



(51) International Patent Classification:

C12N 15/85 (2006.01) C12N 15/867 (2006.01)

(21) International Application Number:

PCT/EP2020/052731

(22) International Filing Date:

04 February 2020 (04.02.2020)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

19305133.1 05 February 2019 (05.02.2019) EP

(71) Applicants: **INSERM (INSTITUT NATIONAL DE LA SANTÉ ET DE LA RECHERCHE MÉDICALE)** [FR/FR]; 101, rue de Tolbiac, 75013 Paris (FR). **ASSISTANCE PUBLIQUE-HÔPITAUX DE PARIS (APHP)** [FR/FR]; 3, avenue Victoria, 75004 Paris (FR). **FONDATION IMAGINE** [FR/FR]; 24, Boulevard du Montparnasse, 75015 Paris (FR). **UNIVERSITÉ DE PARIS** [FR/FR]; 85 Boulevard Saint-Germain, 75006 Paris (FR). **UNIVERSITÉ D'EVRY-VAL-D'ESSONNE** [FR/FR]; 23 Boulevard François Mitterrand, 91000 Evry (FR). **MEDIZINISCHE HOCHSCHULE HANNOVER (MHH)** [DE/DE]; Carl-Neuberg-Straße 1, 30625 Hannover (DE).

(72) Inventors: **ANDRE, Isabelle**; Institut Imagine, 24 Boulevard du Montparnasse, 75015 Paris (FR). **SIX, Emmanuelle**; IMAGINE / UMR 1163 INSERM, 24 Boulevard du Montparnasse, 75015 Paris (FR). **BELLIER, Florence**; IMAGINE / UMR 1163 INSERM, 24 Boulevard du Montparnasse, 75015 Paris (FR). **DELVILLE, Marianne**; IMAGINE / UMR 1163 INSERM, 24 Boulevard du Montparnasse, 75015 Paris (FR). **CAVAZZANA, Marina**; Institut Imagine, 24 Boulevard du Montparnasse, 75015 Paris (FR). **AMENDOLA, Mario**; INSERM U951, 1bis Rue de l'Internationale, 91002 Evry (FR). **SCHAMBACH, Axel**; Institute of Experimental Hematology, Hannover Medical School, Carl Neuberg Strasse 1. OE6960, 30625 Hannover (DE).

(74) Agent: **INSERM TRANSFERT**; 7 rue Watt, 75013 Paris (FR).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: RECOMBINANT VECTORS SUITABLE FOR THE TREATMENT OF IPEX SYNDROME

(57) Abstract: IPEX (Immune dysregulation Polyendocrinopathy X linked) syndrome is a primary immunodeficiency caused by mutations in the gene encoding the transcription factor forkhead box P3 (FOXP3), which leads to the loss of function of thymus-derived CD4+CD25+ regulatory T (tReg) cells. Preclinical and clinical studies suggest that T cell gene therapy approaches designed to selectively restore the repertoire of Treg cells by transfer of wild type FOXP3 gene is a promising potential cure for IPEX. However, there is still a need for a vector that can be used efficiently for the preparation of said Treg cells. The inventors thus compared 6 different lentiviral constructs according to 4 criteria (vector titers, level of transduction of human CD4+ T cells, level of expression of FOXP3 and ΔLNGFR genes, degree of correlation between both expression) and selected one construct comprising a bidirectional EFS-PGK promoter that showed remarkable efficiency.



RECOMBINANT VECTORS SUITABLE FOR THE TREATMENT OF IPEX SYNDROME

5 FIELD OF THE INVENTION:

The present invention relates to recombinant vectors suitable for the treatment of IPEX syndrome.

BACKGROUND OF THE INVENTION:

10 IPEX (Immune dysregulation Polyendocrinopathy X linked) syndrome is a primary immunodeficiency caused by mutations in the gene encoding the transcription factor forkhead box P3 (*FOXP3*) (Wildin et al., 2001) (Bennett et al., 2001), which leads to the loss of function of thymus-derived CD4⁺CD25⁺ regulatory T (tTreg) cells (Yagi et al., 2004) (Fontenot et al., 2003) (Hori et al., 2003) (Khatti et al., 2003) (a small subset of circulating CD4⁺ T
15 lymphocytes dedicated to controlling immune responses to self and foreign antigens). In IPEX patients, the absence of a functional Treg cell compartment leads to the development of multiple autoimmune manifestations (including severe enteropathy, type 1 diabetes and eczema) in the first months or years of life (Barzaghi et al., 2012). IPEX syndrome is often fatal early in infancy, and so a prompt diagnosis is essential for starting treatment as soon as possible (before tissue
20 damage spreads to multiple organs).

The current treatments for IPEX syndrome include supportive therapy, immunosuppressive therapy, hormone replacement therapy and HSCT. Unfortunately, these immunosuppressants are usually only partially effective and the dose is often limited by infectious complications and toxicity. Currently, the only cure for IPEX syndrome is allogeneic
25 HSCT. The absence of an HLA-compatible donor for all patients and their poor clinical condition particularly expose them to a risk of mortality. For all these reasons, effective alternative therapeutic approaches are urgently needed.

Based on the outcome of HSCT in this setting, we learned that partial donor chimerism is sufficient for complete remission – provided that full engraftment is achieved in the Treg
30 compartment. In turn, this suggests that a few Tregs could be enough to control autoimmunity in IPEX syndrome (Horino et al., 2014) (Seidel et al., 2009) (Kasow et al., 2011). Moreover, various studies in the mouse (including the scurfy mouse model) have demonstrated the efficacy of the adoptive transfer of healthy Tregs in curing autoimmune diseases (Fontenot et al., 2003) (Mottet et al., 2003) (Tang et al., 2004). The *in vivo* suppressive capacity of human Tregs

obtained after *ex vivo* expansion has also been demonstrated using humanized mouse model (Wieckiewicz et al., 2010). These various preclinical studies have paved the way for the first clinical trial of the adoptive transfer of *ex vivo*-expanded Treg cells in two patients with GVHD (Trzonkowski et al., 2009).

5 Gene therapy of T cells has been successfully developed for TCR or chimeric antigen receptor gene therapy and effectively targets cancer (Bonini et al., 2011) (Kalos and June, 2013) (Provasti et al., 2012). Previous experience of cell therapy with gene-modified T-cells in ADA-SCID (Aiuti et al., 2002) (Blaese et al., 1995) (Muul et al., 2003) indicates that gene-corrected functional T cells persist for more than 15 years after infusion. Furthermore, it has been
10 demonstrated that LV-mediated *FOXP3* expression in human CD4 T cells, including from IPEX patients enables the generation of regulatory T cells, which exhibited immunosuppressive activity both *in vitro* and *in vivo* in a xenogenic model of GVHD (Aarts-Riemens et al., 2008) (Allan et al., 2008)(Passerini et al., 2013).

 Altogether the results of these preclinical and clinical studies suggest that T cell gene
15 therapy approaches designed to selectively restore the repertoire of Treg cells by transfer of wild type *FOXP3* gene is a promising potential cure for IPEX (Aiuti et al., 2012).

