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(54) Title: OPTIMISATION OF THE SCURFY MODEL FOR IN VIVO TESTING OF INNOVATIVE TREATMENTS OF AUTOIMMUNITY

(57) Abstract: Some rare and very severe autoimmune conditions are of hereditary origin such as APECED and IPEX syndrome due to altered negative selection of autoreactive T cells in the thymus or absence of regulatory T cells (Treg). Innovative strategies based on the use of regulatory T cells have been developed. The preclinical phase of development of these innovative treatments require appropriate animal models. The inventors now provide an optimization of the Scurfy model for in vivo testing of innovative treatments of autoimmunity. In particular, the inventors address all the issues presented by the original Scurfy model.

OPTIMISATION OF THE SCURFY MODEL FOR IN VIVO TESTING OF INNOVATIVE TREATMENTS OF AUTOIMMUNITY

FIELD OF THE INVENTION:

5 The present invention relates to the implementation of an animal model allowing the in vivo testing of innovative treatments of autoimmunity, especially the infusion of regulatory T cells (Tregs).

BACKGROUND OF THE INVENTION:

10 The global frequency of autoimmune diseases is between 3 and 5% in developed countries and has continuously increased in the last years. More than 100 different autoimmune diseases have been reported, which all correspond to chronic diseases triggered by a loss of immune tolerance against self-antigens. The most frequent or described ones are rheumatoid arthritis, systemic lupus erythematosus, due to the production of antibodies directed against self-
15 antigens, inflammatory bowel disease, multiple sclerosis and type 1 diabetes due to aberrant T cell responses. Most have a multifactorial origin involving both genetic (among which polymorphisms in HLA loci), endogenous (chronic inflammation, hormones) and environment (stress, nutrition, viral infections, anti-cancer treatments) factors, as well as diverse targeted organs. Some rare and very severe autoimmune conditions are of hereditary origin such as
20 APECED and IPEX syndrome due to altered negative selection of autoreactive T cells in the thymus or absence of regulatory T cells (Treg). Treatments include replacement therapy (for instance insulin treatment), corticoids, immunosuppressive treatments, immunotherapies (anti-cytokine treatments), and for the most severe cases, autologous or allogenic hematopoietic stem cell transplantation. Very recently, innovative strategies based on the use of regulatory T cells
25 have been developed. The preclinical phase of development of these innovative treatments require appropriate animal models. Several animal models have been implemented to mimic specific autoimmune disorders and specific targets (pancreatic beta cells for type 1 diabetes, central nervous system for multiple sclerosis, intestinal tract for inflammatory bowel disease among others). Scurfy mouse, which bears a loss-of-function mutation in the *FoxP3* gene
30 located on the X chromosome, is one of the best available models of the human IPEX syndrome in peculiar and of multiorgan autoimmune disease in general. Scurfy mutation causes frame-shift and nonsense-mediated decay of mRNA, leading to significantly reduced levels of FoxP3 transcript (Brunkow, M. E. Nat. Genet. 27, 68–73 (2001)). Hence, the mutation results in an absence of Tregs and death at 3-4 weeks of age due to a CD4⁺ T cell-mediated

lymphoproliferative disease. First symptoms appear at 10 days of life: hemizygous males are characterized by runting, scaly, crusty skin on the eyelids, ears and tails, dermal thickening, squinted eyes, cachexia, reddening and swelling of the genital papilla, and small testicles that are retained in the abdominal cavity. Homozygous scurfy females develop the same disease phenotype seen in hemizygous males, but they have a normal reproductive tract. The disease results in anemia, hypergammaglobulinemia, a small, thin thymus, and lymphohistiocytic proliferation in the skin and lymphoid organs, with splenomegaly, lymphadenomegaly, and hepatomegaly, inflammation in the salivary glands, lungs, gastrointestinal tract, skeletal muscle and pancreas. Homozygous scurfy females develop the same disease phenotype seen in hemizygous males, but they have a normal reproductive tract.

Adoptive transfer of murine Treg also efficiently suppresses other autoimmune diseases, such as diabetes (Tang, Q. *J. Exp. Med.* 199, 1455–1465, 2004) or inflammatory bowel disease (Mottet, C., *J. Immunol. Baltim. Md 1950* 170, 3939–3943, 2003). Adoptive transfer of healthy CD4⁺CD25⁺⁺ Treg (4x10⁵ cells) in neonatal scurfy mice (1 or 2 days old) is enough to prevent the development of the disease (Fontenot, J. D., *Nat. Immunol.* 4, 330–336 (2003)). □39. Mottet, C., Uhlig, H. H. & Powrie, F. Cutting edge: cure of colitis by CD4⁺CD25⁺ regulatory T cells. *J. Immunol. Baltim. Md 1950* 170, 3939–3943 (2003)). Moreover, transgenic restoration of FoxP3 expression prevents scurfy disease in FoxP3^{sf/Y} mice (Brunkow ME *Nat Genet* 2001). However, up to date, all studies focused on the prevention and not on the treatment of the disease, which constitutes the final aim of any clinical application. Furthermore, Scurfy mice present some major drawbacks: 1) homozygous females die too early to be used for breeding, 2) scurfy hemizygous males –useful for testing- constitute then only 25% of littermates issued from the breeding of wild type males and heterozygous females, 3) the clinical symptoms and first presentation are – like in IPEX patients- highly variable from one animal to the other, 4) the window for therapeutic intervention is very short (around 15 days) and requires scheduled breedings, taking into account the 25% frequency of useful animals, 5) the injection of Treg may require a pre-treatment to allow their engraftment, 6) Infused Tregs.

SUMMARY OF THE INVENTION:

As defined by the claims, the present invention relates to the implementation of an animal model allowing the in vivo testing of innovative treatments of autoimmunity, especially the infusion of regulatory T cells (Tregs).

DETAILED DESCRIPTION OF THE INVENTION:

The inventors now provide an optimization of the Scurfy model for in vivo testing of innovative treatments of autoimmunity. In particular, the inventors address all the issues presented by the original Scurfy model and listed in the Background section. For instance, issues 1 and 2 were solved by transferring the scurfy colony to a B6 Rag1^{-/-} background, in order to generate viable, disease-free (because of the absence of T cells), homozygous female FoxP3^{sf/sf} Rag1^{-/-} mice and thus double the production of FoxP3^{sf} males per breeding cycle. Thanks to that change, all males of a litter develop the disease. Issue 3 requires to be solved by the implementation of a score allowing to eliminate the variability of the clinical presentations of the disease. Issue 4 and 5 could be solved by a very tightly schedule of pre-and post-treatments and infusion of the Tregs.

Thus the object of the present invention relates to a method of assessing the efficiency of a curative treatment of autoimmunity with a population of Treg cells comprising the step of i) providing a non-human animal deficient in Tregs and that is capable of developing autoimmunity, ii) then administering the animal with an amount of cyclophosphamide, iii) then engrafting the animal with an amount of the population of Treg cells, iv) then administering the animal with an amount of IL-2 and v) finally assessing the severity of the autoimmunity.

As used herein, the term “autoimmunity” has its general meaning in the art and refers to the presence of a self-reactive immune response (e.g., auto-antibodies, self-reactive T-cells). Autoimmune diseases, disorders, or conditions arise from autoimmunity through damage or a pathologic state arising from an abnormal immune response of the body against substances and tissues normally present in the body. Damage or pathology as a result of autoimmunity can manifest as, among other things, damage to or destruction of tissues, altered organ growth, and/or altered organ function. Types of autoimmune diseases, disorders or conditions include type I diabetes, alopecia areata, vasculitis, temporal arteritis, rheumatoid arthritis, lupus, celiac disease, Sjogrens syndrome, polymyalgia rheumatica, and multiple sclerosis.

As used herein, the term “T cell” refers to a type of lymphocytes that play an important role in cell-mediated immunity and are distinguished from other lymphocytes, such as B cells, by the presence of a T-cell receptor on the cell surface.

As used herein, the term “regulatory T cells” or “Treg cells” refers to cells that suppress, inhibit or prevent T cells activity. As used herein, Treg cells have the following phenotype at rest CD4⁺CD25⁺FoxP3⁺ and thus are characterized by the expression of FoxP3.

As used herein, the term “FoxP3” has its general meaning in the art and refers to a transcription factor belonging to the forkhead/winged-helix family of transcriptional regulators. FOXP3 appears to function as a master regulator (transcription factor) in the development and

function of regulatory T cells. FoxP3 confers T cells with regulatory function and increases the expression of CTLA-4 and CD25, but decreases IL-2 production by acting as a transcriptional repressor. FoxP3 binds to and suppresses nuclear factor of activated T cells (NFAT) and nuclear factor-kappaB (NFKB) (Bettelli, E.M. et al, 2005, Proc Natl Acad Sci U S A 102:5138).

5 Typically, the Tregs cells are prepared according to any well-known method in the art. In some embodiments, the Treg cells are prepared by transfecting or transducing a population of T cells in vitro or ex vivo with a vector comprising a nucleic acid encoding for FoxP3. Typically, the vector is a retroviral vector. As used herein, the term "retroviral vector" refers to a vector containing structural and functional genetic elements that are primarily derived from a
10 retrovirus. In some embodiments, the retroviral vector of the present invention derives from a retrovirus selected from the group consisting of alpharetroviruses (e.g., avian leukosis virus), betaretroviruses (e.g., mouse mammary tumor virus), gammaretroviruses (e.g., murine leukemia virus), deltaretroviruses (e.g., bovine leukemia virus), epsilonretroviruses (e.g., Walley dermal sarcoma virus), lentiviruses (e.g., HIV-1, HIV-2) and spumaviruses (e.g., human
15 spumavirus). In some embodiments, the retroviral vector of the present invention is a lentiviral vector. As used herein, the term "lentiviral vector" refers to a vector containing structural and functional genetic elements that are primarily derived from a lentivirus. In some embodiments, the lentiviral vector of the present invention is selected from the group consisting of HIV-1, HIV-2, SIV, FIV, EIAV, BIV, VISNA and CAEV vectors. In some embodiments, the lentiviral
20 vector is a HIV-1 vector.

