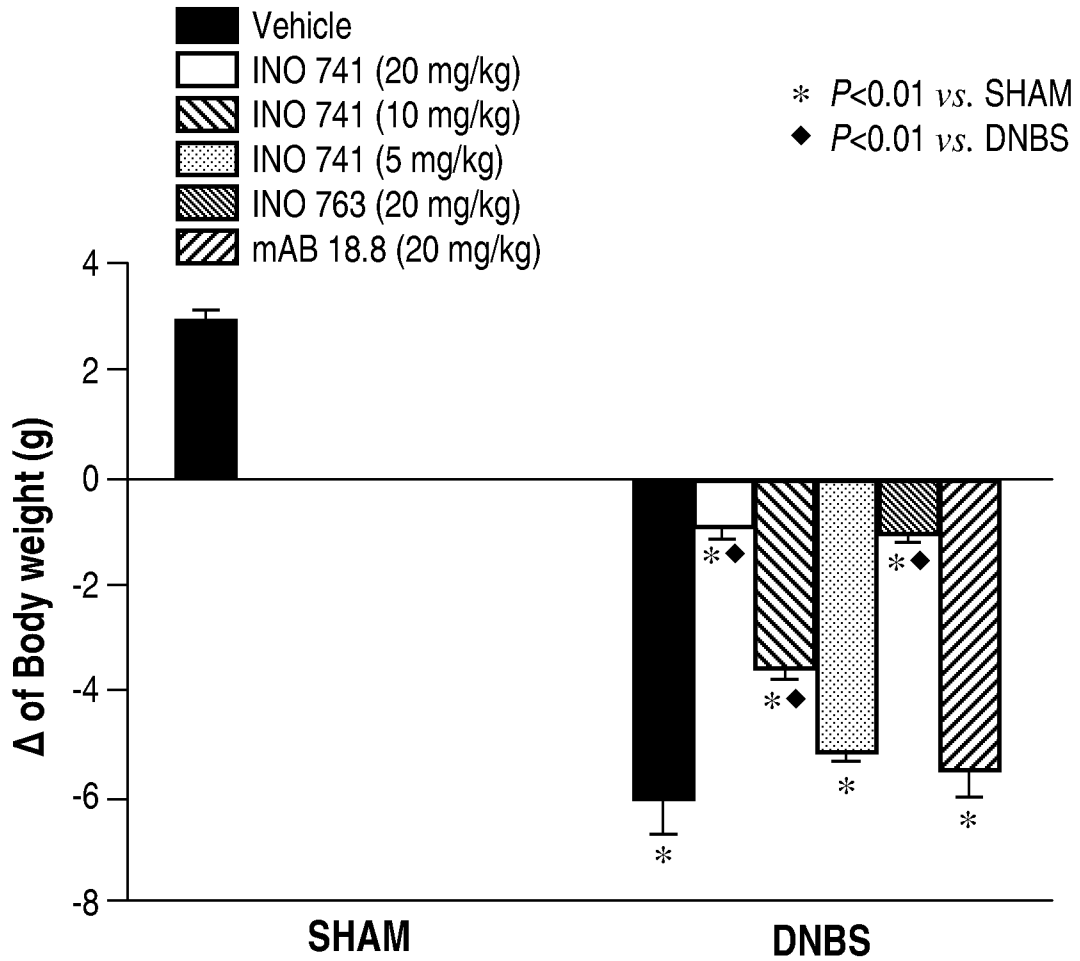
*Fig. 11*

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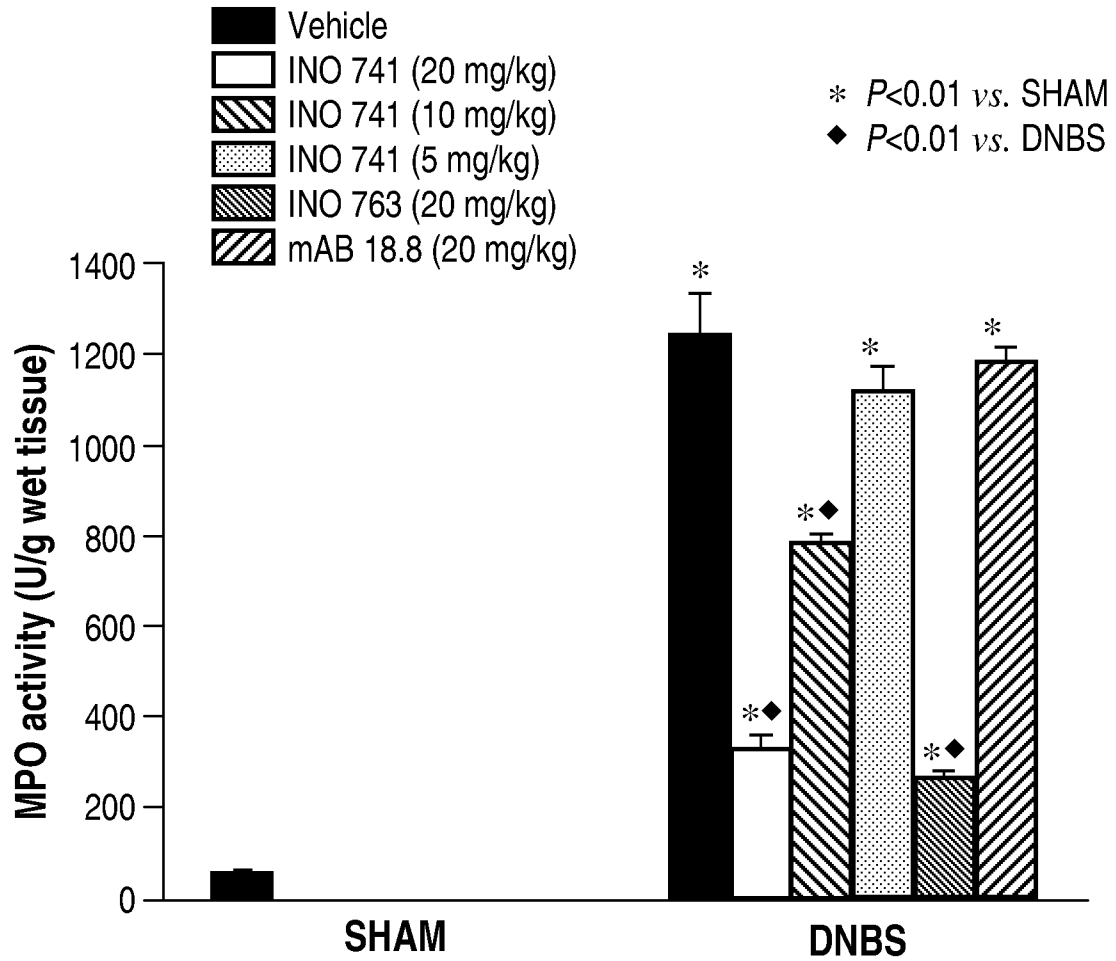
*Fig. 11h*

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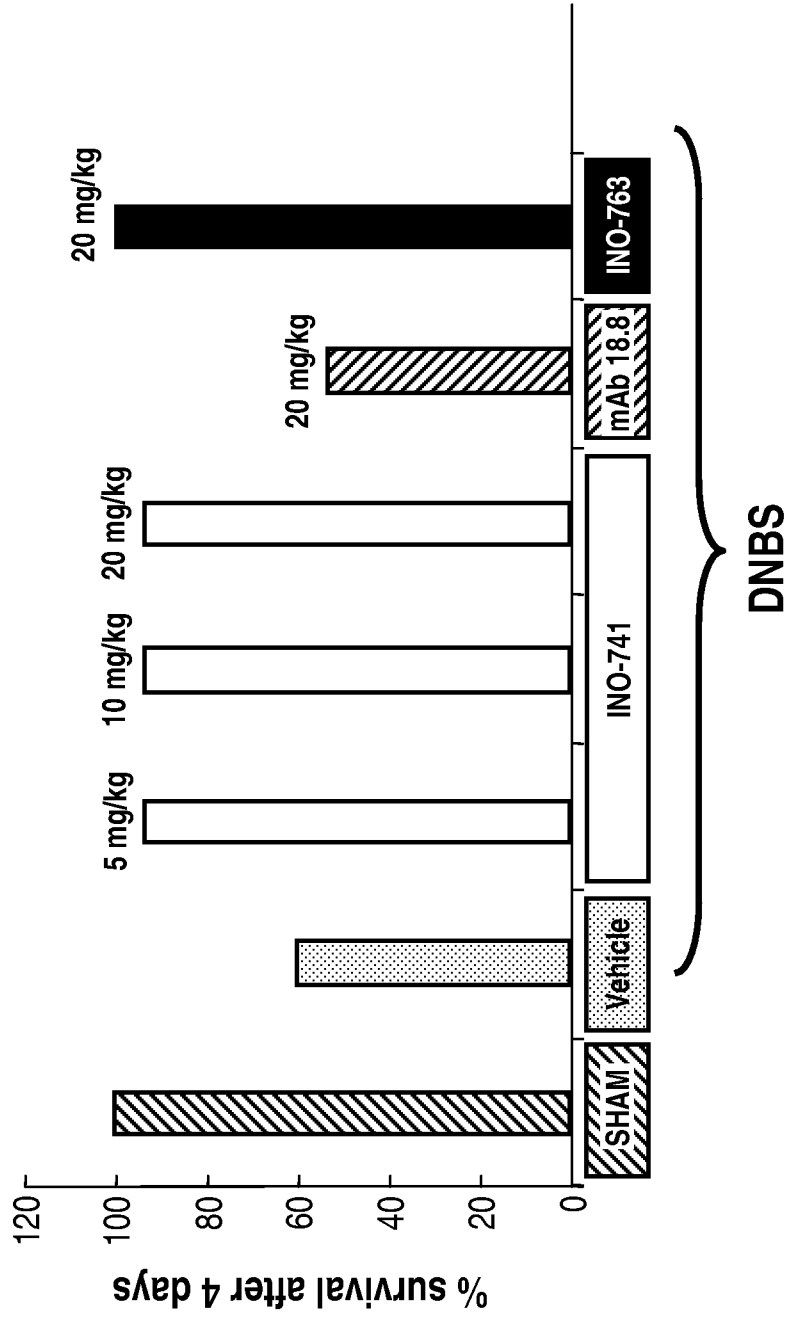


