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(54) **Title:** METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF CROHN'S DISEASE

(57) **Abstract:** The present invention relates to methods and pharmaceutical compositions for the treatment of Crohn's disease. More particularly, the present invention relates to a C-C Chemokine Receptor 1 (CCR1) antagonist for use in the treatment of Crohn's disease.



METHODS AND PHARMACEUTICAL COMPOSITIONS FOR THE TREATMENT OF CROHN'S DISEASE

5 **FIELD OF THE INVENTION:**

The present invention relates to methods and pharmaceutical compositions for the treatment of Crohn's disease.

BACKGROUND OF THE INVENTION:

10 Crohn's disease (CD) is one of the major type of Inflammatory Bowel Disease (IBD) that is a generic classification for a group of nonspecific, idiopathic inflammatory disorders of, the gastrointestinal. CD may occur in any part of the GI tract, but most commonly affects the distal ileum and colon. It is characterized by transmural inflammation of the gastrointestinal wall, interspersed with "skip" areas of normal tissue, leading to the
15 characteristic endoscopic and radiographic appearance of the disease.

The incidence of CD varies within different geographic areas. Northern countries such as the US, UK, Norway and Sweden have the highest rates. The incidence of CD in the US is approximately 7 per 100,000. Countries in southern Europe, South Africa and Australia have lower incidence rates of 0.9 to 3.1 per 100,000. The disease is rare in Asia and South
20 America. The peak age of onset of Crohn's disease occurs between the ages of 15 and 30 years, with a second peak of occurrence between the ages of 60-80 years (Friedman, 2001).

The characteristic inflammatory presentation of Crohn's disease is of abdominal pain, diarrhea, fever and weight loss which may be complicated by intestinal fistulization, obstruction, or both. Fistula formation may occur to the adjacent bowel, the skin, the urinary
25 bladder, or other locations. Obstruction, if present, is initially intermittent due to bowel wall edema and spasm; further progression may lead to chronic scarring and stricture formation. Perianal disease is common and may manifest as anal fissure, perianal fistula, or abscess. In the absence of a key diagnostic test, the diagnosis of Crohn's disease is based on endoscopic, radiographic and pathological findings documenting focal, asymmetric transmural or
30 granulomatous features. Laboratory abnormalities include non-specific markers of inflammation such as elevated sedimentation rate and C-reactive protein (CRP). In more severe cases, finding may include hypoalbuminemia, anemia, and leukkocytosis.

There is no definitive treatment or cure for CD. The major therapeutic goals are the reduction of signs and symptoms, induction and maintenance of remission and most

importantly, the prevention of disease progression and complications. However many of the medicinal products, however, are only moderate efficacious and are associated with challenging side effects

5 The fundamental cause of CD is unknown. There are four basic factors affecting the pathophysiology of CD: genetics, immune dysregulation, epithelial barrier dysfunction and the constitution of microbial flora. Evidence suggests that genetic predisposition leads to an unregulated intestinal immune response to an environmental, dietary or infectious agent. A number of studies suggest that CD is a T-helper 1 (Th-1) mediated disease and that the excessive Th1-cell activity leading to the production of a wide range of proinflammatory
10 cytokines including interleukin IL-1, IL-2 and tumor necrosis factor (TNF)-alpha and an imbalance between proinflammatory and anti-inflammatory reactivity, is a critical component of CD.

Interleukin 15 (IL-15) is a pleiotropic cytokine with a large range of functions at the interface between innate and adaptive immunity. An essential role in the differentiation,
15 survival and/or activation of NK, NK/T cells, TCR $\gamma\delta$ intraepithelial lymphocytes (IEL) and CD8⁺ memory T cells has been firmly established in mice lacking IL-15 or IL-15R α or, conversely, over-expressing IL-15. In humans, IL-15 is also thought to participate in the pathogenesis of a spectrum of inflammatory or autoimmune diseases and different mechanisms have been propounded. In rheumatoid arthritis, increased IL-15 concentrations
20 were found in the synovium, and IL-15 was suggested to exert direct chemoattractant activity toward synovial T cells, to stimulate their proliferation and their production of TNF- α . In addition IL-15-activated T cells were found to stimulate TNF- α production by peripheral blood monocytes and synovial macrophages via a contact-dependent mechanism. A comparable mechanism was suggested in Crohn's disease (CD) where lamina propria (LP) T
25 cells activated by IL-15 could stimulate macrophage production of TNF- α and IL-12 via a CD40-CD40L dependent mechanism. In CD, increased numbers of IL-15-producing mononuclear cells were observed in the intestinal lamina propria (LP) and augmented serum concentrations of IL-15 were found to predict patients' response to anti-TNF treatment. Alternatively, it has been suggested that, in celiac disease, chronic over-expression of IL-15
30 by intestinal epithelial cells drives the expansion and activation of cytotoxic CD8⁺TCR $\alpha\beta$ ⁺ IEL expressing NK receptors, a finding consistent with the expansion of NK1.1 CD8⁺TCR $\alpha\beta$ ⁺ IEL and LPL in mice over-expressing human IL-15 in the gut epithelium. Ex vivo, IL-15 activated IEL from celiac disease patients could destroy epithelial cells via an NK-like mechanism involving the NKG2D receptor and a comparable mechanism was evoked to

explain epithelial destruction in a mouse model of acute intestinal inflammation induced by intra-peritoneal injection of poly-IC. Finally other *ex vivo* studies have suggested that IL-15 might impair immunoregulatory mechanisms and thereby bolster CD8⁺ and CD4⁺ effector responses. However it has never been demonstrated that IL-15 controlled the recruitment of CCR1⁺ inflammatory monocytes (IM) by sustaining the expansion and activation of a subset of gut NK cells producing the CCL3 chemokine.

SUMMARY OF THE INVENTION:

The present invention relates to methods and pharmaceutical compositions for the treatment of Crohn's disease. More particularly, the present invention relates to a C-C Chemokine Receptor 1 (CCR1) antagonist for use in the treatment of Crohn's disease.

DETAILED DESCRIPTION OF THE INVENTION:

In order to delineate the hierarchy of IL-15 effects in intestinal inflammation, the inventors have used the mouse model of acute ileitis induced by oral infection of C57Bl/6 mice with *Toxoplasma gondii*. In this strain of mice, the intestinal immune reaction is necessary to eliminate the parasite but is excessive and results in lethal jejuno-ileitis. Intestinal inflammation depends on the activation of CD4⁺ Th1 intestinal LP cells specific of the triggering antigen but several complementary mechanisms are believed to participate in both pathogen exclusion and inflammation, notably the activation of CD8⁺TCRαβ⁺ IEL and that of inflammatory monocytes. Furthermore, a prior report using IL-15^{-/-} mice has suggested that IL-15 may be necessary for both protection and inflammation during *T. gondii* infection. The inventors show herein that IL-15, although not required to control parasite replication, is necessary for the development of full blown inflammation and tissue destruction. IL-15 pro-inflammatory effect did not depend on IEL activation. Furthermore, IL-15 was dispensable for the development of the CD4⁺Th1 response. In contrast, IL-15 controlled the recruitment of CCR1⁺ inflammatory monocytes (IM) by sustaining the expansion and activation of a subset of gut NK cells producing the CCL3 chemokine. These data identify CCR1 as a major receptor in the recruitment of IM toward the inflamed gut. They also delineate a novel function for gut innate immune cells and identify the IL-15-dependent subset of NK cells as a major source of CCL3 that controls the recruitment of IM. The results suggest that this pathway may operate in Crohn's disease.

Accordingly, the present invention relates to a C-C Chemokine Receptor 1 (CCR1) antagonist for use in the treatment of intestinal inflammation, in a particular for use in the treatment of Crohn's disease.

5 As used herein the term "CCR1" has its general meaning in the art and refers to the CC chemokine receptor 1 (CCR1). CCR1 is a member of the seven transmembranespanning GPCR family. In humans, it is found in monocytes/macrophages, osteoclasts and T cells, and its expression on these cells is upregulated in inflammatory conditions. Eleven different chemokines have been shown to bind and activate CCR1 in vitro, including CCL3, RANTES
10 and leukotactin-1. Furthermore, CCL3 has been shown to recruit monocytes, neutrophils, eosinophils and lymphocytes to the site of intradermal challenge in human subjects. Like many other chemokine receptors, CCR1 is involved in directing leukocyte migration and activation. Disruption of native CCR1 biology by either genetic ablation or pharmacologic intervention has proven efficacious in rodent models of rheumatoid arthritis, MS and solid
15 organ transplantation. Thus, like other chemokine receptors, CCR1 has been considered a potential target for the development of therapies for inflammatory and autoimmune disorders (Carson KG, Jaffee BD, Harriman GCB. CCR1 antagonists. Annu Rep Med Chem 2004;39:149-58; Pease JE, Horuk R. CCR1 antagonists in clinical development. Expert Opin Investig Drugs 2005;14:785-96), but the implication of CCR1 in Cohn's disease, even if
20 suggested, has not yet been demonstrated.

 As used herein the term "CCR1 antagonist" refers to any compound natural or not that is able to inhibit the activation of CCR1 by CCL3 also known as MIP-1alpha. Typically said antagonist can inhibit binding of CCL3 to CCR1. Accordingly, processes or cellular responses
25 mediated by the binding of CCL3 to CCR1 can be inhibited (reduced or prevented, in whole or in part) recruitment of inflammatory monocytes. Antagonistic activities of CCR1 may be determined according to any method well known in the art. Typically the assays may consist in a ligand-binding assay (inhibition of 125I-MIP-1 α binding to membranes prepared from transfected CHO cells) and in a functional assay by measuring inhibition of MIP-1 α induced
30 Ca²⁺ flux in transfected CHO cells loaded with fluo-4 AM. For example a commercially-available cell line (Chemicon HTS001C) that stably co-expresses CCR1 and Gal6 may be used. Said cell may be then loaded with calcium dye, pre-incubated with a test compounds and challenged with CCL3. Intracellular calcium flux may then measured and compared to control (CCL3, but no compound). For example such a method is described in the

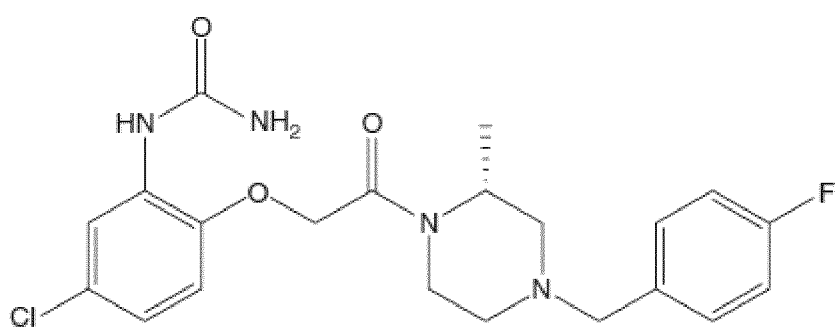
International Patent Application Publications WO 2010/036632, WO 2009/134666 and WO 2009/137338. CCR1 antagonists according to the invention may for example be selected from the group consisting of small organic molecules, antibodies, aptamers and polypeptides.

- 5 In a particular embodiment, the CCR1 antagonist according to the invention is small organic molecule.

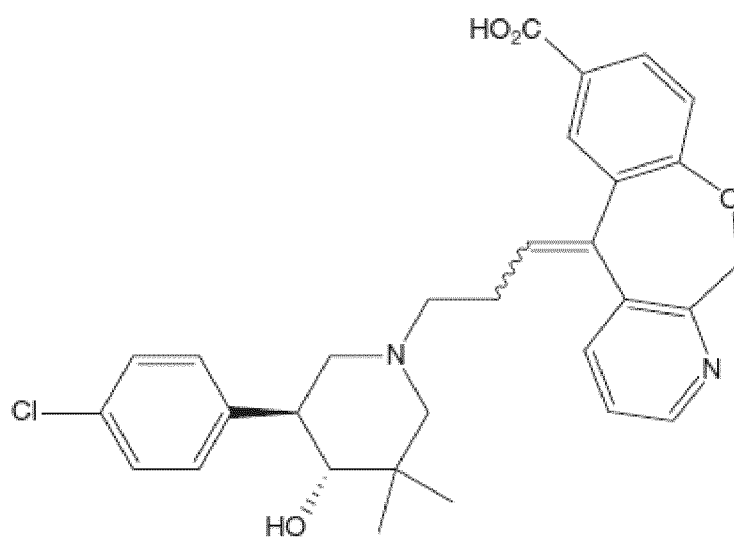
10 The term "small organic molecule" refers to a molecule of a size comparable to those organic molecules generally used in pharmaceuticals. The term excludes biological macromolecules (e. g., proteins, nucleic acids, etc.). Preferred small organic molecules range in size up to about 5000 Da, more preferably up to 2000 Da, and most preferably up to about 1000 Da.

15 Small organic molecules showing CCR1 antagonistic activities are part of the common general knowledge (see for example, Expert Opin. Ther. Patents (2010) 20(11):1609-1618; Expert Opin. Investig. Drugs (2005) 14(7):785-796; Expert Opin. Ther. Patents (2006) 16(3):395-398; Expert Opin. Ther. Patents (2009) 19(11):1629-1633).

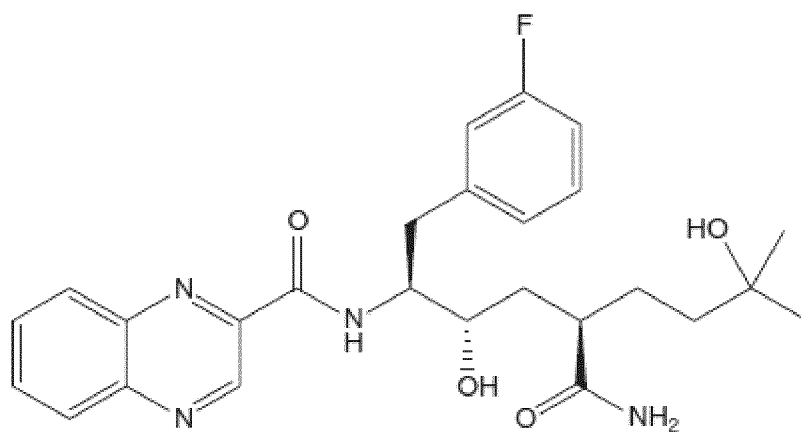
20 Representative CCR1 antagonists that are currently in clinical trials are BX471 ((2R)-1-[[2-[(aminocarbonyl)amino]-4-chlorophenoxy]acetyl]-4-[(4-fluorophenyl)methyl]-2-methylpiperazine monohydrochloride) AZD-4818, CP-481715, MLN-3897, or CCX634 (Expert Opin. Ther. Patents (2010) 20(11):1609-1618).



BX-471



MLN-3897



CP-481715

A CCR1 antagonist according to the invention is, for example, a compound disclosed in WO2001/062728 or WO2001/098273. Also, a CCR1 antagonist is, for example, N-{2-[[[(21S)-3-{[1-(4-chlorobenzyl)piperidin-4-yl]amino}-2-hydroxy-2-methylpropyl]oxy]-4-hydroxyphenyl}acetamide (see WO 2003/051839), or, 2-{2-Chloro-5-{[(2S)-3-(5-chloro-1
5 [Eta],3H-spiro [1-benzofuran-2,4'-piperidin]-r-yl)-2-hydroxypropyl]oxy}-4-[(methylamino)carbonyl]phenoxy}-2-methylpropanoic acid (see PCT publication no. WO 2008/010765), or a pharmaceutically acceptable salt thereof. The Patent cooperation treaty (PCT) application WO 2009/137338 also describes pyrazole compounds as CCR1 antagonists. The PCT applications WO2009/134666 and WO 2010/036632 describe indazole
10 and azaindazole compounds, respectively. The Patent cooperation treaty (PCT) application WO2005080336 describes trans-dimethylpiperazine derivatives. The PCT applications WO2008103126 and WO2009011653, describe spirocyclic piperidine chemokine CCR1 antagonists.

15 In another particular embodiment, the CCR1 antagonist according to the invention is an antibody or a portion thereof. Said antibody may be directed against CCR1 or CCL3.

In one embodiment of the antibodies or portions thereof described herein, the antibody is a monoclonal antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a polyclonal antibody. In one embodiment of the antibodies or portions
20 thereof described herein, the antibody is a humanized antibody. In one embodiment of the antibodies or portions thereof described herein, the antibody is a chimeric antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a light chain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a heavy chain of the antibody.
25 In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fab portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a F(ab')₂ portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a Fc portion of the antibody. In one embodiment of the
30 antibodies or portions thereof described herein, the portion of the antibody comprises a Fv portion of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises a variable domain of the antibody. In one embodiment of the antibodies or portions thereof described herein, the portion of the antibody comprises one or more CDR domains of the antibody.

As used herein, "antibody" includes both naturally occurring and non-naturally occurring antibodies. Specifically, "antibody" includes polyclonal and monoclonal antibodies, and monovalent and divalent fragments thereof. Furthermore, "antibody" includes chimeric antibodies, wholly synthetic antibodies, single chain antibodies, and fragments thereof. The antibody may be a human or nonhuman antibody. A nonhuman antibody may be humanized by recombinant methods to reduce its immunogenicity in man.

