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(54) Title: TR1 CELLS FOR USE IN ATHEROSCLEROSIS

(57) Abstract: The present invention relates to a method of treating or preventing atherosclerosis comprising administering to a mammal in need of such a treatment a) an antigen which has been used to activate *in vitro* a Tri cell population originating from said mammal, and b) the TO cell population activated by said antigen, said TO cell population being obtained from a CD4+ T lymphocyte population of said mammal.

TR1 CELLS FOR USE IN ATHEROSCLEROSIS

The present invention relates to a method of treating or preventing atherosclerosis comprising administering to a mammal in need of such a treatment :

a) an antigen which has been used to activate *in vitro* a Tr1 cell population originating from said mammal, and

b) the Tr1 cell population activated by said antigen, said Tr1 cell population being obtained from a CD4+ T lymphocyte population of said mammal.

BACKGROUND OF INVENTION

Atherosclerosis is a cardiovascular condition occurring as a result of narrowing down of the arterial walls. The narrowing is due to the formation of plaques (raised patches) or streaks in the inner lining of the arteries. These plaques consist of foam cells of low-density lipoproteins, oxidized-LDL, decaying muscle cells, fibrous tissue, clumps of blood platelets, cholesterol, and sometimes calcium. They tend to form in regions of turbulent blood flow and are found most often in people with high concentrations of cholesterol in the bloodstream. The number and thickness of plaques increase with age, causing loss of the smooth lining of the blood vessels and encouraging the formation of thrombi (blood clots). Sometimes fragments of thrombi break off and form emboli, which travel through the bloodstream and block smaller vessels.

The blood supply is restricted to the heart, eventually forming a blood clot leading to death. The major causes of atherosclerosis are hypercholesterolemia and hyperlipidemia is high circulating cholesterol and high lipids like LDL-cholesterol and triglycerides in the blood. These lipids are deposited in the arterial walls, obstructing the blood flow and forming atherosclerotic plaques leading to death.

Atherosclerosis is responsible for more deaths in countries, such as in the Unites States of America, than any other single condition. Atherosclerotic heart

disease involving the coronary arteries is the most common single cause of death, accounting for one third of all deaths. Atherosclerotic interference with blood supply to the brain (causing stroke) is the third most common cause of death after cancer. Atherosclerosis also causes a great deal of serious illness by reducing the blood flow in other major arteries, such as those to the kidneys, the legs and the intestines.

Medication is not a satisfactory treatment because much of the damage to the artery walls has already been done. Anticoagulant drugs have been used to try to minimize secondary clotting and embolus formation, but have little or no effect on the progress of the disease. Vasodilator drugs are used to provide symptom relief, but are of no curative value.

Surgical treatment is available for certain high-risk situations. Balloon angioplasty can open up narrowed vessels and promote an unproved blood supply. The blood supply to the heart muscle can also be restored through a vein graft bypass. Large atheromatous and calcified arterial obstructions can be removed by endarterectomy, and entire segments of diseased peripheral vessels can be replaced by woven plastic tube grafts.

There is a need for a new therapy, possibly to prevent these conditions and insure better health, particularly in people who are genetically predisposed to such conditions. The present invention fulfills these and other needs.

There is now accumulating evidence for a novel functionally distinct subpopulation of T cells, called regulatory T cells that exert important regulatory functions in various immuno-inflammatory diseases (see references 12 to 14 for review). Several subsets of regulatory T (Tr) cells with distinct phenotypes and distinct mechanisms of action have now been identified. These include Tr1 cells (15 to 20), which secrete high levels of interleukin (IL)-10 and low to moderate levels of transforming growth factor (TGF)-β, Th3 cells (21,22), which primarily secrete TGF-β, and CD4+CD25+ T cells, which inhibit immune responses through cell–cell contact (23). We have shown that

repeated stimulation of naive T cells, from OVA T-cell receptor (TCR)-transgenic mice with OVA and IL-10, results in the generation of T-cell clones with a unique cytokine profile distinct from that of Th0, Th1 or Th2 cells (15). These Tr1 cells produce IL-10, IL-5 and IFN-γ, with or without TGF-β, but with little or no IL-2 or IL-4, and proliferate poorly following polyclonal TCR-mediated activation. Functional studies have shown that Tr1 cells have immunosuppressive properties and have been shown to prevent the development of Th1-mediated autoimmune diseases (15). Co-culture of naïve CD4+ T cells and human Tr1 clones in the presence of allogenic antigen-presenting cells (APCs) results in the suppression of proliferative responses (15). Similarly, Tr1 clones specific for filamentous haemaggultinin (HA) from *Bordetella pertussis* suppress proliferation and cytokine production by a Th1 clone against an unrelated antigen, influenza virus HA (20). In both cases, the suppressive effects of Tr1-cell clones are reversed by neutralizing IL-10, suggesting that, regardless of their antigen specificities, Tr1 cell suppression is a bystander effect mediated through the production of IL-10.

Here, we tested whether transfer of Tr1 cells in mammals could reduce immune inflammatory processes and more specifically could reduce risk of artherosclerosis. We show for the first time that transfer of such Tr1 cells to apoE knockout (KO) mice inhibits pathogenic Th responses and reduces the development of atherosclerosis.

DESCRIPTION OF THE INVENTION

A first subject of the present invention is a method of treating or preventing atherosclerosis comprising administering sequencially, simulteanously or separately to a mammal in need of such a treatment :

a) an antigen which has been used to activate *in vitro* a Tr1 cell population originating from said mammal, and

b) the Tr1 cell population activated by said antigen, said Tr1 cell population being obtained from a CD4+ T lymphocyte population of said mammal.

The antigen administered in the method of the present invention is an immunogenic peptide. Immunogenic peptide will be referred herein as non-pathogenic peptides or proteins that can bind to MHCII molecule of an individual and that is recognized by the T cell receptor of said individual. For example, the antigen is a non-allergic food antigen or a non-pathogenic bacterial antigen. Between 0.05 mg/kg to 5 mg/kg, for example 1, 2, 3, 4 mg/kg of the antigen is administered to the mammal in need of treatment. The antigen can be administered once or twice the first month (for example at day 1 and at day 15) and then once a month for about 2 to 3 months. Routes of administration include but are not limited to intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, preferably intravenous.

In another embodiment, the method includes administering from 10^6 to 10^9 antigen-activated Tr1 cells/kg, for example from 0.5 10^7 to 1.5 10^7 cells/kg, preferably 10^7 cells/kg.

