Title: INHIBITION OF FRACALKINE OR OF ITS RECEPTOR FOR THE TREATMENT OF ATOPIC ALLERGIC DISEASES

Abstract: The invention relates to the treatment of atopic allergic diseases in particular respiratory diseases such as allergic rhinitis or asthma, through inhibition of the expression of fractalkine or of its receptor CX3CR1, or through inhibition of their interaction.
INHIBITION OF FRACTALKINE OR OF ITS RECEPTOR  
FOR THE TREATMENT OF ATOPIC ALLERGIC DISEASES.

The invention relates to the treatment of any disease which results from the development of an inappropriate T cell-dependent immune response including atopic allergy, airway inflammation and allergic asthma, through regulation of the interaction between fractalkine (CX3CL1) and its receptor (CX3CR1).

Atopic allergic diseases are characterized by inflammatory reactions resulting from inappropriate IgE antibody response to common environmental antigens. Once IgE antibodies directed against a given allergen have been produced, they bind to mast cells. Later exposure to the same allergen induces mast cells degranulation, and release of numerous pro-inflammatory substances, such as histamine, prostaglandins, leukotrienes, and chemotactic factors for inflammatory cells such as eosinophils.

The clinical expression of atopic allergy depends on the organ wherein the inflammatory reaction takes place. A reaction located to the respiratory tract takes the form of asthma when the lungs are affected, or allergic rhinitis when it is the nose. Allergic rhinitis is often accompanied by allergic conjunctivitis, resulting in so-called “hay fever”. A reaction located to the skin induces atopic dermatitis, often under, the form of eczema. Food allergy may induce a local reaction in the gastrointestinal tract, and/or distant reactions concerning mainly the skin, as eczema or urticaria, or less frequently, as Quinck oedema. The most dramatic form of atopic allergy is anaphylactic shock, associated with a sudden and generalized allergic reaction which can be fatal if not treated immediately.

Among these diseases, those affecting the respiratory tract and in particular asthma, represent a growing public health problem, since they can be invalidating, and become more and more frequent worldwide.

Atopic allergy is believed to result from an increase in the T helper type 2 (Th2) lymphocytes combined with a decrease in the T helper type 1 (Th1) lymphocytes. Th1 and Th2 cells arise from antigen-stimulated precursor CD4+T cells, that differentiate to either the Th1 or the Th2 phenotype. Although the mechanism of Th1 versus Th2 differentiation is not completely understood, it appears that IL-12 and IL-4 play a major part: IL-12 stimulates the differentiation toward a Th1 phenotype, whereas IL-4 stimulates the differentiation toward a Th2 phenotype.

Th1 and Th2 cells display different profiles of cytokine secretion and are involved in different types of immune response. The Th1 and Th2 cytokines are mutually inhibitory for the functions of the other phenotype.
Th1 cells are characterized by the production of IL-2, IFNγ and TNFβ and are associated with cell-mediated immunity, delayed-type hypersensitivity, macrophage activation, and production of opsonizing antibodies (IgG1 and IgG3). Th2 cells produce in particular IL-4, IL-5, and IL-13, and are associated with humoral response and immediate hypersensitivity, including production of IgE antibodies and recruitment and activation of effector cells such as mast cells and eosinophils.

Recent approaches to treatment of atopic allergic diseases aim at decreasing the Th2 response, by use of Th2 cytokines antagonists, or by correcting the disequilibrium in the Th1/Th2 balance in favour of the Th1 response.

By way of example, in the treatment of asthma, it has been proposed to use cytokines stimulating the Th1 response, such as IL-12 or IFNγ, or inhibitors of cytokines secreted by the Th2 lymphocytes, such as IL-4, IL-5, IL-13, and IL-9 (for review, cf. Renauld 2001).

Other therapeutic targets that are currently the subject of many studies are chemokines (chemotactic cytokines), that are involved in the inflammatory response by attracting effector cells.

Chemokines are involved in the control of leukocyte traffic and inflammation. They are characterized by a specific pattern of four conserved cysteine residues. They have been classified into four families, CXC, CX3C, CC and C, dependent on the number and spacing of the first two conserved cysteine residues.

The CXC group includes interleukin 8 (IL-8), MGSA (melanoma growth stimulatory activity) and neutrophil activating peptide 2 (NAP-2); the CC group includes RANTES, MCP-1 (monocyte chemotactic protein 1) and MIP-1α (macrophage inflammatory protein 1α). The only representatives of the CX3C group and the C group identified so far are respectively fractalkine, which is chemotactic for T cells and monocytes, and lymphotactin, which is chemotactic for lymphocytes.

The effects of chemokines on leukocytes are mediated by seven-transmembrane domain G protein–coupled receptors. Chemokine receptors are expressed in a different way on different types of leucocytes. Some of them are widely expressed on many leucocytes, while others have a more limited expression. In addition, the expression of chemokine receptors can depend on the cellular activation or the state of differentiation.

For instance, it has been shown that Th1 and Th2 populations have a different chemokine receptor repertoire. Although some chemokine receptors are expressed by both subsets, Th2 cells were reported to express preferentially the receptors CCR3, CCR4, and CCR8, while Th1 cells were reported to express
preferentially the chemokine receptors CCR5, CXCR3, and CX3CR1 (Bonecchi, Bianchi et al. 1998; Zingoni, Soto et al. 1998; Fraticelli, Sironi et al. 2001).

Chemokine receptors that are preferentially expressed by Th2 cells, mast cells and/or eosinophils are considered as promising therapeutic targets for allergy. One example is CXCR3: this receptor and its ligands (in particular eotaxin) have been shown to play a major part in eosinophils migration and antagonists thereof are attractive candidates for the treatment of atopic allergy.

On the other hand, consistently with their reported role in the amplification of Th1 response (FRATICELLI, SIRONI ET AL. 2001) fractalkine (CX3CL1) and its receptor (CX3CR1) have been proposed as therapeutic targets for treating Th1-mediated pathologic conditions such as rheumatoid arthritis (PCT WO 01/60406; Ruth, Volin et al. 2001), or more generally, diseases involving killer lymphocytes (US application 2002/0192212). Fractalkine and CX3CR1 have also been associated with inflammatory conditions such as renal inflammation (Chen, Bacon et al. 1998; Cockwell, Chakravorty et al. 2002), allograft rejection (Robinson, Nataraj et al. 2000), neurodegenerative disorders, demyelinating diseases and pain (PCT WO 02/076990).

Concerning the involvement of CX3CR1 in airway diseases, it has been reported that G glycoprotein of respiratory syncytial virus (RSV) can bind and activate CX3CR1, and compete with fractalkine for the occupancy of said receptor, resulting in an alteration of Th1 and NK cell responses through interference with fractalkine activity (Tripp, Jones et al. 2001; Haynes, Jones et al. 2003).