Antibodies are prepared according to conventional methodology. Monoclonal antibodies may be generated using the method of Kohler and Milstein (*Nature*, 256:495, 1975). To prepare monoclonal antibodies useful in the invention, a mouse or other appropriate host animal is immunized at suitable intervals (e.g., twice-weekly, weekly, twice-monthly or monthly) with antigenic forms of CCR1 or CCL3. The animal may be administered a final "boost" of antigen within one week of sacrifice. It is often desirable to use an immunologic adjuvant during immunization. Suitable immunologic adjuvants include Freund's complete adjuvant, Freund's incomplete adjuvant, alum, Ribi adjuvant, Hunter's Titermax, saponin adjuvants such as QS21 or Quil A, or CpG-containing immunostimulatory oligonucleotides. Other suitable adjuvants are well-known in the field. The animals may be immunized by subcutaneous, intraperitoneal, intramuscular, intravenous, intranasal or other routes. A given animal may be immunized with multiple forms of the antigen by multiple routes. Briefly, the recombinant CCR1 proteins may be provided by surface expression on recombinant cell lines. CCR1 may be provided in the form of human cells that express CCR1. Recombinant forms of CCR1 may be provided using any previously described method. Following the immunization regimen, lymphocytes are isolated from the spleen, lymph node or other organ of the animal and fused with a suitable myeloma cell line using an agent such as polyethylene glycol to form a hybridoma. Following fusion, cells are placed in media permissive for growth of hybridomas but not the fusion partners using standard methods, as described (Coding, *Monoclonal Antibodies: Principles and Practice: Production and Application of Monoclonal Antibodies in Cell Biology, Biochemistry and Immunology*, 3rd edition, Academic Press, New York, 1996). Following culture of the hybridomas, cell supernatants are analyzed for the presence of antibodies of the desired specificity, i.e., that selectively bind the antigen. Suitable analytical techniques include ELISA, flow cytometry, immunoprecipitation, and western blotting. Other screening techniques are well-known in the field. Preferred techniques are those that confirm binding of antibodies to conformationally intact, natively folded antigen, such as non-denaturing ELISA, flow cytometry, and immunoprecipitation.

Significantly, as is well-known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W. R. (1986) *The Experimental Foundations of Modern Immunology* Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The Fc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an F(ab')₂ fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

It is now well-established in the art that the non CDR regions of a mammalian antibody may be replaced with similar regions of conspecific or heterospecific antibodies while retaining the epitopic specificity of the original antibody. This is most clearly manifested in the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody.

This invention provides in certain embodiments compositions and methods that include humanized forms of antibodies. As used herein, "humanized" describes antibodies wherein some, most or all of the amino acids outside the CDR regions are replaced with corresponding amino acids derived from human immunoglobulin molecules. Methods of humanization include, but are not limited to, those described in U.S. Pat. Nos. 4,816,567, 5,225,539, 5,585,089, 5,693,761, 5,693,762 and 5,859,205, which are hereby

incorporated by reference. The above U.S. Pat. Nos. 5,585,089 and 5,693,761, and WO 90/07861 also propose four possible criteria which may be used in designing the humanized antibodies. The first proposal was that for an acceptor, use a framework from a particular human immunoglobulin that is unusually homologous to the donor immunoglobulin to be humanized, or use a consensus framework from many human antibodies. The second proposal was that if an amino acid in the framework of the human immunoglobulin is unusual and the donor amino acid at that position is typical for human sequences, then the donor amino acid rather than the acceptor may be selected. The third proposal was that in the positions immediately adjacent to the 3 CDRs in the humanized immunoglobulin chain, the donor amino acid rather than the acceptor amino acid may be selected. The fourth proposal was to use the donor amino acid residue at the framework positions at which the amino acid is predicted to have a side chain atom within 3 Å of the CDRs in a three dimensional model of the antibody and is predicted to be capable of interacting with the CDRs. The above methods are merely illustrative of some of the methods that one skilled in the art could employ to make humanized antibodies. One of ordinary skill in the art will be familiar with other methods for antibody humanization.

In one embodiment of the humanized forms of the antibodies, some, most or all of the amino acids outside the CDR regions have been replaced with amino acids from human immunoglobulin molecules but where some, most or all amino acids within one or more CDR regions are unchanged. Small additions, deletions, insertions, substitutions or modifications of amino acids are permissible as long as they would not abrogate the ability of the antibody to bind a given antigen. Suitable human immunoglobulin molecules would include IgG1, IgG2, IgG3, IgG4, IgA and IgM molecules. A "humanized" antibody retains a similar antigenic specificity as the original antibody. However, using certain methods of humanization, the affinity and/or specificity of binding of the antibody may be increased using methods of "directed evolution", as described by Wu et al., *J. Mol. Biol.* 294:151, 1999, the contents of which are incorporated herein by reference.

Fully human monoclonal antibodies also can be prepared by immunizing mice transgenic for large portions of human immunoglobulin heavy and light chain loci. See, e.g., U.S. Pat. Nos. 5,591,669, 5,598,369, 5,545,806, 5,545,807, 6,150,584, and references cited therein, the contents of which are incorporated herein by reference. These animals have been genetically modified such that there is a functional deletion in the production of endogenous (e.g., murine) antibodies. The animals are further modified to contain all or a portion of the human germ-line immunoglobulin gene locus such that immunization of these animals will

result in the production of fully human antibodies to the antigen of interest. Following immunization of these mice (e.g., XenoMouse (Abgenix), HuMAb mice (Medarex/GenPharm)), monoclonal antibodies can be prepared according to standard hybridoma technology. These monoclonal antibodies will have human immunoglobulin amino acid sequences and therefore will not provoke human anti-mouse antibody (KAMA) responses when administered to humans.

In vitro methods also exist for producing human antibodies. These include phage display technology (U.S. Pat. Nos. 5,565,332 and 5,573,905) and in vitro stimulation of human B cells (U.S. Pat. Nos. 5,229,275 and 5,567,610). The contents of these patents are incorporated herein by reference.

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')₂ Fab, Fv and Fd fragments; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric F(ab')₂ fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences; and chimeric Fd fragment antibodies in which the FR and/or CDR1 and/or CDR2 regions have been replaced by homologous human or non-human sequences. The present invention also includes so-called single chain antibodies.

The various antibody molecules and fragments may derive from any of the commonly known immunoglobulin classes, including but not limited to IgA, secretory IgA, IgE, IgG and IgM. IgG subclasses are also well known to those in the art and include but are not limited to human IgG1, IgG2, IgG3 and IgG4.

In another embodiment, the antibody according to the invention is a single domain antibody. The term "single domain antibody" (sdAb) or "VHH" refers to the single heavy chain variable domain of antibodies of the type that can be found in Camelid mammals which are naturally devoid of light chains. Such VHH are also called "nanobody®". According to the invention, sdAb can particularly be llama sdAb.

Antibodies showing CCR1 antagonistic activities are part of the common general knowledge since US patent 6,723,570 disclose CCR1 antibodies.

In another embodiment the CCR1 antagonist according to the invention is an aptamer. Aptamers are a class of molecule that represents an alternative to antibodies in term of molecular recognition. Aptamers are oligonucleotide or oligopeptide sequences with the capacity to recognize virtually any class of target molecules with high affinity and specificity.

5 Such ligands may be isolated through Systematic Evolution of Ligands by EXponential enrichment (SELEX) of a random sequence library, as described in Tuerk C. and Gold L., 1990. The random sequence library is obtainable by combinatorial chemical synthesis of DNA. In this library, each member is a linear oligomer, eventually chemically modified, of a unique sequence. Possible modifications, uses and advantages of this class of molecules have
10 been reviewed in Jayasena S.D., 1999. Peptide aptamers consists of a conformationally constrained antibody variable region displayed by a platform protein, such as E. coli Thioredoxin A that are selected from combinatorial libraries by two hybrid methods (Colas et al., 1996).

Then after raising aptamers directed against the CCR1s as above described, the skilled
15 man in the art can easily select those modulating the CCR1.

A further object of the invention relates to an inhibitor of CCR1 or CCL3 gene expression for use in the treatment of intestinal inflammation in particular for use in the treatment of Crohn's disease.

20 Inhibitors of expression for use in the present invention may be based on anti-sense oligonucleotide constructs. Anti-sense oligonucleotides, including anti-sense RNA molecules and anti-sense DNA molecules, would act to directly block the translation of CCR1 (or CCL3) mRNA by binding thereto and thus preventing protein translation or increasing mRNA
25 degradation, thus decreasing the level of CCR1 (or CCL3), and thus activity, in a cell. For example, antisense oligonucleotides of at least about 15 bases and complementary to unique regions of the mRNA transcript sequence encoding CCR1 (or CCL3) can be synthesized, e.g., by conventional phosphodiester techniques and administered by e.g., intravenous injection or infusion. Methods for using antisense techniques for specifically inhibiting gene
30 expression of genes whose sequence is known are well known in the art (e.g. see U.S. Pat. Nos. 6,566,135; 6,566,131; 6,365,354; 6,410,323; 6,107,091; 6,046,321; and 5,981,732).

Small inhibitory RNAs (siRNAs) can also function as inhibitors of expression for use in the present invention. CCR1 gene expression can be reduced by contacting a subject or cell with a small double stranded RNA (dsRNA), or a vector or construct causing the production

of a small double stranded RNA, such that CCR1 (or CCL3) gene expression is specifically inhibited (i.e. RNA interference or RNAi). Methods for selecting an appropriate dsRNA or dsRNA-encoding vector are well known in the art for genes whose sequence is known (e.g. see Tuschl, T. et al. (1999); Elbashir, S. M. et al. (2001); Hannon, GJ. (2002); McManus, MT. et al. (2002); Brummelkamp, TR. et al. (2002); U.S. Pat. Nos. 6,573,099 and 6,506,559; and International Patent Publication Nos. WO 01/36646, WO 99/32619, and WO 01/68836). All or part of the phosphodiester bonds of the siRNAs of the invention are advantageously protected. This protection is generally implemented via the chemical route using methods that are known by art. The phosphodiester bonds can be protected, for example, by a thiol or amine functional group or by a phenyl group. The 5'- and/or 3'- ends of the siRNAs of the invention are also advantageously protected, for example, using the technique described above for protecting the phosphodiester bonds. The siRNAs sequences advantageously comprises at least twelve contiguous dinucleotides or their derivatives.

As used herein, the term "siRNA derivatives" with respect to the present nucleic acid sequences refers to a nucleic acid having a percentage of identity of at least 90% with erythropoietin or fragment thereof, preferably of at least 95%, as an example of at least 98%, and more preferably of at least 98%.

As used herein, "percentage of identity" between two nucleic acid sequences, means the percentage of identical nucleic acid, between the two sequences to be compared, obtained with the best alignment of said sequences, this percentage being purely statistical and the differences between these two sequences being randomly spread over the nucleic acid acids sequences. As used herein, "best alignment" or "optimal alignment", means the alignment for which the determined percentage of identity (see below) is the highest. Sequences comparison between two nucleic acids sequences are usually realized by comparing these sequences that have been previously align according to the best alignment; this comparison is realized on segments of comparison in order to identify and compared the local regions of similarity. The best sequences alignment to perform comparison can be realized, beside by a manual way, by using the global homology algorithm developed by SMITH and WATERMAN (Ad. App. Math., vol.2, p:482, 1981), by using the local homology algorithm developped by NEDDLEMAN and WUNSCH (J. Mol. Biol., vol.48, p:443, 1970), by using the method of similarities developed by PEARSON and LIPMAN (Proc. Natl. Acad. Sci. USA, vol.85, p:2444, 1988), by using computer softwares using such algorithms (GAP, BESTFIT, BLAST P, BLAST N, FASTA, TFASTA in the Wisconsin Genetics software Package, Genetics Computer Group, 575 Science Dr., Madison, WI USA), by using the MUSCLE multiple

alignment algorithms (Edgar, Robert C., Nucleic Acids Research, vol. 32, p:1792, 2004). To get the best local alignment, one can preferably used BLAST software. The identity percentage between two sequences of nucleic acids is determined by comparing these two sequences optimally aligned, the nucleic acids sequences being able to comprise additions or deletions in respect to the reference sequence in order to get the optimal alignment between these two sequences. The percentage of identity is calculated by determining the number of identical position between these two sequences, and dividing this number by the total number of compared positions, and by multiplying the result obtained by 100 to get the percentage of identity between these two sequences.

shRNAs (short hairpin RNA) can also function as inhibitors of expression for use in the present invention.

Ribozymes can also function as inhibitors of expression for use in the present invention. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific hybridization of the ribozyme molecule to complementary target RNA, followed by endonucleolytic cleavage. Engineered hairpin or hammerhead motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of CCR1 (OR CCL3) mRNA sequences are thereby useful within the scope of the present invention. Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites, which typically include the following sequences, GUA, GUU, and GUC. Once identified, short RNA sequences of between about 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site can be evaluated for predicted structural features, such as secondary structure, that can render the oligonucleotide sequence unsuitable.

Both antisense oligonucleotides and ribozymes useful as inhibitors of expression can be prepared by known methods. These include techniques for chemical synthesis such as, e.g., by solid phase phosphoramidite chemical synthesis. Alternatively, anti-sense RNA molecules can be generated by in vitro or in vivo transcription of DNA sequences encoding the RNA molecule. Such DNA sequences can be incorporated into a wide variety of vectors that incorporate suitable RNA polymerase promoters such as the T7 or SP6 polymerase promoters. Various modifications to the oligonucleotides of the invention can be introduced as a means of increasing intracellular stability and half-life. Possible modifications include but are not limited to the addition of flanking sequences of ribonucleotides or

deoxyribonucleotides to the 5' and/or 3' ends of the molecule, or the use of phosphorothioate or 2'-O-methyl rather than phosphodiesterase linkages within the oligonucleotide backbone.

Antisense oligonucleotides, siRNAs, shRNAs and ribozymes of the invention may be delivered in vivo alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating the transfer of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid to the cells and preferably cells expressing CCR1 (OR CCL3). Preferably, the vector transports the nucleic acid to cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. In general, the vectors useful in the invention include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequences. Viral vectors are a preferred type of vector and include, but are not limited to nucleic acid sequences from the following viruses: retrovirus, such as moloney murine leukemia virus, harvey murine sarcoma virus, murine mammary tumor virus, and rous sarcoma virus; adenovirus, adeno-associated virus; SV40-type viruses; polyoma viruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; polio virus; and RNA virus such as a retrovirus. One can readily employ other vectors not named but known to the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses (e.g., lentivirus), the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes in vivo. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell lined with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, 1990 and in Murry, 1991).

Preferred viruses for certain applications are the adenoviruses and adeno-associated (AAV) viruses, which are double-stranded DNA viruses that have already been approved for human use in gene therapy. Actually 12 different AAV serotypes (AAV1 to 12) are known,

each with different tissue tropisms (Wu, Z Mol Ther 2006; 14:316-27). Recombinant AAV are derived from the dependent parvovirus AAV2 (Choi, VW J Virol 2005; 79:6801-07). The adeno-associated virus type 1 to 12 can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species (Wu, Z Mol Ther 2006; 14:316-27). It further has advantages such as, heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the adeno-associated virus can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression characteristic of retroviral infection. In addition, wild-type adeno-associated virus infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the adeno-associated virus genomic integration is a relatively stable event. The adeno-associated virus can also function in an extrachromosomal fashion.

Other vectors include plasmid vectors. Plasmid vectors have been extensively described in the art and are well known to those of skill in the art. See e.g. Sambrook et al., 1989. In the last few years, plasmid vectors have been used as DNA vaccines for delivering antigen-encoding genes to cells in vivo. They are particularly advantageous for this because they do not have the same safety concerns as with many of the viral vectors. These plasmids, however, having a promoter compatible with the host cell, can express a peptide from a gene operatively encoded within the plasmid. Some commonly used plasmids include pBR322, pUC18, pUC19, pRC/CMV, SV40, and pBlueScript. Other plasmids are well known to those of ordinary skill in the art. Additionally, plasmids may be custom designed using restriction enzymes and ligation reactions to remove and add specific fragments of DNA. Plasmids may be delivered by a variety of parenteral, mucosal and topical routes. For example, the DNA plasmid can be injected by intramuscular, intradermal, subcutaneous, or other routes. It may also be administered by intranasal sprays or drops, rectal suppository and orally. It may also be administered into the epidermis or a mucosal surface using a gene-gun. The plasmids may be given in an aqueous solution, dried onto gold particles or in association with another DNA delivery system including but not limited to liposomes, dendrimers, cochleate and microencapsulation.

In a preferred embodiment, the antisense oligonucleotide, siRNA, shRNA or ribozyme nucleic acid sequence is under the control of a heterologous regulatory region, e.g., a heterologous promoter. The promoter can also be, e.g., a viral promoter, such as CMV promoter or any synthetic promoters.

Another object of the invention relates to a method for treating intestinal inflammation (in particular Crohn's disease) comprising administering a subject in need thereof with a CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression, as above described.

5 The CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression may be administered in the form of a pharmaceutical composition, as defined below.

Preferably, said inhibitor is administered in a therapeutically effective amount.

By a "therapeutically effective amount" is meant a sufficient amount of the CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression to treat Crohn's disease at a
10 reasonable benefit/risk ratio applicable to any medical treatment.