In the method as depicted above, the antigen-activated Tr1 cell population of step b) is obtainable by an *in vitro* preparation process comprising the following steps:

i) obtaining a Tr1 cell population from the CD4+ T lymphocyte population of the mammal in need of the treatment;

ii) *in vitro* activating said Tr1 cell population by contacting with an antigen; and

iii) recovering the antigen-activated Tr1 cell population.

In a preferred embodiment, step i), which is obtaining the Tr1 cell population, comprises the following steps:

a) isolating a progenitor cell population from said mammal;

b) obtaining a population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10);

c) contacting cells of step b) with the CD4+ T lymphocyte population isolated from said mammal to allow differenciation of said CD4+ T lymphocytes into the Tr1 cell population; and

d) recovering the Tr1 cell population from the step c).

In step b), IL-10 is from 50 to 250 Uml^{-1}, preferably at 100 Uml^{-1} in the culture medium.

The obtention of the Tr1 cell population with steps comprising contacting dendritic cells with a CD4+ T lymphocyte population, and obtening the population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10), are described in the paragraphs "Results" and "Experimental procedures" of the publication Wakkach et al. (Immunity. 2003 May;18(5):605-17), incorporated herein by reference.

In yet another preferred embodiment, step i) which is obtaining the Tr1 cell population comprises the following steps:

a) culturing the CD4+ T lymphocyte population in a media with an appropriate amount of alpha-interferon (α-IFN), and

b) recovering the Tr1 cell population.

α-IFN is preferably at 5 ng/ml in the media.
In the step a), the media may further comprise an appropriate amount of IL-10, which is preferably at 100 Uml-1.

In step b), the Tr1 cell population is cultured in a media comprising interleukine 15 (IL-15) to allow proliferation. IL-15 is preferably at 5 ng/ml in the media.

The process of obtaining a Tr1 cell population by contacting a CD4+ T lymphocyte population with an appropriate amount of alpha-interferon (α-IFN) is described in the paragraph "Tr1 cell differentiation" of the american patent application US 2002/0034500, which was published on March 21, 2002

(LEVINGS et al) (see from p. 2, col. 2, L.33 to p.6, col.1, L. 22 and claims, which are incorporated herein by reference).

In still another embodiment, the invention is directed to a method as defined above wherein the antigen-activated Tr1 cell population of step b) is obtained by an *in vitro* preparation process comprising the following steps:

i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen, presented by artificial antigen presenting cells; and

ii) recovering an activated CD4+ T lymphocyte population comprising at least 10% of antigen-activated Tr1 cells.

Preferably, the artificial antigen presenting cells express a HLA II system molecule and a human LFA-3 molecule, and don't express the co-stimulation molecules B7-1, B7-2, B7-H1, CD40, CD23 and ICAM-1.

The preparation process of obtaining the antigen-activated Tr1 cell population wherein artificial antigen presenting cells are used is described in the international patent application WO 02/092793 published on November 21, 2002, from page 5, L. 8 to 14, L. 25, which passage is incorporated herein by reference. The figure 1 of this patent application is also incorporated herein by reference.

In still another embodiment, the invention is directed to a method as defined above wherein the antigen-activated Tr1 cell population of step b) is obtained by an *in vitro* preparation process comprising the following steps:

i) *in vitro* activating the CD4+ T lymphocyte population in presence of an antigen and an appropriate amount of interleukine -10 (IL-10); and

ii) recovering the antigen-activated Tr1 cell population.

Preferably, IL-10 is present in the culture media at a 100 Uml^{-1}.

The preparation process of obtaining the antigen-activated Tr1 cell population wherein IL-10 is used is described in the scientific publication Groux et al

(Nature. 1997 Oct 16;389(6652):737-42) in the paragraph "Methods", which is incorporated herein by reference.

The method as described above is particularly useful for preventing, delaying the onset and/or treating humans.

A second aspect of the present invention is aimed at a product comprising :

a) an antigen which has been used to activate *in vitro* a Tr1 cell population originating from a mammal, and

b) the Tr1 cell population activated by said antigen, said Tr1 cell population being obtained from a CD4+ T lymphocyte population of said mammal,

as a combined preparation for simultaneous, separate or sequential use for treating or preventing atherosclerosis.

Preferably, the combined preparation is for treating or preventing atherosclerosis in said mammal.

More preferably, said product is a combined preparation for simultaneous, separate or sequential use for treating or preventing atherosclerosis in humans.

The antigen in the product may be at a dose ranging from 0.05 mg/kg to 5 mg/kg, for example 1, 2, 3, 4 mg/kg.

In one particular embodiment, the product is suitable for administration once or twice the first month (for example at day 1 and at day 15) and then once a month for about 2 to 3 months. It canbe formulated for different routes of administration including intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, preferably intravenous.

The product of the invention also comprises from 10^6 to 10^9 antigen-activated Tr1 cells/kg, for example from 0.5 10^7 to 1.5 10^7 cells/kg, preferably 10^7 cells/kg.

In the product of the invention, the antigen-activated Tr1 cell of step b) population is obtainable by an *in vitro* preparation process comprising the following steps:

i) obtaining a Tr1 cell population from the CD4+ T lymphocyte population of the mammal in need of treatment;

ii) *in vitro* activating the Tr1 cell population by contacting it with the antigen; and

iii) recovering the antigen-activated Tr1 cell population.

In another embodiment, the product comprises Tr1 cells obtainable by a process comprising the following steps:

a) isolating a progenitor cell population from said mammal;

b) obtaining a population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10);

c) contacting cells of step b) with the CD4+ T lymphocyte population isolated from said mammal to allow differenciation of said CD4+ T lymphocytes into the Tr1 cell population; and

d) recovering the Tr1 cell population from the step c).

The obtention of the Tr1 cell population with a step of contacting dendritic cells with a CD4+ T lymphocyte population, and the obtention of the population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10), are described in the paragraphs "Results" and "Experimental procedures" of the scientific publication Wakkach et al. (Immunity. 2003 May;18(5):605-17), incorporated herein by reference.

Preferably, step i) of obtaining the Tr1 cell population comprises the following steps:

a) culturing the CD4+ T lymphocyte population with an appropriate amount of alpha-interferon (α-IFN); and

b) recovering the Tr1 cell population.

Step a) may also comprises culturing in combination with an appropriate amount of IL-10. In addition, the Tr1 cell population may be further expanded with interleukine 15 (IL-15).