In some embodiments, the population of Treg cells is genetically modified to encode desired expression products, as will be further described below. The term "genetically modified" indicates that the cells comprise a nucleic acid molecule not naturally present in non-modified population of Treg cells, or a nucleic acid molecule present in a non-natural state in
25 said population of Treg cells (e.g., amplified). The nucleic acid molecule may have been introduced into said cells or into an ancestor thereof. A number of approaches can be used to genetically modify a population of cells, such as virus-mediated gene delivery, non-virus-mediated gene delivery, naked DNA, physical treatments, etc. To this end, the nucleic acid is usually incorporated into a vector, such as a recombinant virus, a plasmid, phage, episome, artificial chromosome, etc. Examples of means by which the nucleic acid carrying the gene may
30 be introduced into the cells include, but are not limited to, microinjection, electroporation, transduction, or transfection using DEAE-dextran, lipofection, calcium phosphate or other procedures known to one skilled in the art.

The nucleic acid used to genetically modify the population of Treg cells may encode various biologically active products, including polypeptides (e.g., proteins, peptides, etc.), RNAs, etc. In some embodiments, the nucleic acid encodes a polypeptide having an immuno-suppressive activity. Another preferred category of nucleic acids are those encoding a T cell
5 receptor or a subunit or functional equivalent thereof such as a chimeric antigen receptor (CAR) specific to an antigen of interest or a chimeric autoantibody receptor (CAAR) comprising an auto-antigen. For instance, the expression of recombinant TCRs or CARs specific for an antigen produces human Treg cells which can act more specifically and efficiently on effector T cells to inhibit immune responses in a patient in need thereof. The basic principles of chimeric
10 antigen receptor (CAR) design have been extensively described (e.g. Sadelain et al., 2013). Thus, in some embodiments, the Treg cells of the invention are genetically modified and express at least one CAR, one CAAR and/or one native receptor linked to intracellular signaling molecules. Examples of CAR included, without being limited to, first generation CARs, second generation CARs, third generation CARs, CARs comprising more than three signaling domains
15 (co-stimulatory domains and activation domain), and inhibitory CARs (iCARs).

As used herein, the term "non-human animal" means an animal excluding human, and is intended to include any vertebrate such as mammals, birds, reptiles, amphibians and fish. Suitable mammals include rodents, non-human primates, sheep, dogs and cattle. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably
20 mouse.

In some embodiments, the animal is characterized by a FoxP3 gene that is not expressed and that is rendered not functional by the present of at least one mutation. In some embodiments, the animal bears a loss-of-function mutation in the FoxP3 gene. In some embodiments, the animal is also characterized by a deficiency in a gene coding for a protein that is involved in
25 activation of immunoglobulin V-D-J recombination. In some embodiments, the animal is deficient for a *RAG1* (recombination activating gene 1) gene. The RAG-1 gene sequence is described in Schatz, et al., Cell 59, 1035-1048 (1989), the teachings of which are incorporated herein. Accordingly, the double transgenic animal is thus characterized by Treg deficiency and by an absence of mature B and T lymphocytes. In some embodiments, the non-human animal
30 is a FoxP3^{sf/sf} Rag1^{-/-} mouse that is produced as described in the EXAMPLE.

In some embodiments, the term "cyclophosphamide" has its general meaning in the art and refers to the generic name for 2-[bis(2-chloroethyl)amino]-tetrahydro-2H-1,3,2-oxazaphosphorine-2-oxide monohydrate.

The inventors have found that an amount of cyclophosphamide of between 40 and 150 mg/kg may be used. In some embodiments, the an amount of about 40; 41; 42; 43; 44; 45; 46; 47; 48; 49; 50; 51; 52; 53; 54; 55; 56; 57; 58; 59; 60; 61; 62; 63; 64; 65; 66; 67; 68; 69; 70; 71; 72; 73; 74; 75; 76; 77; 78; 79; 80; 81; 82; 83; 84; 85; 86; 87; 88; 89; 90; 91; 92; 93; 94; 95; 96; 97; 98; 99; 100; 101; 102; 103; 104; 105; 106; 107; 108; 109; 110; 111; 112; 113; 114; 115; 116; 117; 118; 119; 120; 121; 122; 123; 124; 125; 126; 127; 128; 129; 130; 131; 132; 133; 134; 135; 136; 137; 138; 139; 140; 141; 142; 143; 144; 145; 146; 147; 148; 149; or 150 mg/kg is used. Preferably an amount of 50 mg/kg is used.

As used herein, the term “about,” as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In some embodiments, the term “about” refers to a range of values that fall within 25%, 20%, 19%, 18%, 17%, 16%, 15%, 14%, 13%, 12%, 11%, 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, or less in either direction of the stated reference value unless otherwise stated or otherwise evident from the context.

In some embodiments, the amount of cyclophosphamide is administered to the mouse as herein disclosed between 8 and 12 days (i.e. 8, 9, 10, 11, or 12 days) after birth. Preferably, the amount of cyclophosphamide is administered to the mouse 10 days after birth.

The inventors have found that an amount of between 4×10^5 Treg cells and 1×10^6 Treg cells is engrafted in the animal. In some embodiments, an amount of 4×10^5 , 5×10^5 , 6×10^5 , 7×10^5 , 8×10^5 , 9×10^5 or 10^6 Treg cells is engrafted in the animal. Preferably, an amount of about 5×10^5 of Treg is engrafted in the animal.

In some embodiments, the engraftment in the mouse as herein disclosed is performed between 12 to 16 days (i.e. 12, 13, 14, 15, or 16 days) after birth. Preferably, the engraftment is performed 14 days after birth.

Typically, the typically the engraftment is performed by an intraperitoneal administration of the Treg cells.

In some embodiments, the engraftment is performed in combination with another biologically active agent. As used herein, the term “biologically active agent” is an agent, or its pharmaceutically acceptable salt, or mixture of compounds, which has therapeutic, prophylactic, pharmacological, or physiological effects on a mammal. Typically the biological agent is deemed to potentiate the immunosuppressive properties of the Tregs. The biological active agent may be selected from the group of (a) proteins or peptides, (b) nucleic acids and (c) organic or chemical substances.

As used herein, the term “IL-2” has its general meaning in the art and refers to the interleukin-2 that is typically required for T-cell proliferation and other activities crucial to

regulation of the immune response. An exemplary human amino acid sequence for IL-2 is represented by SEQ ID NO:1.

SEQ ID NO:1 >sp|P60568|IL2_HUMAN Interleukin-2 OS=Homo sapiens
OX=9606 GN=IL2 PE=1 SV=1
MYRMQLLSICIALSLALVTNSAPTSSSTKKTQLQLEHLLLDLQMI L N G I N N Y K N P K L T R M L
TFKFYMPKKATELKHLQCLEEEELKPLEEVNLNAQSKNFHLRPRDLISNINVIVLELKGSE
TTFMCEYADETATIVEFLNRWITFCQSIISTLT

The inventors have found that an amount of IL-2 of between 500 UI/g and 2000 UI/g may be used. In some embodiments, an amount of 500; 550; 600; 650; 700; 750; 800; 850; 900; 950; 1000; 1050; 1100; 1150; 1200; 1250; 1300; 1350; 1400; 1450; 1500; 1550; 1600; 1650; 1700; 1750; 1800; 1850; 1900; 1950; or 2000 UI/g is used. Preferably an amount of 1000 UI/g is used.

In some embodiments, the amount of IL-2 is administered to the mouse daily for 2, 3, 4, 5, 6, or 7 days after the engraftment. In some embodiments, the amount of IL-2 is administered to the mouse from day 14 to day 18 after birth. In some embodiments, further administrations of IL-2 are performed weekly after the daily administrations.

Assessing the severity of autoimmunity may be performed by any well-known method in the art. For instance a score that is composite of different symptoms may be determined. Typically, the symptoms include general appearance, behaviour, weight loss, degree of desquamation of the tail, blepharitis, crusting of the ears and eczema. In some embodiments, a scurfy score as described in EXAMPLE and Table 1 may be assessed. In particular, the score established for a particular treatment may be compared to another one treatment wherein the lowest score indicates the most efficient treatment.

The method of the present invention is thus particularly suitable for screening population of Treg cells that can be suitable for the treatment of autoimmunity. In particular, the method of the present invention suitable for screening population of Treg cells useful for the treatment of IPEX syndrome. As used herein, the term "IPEX syndrome" has its general meaning in the art and a disease that results in most cases from mutations in FoxP3. IPEX syndrome usually develops during the first few days or weeks of life and affects exclusively boys. It manifests with the sequential appearance of the triad of enteropathy, autoimmune disease, and cutaneous involvement, but the clinical features and severity of the disease can vary considerably between individuals. Severe autoimmune enteropathy manifests with intractable secretory diarrhea leading to malabsorption, electrolyte disturbance and failure to thrive. Vomiting, ileus, gastritis or colitis can also be observed. Patients also present with autoimmune endocrinopathies, generally insulin-dependent diabetes mellitus (type 1 DM), but

also thyroiditis leading to hypothyroidism or hyperthyroidism. Skin involvement consists of a generalized pruriginous eruption resembling eczema, psoriasis, and/or atopic or exfoliative dermatitis. Less frequently, alopecia or onychodystrophy can be observed. Patients may develop autoimmune cytopenias, thrombocytopenia, hemolytic anemia and neutropenia.