*Fig. 12*

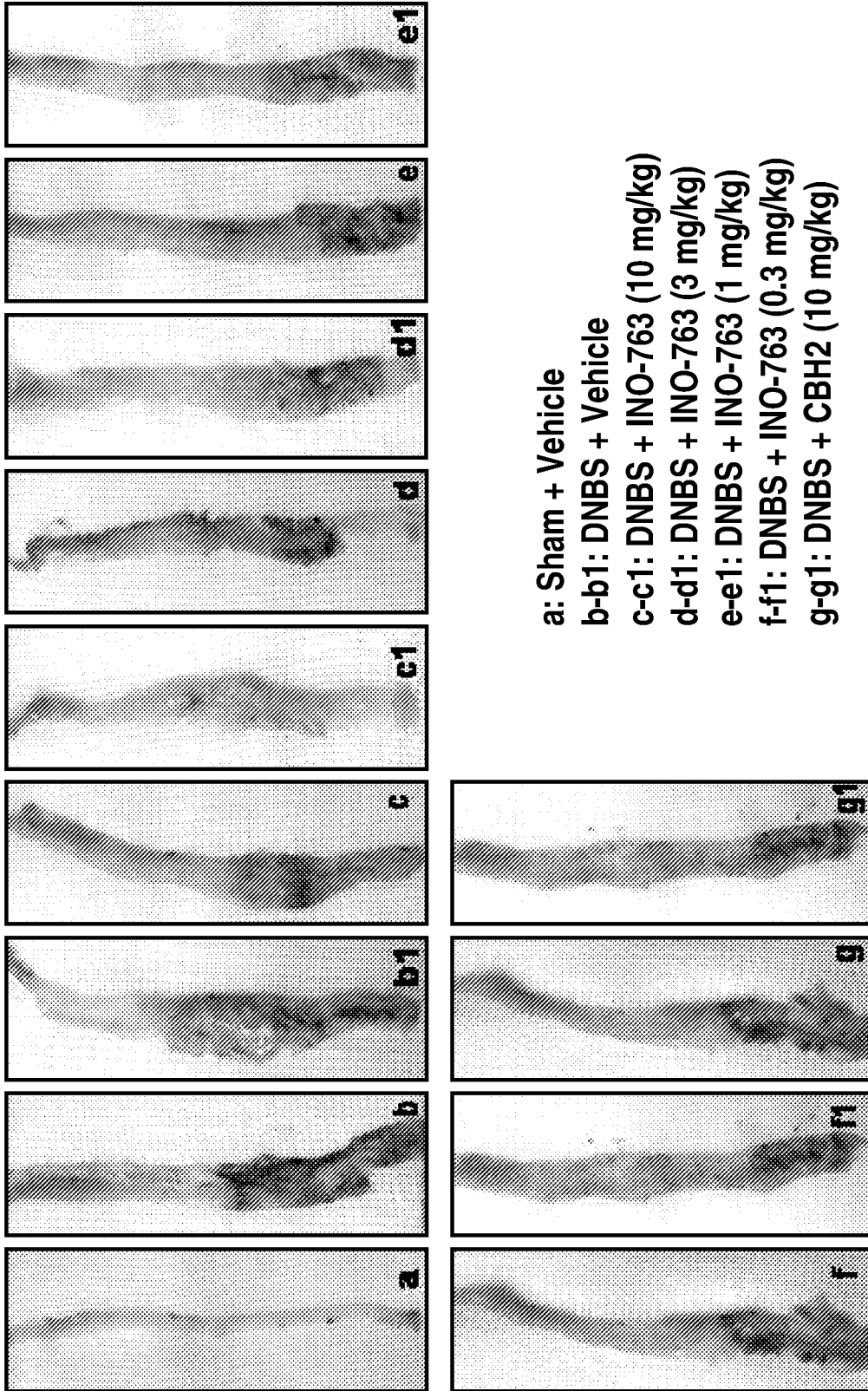
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*Fig. 13*



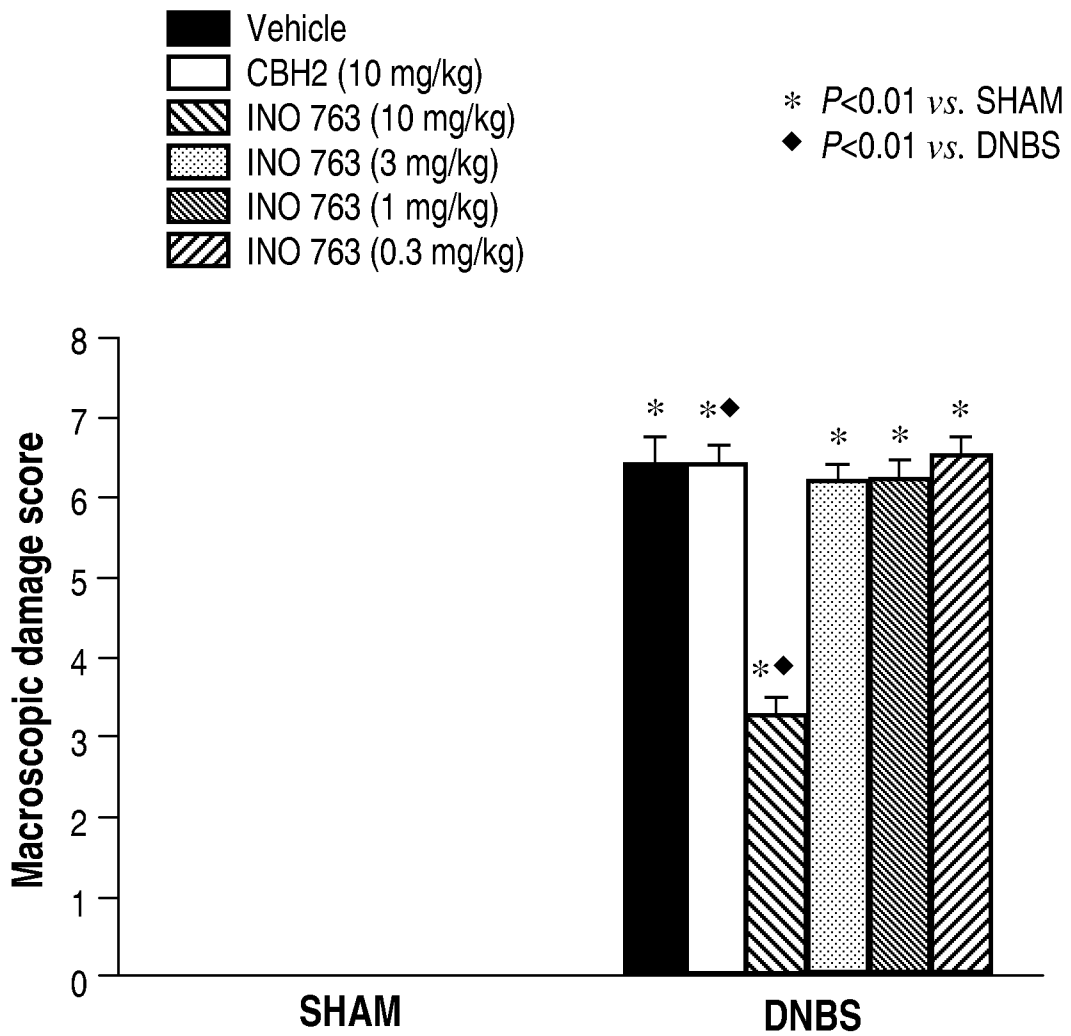
*Fig. 14*



**a:** Sham + Vehicle  
**b-b1:** DNBS + Vehicle  
**c-c1:** DNBS + INO-763 (10 mg/kg)  
**d-d1:** DNBS + INO-763 (3 mg/kg)  
**e-e1:** DNBS + INO-763 (1 mg/kg)  
**f-f1:** DNBS + INO-763 (0.3 mg/kg)  
**g-g1:** DNBS + CBH2 (10 mg/kg)

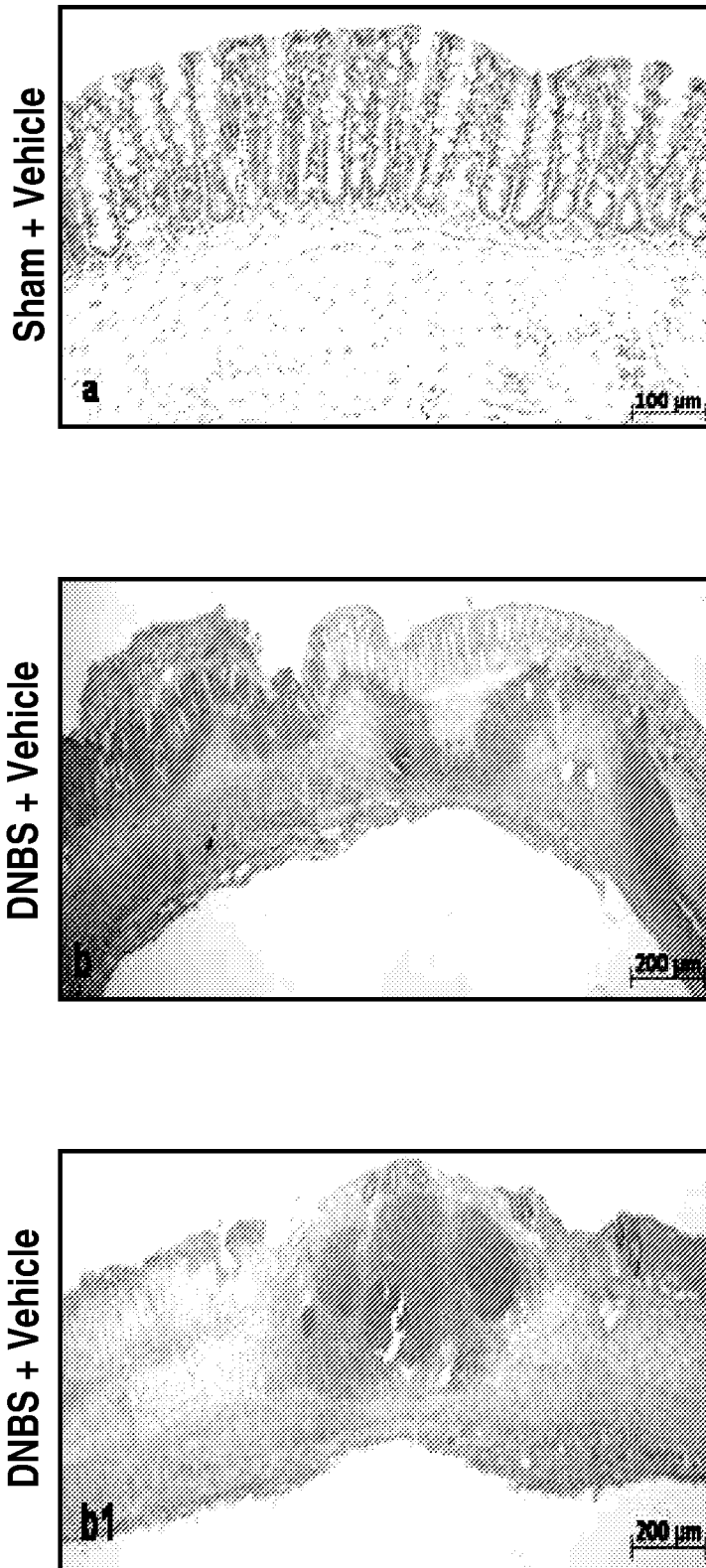
*Fig. 15*

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*Fig. 15h*

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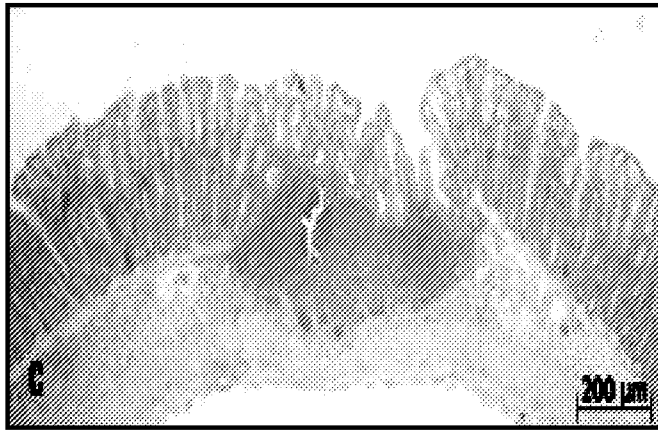