In analyzing the immune response in mice sensitized by Leishmania LACK allergen (which induces a Th2 response), the inventors have observed that LACK-specific CD4+ T cells generated upon challenge with LACK aerosols expressed higher levels of CX3CR1 chemokine receptor in peri-bronchial lymph nodes (PBLN) than in the spleen. The inventors have then undertaken to investigate the role of CX3CR1 in the migration of LACK-specific CD4+ T cells to the airways, using CX3CR1-deficient mice in which both alleles of the CX3CR1 gene have been replaced by the eGFP reporter gene (CX3CR1^eGFP/+ mice). They found that, surprisingly, these CX3CR1-deficient mice were impaired in their ability to develop a Th2 response (and in particular to secrete IL-4, IL-5 and IL-13) upon antigenic restimulation.

These findings allow to propose new means of treatment of atopic allergic diseases by use of fractalkine/CX3CR1 antagonists, or by use of inhibitors of the expression of fractalkine or of CX3CR1.
Inhibitors of the expression of fractalkine or of CX3CR include for instance antisense oligonucleotides, interfering RNAs, or ribozymes, targeting the fractalkine gene or the CX3CR1 gene.

A "fractalkine/CX3CR1 antagonist" is herein defined as a compound able to inhibit the activation of CX3CR1 by fractalkine, without being able to activate CX3CR1 on its own. This includes in particular compounds able to interact with fractalkine, and to inhibit its binding with CX3CR1 or to inhibit the activation of CX3CR1 resulting from said binding, as well as compounds able to interact with CX3CR1, and to inhibit its binding with fractalkine or its activation resulting from said binding.

The invention thus proposes the use of an inhibitor of the expression of fractalkine or of CX3CR1 or of a fractalkine/CX3CR1 antagonist for preparing a medicament for treating an atopic allergic disease.

As indicated above, atopic allergic diseases include in particular asthma, allergic rhinitis, allergic conjunctivitis, hay fever, atopic dermatitis, Quincke oedema, and anaphylactic shock.

According to a preferred embodiment of the invention, said atopic allergic disease is an airway disease, in particular asthma or allergic rhinitis.

In one embodiment, the fractalkine/CX3CR1 antagonist consists in an organic molecule. For example, PCT WO 02/076990 describes thiazolopyrimidine derivatives as antagonists of the CX3CR1 receptor.

Antagonists according to the invention can also include peptidomimetic. The term "peptidomimetic", refers to molecules which are not peptides, but which mimic aspects of their structures. Peptidomimetics can be designed, for example, by establishing the three dimensional structure of a peptide agent in the environment in which it is bound or will bind to CX3CR1. PCT WO 01/60406 discloses methods for obtaining such peptidomimetics.

In a preferred embodiment, the fractalkine/CX3CR1 antagonist is an antibody which can be polyclonal or monoclonal. The term "antibody" as used herein also encompasses functional fragments of antibodies, including fragments of chimeric, humanized, single chain antibodies or fragments thereof. Suitable antibodies are those which are directed to fractalkine or to CX3CR1. In preferred embodiment, the fractalkine/CX3CR1 antagonist is a monoclonal antibody, or fragment thereof.

Laboratory methods for preparing monoclonal antibodies are well known in the art. Monoclonal antibodies may be prepared by immunizing purified CX3CR1, fractalkine or portions thereof into a mammal, e.g., a mouse, rat, human and
the like mammals. The antibody-producing cells in the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. Phage display technology is also a useful technology for the production of monoclonal antibodies.

For instance, antibody fragments capable of binding to CX3CR1 or fractalkine, include, but are not limited to Fv, Fab, Fab' and F(ab') 2 fragments. Such fragments can be produced by enzymatic cleavage or by recombinant techniques. For example, papain or pepsin cleavage can generate Fab or F (ab') 2 fragments, respectively.

By way of example, PCT WO 01/60406 discloses methods to produce antibodies suitable for use according to the invention.

Alternatively, aptamers recognizing either fractalkine or CX3CR1 can be used to antagonize the fractalkine/CX3CR1 interaction. Aptamers are single stranded nucleic acid molecules (DNA or RNA) that are selected in vitro for their ability to bind to a target molecule; this selection can be performed, for example, by the SELEX method (Systematic Evolution of Ligands by Exponential Enrichment) described in US 5,270,163.

Other fractalkine/CX3CR1 antagonists can be identified, for example, by screening a collection of candidate compounds for their ability to inhibit the activation of CX3CR1 in presence of fractalkine. Methods for measuring the activation of G protein–coupled receptors that are known in themselves, and are for instance commonly used in high–throughput screening assays, can be used for evaluating the activation of CX3CR1. Examples of methods for assessing the activity of such antagonists are described in WO 01/60406, from page 17, line 24, to page 24, line 14.

Said fractalkine/CX3CR1 antagonists can be administered by themselves, or mixed with suitable carriers or excipient(s). They can be used systemically or locally. Local administration may be preferred in the case of airway diseases such as asthma or allergic rhinitis, or in the case of cutaneous diseases such as atopic dermatitis. In these cases, one can use any formulation suitable for local administration, such as a solution, a suspension, a gel, an ointment, for administration on the skin, or for instance a spray or an aerosol for administration in the respiratory tract.
The present invention will be further illustrated by the additional description which follows, which refers to non-limitative examples illustrating the effects of the inactivation of CX3CR1 on the immune response to an allergen in mice.

MATERIALS AND METHODS

5 Mice and induction of allergic asthma

CX3CR1<sup>eGFP</sup>+/+ mice on BALB/c background have been previously described (Jung, Aliberti et al. 2000). WT15 TCR transgenic mice have been described (Wang, Strong et al. 2001). Female BALB/c ByJ mice were purchased from Charles River (Lyon, France). CX3CR1<sup>eGFP</sup>−/− mice were obtained by crossing CX3CR1<sup>eGFP</sup>+/+ mice to BALB/c mice. CX3CR1<sup>eGFP</sup>+/+ Thy1.1<sup>+/+</sup> congenic mice were obtained by crossing CX3CR1<sup>eGFP</sup>+/+ mice to Thy1.1<sup>+/−</sup> BALB/c congenic mice. Mice were raised in a specific pathogen-free animal facility and used between 6 and 8 weeks of age. Sensitization was performed by 2 i.p. injections of 10 μg LACK protein precipitated in 2 mg of aluminum hydroxide (ALUM) (PerBio Science France SAS, Brebières, France) at day 0 and 7. At day 14, 15, 16, 17 and 18, sensitized mice were exposed to LACK aerosols (1.5 mg/ml) for 30 minutes. Aerosol nebulization was performed using an Ultramed, ultrasonic nebulizer (Medicatia, Italy) connected to a 6500 cm<sup>3</sup> box that was used as a nebulization chamber.