In the product of the invention, the antigen-activated Tr1 cell population of step b) may also be obtained by an *in vitro* preparation process comprising the following steps:

i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen, presented by artificial antigen presenting cells; and

ii) recovering an activated CD4+ T lymphocyte population comprising at least 10% of the antigen-activated Tr1 cell population.

Preferably, the artificial antigen presenting cells express a HLA II system molecule and a human LFA-3 molecule, and don't express the co-stimulation molecules B7-1, B7-2, B7-H1, CD40, CD23 and ICAM-1.

In the product of the present invention, the antigen-activated Tr1 cell population of step b) may also be obtained by an *in vitro* preparation process comprising the following steps:

i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen and and appropriate amount of interleukine -10 (IL-10); and

iii) recovering the antigen-activated Tr1 cell population.

The preparation process of obtaining the antigen-activated Tr1 cell population wherein IL-10 is used is described in the scientific publication Groux et al (Nature. 1997 Oct 16;389(6652):737-42) in the paragraph "Methods", which is incorporated herein by reference.

The invention is also aimed at the use of a product as defined above for the manufacture of a medicament for preventing or treating artherosclerosis.

In the method and product as depicted above, Tr1 cells are featured by the following specific combination of surface markers : CD4, CD18 et/ou CD11a, et CD49b. CD3 can also be contemplated as marker.

Therefore, in another embodiment, the invention is directed to a method and product as described above, wherein Tr1 cells are obtainable by any method using said markers. For example, Tr1 cells can be identified and/or purified by Elisa, flowcytometry, immunoaffinity chromatography with antibodies directed against said markers, for example with :

APC- conjugated anti-CD4 (RPA-T4) – Becton Dickinson

PC5- conjugated anti-CD3 (UCHT-1) - Caltag

PE- conjugated anti-CD18 (6.7) - Becton Dickinson

FITC- conjugated anti-CD49b (AK-7) - Becton Dickinson

Purification of CD3+CD4+CD18brightCD49b+ cells :

Enrichment of CD3+CD4+CD18brightCD49b+ cells from lymphocytes can be performed with magnetic beads in two steps:

- depletion of the total population with anti-human Ig-magnetic beads of cells bound with human anti-CD8, anti-CD14, anti-CD56 and anti-CD19.
- Selection of CD49b+ cells bound to an anti-CD49b human antibody with anti-human Ig-magnetic beads.

Further purification is possible with flowcytometry or beads with CD3, CD18 et CD49b antibodies.

ELISA tests may also be used to mesure IL-4, IL-10, and l'IFN-alpha expression.

In this regard, claims and example 5 of PCT/FR 004/001583 is incorprated herein by reference.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 depicts representative photomicrographs show sections of advanced atherosclerotic plaques (Oil red O staining) from the aortic sinus of mice treated with Tr1, OVA/CFA, or (OVA/CFA)+Tr1. There was a substantial reduction in lesion size in OVA/CFA+Tr1 group. There was also a reduction in macrophage infiltration (red MOMA-2 staining) within lesions of mice treated with OVA/CFA+Tr1 compared with either Tr1- or OVA/CFA-treated mice. Original magnifications: x40 for Oil red O staining; x400 for MOMA-2 staining.

Figure 2 depicts representative photomicrographs show IL-10 red staining (arrows) in sections of advanced atherosclerotic plaques from the aortic sinus of mice treated with OVA/CFA (a, c), or (OVA/CFA)+Tr1 (b, d). Intense IL-10 staining (arrows in b and d) was found in plaques of mice treated with OVA/CFA+Tr1. Original magnifications: a and b, x200; c and d, x400.

EXAMPLES

EXAMPLE 1 – METHODS

1.1 Generation, expansion and transfer of ovalbumin (OVA)-specific Tr1 clones

The mouse T-cell clones were obtained from DO11-10 BALB/c mice after *in vitro* differentiation as previously described (15). Naive (MEL-14^{bright}) $CD4^+$, KJ-1.26^+ cells were stimulated repeatedly with OVA peptide 323-339 every week for 3 weeks, in the presence of IL-10. T-cell clones were generated as previously described (15). Clones were expanded and analyzed for cytokine secretion after activation with APCs and OVA peptide. Selected clones were then stimulated with irradiated splenocytes and OVA peptide every 2 weeks

and further expanded with IL-2 (R&D systems). T-cell clones were used at least 10 days after the last stimulation. Here we used clone A-10-9 that has been previously described and shown to prevent Th1- and Th2-mediated immune responses (15, 24).

Fifteen week-old female apoE KO C57Bl/6 mice were immunized subcutaneously with either saline (n=11) or 50 µg of OVA in CFA (both from Sigma) (n=18) every 12 days for 9 weeks. The saline-treated mice were then divided into 2 groups that received at day 0, in addition to subcutaneous saline, a unique intra-peritoneal injection (200 µl) of either saline (n=5) or 10^6 OVA-specific Tr1 cells without OVA/CFA (n=6). The OVA/CFA-immunized group was divided into 2 other groups of mice which received at day 0 an intra-peritoneal injection (200 µl) of either saline (OVA/CFA group, n=9) or 10^6 OVA-specific Tr1 cells (OVA/CFA+Tr1 group, n=9).

1.2 Cytokine assays

In brief, ELISA plates (Polylabo) were coated with the appropriate coating anti-cytokine mAbs (11B11, 2A5, TRFK4 and XGM-1 for IL-4, IL-10, IL-5 and IFN-γ respectively) in carbonate buffer and incubated at 4°C overnight. Sandwich ELISA were performed as previously described (24). Plates were read on an ELISA reader at a wavelenth of 405nm after color development (Labsystems iEMS reader).

1.3 Analysis of OVA-specific serum IgE, IgG1 and IgG2a

OVA-specific Igs levels were measured using a two-step sandwich ELISA as described (24). To detect IgG1 and IgG2a we used biotinylated rat mAb (A85-1, and R19-15 for IgG1 and IgG2a respectively, Becton Dickinson) at 2 µg/ml. Standards for OVA-specific IgG1 were pooled sera from hyperimmunised BALB/c mice.

1.4 Purification and culture of spleen and lymph node cells

T cells were purified from spleen or draining lymph nodes by negative selection using anti-CD11b (M1/70), anti-B220 and anti-NK cells (DX5) followed by depletion with a mixture of magnetic beads coated with anti-rat Ig (Dynal).

For cytokine measurements, purified T cells (10^5) were mixed with irradiated splenic APCs ($4x10^5$) and OVA/CFA (0.25 mg/ml) ConA (2 μg/ml) in 96 well plates. Supernatants were collected at 24 h (for IL-4 measurements) and at 48 h (for IL-5, IL-10, and IFN-γ measurements) and assayed for cytokine levels by ELISA as previously described (24).