*Fig. 16*

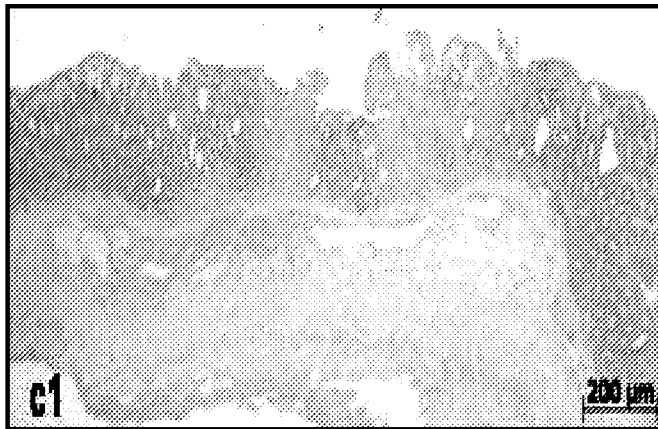


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DNBS + INO 763  
(10 mg/kg)



DNBS + INO 763  
(10 mg/kg)



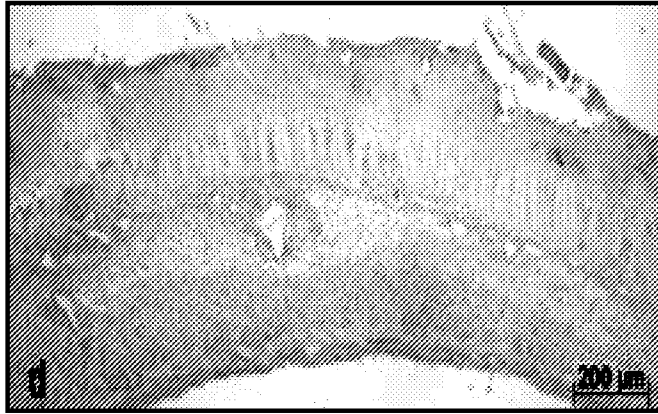
DNBS + INO 763  
(10 mg/kg)



*Fig. 16*

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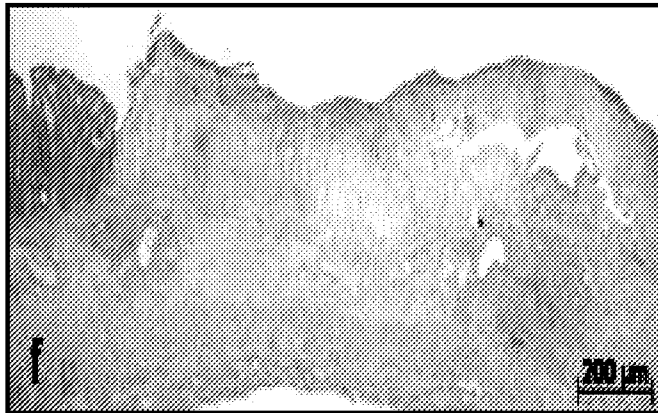
DNBS + INO 763  
(3 mg/kg)



DNBS + INO 763  
(1 mg/kg)



DNBS + INO 763  
(0.3 mg/kg)

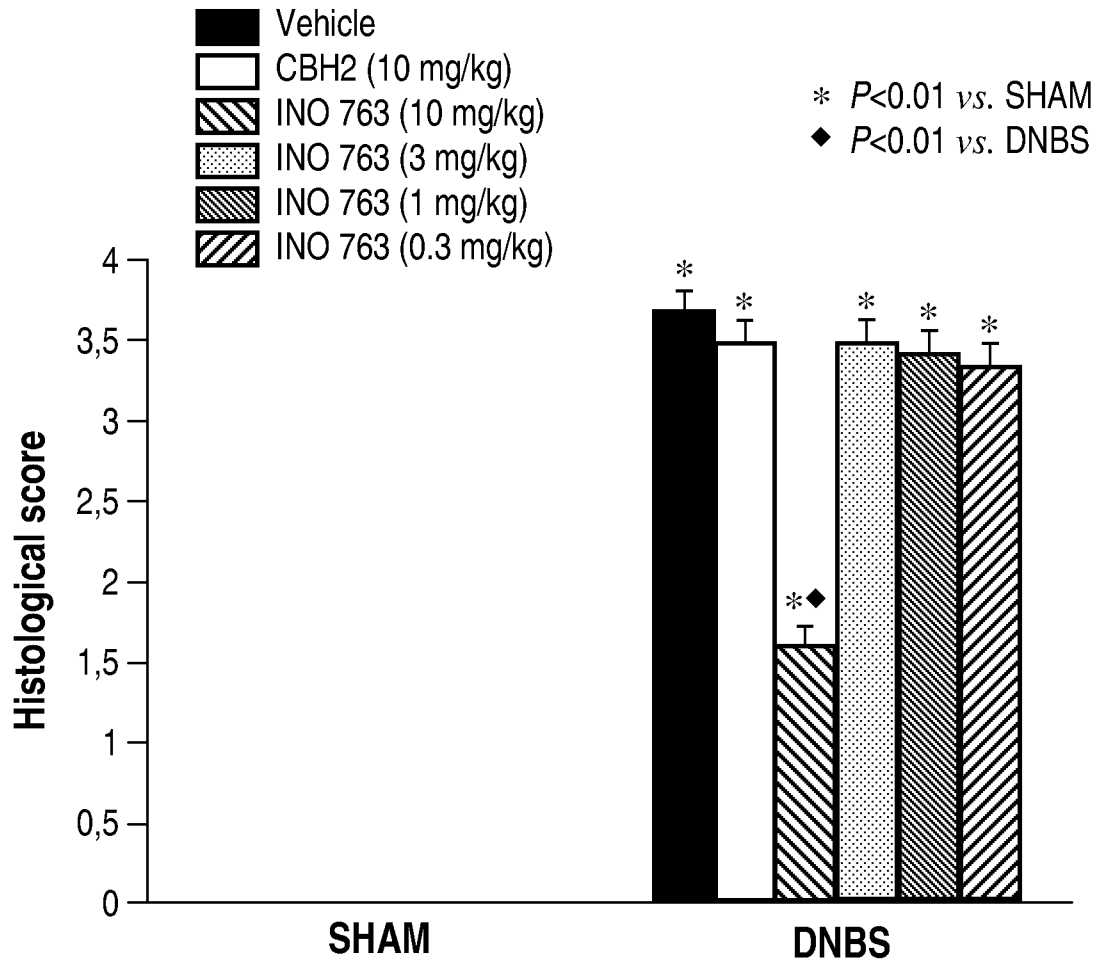


DNBS + CBH2  
(10 mg/kg)