Reagents and antibodies

LACK peptide (aa 156 through 173; ICFSPSLEHPIVVSGLSWD) was purchased from Mimotopes (Paris, France). LACK recombinant protein was produced in E. Coli and purified as described (Mougeau, Altare et al. 1995). Soluble I-A<sup>d</sup>/LACK dimers were produced and used as described (Julia, Hessel et al. 2002). Briefly, staining reagent was prepared by incubating 0.7 μl of protein A coupled to the indicated Alexa Fluor<sup>®</sup> reagent (Molecular Probes Inc., Interchim, Montluçon, France) at the concentration of 0.5 mg/ml in PBS with 4 μg of I-A<sup>d</sup>/LACK dimers for 30 min at room temperature. The following mAbs were used for cell purification, or flow-cytometry analysis: GK1.5, anti-CD4; 53-6.7, anti-CD8α; M1/170, anti-CD11b; RA3-6B2, anti-B220; AMS-32.1, anti-I-A<sup>d</sup>; HL3, anti-CD11c; RB6-8C5, anti-Gr-1; 5B11, 2.4G2, anti-FcγIII/II receptor, H1-2F3, anti-CD69; DX5, anti-CD49b/Pan-NK cells; OX-7, anti-Thy1.1; 53-2.1 anti-Thy1.2. All mAbs were purchased from BD Biosciences (Le Pont de Claix, France). Anti-IgE (R35-118) coupled to biotin, anti-IgG1 (A85-1) and anti-IgG1 (A85-3) coupled to biotin, anti-IgG2a (R11-89) and anti-IgG2a (R19-15) coupled to biotin mAbs, were purchased from BD Biosciences. Anti-IgE (EM-95) mAbs were a gift from DNAX (Palo Alto, CA, USA).
**Antibody titers**

Mice were bled 7 days after the second immunization. LACK-specific IgG1 and IgG2a were measured by ELISA as described (McSorley, Soldera et al. 1997). Total IgE were quantified by ELISA using rat EM-95 anti-IgE mAbs as coating antibody and rat anti-IgE mAbs coupled to biotin as second antibody.

**Analysis of bronchoalveolar lavage (BAL) cells**

Mice were bled and a canula was inserted into the trachea. Lungs were washed 3 times with 1 ml of warmed PBS. Cells were washed with PBS, resuspended in 500 µl, and counted using a Burker-Turk chamber. For differential BAL cell counts, cytospin preparations were made and stained with Wright/Giemsa. At least 400 cells were scored for each slide, and the numbers of lymphocytes, neutrophils, eosinophils, and other mononuclear cells were determined by microscopic examination.

**Airway hyperresponsiveness (AHR)**

AHR was measured one day after the last antigen challenge by whole-body plethysmography (Emka) in response to increasing concentrations (6–25 mg/ml) of inhaled methacholine (acetyl methyl choline, Sigma) (Woerly, Honda et al. 2003). AHR is expressed as enhanced pause (Penh), a dimensionless parameter correlated to airway compliance and resistance calculated value, which correlates with measurement of airway resistance, impedance, and intrapleural pressure in the same mouse.

**Histology**

Lung samples (left lungs) from mice that have not been used for BAL cell content analysis were harvested and fixed with Immunohistofix™ and embedded in Immunohistowax™. 4-µm sections were performed and stained with May Grünwald Giemsa.

**Purification of cells**

For purification of CD4+ T cells, LN and spleen cells were depleted of I-A^d+, B220+, CD11b+, CD8α+, DX5+ and Gr-1+ cells by negative depletion using sheep anti-rat coated Dynabeads (Dynal, Compiègne, France) as described (Julia, Rassoulzadegan et al. 1996). For staining with I-A^d/LACK multimers, lung T cells were enriched through a Percoll gradient as described (Julia, Hessel et al. 2002). Briefly, cell suspensions were depleted of red blood cells, washed and resuspended in 6 ml of 90% Percoll. A 6 ml layer of Percoll 40% was added delicately on the top.
After centrifugation at 1000 rpm for 30 minutes, cells at the interface were collected. For purification of spleen and LN I-A$^d$/LACK$^+$ and I-A$^d$/LACK$^-$ cells, 120 mice that had been previously sensitized and challenged were sacrificed 2 days after the last aerosol. Briefly, spleen and LN CD4$^+$ cells were stained with I-A$^d$/LACK multimers, cychrome (CyCR)-conjugated anti-CD4 mAbs, and with a cocktail of phycoerythrin (PE)-conjugated mAbs: anti-B220, anti-CD11c, anti-CD11b, anti-Gr-1 and anti-CD8α mAbs. I-A$^d$/LACK$^+$CD4$^+$PE$^-$ and I-A$^d$/LACK$^-$CD4$^+$PE$^-$ cells were sorted on a high-speed sorter VANTAGE SECTLO$^+$ flow cytometer (Becton Dickinson, Le Pont de Claix, France). For purification of lung and BAL I-A$^d$/LACK$^+$ and I-A$^d$/LACK$^-$ CD4$^+$ cells, 300 mice were sacrificed 4 and 7 days after the last aerosol, respectively. To prepare lung cells, lungs were washed 3 times with PBS and cell suspensions were prepared as described below. Cells were depleted from red blood cells, washed, incubated with 2.4G2 mAbs, and stained with CyCR-conjugated anti-CD4 mAbs. CD4$^+$ T cells were sorted by FACS, and further stained with I-A$^d$/LACK multimers and the cocktail of lineage-specific PE-conjugated mAbs described above. I-A$^d$/LACK$^+$ and I-A$^d$/LACK$^-$ CD4$^+$ T cells were sorted by FACS after gating on CD4$^+$ PE$^-$ cells.

For CD4+GFP+ and CD4+GFP- T cell purification in Example 6, lung T cells were enriched through a Percoll gradient as described above and sorted by FACS after staining with anti-CD4 mAbs.

**Flow cytometry**

For staining with I-A$^d$/LACK multimers, 10$^6$ CD4$^+$ T cells were preincubated with 2.4.G2 anti-FcγRII/III mAbs, and stained with 4 μg/ml of I-A$^d$/LACK multimers for 30 min at room temperature in PBS supplemented with 0.5% of BSA. Cells were washed and incubated with anti-CD4 mAbs, and with the cocktail of PE-conjugated mAbs described above. For adoptive transfer, cells from BAL, lung and PBLN were preincubated with anti-FcγRII/III mAbs, and stained with anti-CD4, anti-Thy1.1 and anti-Thy1.2 mAbs. Lymphocytes gated by forward- and side-scatter parameters were analyzed on a FACScalibur flow cytometer (Becton Dickinson).

**Cytokine assays**

Lungs and PBLN were removed separately, cut into small pieces, and digested with collagenase D (Roche Diagnostics, Meylan, France) for 30 min at 37°C. Cell suspensions were filtered through a 70 μm cell strainer and depleted of red blood cells using lysis buffer. Cells were washed with Hanks BSS solution (Invitrogen SARL, Cergy-pontoise, France). 5 X 10$^6$ cells were incubated for 72 hrs in either 24-
well plates for lung cells, or in 96-well plates for PBLN cells, in medium containing either 0.25 mg/ml LACK protein or 20 μM LACK peptide. Purified lung or LN T cells were restimulated with mitomycin-C treated splenocytes (20 min at 37°C). Supernatants were analyzed for IL-4, IL-5, and IFN-γ contents by ELISA as described (Julia, Hessel et al. 2002). Supernatants were analyzed for IL-13 content by ELISA, using a quantitative mouse IL-13 kit according to the manufacturer’s instructions (R&D Systems, Inc., Lille, France). RPMI 1640 containing Glutamax I (Invitrogen), 10% heat-inactivated FCS (Perbio), 0.05 μM 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen), was used as medium for all assays.