For the cell proliferation assay, purified T cells were mixed with irradiated splenic and ConA or OVA. One μCi of ^{3}-thymidine (Perkin Elmer) was added for the last 12 h of cell culture.

1.5 Analysis of atherosclerotic plaque size and composition

Mice were sacrificed at 24 weeks of age. Plasma total and HDL-cholesterol were measured with a commercially available Cholesterol kit (Sigma). Morphometric and immunohistochemical studies were performed in the aortic sinus and the thoracic aorta (spanning from the brachiocephalic artery to the renal arteries and including the first 3 mm^2 of the brachiocephalic artery) as previously described (9).

Collagen fibers were stained with Sirius red. Immunohistochemical analysis was performed as previously described (9). The following primary antibodies were used: MOMA-2 (BioSource Int.) as a specific marker for macrophage; anti-mouse CD3-θ (Santa Cruz), anti-α-smooth muscle actin, alkaline phosphatase conjugate, clone 1A4 (Sigma), and anti-IL-10 antibody (Santa Cruz). At least four sections per animal were analyzed for each immunostaining. Morphometric analysis was performed with an automated image processor (Histolab, Microvision) as described (9).

1.6 Statistical Analysis

The effects of treatment on lesion area, plaque composition, serum Ig levels and cytokine production by spleen and lymph T cells were evaluated using ANOVA and Bonferroni/Dunn test or a Student t test. A value of $P<0.05$ was considered to be statistically significant.

EXAMPLE 2 - RESULTS

2.1 Transfer of Tr1 cells with their cognate antigen induces immune suppression

As expected (Table 1), treatment with OVA in complete Freund's adjuvant resulted in elevated serum levels of OVA-specific antibodies, predominantly IgG2a, and to a much lesser extent IgE, in comparison with the saline-treated group where OVA-specific IgGs were undetectable. Treatment of mice with OVA/CFA induced a Th1-like response as revealed by the production of high levels of IFN-γ (Th1 cytokine), but low levels of IL-10 and no IL-4 (Th2 cytokines), by OVA-stimulated lymph T cells (Table 1). Injection of regulatory Tr1 cells alone did not alter the immune response (Table 1). However, administration of Tr1 cells to OVA/CFA-immunized animals significantly inhibited the secretion of OVA-specific IgG2a antibodies as previously reported in a model using Alum as an adjuvant (24). Moreover, the suppressive effect of Tr1 cells was confirmed by the marked decrease in IFN-γ production and proliferative response observed in the (OVA/CFA)+Tr1 group as compared with the OVA/CFA after *in vitro* recall response of T cells ($P<0.001$). This was associated with enhanced IL-10 production by T cells stimulated with OVA in vitro ($P<0.001$), suggesting that the transferred OVA-specific Tr1 cells were functionally active (Table 1). TGF-β in the supernatants was below detectable levels.

IL-10 is a cytokine with important immunosuppressive effect *in vivo*. Therefore, to analyze the consequence of high IL-10 secretion in mice transferred with Tr1 cells and treated with OVA/CFA, we analyzed the cytokine response of T cells after polyclonal activation with ConA. As expected, ConA-stimulation induced a Th0 type response in T cells from

saline- or Tr1-treated mice and a Th1-type response in mice treated with OVA/CFA (Table 2). In contrast, in mice treated with regulatory Tr1 cells and OVA/CFA, we observed a marked decrease in IFN-γ production and an increase in IL-10 production by conA-stimulated T cells (Table 2). Interestingly, the ratio of INF-γ to IL-10 was reduced by 3 to 4-fold in (OVA/CFA)+Tr1 group compared to the other groups of mice, suggesting that the transfer of regulatory Tr1 cells followed by a systemic delivery of their specific antigen induced a bystander non specific regulatory immune response due to the chronic stimulation of Tr1 cells with OVA/CFA. However, as previously shown in other inflammatory models, the inhibitory function of Tr1 cells is local (15, 25), and chronic stimulation of Tr1 cells did not result in systemic secretion of IL-10 in the serum as <30 pg/ml of IL-10 was measured in the serum of the four different groups of mice.

2.2 Transfer of Tr1 cells with their cognate antigen reduces atherosclerotic lesion size in apoE deficient mice

Serum total cholesterol and high density lipoprotein (HDL) levels did not differ between groups (Table 3). Atherosclerotic lesion size in the thoracic aorta did not differ between saline and Tr1 groups (Table 3). There was a modest but not statistically significant reduction in lesion size in the thoracic aorta of mice treated with OVA/CFA in comparison with saline or Tr1 group (P=0.09 and P=0.07, respectively). In contrast, there was a marked and highly significant redcution in lesion size in the (OVA/CFA)+Tr1 group in comparison with either OVA/CFA (42% reduction, P=0.008), Tr1 alone or saline (55% reduction, $P \leq 0.001$) (Table 3).

Atherosclerotic lesion size in the aortic sinus did not differ between saline, Tr1 and OVA/CFA-treated animals. However, we found a highly significant decrease of plaque size in the (OVA/CFA)+Tr1 group compared with either saline (P=0.002), Tr1 (P<0.0001) or OVA/CFA group (P=0.001) (Table 3 and Figure 1). Despite the significant decrease in lesion size, plaques of mice treated with (OVA/CFA)+Tr1 showed a marked decrease in relative macrophage infiltration (32% to 46% reduction, Table 3 and Figure 1), and T

cell accumulation (37% to 50% reduction, Table 3), with no change in smooth muscle cell or collagen content (Table 3). In addition, we found intense IL-10 staining in the plaques of mice treated with (OVA/CFA)+Tr1 whereas no or barely detectable levels of IL-10 were found in plaques from the other 3 groups (Figure 2). IL-10 staining was also detected in the adventitia of all study groups but was clearly enhanced in (OVA/CFA)+Tr1 group (Figure 2). Overall, these results suggest that a regulatory T cell response is associated with a less pronounced inflammatory plaque phenotype.