5 Autoimmune involvement may also lead to pneumonitis, hepatitis, nephritis, myositis, splenomegaly and/or lymphadenopathy. Local or systemic infections (e.g. pneumonia, *Staphylococcus aureus* infections, candidiasis) may occur but seem to be due to loss of skin and gut barriers, immunosuppressive therapies, and poor nutrition rather than a primary immunodeficiency. IPEX syndrome is caused by mutations in the FOXP3 gene (Xp11.23).

10 More than 20 mutations of FOXP3 are reported in IPEX, and the syndrome is lethal if untreated. Diagnosis is based on clinical examination, family history, and laboratory findings revealing autoimmune enteropathy (anti-enterocyte, harmonin and villin autoantibodies), type 1 DM (antibodies against insulin, pancreatic islet cells, or anti-glutamate decarboxylase), thyroiditis (anti-thyroglobulin and anti-microsome peroxidase antibodies) and cytopenia (anti-platelets

15 and anti-neutrophils antibodies, positive Coombs test). Molecular genetic testing confirms the diagnosis.

In some embodiments, the method of the present invention is particularly suitable for assessing the efficiency of a protocol for preparing Treg cells in particular, for assessing the efficiency of a method of converting a population of T cells into a population of Treg cells by

20 transducing said T cells with a nucleic acid encoding for e.g. an immunosuppressive factor, such as FoxP3. In some embodiments, the method of the present invention is particularly suitable for determining the optimal doses for the administration of Treg cells. In some embodiments, the method of the present invention is particularly suitable for the determining the most efficient combination of Treg cells and biologically active agents as described above

25 for proving an immunosuppressive activity.

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

FIGURES:

- 30 **Figure 1 depicts the experiment 1 tested by the inventors.**
- Figure 2 depicts the experiment 2 tested by the inventors.**
- Figure 3 depicts the experiment 3 tested by the inventors.**
- Figure 4 depicts the experiment 4 tested by the inventors.**
- Figure 5 depicts the experiment 6 tested by the inventors.**

Figure 6 depicts the experiment 6 tested by the inventors.

Figure 7 depicts the experiment 7 tested by the inventors.

MATERIAL AND METHODS

Mice

Scurfy phenotype was obtained by backcrossing on B6.129S7-Rag1tm1Mom/J background, allowing generation of homozygous XSf/ XSf.Rag1-/- female. Crossing of these female with WT C57BL/6J mice result in the birth only of diseased XSf/Y.Rag1-/+ male.

WT CD4 T cells

Splenocytes were harvested from C57BL/6J by aseptic removal. After gentle crushing of spleens through a 70 μ M mesh filter, CD4+ T cells were isolated by negative selection using EasySep Mouse CD4+ T cell Isolation Kit (StemCell Technologies, Grenoble, France). Purity exceeded 90%.

Scurfy CD4 T cells

From XSf/Y.Rag1-/+ mice of 10 days, lymph nodes were collected and CD4+ T cells were separated using Murine CD4+ T cell Isolation kit (Miltenyi Biotec, Paris, France). Briefly, CD4+ collected from lymph nodes were labeled with a cocktail of biotinylated antibodies targeting CD4- cells, followed by labeling with anti-biotin magnetic beads. Cells were separated on an LS column (Miltenyi Biotec) and CD4+ cells were collected in the flow through. Purity exceeded 90%.

WT Tregs CD4+CD25+

Splenocytes and lymph nodes were harvested from B6LY5.1 CD45.1 (8-12 weeks) and CD4+ T cells were isolated using EasySep Mouse CD4+ T cell Isolation Kit. A staining of CD25+ cells was performed with an anti-CD25 PE antibody (clone PC61, BD Biosciences, Le Pont de Claix, France), and then CD4+CD25+ cells were sorted on SH800 (Sony Biotechnology, Weybridge, UK) or ARIA II (BD Biosciences) cells sorters with a nozzle of 100 μ m. For Treg suppression assay, CD4+CD25- cells were also sorted.

Lentiviral vector

The cDNA for a truncated codon-optimized human Δ LNGFR and/or a codon optimized human FOXP3 was cloned in a pCCL backbone with different designs. Bidirectional vectors with the bidirectional promoters architecture, one allowing FOXP3 expression under the control of the ubiquitous elongation factor 1 alpha (EF1 α) and Δ LNGFR under the control of phosphoglycerate kinase (PGK) human promoter and their mock counterpart containing only the Δ LNGFR reporter (LNGFRp-eFOXP3 and LNGFRp-e) and one allowing FOXP3

expression under the control of PKG and Δ LNGFR under the control of a short version of EF1 α (EFS) LNGFR α -pFOXP3 and LNGFR α -p).

In T2A designs, expression is under the control of EF1 α . Two constructs were built: Δ LNGFR followed by the T2A sequence and FOXP3 or FOXP3 followed by the T2A and Δ LNGFR.

T cell Transduction

Freshly isolated CD4⁺ T cells were plated at 1.10^6 cells/mL in round bottom plate in RPMI 1640 medium + GlutaMax (GIBCO, Thermo Fisher Scientific, Montigny-Le-Bretonneux, France) supplemented with 10% fetal bovine serum (GIBCO), 1% Penicillin-Streptomycin (GIBCO), 0,1% 2-mercaptoethanol (GIBCO). Medium was supplemented with recombinant murine IL-2 (Peprotech, Rocky Hill, USA) at a concentration of 100 UI/ml for WT CD4 T cells or 300 UI/ml for Scurfy CD4 T cells. Cells were activated and expanded with anti-CD3/CD28 Dynabeads (GIBCO) at a 1:1 bead:cell ratio. Transduction was performed according the protocol previously described ⁴³ (ref article LB). Briefly transduction medium (RPMI supplemented with 0,25mg/ml Lentiboost (Sirion Biotech, FlashTherapeutics, Toulouse, France)) was added to cells with lentiviral vector at a MOI 10 concomitantly with activation and incubated overnight. Transduced cells were stained at day 5 after transduction by Δ LNGFR PE antibodies (clone ME20.4-1.H4, Miltenyi Biotec) and sorted on SH800 (Sony Biotechnology).

Adoptive T cells transfer

First, scurfy mice were treated with an immunosuppressor: Temsirolimus (LC laboratories, Woburn, USA) was injected S.C at the dose of 2 mg/kg at day 8 and day 10 after birth. This treatment was continued twice a week in some experiment. Anti-CD3 Fab'2 (clone 145-2C11, BioXCell, West Lebanon, USA) was injected S.C at 20 μ g/day during 5 days at day 8 after birth. Cyclophosphamide (European Pharmacopoeia (EP) Reference Standard, Merck KGaA, Darmstadt, Germany) was injected I.P. at 50, 100, or 150mg/kg 10 days after birth. At day 10 or day 14, CD4⁺CD25⁺ CD45.1⁺ cells (containing putative Tregs) or engineered CD4⁺ T cells (Foxp3.LNGFR or LNGFR) from Scurfy mice were injected at 0.5×10^6 , 0.75×10^6 or 1×10^6 of cells respectively via I.P injections. Vehicle (ie. PBS) was injected in the same volume IP for the mice that did not receive cells injection. Because of low titer of the mock LNGFR α -p vector, which hampered the production of high numbers of CD4^{LNGFR} cells, LNGFR α -p transduced Scurfy CD4 T cells were used to complete the group of CD4^{LNGFR} treated mice. In indicated experiments, together with cells, Proleukin (human IL-2, aldesleukine, Novartis) was injected at 1.000UI/g via I.P and during 5 days then once a week.

Flow cytometry

Single cell suspensions from spleen and lymph nodes were obtained by gentle crushing of spleens through a 70 μ M mesh filter. Samples from the lung and the liver were prepared after digestion with Collagenase IV (Thermo Fischer Scientific) followed by gentle crushing of spleens through a 100 μ M mesh filter.

Samples were prepared for flow cytometry using the following method: Cells were resuspended in 100 μ L of FACS buffer (phosphate buffered saline (PBS, Corning) /2% Fetal Bovine Serum [GIBCO]) and incubated with 2 μ L of each antibody 7AAD (Miltenyi Biotec,) for 20-30 min at 4 C.

Cells were washed once in FACS buffer prior to analysis. For intracellular FoxP3 staining, cells were first stained with cell surface markers and fixable viability dye eF780 (eBioscience, Thermo Fischer Scientific) as described above. After washing, cells were fixed and permeabilized using the FoxP3 staining buffer set eBioscience, Thermo Fischer Scientific) according to manufacturers' directions. Human FoxP3-APC (eBioscience, Thermo Fischer Scientific) was added for 30-60 min at RT. Samples were acquired on a MACSquant flow cytometer (Miltenyi Biotec), BD LSR Fortessa cytometer (BD Biosciences) or a Sony Spectral SH6800 (Sony Biotechnology). Data were analyzed using FlowJo V10 (TreeStar). The following antibodies were used: anti-mouse CD62L APC-Cy7 clone MEL-14, CD44 APC clone IM7 (BD Biotechnology), CD45.1 APC-Cy7 clone A20, CD45.2 PeCy7 clone 104, CD134 clone OX-40 Brilliant Violet 421, CD279 (PD-1) clone 29F.1A12 Brilliant Violet 605, CD25 clone PC61 Brilliant Violet 711, TIGIT clone Vstm3 1G9 PE, CD357 (GITR) clone DTA-1 PerCP/Cy5.5, CD39 clone Duha59 PE/Cy7 and CD152 clone UC10-4B9 PE/Dazzle (Sony Biotechnology) and human Δ LNFR PE clone ME20.4-1.H4 (Miltenyi Biotec), Helios clone 22F6 eF450 and human FOXP3 APC Clone PCH101 (eBioscience, Thermo Fischer Scientific)

Histology

Lung, liver and ear was collected after mice euthanasia and fixed in PFA 4% (Sigma). Tissues section was stained with HE and inflammation was analyzed as described by Workman and al.