Measurement of chemokine and chemokine receptor mRNA expression

Sorted I-A^d/LACK^+/CD4^+ and I-A^d/LACK^-CD4^+ cells were frozen in RNaseasy® lysis buffer (Qiagen Inc.). Total RNA was isolated using the RNaseasy® 96 kit (Qiagen Inc.) according to the manufacturer’s instructions. RNA was transcribed into cDNA by incubation with random hexamers (Promega Corporation, Madison, WI) and oligo(dT)_{12-18} (Pharmacia Biotech Inc., Piscataway, NJ), followed by incubation for 50 min at 45°C in 100 μl containing superscript II reverse transcriptase (Gibco BRL), acetylated BSA (Gibco BRL), dNTPs (Amersham Pharmacia Biotech AB), DTT (Gibco BRL), rRNasin® (Promega), and 1X synthesis buffer (Gibco BRL). Samples were incubated for 45 min at 37°C with ribonuclease H (Gibco BRL) and the samples were stored at -20°C until later use. Real-time quantitative PCR was performed using the ABI Prism® 7700 sequence detection system (Perkin Elmer Applied Biosystems, Foster City, CA) as described (Scheerens, Hessel et al. 2001). Sense and anti-sense primers, and probes were pre-developed TaqMan® assay reagents (Perkin Elmer Applied Biosystems). 18S rRNA was used as an internal control to measure the amount of RNA in each sample. Data were expressed in arbitrary units normalized to the expression of ubiquitin mRNA.

Statistical analysis

ANOVA for repeated measures was used to determine the levels of difference between groups of mice for plethysmography measurements. Comparisons for all pairs were performed by unpaired two-tailed Student’s t-test. Significance levels were set at a P value of 0.05.
RESULTS

EXAMPLE 1: CHEMOKINE RECEPTOR mRNA EXPRESSION IN ALLERGEN-SPECIFIC T CELLS

To measure the level of expression of chemokine receptors mRNA in allergen-specific CD4⁺ T cells, BALB/c mice were sensitized and challenged with LACK, and sacrificed 4 days after the last aerosol. PBLN, lungs, BAL and spleen cells were harvested and both I-A²/LACK⁺ and I-A²/LACK⁻ CD4⁺ T cells were purified by flow cytometry after staining with I-A²/LACK multimers. The purified cells were used to prepare RNA and levels of chemokine receptors mRNA were measured using quantitative real time PCR using pre-developed TaqMan® assay reagents.

The results are shown in Figure 1.

Legend of figure 1: RNA expression is shown for the indicated chemokine receptors in I-A²/LACK⁺ (filled bars) and I-A²/LACK⁻ (empty bars) CD4⁺ T cells. Data are expressed as arbitrary units after normalization to the level of ubiquitin mRNA. Numbers indicate the ratio between mRNA expression in I-A²/LACK⁺ as compared to I-A²/LACK⁻ cells.

These results show that all tested chemokine receptors mRNA were expressed at the same levels in spleen I-A²/LACK⁺ and LACK⁻ CD4⁺ T cells. In contrast, I-A²/LACK⁺ CD4⁺ T cells purified from PBLN contained 10-30 fold more CCR3, CCR4, CCR8, CCR5, CXCR3 and CXCR6 mRNA than PBLN I-A²/LACK⁻ CD4⁺ T cells. PBLN I-A²/LACK⁺ CD4⁺ T cells also expressed 50 fold more CX3CR1 mRNA than I-A²/LACK⁻ CD4⁺ T cells. As compared to PBLN I-A²/LACK⁻ CD4⁺ T cells, I-A²/LACK⁺ CD4⁺ T cells also expressed 2 and 6 fold more CCR7 and CXCR4 mRNA respectively.

Lung and BAL I-A²/LACK⁺ CD4⁺ T cells expressed similar amounts of CX3CR1 mRNA, and 2-3 fold more CCR3, CCR4, CCR8, CCR7, CXCR4, CXCR3 and CXCR6 mRNA than I-A²/LACK⁻ CD4⁺ T cells purified from the same compartments. Also, for each chemokine receptor and for both I-A²/LACK⁺ and I-A²/LACK⁻ CD4⁺ T cells, the amount of chemokine receptor mRNA was higher in airway associated tissues as compared to spleen.

EXAMPLE 2: I-A²/LACK⁺ CD4⁺ T CELLS EXPRESS CX3CR1 IN BAL AND LUNG

To further investigate the expression of CX3CR1 at the surface of allergen-specific T cells, CX3CR1-deficient CX3CR1eGFP⁺/⁻ mice in which both
alleles of the CX3CR1 gene have been replaced by the eGFP reporter gene, were crossed with BALB/c mice, resulting in CX3CR1\(^{eGFP+/+}\) mice which carried one copy of the eGFP reporter gene and one copy of the CX3CR1 wt gene. Syngenic BALB/c mice were used as controls.

Mice (5 mice per group) were sensitized and challenged with LACK and sacrificed 1 day after the last aerosol. CD4\(^+\) T cells were partially purified from blood, spleen, PBLN, lungs, and BAL, and analyzed by FACS after staining with Alexa-Fluor\(^{\text{®-633}}\) conjugated protein A bound I-A\(^d\)/LACK dimers and PerCP-anti-CD4 mAb.

Figure 2 shows the frequency of eGFP\(^+\) cells among CD4\(^+\) T cells after gating on lymphocytes, in lung (A) and BAL (B).

As expected, eGFP\(^+\) CD4\(^+\) T cells were not detected in wt BALB/c mice. In contrast, 5-10% of total CD4\(^+\) T cells and 20-30% of I-A\(^d\)/LACK\(^+\) cells were eGFP\(^+\) respectively, in the lungs and BAL of CX3CR1\(^{eGFP+/+}\) mice. eGFP\(^+\) CD4\(^+\) T cells were not detected in blood, spleen and PBLN (data not shown).

**EXAMPLE 3: LACK-SENSITIZED CX3CR1\(^{eGFP+/+}\) MICE DO NOT EXHIBIT AIRWAY EOSINOPHILIA UPON CHALLENGE WITH LACK AEROSOLS**

To investigate the role of CX3CR1 in allergic airway inflammation, CX3CR1-deficient CX3CR1\(^{eGFP+/+}\) and control wt BALB/c mice (5 mice per group) were sensitized and challenged or not with LACK and sacrificed 1 day after the last aerosol. BAL cells were harvested from individual mouse, centrifugated onto a glass slide using a cystospin centrifuge, and analyzed for cellular contents by optical microscopy after Wright/Giemsa coloration.