It will be understood that the total daily usage of the compounds and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of
15 the disorder; activity of the specific compound employed; the specific composition employed, the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific polypeptide employed; and like factors well known in the medical arts. For example, it is well
20 within the skill of the art to start doses of the compound at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved. However, the daily dosage of the products may be varied over a wide range from 0.01 to 1,000 mg per adult per day. Preferably, the compositions contain 0.01, 0.05, 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, 25.0, 50.0, 100, 250 and 500 mg of the active ingredient for the
25 symptomatic adjustment of the dosage to the patient to be treated. A medicament typically contains from about 0.01 mg to about 500 mg of the active ingredient, preferably from 1 mg to about 100 mg of the active ingredient. An effective amount of the drug is ordinarily supplied at a dosage level from 0.0002 mg/kg to about 20 mg/kg of body weight per day, especially from about 0.001 mg/kg to 7 mg/kg of body weight per day.

30 The CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression may be combined with pharmaceutically acceptable excipients, and optionally sustained-release matrices, such as biodegradable polymers, to form therapeutic compositions.

In the pharmaceutical compositions of the present invention for oral, sublingual, subcutaneous, intramuscular, intravenous, transdermal, local or rectal administration, the active principle, alone or in combination with another active principle, can be administered in a unit administration form, as a mixture with conventional pharmaceutical supports, to
5 animals and human beings. Suitable unit administration forms comprise oral-route forms such as tablets, gel capsules, powders, granules and oral suspensions or solutions, sublingual and buccal administration forms, aerosols, implants, subcutaneous, transdermal, topical, intraperitoneal, intramuscular, intravenous, subdermal, transdermal, intrathecal and intranasal administration forms and rectal administration forms.

10 Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized
15 water or physiological saline, permit the constitution of injectable solutions.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy
20 syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions comprising compounds of the invention as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as
25 hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression of the invention can be formulated into a composition in a neutral or salt form. Pharmaceutically
30 acceptable salts include the acid addition salts (formed with the free amino groups of the protein) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups can also be derived from inorganic bases such as, for

example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, histidine, procaine and the like.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. 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. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the use in the compositions of agents delaying absorption, for example, aluminium monostearate and gelatin.

Sterile injectable solutions are prepared by incorporating the active polypeptides in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is therapeutically effective. The formulations are easily administered in a variety of dosage forms, such as the type of injectable solutions described above, but drug release capsules and the like can also be employed.

For parenteral administration in an aqueous solution, for example, the solution should be suitably buffered if necessary and the liquid diluent first rendered isotonic with sufficient saline or glucose. These particular aqueous solutions are especially suitable for intravenous, intramuscular, subcutaneous and intraperitoneal administration. In this connection, sterile aqueous media which can be employed will be known to those of skill in the art in light of the present disclosure. For example, one dosage could be dissolved in 1 ml of isotonic NaCl solution and either added to 1000 ml of hypodermoclysis fluid or injected at the proposed site of infusion. Some variation in dosage will necessarily occur depending on the condition of the

subject being treated. The person responsible for administration will, in any event, determine the appropriate dose for the individual subject.

The CCR1 antagonist or inhibitor of CCR1 (or CCL3) gene expression of the invention may be formulated within a therapeutic mixture to comprise about 0.0001 to 1.0 milligrams, or about 0.001 to 0.1 milligrams, or about 0.1 to 1.0 or even about 10 milligrams per dose or so. Multiple doses can also be administered.

In addition to the compounds of the invention formulated for parenteral administration, such as intravenous or intramuscular injection, other pharmaceutically acceptable forms include, e.g. tablets or other solids for oral administration ; liposomal formulations ; time release capsules ; and any other form currently used.

The invention will be further illustrated by the following figures and examples. However, these examples and figures should not be interpreted in any way as limiting the scope of the present invention.

FIGURES:

Figure 1: The recruitment of inflammatory monocytes during *T. gondii*-induced ileitis depends on CCR1/CCL3 interactions

(A,D) qRT-PCR quantification of *ccl2*, *ccl3*, *ccl4*, *ccl5* (A) or *ccr1*, *ccr5* (D) transcripts in LPL from IL-15^{-/-} and WT mice either naïve or on day 7 PI. (B,E) Percentage and absolute numbers of LP Ly6C^{hi} F4/80⁺ IM on day 7 PI in IL-15^{-/-} and WT mice treated or not with anti-CCL3 mAb (B) or in IL-15^{-/-}, WT, CCR1^{-/-} mice (E).

(C,G) Spontaneous secretion of IL-1 β , TNF α evaluated by ELISA in 72h supernatants of LP cells isolated from IL-15^{-/-}, WT mice treated or not with anti-CCL3 mAb (C) or from IL-15^{-/-}, WT, CCR1^{-/-} mice (G) either naïve or on day 7 PI. (F) Percentage of LP cells intracellularly stained with anti-TNF α mAb on day 7 PI in IL-15^{-/-}, WT and CCR1^{-/-} mice. (*, p<0.05 ; **, p<0.005 ; ***, p<0.0005)

Figure 2: Intestinal recruitment of inflammatory monocytes depends on CCR1/CCL3 interactions during *T. gondii*-induced ileitis

(A) qRT-PCR quantification in jejunal biopsies of *ccl2*, *ccl4*, *ccl5* and *cxcl10* transcripts from IL-15^{-/-} and WT mice naïve or on day 7 PI. (B) qRT-PCR quantification of *cxcl10* transcripts in LP cells from IL-15^{-/-} and WT mice naïve or on day 7 PI. (C) qRT-PCR

quantification of *cc13* transcripts from IL-15^{-/-} and WT mice naïve on days 3, 5 and 7 PI in jejunal biopsies (left) or in LP cells (right). (D) Spontaneous secretion of CCL3 evaluated by ELISA in 72h supernatants of LP cells from IL-15^{-/-}, WT and CCR1^{-/-} mice either naïve or on day 7 PI. (E,H) Spontaneous secretion of IL-1 β , TNF α evaluated by ELISA in 24h supernatants of jejunal biopsies from WT mice treated or not with anti-CCL3 mAb (E) and from IL-15^{-/-}, WT and CCR1^{-/-} mice (H) either naïve or on day 7 PI. (F) qRT-PCR quantification of *ccr1* transcripts in LP cells from WT mice treated or not with anti-CCL3 mAb (*, p<0.05 ; **, p<0.005 ; ***, p<0.0005).

Figure 3: CCL3 and CCR1 are increased in Crohn's disease

(A,B,C,E) qRT-PCR quantification of *il1b*, *tnfa*, *il6* (A), *ccl3*, *ccl5* (B), *ccr1*, *ccr5* (C), *il15*, *il15ra* (E) transcripts in intestinal biopsies from controls (n=15) and from CD patients (n=19). (D) Correlation between *ccl3* and *ccr1* transcripts (r=0.76, p=0.0002) and *ccl3* and *ccr5* transcripts (r= 0.35, p= 0.1366). (F) qRT-PCR quantification of *ccl3* transcripts in PBL and FACS-sorted human blood lymphocyte subsets (CD19⁺, CD3⁺ CD56⁻, NKP46⁺ CD56⁺ CD3⁻) stimulated or not with 500 ng/ml human IL15, and/or 10ng/mL human IL-12 or anti-CD3 and anti-CD28 antibodies as indicated in methods. (*, p<0.05 ; **, p<0.005 ; ***, p<0.0005). (G) Medians of percentages of CCL3+CD3-NKP46+ cells among CD45+ PBL after 24h stimulation with indicated concentrations of human IL-15 (data from 3 independent experiments).

EXAMPLE: IL-15-DEPENDENT GUT NK CELLS CONTROLS INTESTINAL INFLAMMATION VIA CCL3-CCR1- DEPENDENT RECRUITMENT OF INFLAMMATORY MONOCYTES

Material & Methods

Mouse models

Eigth to 12-week-old inbred male C57Bl/6, IL-15^{-/-}, perforin^{-/-}, β_2m ^{-/-}, CCR1^{-/-} and transgenic mice expressing human IL-15 under the T3b promoter (gift from H. Kiyono, Tokyo University), all backcrossed onto a C57Bl/6 background, were raised under specific pathogen-free conditions in animal facilities of Institut Pasteur in accordance with European guidelines. For adoptive transfer, IEL were isolated from WT mice and/or not sorted using FITC-conjugated anti-TCR $\gamma\delta$ antibody and anti-FITC beads and AutoMACS (Myltenyi

Biotech) according to manufacturers' instructions; 5×10^6 cells were intravenously injected into IL-15^{-/-} mice one day before oral infection. To produce hematopoietic chimeras, WT (CD45.1) or IL-15^{-/-} (CD45.2) recipient mice were lethally irradiated at 900 rads with a Ce¹³⁷ source and intravenously injected with 5×10^6 bone marrow cells from either WT or IL-15^{-/-} donor mice. After 8 weeks, reconstitution was controlled using CD45.2 and CD45.1 markers on blood lymphocytes. For infection, mice were intragastrically gavaged with 35 cysts from *T. gondii* 76K strain and their weight and survival were monitored. Histology was evaluated on paraffin sections and parasitic load determined by quantitative PCR on day 7 PI (Burg et al., 1988). For in vivo blocking or depletion experiments, WT mice received intraperitoneal injections of 100 µg of control isotypes or of anti-NKG2D (clone CX5, IgG1 gift from Novo Nordisk, Denmark) on days -1 and 5 or of anti-CCL3 (MAB450, IgG2a, R&D systems) on days 4 and 5 PI, or anti-NK1.1 (clone PK136, IgG2a, AbDserotec) on days -1, 1 and 5.

Controls and patients

Peripheral blood was obtained from healthy adult volunteers. Intestinal biopsies were obtained from 19 patients with Crohn's disease and 15 controls (age: 7-15 years) according to protocol AOM08087 approved by Ethical Committee Ile de France II.

Lymphocyte isolation and cell cultures

Mouse lymphocytes were isolated from blood, spleen, MLN, small intestinal epithelium and LP as previously described (Mennechet et al., 2004). Peritoneal mononuclear cells were harvested in cold PBS. Human PBL were isolated on Ficoll Hypaque gradient according to standard procedures.

All cultures were performed in RPMI supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, 30 mM HEPES (Gibco) and 0.05 mM β-mercaptoethanol (Biorad) at 37°C and in 5% CO₂. Intestinal biopsies (5mm) were cultured alone for 24h in 48 well plates. Lymphocytes and peritoneal mononuclear cells (1×10^6 /mL) were cultured in 96 well plates alone or in the presence of human IL-15 (R&D Systems) or of 10 µg/mL STAg (Grunvald et al., 1996). Production of IL-1β, IL-6, TNFα, IFNγ or CCL3 was measured in culture supernatants by ELISA (R&D Systems). Stimulation of human PBL was performed using human IL-15 and/or coated anti-CD3 (5 µg/well) and soluble anti-CD28 (0.25 µg/ml) antibodies (BD Biosciences).

Phenotyping and lymphocyte subset isolation by flow cytometry

Isolated cells were incubated with anti-FcγRII/III mAb (Fcblock, 2.4G2 ; BD Pharmingen) for 10 min and then stained with various mixes of directly coupled mAbs : Ly6C-FITC, Ly6G-PE, F4/80-PECy5, CD3-PE, CD3-APCCy7, NK1.1-APC, CD127-PECy7, NKP46-PECY5, CD11b-APCH7, CD11c-PECY7, TCRαβ-APC (BD Pharmingen), CD45-eFluor450 (eBiosciences), Aqua view blue (Invitrogen) or control isotypes. For intracellular staining, LPL suspensions were incubated for 4 h in culture medium added with 2μl/ml Brefeldin A (Sigma-Aldrich) at 37°C. After surface staining, cells were fixed with 2% paraformaldehyde (Euromedex), permeabilized using 0.5% saponin (Sigma) and stained intracellularly with anti-IFNγ-APC, anti-TNFα-APC (BD Pharmingen) or anti-CCL3-PE mAbs (R&D systems). Cells were analysed on a FACScanto II or sorted using a FACS Aria cell sorter III (BD Biosciences).

Quantitation of Gene Expression by Real-Time PCR

Tissues were harvested in RNA later (Qiagen, disrupted in RLT buffer (Qiagen) using a FastPrep machine (MP Biomedicals). Cells were lysed in RLT (Quiagen). RNA was extracted with RNAeasy kit (Qiagen) and 1μg was reversed transcribed to cDNA with Superscript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed using TaqMan gene expression assays (*hpert*, *ifng*, *il1b*, *tnfa*, *il6*, *ccl2*, *ccl3*, *ccl4*, *ccl5*, *ccr1*, *ccr2*, *ccr5*) and TaqMan Universal PCR master mix and GeneAmp 7000 machine (Applied Biosystems). CDNA samples were assayed in duplicates and expression levels were normalized relative to HPRT with Ct calculation.

Statistical Analysis

Groups were compared with GraphPad Prism software using unpaired Mann-Whitney test.

Results

IL-15 controls the severity of intestinal inflammation induced by *T. gondii* in C57BL/6 mice: In wild type male C57BL/6 mice, oral gavage with 35 cysts of *T. gondii* strain 76 K induced a severe jejuno-ileitis leading to animal death between days 10 and 13. In IL-15^{-/-} mice, oral infection was associated with a significant weight loss, but all animals survived the early phase of infection and the severity of ileal lesions was markedly attenuated. In contrast, no significant increase in parasite load was observed in the intestine, mesenteric

lymph nodes (MLN), liver and spleen of IL-15^{-/-} mice indicating that, in this model, IL-15 is not necessary to control parasite replication but rather contributes to intestinal inflammation. These results differentiate the role of IL-15 from that of IFN γ which is necessary not only to induce intestinal inflammation (Liesenfeld et al., 1996) but also to control local and systemic replication of the parasite.

IL-15 can be produced by many cell types including enterocytes, dendritic cells (DC) and macrophages. Accordingly, up-regulation of *il15* mRNA following *T. gondii* infection was observed in enterocytes as well as in CD11c⁺ CD11b⁺ DC isolated from LP and MLN. To define which cellular source of IL-15 determines the severity of the ileitis, the outcome of *T. gondii* infection was compared in hematopoietic chimeras. All irradiated WT mice reconstituted with IL-15^{-/-} bone marrow developed a lethal ileitis while 80% of IL-15^{-/-} mice reconstituted with WT bone marrow survived, indicating that a non hematopoietic source of IL-15, presumably epithelial cells, controls the intensity of intestinal inflammation. Consistent with this hypothesis, IL-15T_{GE} mice that constitutively express human IL-15 in their gut epithelium developed an even most severe ileitis and died 2-3 days before WT animals (Ohta et al., 2002). Altogether, these data indicate that stroma-derived IL-15 potentiates inflammation during *T.gondii*-induced ileitis.

The enhancing effect of IL-15 on intestinal inflammation does not depend on IEL during *T. gondii*-induced ileitis: A stromal source of IL-15 is necessary for the expansion of CD8 $\alpha\alpha$ IEL and more particularly of TCR $\gamma\delta$ IEL (Schluns et al., 2004b). Accordingly, TCR $\gamma\delta$ IEL were almost absent in IL-15^{-/-} mice reconstituted or not with WT bone marrow which developed an attenuated form of ileitis. Conversely, they were present in WT chimeras reconstituted with IL-15^{-/-} bone marrow, in which *T. gondii* induced a lethal ileitis, suggesting their possible contribution to IL-15-driven inflammation. Yet, adoptive transfer of 5x10⁶ TCR $\gamma\delta$ IEL did not aggravate the course of the ileitis in IL-15^{-/-} recipients while, conversely, all TCR δ ^{-/-} mice developed a lethal ileitis, a result in keeping with previous observations (Liesenfeld et al., 1996).

Epithelium-derived IL-15 might also activate cytotoxic CD8⁺ TCR $\alpha\beta$ ⁺ IEL. After oral infection with *T. gondii*, this subset of IEL can *in vitro* kill *T. gondii*-infected targets (Chardes et al., 1994). Moreover, IL-15 can stimulate IEL cytotoxicity by inducing perforin and granzymes (Mention et al., 2003) or by enhancing expression of and/or signaling via the activating NKG2D receptor (Hue et al., 2003; Meresse et al., 2004). NKG2D expression

increased significantly in LP CD8⁺ TCRαβ⁺ lymphocytes from WT but not from IL-15^{-/-} mice but IEL remained largely NKG2D negative during *T.gondii* ileitis. Moreover intravenous treatment with a blocking (but non-depleting) anti-NKG2D antibody (Ogasawara et al., 2004) did not modify the course of the disease in WT mice. The ratio of granzyme B and perforin to CD3ε mRNA expression increased during infection in WT but not in IL-15^{-/-} mice, so that a significant difference was observed on day 7. Yet, neither the course of the lethal ileitis, nor the parasitic load were modified in either perforin^{-/-} or β2m^{-/-} mice arguing against a major contribution of cytotoxic CD8⁺ T cells to early protection against the parasite, as well as to IL-15-mediated amplification of intestinal inflammation in this acute model.