 However, several prerequisites are absolutely required before any clinical application. First, *FOXP3* controls partly the transcriptional signature – and therefore the suppressive function- of Tregs. It has to be expressed at sufficient level to ensure this function and stably to
20 avoid any conversion from Treg to T effector cells and loss of suppressive ability. Secondly, the *in vitro* generated Tregs must be sorted before their infusion to the patients to avoid any side-effect of non-corrected contaminant effector T cells. As *FOXP3* protein is located in the nucleus, it cannot be used to sort *FOXP3*⁺ expressing cells. Therefore, it has to be co-expressed with a surface marker. A truncated form of the p75 low-affinity nerve growth factor receptor
25 (Δ LNGFR) with most of the intracytoplasmic tail deleted (from residue 248) has been used as a surface marker in T-cell targeted gene therapy approaches without any side effect (Bonini et al., 1997). Furthermore, the expression of surface Δ LNGFR allows the sorting of transduced cells in clinically applicable conditions. However, to be applicable, the correlation between Δ LNGFR and *FOXP3* expression has to be perfect and Δ LNGFR expression should be
30 sufficient to allow the sorting of Δ LNGFR⁺ cells. The different localization of the two proteins, *FOXP3* in the nucleus and Δ LNGFR at the membrane might hamper a strict correlation between both expressions. Thirdly, another obstacle to gene therapy is the efficacy of lentiviral vector production (measured by titrating the vector), which is highly variable and depends on the vector construct and the transgenes. From our knowledge and long-lasting experience, it is

impossible to predict the titer. Accordingly, there is still a need for a vector that addresses all these obstacles.

SUMMARY OF THE INVENTION:

5 The present invention relates to recombinant vectors suitable for the treatment of IPEX syndrome. In particular, the present invention is defined by the claims.

DETAILED DESCRIPTION OF THE INVENTION:

10 As shown in the EXAMPLE, the inventors compared 6 different lentiviral constructs according to 4 criteria (vector titers, level of transduction of human CD4⁺ T cells, level of expression of *FOXP3* and *ΔLNGFR* genes, degree of correlation between both expression) and selected one construct comprising a bidirectional EFS-PGK promoter that showed remarkable efficiency.

15 Accordingly, the first object of the present invention relates to a recombinant nucleic acid molecule comprising a bidirectional EFS-PGK promoter operably linked to a first transgene in one direction and to a second transgene in the opposite direction wherein the first transgene that is under the control of the EFS portion of the bidirectional promoter encodes for a protein that is not constitutively expressed by a T cell and the second transgene that is under the control of the PGK portion of the bidirectional promoter encodes for a transcription factor.

20 As used herein, the term "nucleic acid molecule" has its general meaning in the art and refers to a DNA molecule.

25 As used herein, the terms "promoter" has its general meaning in the art and refers to a segment of a nucleic acid sequence, typically but not limited to DNA that controls the transcription of the nucleic acid sequence to which it is operatively linked. The promoter region includes specific sequences that are sufficient for RNA polymerase recognition, binding and transcription initiation. In addition, the promoter region can optionally include sequences which modulate this recognition, binding and transcription initiation activity of RNA polymerase. The skilled person will be aware that promoters are built from stretches of nucleic acid sequences and often comprise elements or functional units in those stretches of nucleic acid sequences, such as a transcription start site, a binding site for RNA polymerase, general transcription factor binding sites, such as a TATA box, specific transcription factor binding sites, and the like. Further regulatory sequences may be present as well, such as enhancers, and sometimes introns at the end of a promoter sequence.

30

As used herein, the term “EFS promoter” has its general meaning in the art and refers to the promoter of the gene encoding for an intron-less promoter derived from elongation factor-1 alpha promoter. An exemplary nucleic acid sequence for the EFS promoter is represented by SEQ ID NO:1.

5

```
SEQ ID NO:1 >EFS promoter
ggctccggtgcccgtcagtgggcagagcgcacatcgcccacagtccccgagaagttggggggaggggtc
ggcaattgaaccggtgcctagagaaggtggcgcggggtaaaactgggaaagtgatgtcgtgtactggctc
cgcctttttcccgagggtgggggagaaccgtatataagtgcagtagtcgcctgaacgttcttttttcgc
aacgggtttgccgccagaacacaggtgtcgtgacgc
```

10

As used herein, the term “PGK promoter” has its general meaning in the art and refers to the promoter of the gene encoding for phosphoglycerate kinase. An exemplary nucleic acid sequence for the PGK promoter is represented by SEQ ID NO:2.

15

```
SEQ ID NO: 2 >PGK promoter
ccacgggggttgggggttgcccttttccaaggcagccctggggtttgcgccaggacgcggctgctctgggc
gtggttccgggaaacgcagcggcgccgacccctgggtctcgcacattcttcacgtccgttcgcagcgtca
cccggatcttcgccgctacccttgtgggccccccggcgacgttctctgctccgcccctaagtccgggaag
gttccttgcggttcgcggcggtgccggacgtgacaaacggaagccgcacgtctcactagtaccctcgca
acggacagcgccaggagcaatggcagcgcgcgacccgcgatgggctgtggccaatagcggctgctcag
cggggcgcgccgagagcagcggccgggaagggcggtgcgggagggcggggtgtggggcggtagtgtggg
ccctgttctgcccgcgcgggtgttcgcattctgcaagcctccggagcgacgtcggcagtcggctccc
tcgttgaccgaatcaccgacctctctcccc
```

20

25

As used herein, the term “bidirectional promoter” has its general meaning in the art and refers to a promoter which directs transcription of at least 2 transgenes in opposite orientations. Accordingly, a bidirectional promoter according to the present invention directs transcription of a first transgene which lies 5' to 3' in the same 5' to 3' direction as said promoter (“forward orientation”) and also directs transcription of another transgene which lies 5' to 3' in a direction opposite from the 5' to 3' direction of said promoter (“reverse orientation”). The bidirectional promoter of the present invention directs gene expression in a bidirectional fashion controlling expression for transgenes placed on both sides of the bidirectional promoter sequence. Thus, the recombinant nucleic acid molecule of the present invention comprises two transgenes, wherein the transcriptional direction (5' to 3') of the EFS and PGK portions of the EFS-PGK bidirectional promoter point away from each other (head to head configuration), wherein a first transgene is operably linked in one direction on the left side (i.e. in a reverse orientation), with expression controlled by the EFS portion of the bidirectional promoter, and a second transgene

30

35

is operably linked in the opposite direction on the right side (i.e. in a forward orientation), with expression controlled by the PGK portion of the bidirectional promoter.

According to the present invention, the bidirectional promoter of the present invention comprises a first portion that derives from the EFS promoter and a second portion derives from the PGK promoter.

In some embodiments, the first portion that derives from the EFS promoter comprises a nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:3 (i.e. the nucleic acid sequence as set forth in SEQ ID NO:1 that is reverse orientated).

SEQ ID NO:3 >EFS promoter in reverse orientation
gcgtcacgacacctgtgttctggcggcaaacccgttgcaaaaagaacgttcacggcgactactgcact
tatatacggtttctccccaccctcgggaaaaaggcggagccagtacacgacatcactttccagtttac
cccgcgccaccttctctagggaccggttcaattgccgacccctcccccaacttctcggggactgtggg
cgatgtgcgctctgcccactgacggggaccggagcc

In some embodiments, the second portion that derives from the PGK promoter comprises a nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:2.

In some embodiments, the first portion that derives from the EFS promoter and the second portion derives from the PGK promoter are separated by a spacer sequence. In some embodiments, the spacer sequence comprises a nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:4.

SEQ ID NO: 4 >linker
tttaattaaacgcctaccctcgagtagcttgatatgctagc

In some embodiments, the bidirectional promoter of the present invention comprises a nucleic acid sequence having at least 80% of identity with the sequence as set forth in SEQ ID NO:5.