Statistical analysis

Values are represented as means $\pm$ SD, unless stated otherwise. GraphPad Prism 6.0 was used for all statistical analyses. P value was calculated with a confidence interval of 95% to indicate the statistical significance between groups. Statistical test included non-parametric Mann-Whitney test, Fischer exact test or two ways ANOVA depending on the dataset. A P

value < 0.05 was considered statistically significant. Statistically significant differences between groups are noted in figures with asterisks (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Correlations were performed with a non-parametric Spearman correlation. Survival was analyzed with Log-rank test (Mantel-Cox).

Ethics

Animal procedure received our institution ethics committee agreement and Ministère de l'Agriculture agreement according to European directive **2010/63/UE**.

EXAMPLE 1:

As shown in the EXAMPLE 1 (Table 1), the inventors established a **scurfy score** based on sub-scores for each type of clinical symptom: general appearance, behavior, weight loss, degree of desquamation of the tail, blepharitis, crusting of the ears and eczema. Those 7 symptoms do not require any manipulation or sampling and are thus in agreement with the 3R rules. The data are easy to collect and allow to prevent the variability between animals.

Table 1: Scurfy score of the present invention

Parameters	Observation	Score
General appearance	Normal	0
	Lack of grooming	1
	Bristling hair (piloerection)	2
	Previous observations + crooked back	3
Behavior	Normal	0
	Slightly modified	1
	Less mobile and isolated but reactive	2
	Motionless, prostrate, loss of reflexes of alert, reflex pedaling absent	3
Weightless	Value (g)	
	Growth/stagnation	0
	Loss $< 10\%$	1
	Loss between 10-20%	2
	Loss $> 20\%$	3

Tail	Normal	0
	Focal or moderate desquamation	1
	Severe desquamation + ring constrictions / loss of part of the tail / Focal inflammation	2
	Ulceration / loss of the entire tail	3
Eyes	Normal	0
	Blepharitis	1
	Eyes closed	2
Ears	Normal	0
	Flattening of the lobes	1
	Crusting	2
Eczema	Absent	0
	Localized	1
	Diffuse	2
	Sores / severe lesions scratching	3

As shown in **Figures 1-7**, the inventors compared 7 different experimental protocols to identify the one allowing to test the efficacy of Treg to treat Scurfy autoimmune syndrome. **Table 2** summarizes the different experiments. Tregs were sorted on the basis of CD4 and CD25 expression from CD45.1 congenic B6 mice and injected at a dose of 5×10^5 intra-peritoneally at day 10 or 14. The criteria was the scurfy score measured every 3 to 4 days from birth and when signs of efficiency evidenced improved scores for mice transplanted with Tregs, survival was followed. Three drugs were tested as conditioning regimens: Temsirolimus, subcutaneously injected at a dose of 2mg/kg daily from day 4 to day 28 or from day 4 to day 9 (post-birth), anti-CD3 Fab'2, subcutaneously injected at a dose of 20 micrograms/recipient, daily from day 8 to day 12, Cyclophosphamide at 3 different doses (50, 100 and 150mg/kg) injected at day 10. As shown in **Figures 1-3**, Temsirolimus and anti-CD3 did not allow to reveal any improvement of Scurfy score after the infusion of Tregs. As shown in Figure 4 cyclophosphamide at all doses delayed the death of scurfy mice to more than 60 days. However, 100 and 150mg/kg led to general toxicity revealed by the health status of the mice. This toxicity was absent at the dose of 50mg/kg. The dose of 50mg/kg led to a more stable scurfy score and allowed to see the benefit of infused Tregs in delaying the worsening of scurfy score and death.

The final conditioning regimen thus consists in the injection of 50mg/kg of cyclophosphamide at day 10 before the injection of Tregs at day 14.

Tregs require IL-2 for their survival (Fontenot, Jason D., et al. "A function for interleukin 2 in Foxp3-expressing regulatory T cells." *Nature immunology* 6.11 (2005): 1142.

5 And Setoguchi, Ruka, et al. "Homeostatic maintenance of natural Foxp3+ CD25+ CD4+ regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization." *Journal of Experimental Medicine* 201.5 (2005): 723-735). Human proleukine 2 was injected intraperitoneally at a dose of 1000UI/g, daily from day 14 to day 18, and once per week thereafter. As shown in **Figure 5**, following the protocol including IL-2 and
10 cyclophosphamide, T regs delay the death of the animals and are detected in all organs tested, demonstrating that this conditioning regimen allowed their engraftment and persistence in the recipients.

Table 2: Summary of the different experiments:

Experiment	Drug Name	Site of injection	Dose	Delay of injection	Cell Type	Site of injection	Dose	Delay	Maintenance	Read-out
1	Temsirolimus	Subcutaneous	2 mg/kg	Twice a week, starting from day 4	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 10	Temsirolimus, 2 mg/kg, subcutaneously, twice a week	Scurfy score, weight, cell subset count, histology
2	Temsirolimus	Subcutaneous	2 mg/kg	Day 4 and day 8	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 10	None	Scurfy score, weight, cell subset count, histology
3	Abt-CD3 Fab2	Subcutaneous	20 ug/mice	Every day from day 4 to day 12	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 14	None	Scurfy score, weight, cell subset count, histology
4	Cyclophosphamide	Intraperitoneously	50, 100 and 150 mg/kg	Day 10	CD4+CD25+ (CD45.1+)	None				Scurfy score, weight, survival
5	Cyclophosphamide	Intraperitoneously	50 mg/kg	Day 10	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 10	Human prol2, 1000U/g, intraperitoneously; daily for 5 days after T cell injection, then weekly	Scurfy score, weight, survival
6 A	Cyclophosphamide	Intraperitoneously	50 mg/kg	Day 10	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 10	Human prol2, 1000U/g, intraperitoneously; daily for 5 days after T cell injection, then weekly	Scurfy score, weight, cell subset count, histology
6 B	Cyclophosphamide	Intraperitoneously	50 mg/kg	Day 10	CD4+CD25+ (CD45.1+)	Intraperitoneously	5.E+05	Day 10	Human prol2, 1000U/g, intraperitoneously; daily for 5 days after T cell injection, then weekly	Scurfy score, weight, cell subset count, histology

EXAMPLE 2

A specific combination of cyclophosphamide, IL-2 and Tregs cures Scurfy syndrome

Scurfy phenotype included blepharitis, tail and ear eczema and failure to thrive. $X^{Sf}/Y.Rag1^{-/+}$ male mice develop a Scurfy phenotype similar to $X^{Sf}/Y.Rag1^{+/+}$ males with a disease onset at day 8 of life (data not shown). To allow a reproducible evaluation of Scurfy mice phenotype, we developed a specific method of scoring including signs of Scurfy disease (blepharitis, ear and whole body eczema, tail eczema, limbs edema, body weight, mice appearance and behavior) on a scale from 0 to 21 (Supplemental Table 1). The weight of each criterion in this Scurfy severity score was adjusted depending on the severity of injuries. This method was validated on more than 50 mice and by two independent investigators.

Different immunosuppressive strategies including Temsirolimus (a prodrug of Rapamycin that increases Scurfy life expectancy), anti-CD3 antibody and cyclophosphamide (Cy); were evaluated in order to (i) control Scurfy symptoms, increase mice survival and thus therapeutic window, (ii) mimic IPEX patients conventional immunosuppressive treatments, and (iii) deplete the activated Tconv compartment to favor engraftment of Tregs. The experimental scheme we used included injection of the immunosuppressive drug, followed by the transplantation of $5 \cdot 10^5$ congenic CD45.1 WT $CD4^+CD25^{high}$ Tregs (**Figure 3** and data not shown). Scurfy score was evaluated every two days. Engraftment of WT Treg was quantified in various tissues at study endpoint. Injections of Temsirolimus twice a week starting at disease onset (i.e. at day 8) resulted in reduction of Scurfy score and a doubling of life expectancy as shown by Cheng and coll. (data not shown). However, Scurfy score was not improved by WT Treg transfer at day 10 in accordance with less than 1% chimerism (data not shown). Anti-CD3 Fab'2 injected in a single dose of 20 μ g resulted in a nadir of depletion after 5 days and $CD4^+$ T cells recovery starting after 10 days (data not shown). Anti-CD3 Fab'2 was injected for 5 consecutive days and Treg were transferred at day 14 of life. Despite a higher engraftment rate (1.9 ± 0.3 % CD45.1⁺ in $CD4^+$ T cells in lymph nodes and 1.0 ± 0.4 % CD45.1⁺ in spleen), Scurfy score was not improved by Treg transfer with this anti-CD3 based conditioning regimen (**Figure 3**). Cyclophosphamide (Cy) was administered to Scurfy males at day 10 of life at doses of 50, 100 or 150 mg/mg of body weight. T cells depletion was not different with the three doses. Depletion nadir was observed between day 3 and 5 after Cyclophosphamide injection. Survival was significantly increased following Cy injection from 30 to 90 days as compared to untreated mice (data not shown, $p=0.01$), allowing a significant increase of the therapeutic window. However, some toxicity was observed including growth retardation and a transient

alopecia correlated with Cy doses. Based on these safety and efficacy criteria, we chose the lowest dose of Cy (ie 50 mg/kg) to limit toxicity. Transfer of Treg was performed at day 14 in mice conditioned by a single Cy injection at day 10 (Data not shown). We observed a trend toward a decreased severity score in mice that received Cy and Tregs (Data not shown).