IL-15 is dispensable for the induction of *T. gondii*-specific Th1 response: A previous study has suggested that IL-15 promotes specific Th1 response during *T. gondii* infection (Combe et al., 2006). This response is thought to be initiated by mucosal CD11c⁺ DC cells which are infected by the parasite in the mucosa within the first two days of infection and then emigrate to MLN where they can be detected between days 3 to 7 post-infection (Courret et al., 2006). In MLN, DC elicit *T. gondii*-specific CD4⁺ Th1 cells which then home into LP. To analyze the contribution of IL-15 to *T.gondii*-induced intestinal Th1 response, *ifng* mRNA and IFNγ protein expression were compared on days 0, 3, 5 and 7 in jejunal biopsies and in cells isolated from MLN and LP of IL-15^{-/-} and WT mice and stimulated or not with soluble *T.gondii* antigen (STAg). A slight delay was observed in the up-regulation of *ifng* mRNA in MLN lymphocytes and of IFNγ secretion by LPL cultured with STAg. Yet, on day 7, LPL from both groups of animals spontaneously produced massive and comparable amounts of IFNγ which were not further increased in the presence of STAg. Furthermore, no significant difference could be demonstrated between WT and IL-15^{-/-} mice in the percentages of IFNγ-producing CD4⁺ cells in either MLN or LPL at all time points. Altogether, these results indicate that WT and IL-15^{-/-} mice develop comparable CD4⁺ Th1 responses, a finding in keeping with comparable parasitic burden in both animal groups.

The recruitment of inflammatory monocytes is impaired in IL-15^{-/-} mice during *T. gondii*-induced ileitis: Inflammation in WT and IL-15^{-/-} mice was next assessed by comparing intestinal IL-1β, TNFα, IL-6 production during infection. Up-regulation of *il1b*, *tnfa*, *il6* mRNA was first observed in intestinal biopsies and in isolated LP cells on day 5 post-infection and increased markedly on day 7 when copious amounts of IL-1β, TNFα, IL-6 were

spontaneously released in the supernatants of either biopsies or isolated LP. Up-regulation of inflammatory cytokines was observed in both animal groups but was significantly higher in WT than in IL-15^{-/-} mice.

To identify the cellular source of inflammatory cytokines, LP cells were isolated from infected WT and IL-15^{-/-} mice on day 7 and sorted into CD3⁺, CD19⁺, CD3⁻NK1.1⁺, CD45⁺ CD3⁻CD19⁻NK1.1⁻ cells. RT-PCR analysis of sorted cells showed that *il1b*, *tnfa*, *il6* mRNA were only up-regulated in CD45⁺ CD3⁻CD19⁻NK1.1⁻ cells and confirmed the difference between IL-15^{-/-} and WT mice. ELISA authenticated the secretion of IL-1 β and TNF α by LP CD45⁺ CD3⁻CD19⁻NK1.1⁻ cells isolated from WT mice and showed that the secretion was below the detection limit in the same fraction isolated from IL-15^{-/-} mice. To further identify the cellular origin of inflammatory cytokines, LP cells from WT mice were isolated at day 7 post-infection (PI) and analyzed by flow cytometry after staining with a panel of surface markers and intracellular TNF α . TNF- α was absent in CD3⁺ and CD11c⁺ LP cells and was exclusively detected in Ly6C^{hi} F4/80⁺ Ly6G⁻ CD11b⁺ CD11c⁻ LP cells, a phenotype typical of inflammatory monocytes (IM) (Auffray et al., 2009; Dunay et al., 2008).

Altogether, these data suggested that IL-15 was necessary to promote the activation and/or the recruitment of IM. IL-15 alone did not induce the production of TNF α and/or IL-1- β in peritoneal macrophages from WT mice either naive or infected peritoneally with *T. gondii* for 3 days and did not synergize with LPS. IL-15, even at high concentrations, also failed to stimulate the production of inflammatory cytokines by LP cells from naive WT mice even after stimulation by anti-CD3 and anti-CD28 antibodies. In contrast the percentages as well as the absolute numbers of Ly6C^{hi} F4/80⁺ Ly6G⁻ CD11b⁺ CD11c⁻ cells isolated on day 7 were significantly higher in WT than in IL-15^{-/-} mice, suggesting that IL-15 controls the recruitment of IM during infection by *T. gondii*.

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The recruitment of inflammatory monocytes during *T. gondii*-induced ileitis depends on CCR1/CCL3 interactions: One attractive hypothesis to explain the role of IL-15 was the induction of chemokines able to stimulate intestinal migration of IM. *Ccl2*, *-3*, *-4*, *-5* and *cxcl10* mRNA levels were markedly increased in intestinal biopsies and in isolated LP cells on day 7 PI compared to naive animals. The increase was comparable in WT and IL-15^{-/-} mice for all tested chemokines except for *ccl3* mRNA levels which were significantly less induced in IL-15^{-/-} than in WT mice (Figures 1A). Accordingly, CCL3 concentrations were significantly reduced in supernatants of LP cells from infected IL-15^{-/-} mice. To demonstrate that CCL3 participates in the recruitment of IM during *T. gondii* infection, WT mice were

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injected on days 4 and 5 with a blocking anti-CCL3 antibody. This treatment strongly reduced the percentage and absolute numbers of Ly6C^{hi} F4/80⁺ LP cells as well as the production of IL-1 β and TNF α (Figures 1B, 1C, 2E), confirming the role of CCL3.

CCL3 binds two distinct receptors, CCR1 and CCR5, both of which can be expressed by IM. On day 7 PI, *ccr5* mRNA levels were similarly increased in jejunal biopsies and in LP cells from WT and IL-15^{-/-} mice. In contrast, *ccr1* mRNA was significantly lower in IL-15^{-/-} than in WT mice (Figure 1D) and were almost undetectable in mice treated by anti-CCL3 antibody (Figure 2F). The role of CCR1 in the recruitment of IM was therefore assessed using CCR1^{-/-} mice. Contrasting with their recruitment in infected WT mice, no increase in Ly6C^{hi} F4/80⁺ LP cells was observed in CCR1^{-/-} mice during *T. gondii* infection and the percentage and absolute numbers of such cells on day 7 were even lower than in IL-15^{-/-} mice (Figure 1E). Accordingly, no increase in TNF α -producing LP cells was observed in infected CCR1^{-/-} mice (Figure 1F), and the production of TNF α and IL-1 β by biopsies or LP cells remained very low (Figures 1G), a result that contrasted with the comparable production of IFN γ and CCL3 by LP cells from CCR1^{-/-} and WT mice (Figure 2H). As already described in CCR1^{-/-} mice (Khan et al., 2001), intestinal lesions were less severe on day 7 PI than in WT mice but the parasitic load was increased 20 to 100 fold in liver and/or spleen compared to WT and IL-15^{-/-} mice. Altogether these results indicated that the intestinal recruitment of Ly6C^{hi} F4/80⁺ IM during *T. gondii*-induced ileitis depends on CCL3/CCR1 interactions and can be enhanced by IL-15 through the control of CCL3 production.

CCL3 production during *T. gondii*-induced ileitis depends on intestinal IL-15-dependent NKP46⁺ NK1.1⁺ CD127⁻ cells: CCL3 can be produced by cells of hematopoietic or epithelial origin. Accordingly, an increase in *ccl3* transcripts was observed in both enterocyte and LP cell fractions isolated from WT mice on day 7 PI. Yet, *ccl3* transcripts was 100-200 fold more increased in WT LP cells than in enterocytes, indicating that, during *T. gondii* infection, CCL3 was mainly produced by LP cells. Analysis of sorted LP cell subsets showed that, on day 7 PI, *ccl3* mRNA was strongly expressed by a minor subset of NK1.1⁺ CD3⁻ cells. Consistent with the hypothesis that the IL-15 dependent source of CCL3 may be NK cells, absolute numbers of NKP46⁺ CD3⁻ cells were significantly decreased in naive and infected IL-15^{-/-} mice compared to WT mice. Recent work indicates that LP contains two main subsets of NK cells. A first subset of NKP46⁺ NK1.1⁻ CD127⁺ cells depends on IL-7 and secretes IL-22, a cytokine that strengthens the intestinal barrier. A second subset of NKP46⁺ NK1.1⁺ CD127⁻ cells depends on IL-15 and thus resembles classical splenic NK cells. Yet, its

function in intestine remains ill-defined (Satoh-Takayama et al.). Intracellular staining of spleen cells stimulated by IL-15 showed a selective induction of CCL3 in NKP46⁺ NK1.1⁺ CD127⁻ cells, indicating that they can be a source of CCL3. Contrary to NKP46⁺ NK1.1⁻ CD127⁺ cells, NKP46⁺ NK1.1⁺ CD127⁻ cells were absent in the LP of naive and infected IL-15^{-/-} mice, confirming their dependence on IL-15.

Analysis of hematopoietic chimeras further indicated that a stromal source of IL-15, presumably epithelial cells, was necessary for the optimal differentiation and/or local homeostasis of NKP46⁺ NK1.1⁺ CD127⁻ cells. Thus, efficient reconstitution of LP CD3⁻ NK1.1⁺ cells was observed in irradiated WT mice reconstituted with IL-15^{-/-} bone marrow (WT+BM IL-15^{-/-}) but not in irradiated IL-15^{-/-} mice receiving WT bone marrow (IL-15^{-/-}+BM WT). Interestingly, *ccl3* and *ccr1* mRNA levels and percentages of LP Ly6C^{hi} F4/80⁺ IM remained as low in infected IL-15^{-/-}+BM WT chimeras as in IL-15^{-/-} mice and significantly less than in WT mice and WT+BM IL-15^{-/-} chimeras. Consequently, a positive correlation was observed between percentages of LP CD3⁻ NK1.1⁺ cells and level of *ccl3* mRNA or of LP Ly6C^{hi} F4/80⁺ IM. Moreover the lack of LP CD3⁻ NK1.1⁺ cells in IL-15^{-/-}+BM WT chimeras coincided with a lesser severity of the ileitis. Altogether these data suggested that LP NKP46⁺ NK1.1⁺ CD127⁻ cells depend on a stromal source of IL-15 for their differentiation/homeostasis and are an important source of CCL3 during *T. gondii* infection that might stimulate the recruitment of CCR1⁺ IM.

To confirm this hypothesis, mice were treated with a depleting anti-NK1.1 antibody on day -1 before infection, and on days 1 and 4 PI. At day 7 PI, this treatment had efficiently depleted LP NKP46⁺ NK1.1⁺ CD127⁻ cells and resulted in a significant decrease in CCL3 production and *ccr1* mRNA levels. Accordingly, the numbers of LP Ly6C^{hi} F4/80⁺ IM and the spontaneous production of IL-1 β and TNF α by isolated LP cells or whole intestinal tissue were also significantly reduced.

CCL3 and CCR1 expression are up-regulated in Crohn's disease: In Crohn's disease (CD), TNF α -producing inflammatory macrophages play a central role in gut inflammation attested by the efficiency of therapeutic administration of anti-TNF α antibodies. Recent work has identified their CD33⁺ CD14⁺ phenotype and shown their expression of several chemokine receptors, including CCR1 which is absent on gut resident CD33⁺ CD14⁻ myeloid cells (Kamada et al., 2008). In keeping with these data, *il1b*, *tnfa*, *il6* and *ccr1* mRNA levels were significantly up-regulated in inflamed biopsies from CD patients. (Figures 3A, 3C). Interestingly, *ccl3* (but not *ccl5*) mRNA was also significantly increased in the same

biopsies (Figures 3B) and there was a positive correlation between the level of *ccl3* and *ccr1* (spearman coefficient r : 0.64) but not of *ccl3* and *ccr5* mRNA (Figure 3D). Recent work has also suggested a selective increase in the subset of NKP46⁺ CD56⁺ CD127⁻ CD22⁺ NK cells in the intestine of CD patients (Kamada et al.). Sustaining the hypothesis that the later cells may be the source of CCL3 in CD, we observed a 1000 fold increase in *ccl3* transcripts in FACS-sorted CD45⁺ CD3⁻ NKP46⁺ CD56⁺ cells stimulated with IL-15 which contrasted with the lack of *ccl3* mRNA induction in purified CD3⁺ T cells stimulated by IL-15 and/or anti-CD3/CD28 antibodies (Figure 3E). Previous reports have suggested that IL-15 is up-regulated in CD. Consistent with the mainly post-transcriptional control of IL-15 in humans (Fehniger and Caligiuri, 2001), no change in *il15* mRNA could be demonstrated in CD biopsies. Yet, there was a significant increase in mRNA encoding IL15R α (Figure 3D), a receptor necessary for transpresentation of IL-15 to T and NK cells expressing IL15R β / γ_c (CD122/CD132), the signaling module activated by IL-15 in lymphocytes (Sandau et al., 2004; Schluns et al., 2004a).

Discussion:

Herein we describe a new mechanism underlying the role of IL-15 in inflammation and show that IL-15 orchestrates a cross-talk between CCL3-producing NK cells and inflammatory CCR1⁺ monocytes which stimulates the recruitment of the latter cells in the inflamed gut of mice orally infected by *T. gondii*. Our data also provide the first demonstration that CCR1 is central to the intestinal recruitment of IM and uncover a novel function for the NKP46⁺ NK1.1⁺ CD127⁻ subset of gut innate immune cells. Preliminary data suggest that a comparable mechanism may operate in the inflamed gut of CD patients.

In response to oral infection by the virulent 76K strain of *T. gondii*, C57Bl/6 mice develop an immune response which is a two-edge sword as it permits early control of parasite replication but results in uncontrolled intestinal inflammation and animal death within 10 days (Liesenfeld et al., 1996; Mennechet et al., 2002). The role of IL-15 in the immune response against *T. gondii* infection is controversial. Combe et al have suggested that intestinal inflammation is reduced and *T. gondii* infection less well controlled in orally infected IL-15^{-/-} mice (Combe et al., 2006). In contrast, Lieberman *et al* did not detect any increase in cyst burden in IL-15^{-/-} mice after intraperitoneal infection (Lieberman et al., 2004). Our results confirm that intestinal inflammation is markedly attenuated in infected IL-15^{-/-} mice. Yet, no difference could be demonstrated in parasitic load in intestine, liver and spleen between IL-

15^{-/-} and WT mice suggesting that, in our conditions of infection, IL-15 did not impair early parasite control and therefore that this model was valuable to analyze the mechanisms underlying the role of IL-15 in intestinal inflammation.

Following oral infection with *T. gondii*, IL-15 transcription was up-regulated in both
5 in epithelial and dendritic cells. The dramatic course of the ileitis in irradiated WT mice reconstituted with IL-15^{-/-} bone marrow and in IL-15Tg_E mice over-expressing IL-15 in the gut epithelium contrasted with the attenuated ileitis observed in IL-15^{-/-} mice either or not irradiated and reconstituted with WT bone marrow, altogether pointing to a central role of epithelium-derived IL-15 in shaping the severity of intestinal inflammation. It has recently
10 been suggested that acute induction of IL-15 in intestinal epithelial cells following intra-peritoneal injection of poly-IC can stimulate NKG2D expression on IEL and results in a cytolytic attack of Rae1⁺ enterocytes (Zhou et al., 2007). Contrasting with the marked NKG2D up-regulation observed in IEL from IL-15Tg_E mice which are chronically exposed to epithelium-derived IL-15 (data not shown), IEL remained largely NKG2D negative during the
15 acute course of *T.gondii*-induced ileitis, and a modest up-regulation of NKG2D was only observed on CD8⁺ TCRαβ⁺ LPL. In addition, intraperitoneal administration of a blocking NKG2D antibody did not modify the course of the ileitis. Finally, this course was also unchanged in TCRδ^{-/-}, β₂m^{-/-} and perforin^{-/-} mice, indicating that TCRγδ and CD8⁺ TCRαβ⁺ IEL do not play a determinant role in ileitis severity. This conclusion is consistent with two
20 previous studies (Denkers et al., 1997) but is at odd with a recent report suggesting the contribution of CCR2-dependent CD103⁺ CD11c⁺ IEL to *T. gondii*-induced gut inflammation (Egan et al., 2009). Yet, it is noticeable that, during *T.gondii* infection, CCR2 may be involved not only in the recruitment of IEL but also in the migration of IM which, as discussed below, play a central role in *T.gondii*-induced ileitis.

25 An alternative hypothesis suggested by the work of Combe et al was a role of IL-15 in the amplification of the *T. gondii*-specific Th1 response (Combe et al., 2006). Based on the analysis of MLN at day 10 post-infection, these authors have indeed suggested that IL-15 is necessary for optimal priming of Th1 responses. Day 10 however appears a late time point to evaluate priming since mucosal dendritic cells infected by the parasite are first detected in
30 MLN on day 3 (Courret et al., 2006) and ileitis is full blown by day 7. Furthermore, another report has suggested that the serum IFNγ response is not altered in IL-15^{-/-} mice after intraperitoneal infection (Lieberman et al., 2004). To assess precisely the influence of IL-15 on the course and intensity of the Th1 response, this response was compared in WT and IL-15^{-/-} in MLN and LP on days 3, 5 and 7. No significant difference in CD4⁺ (or CD8⁺) Th1

response could be demonstrated in MLN and LP, a finding consistent with the normal control of the parasitic load. A small up-regulation of IFN γ mRNA in MLN on day 3 and a modest secretion of IFN γ by STAg-stimulated LPL on day 5 were observed in WT but not IL-15^{-/-} mice. In LP, this early production may derive from the NKP46⁺ NK1.1⁺ CD127⁻ NK cells which can produce IFN γ (Sato-Takayama et al., 2008).