2.3 Discussion

A substantial body of evidence suggests an important role for Th1-mediated immuno-inflammatory responses in the development and progression of atherosclerotic lesions, at least in experimental models of human-like atherosclerosis (3 to 10). There are also reports from human studies suggesting a strong association between Th1-related responses and plaque instability (9, 26 to 29). Therefore, much of the recent research in the field of atherosclerosis, based on current dogma suggesting that the immuno-inflammatory response in atherosclerosis is controlled by distinct Th1 and Th2 subpopulations of T cells, has focused on the potential role of Th2-mediated responses in the modulation of the atherosclerotic process. The finding by several independent groups that IL-10, a Th2-related cytokine, exerts major anti-atherogenic effects, has particularly reinforced the Th1/Th2 paradigm in immunity to atherosclerosis (11, 30 to 33). However, IL-10 is not specific of Th2 cells. Interestingly, another subtype of T cells, Tr1, with cytokine profiles distinct from either Th1 or Th2 cells, has been identified and shown to play an important role in the regulation and protection against various Th1- and Th2-mediated immuno-inflammatory diseases (see 12 to 14 for review). Therefore, we hypothesized that, in atherosclerosis, there may be an imbalance between the effector and the regulatory (Tr1 for instance) arms of the immune response, and suggested that supplementation with Tr1 cells may lead to the induction of a regulatory immune phenotype and a reduction in Th1- (and Th2) mediated responses, ultimately altering plaque development and/or composition.

Here, we showed that these regulatory Tr1 cells, when transferred into mice with their cognate antigen, induced a significant suppression of Th1-mediated responses and led to an increase in IL-10 production by stimulated peripheral T cells. Tr1-mediated immune suppression was observed in response to stimulation with either OVA or ConA, suggesting the induction of a systemic bystander immune suppression, which most likely resulted from repetitive administration of OVA/CFA. Interestingly, the induction of Tr1 responses was associated with a significant reduction in atherosclerotic plaque size, a marked reduction in the relative accumulation of inflammatory macrophages and T lymphocytes with a preservation of smooth muscle cell and collagen contents. It is noteworthy that the transfer and in vivo activation of Tr1 cells led to a profound reduction in the ratio of IFN-γ to IL-10 compared with the other groups (Table 2), and to the appearance of intense IL-10 staining within the plaques, suggesting that this change in the balance between pro- and anti-atherogenic mediators may have contributed to the alterations in lesion development and composition. These results show that modulation of the immune response is achievable by transfer and activation of Tr1 cells, and leads to limitation of the development of atherosclerosis in apoE deficient mice. It could be argued that our data may simply be compatible with a systemic effect of circulating IL-10 and that such effect may be independent of any regulatory or immuno-suppressive function. Although it is very difficult to separate the regulatory functions of Tr1 clones from their capacity to produce IL-10 (several studies have established the critical contribution of the immuno-modulatory cytokine IL-10 to the regulatory potential of Tr1, reviewed in (12, 13), we believe that several data argue against such hypothesis. First, we have been unable to detect any substantial production or difference in serum IL-10 levels between the various study groups whereas IL-10 protein accumulation was increased locally within the plaques of OVA/CFA+Tr1 group. Second, not all IL-10-producing T cells are regulatory T cells. Pinderski et al (33) have recently shown that overexpression of IL-10 by T lymphocytes (IL-10 was placed under the control of the IL-2 promoter) led to a switch toward a Th2 phenotype, which is phenotypically and functionally distinct from all Tr1 clones. Unlike the results obtained with the overexpression of IL-10 in the

latter study (33), the inventors have shown in their work that appropriate activation of Tr1 led to significant reductions in both Th1 (IFN-γ) and Th2-related cytokines (IL-4 and IL-5) and immunoglobulins (IgG2a and IgE, respectively) (Tables 1 and 2). This is consistent with previous studies showing that only regulatory T cells, like Tr1, are able to suppress both Th1 and Th2-related responses (12, 13, 20, 24).

Another potential limitation to our study is the confounding effect of CFA on atherosclerotic lesion development. Indeed, previously published studies have reported a reduction in atherosclerosis with CFA/IFA (34, 35). These reductions in the aortic arch or the thoracic aorta were moderate (18% and 19%) and were accompanied with changes in total and/or HDL-cholesterol levels that may have contributed to limitation of plaque progression. It is noteworthy that in our study, the OVA/CFA group showed a similar reduction in atherosclerotic lesion size in the thoracic aorta (23%) as that reported in studies using CFA/IFA, even in the absence of changes in cholesterol levels. However, in our study the additional transfer of Tr1 cells resulted in a much more important and marked 55% reduction in atherosclerosis.

In conclusion, our results provide the first proof of concept that cell transfer of a novel subpopulation of T cells with regulatory functions, here Tr1 cells, is able to modulate the in vivo immune response and leads to substantial limitation of plaque size while inducing changes in plaque composition indicative of a less inflammatory plaque phenotype. This work paves the way for the development and testing of novel therapeutic strategies based on the enhancement and/or transfer of specific regulatory immune cells (i.e., Tr1 cells specific for a plaque-derived antigen) to limit the development and prevent the complications of atherosclerosis.

Table 1. Proliferation and cytokine production by OVA-stimulated lymph nodes T cells, and serum levels of OVA-specific IgG2a and IgE.

	Saline	OVA/CFA	Tr1	Tr1+OVA/CFA
	(n=5)	(n=9)	(n=6)	(n=9)
IFN-γ (ng/ml	0.01 ± 0.001	3.34 ± 1.2*	0.02 ± 0.001	1.4 ± 0.2†
IL-10 (ng/ml)	0.1±0.02	0.34±.0.09*	0.09 ± 0.01	1.3 ± 0.3†
IL-4 (pg/ml)	0	0	0	0
IL-5 (ng/ml)	0.01±0.001	0.16 ± 0.02*	0	0.1± 0.01†
Proliferation (cpm)	1 231 ± 136	25 147 ± 1 267	1 567 ± 637	9 547 ± 957†
IgG2a (ng/ml)	0.677 ± 0.09	10,555 ± 2,254*	0.11 ± 0.01	3,567 ± 985†
IgE (pg/ml)	0	57 ± 12*	0	29,1 ± 9†

* $P<0.001$ vs saline, †$P<0.001$ vs OVA/CFA

Results are expressed as mean ±SD.

Table 2. Cytokine production by conA-stimulated splenic T cells.

	Saline	OVA/CFA	Tr1	Tr1+OVA/CFA
	(n=5)	(n=9)	(n=6)	(n=9)
IFN-γ (ng/ml)	2.63 ± 1.6	3.39 ± 1.5*	2.6 ± 1.3	1.7 ± 0.3†
IL-10 (ng/ml)	0.5 ± 0.05	1.05 ± 0.2*	0.58±.0.1	1.2 ± 0.2
IL-4 (pg/ml)	137 ± 27	54 ± 12	148 ± 31	0
IL-5 (ng/ml)	0.68 ± 0.03	0.3 ± 0.1*	0.69 ± 0.08	0.12 ± 0.05†

*P<0.001 vs saline, †P<0.001 vs OVA/CFA

Results represent mean±SD.