5 However CD45.1⁺ cells were not detectable at sacrifice in lymph nodes and spleen (Data not shown). On the basis of previous reports showing the beneficial effect of low dose IL-2 on Treg expansion in murine and human models of autoimmunity and transplantation²⁷⁻²⁹. Scurfy recipients were treated with IL-2 once a day for 5 days then once a week (Data not shown). With the association of Cy, Tregs and IL-2, Scurfy score started to decline after day 28 post-

10 transplantation (p= 0.007) (Data not shown). Then, 49 days after Tregs transfer, CD45.1⁺ chimerism in CD4⁺ T cells reached 2.2% ± 0.6 in lymph nodes, 3.7% ± 1.4 in spleen, 2.1% ± 0.5 in blood, 3.5% ± 3.2 in liver and 3.3% ± 0.8 in lung (Data not shown). Finally, mice life expectancy was increased with a mean survival of 69 days as compared to 39 days in mice that did not receive Tregs (p=0.0004). Interestingly, IL-2 by itself increased survival from a mean

15 survival to 51 days (p=0.03) (Data not shown). Moreover, CD62L staining was increased on CD4 T cells from mice treated with Tregs demonstrating the restoration of a naïve CD4 population in lymph nodes (Data not shown).

Thus, the combination of a low dose Cy conditioning with IL-2 treatment post-transplant allowed not only the delay of the disease symptoms, hence increasing the therapeutic window,

20 but also the increase of Tregs engraftment. These results showed for the first time the beneficial effect of Tregs on Scurfy disease after its onset. This optimized murine model was then used to test the suppressive functionality of gene corrected scurfy CD4 T cells.

Engineered Scurfy CD4 T cells with LNGFR.FOXP3 vector rescue Scurfy mice after the onset of the disease

25 To test the ability of CD4^{LNGFR.FOXP3} required to control Scurfy disease, we treated X^{Sf}/Y.Rag1^{-/+} male with one I.P injection of Cy at day 10 as previously described followed by an injection of congenic 5.10⁵ CD45.1 Tregs or 5.10⁵, 7.5.10⁵ or 1.10⁶ scurfy CD4^{LNGFR.FOXP3} at 14 days of life. Follow-up of Scurfy score showed a dose-dependent inhibition of Scurfy symptoms according to the number of injected CD4^{LNGFR.FOXP3} (ANOVA test, p-value= 0.0039,

30 Data not shown). Moreover, after 50 days of follow-up, injection of 7.5.10⁵ CD4^{LNGFR.FOXP3} demonstrated similar results in term of Scurfy score but also percentage of chimerism as compared to 5.10⁵ Tregs (Data not shown). Therefore, the dose of 7.5.10⁵ CD4^{LNGFR.FOXP3} was selected for further evaluation. Surprisingly, CD62L staining was not different in the three doses of CD4^{LNGFR.FOXP3} (Data not shown).

Then, in order to compare CD4^{LNGFR.FOXP3} efficacy to Tregs, adoptive transfer of CD45.1 WT Tregs, CD4^{LNGFR.FOXP3} and its mock counterpart, CD4^{LNGFR} or vehicle (PBS) was performed. Mice were carefully followed until their sacrifice at day 50 of life. To note, VCN were similar between the three vectors (1.3 for LNGFRp-eFOXP3, 1.2 for LNGFRp-e and 1.5 for LNGFRp-e vectors). Scurfy score increased similarly in all groups up to day 27 (Data not shown). At day 32, while it continues to rise in the same way in mice that received Cy and IL-2 alone or with CD4^{LNGFR}, we observed a significant decrease in mice treated with WT Tregs and CD4^{LNGFR.FOXP3} (p-value=0.007 and 0.0008 respectively). More specifically, WT Tregs and CD4^{LNGFR.FOXP3} treated mice recovered of alopecia induced by Cy, presented a mild eczema of the tail, low level of blepharitis and gained weight whereas diluent and CD4^{LNGFR} treated mice presented failure to thrive and severe eczema of the whole body (Data not shown).

Analysis of chimerism showed a mean percentage of CD45.1 Treg of 5.0 ranging from 1.7 to 12.6% in lymph nodes, spleen, blood, liver and lung CD4 T cells (Data not shown). Δ LNGFR⁺ chimerism in mice treated with CD4^{LNGFR.FOXP3} T cells were slightly lower with a mean of 3.4% ranging from 0.2 to 4.6% in lymph nodes, spleen, blood, liver and lung (Data not shown). On the opposite, CD4^{LNGFR} T cells did not expand and percentage remained inferior to 1.5% in all tissues. Importantly, human FOXP3 expression persisted in Δ LNGFR⁺ CD4⁺ collected from lymph nodes of CD4^{LNGFR.FOXP3} treated mice (Data not shown). Δ LNGFR⁺ cells were sorted from lymph nodes lymphocytes and VCN analysis were quantified at 2.3±0.8 for LNGFR.FOXP3 vector and 1.6±0.7 for LNGFR vector (Data not shown).

Analysis of CD62L staining in lymph nodes demonstrated that a subset of naïve CD4⁺ T cells was restored in mice treated with Tregs and CD4^{LNGFR.FOXP3} cells. CD4⁺ T cells in lymph nodes contained 15.7±0.6% of CD62L⁺ cells in mice treated by Cy and IL-2 against 78.1±2.4% in WT mice. Treatment with Tregs increased this level to 44.0±6.2% and with Scurfy CD4^{LNGFR.FOXP3} T cells to 31.1±11.8%, as compared to 20.8±2.5 CD62L⁺ T CD4 after transplantation of CD4^{LNGFR} T cells. Histology analysis showed no significant difference in the inflammation score in the lung, liver and skin (data not shown).

Survival was significantly increased following Cy+IL-2+CD4^{LNGFR.FOXP3} treatment as compared to Cy treated Scurfy mice with respectively a mean survival of 64 days to 47 days (p= 0.0195). Similarly, mean survival was significantly increased with Tregs as compared to Cy with a survival above 89 days (p-value<0.0001). Mean survival for Cy+IL-2+Treg treated mice was not significantly different as compared to Cy+IL-2+ CD4^{LNGFR.FOXP3} treated mice (p=0.1047). Survival was not different between Cy+IL-2+PBS and Cy+IL-2+CD4^{LNGFR} treated mice with a mean survival of respectively of 54.5 days and 53 day (p=0.8729) (Data not shown).

After 90 days of follow-up, CD45.1 Tregs and CD4^{LNGFR.FOXP3} chimerism in CD4⁺ T cells decreased as compared to day 50 analyses with a mean percentage of 1.5% and 1.1% respectively. Importantly, hFOXP3 was still detectable in CD4^{LNGFR.FOXP3} demonstrating the stability of hFOXP3 in transduced CD4⁺ T cells *in vivo*.

5 These results demonstrated that FOXP3 expression in Scurfy CD4⁺ T cells recapitulated a suppressive function and transduced CD4^{LNGFR.FOXP3} were able to rescue the severe Scurfy autoimmune disease.

CONCLUSION:

10 Transfer of splenocytes or sorted Tregs in Scurfy deficient mice has been shown to prevent Scurfy symptoms when transferred within the two first days of live ^{3,11}. In this study, we demonstrated that Treg transfer at day 14 of life allowed long-term rescue of Scurfy symptoms if combined with Cy conditioning followed by low dose of IL-2 injections. This was demonstrated with robust parameters including a clinical score, staining of CD4⁺ T cells, analysis of chimerism and survival. In the different strategies tested for Treg adoptive transfer, 15 Cy allowed the best control of autoimmunity. Cy has been shown to deplete the T cell niche in mice ^{31,32}. CD4 T cells nadir was obtained 4 days after injection and cell count normalized at 10 days. Moreover, Cy allows a functional impairment of activated T cells resulting in a relative enrichment in Tregs ^{33,34}. However, in young animals, even low dose of Cy resulted in toxicities 20 as alopecia and growth retardation. Other conditioning to tip the balance between Tregs and Tconvs based on a more specific depletion of activated T cells would be a high requirement for clinical application. For example non-mitogenic anti-CD3 antibodies could be interesting ^{16,17}. However in our Scurfy mice model, we were not able to demonstrate its efficacy to allow an appropriated engraftment of Tregs. Then, low dose IL-2 has been shown to favor Treg 25 expansion and thus has beneficial therapeutic effects in the context of several autoimmune diseases such as type I diabetes ³⁵, systemic lupus erythematosus ³⁶ and others ³⁷⁻³⁹. IL-2 also enhances proliferation of donor-specific Tregs and promotes tolerance in allogeneic transplantation ^{40,41}. In 10 weeks-old NOD mice, it has been demonstrated that daily injection of 25.000UI/day during five day was able to reverse diabetes ²⁹. In order to adapt this low dosing 30 of IL-2 to 14 days-old Scurfy mice, we decided to use 1.000UI/g of mice. Moreover, based on the TRANSREG study, this induction therapy was completed by a maintenance therapy of weekly IL-2 injections ³⁸. Importantly, treatment with low dose IL-2 did not worsen autoimmunity in Scurfy settings. The remaining question would concern the end of IL-2 treatment.

Altogether, this therapeutic strategy allowed evaluating cells suppressive activity in the *in vivo* Scurfy model of autoimmunity.