Figure 3 shows (A) the total number of cells recovered from BAL, (B) the frequency of each cell type among total cells and (C) the number of each cell types in BAL.

As previously observed, the numbers of lymphocytes, DC, macrophages eosinophils and neutrophils were higher in wt mice upon challenge with LACK (Julia, Hessel et al. 2002). LACK aerosols induced airway cellular infiltration in both CX3CR1\(^{eGFP+/+}\) mice and wt mice. However, in contrast to wt mice, BAL from CX3CR1\(^{eGFP+/+}\) mice contained almost no eosinophils and neutrophils. Furthermore, BAL from CX3CR1\(^{eGFP+/+}\) mice contained 4-fold more lymphocytes than those from wt mice.
EXAMPLE 4: T CELL RESPONSES ARE IMPAIRED IN THE AIRWAYS OF CX3CR1<sup>eGFP<sup>+/+</sup></sup> MICE

To further characterize T cells in CX3CR1<sup>eGFP<sup>+/+</sup></sup> mice, WT, CX3CR1<sup>eGFP<sup>+/+</sup></sup>, and CX3CR1<sup>eGFP<sup>+</sup></sup> mice were sensitized and challenged with LACK, and sacrificed 1 day after the last aerosol. PBLN and lung cells were harvested and incubated <i>in vitro</i> with or without LACK peptide for 72 hours. Supernatants were harvested and analyzed for cytokine (IL-4, IL-13, IL-5 and IFN-γ) content by ELISA.

The results are shown in Figure 4.

Legend of figure 4: (A) Lung cells; (B) PBLN cells from WT (empty bars), CX3CR1<sup>eGFP<sup>+/+</sup></sup> (filled bars), and CX3CR1<sup>eGFP<sup>+</sup></sup> (dashed bars) mice were sensitized and challenged with LACK, and sacrificed 1 day after the last aerosol. Lung and PBLN were harvested and cells were incubated with or without LACK peptide for 72 hours. Cytokine contents were measured by ELISA. Data show the amount of IL-4, IL-13, IL-5 and IFN-γ upon restimulation with LACK. None of these cytokines was detected when cells were incubated without LACK (data not shown). The limit of detection for IL-4, IL-13, IL-5 and IFN-γ were 200 U/ml, 150 pg/ml, 15 U/ml and 0.2 ng/ml respectively. IFN-γ was below the level of detection in all PBLN samples.

While both lung and PBLN cells from CX3CR1<sup>eGFP<sup>+/+</sup></sup> and wt mice secreted detectable amounts of IL-4, IL-5 and IL-13 in response to LACK, these cytokines were not detectable in the supernatants of cells from CX3CR1<sup>eGFP<sup>+/+</sup></sup> mice (Figure 4A). In contrast, lung cells from all mice secreted similar low levels of IFN-γ in response to LACK (Figure 4A). Thus, lung T cells from CX3CR1<sup>eGFP<sup>+/+</sup></sup> mice were selectively impaired in their ability to secrete Th2 cytokines upon antigenic restimulation. This phenomenon was unlikely to result from a difference in T cell priming because sera from CX3CR1<sup>eGFP<sup>+/+</sup></sup>, CX3CR1<sup>eGFP<sup>+</sup></sup> and wt mice exhibited similar titers of LACK-specific IgG1 and IgG2a (data not shown).

EXAMPLE 5: THE FRACTALKINE RECEPTOR CX3CR1 IS CRITICAL FOR THE DEVELOPMENT OF ALLERGIC ASTHMA IN MICE

To investigate the role of CX3CR1 in allergic asthma, the same CX3CR1<sup>eGFP<sup>+/+</sup></sup> mice were used. CX3CR1<sup>eGFP<sup>+/+</sup></sup> and syngenic BALB/c wild type (wt) mice were sensitized by intraperitoneal injections of the <i>Leishmania major</i> LACK antigen (Jüla, Hessel et al. 2002) and further challenged with LACK aerosols. One day after the last aerosol, AHR was measured by whole-body plethysmography in response to increasing concentrations of methacholine.
The results are shown in figure 5.

Legend of figure 5: (a) Wild type (filled symbols) and CX3CR1<sup>GFP</sup><sup>+/+</sup> (empty symbols) mice were sensitized with LACK and challenged with either LACK (circles) or saline buffer (squares). AHR was measured 1 day after the last aerosol by whole-body plethysmography in response to either nothing (baseline) or the indicated concentrations of inhaled methacholine in PBS. AHR is expressed as enhanced pause (Penh) arbitrary units. Data are expressed as mean values of individual mice (8 per group) ± SEM. (b, c, d, e) Wt (filled bars) and CX3CR1<sup>GFP</sup><sup>+/+</sup> (empty bars) mice (8 mice per group) were sensitized and challenged with LACK and sacrificed one day after the last aerosol. (b) BAL cells were harvested from individual mice and cytospins were analyzed by optical microscopy after Wright-Giemsa staining. Data show total number of cells, frequency and number of each cell type in wt and CX3CR1<sup>GFP</sup><sup>+/+</sup> mice. Data show mean value ± SD for a representative (out of 5) experiment. (c) Lungs from wt (upper panels) and CX3CR1<sup>GFP</sup><sup>+/+</sup> mice (lower panels) were fixed with Immunofix<sup>TM</sup>, embedded in Immunohistowax<sup>TM</sup>, and 4 μm-sections were prepared and stained with May-Grünwald-Giemsa. Data show representative microscopic images at a 10- (left) and 63-fold (right) magnification. (d) Total lung cells were restimulated in vitro with LACK. Supernatants were harvested 3 days later and analyzed for IL-4, IL-5, IL-13 and IFN-γ contents by ELISA. Data show mean ± SD for 5 mice per group, and are representative of one out of four experiments. (e) Lung T cells were purified and 1.5 x 10<sup>5</sup> cells were incubated in vitro with LACK in the presence of 10<sup>8</sup> BALB/c mitomycin C-treated syngenic splenocytes. Supernatants were harvested 3 days later and analyzed for IL-5 and IL-13 contents by ELISA. Data show mean ± SD for 5 mice per group, and are representative of one out of two experiments. * P < 0.05.