Table 3. Serum cholesterol levels, atherosclerotic lesion size and composition (mean±SEM) in the aortic sinus of apoE deficient mice.

	Saline	OVA/CFA	Tr1	Tr1+OVA/CFA
	(n=5)	(n=9)	(n=6)	(n=9)
Total cholesterol (g/l)	4.22 ± 0.2	4.03 ± 0.13	4.05 ± 0.19	3.90 ± 0.15
HDL cholesterol (g/l)	0.17 ± 0.04	0.11 ± 0.02	0.14 ± 0.02	0.13 ± 0.01
Lesion size in thoracic aorta (%)	8.4 ± 0.9	6.5 ± 0.7	8.5 ± 1.0	3.8 ± 0.3*
Lesion size in aortic sinus (μm^2)	323 564 ± 28 956	317 120 ± 13 464	376 102 ± 22 697	234 511 ± 11 797†
MOMA2-positive area (%)	34.6 ± 1.8	27.3 ± 2.3	31.4 ± 3.6	18.6 ± 1.4‡
CD3-positive (cells/mm^2)	283.5 ± 31.7	314.5 ± 39.6	257.2 ± 29.4	159.2 ± 25.3§
α-actine positive area (%)	5.0 ± 1.1	4.9 ± 0.9	5.8 ± 1.0	4.6 ± 0.9
Sirius red-positive area (%)	37.5 ± 2.4	39.6 ± 2.3	39.6 ± 3.9	35.4 ± 2.2

*P=0.0002 vs Tr1; P=0.008 vs OVA/CFA and P=0.0003 vs saline.

†P<0.0001 vs Tr1; P=0.001 vs OVA/CFA and P=0.002 vs saline.

‡P=0.0006 vs Tr1; P=0.006 vs OVA/CFA and P<0.0001 vs saline.

§P<0.05 vs Tr1; P=0.001 vs OVA/CFA and P=0.02 vs saline.

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CLAIMS

1. A method of treating or preventing atherosclerosis comprising administering sequencially, simulteanously or separately to a mammal in need of such a treatment :
   a) an antigen which has been used to activate *in vitro* a Tr1 cell population originating from said mammal, and
   b) the Tr1 cell population activated by said antigen, said Tr1 cell population being obtained from a CD4+ T lymphocyte population of said mammal.

2. The method of claim 1, wherein said antigen is an immunogenic peptide or protein.

3. The method of claim 1, wherein between 0.05 mg/kg to 5 mg/kg of said antigen is administered to said mammal.

4. The method of claim 1, wherein the antigen is administered once or twice the first month and then once a month for about 2 to 3 months.

5. The method of claim 1, wherein step b) comprises administering from 10^6 to 10^9 antigen-activated Tr1 cells/kg, for example from 0.5 10^7 to 1.5 10^7 cells/kg, preferably 10^7 cells/kg.

6. The method of claim 1, comprising intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, or subcutaneous administration of said antigen and antigen-activated Tr1 cells.

7. The method of claim 1, wherein Tr1 cells are CD3+CD4+CD18brightCD49b+ cells.

8. The method of claim 1, wherein the antigen-activated Tr1 cell population of step b) is obtained by an *in vitro* preparation process comprising the following steps:
   i) obtaining a Tr1 cell population from the CD4+ T lymphocyte population of the mammal in need of the treatment;
   ii) *in vitro* activating the Tr1 cell population by contacting it with the antigen; and
   iii) recovering the antigen-activated Tr1 cell population.

9. The method of claim 8, wherein the step i) of obtaining the Tr1 cell population comprises the following steps:
   a) isolating a progenitor cell population from said mammal;
   b) obtaining a population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10);
   c) contacting cells of step b) with the CD4+ T lymphocyte population isolated from said mammal to allow differenciation of said CD4+ T lymphocytes into the Tr1 cell population; and
   d) recovering the Tr1 cell population from the step c).

10. The method of claim 8, wherein the step i) of obtaining the Tr1 cell population comprises the following steps:
   a) contacting the CD4+ T lymphocyte population with an appropriate amount of alpha-interferon (α-IFN); and
   c) recovering the Tr1 cell population.

11. The method of claim 10, wherein the step a) of contacting is in combination with an appropriate amount of IL-10, such as 100 Uml^{-1}.

12. The method of claim 10, wherein the Tr1 cell population is further proliferated in interleukine 15 (IL-15).

13. The method of claim 1, wherein the antigen-activated Tr1 cell population of step b) is obtained by an *in vitro* preparation process comprising the following steps:
    i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen, presented by artificial antigen presenting cells; and
    ii) recovering an activated CD4+ T lymphocyte population comprising at least 10% of the antigen-activated Tr1 cell population.

14. The method of claim 13, wherein the artificial antigen presenting cells express a HLA II system molecule and a human LFA-3 molecule, and don't express the co-stimulation molecules B7-1, B7-2, B7-H1, CD40, CD23 and ICAM-1.

15. The method of claim 1, wherein the antigen-activated Tr1 cell population of step b) is obtained by an *in vitro* preparation process comprising the following steps:
    i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen and an appropriate amount of interleukine -10 (IL-10); and
    ii) recovering the antigen-activated Tr1 cell population.

16. The method of claim 1, wherein the mammal in need of such a treatment is a human being.

17. A product comprising:
    a) an antigen which has been used to activate *in vitro* a Tr1 cell population originating from a mammal, and
    b) the Tr1 cell population activated by said antigen, said Tr1 cell population being obtained from a CD4+ T lymphocyte population of said mammal,

    as a combined preparation for simultaneous, separate or sequential use for treating or preventing atherosclerosis.

18. The product of claim 17, wherein the combined preparation is for treating or preventing atherosclerosis to said mammal.

19. The product of claim 17 as a combined preparation for simultaneous, separate or sequential use for treating or preventing atherosclerosis in humans.

20. The product of claim 17, wherein said antigen is an immunogenic peptide or protein.

21. The product of claim 18, comprising from 0.05 mg/kg to 5 mg/kg of the antigen.

22. The product of claim 17, suitable for intravenous, intramuscular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, or subcutaneous administration.