In these settings, Scurfy CD4 T cells transduced with LNGFR.FOXP3 vector were able to rescue Scurfy disease, demonstrating the efficiency of the lentiviral vector to induce regulatory functions with a mean of 2 VCN per cell. This suggested that the homology between human and murine *FOXP3* allows the expression of human *FOXP3* in murine CD4⁺ T cells by itself to be sufficient to induce suppressive function to CD4⁺ cells. Interestingly Scurfy CD4⁺ T cells transduced with LNGFR.FOXP3 vector expanded preferentially as compared to Scurfy CD4⁺ T transduced with the mock LNGFR vector. This could be explained by their increased sensibility to IL-2 due to higher level of CD25. Moreover these adoptively transferred Tregs were stably maintained until 90 days *in vivo* in an inflammatory context, and expressed durably FOXP3. In our assay, transfer of *bona fide* Tregs allowed a slightly better control of Scurfy disease as compared to genetically engineered CD4⁺ T cells. Several factors could explain those differences. First, the level of chimerism was half the one of WT Tregs at day 50 and also in long-term follow-up at day 90. Consequently, increasing the cell dose or recurrent infusion could improve outcome. Then, our vector allowed the expression of human FOXP3 protein, which present 86% of homology with murine FOXP3 and may be do not fully recapitulate Tregs transcriptomic program. Moreover, engineered regulatory CD4^{LNGFR.FOXP3} were collected from Scurfy mice and cultured for 5 days. Consequently, they might be more sensitive to sorting and manipulation. Beyond, preselecting naïve T cell might result in more efficient suppressive cells as demonstrated by Passerini and al. CD4^{LNGFR.FOXP3} ¹⁴. In our work, after adoptive transfer of Treg or CD4^{LNGFR.FOXP3} some Scurfy mice died mostly of Scurfy symptoms recurrence. Therefore multiple injections of Tregs or increasing of IL-2 dose or frequencies of injection could be considered to improve Scurfy disease control.

REFERENCES:

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

CLAIMS:

1. A method of assessing the efficiency of a curative treatment of autoimmunity with a population of Treg cells comprising the step of i) providing a non-human animal deficient in Tregs and that is capable of developing autoimmunity, ii) then administering
5 the animal with an amount of cyclophosphamide, iii) then engrafting the animal with an amount of the population of Treg cells, iv) then administering the animal with an amount of IL-2 and v) finally assessing the severity of the autoimmunity.
2. The method of claim 1 wherein the Treg cells are prepared by transfecting or transducing a population of T cells in vitro or ex vivo with a vector comprising a nucleic acid
10 encoding for FoxP3.
3. The method of claim 1 Treg cells express a chimeric antigen receptor (CAR).
4. The method of claim 1 wherein the animal bears a loss-of-function mutation in the FoxP3 gene.
5. The method of claim 1 wherein the animal is also characterized by a deficiency in a gene
15 coding for a protein that is involved in activation of immunoglobulin V-D-J recombination.
6. The method of claim 5 wherein the animal is deficient for a *RAG1* (recombination activating gene 1) gene.
7. The method of claim 1 wherein the non-human animal is a FoxP3sf/sf Rag1^{-/-} mouse
- 20 8. The method of claim 1 wherein an amount of cyclophosphamide of between 40 and 150 mg/kg is used, preferably an amount of 50 mg/kg is used.
9. The method of claim 1 wherein the amount of cyclophosphamide is administered to the mouse 8 and 12 days after birth, preferably 10 days after birth.
10. The method of claim 1 wherein an amount of between 4×10^5 Treg cells and 1×10^6 Treg
25 cells is engrafted in the animal, preferably an amount of about 5×10^5 of Treg.
11. The method of claim 1 wherein the engraftment in the mouse is performed between 12 to 16 days (after birth, preferably 14 days after birth).

12. The method of claim 1 wherein the engraftment is performed by an intraperitoneal administration of the Treg cells.
13. The method of claim 1 wherein, the engraftment is performed in combination with another biologically active agent.
- 5 14. The method of claim 1 wherein an amount of IL-2 of between 500 UI/g and 2000 UI/g may be used. In some embodiments, an amount of 500; 550; 600; 650; 700; 750; 800; 850; 900; 950; 1000; 1050; 1100; 1150; 1200; 1250; 1300; 1350; 1400; 1450; 1500; 1550; 1600; 1650; 1700; 1750; 1800; 1850; 1900; 1950; or 2000 UI/g is used, preferably an amount of 1000 UI/g is used.
- 10 15. The method of claim 1 wherein the amount of IL-2 is administered to the mouse from day 14 to day 18 after birth and further administrations of IL-2 are performed weekly after the daily administrations.
16. The method of claim 1 wherein a score that is composite of different symptoms is determined for assessing the severity of autoimmunity.
- 15 17. The method of claim 16 wherein a scurfy score as described Table 1 is assessed.