In striking contrast to antigen-challenged wt mice, antigen-challenged CX3CR1<sup>GFP</sup><sup>+/+</sup> mice did not develop AHR (Fig. 5a). As expected, neither wt nor CX3CR1<sup>GFP</sup><sup>+/+</sup> mice developed AHR when challenged with saline. The number of cells recovered from BAL was two-fold lower in CX3CR1<sup>GFP</sup><sup>+/+</sup> than in wt mice (Fig. 5b). Frequency and total number of eosinophils in BAL were respectively reduced 3- and 6-fold in CX3CR1<sup>GFP</sup><sup>+/+</sup> mice as compared to wt mice (Fig. 5b). Furthermore, lymphocytes were present in the BAL from wt mice but hardly detectable in CX3CR1<sup>GFP</sup><sup>+/+</sup> animals (Fig. 5b). May-Grünwald-Giemsa staining of histological lung sections from CX3CR1<sup>GFP</sup><sup>+/+</sup> mice revealed reduced peribronchial and perivascular inflammatory infiltrates and greatly reduced numbers of eosinophils as compared to prominent inflammation and eosinophilia in wt animals (Fig. 5c).
Upon *in vitro* restimulation with LACK, lung cells from CX3CR1<sup>GFP<sup>+/+</sup> mice secreted reduced amounts of IL-4 and IL-13, but similar quantities of IFN-γ as compared to those from wt mice (Fig. 5d). To rule out that differences between APC from CX3CR1<sup>GFP</sup>+/+ and wt mice were responsible for this phenomenon, lung T cells from sensitized and challenged mice were purified and incubated with wt splenocytes and LACK. While lung T cells from wt mice secreted large amounts of IL-5 and IL-13 in response to LACK, those from CX3CR1<sup>GFP</sup>+/+ mice did not produce detectable levels of these cytokines (Fig. 5e).

CX3CR1 is expressed by several cell types including monocytes, NK and subsets of T cells (Imai, Hieshima et al. 1997). Therefore, the inventors investigated whether the impaired ability of CX3CR1<sup>GFP</sup>/+ mice to develop allergic asthma resulted from a defect within the T cell or the non-T cell compartment. To address this issue, CD4<sup>+</sup> T cells from either CX3CR1<sup>GFP</sup>/+ or wt mice were adoptively transferred into CX3CR1<sup>GFP</sup>/+ recipients that were further sensitized and challenged with LACK. The results are shown on figure 6.

Legend of figure 6: Wt (filled symbols) and CX3CR1<sup>GFP</sup>/+ (empty symbols) mice (8 mice per group) were injected or not with 2 x 10<sup>6</sup> CD4<sup>+</sup> T cells from either wt or CX3CR1<sup>GFP</sup>/+ mice. Mice were sensitized with LACK, and challenged with LACK or saline buffer. (a) AHR was measured 1 day after the last aerosol by whole-body plethysmography in response to nothing (baseline) or the indicated concentrations of inhaled methacholine in PBS. (b) BAL cells were harvested from individual mice and cytopsins were analysed by optical microscopy after Wright-Giemsa staining. Data show frequency and number of each cell type for the indicated mice. Data show mean value ± SD for a representative (out of 2) experiments.

The results show that while wt CD4<sup>+</sup> T cells fully restored the ability of CX3CR1<sup>GFP</sup>/+ mice to develop AHR, CD4<sup>+</sup> T cells from CX3CR1<sup>GFP</sup>/+ mice were unable to do so (Fig. 6a). Likewise, wt, but not CX3CR1-deficient, CD4<sup>+</sup> T cells induced an increase in both the frequency and the number of eosinophils in BAL as compared to control CX3CR1<sup>GFP</sup>/+ mice that had not been injected with CD4<sup>+</sup> T cells (Fig. 6b). Therefore, the impaired ability of CX3CR1<sup>GFP</sup>/+ mice to develop allergic asthma resulted solely from a defect within the T cell compartment.

CX3CR1 could play a role in T cell activation, expansion or differentiation. The inventors have hence investigated the phenotype of CX3CR1-deficient T cells. The results are shown in figure 7, in which: (a) Wt (filled circles) and CX3CR1<sup>GFP</sup>/+ (empty circles) mice (4 mice per group) were sensitized twice with LACK. Splenocytes were harvested 6 days after the last immunization and
incubated with the indicated concentrations of LACK. Cellular supernatants were harvested 72 hours later and analyzed for IL-5 and IL-13 contents by ELISA. (b) Wt (filled bars) and CX3CR1<sup>GFP<sup>+/+</sup> (empty bars) mice (4 mice per group) were sensitized and challenged with LACK. Sera were collected 24 hours after the last aerosol and analyzed for the presence of LACK-specific IgG1 and total IgE by ELISA. Data show mean ± S.D. for individual mice. (c) CD4<sup>+</sup> T cells from either Thy1.1<sup>+</sup> WT15 TCR transgenic mice or Thy1.1<sup>−/−</sup> CX3CR1<sup>GFP<sup>+/+</sup> WT15 TCR transgenic mice were labeled with CFSE and injected into Thy1.1<sup>−/−</sup> BALB/c mice. Animals were sensitized with LACK one day after and mesenteric LN were analyzed 6 days later by flow cytometry after staining with anti-CD4, anti-Thy1.1 and anti-Thy1.2 mAb. Data show representative FACS profiles after gating on Thy1.1<sup>+</sup> Thy1.2<sup>−</sup> CD4<sup>+</sup> T cells (CX3CR1-deficient) or Thy1.1<sup>+</sup> Thy1.2<sup>−</sup> CD4<sup>+</sup> T cells.

Spleen cells from LACK-sensitized CX3CR1<sup>GFP<sup>+/+</sup> </sup> and wt mice secreted similar amounts of IL-13 and IL-5 when incubated with LACK (Fig. 7a). CX3CR1<sup>GFP<sup>+/+</sup> </sup> and wt mice exhibited similar titers of LACK-specific IgG1 and total IgE upon sensitization with LACK (Fig. 7b). Lastly, adoptively transferred CX3CR1-deficient LACK-specific CD4<sup>+</sup> TCR transgenic T cells were not impaired in their ability to proliferate in vivo in syngeneic wt recipients upon LACK sensitization (Fig. 7c). Therefore, CX3CR1-deficient CD4<sup>+</sup> T cells were not impaired in their ability to proliferate and to differentiate into Th2 cells in vivo.

To investigate CX3CR1 expression by CD4<sup>+</sup> T cells, wt mice were sensitized and challenged with LACK, and total and I-A<sup>β</sup>/LACK<sup>+</sup> CD4<sup>+</sup> T cells were purified by FACS after staining with I-A<sup>β</sup>/LACK multimers. Levels of CX3CR1 mRNA were measured using quantitative real-time PCR. In total CD4<sup>+</sup> T cells, CX3CR1 mRNA was expressed at higher levels in BAL and lungs as compared to either spleen or PBLN. In PBLN, I-A<sup>β</sup>/LACK<sup>+</sup> cells expressed 50-fold more CX3CR1 mRNA than total CD4<sup>+</sup> T cells, further suggesting that CX3CR1 was upregulated upon antigen-driven T cell activation (Fig. 8a). As observed for total CD4<sup>+</sup> T cells, I-A<sup>β</sup>/LACK<sup>+</sup> cells expressed higher levels of CX3CR1 mRNA in lungs and BAL as compared to spleen. Because GFP expression was found to correlate with CX3CR1 surface expression in CX3CR1<sup>GFP<sup>+/+</sup> </sup> heterozygote mice (Jung, Aliberti et al. 2000; Geissmann, Jung et al. 2003), CX3CR1<sup>GFP<sup>+/+</sup> </sup> mice were sensitized and challenged with LACK, and CD4<sup>+</sup> T cells were analyzed for GFP expression. While less than 1.2% of CD4<sup>+</sup> T cells in blood, spleen and PBLN were GFP<sup>+</sup>, between 5 and 10% of lung and BAL CD4<sup>+</sup> T cells were GFP<sup>+</sup> (Fig. 8b). In addition, lung purified CD4<sup>+</sup>GFP<sup>+</sup> cells secreted 1.5-, 4.5- and 7-fold more IL-5, IL-13 and IL-4,
respectively, than CD4+GFP+ T cells, when restimulated with LACK in vitro (Fig. 8c). Therefore, lung GFP+CD4+ T cells were enriched in LACK-specific Th2 cells.