23. The product of claim 17 comprising from 10^6 to 10^9 antigen-activated Tr1 cells/kg, for example from 0.5 10^7 to 1.5 10^7 cells/kg, preferably 10^7 cells/kg.

24. The product of claim 17, wherein the administration of the antigen-activated Tr1 cell population is a unique intra-peritoneal injection.

25. The product of claim 17, wherein Tr1 cells are are CD3+CD4+CD18brightCD49b+ cells.

26. The product of claim 18, wherein the antigen-activated Tr1 cell population is obtainable by an *in vitro* preparation process comprising the following steps:

i) obtaining a Tr1 cell population from the CD4+ T lymphocyte population of the mammal in need of the treatment;

ii) *in vitro* activating the Tr1 cell population by contacting it with the antigen; and

iii) recovering the antigen-activated Tr1 cell population.

27. The product of claim 26, wherein the Tr1 cell population of step i) is obtainable by a process which comprises the following steps:
    a) isolating a progenitor cell population from said mammal;
    b) obtaining a population of dendritic cells by culturing said progenitor cell population in presence of interleukine -10 (IL-10);
    c) contacting cells of step b) with the CD4+ T lymphocyte population isolated from said mammal to allow differenciation of said CD4+ T lymphocytes into the Tr1 cell population; and
    d) recovering the Tr1 cell population from the step c).

28. The product of claim 26, wherein the Tr1 cell population of step i) is obtainable by a process which comprises the following steps:
    a) culturing the CD4+ T lymphocyte population with an appropriate amount of alpha-interferon (α-IFN); and
    b) recovering the Tr1 cell population.

29. The product of claim 28, wherein the step a) furhter comprises culturing the CD4+ T lymphocyte population with an appropriate amount of IL-10, such as 100 Uml^{-1}.

30. The product of claim 28, wherein step b) further comprises expanding the Tr1 cell population with interleukine 15 (IL-15).

31. The product of claim 17, wherein the antigen-activated Tr1 cell population of step b) is obtainable by an *in vitro* preparation process comprising the following steps:
    i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen, presented by artificial antigen presenting cells; and
    ii) recovering an activated CD4+ T lymphocyte population comprising at least 10% of the antigen-activated Tr1 cells.

32. The product of claim 31, wherein the artificial antigen presenting cells express a HLA II system molecule and a human LFA-3 molecule, and don't express the co-stimulation molecules B7-1, B7-2, B7-H1, CD40, CD23 and ICAM-1.

33. The product of claim 17, wherein the antigen-activated Tr1 cell population of step b) is obtainable by an *in vitro* preparation process comprising the following steps:

i) *in vitro* activating the CD4+ T lymphocyte population in presence of the antigen and and appropriate amount of interleukine -10 (IL-10); and

iii) recovering the antigen-activated Tr1 cell population.

34. The use of a product as defined in one of claims 17 to 33 for the manufacture of a medicament for preventing or treating artherosclerosis.

35. The method of claim 1, wherein Tr1 cells are purified with Elisa, flowcytometry and/or immunoaffinity using the following antibodies :

- anti-CD4
- anti-CD3
- anti-CD18
- anti-CD49b

36. The method of claim 35, wherein enrichment of CD3+CD4+CD18brightCD49b+ cells from lymphocytes comprises the following steps :

- depletion of the total population with anti-human Ig-magnetic beads of cells bound with human anti-CD8, anti-CD14, anti-CD56 and anti-CD19.
- Selection of CD49b+ cells bound to an anti-CD49b human antibody with anti-human Ig-magnetic beads.