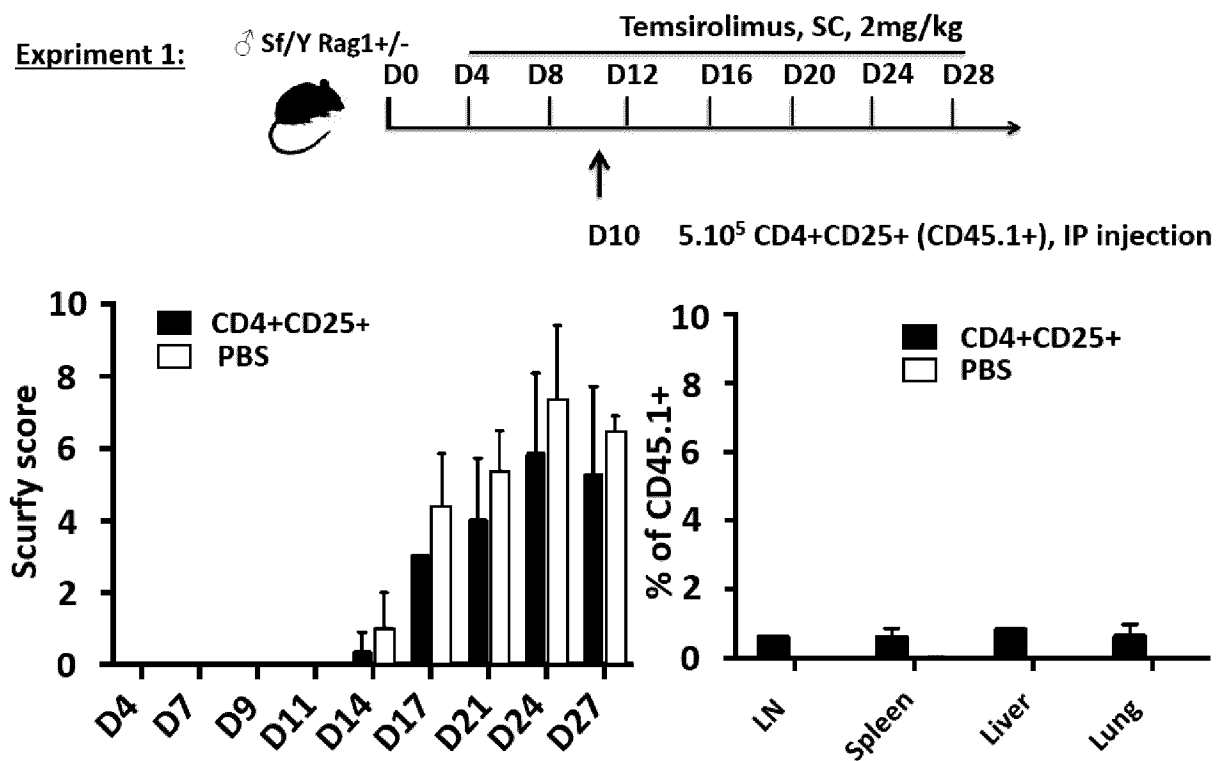


Figure 1

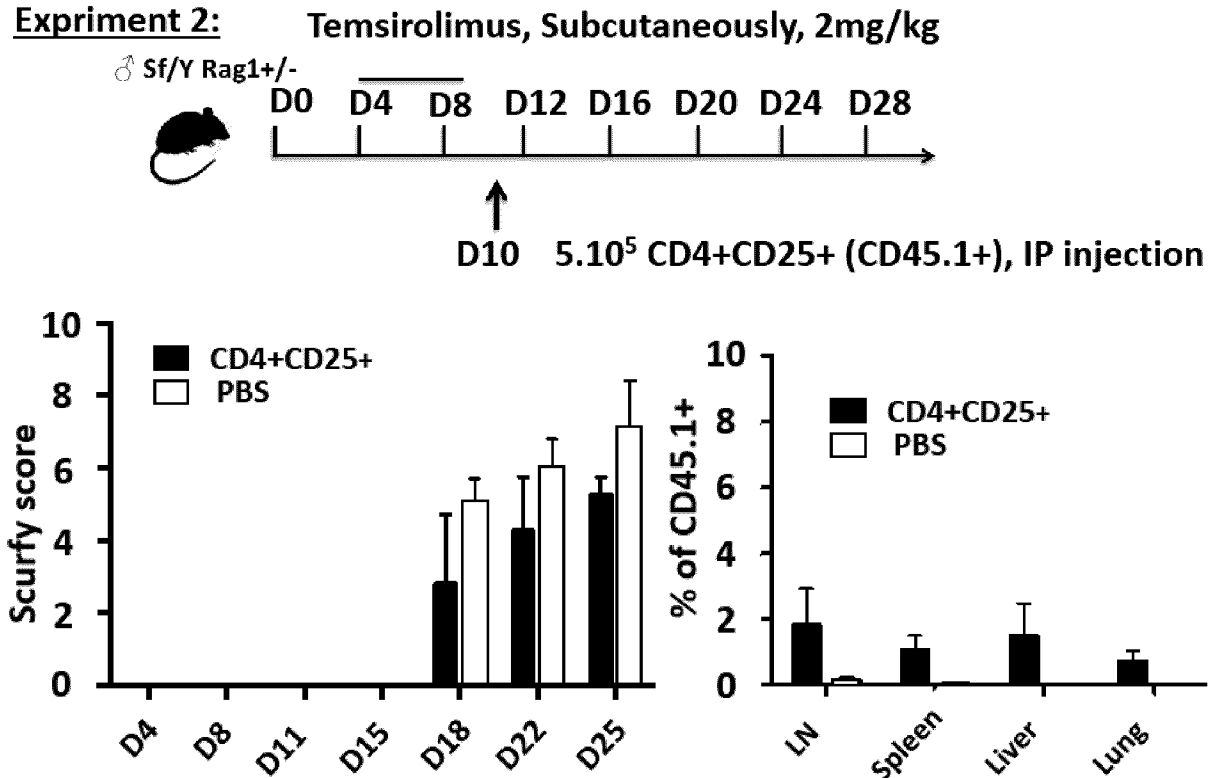


Figure 2

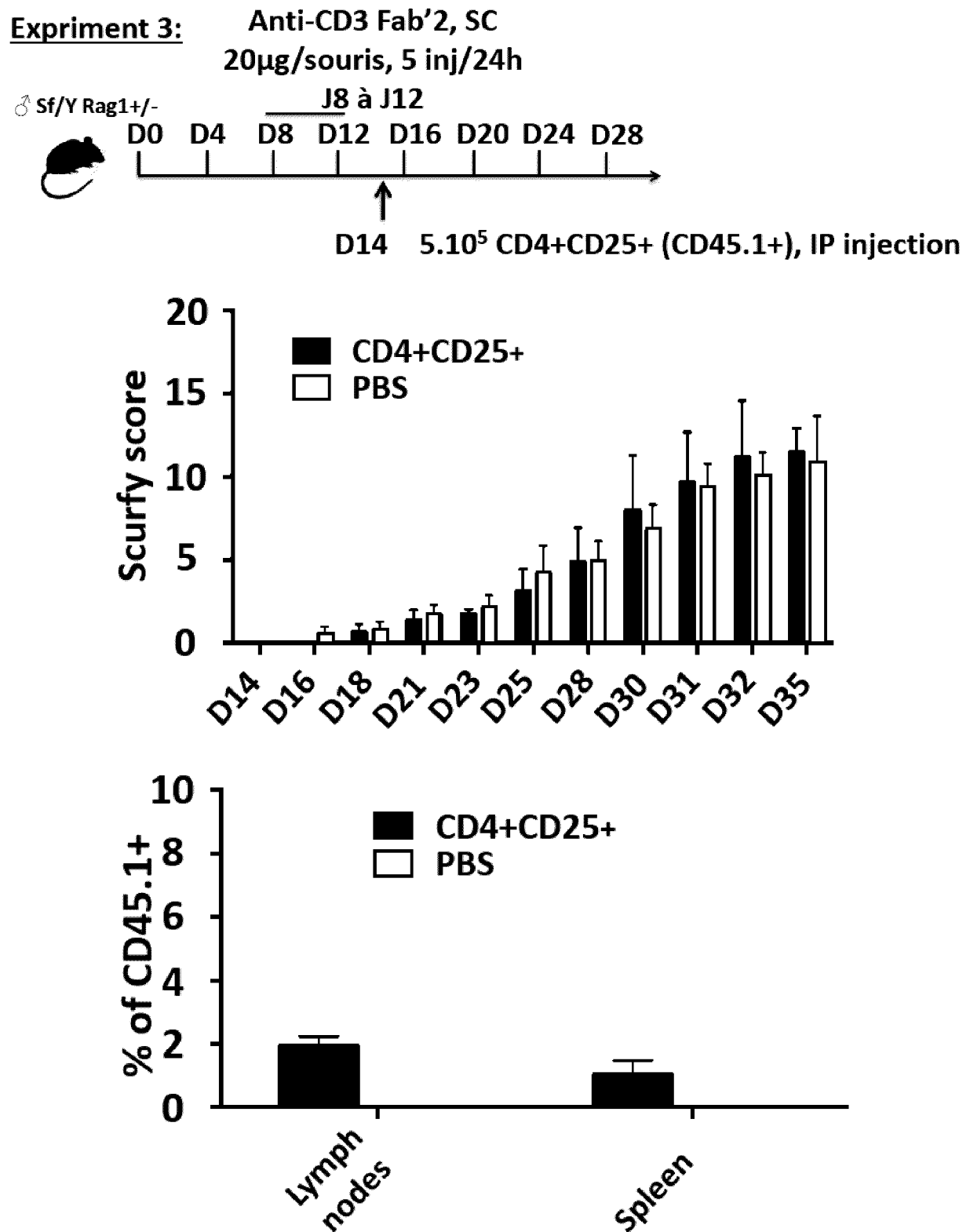


Figure 3

Experiment 4:**D10****Cyclophosphamide**
150, 100 ou 50 mg/kg

♂ Sf/Y Rag1+/-

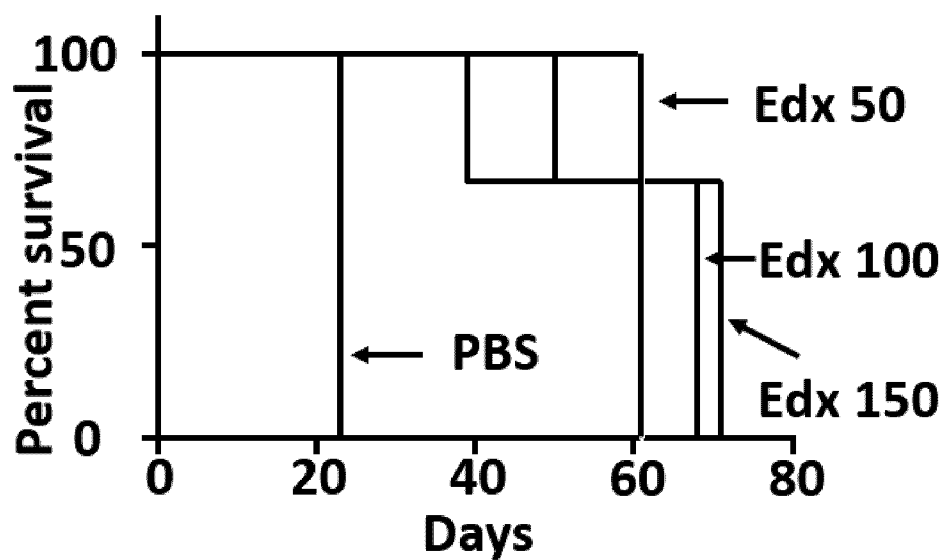
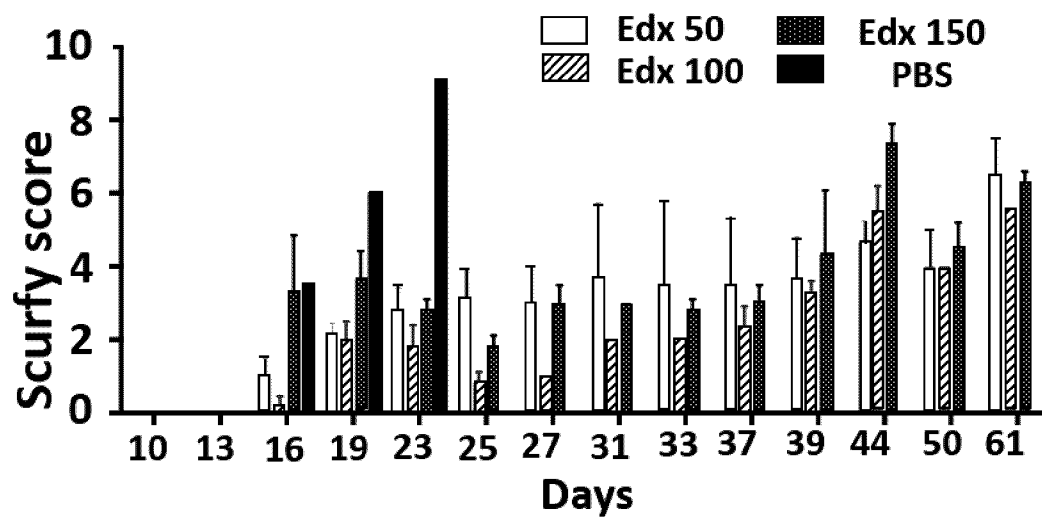
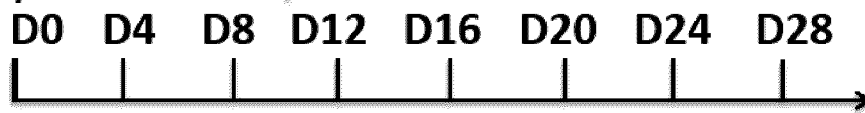