SEQ ID NO: 5 > bidirectional promoter
gcgtcacgacacctgtgttctggcggcaaacccgttgcaaaaagaacgttcacggcgactactgcact
tatatacggtttctccccaccctcgggaaaaaggcggagccagtacacgacatcactttccagtttac
cccgcgccaccttctctagggaccggttcaattgccgacccctcccccaacttctcggggactgtggg
cgatgtgcgctctgcccactgacggggaccggagcc**tttaattaaacgcctaccctcgagtagcttgata**
tgctagcccacgggggttgggggttgcgccttttccaaggcagccctgggtttgcgcagggacgcggctgc

tctgggcgtgggttcgggaaacgcagcggcgccgaccctgggtctcgcacattcttcacgtccgttcgc
agcgtcaccgggatcttcgccgctacccttggtgggccccccggcgacgcttcctgctccgccccctaagt
cgggaaggttccttgcggttcgcggcgtgccggacgtgacaaacggaagccgcacgtctcactagtacc
ctcgacagcggacagcgccagggagcaatggcagcgccgacccgatgggctgtggccaatagcggc
tgctcagcggggcgccgagagcagcggccgggaagggcggtgcgggagggcggtgtggggcggtc
gtgtgggcccctgttcctgcccgcgcggtgttcgcattctgcaagcctccggagcgacgtccgcagtc
ggctccctcggttgaccgaatcaccgacctctctcccc

According to the invention a first nucleic acid sequence having at least 80% of identity
 10 with a second nucleic acid sequence means that the first sequence has 80, 81, 82, 83, 84, 85, 86,
 87, 88, 89, 90; 91; 92; 93; 94; 95; 96; 97; 98; 99 or 100% of identity with the second nucleic
 acid sequence.

As used herein, the term “sequence identity,” as used herein, has the standard meaning
 in the art. As is known in the art, a number of different programs can be used to identify whether
 15 a nucleic acid sequence has sequence identity or similarity to another nucleic acid sequence.
 Sequence identity or similarity may be determined using standard techniques known in the art,
 including, but not limited to, the local sequence identity algorithm of Smith & Waterman, Adv.
 Appl. Math. 2:482 (1981), by the sequence identity alignment algorithm of Needleman &
 Wunsch, J. Mol. Biol. 48:443 (1970), by the search for similarity method of Pearson & Lipman,
 20 Proc. Natl. Acad. Sci. USA 85:2444 (1988), by computerized implementations of these
 algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software
 Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.), the Best Fit sequence
 program described by Devereux et al., Nucl. Acid Res. 12:387 (1984), preferably using the
 default settings, or by inspection. An example of a useful algorithm is the BLAST algorithm,
 25 described in Altschul et al., J. Mol. Biol. 215:403 (1990) and Karlin et al., Proc. Natl. Acad.
 Sci. USA 90:5873 (1993). A particularly useful BLAST program is the WU-BLAST-2 program
 which was obtained from Altschul et al., Meth. Enzymol., 266:460 (1996);
 blast.wustl.edu/blast/README.html. WU-BLAST-2 uses several search parameters, which are
 preferably set to the default values. The parameters are dynamic values and are established by
 30 the program itself depending upon the composition of the particular sequence and composition
 of the particular database against which the sequence of interest is being searched; however,
 the values may be adjusted to increase sensitivity.

As used herein, the term “transgene” refers to any nucleic acid that shall be expressed
 in a mammal cell, in particular a T cell.

In some embodiments, the sequence of the transgenes is codon-optimized. As used herein, the term “codon-optimized” refers to nucleic sequence that has been optimized to increase expression by substituting one or more codons normally present in a coding sequence (for example, in a wildtype sequence, including, e.g., a coding sequence for LNGFR or FoxP3) with a codon for the same (synonymous) amino acid. In this manner, the protein encoded by the gene is identical, but the underlying nucleobase sequence of the gene or corresponding mRNA is different. In some embodiments, the optimization substitutes one or more rare codons (that is, codons for tRNA that occur relatively infrequently in cells from a particular species) with synonymous codons that occur more frequently to improve the efficiency of translation. For example, in human codon-optimization one or more codons in a coding sequence are replaced by codons that occur more frequently in human cells for the same amino acid. Codon optimization can also increase gene expression through other mechanisms that can improve efficiency of transcription and/or translation. Strategies include, without limitation, increasing total GC content (that is, the percent of guanines and cytosines in the entire coding sequence), decreasing CpG content (that is, the number of CG or GC dinucleotides in the coding sequence), removing cryptic splice donor or acceptor sites, and/or adding or removing ribosomal entry sites, such as Kozak sequences. Desirably, a codon-optimized gene exhibits improved protein expression, for example, the protein encoded thereby is expressed at a detectably greater level in a cell compared with the level of expression of the protein provided by the wildtype gene in an otherwise similar cell.

According to the present invention, the first transgene that is under the control of the EFS portion of the bidirectional promoter thus encodes for a protein that is not constitutively expressed by a T cell. Typically, the expression of said protein will be suitable for the cell sorting of the transformed cell with the recombinant nucleic acid molecule of the present invention as described herein after. Typically, said protein is a cell surface marker so that use of binding partners specific for this protein can be used for cell sorting. In some embodiments, the protein is a receptor that will be expressed at the surface of the T cell. In some embodiments, the protein derives from the LNGFR. As used herein, the term “LNGFR” has its general meaning in the art and refers to the low-affinity nerve growth factor receptor. It is a member of the Tumor Necrosis Factor receptor (TNFR) superfamily, and thus anonymously called TNFRSF16. ΔLNGFR consists of 427-amino-acid in overall length, and possesses an extracellular region with four 40 amino acid repeats with 6 cysteins at conserved positions followed by a serine/threonine-rich region, a single transmembrane domain, and a 155 amino acid cytoplasmic domain. LNGFR is expressed in a wide variety of tissues, such as brain,

peripheral neurons, Schwann cells, liver, esophagus and oral epithelium and the mesenchyme. However, Δ LNGFR is not expressed in T cells. In some embodiments, the proteins consists of the LNGFR truncated of its intracytoplasmic part. This protein is named " Δ LNGFR". In some embodiments, the first transgene comprises a nucleic acid sequence having at least 80% identity with the nucleic acid sequence as set forth in SEQ ID NO:6.

SEQ ID NO: 6 >deltaLNGFRco sequence

```
atggatggcctagactcctccttctcctgctgctgggcgtgtcactgggcggagccaaagaggcctgt
cctaccggcctgtacacacacagcggcgagtgctgcaaggcctgcaatctgggagaaggcgtggcccag
ccttgccggcgctaatacagaccgtgtgcgagccctgctggacagcgtgacctttagcgacgtggtgtcc
gccaccgagccttgcaagccttgtagcgagtggtggtggcctgcagagcatgagcgcccccttgctggaa
gccgacgatgccgtgtgcagatgcgcctacggctactaccaggacgagacaaccggcagatgcgaggcc
tgtagagtgtgcgaggccggatctggcctggtgttcagttgtcaagacaagcagaacaccgtgtgtgaa
gagtgcctcgacggcacctacagcgacgaggccaatcacgtggaccctgctgccatgcacagtgtgc
gaagataccgagcggcagctgctgcgagtgtagcagatgggcccagtgccgagtgcaagagatccctggc
agatggatcaccagaagcaccctcctgagggcagcgatagcacagccctagcaccaggaacctgag
gccccctcctgagcaggatctgatcgccctctacagtggccggcgctcgtgaccacagtgtgggcagttct
cagcccgctcgtgacaagaggcaccaccgacaacctgatccccgtgtactgcagcatcctggccgctgtg
gtcgtgggcctggtggcctatatcgccctcaagcgggtggaaccggggcatcctgtga
```

In some embodiments, the second transgene that is under the control of the PGK portion of the bidirectional promoter encodes for a transcription factor. In some embodiments, the transcription factor is 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). In some embodiments, the second transgene comprises a nucleic acid sequence having at least 80% identity with the nucleic acid sequence as set forth in SEQ ID NO:7.