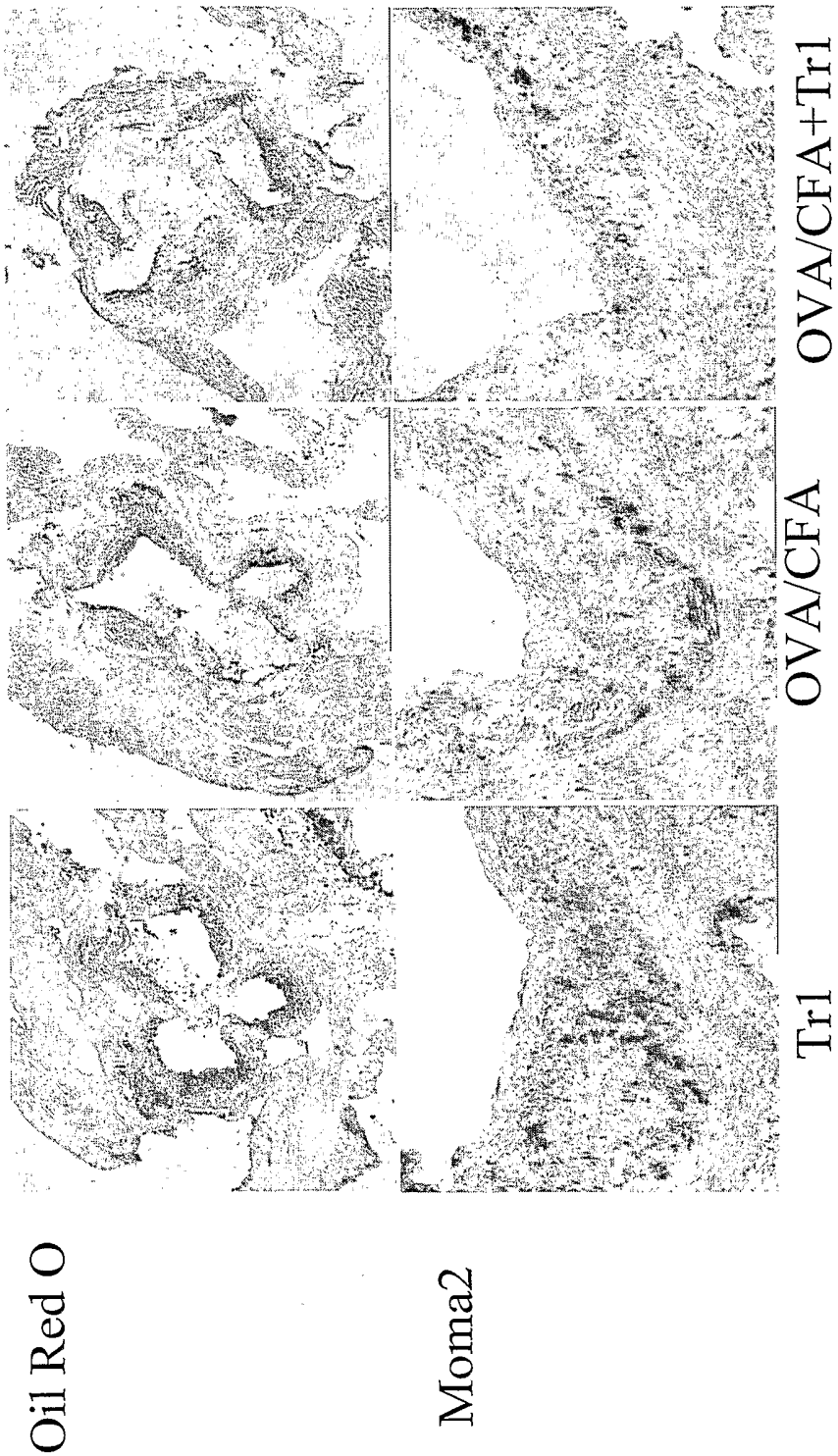


Figure 1

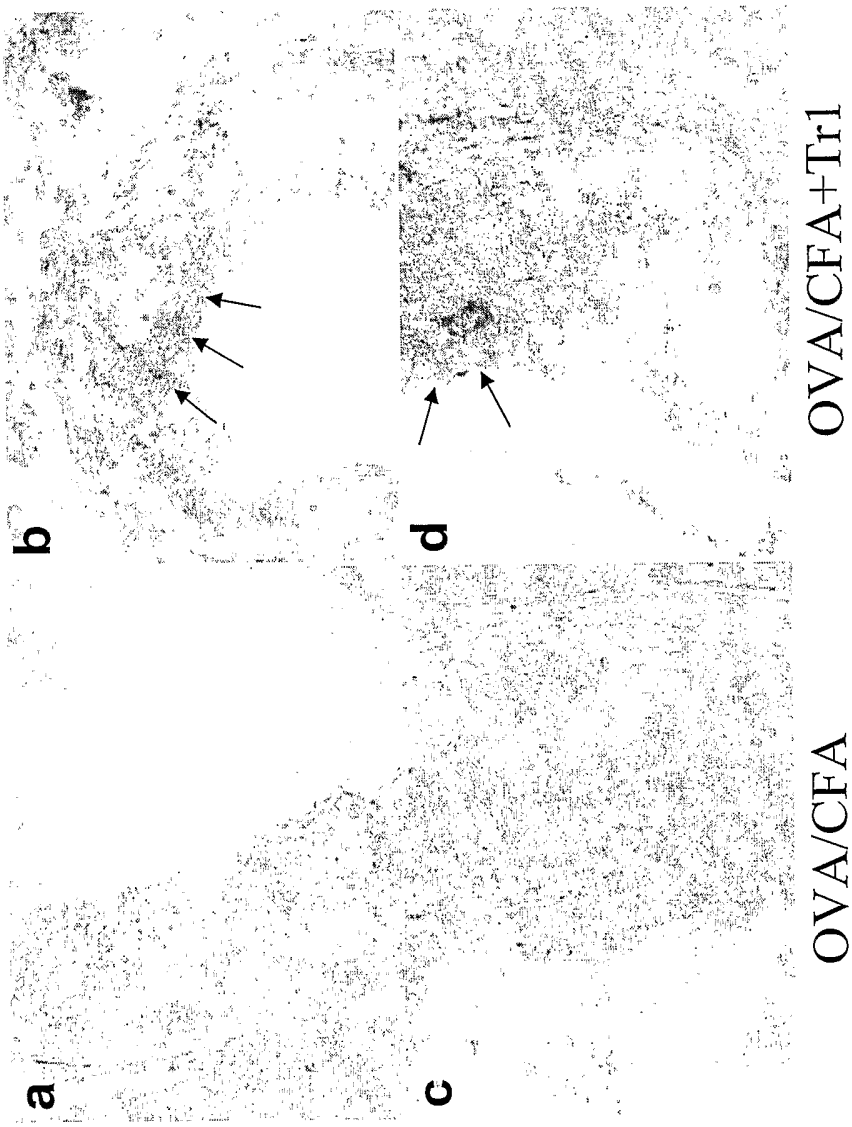


Figure 2

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/IB2004/002858

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 A61K39/00 A61K35/14

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 7 C12N A61K

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 practical, search terms used)
EPO-Internal, EMBASE, PAJ, WPI Data, FSTA, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MALLAT Z ET AL: "Induction of a regulatory T cell type 1 response reduces the development of atherosclerosis in apolipoprotein E-knockout mice" CIRCULATION, AMERICAN HEART ASSOCIATION, DALLAS, TX, US, vol. 108, no. 10, 9 September 2003 (2003-09-09), pages 1232-1237, XP009028370 ISSN: 0009-7322 the whole document ----- -/--	1-36

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in 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 document but published on or after the international filing date

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 November 2004	29/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Seroz, T

INTERNATIONAL SEARCH REPORT

Interr al Application No
PCT/IB2004/002858

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GROUX HERVE ET AL: "A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis" NATURE, MACMILLAN JOURNALS LTD. LONDON, GB, vol. 389, no. 6652, 16 October 1997 (1997-10-16), pages 737-742, XP002164695 ISSN: 0028-0836 *Methods*	1-36
Y	WO 02/092793 A (COTTREZ FRANCOISE ; INST NAT SANTE RECH MED (FR); WAKKACH ABDELILAH (F) 21 November 2002 (2002-11-21) page 5, line 8 - page 14, line 25	1-36
Y	WAKKACH A ET AL: "Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo" IMMUNITY, CELL PRESS, US, vol. 18, no. 5, May 2003 (2003-05), pages 605-617, XP002265204 ISSN: 1074-7613 page 615, left-hand column, last paragraph - page 616, left-hand column, paragraph 7 left-hand column	1-36
Y	US 2002/034500 A1 (LEVINGS MEGAN K ET AL) 21 March 2002 (2002-03-21) page 2, right-hand column, paragraph 3 - page 6, left-hand column, paragraph 2	1-36
A	MCGUIRK P ET AL: "Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: A novel strategy for evasion of protective T helper type 1 responses by Bordetella pertussis" JOURNAL OF EXPERIMENTAL MEDICINE, TOKYO, JP, vol. 195, no. 2, 21 January 2002 (2002-01-21), pages 221-231, XP002271535 ISSN: 0022-1007	

INTERNATIONAL SEARCH REPORT

Ir ational application No.
PCT/IB2004/002858

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 1-17, 35, 36 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

rmation on patent family members

Intern al Application No
PCT/IB2004/002858

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