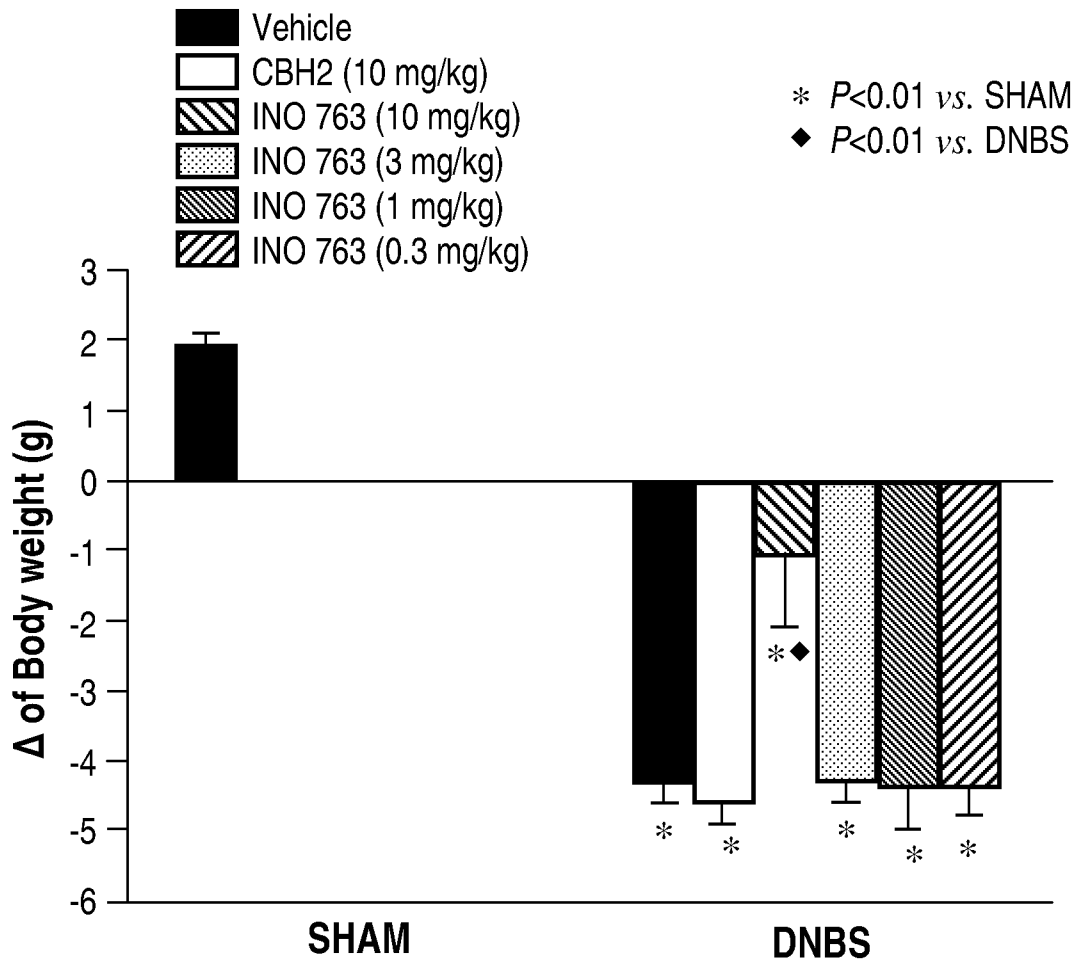
*Fig. 16*

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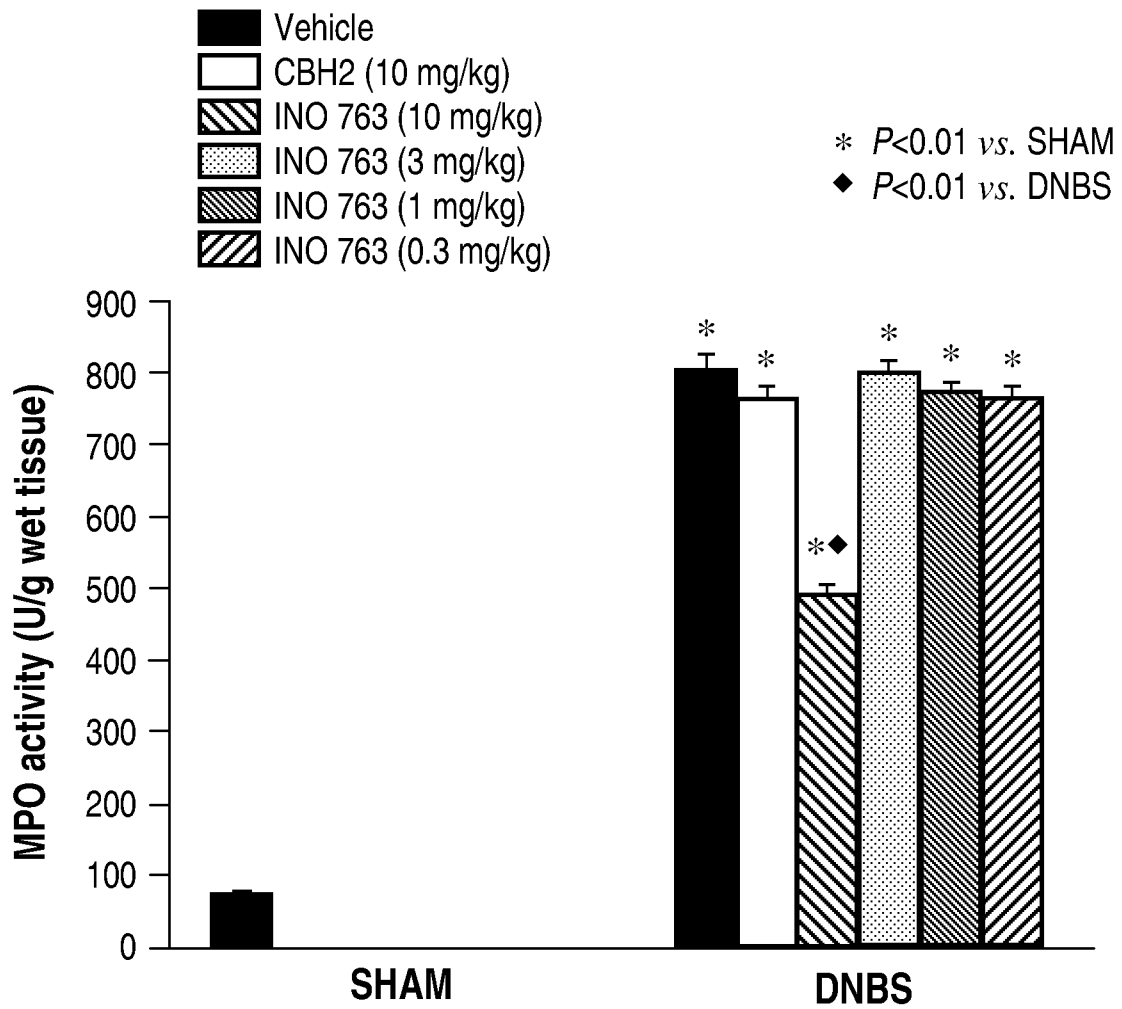
*Fig. 16h*

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*Fig. 17*

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*Fig. 18*

**Comparison of INO 763 vs. Infliximab on  
DNBS-induced mediator production from colon extracts**

Monoclonal Antibody	DNBS	MDA (mM/mg wet tissue)	IL-1 $\beta$ (pg/100 mg)	TNF $\alpha$ (pg/100 mg)	MIP-1 (pg/mg)	MIP-2 (pg/mg)	IL-8 (pg/100 mg)
saline	no	26.00 $\pm$ 1.58	34.40 $\pm$ 4.56	26.20 $\pm$ 2.88	19.40 $\pm$ 1.80	16.00 $\pm$ 1.22	46.60 $\pm$ 2.24
saline	yes	153.00 $\pm$ 6.74	195.80 $\pm$ 23.94	131.80 $\pm$ 15.60	104.60 $\pm$ 7.06	146.00 $\pm$ 17.20	143.40 $\pm$ 3.17
mAb 763 (10 mg/kg)	yes	89.80 $\pm$ 8.07	116.60 $\pm$ 7.41	62.80 $\pm$ 14.36	61.80 $\pm$ 4.29	57.60 $\pm$ 5.52	87.20 $\pm$ 3.67
mAb 763 (20 mg/kg)	yes	41.40 $\pm$ 3.17	73.20 $\pm$ 4.58	46.20 $\pm$ 8.51	38.00 $\pm$ 3.64	25.40 $\pm$ 8.42	69.60 $\pm$ 3.61
CBH2 (10 mg/kg)	yes	153.00 $\pm$ 2.98	205.00 $\pm$ 11.82	125.20 $\pm$ 8.35	106.40 $\pm$ 4.46	137.80 $\pm$ 4.30	138.40 $\pm$ 1.36
CBH2 (20 mg/kg)	yes	152.20 $\pm$ 4.28	202.8 $\pm$ 17.04	119.80 $\pm$ 11.82	105.00 $\pm$ 4.88	145.20 $\pm$ 5.09	138.20 $\pm$ 1.42
Infliximab (5 mg/kg)	yes	59.00 $\pm$ 7.64	80.80 $\pm$ 10.62	20.80 $\pm$ 4.21	47 $\pm$ 2.34	16.60 $\pm$ 2.48	63.80 $\pm$ 1.82

*Fig. 19*

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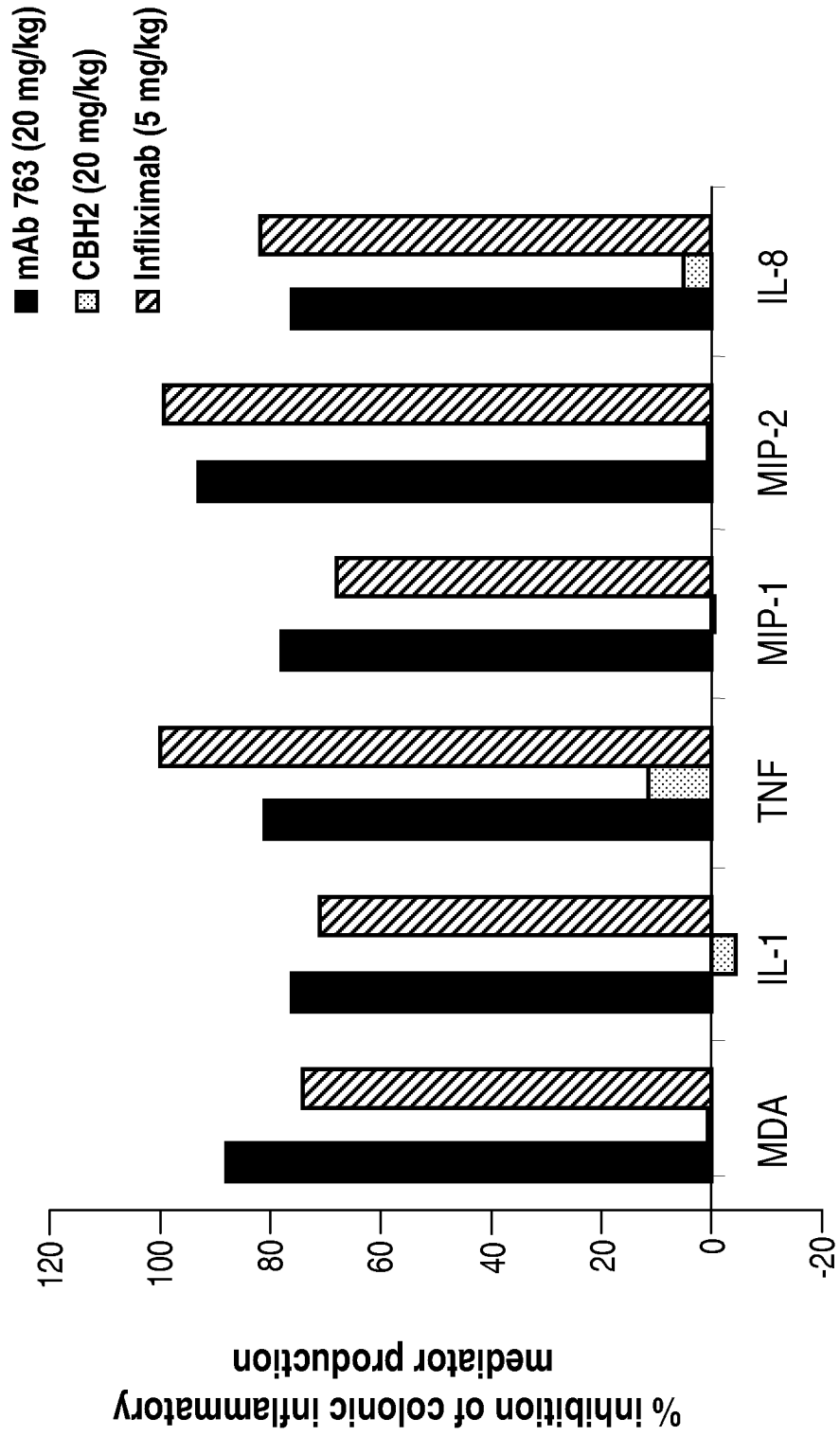
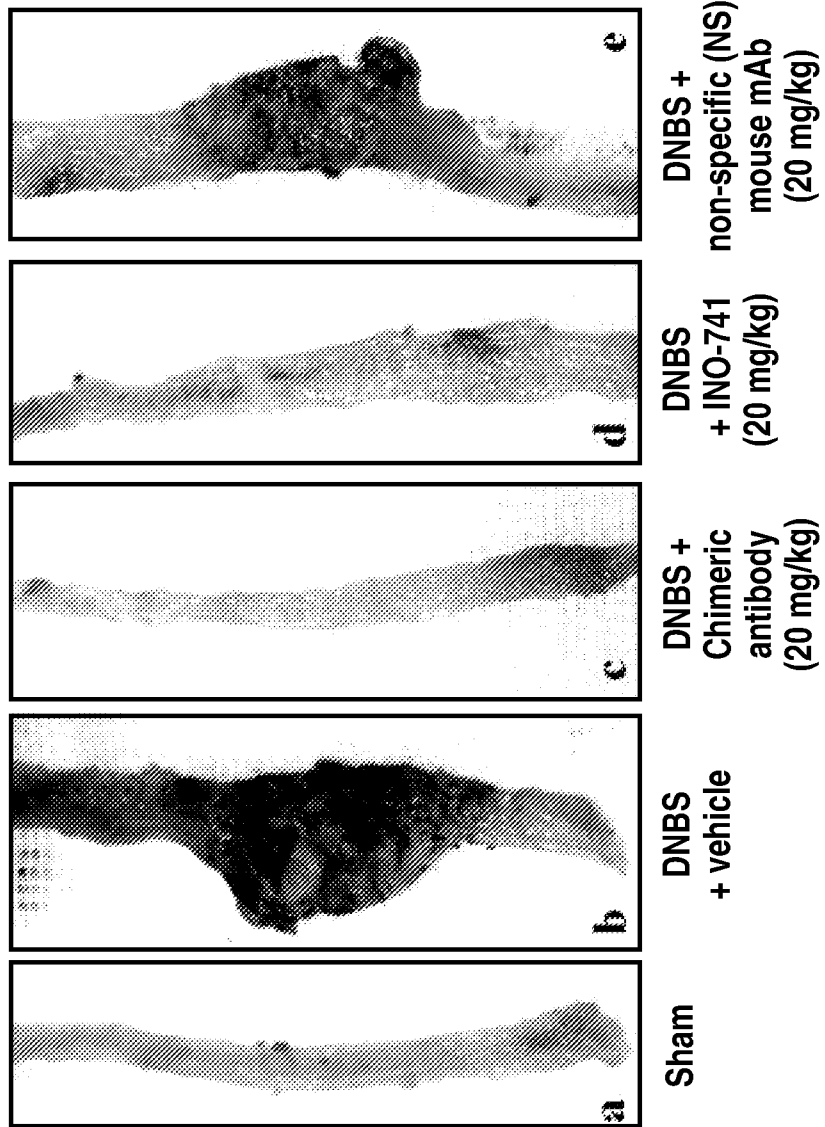