Legend of figure 8: (a) BALB/c mice or (b, c) CX3CR1eGFP+/− were sensitized and challenged with LACK. Mice were sacrificed one (b) or two days (a, c) after the last aerosol. (a) Spleen, PBLN, lung and BAL cells were stained with I-A^B/LACK multimers and anti-CD4 mAbs, and I-A^B/LACK^+ (filled bars) and total (empty bars) CD4+ T cells were sorted by flow cytometry. Cells were used as a source of mRNA and levels of CX3CR1 mRNA was measured by quantitative RT-PCR using pre-calibrated TaqMan reagents. Data are expressed as arbitrary units. (b) Blood, spleen, PBLN, lung and BAL cells were prepared and analyzed by flow cytometry after staining with anti-CD4 mAbs. Data show representative FACS profiles after gating on CD4+ T cells. Numbers indicate the frequency of GFP+ cells among CD4+ T cells. (c) Lung cells were prepared, stained with anti-CD4 mAbs, and CD4+GFP+ (filled bars) and CD4+GFP− (empty bars) cells were sorted by flow cytometry. 2 x 10^4 sorted cells were incubated with 5 x 10^5 mitomycin C-treated syngenic splenocytes and LACK protein. Supernatants were harvested 3 days later and analyzed for IL-5, IL-13 and IL-4 contents by ELISA. Data are representative of one out of two experiments.

Earlier studies using CX3CR1 and CX3CL1-deficient mice have shown that CX3CL1/CX3CR1 interactions were not required for monocyte recruitment in peritonitis, DC differentiation, and DC migration in response to microbial antigens or skin-sensitizing agents (Jung, Aliberti et al. 2000; Cook, Chen et al. 2001). In striking contrast, the above results show that CX3CR1 is required for the development of allergic asthma in mice that have been sensitized and challenged with LACK. This phenomenon was also observed in mice that had been sensitized and challenged with OVA (ovalbumin antigen), further suggesting that the role of CX3CR1 is not restricted to specific antigens. The results are shown in figure 9, in which: (a) Wild type (filled symbols) and CX3CR1eGFP+/− (empty symbols) mice were sensitized with OVA and challenged with either OVA (circles) or saline buffer (squares). AHR was measured 1 day after the last aerosol by whole-body plethysmography in response to either nothing (baseline) or the indicated concentrations of inhaled methacholine in PBS. AHR is expressed as enhanced pause (Penh) arbitrary units. Data are expressed as mean values of individual mice (8 per group) ± SEM. (b, c) Wt (filled bars) and CX3CR1eGFP+/− (empty bars) mice (8 mice per group) were sensitized and challenged with OVA and sacrificed one day after the last aerosol. (b) BAL cells were harvested from individual mice and cystospins were
analyzed by optical microscopy after Wright-Giemsa staining. Data show total number of cells, frequency and number of each cell type in wt and CX3CR1<sup>GFP/+</sup> mice. Data show mean value ± SD for a representative (out of 5) experiment. (c) Lungs from wt (upper panels) and CX3CR1<sup>GFP/+</sup> mice (lower panels) were fixed with Immunofix<sup>TM</sup>, embedded in Immunohistowax<sup>TM</sup>, and 4 μm-sections were prepared and stained with May-Grünwald Giemsa. Data show representative microscopic images at a 10- (left) and 63-fold (right) magnification.

The present results can most probably be extrapolated to the development of allergic asthma in humans. Indeed, asthmatic patients were found to exhibit both increased levels of CX3CL1 in BAL upon bronchial segmental allergen challenge and increased CX3CR1 activity on peripheral CD4<sup>+</sup> effector T cells (Rimaniol, Till et al. 2003). Furthermore, CX3CR1 was found to be increased in atopic dermatitis, another Th2-associated pathology (Echigo, Hasegawa et al. 2004) and in psoriasis, rather associated to a Th1 profile, while CX3CL1 was only increased in atopic dermatitis. Therefore, a specific increase of CX3CR1-CX3CL1 is most probably not merely restricted to asthma but extended to other Th2 pathologies.

In contrast to other chemokine and chemokine receptors, CX3CR1 has only one ligand, and is the sole receptor for CX3CL1. Any treatment blocking CX3CR1/CX3CL1 interactions could hence be beneficial for patients with atopic allergies, in particular atopic asthma and rhinitis.

**EXAMPLE 6: TREATMENT AND PREVENTION OF AIRWAY INFLAMMATORY DISEASES BY BLOCKING CX3CR1/CX3CL1 INTERACTIONS**

Experiments are conducted in order to analyze the effect of anti-CX3CL1 antibodies on the development of AHR, eosinophilia, and antigen-specific T cell responses in mice.

BALB/c mice (20 mice) are sensitized with LACK on day 0 and day 7 as described (Julia, Hessel et al. 2002). Briefly, female BALB/c ByJ mice purchased from Charles River (Lyon, France) are used between 6 and 8 weeks of age. Sensitization is performed by 2 intra-peritoneal injections of 10 micrograms LACK protein precipitated in 2 mg of aluminum hydroxide (Alum) (PerBio Science France SAS, Brebières, France) at day 0 and 7. Mice (10 per group) are treated with either anti-CX3CL1 antibodies or with control isotypic antibodies on day 13. On day 14, 15, 16, 17 and 18, sensitized mice are exposed to LACK aerosols (1.5 mg/ml) for 30 minutes. Aerosolization is performed using an Ultramed, ultra-son nebulizator (Medicalia, Italy) connected to a 6500 cm<sup>3</sup> box that is used as a nebulization chamber.
As a control, CX3CR1\textsuperscript{GFP}+ mice (10 mice) are sensitized and challenged with LACK.

Five mice in each group are analyzed on day 19 for AHR by whole-body plethysmography in response to increasing concentrations (6-25 mg/ml) of inhaled methacholine (acetyl methyl choline). AHR is expressed as enhanced pause (Penh), a dimensionless parameter that correlates to airway compliance and resistance calculated value, which correlates with measurement of airway resistance, impedance, and intra-pleural pressure in the same mouse. ANOVA is used to determine the levels of difference between groups of mice for plethysmography values. Comparisons for all pairs are performed by unpaired two-tailed Student's t-test. Significance level is set at a P value of 0.05.