Contrasting with the comparable production of IFN γ in the intestinal mucosa at day 7 in both animal groups, the production of IL-1 β , TNF α and IL-6 was significantly diminished in IL-15^{-/-} mice. Analysis of the cellular source of inflammatory cytokines unambiguously demonstrated their production by a subset of Ly6C^{hi} F4/80⁺ Ly6G⁻ CD11b⁺ CD11c⁻ IM. *In vitro* studies have suggested that IL-15 enhances the secretion of TNF α by human monocytes via a T cell contact-dependent mechanism (Liu et al., 2000; McInnes et al., 1996). Yet, adding IL-15 alone or in combination with other stimuli to *in vitro* culture of peritoneal or LP cells failed to enhance TNF α and IL-1 β secretion, arguing against a role of IL-15 in IM differentiation. In contrast, IL-15 stimulated *in vivo* recruitment of IM, as their numbers increased significantly less in the LP of IL-15^{-/-} than of WT infected mice. A central role of IM in oral toxoplasmosis was recently demonstrated in CCR2^{-/-} mice which displayed normal Th1 response but were unable to recruit IM and to control parasite replication. Yet, CCR2 was dispensable for IM migration into the inflamed gut and it was suggested that CCR2 might rather control monocyte emigration from bone marrow (Dunay et al., 2008). Herein, the lesser levels of *ccr1* and *ccl3* mRNA in the intestine of infected IL-15^{-/-} than WT mice pointed to their possible role in the local recruitment of IM. Increased susceptibility to *T. gondii* has already been reported in CCR1^{-/-} mice and ascribed to the impaired recruitment of cells defined as neutrophils on the basis of their staining with the anti-Ly6 RB6-8C5 antibody (Khan et al., 2001). Yet this antibody does not discriminate between Ly6G, only expressed on neutrophils, and Ly6C expressed on both neutrophils and monocytes. Moreover it was recently shown that the recruitment of IM predominates over that of neutrophils during *T. gondii*-induced ileitis (Dunay et al.). The respective role of CCR1 and CCR2 in tissue migration of IM is controversial but CCR1 plays a prominent role in inflamed joints (Schall and Proudfoot). Demonstrating that CCR1 and CCL-3 interactions are instrumental for IM recruitment in inflamed intestine, LP IM were markedly reduced in infected WT mice treated by a blocking anti-CCL3 antibody and almost undetectable in infected CCR1^{-/-} mice. Furthermore, the production of IL-1 β and TNF α by LP cells was abolished in the latter mice and the lack of IM was associated with a markedly increased parasitic load and a rapid death

of infected animals (data not shown). In IL-15^{-/-} mice, the recruitment of IM may be sufficient to control parasite replication but insufficient to induce irreversible intestinal lesions.

A clue to link IL-15 and CCL3-dependent recruitment of CCR1⁺ monocytes was the demonstration that LP NKP46⁺ NK1.1⁺ CD127⁻ NK cells are an important source of CCL3 during *T. gondii* infection. Indeed, it was recently shown that this subset of gut innate cells, alike peripheral NK cells, depends on IL-15 for its differentiation and maturation (Sato-Takayama et al.). Previous work has shown that NK depletion using the anti-asialoGM1 antibody markedly reduced intestinal inflammation in WT mice but the role of gut NK cells was not studied (Khan et al., 2006). Our data combining the analysis of IL-15^{-/-} mice, of hematopoietic chimera and of WT mice selectively depleted in NK1.1⁺ cells indicate that LP NKP46⁺ NK1.1⁺ CD127⁻ NK cells contribute, via their production of CCL3, to the recruitment of IM and to the severity of the ileitis after oral infection with *T. gondii*. The analysis of hematopoietic chimeras further indicates that a stromal source of IL-15, presumably epithelial cells, is necessary to drive their differentiation and/or maintain their survival. Whether IL-15 participates in the local induction of CCL3 during infection is unclear. *In vitro*, IL-15 could stimulate CCL3 synthesis by spleen NKP46⁺ NK1.1⁺ CD127⁻ NK cells. *In vivo* during *T. gondii* infection, CCL3 induction was maximal at late time points (after day 5 PI), when the level of IL-15 mRNA had returned to the baseline. Moreover, administration of a blocking anti-IL-15 antibody prevented the death of only 50% of infected WT mice (data not shown). Although we cannot exclude that IL-15 blockade was incomplete, our results suggest that IL-15 is not indispensable for the induction of CCL3 and that other stimuli, yet to be identified, participate in the local induction of CCL3 secretion by NK cells.

Interestingly, it was recently shown that LP NKP46⁺ CD56⁺ CD127⁻ NK cells are selectively increased in the intestine of Crohn's disease patients (Takayama et al.) and that serum detection of IL-15 in CD patients can predict their response to anti-TNF α antibodies (Bouchaud et al.). In keeping with an ancient study (McInnes et al., 1996), IL-15 selectively and strongly stimulated the transcription of *ccl3* in human peripheral NKP46⁺ CD56⁺ NK cells. Furthermore there was a positive correlation between the levels of *ccl3* and of *ccr1* mRNA in intestinal biopsies from CD patients. It is therefore tempting to hypothesize that, in CD patients alike in mice infected by *T. gondii*, intestinal NKP46⁺ CD56⁺ NK cells might produce CCL3, and thereby recruit TNF α producing IM and enhance intestinal inflammation. The increased transcription of IL-15 receptor α observed in inflamed intestine might promote transpresentation of IL-15 to NK cells.

Recent work has enlightened the role of a spectrum of intestinal innate cells in the early response to pathogens and /or intestinal inflammatory responses. A particular emphasis has been put on a novel subset of IL-7 dependent LP NKP46⁺ NK1.1⁻ CD127⁺ NK cells producing IL-22 in the defense against invasive bacteria (Satoh-Takayama et al., 2008). Our results indicate that the second subset of IL-15-dependent gut NK cells may be activated in response to intracellular pathogens and can, via CCL3, participate in the recruitment of IM. IL-15- dependent NK cells may thereby participate in gut defense but also induce deleterious inflammation in and perhaps beyond the gut.

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Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.

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CLAIMS:

1. A method for the treatment of Crohn's disease comprising administering a subject in
5 need thereof with a CCR1 antagonist or an inhibitor of CCR1 or CCL3 gene
 expression.

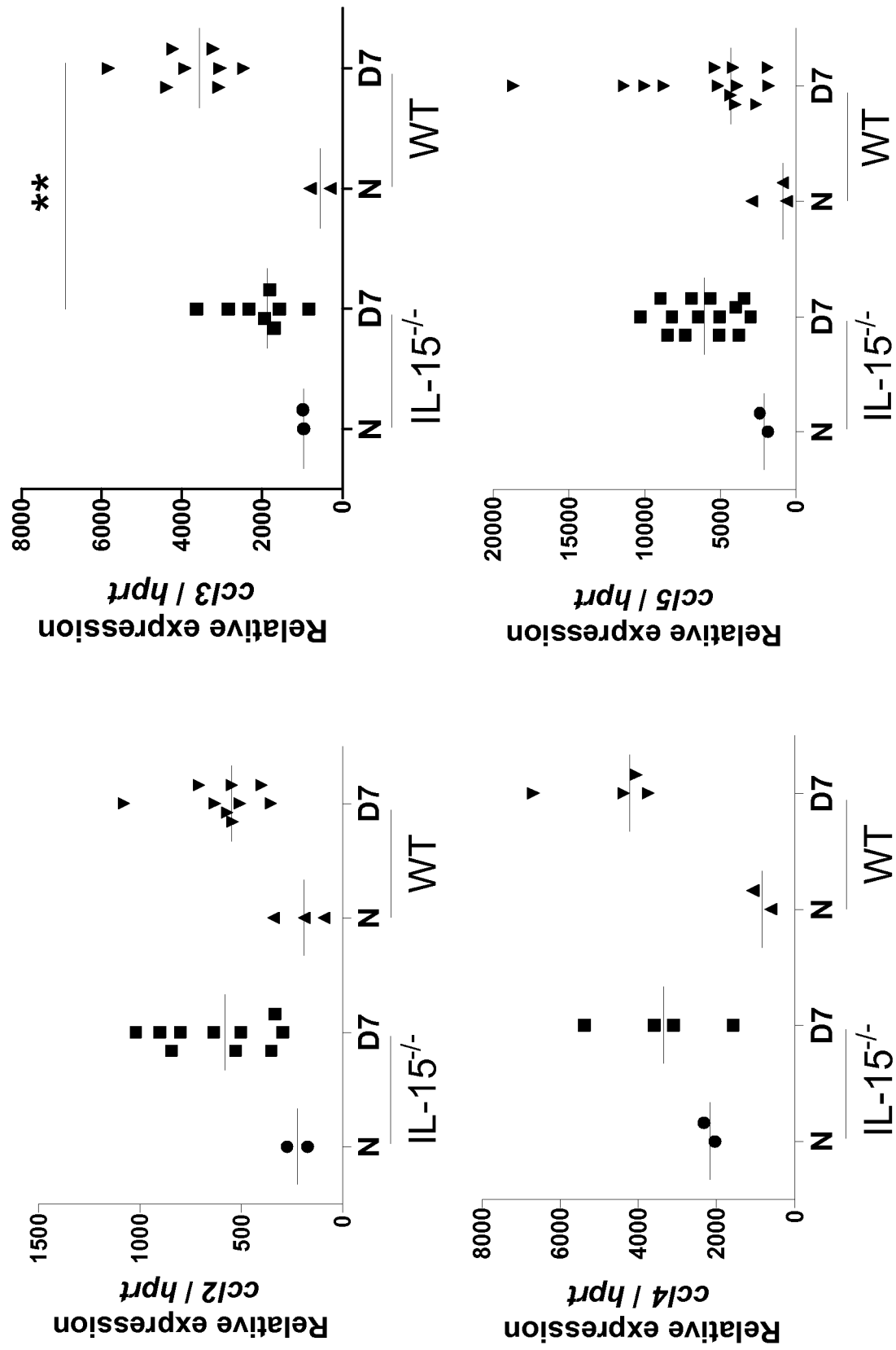


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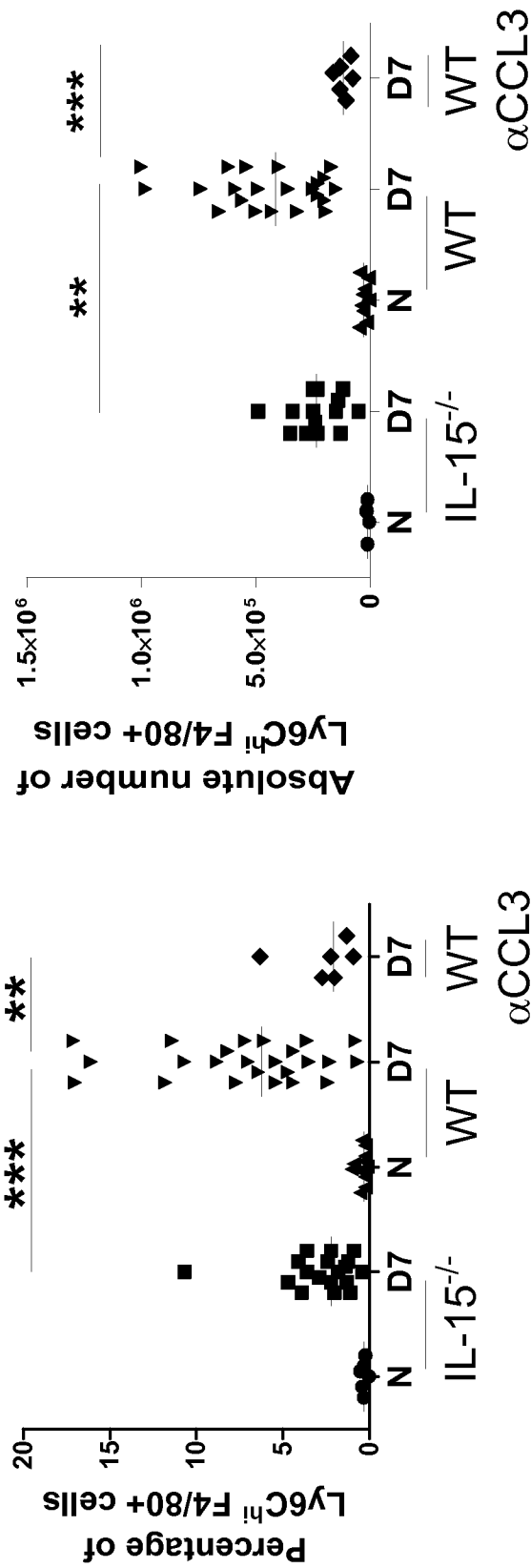