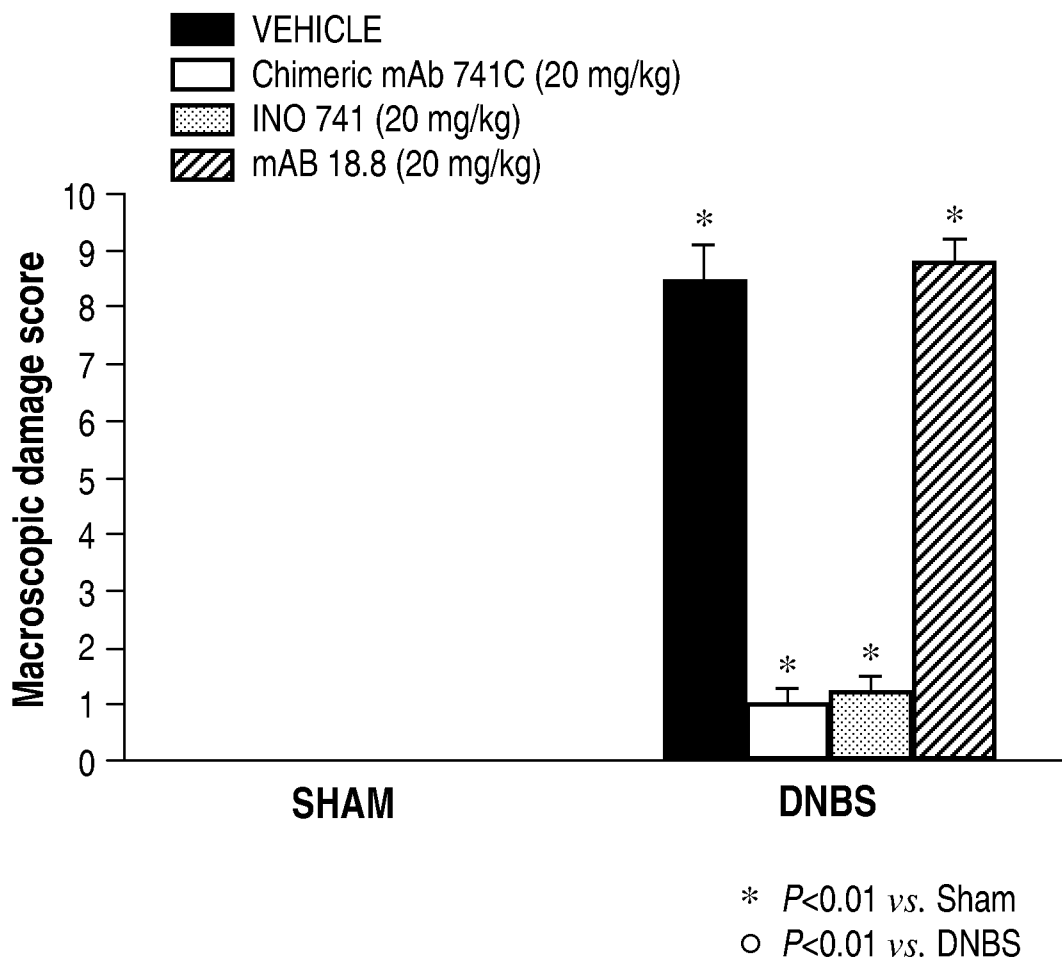
Fig. 20



*Fig. 21*

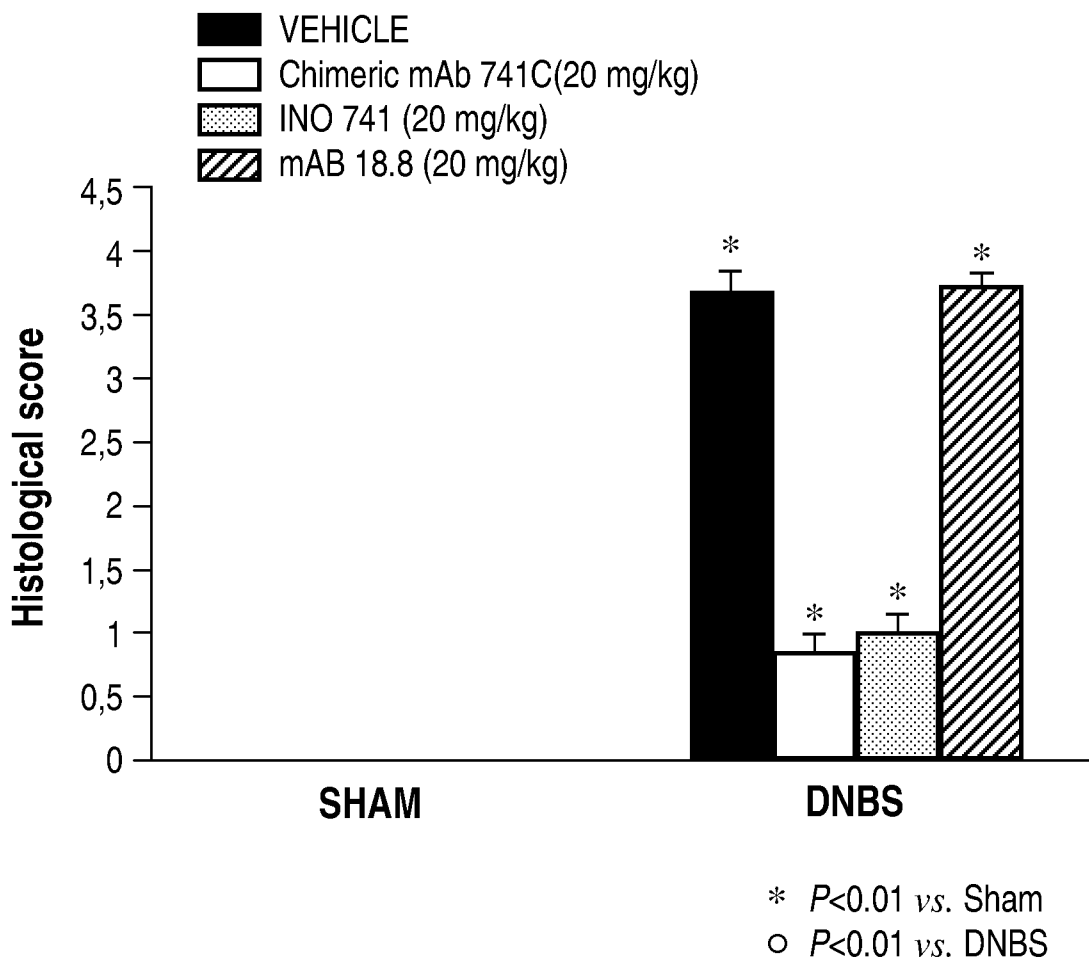


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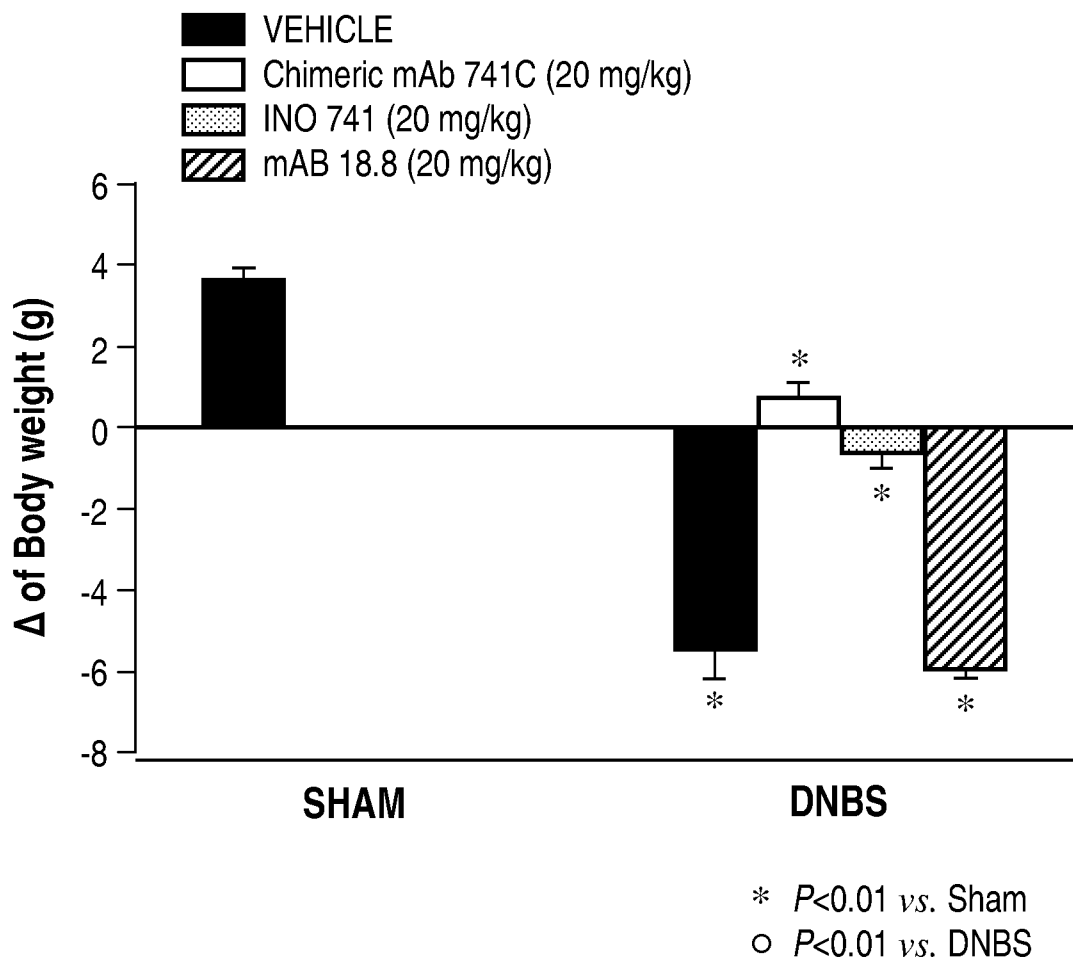
*Fig. 22*