Figure 4

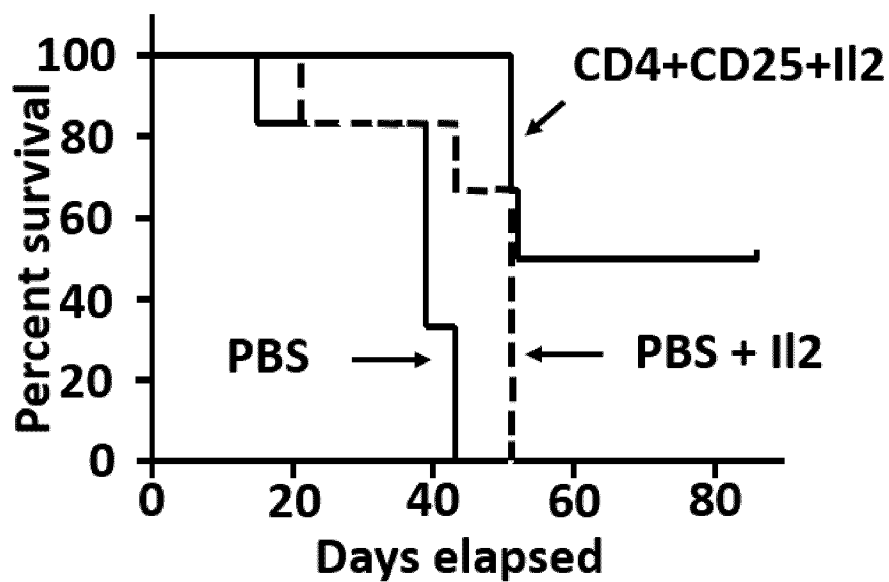
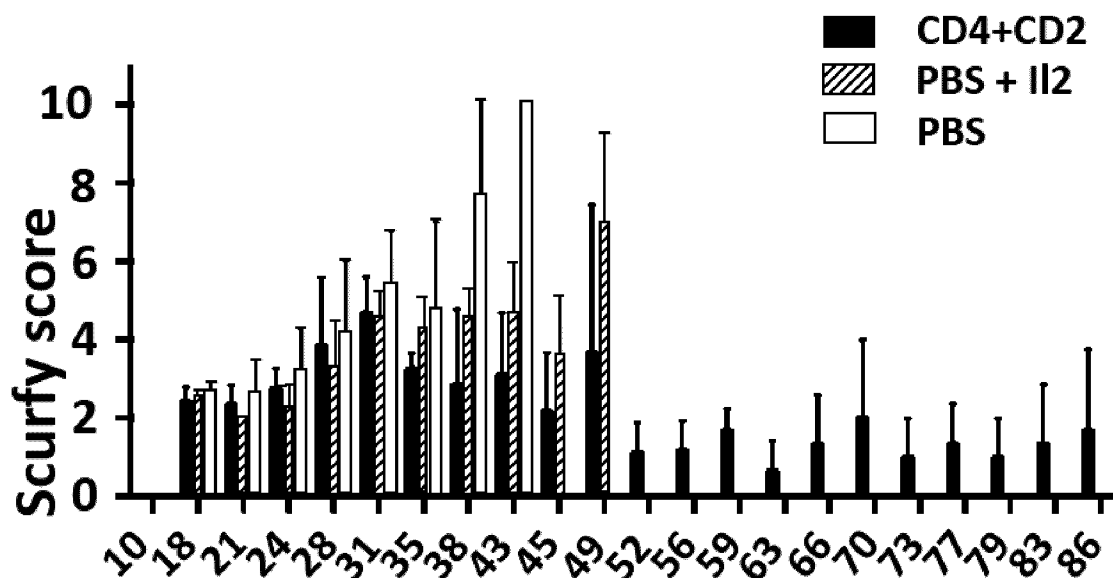
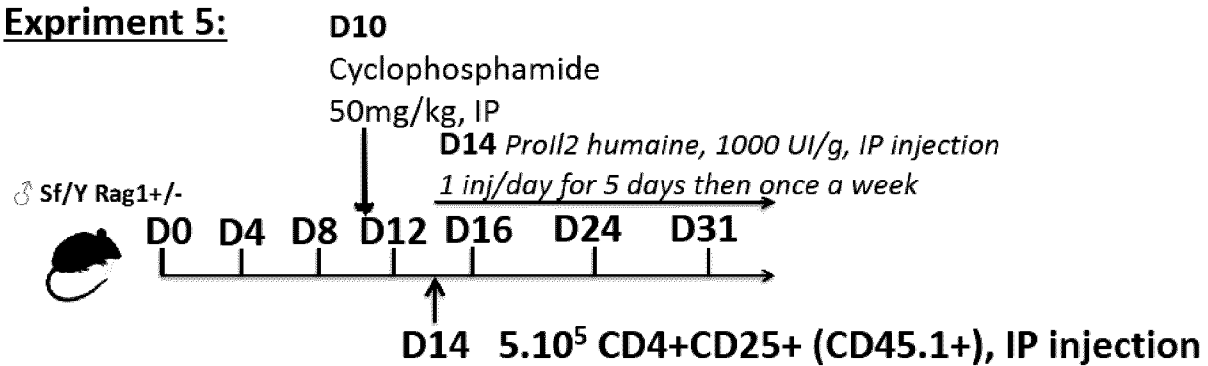
Exprimment 5:

Figure 5

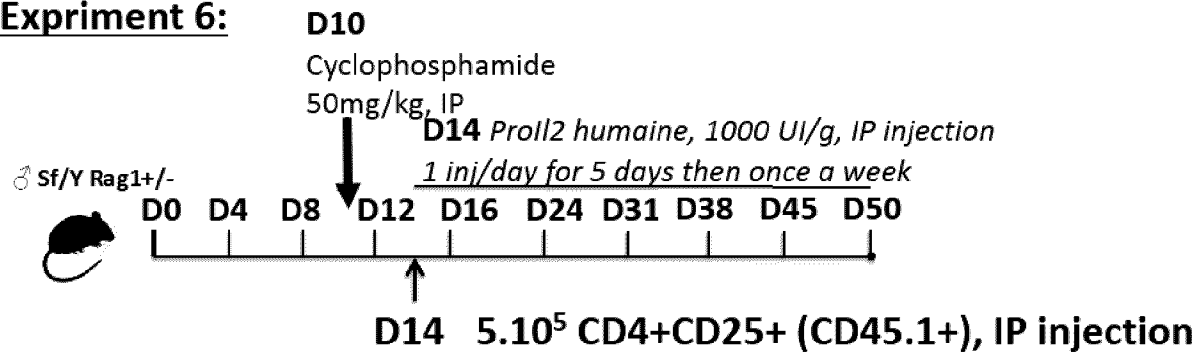
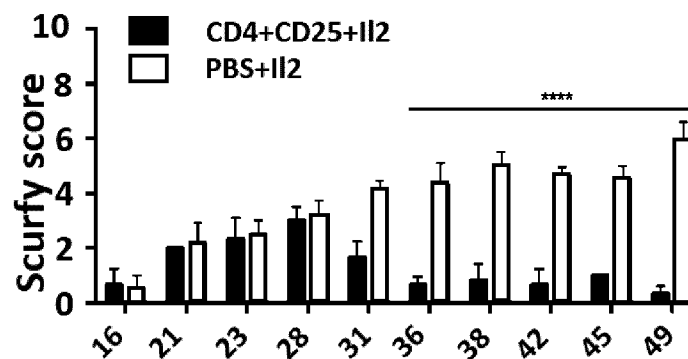
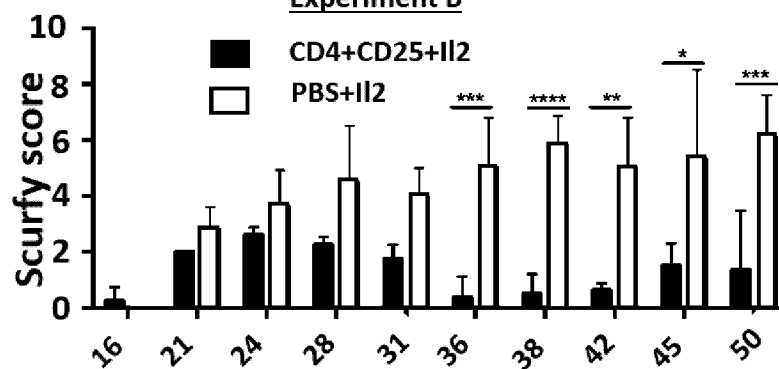
Experiment 6:**Experiment A****Experiment B**

Figure 6

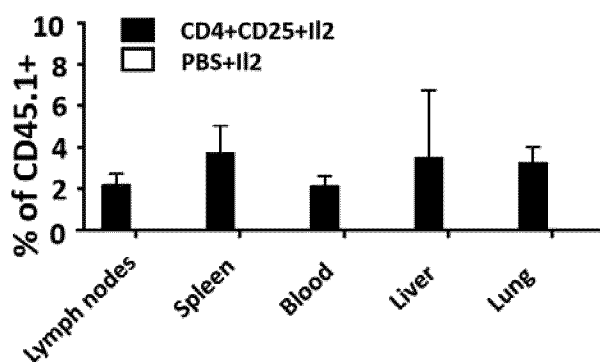
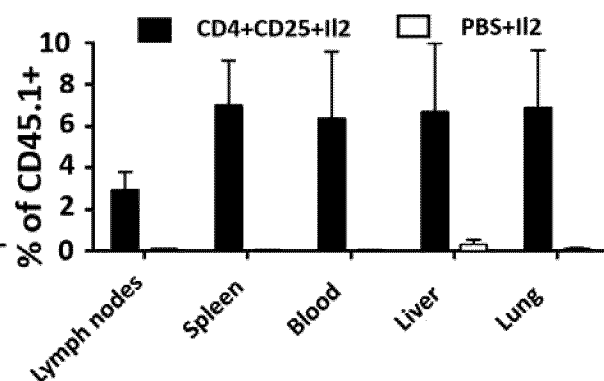
Experiment A**Experiment B**

Figure 7

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/052162

A. CLASSIFICATION OF SUBJECT MATTER INV. C12N5/0783 A61K35/12 A61K35/17 A61K38/20 A61P37/02 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C12N A61K A01K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data		
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* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 11 May 2020		Date of mailing of the international search report 28/05/2020
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Trommsdorff, Marion

INTERNATIONAL SEARCH REPORT

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