SEQ ID NO:7 >hFoxp3co sequence

```
atgcccacccccagaccggaaagcctagcgcccccttctctggccctgggaccttctcctggcgccctcc
ccatcttgtagagccgccccctaaagccagcgatctgctgggagctagaggccctggcggcacattccag
ggcagagatctgagaggcggagcccacgcctctagcagcagcctgaatcccatgccccctagccagctg
cagctgcctacactgcctctcgtgatggtggccccctagcggagctagactgggccccctctgcctcatctg
caggccctgctgcaggacagacccccacttcatgcaccagctgagcaccgtggatgcccacgccagaaca
cctgtgctgcagggtgcacccccctggaaagccctgccatgatcagcctgacccctccaaccacagccacc
ggcgtgttcagcctgaaggccagacctggactgccccctggcatcaatgtggccagcctggaatgggtg
tccccgcgaacctgcctgctgtgcaccttcccccaatcccagcgccccccagaaaggacagcacactgtct
gccgtgccccagagcagctatccccctgctggtgctaacggcgctgtgcaagtggcctggctgcgagaagggtg
ttcgaggaacccgaggacttctgaagcactgccaggccgacatctgctggacgagaaaggcagagcc
cagtgctctgctgcagcgcgagatggtgcagagcctggaacagcagctgggtgctggaaaaagaaaagctg
```

agcgccatgcaggcccacctggccggaataatggccctgacaaaggccagcagcgtggccagctctgac
aagggcagctgctgcattgtggccgctggctctcagggacctgtggtgacctgcttgagcggacctaga
gagggcccgatagcctgtttgccgtgaggagacacctgtggggcagccacggcaactctaccttcccc
5 gagttcctgcacaacatggactacttcaagttccacaacatgaggcccccttcacctacgccaccctg
atcagatgggcatcttctggaagcccccgagaagcagcggaccctgaacgagatctaccactggtttacc
cggatgttcgccttcttccggaaccaccccgccacctggaagaacgccatccggcacaatctgagcctg
cacaagtgttcgtgcggtggaaagcagagaagggcgccgtgtggacagtggaacgagctggaatttcgg
aagaagcgggtcccagaggcccgccggtgtagcaatcctaccttggcccttga

- 10 In some embodiments, the nucleic acid molecule of the present invention comprises:
- i) a first nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID: 8 (which corresponds to the nucleic acid sequence encoding for Δ LNGFR (i.e. SEQ ID NO:6) a in reverse orientation),
 - ii) a second nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:5 (which corresponds to the bidirectional promoter) and
 - iii) a third nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:6 (which corresponds to the nucleic acid sequence encoding for FoxP3).

20

SEQ ID NO:8 >deltaLNGFRco sequence in reverse orientation
tcacaggatgccccggttccaccgcttgaaggcgatataggccaccaggccacgaccacagcggccag
gatgctgcagtacacggggatcaggttgctgggtgaccttctgtcacgacgggctgagaactgcccat
25 cactgtggtcacgacgccccgactgtagaggcgatcagatcctgctcaggaggggctcaggttcctg
ggtgctaggggctgtgctatcgctgccctcagggggggtgcttctggtgatccatctgccagggatctc
ttcgcaactcggcacatcgcccatctggtacactcgcgagctgccgctcggtatcttcgcacactgtgca
tggcaggcaggggtccacgtgattggcctcgctcgctgtaggtgccgctcggggcaactcttcacacaggt
gttctgcttgtcttgacaactgaacaccaggccagatccggcctcgcaactctacaggcctcgcatct
30 gccggttgtctcgctcctggtagtagccgtaggcgcatctgcacacggcatcgctcggttccacgcaagg
ggcgctcatgctctgcaggccacacactcggtacaaggcttgcaaggctcggtggcggaacacacgctc
gctaaaggtcacgctgtccaggcagggctcgcacacgggtctgattagcgccgcaaggctgggccacgcc
ttctcccagattgcaggccttgagcactcgccgctgtgtgtgtacaggccggtaggacaggcctcttt
ggctccgcccagtgacacgcccagcagcaggagaaggaggagtctagggccatccat

- 35 As used herein, the terms "operably linked", or "operatively linked" are used interchangeably herein, and refer to the functional relationship of the nucleic acid sequences with regulatory sequences of nucleotides, such as promoters, enhancers, transcriptional and translational stop sites, and other signal sequences and indicates that two or more DNA segments are joined together such that they function in concert for their intended purposes. For
- 40 example, operative linkage of nucleic acid sequences, typically DNA, to a regulatory sequence or promoter region refers to the physical and functional relationship between the DNA and the regulatory sequence or promoter such that the transcription of such DNA is initiated from the regulatory sequence or promoter, by an RNA polymerase that specifically recognizes, binds

and transcribes the DNA. In order to optimize expression and/or in vitro transcription, it may be necessary to modify the regulatory sequence for the expression of the nucleic acid or DNA in the cell type for which it is expressed. The desirability of, or need of, such modification may be empirically determined.

Further regulatory sequences may also be added to the recombinant nucleic acid molecule of the present invention. As used herein, the term "regulatory sequence" is used interchangeably with "regulatory element" herein and refers to a segment of nucleic acid, typically but not limited to DNA, that modulate the transcription of the nucleic acid sequence to which it is operatively linked, and thus acts as a transcriptional modulator. A regulatory sequence often comprises nucleic acid sequences that are transcription binding domains that are recognized by the nucleic acid-binding domains of transcriptional proteins and/or transcription factors, enhancers or repressors etc. In some embodiments, the nucleic acid molecule of the present invention comprises a Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) sequence that is a DNA sequence that, when transcribed creates a tertiary structure enhancing expression, by stabilization of the messenger RNA. Typically, the WPRE sequence is inserted downstream to the second transgene (e.g. FoxP3). In some embodiments, the recombinant acid molecule of the present invention comprises a WPRE sequence devoid of X protein open reading frames (ORFs), that allows to remove oncogenic side effect without significant loss of RNA enhancement activity (Schambach, A. et al. Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther. 13, 641–645 (2006)). In some embodiments, the WPRE sequence comprises nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO: 9.

SEQ ID NO :9 > WPRE sequence, LPREm6 [Sequence derived from WPRE J02442.1 region 1093-1684 with point mutations as described in Schambach et al Gene Therapy 2006]

AATCAACCTCTGGATTACAAAATTTGTGAAAGATTGACTGGTATTCTTAAGTATGTTGCTCCTTTTACG
 CTATGTGGATACGCTGCTTTAATGCCTTTGTATCATGCTATTGCTTCCCGTATGGCTTTCATTTTCTCC
 TCCTTGATAAATCCTGGTTGCTGTCTCTTTATGAGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTG
 GTGTGCACTGTGTTTGCTGACGCAACCCCCACTGGTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCC
 GGGACTTTCGCTTTCCCCCTCCCTATTGCCACGGCGGAATCATCGCCGCCTGCCTTGCCCGCTGCTGG
 ACAGGGGCTCGGCTGTTGGGCACTGACAATTCCGTGGTGTGTCGGGGAATCATCGTCCCTTTCCTTGG
 CTGCTCGCCTGTGTTGCCACCTGGATTCTGCGCGGGACGTCTTCTGCTACGTCCCTTCGGCCCTCAAT
 CCAGCGGACCTTCCTTCCCGCGGCCTGCTGCCGCTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCT
 CAGACGAGTCGGATCTCCCTTTGGGCCGCTCCCCGCGCTG

In some embodiments, the recombinant nucleic acid molecule of the present invention comprises a polyadenylation signal sequence inserted downstream to the first transgene (e.g. Δ LNGFR). As used herein, the term “polyadenylation signal sequence” has its general meaning in the art and refers to a nucleic acid sequence that mediates the attachment of a polyadenine stretch to the 3' terminus of the mRNA. Suitable polyadenylation signals include the SV40 early polyadenylation signal, the SV40 late polyadenylation signal, the HSV thymidine kinase polyadenylation signal, the protamine gene polyadenylation signal, the adenovirus 5 E1b polyadenylation signal, the bovine growth hormone polyadenylation signal, the human variant growth hormone polyadenylation signal and the like. In some embodiments, the polyadenylation sequence comprises nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO: 10.