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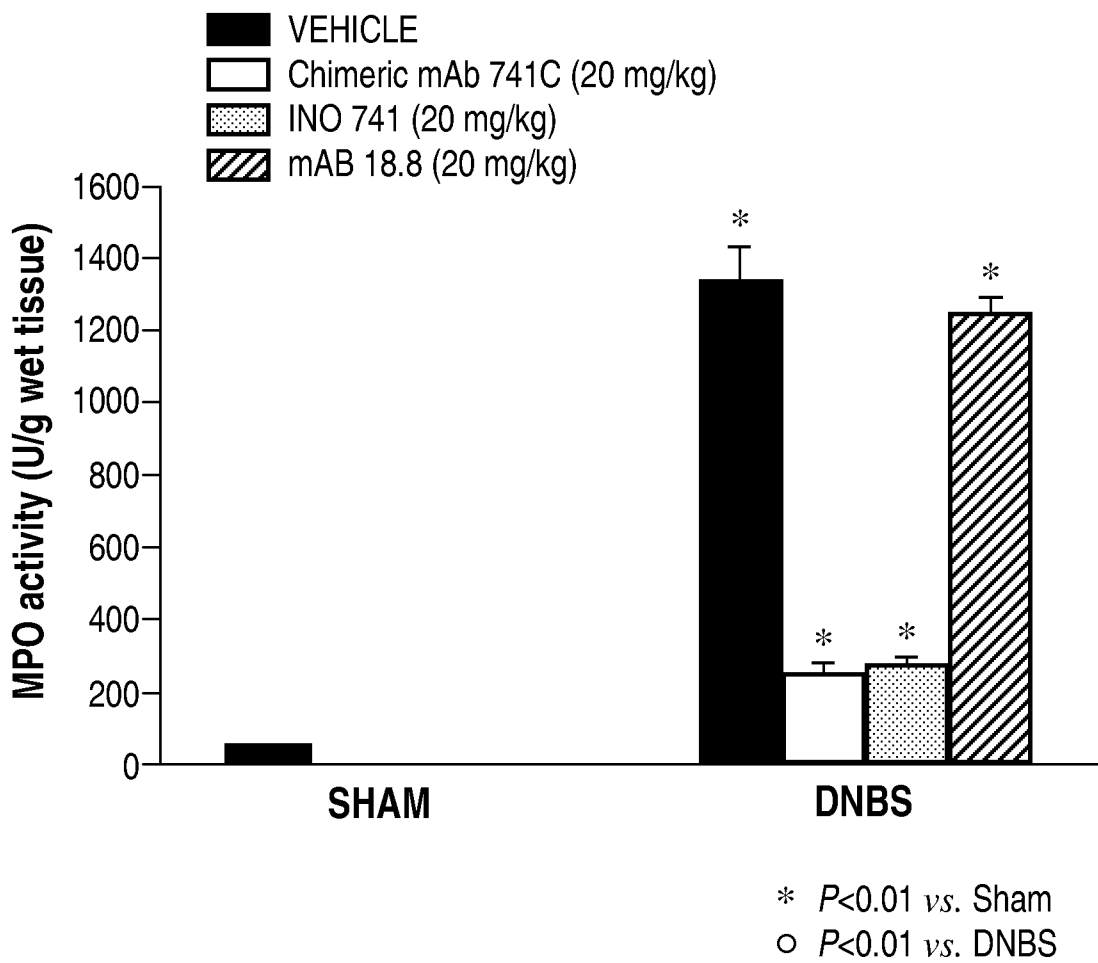
*Fig. 23*

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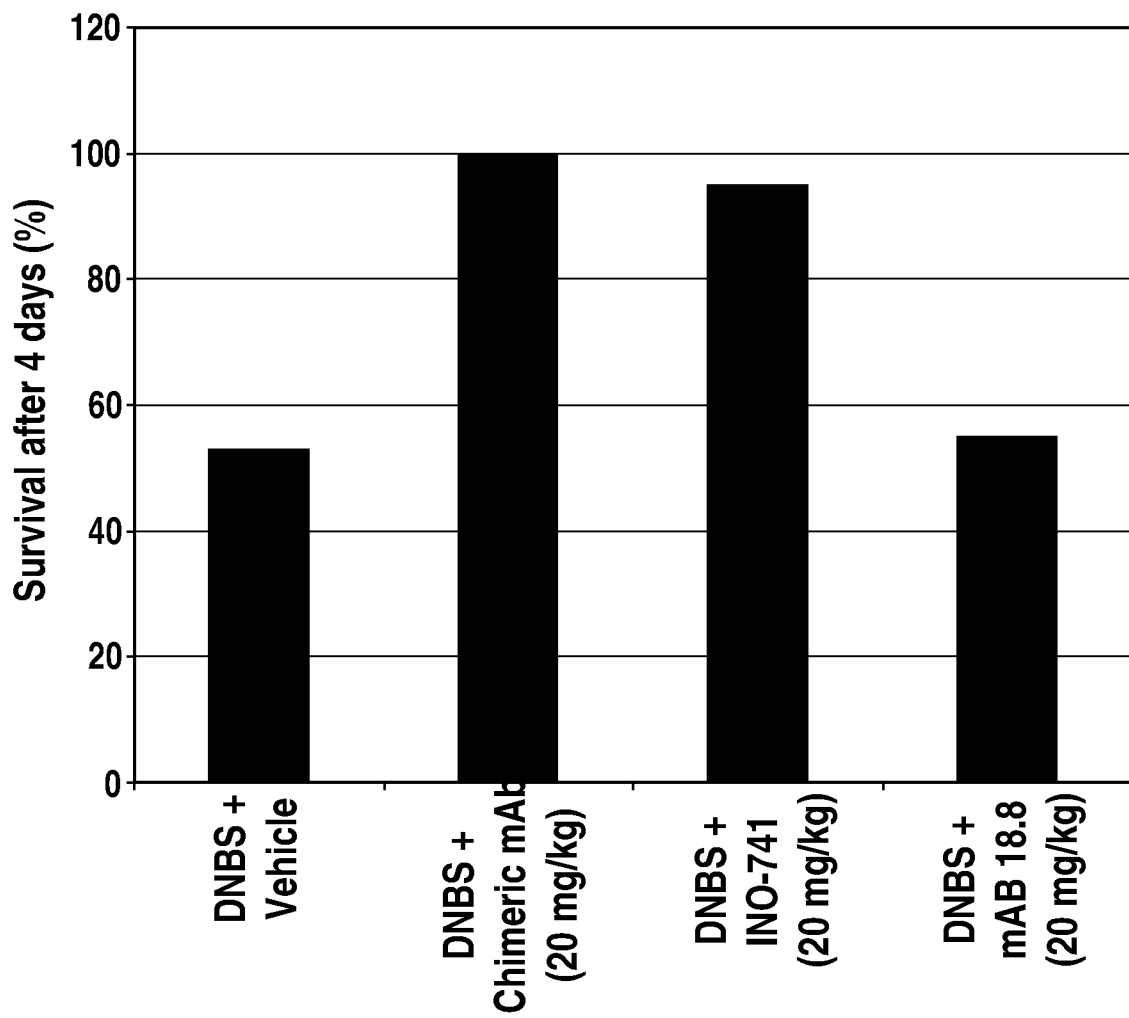
*Fig. 24*