Figure 1B

3/20

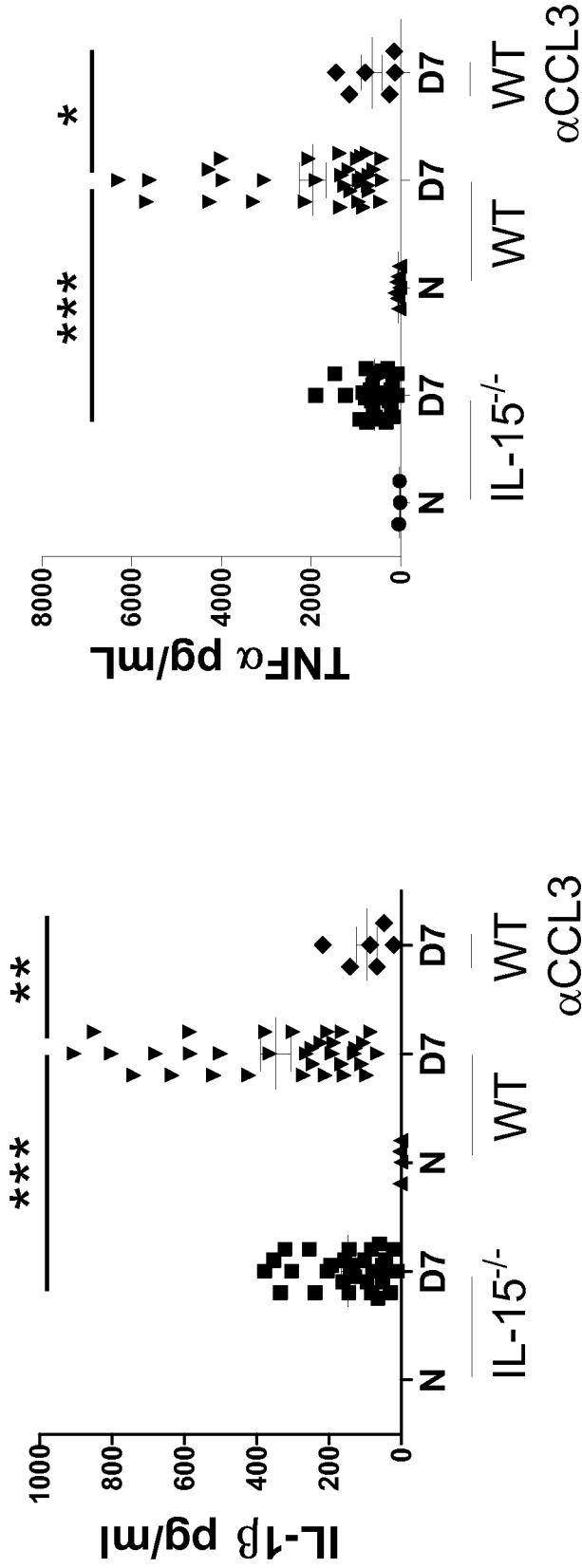


Figure 1C



Figure 1D

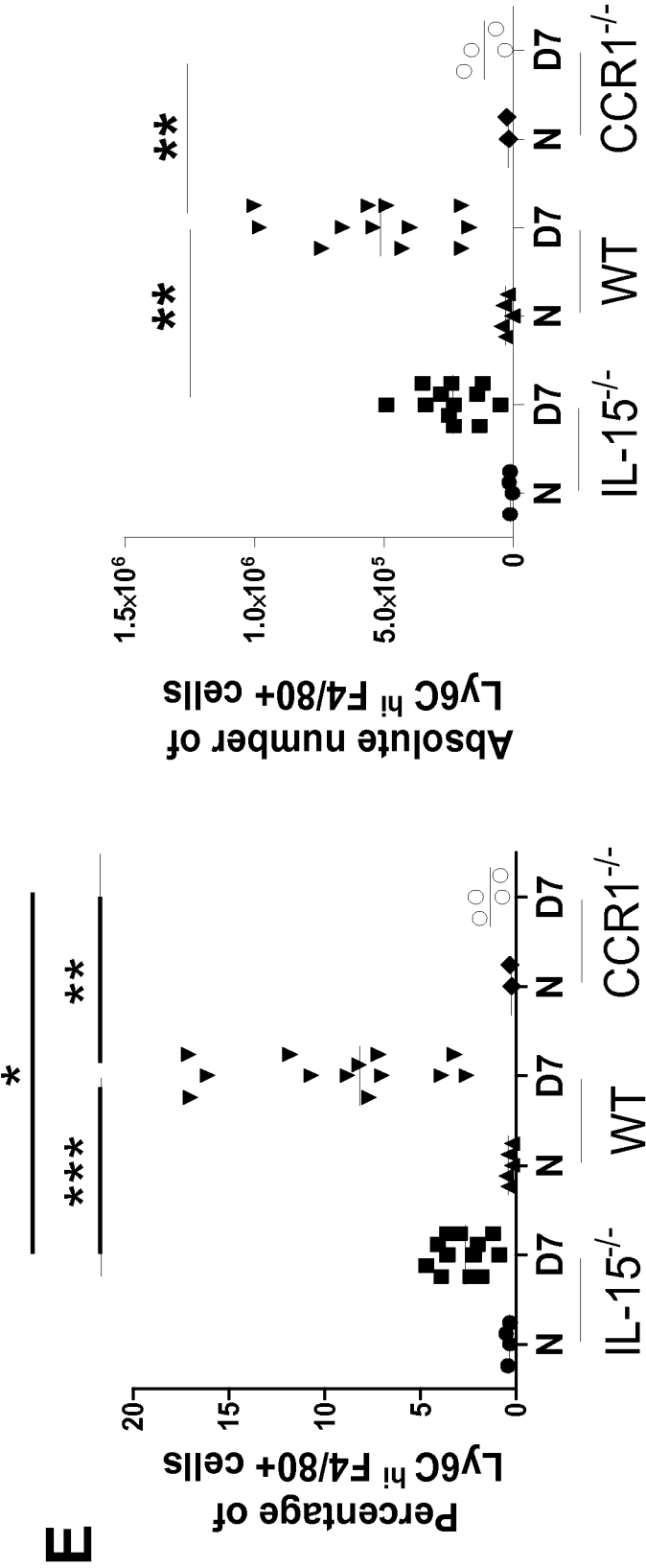


Figure 1E

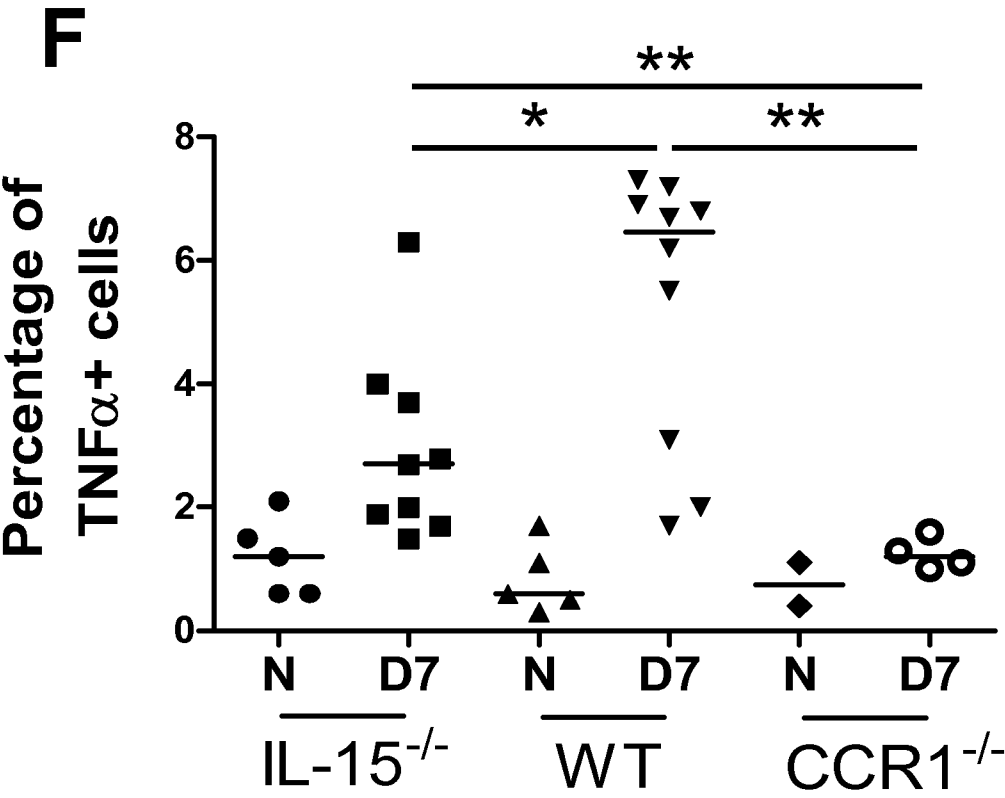


Figure 1F

Figure 1G

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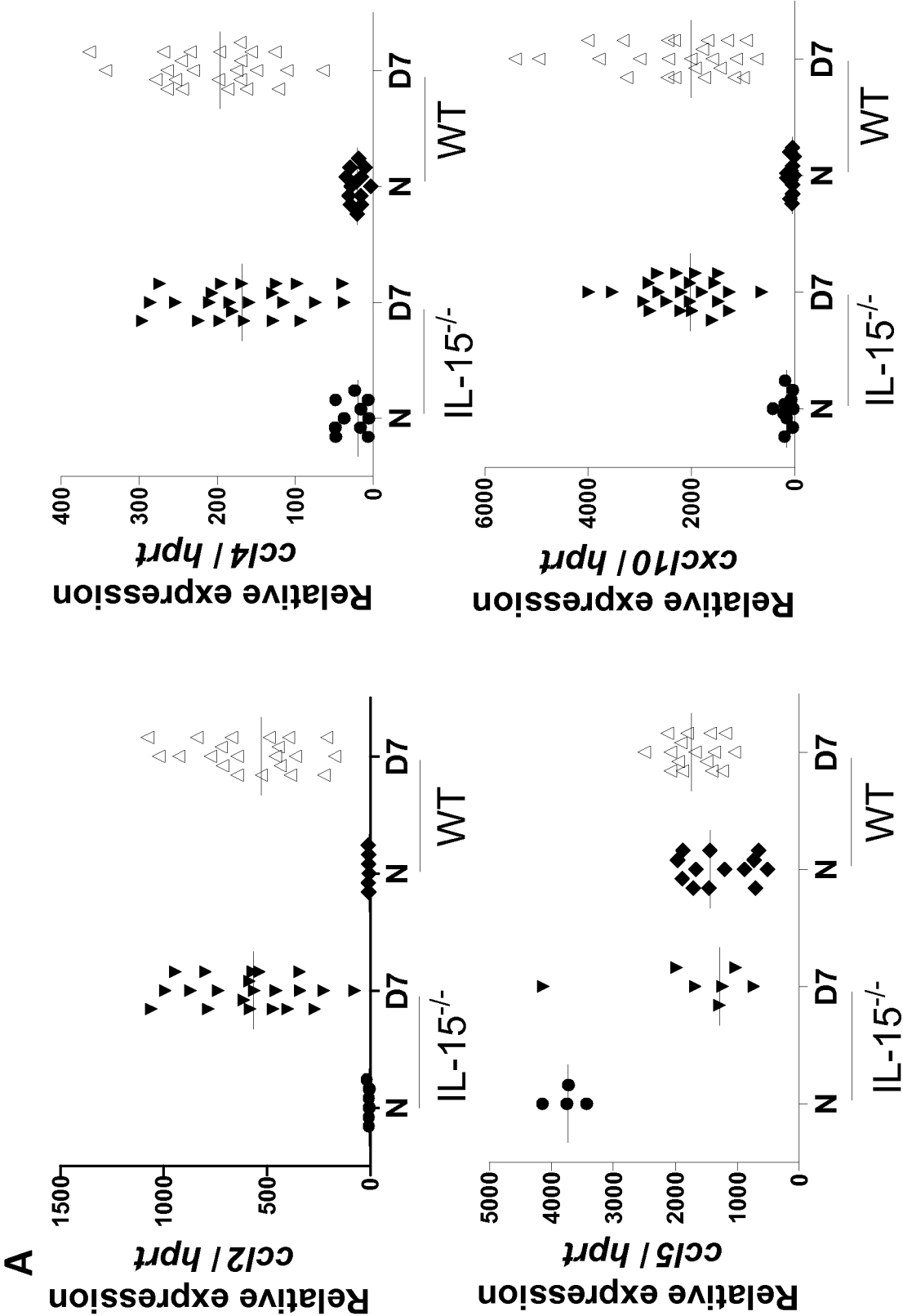


Figure 2A

9/20

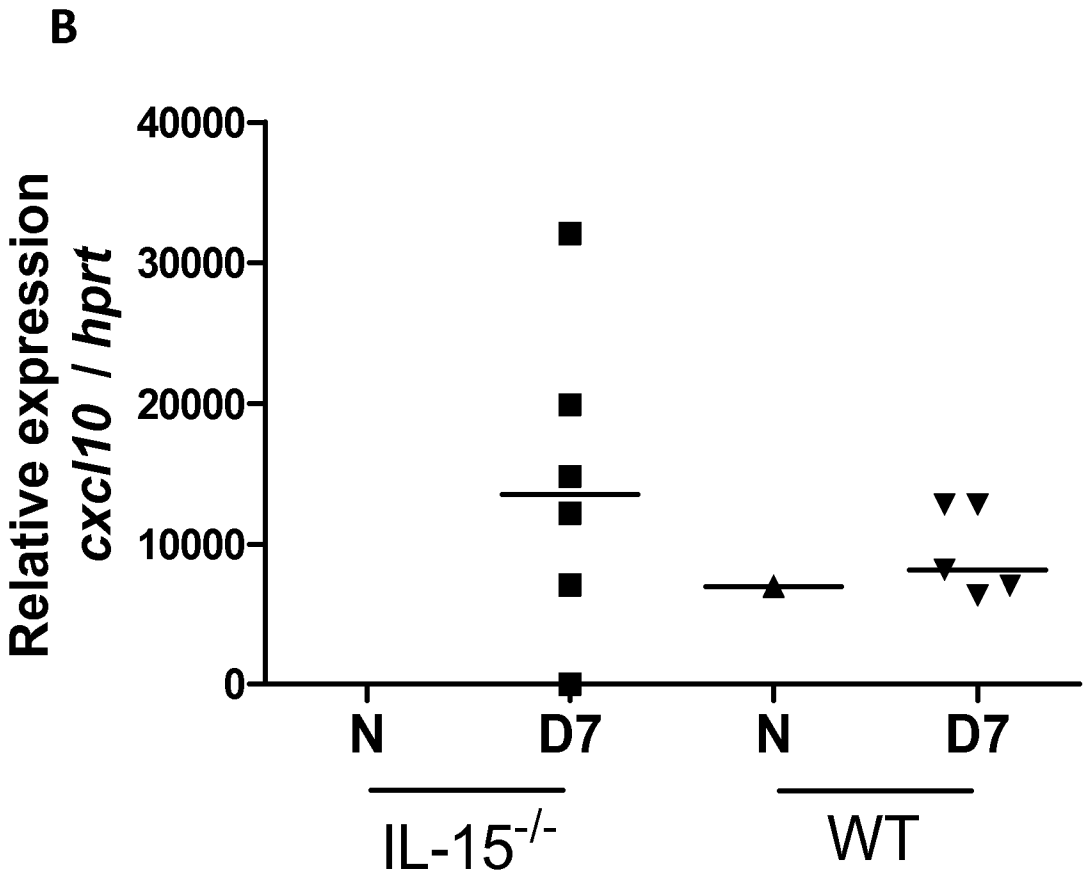


Figure 2B

10/20

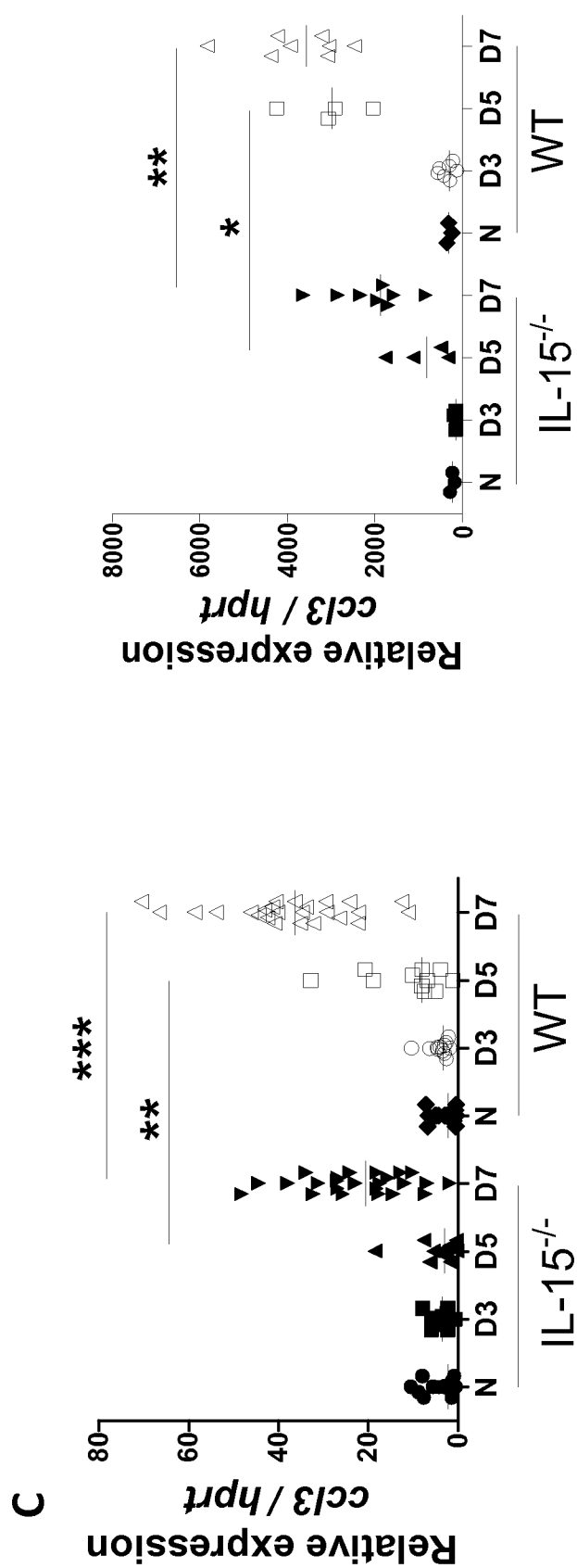


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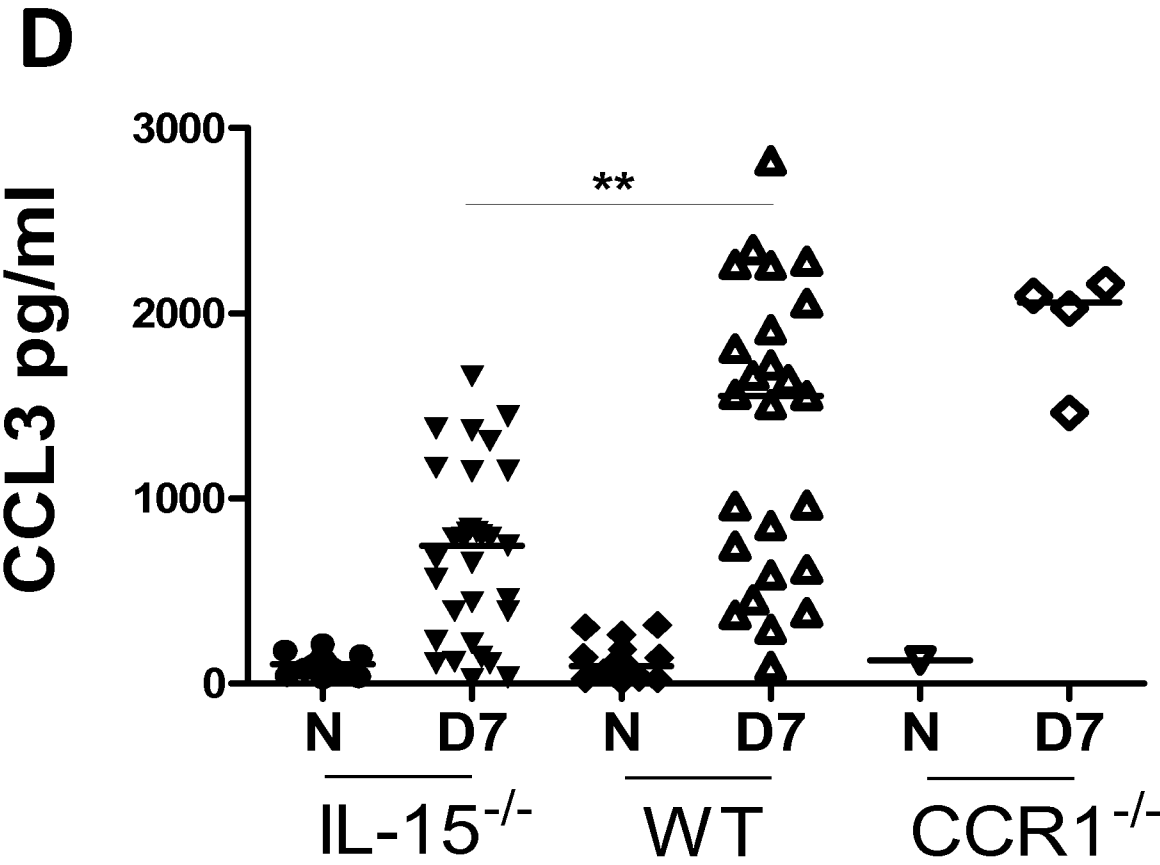