SEQ ID NO:10 >polyadenylation signal in reverse orientation
 cagatctgatcataatcagccataccacatttgttagagggttttacttgctttaaaaaacctcccacacc
 tccccctgaacctgaaacataaaatgaatgcaattgttgttgaacttgctttattgcagcttataatg
 gttacaaataaggcaatagcatcacaaatttcacaaataaggcatttttttactgcattctagttttg
 gtttgtccaaactcatcaatgtatcttatcatgtctggtatctc

In some embodiments, the recombinant acid molecule of the present invention comprises a nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:11.

SEQ ID NO:11 > Whole sequence including the 5' and 3' LTR sequences
 ccattgcatacgttgtatccatatcataatatgtacatttatattggctcatgtccaacattaccgcca
 tgttgacattgattattgactagttattaatagtaatcaattacggggtcatttagttcatagcccatat
 atggagttccgcgttacataacttacggtaaatggcccgccctggctgacccgccaacgacccccgcca
 ttgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtg
 gagtatttacggtaaaactgccacttggcagtagcatcaagtgtatcatatgccagtagcggccctatt
 gacgtcaatgacggtaaatggcccgccctggcattatgccagtagcatgaccttatgggactttcctact
 tggcagtagcatctacgtattagtcacgtattaccatgggtgatgcggttttggcagtagcatcaatggg
 cgtggatagcggtttgactcacggggatttccaagtctccacccattgacgtcaatgggagtttgttt
 tggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaattgggcggt
 aggcgtgtacggtgggaggtctatataagcagagctcgtttagtgaaccgggggtctctctggttagacc
 agatctgagcctgggagctctctggctaactagggaacccactgcttaagcctcaataaagcttgccct
 gagtgcttcaagtagtgtgtgcccgtctgttgtgtgactctggtaactagagatccctcagaccctttt
 agtcagtggtgaaaatctctagcagtgggcgccgaacagggacttgaaagcgaaaggggaaaccagagga
 gctctctcgacgcaggactcggcttgctgaagcgcgcacggcaagaggcgagggcgggcgactggtgag
 tacgccccaaaattttgactagcggaggctagaaggagagagatgggtgagagagcgtcagttattaagcg
 ggggagaattagatcgcgatgggaaaaaattcgggttaaggccaggggggaaagaaaaatataaattaaa

acatatagtagtgggcaagcagggagctagaacgattcgcagttaatcctggcctgttagaaacatcaga
 aggctgtagacaaatactgggacagctacaacccatcccttcagacaggatcagaagaacttagatcatt
 atataatacagtagcaaccctctattgtgtgcatcaaaggatagagataaaagacaccaaggaagcttt
 agacaagatagaggaagagcaaaacaaaagtaagaccaccgcacagcaagcgccgctgatcttcagac
 5 ctggaggaggagatatgagggacaattggagaagtgaattatataaatataaagtagtaaaaattgaac
 cattaggagtagcacccaccaaggcaaagagaagagtgggtgcagagagaaaaagagcagtgggaatag
 gagctttgttccttgggttcttgggagcagcaggaagcactatgggcgagcctcaatgacgctgacgg
 tacaggccagacaattattgtctggtatagtgcagcagcagaacaatttgctgagggctattgagggcg
 aacagcatctgttgcaactcacagtctggggcatcaagcagctccaggcaagaatcctggctgtggaaa
 10 gatacctaaaggatcaacagctcctggggatttgggggttgcctctggaaaactcatttgcaccactgctg
 tgccttggaaatgctagttggagtaataaatctctggaacagatttggaaatcacacgacctggatggagt
 gggacagagaaattaacaattacacaagcttaatacactccttaattgaagaatcgcaaaaccagcaag
 aaaagaatgaacaagaattattggaattagataaatgggcaagtttgtggaattggtttaacatacaa
 attggctgtggtatataaaattattcataatgatagtaggaggcttggtaggtttaagaatagtttttg
 15 ctgtactttctatagtgaatagagttaggcagggatattcaccattatcgtttcagacccacctcccaa
 ccccgaggggacccgcagggccgaaggaatagaagaagaaggtggagagagagacagagacagatcca
 ttcgattagtgaacggatctcgacggtatcgggttaacttttaaaagaaaaggggggattggggggtaca
 gtgcaggggaaagaatagtagacataatagcaacagacatacaaaactaaagaattacaaaaacaaatta
 caaaaattcaaaattttatcgattagaccagaaatagttcgtttaaacagatctgatcataatcagcc
 20 ataccacattttagtagaggttttacttgcctttaaanaacctcccacacctccccctgaacctgaaacata
 aatgaatgcaattgttgttgaacttgtttattgcagcttataatgggttacaataaggcaatagca
 tcacaaatttcacaaataaggcatttttttcaactgcattctagttttggtttgcctaaactcatcaatg
 tatcttatcatgtctggatctcaaaccctcggaagctgcgcctgtcatcaattcctgcagcccggtgc
 atgactaatcagttagcctccccatctccctcgactcctgcaggctatcacaggatgccccggttcca
 25 ccgcttgaaggcgatataggccaccaggcccaagaccacagcggccaggatgctgcagtacacggggat
 caggttgtcggtggtgcctcttgtcacgacgggctgagaactgcccactcactgtggtcacgacgcccgc
 cactgtagaggcgatcagatcctgctcaggaggggctcagggttcctgggtgctaggggctgtgctatc
 gctgccctcaggggggggtgcttctggtgatccatctgccagggatctcttcgcaactcggcacgccc
 tctggtacactcgcgagctgccgctcggtatcttcgcacactgtgcatggcaggcaggggtccacgtg
 30 attggcctcgctcgctgtaggtgccgtcggggactcttcacacacgggtgttctgcttgtcttgacaact
 gaacaccaggccagatccggcctcgacactctacaggcctcgcatctgccggttgtctcgctcctggta
 gtagccgtaggcgcatctgcacacggcatcgctcggttccacgcaaggggcgctcatgctctgcaggcc
 cacacactcgggtacaaggcttgcaaggctcggtggcggaacacagtcgctaaagggtcacgctgtccag
 gcagggtcgcacacgggtctgattagcgccgcaaggctggggccacgccttctcccagattgcaggcctt
 35 gcagcactcgccgctgtgtgtgtacaggccggttaggacaggcctcttgggtcggcccagtgacacgcc
 cagcagcaggagaaggaggagtctagggccatccatgggtggcacgcgtcgcgtaacgacacctgtgttc
 tggcggaaccccggttgcgaaaaagaacgttcacggcgactactgcacttatatacgggttctccccac
 cctcgggaaaaaggcgagccagtagcacgacatcactttcccagtttaccggcgccaccttctctag
 caccggttcaattgccgacccctcccccaacttctcggggactgtggggcatgtgcgctctgccact
 40 gacgggacccggagccttaattaaacgcctaccctcgagtagcttgatatgctagcccacgggggtggg
 gttgcgccttttccaaggcagccctgggttgcgcagggaacgcggctgctctgggcgtggttccgggaa