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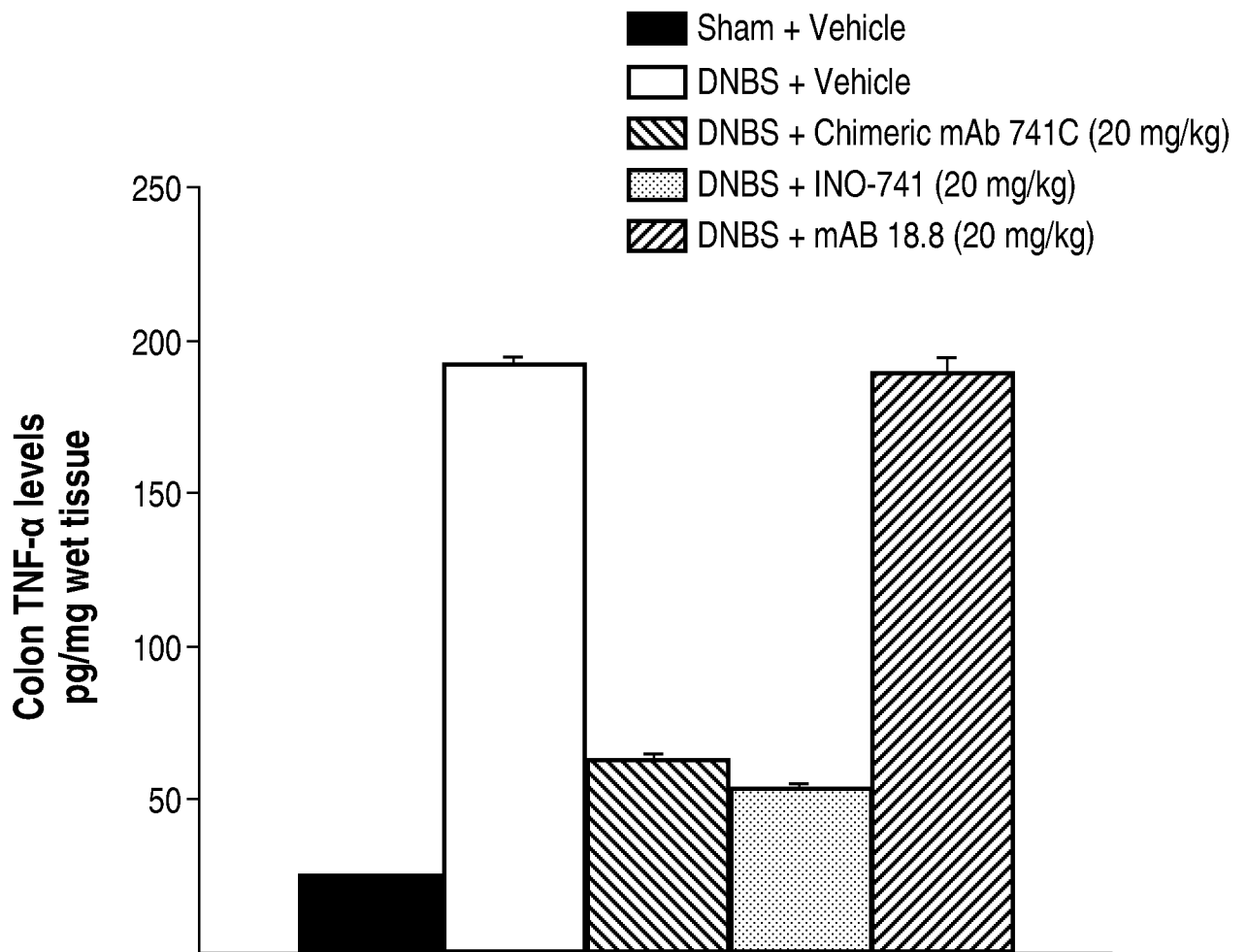


*Fig. 25*

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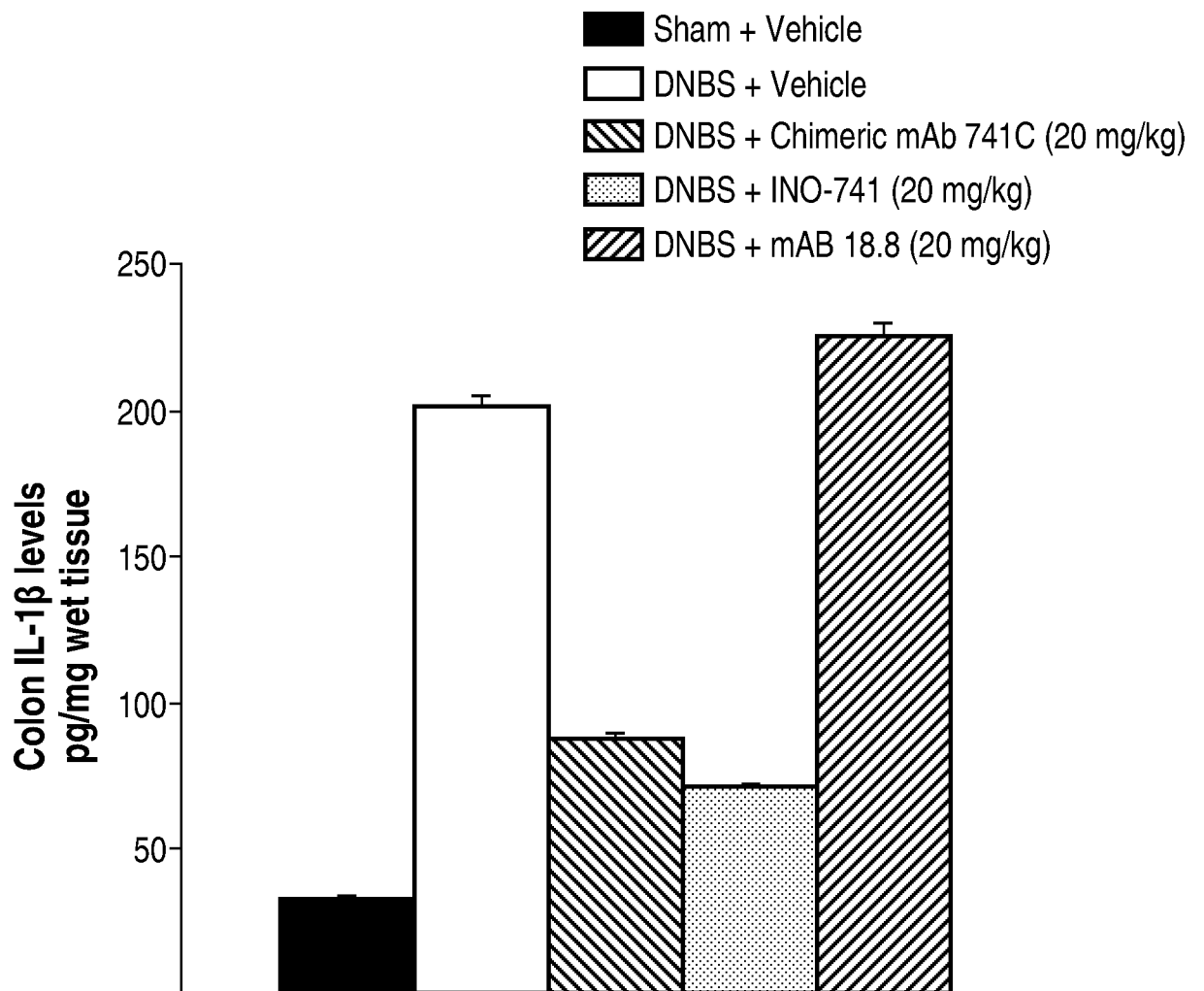
*Fig. 26*

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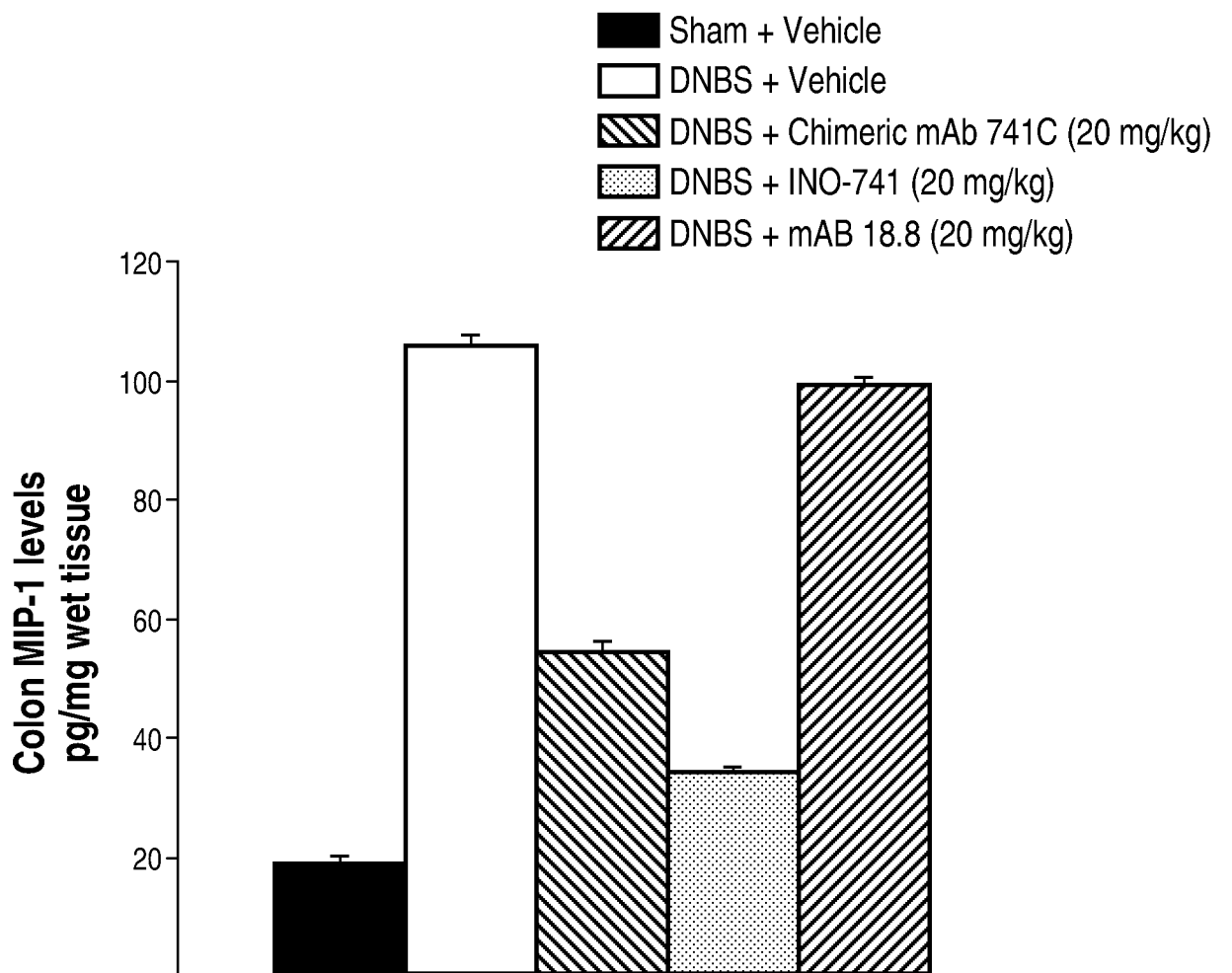


*Fig. 27*

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*Fig. 28*

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*Fig. 29*



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Bacterial Cell ELISA with mAb 741