Figure 2D

12/20

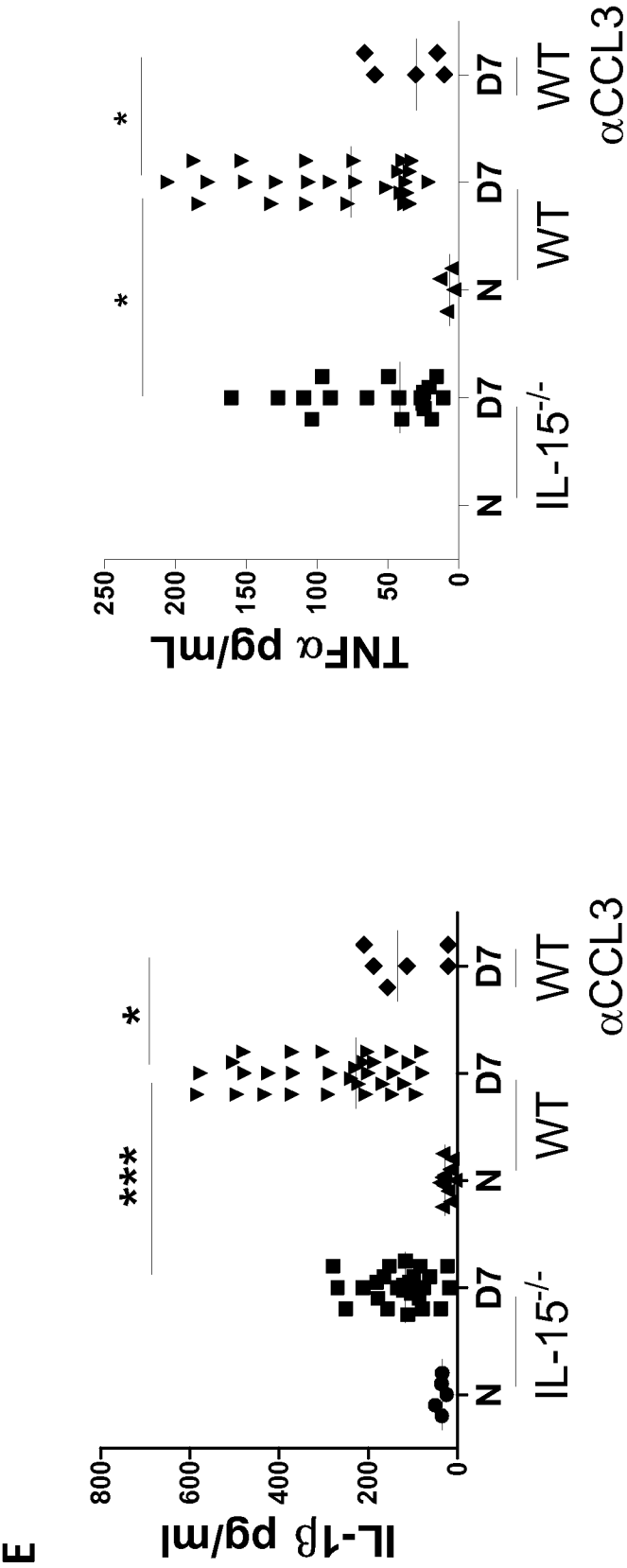


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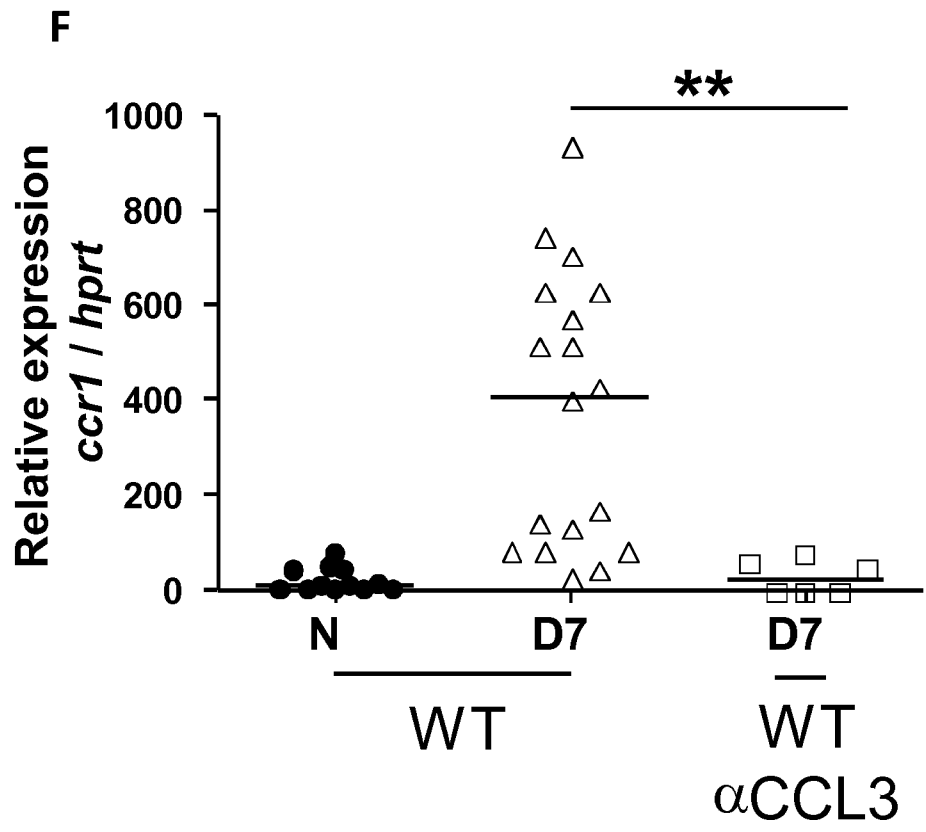


Figure 2F

14/20

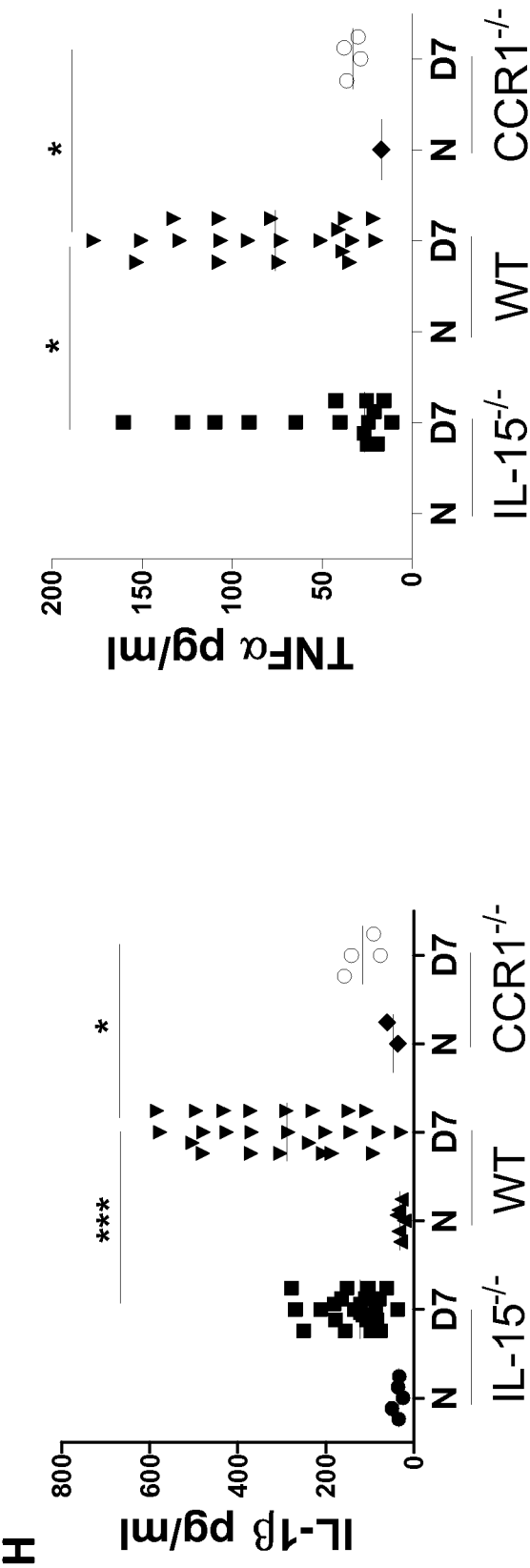


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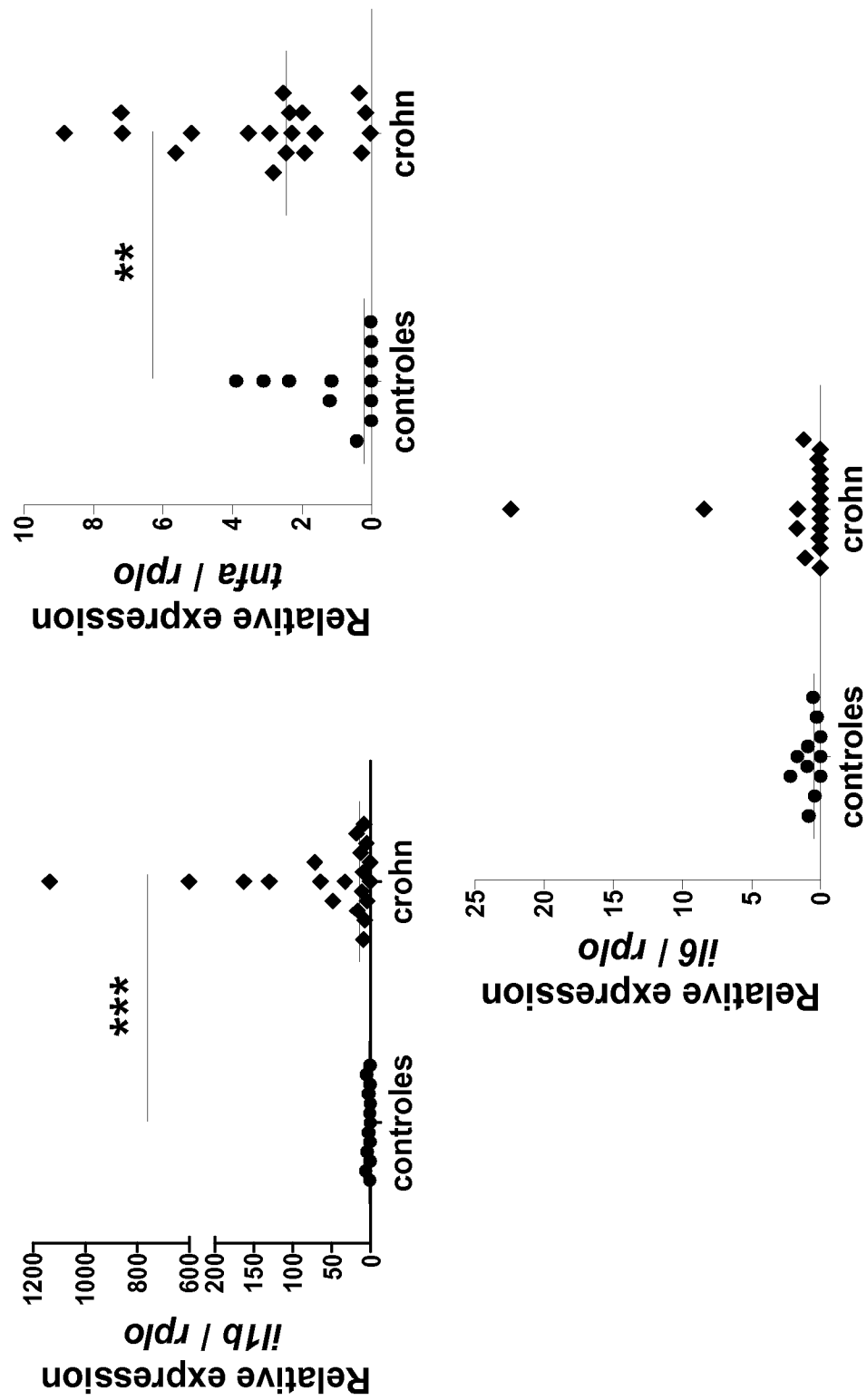


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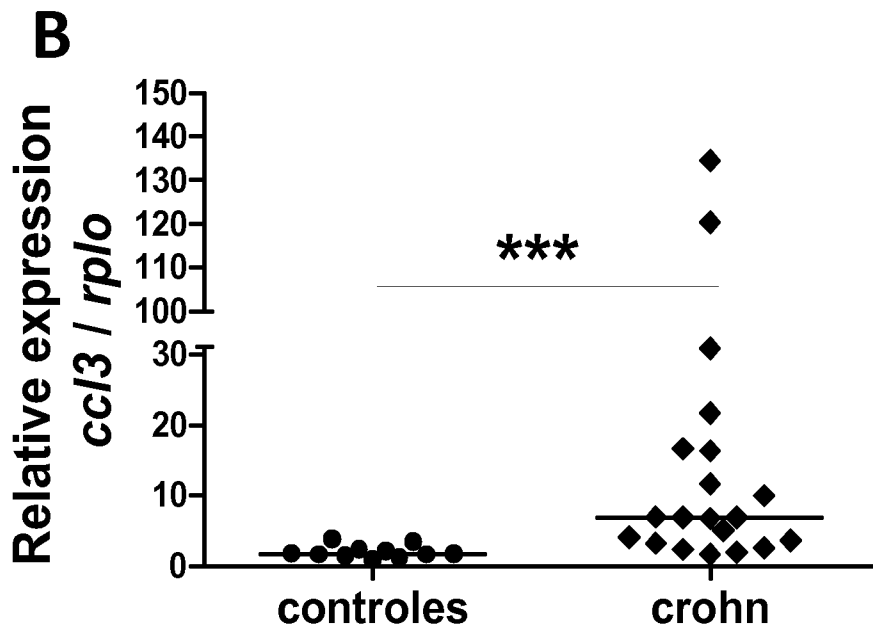


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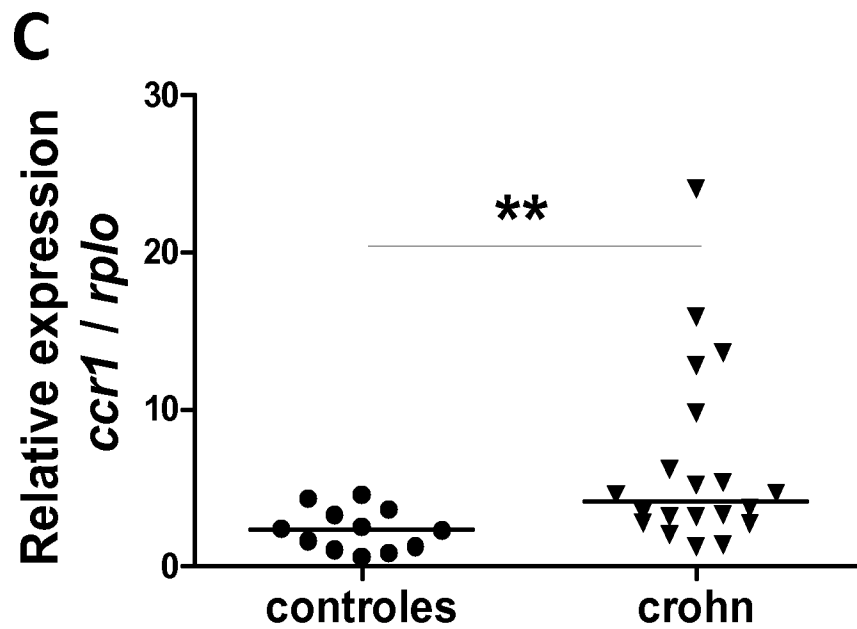