Penh values measured in mice treated with anti-CX3CL1 antibodies are lower than those measured in mice treated with control isotypic antibodies, further suggesting that blocking CX3CL1 results in decreased AHR.

Five mice in each group are bled and a canula is inserted into the trachea. Lungs are washed 3 times with 1 ml of warmed PBS. Cells are washed with PBS, resuspended in 500 µl, and counted using a Burker-Turk chamber. For differential BAL cell counts, cytospin preparations are made and stained with Wright/Giemsa. At least 400 cells are scored for each slide, and the numbers of lymphocytes, neutrophils, eosinophils, and others cells are determined by microscopic examination. Lung samples are harvested and fixed with ImmunohistofixTM and embedded in Immunohistowax\textsuperscript{TM}. 4 µm sections are performed and stained with May Grünwald Giemsa. PBLN are removed, cut into small pieces, and digested with collagenase D (Roche Diagnostics, Meylan, France) for 1 hour at 37°C. Cell suspensions are filtered through a 70 µm cell strainer and depleted of red blood cells using lysis buffer. Cells are washed with Hanks BSS solution (Invitrogen SARL, Cergy-pontoise, France). 5 x 10^6 cells are incubated for 72 hours in either 24-well plate for lung cells, or in 96-well plates for PBLN cells, in medium containing 0.25 mg/ml LACK protein. Purified lung or LN T cells are restimulated with mitomycin-C treated splenocytes (20 minutes at 37°C). Supernatants are analyzed for IL-4, IL-5, and IFN-gamma contents by ELISA as described 3, and IL-13 content is measured using quantitative mouse IL-13 kit according to the manufacturer's instructions (R&D Systems, Inc., Lille, France). RPMI 1640 containing Glutamax I (Invitrogen), 10% heat-inactivated FCS (Perbio), 0.05 micromolar 2-mercaptoethanol (Sigma), 2 mM L-glutamine (Invitrogen) and penicillin/streptomycin (Invitrogen), are used as medium for all assays. Quantification of cytokines in lung tissue is performed as described
(Julia, Hessel et al. 2002). In brief, samples are homogenized in 400 µl of buffer (per right lung) containing 1% NP-40, 0.5 M NaCl, 50 mM Hepes, 0.1 mM PMSF, 1 mM iodoacetamide and Complete protease inhibitor cocktail (Roche). IL-13 and eotaxin/CCL11 are measured using quantitative mouse IL-13 and eotaxin kit (R&D Systems, Inc., Lille, France).

Results show that the BAL from mice treated with anti-CX3CL1 antibodies contain less cells than those from mice treated with isotype control antibodies further suggesting that neutralization of CX3CL1 results in decreased cell infiltration in the airways. Results show that the BAL from mice treated with anti-CX3CL1 antibodies contain less eosinophils than those from mice treated with isotype control antibodies, further suggesting that neutralization of CX3CL1 results in decreased eosinophilia. Moreover, both lungs cells and PBLN cells from mice treated with anti-CX3CL1 antibodies secrete less IL-4, IL-5 and IL-13 when incubated with LACK in vitro than those from mice treated with isotype control antibodies, suggesting that neutralization of CX3CL1 results in decreased Th2 response to LACK.

**EXAMPLE 7: CX3CR1 IS EXPRESSED BY T CELLS INFILTRATING THE AIRWAYS**

Experiments are conducted in order to monitor the expression of CX3CR1 at the surface of the T cells which infiltrate the airways in sensitized and challenged mice.

BALB/c mice (20 mice) are sensitized with LACK on day 0 and day 7 as described (Julia, Hessel et al. 2002). Briefly, female BALB/c ByJ mice purchased from Charles River (Lyon, France) are used between 6 and 8 weeks of age. Sensitization is performed by 2 intra-peritoneal injections of 10 micrograms LACK protein precipitated in 2 mg of aluminum hydroxide (Alum) (PerBio Science France SAS, Brebières, France) at day 0 and 7. Mice (10 per group) are treated with either anti-CX3CL1 antibodies or with control isotypic antibodies on day 13. On day 14, 15, 16, 17 and 18, sensitized mice are exposed to LACK aerosols (1.5 mg/ml) for 30 minutes. Aerosolization is performed using an Ultramed, ultra-son nebulizator (Medicalia, Italy) connected to a 6500 cm³ box that is used as a nebulization chamber. As a control, CX3CR1 KO mice (10 mice) are sensitized and challenged with LACK.

Ten mice in each group are bled and a canula is inserted into the trachea. Lungs are washed 3 times with 1 ml of warmed PBS. Cells are washed with PBS, resuspended in 500 µl, and counted using a Burker-Turk chamber. PBLN are removed, cut into small pieces, and digested with collagenase D (Roche Diagnostics, Meylan, France) for 1 hour at 37°C. Cell suspensions are filtered through a 70 µm cell
strainer and depleted of red blood cells using lysis buffer. Cells are washed with Hanks BSS solution (Invitrogen SARL, Cergy-pontoise, France) and analyzed by flow cytometry after staining with reagents which bind to CX3CR1 and anti-CD3 and anti-CD4 antibodies.

5 Results show that CD3+ CD4+ T cells in BAL and lungs are stained with the CX3CR1 staining reagent, further suggesting that these cells express CX3CR1. In contrast, no staining is observed in CX3CR1<sup>gfp<sup>-/-</sup> mice, further confirming the specificity of the staining reagent used in these experiments.
REFERENCES


CLAIMS

1) Use of a fractalkine/CX3CR1 antagonist or of an inhibitor of the expression of fractalkine or of CX3CR1, for preparing a medicament for treating an atopic allergic disease.

2) Use according to claim 1, wherein said atopic allergic disease is a respiratory disease.

3) Use according to claim 2, wherein said disease is asthma

4) Use according to claim 2 wherein said disease is allergic rhinitis.
Figure 1
Figure 2
Figure 4

A- Lung

- IL-4 (U/ml)
- IL-13 (pg/ml)
- IL-5 (U/ml)
- IFN-γ (ng/ml)

B- PBLN

- IL-4 (U/ml)
- IL-13 (pg/ml)

Legend:
- WT
- CX3CR1<sup>+</sup>GFP<sup>+</sup>
- CX3CR1<sup>+</sup>GFP<sup>-/-</sup>
Fig. 5 c
Fig. 7
Fig. 8
Fig. 9
### A. CLASSIFICATION OF SUBJECT MATTER

| IPC | A61K38/17 | A61K39/395 | A61P11/06 |

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)

| IPC | A61K | A61P |

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

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, MEDLINE, EMBASE, WPI Data, PAJ, BIOSIS, PASCAL

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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Further documents are listed in the continuation of box C.

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Date of the actual completion of the international search: 12 January 2005

Date of mailing of the international search report: 19/01/2005

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