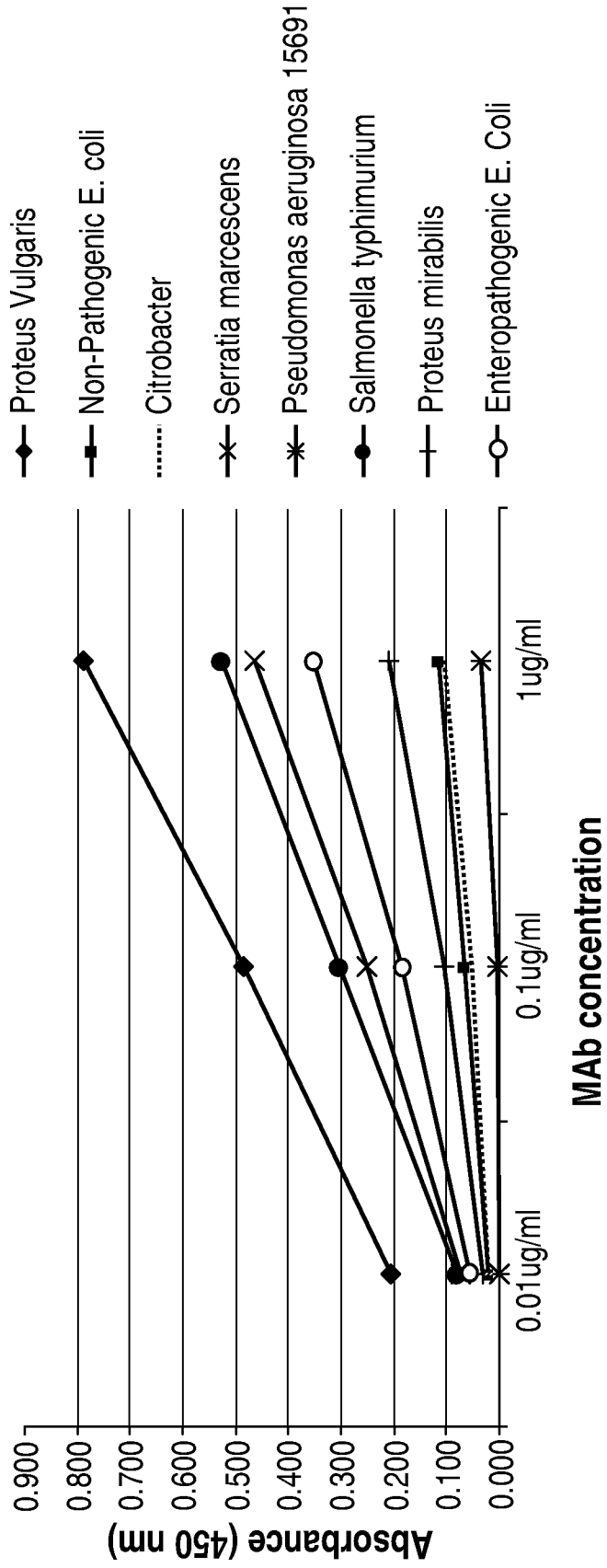
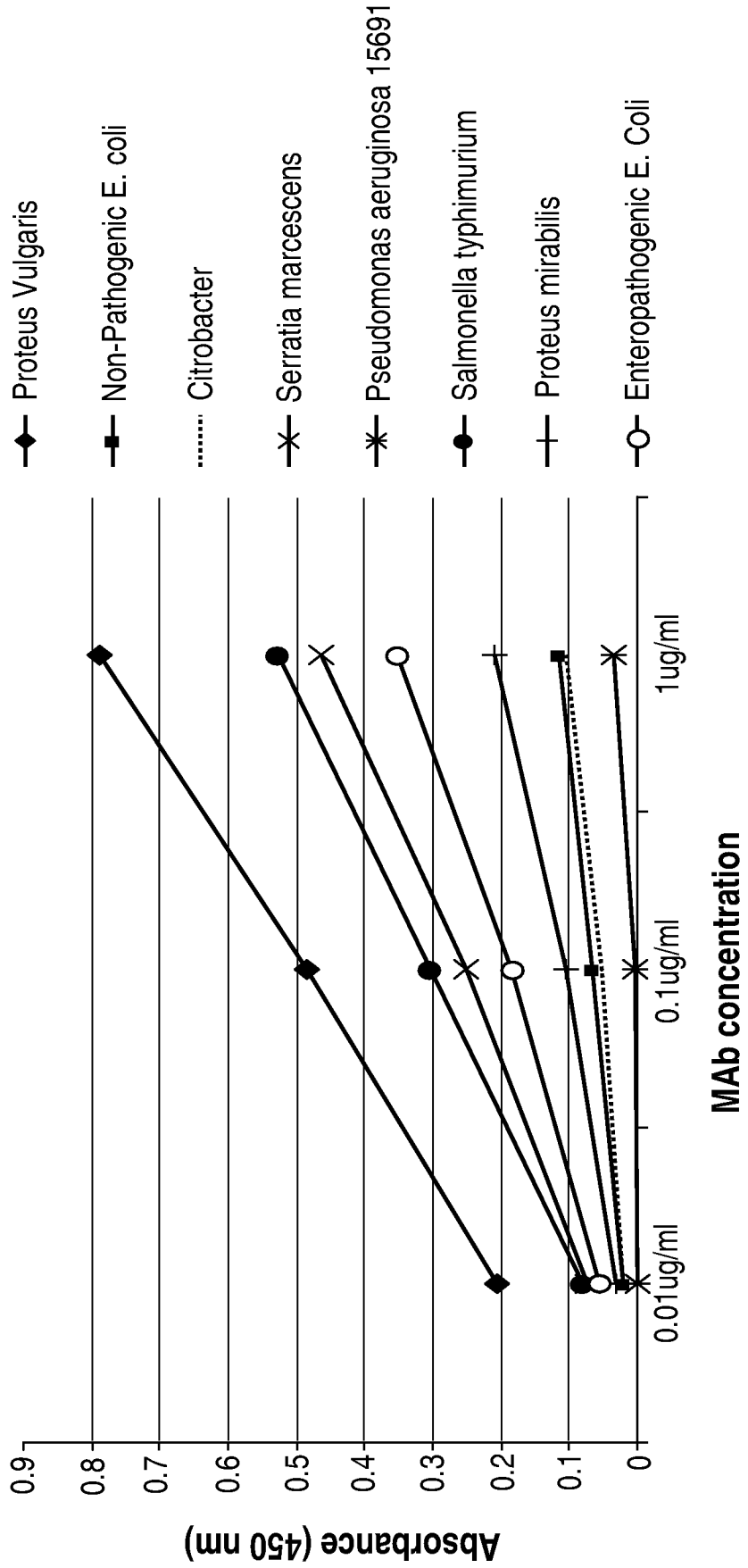


Fig. 30

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**Bacterial Cell ELISA with mAb 763**



*Fig. 31*

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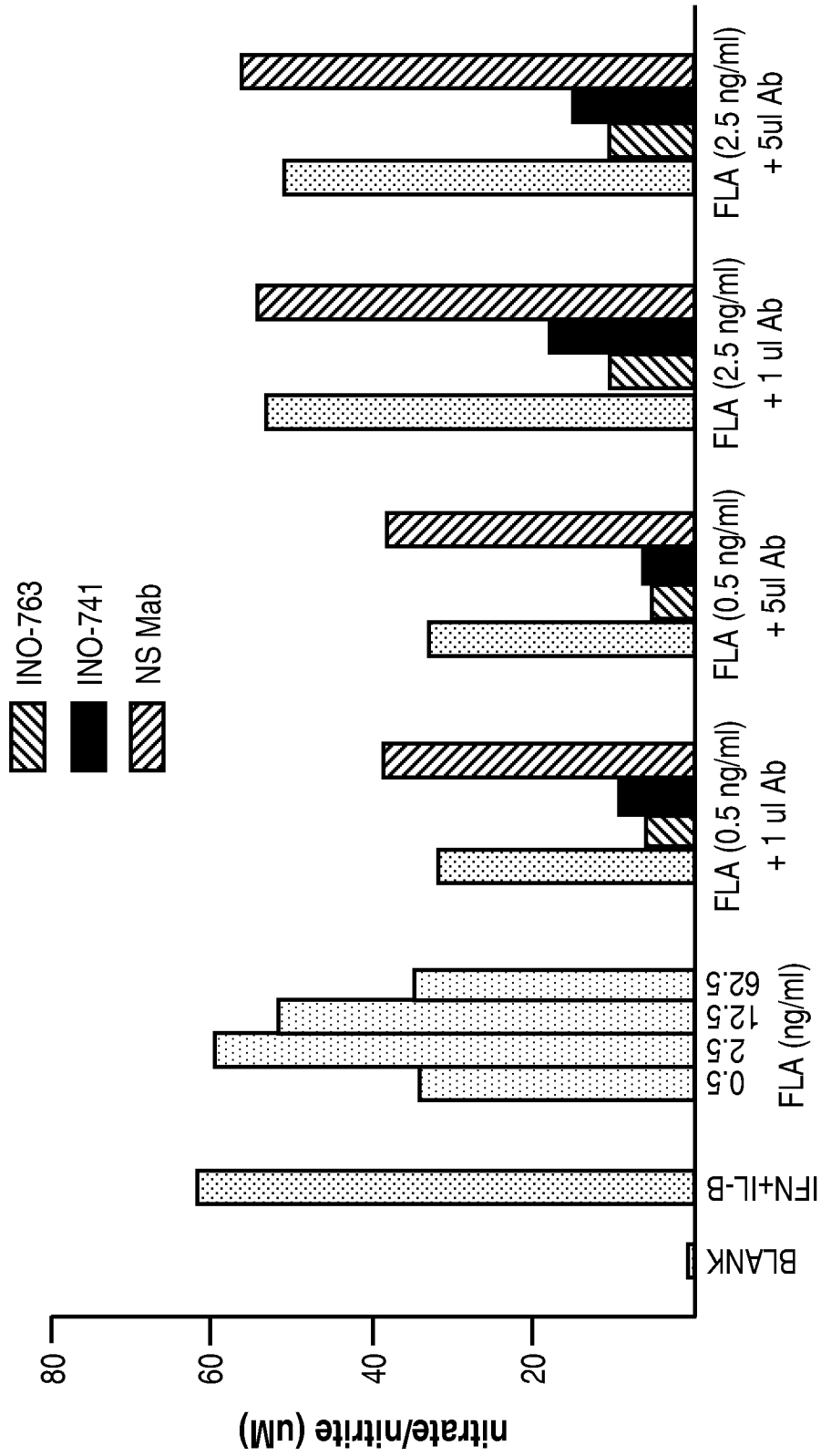


Fig. 32