Figure 3C

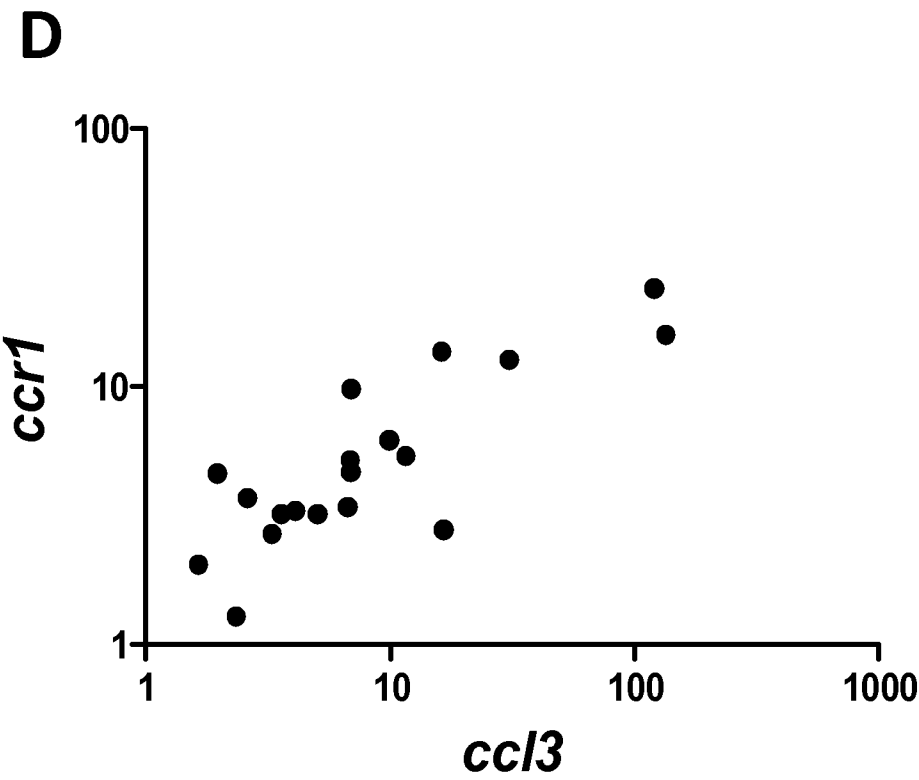


Figure 3D

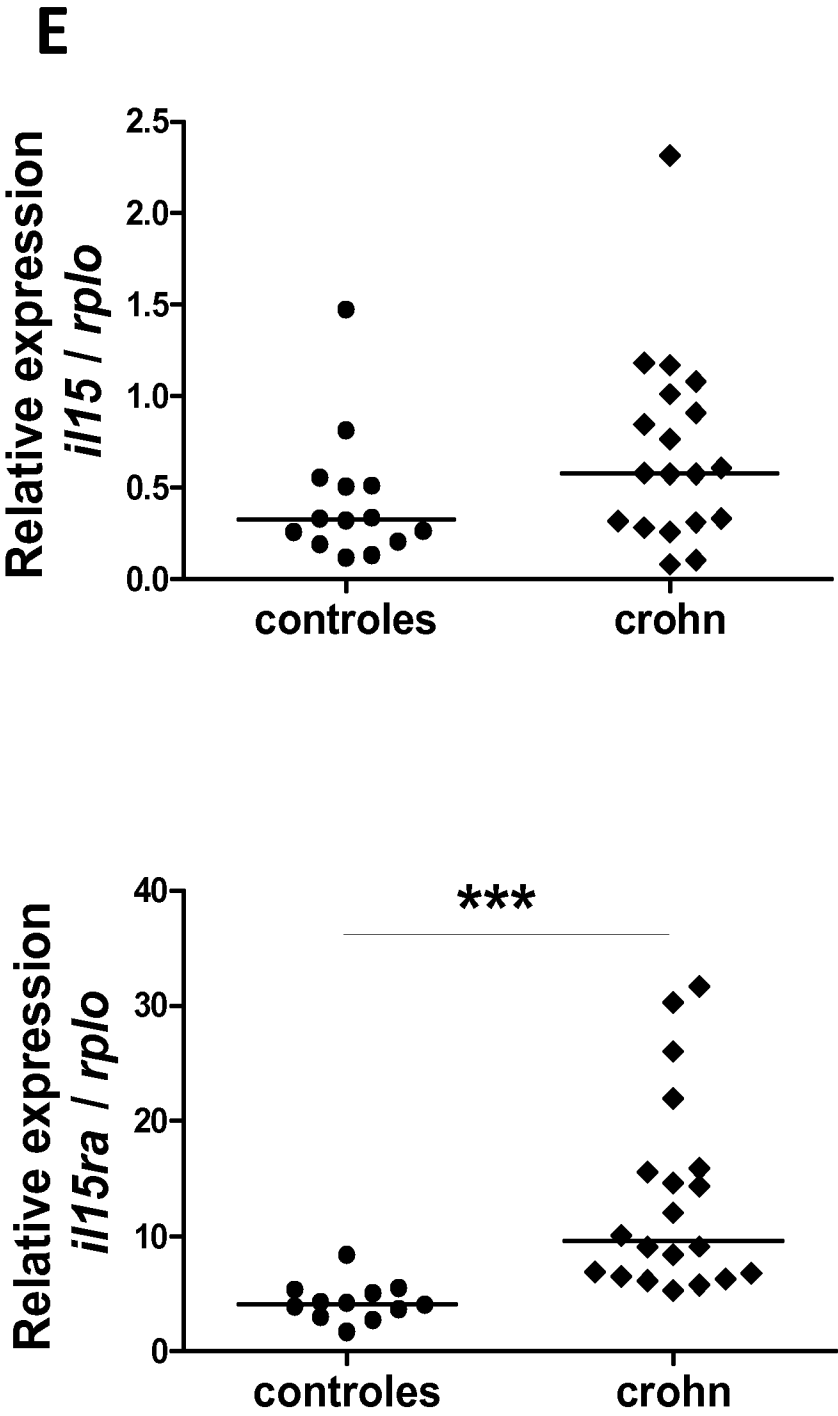


Figure 3E

F

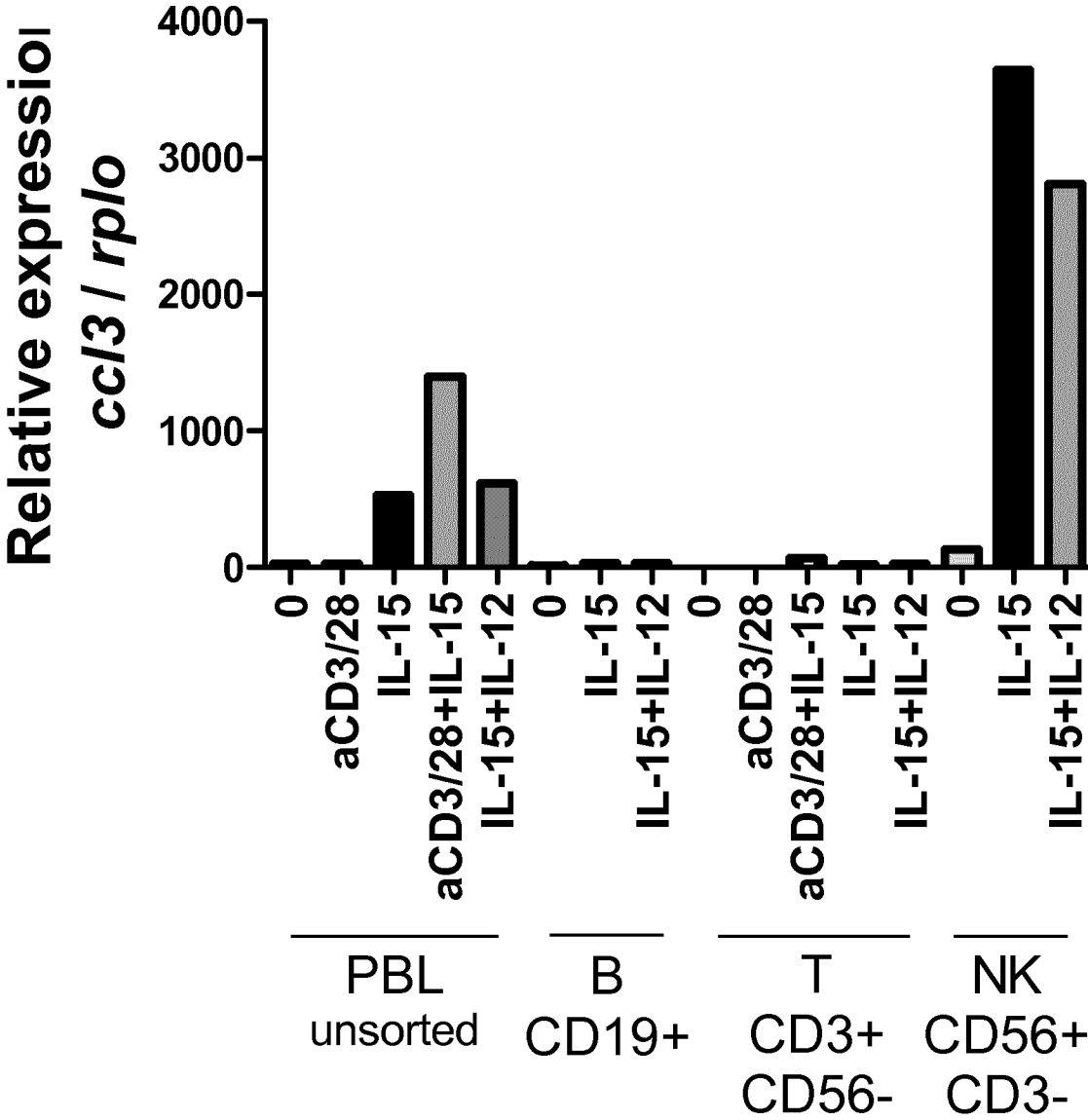


Figure 3F

20/20

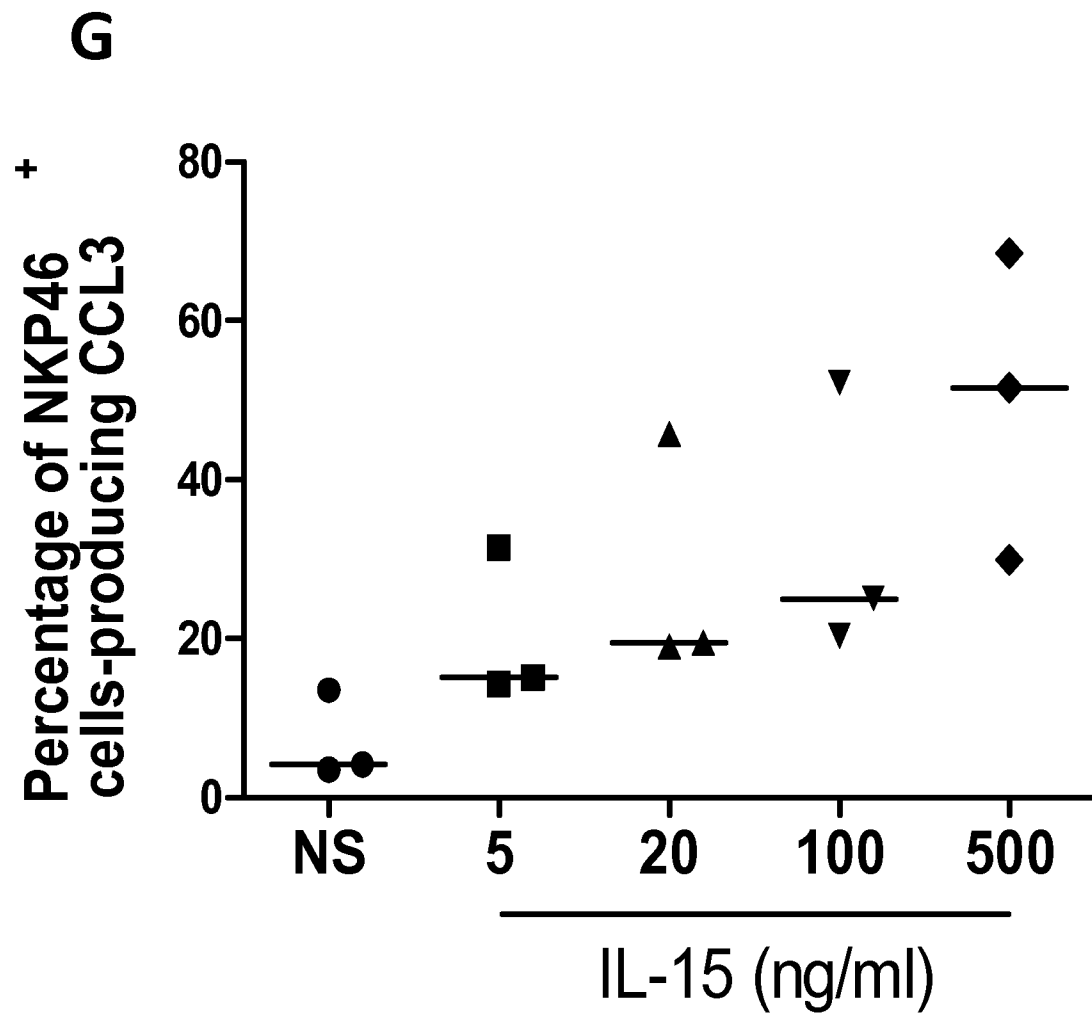


Figure 3G

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059880

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K39/395 A61P1/04
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61P

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

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

EPO-Internal, BIOSIS, CHEM ABS Data, EMBASE, WPI Data, BEILSTEIN Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2011/056440 A1 (BOEHRINGER INGELHEIM INT [DE]; COOK BRIAN NICHOLAS [US]; KUZMICH DANIE) 12 May 2011 (2011-05-12)	1
Y	abstract claims 1-8,9	1
X	WO 2011/044197 A1 (SQUIBB BRISTOL MYERS CO [US]; DHAR T G MURALI [US]; DUNCIA JOHN V [US]) 14 April 2011 (2011-04-14)	1
Y	abstract claims 1-4,6-8,10-11,13-14	1
X	WO 2009/054873 A2 (NOVIMMUNE SA [CH]; FISCHER NICOLAS [CH]; KOSCO-VILBOIS MARIE [FR]; MAC) 30 April 2009 (2009-04-30)	1
Y	abstract page 64, line 25 claims	1
	----- -/-	



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

28 June 2012

Date of mailing of the international search report

05/07/2012

Name and mailing address of the ISA/

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Authorized officer

Garabatos-Perera, J

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/059880

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6 329 510 B1 (QIN SHIXIN [US] ET AL) 11 December 2001 (2001-12-11)	1
Y	abstract column 27, line 61	1
X	US 6 613 905 B1 (LULY JAY R [US] ET AL) 2 September 2003 (2003-09-02)	1
Y	abstract paragraph [0016] figure 6	1
X	AJUEBOR M N ET AL: "The role of CCL3/macrophage inflammatory protein-1alpha in experimental colitis", EUROPEAN JOURNAL OF PHARMACOLOGY, ELSEVIER SCIENCE, NL, vol. 497, no. 3, 30 August 2004 (2004-08-30), pages 343-349, XP004546300, ISSN: 0014-2999, DOI: 10.1016/J.EJPHAR.2004.07.005	1
Y	abstract page 343, left-hand column, lines 1-4 page 343, right-hand column, line 7 - page 344, left-hand column, line 11 page 344, right-hand column, paragraph 4 page 348, left-hand column, lines 4-10 page 348, left-hand column, line 22 - right-hand column, paragraph 3	1
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Y	abstract page 77, left-hand column, paragraph 1 page 78, left-hand column, paragraph 2 page 82, left-hand column, paragraph 2-3	1
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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2012/059880

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	AJUEBOR M N ET AL: "The chemokine RANTES is a crucial mediator of the progression from acute to chronic colitis in the rat.", JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 1 JAN 2001 LNKD- PUBMED:11123336, vol. 166, no. 1, 1 January 2001 (2001-01-01), pages 552-558, XP002661768, ISSN: 0022-1767	1
Y	abstract page 552, left-hand column, paragraph 1 - right-hand column, paragraph 2 page 553, left-hand column, paragraph 4 page 554, right-hand column, paragraph 3 - page 555, left-hand column, paragraph 1 page 555, right-hand column, lines 6-26 -----	1
X	MORTEAU ET AL: "Genetic deficiency in the chemokine receptor CCR1 protects against acute Clostridium difficile toxin A enteritis in mice", GASTROENTEROLOGY, ELSEVIER, PHILADELPHIA, PA, vol. 122, no. 3, 1 March 2002 (2002-03-01), pages 725-733, XP005752856, ISSN: 0016-5085, DOI: 10.1053/GAST.2002.31873	1
Y	abstract page 725, left-hand column, line 10 - page 726, left-hand column, paragraph 2 page 728, left-hand column, paragraphs 1,2,3 page 729, left-hand column, paragraph 2 - page 732, right-hand column, line 3 -----	1
X	KRISTENSEN NANNA NY ET AL: "Chemokines involved in protection from colitis by CD4+CD25+ regulatory T cells.", INFLAMMATORY BOWEL DISEASES JUL 2006 LNKD- PUBMED:16804399, vol. 12, no. 7, July 2006 (2006-07), pages 612-618, XP002661769, ISSN: 1078-0998	1
Y	abstract page 617, left-hand column, paragraph 3 page 618, left-hand column, last paragraph table 1; page 615 ----- -/--	1

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2012/059880

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	AJUEBOR M N ET AL: "Role of chemokines and chemokine receptors in the gastrointestinal tract", IMMUNOLOGY, BLACKWELL PUBLISHING, OXFORD, GB, vol. 105, no. 2, 1 February 2002 (2002-02-01), pages 137-143, XP002263039, ISSN: 0019-2805, DOI: 10.1046/J.1365-2567.2002.01309.X abstract page 140, left-hand column, paragraph 2 - right-hand column, line 2 -----	1
Y	KHAN I A ET AL: "Mice lacking the chemokine receptor CCR1 show increased susceptibility to Toxoplasma gondii infection.", JOURNAL OF IMMUNOLOGY (BALTIMORE, MD. : 1950) 1 FEB 2001 LNKD- PUBMED:11160241, vol. 166, no. 3, 1 February 2001 (2001-02-01), pages 1930-1937, XP002661770, ISSN: 0022-1767 abstract page 1935, left-hand column, lines 44-59 page 1936, left-hand column, last paragraph -----	1
Y	RAMOS CLEBER D L ET AL: "MIP-1 alpha-[CCL3] acting on the CCR1 receptor mediates neutrophil migration in immune inflammation via sequential release of TNF-alpha and LTB4", JOURNAL OF LEUKOCYTE BIOLOGY, vol. 78, no. 1, July 2005 (2005-07), pages 167-177, XP002661771, ISSN: 0741-5400 abstract page 174, left-hand column, lines 7-11 page 175, left-hand column, last paragraph -----	1
Y	PENDER S L-F ET AL: "Systemic administration of the chemokine macrophage inflammatory protein 1 alpha exacerbates inflammatory bowel disease in a mouse model", GUT, vol. 54, no. 8, August 2005 (2005-08), pages 1114-1120, XP002661772, ISSN: 0017-5749 the whole document -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2012/059880

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2011056440	A1	12-05-2011	NONE
WO 2011044197	A1	14-04-2011	NONE
WO 2009054873	A2	30-04-2009	AU 2008317495 A1 30-04-2009 CA 2695237 A1 30-04-2009 CN 101802211 A 11-08-2010 EP 2185719 A2 19-05-2010 JP 2010535763 A 25-11-2010 US 2009148455 A1 11-06-2009 WO 2009054873 A2 30-04-2009
US 6329510	B1	11-12-2001	US 6329510 B1 11-12-2001 US 2002037539 A1 28-03-2002 US 2002061305 A1 23-05-2002 US 2004265304 A1 30-12-2004
US 6613905	B1	02-09-2003	JP 4853934 B2 11-01-2012 US 6613905 B1 02-09-2003