acgcagcggcgccgacccctgggtctcgcacattcttcacgtccgttcgcagcgtcaccgcgatcttcgc
 cgctacccttgtgggccccccggcgacgcttctgctccgcccctaagtcgggaaggttccttgcggtt
 cgcgcggtgccggacgtgacaaacggaagccgcacgtctcactagtagccctcgcagacggacagcgcca
 5 gggagcaatggcagcgcgcgcgacccgcgatgggctgtggccaatagcggctgctcagcggggcgcgccga
 gagcagcggccgggaagggcggtgcgggagggcggggtgtggggcggtagtgtgggccctgttcctgcc
 cgcgcggtgttcgcattctgcaagcctccggagcgcacgtcggcagtcggctccctcggtgaccgaat
 caccgacctctctccccgggatccgccaccatgcccaccccagaccccgaaagcctagcgcccttct
 ctggccctgggaccttctcctgggcgctcccatcttgagagccgcccctaaagccagcgatctgctg
 ggagctagaggccctggcggcacattccagggcagagatctgagaggcggagccacgcctctagcagc
 10 agcctgaatcccatgccccctagccagctgcagctgcctacactgcctctcgatggtggcccttagc
 ggagctagactgggcccctctgcctcatctgcaggcccctgctgcaggacagaccccacttcatgcaccag
 ctgagcacctggtgatgcccacgccagaacacctgtgctgcaggtgcacccccctggaaagccctgccatg
 atcagcctgacccctccaaccacagccaccggcggtgttcagcctgaaggccagacctggactgcccct
 ggcatcaatgtggccagcctggaatgggtgtccgcgaacctgcccctgctgtgcaccttcccacatccc
 15 agcgcccccagaaaggacagcacactgtctgcctgccccagagcagctatcccctgctggctaaccgpc
 gtgtgcaagtggcctggctgcgagaaggtgttcgaggaacccgaggacttctgaagcactgccaggcc
 gaccatctgctggacgagaaaggcagagcccagtgctgtgctgcagcgcgagatggtgcagagcctggaa
 cagcagctggtgctggaaaaagaaaagctgagcgccatgcaggcccacctggccggaaaaatggccctg
 acaaaggccagcagcgtggccagctctgacaagggcagctgctgcattgtggccgctggctctcaggga
 20 cctgtggtgacctgcttgagcgccagcctagagaggcccccgatagcctgtttgccgtgaggagacacctg
 tggggcagccacggcaactctaccttccccgagttcctgcacaacatggactacttcaagttccacaac
 atgaggcccccttccacctacgccaccctgatcagatgggcccattctggaagcccccgagaagcagcgg
 accctgaacgagatctaccactgggtttaccgcgatgttcgccttcttccggaaccaccccgccacctgg
 aagaacgccatccggcacaatctgagcctgcacaagtgttcgtgcggtggaaagcgagaagggcgcc
 25 gtgtggacagtggacgagctggaatttcggaagaagcgggtcccagaggcccagccggtgtagcaatcct
 acccctggcccttgataggcatgcatatgTGCACAATCAACCTCTGGATTACAAAATTTGTGAAAGAT
 TGA CTGGTATTCTTA ACTATGTTGCTCCTTTTACGCTATGTGGATACGCTGCTTTAATGCCTTTGTATC
 ATGCTATTGCTTCCCGTATGGCTTTTCA TTTTCTCCTCCTTGTATAAATCCTGGTTGCTGTCTCTTTATG
 AGGAGTTGTGGCCCGTTGTCAGGCAACGTGGCGTGGTGTGCACTGTGTTTGCTGACGCAACCCCCACTG
 30 GTTGGGGCATTGCCACCACCTGTCAGCTCCTTTCCGGGACTTTTCGCTTTCCCCCTCCCTATTGCCACGG
 CGGA ACTCATCGCCGCCTGCCTTGCCCGCTGCTGGACAGGGGCTCGGCTGTTGGGCACTGACAATTCCG
 TGGTGTGTCGGGGAAATCATCGTCCTTTTCTTGCTGCTCGCTGTGTTGCCACCTGGATTCTGCGCG
 GGACGTCCTTCTGCTACGTCCCTTCGGCCCTCAATCCAGCGGACCTTCCTTCCCGGGCCTGCTGCCGG
 CTCTGCGGCCTCTTCCGCGTCTTCGCCTTCGCCCTCAGACGAGTCGGATCTCCCTTTGGGCGGCCTCCC
 35 CGCCTGGAATTGAGCTCGGTACCTtttaagaccaatgacttacaaggcagctgtagatcttagccactt
 tttaaaagaaaaggggggactggaagggctaattcactcccaacgaagacaagatctgctttttgcttg
 tactgggtctctctggttagaccagatctgagcctgggagctctctggctaactagggaacccactgct
 taagcctcaataaagccttgcttgagtgttcaagtagtgtgtgcccgtctgttggtgtgactctggtaa
 ctagagatccctcagacccttttagtcagtggtgaaaatctctagcagtagtagttcatgtcatcttat
 40 tattcagtatttataacttgcaagaaatgaatatcagagagtgagaggaacttggtttattgcagctta
 taatggttacaaataaagcaatagcatcacaatttcacaaataaagcatttttttactgcattctag

ttgtggtttgtccaaactcatcaatgtatcttatcatgtctggctctagctatcccgcccctaactccg
 ccagttccgcccattctccgcccattggtgactaattttttttatttatgcagaggccgaggccgccc
 tcggcctctgagctattccagaagtagtgaggaggcttttttggaggcctagggacgtacccaattcgc
 cctatagttagtcgtattacgcgcgctcactggcgcgtgttttacaacgtcgtgactgggaaaaacctg
 5 gcgttaccaacttaatcgcccttcagcacatcccccttcgccagctggcgtaatagcgaagaggccc
 gcaccgatcgcccttcccaacagttgcgagcctgaatggcgaatgggacgcgcctgtagcggcgcat
 taagcgcggcggtgtggtggttacgcctgaatggcgaatgggacgcgcctgtagcggcgcat
 gggcggtgtggtggttacgcgcagcgtgaccgctacacttgccagcgccttagcgcgcctcctttc
 gctttcttcccttcccttctcgccacgttcgcgcgctttcccgctcaagctctaaatcgggggctccct
 10 ttagggttccgatttagtgctttacggcacctcgacccccaaaaacttgattagggatggttcacgt
 agtggggccatcgccctgatagacgggtttttcgccctttgacggttgagtcacggttctttaatagtga
 ctcttggttccaaactggaacaacactcaaccctatctcggtctattcttttgatttataagggttttg
 ccgatttcggcctattggttaaaaaatgagctgatttaacaaaaatttaacgcgaattttaacaaaaa
 ttaacgcttacaatttaggtggcacttttcggggaaatgtgcgcggaacccctatttggtttattttct
 15 aaatacattcaaataatgtatccgctcatgagacaataaccctgataaatgcttcaataatattgaaaaa
 ggaagagtatgagtattcaacatttcggtgctgcgccttattcccttttttgcggcattttgccttcctg
 tttttgctcaccagaaacgctggtgaaagtaaaagatgctgaagatcagttgggtgcacgagtgggtt
 acatcgaactggatctcaacagcggtaagatccttgagagttttcgccccgaagaacgttttccaatga
 tgagcacttttaagttctgctatgtggcgcggtattatcccgctattgacgcggggcaagagcaactcg
 20 gtcgcgcgcatacactattctcagaatgacttggttgagtactcaccagtcacagaaaagcatcttacgg
 atggcatgacagtaagagaattatgcagtgctgccataaccatgagtgataaacactgcggccaacttac
 ttctgacaacgatcggaggaccgaaggagctaaccgcttttttgcaaacatgggggatcatgtaactc
 gccttgatcggttggaacgggagctgaatgaagccataccaaacgacgagcgtgacaccacgatgcctg
 tagcaatggcaacaacggttcgcgcaactattaactggcgaactacttactctagcttcccggaacaat
 25 taatagactggatggaggcggataaagttgcaggaccacttctgcgctcggcccttccggctggctggt
 ttattgctgataaatctggagccggtgagcgtgggtctcgcggtatcattgcagcactggggccagatg
 gtaagccctcccgctatcgtagttatctacacgacggggagtcaggcaactatggatgaacgaaatagac
 agatcgctgagataggtgcctcactgattaagcattggtaactgtcagaccaagtttactcatatatac
 tttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatcctttttgataatctca
 30 tgacaaaaatcccttaacgtgagttttcggtccactgagcgtcagaccccgtagaaaagatcaaaggat
 cttcttgagatccttttttctgcgcgtaactctgctgcttgcaaacaaaaaaaccaccgctaccagcgg
 tgggttgggttgcgggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcaga
 taccaaatactgttcttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgccta
 catacctcgctctgctaactcctgttaccagtggtgctgccagtgggcgataagtcgtgtcttaccgggt
 35 tggactcaagacgatagttaccggataaggcgcagcggtcgggctgaacgggggggttcgtgcacacagc
 ccagcttgagcgaacgacctaaccgaactgagatacctacagcgtgagctatgagaaagcgccacgc
 tccccgaaggagaaaggcggacaggtatccggtaagcggcagggtcggaaacaggagagcgcacgaggg
 agcttcagggggaaacgcctgggtatctttatagtcctgtcggttttcgccacctctgacttgagcgtc
 gatTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGT
 40 TCCTGGCCTTTTGTGGCCTTTTGTCTCATGTTCTTTCTCGTTCATCCCTGATTCTGTGGATAACC
 GTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGACGCCGAACGACCGAGCGCAGCGAGTCAGTGA

gcgaggaagcggaagagcgcccaatacgcaaaccgcctctccccgcgcgttgccgattcattaatgca
 gctggcagcagcaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagttagctca
 ctcataggcaccccagggtttacactttatgcttcggctcgtatggtgtgtggaattgtgagcggat
 aacaatttcacacaggaacagctatgaccatgattacgccaagcgcgcaattaaccctcactaaaggg
 5 aacaaaagctggagctgcaagcttgg

In some embodiments, the recombinant nucleic acid molecule of the present invention is inserted in a viral vector, and in particular in a retroviral vector.

As used herein, the term “viral vector” refer to a virion or virus particle that functions
 10 as a nucleic acid delivery vehicle and which comprises a vector genome packaged within the virion or virus particle.

As used herein, the term “retroviral vector” refers to a vector containing structural and functional genetic elements that are primarily derived from a retrovirus.

In some embodiments, the retroviral vector of the present invention derives from a
 15 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), epsilon retroviruses (e.g., Walley dermal sarcoma virus), lentiviruses (e.g., HIV-1, HIV-2) and spumaviruses (e.g., human spumavirus).

20 In some embodiments, the retroviral vector of the present invention is a replication deficient retroviral virus particle, which can transfer a foreign imported RNA of a gene instead of the retroviral mRNA. 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
 25 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 vector is a HIV-1 vector.

The structure and composition of the vector genome used to prepare the retroviral
 30 vectors of the present invention are in accordance with those described in the art. Especially, minimum retroviral gene delivery vectors can be prepared from a vector genome, which only contains, apart from the recombinant nucleic acid molecule of the present invention, the sequences of the retroviral genome which are non-coding regions of said genome, necessary to provide recognition signals for DNA or RNA synthesis and processing. In some embodiment,
 35 the retroviral vector genome comprises all the elements necessary for the nucleic import and

the correct expression of the polynucleotide of interest (i.e. the transgene). As examples of elements that can be inserted in the retroviral genome of the retroviral vector of the present invention are at least one (preferably two) long terminal repeats (LTR), such as a LTR5' and a LTR3', a psi sequence involved in the retroviral genome encapsidation, and optionally at least one DNA flap comprising a cPPT and a CTS domains. In some embodiments of the present invention, the LTR, preferably the LTR3', is deleted for the promoter and the enhancer of U3 and is replaced by a minimal promoter allowing transcription during vector production while an internal promoter is added to allow expression of the transgene. In particular, the vector is a Self-INactivating (SIN) vector that contains a non-functional or modified 3' Long Terminal Repeat (LTR) sequence. This sequence is copied to the 5' end of the vector genome during integration, resulting in the inactivation of promoter activity by both LTRs. Hence, a vector genome may be a replacement vector in which all the viral coding sequences between the 2 long terminal repeats (LTRs) have been replaced by the recombinant nucleic acid molecule of the present invention.

In some embodiments, the retroviral vector genome is devoid of functional gag, pol and/or env retroviral genes. By "functional" it is meant a gene that is correctly transcribed, and/or correctly expressed. Thus, the retroviral vector genome of the present invention in this embodiment contains at least one of the gag, pol and env genes that is either not transcribed or incompletely transcribed; the expression "incompletely transcribed" refers to the alteration in the transcripts gag, gag-pro or gag-pro-pol, one of these or several of these being not transcribed. In some embodiments, the retroviral genome is devoid of gag, pol and/or env retroviral genes.

In some embodiments the retroviral vector genome is also devoid of the coding sequences for Vif-, Vpr-, Vpu- and Nef-accessory genes (for HIV-1 retroviral vectors), or of their complete or functional genes.

Typically, the retroviral vector of the present invention is non replicative i.e., the vector and retroviral vector genome are not able to form new particles budding from the infected host cell. This may be achieved by the absence in the retroviral genome of the gag, pol or env genes, as indicated in the above paragraph; this can also be achieved by deleting other viral coding sequence(s) and/or cis-acting genetic elements needed for particles formation.

The retroviral vectors of the present invention can be produced by any well-known method in the art including by transfection (s) transient (s), in stable cell lines and / or by means of helper virus. Use of stable cell lines may also be preferred for the production of the vectors (Greene, M. R. et al. Transduction of Human CD34 + Repopulating Cells with a Self-Inactivating Lentiviral Vector for SCID-X1 Produced at Clinical Scale by a Stable Cell Line.

Hum. Gene Ther. Methods 23, 297–308 (2012).) For instance, the retroviral vector of the present invention is obtainable by a transcomplementation system (vector/packaging system) by transfecting in vitro a permissive cell (such as 293T cells) with a plasmid containing the retroviral vector genome of the present invention, and at least one other plasmid providing, in

5 trans, the gag, pol and env sequences encoding the polypeptides GAG, POL and the envelope protein(s), or for a portion of these polypeptides sufficient to enable formation of retroviral particles. As an example, permissive cells are transfected with a) transcomplementation plasmid, lacking packaging signal psi and, the plasmid is optionally deleted of accessory genes vif, nef, vpu and / or vpr, b) a second plasmid (envelope expression plasmid or pseudo-typing env

10 plasmid) comprising a gene encoding an envelope protein(s) and c) a plasmid vector comprising a recombinant genome retroviral, optionally deleted from the promoter region of the 3' LTR or U3 enhancer sequence of the 3' LTR, including, between the LTR sequences 5' and 3' retroviral, a psi encapsidation sequence, a nuclear export element (preferably RRE element of HIV or other retroviruses equivalent), comprising the nucleic acid molecule of the present invention

15 and optionally a promoter and / or a nuclear import sequence (cPPT sequence eg CTS) of the RNA. Advantageously, the three plasmids used do not contain homologous sequence sufficient for recombination. Nucleic acids encoding gag, pol and env cDNA can be advantageously prepared according to conventional techniques, from viral gene sequences available in the prior art and databases. The trans-complementation plasmid provides a nucleic acid encoding the

20 proteins retroviral gag and pol. These proteins are derived from a lentivirus, and most preferably, from HIV-1. The plasmid is devoid of encapsidation sequence, sequence coding for an envelope, accessory genes, and advantageously also lacks retroviral LTRs. Therefore, the sequences coding for gag and pol proteins are advantageously placed under the control of a heterologous promoter, eg cellular, viral, etc., which can be constitutive or regulated, weak or strong. It is

25 preferably a plasmid containing a sequence transcomplémentant Δ psi-CMV-gag-pol-PolyA. This plasmid allows the expression of all the proteins necessary for the formation of empty virions, except the envelope glycoproteins. The plasmid transcomplementation may advantageously comprise the TAT and REV genes. Plasmid transcomplementation is advantageously devoid of vif, vpr, vpu and / or nef accessory genes. It is understood that the

30 gag and pol genes and genes TAT and REV can also be carried by different plasmids, possibly separated. In this case, several plasmids are used transcomplementation, each encoding one or more of said proteins. The promoters used in the plasmid transcomplementation, the envelope plasmid and the plasmid vector respectively to promote the expression of gag and pol of the coat protein, the mRNA of the vector genome and the transgene are promoters identical or

different, chosen advantageously from ubiquitous promoters or specific, for example, from viral promoters CMV, TK, RSV LTR promoter and the RNA polymerase III promoter such as U6 or H1 or promoters of helper viruses encoding env, gag and pol (i.e. adenoviral, baculoviral, herpes viruses). For the production of the retroviral vector of the present invention, the plasmids described above can be introduced into competent cells and viruses produced are harvested. The cells used may be any cell competent, particularly eukaryotic cells, in particular mammalian, eg human or animal. They can be somatic or embryonic stem or differentiated. Typically the cells include 293T cells, fibroblast cells, hepatocytes, muscle cells (skeletal, cardiac, smooth, blood vessel, etc.), nerve cells (neurons, glial cells, astrocytes) of epithelial cells, renal, ocular etc.. It may also include, insect, plant cells, yeast, or prokaryotic cells. It can also be cells transformed by the SV40 T antigen. The genes gag, pol and env encoded in plasmids or helper viruses can be introduced into cells by any method known in the art, suitable for cell type considered. Usually, the cells and the vector system are contacted in a suitable device (plate, dish, tube, pouch, etc...), for a period of time sufficient to allow the transfer of the vector system or the plasmid in the cells. Typically, the vector system or the plasmid is introduced into the cells by calcium phosphate precipitation, electroporation, transduction or by using one of transfection-facilitating compounds, such as lipids, polymers, liposomes and peptides, etc.. The calcium phosphate precipitation is preferred. The cells are cultured in any suitable medium such as RPMI, DMEM, a specific medium to a culture in the absence of fetal calf serum, etc. Once transfected the retroviral vectors of the present invention may be purified from the supernatant of the cells. Purification of the retroviral vector to enhance the concentration can be accomplished by any suitable method, such as by density gradient purification (e.g., cesium chloride (CsCl)) or by chromatography techniques (e.g., column or batch chromatography). For example, the vector of the present invention can be subjected to two or three CsCl density gradient purification steps. The vector, is desirably purified from cells infected using a method that comprises lysing cells infected with adenovirus, applying the lysate to a chromatography resin, eluting the lentivirus from the chromatography resin, and collecting a fraction containing the retroviral vector of the present invention.

The vector of the present invention is particularly suitable for driving the targeted expression of the transgenes in T cells. According to the present invention, the expression of the transgenes is balanced from both sides of the bidirectional EFS-PGK promoter. As used herein, "balanced expression", "balance of expression", "expression balance", or "balanced" as it refers to expression, mean that the expression from one side of the bidirectional promoter, as measured for example by different protein expression detection techniques such as Western

Blot, FACS analysis, or other assays using luminescence or fluorescence, is comparable to the expression from the other side of the bidirectional promoter. Therefore, balanced expression of the 2 transgenes expressed by a bidirectional EFS-PGK promoter of the present invention is expected to generate comparable expression of both proteins.

5 In particular, the vector of the present invention is particularly suitable for obtaining a population of Treg cells that express LNGFR at their cell surface.

Thus a further object of the present invention relates to a method of producing a population of Treg cells, which comprises the step of transfecting or transducing a population of T cells in vitro or ex vivo with the vector of the present invention.

10 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
15 rest CD4⁺CD25⁺FoxP3⁺.

The term "transformation" means the introduction of a "foreign" (i.e. extrinsic or extracellular) gene, DNA or RNA sequence to a host cell, so that the host cell will express the introduced gene or sequence to produce a desired substance, typically a protein or enzyme coded by the introduced gene or sequence. A host cell that receives and expresses introduced
20 DNA or RNA has been "transformed".

In some embodiments, the population of T cells is isolated from a subject to whom the genetically modified population of T cells is to be adoptively transferred. In some embodiments, a population of T cells of the present invention are obtained by isolating a population of T-cells from a subject, and by subsequently proceeding with FoxP3 gene transfer ex vivo with the viral
25 vector of the present invention and subsequent immunotherapy of the subject by adoptive transfer of the transduced T cells. Alternatively, the population of T cells is isolated from a different subject, such that it is allogeneic. In some embodiments, the population of T cells is isolated from a donor subject.

Typically, the population of Treg cells is prepared as described in the EXAMPLE. The
30 population of T cells is preactivated in an appropriate culture medium that contains an amount of a recombinant human IL-2 in the presence of anti-CD3/CD28 microbeads. T cells are then infected with the vector of the present invention. LNGFR⁺ transduced cells were purified by cell sorting. As used herein, the term "cell sorting" is used to refer to a method by which cells are mixed a binding partner (e.g., a fluorescently detectable antibody) in solution. According to

the invention, any conventional cell sorting method may be used and typically involve use of anti-LNGFR antibodies. For instance, magnetic bead selection is suitable. Finally, the sorted T cells are expanded in presence of an amount of IL-2.

The population of Treg cells prepared as described above can be utilized in methods and compositions for adoptive immunotherapy in accordance with known techniques, or variations thereof that will be apparent to those skilled in the art based on the instant disclosure.

In particular, the population of Treg cells of the present invention is particularly suitable for the treatment of autoimmune diseases. The Treg cells as prepared by the method of the present invention may be administered for the purpose of suppressing autoimmune activity in a subject. 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.

In particular, the Treg cells of the present invention are particularly suitable 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. 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). 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 and anti-neutrophils antibodies, positive Coombs test). Molecular genetic testing confirms the diagnosis.

Accordingly, a further object of the present invention relates to a method of treating an autoimmune disease (e.g. IPEX syndrome) in a subject in need thereof, comprising administering to the subject a therapeutically effective amount of a population of Treg cells of the present invention.

As used herein, the term "treatment" or "treat" refer to both prophylactic or preventive treatment as well as curative or disease modifying treatment, including treatment of patient at risk of contracting the disease or suspected to have contracted the disease as well as patients who are ill or have been diagnosed as suffering from a disease or medical condition, and includes suppression of clinical relapse. The treatment may be administered to a subject having a medical disorder or who ultimately may acquire the disorder, in order to prevent, cure, delay the onset of, reduce the severity of, or ameliorate one or more symptoms of a disorder or recurring disorder, or in order to prolong the survival of a subject beyond that expected in the absence of such treatment. By a "therapeutically effective amount" is meant a sufficient amount of cells generated with the present invention for the treatment of the disease at a reasonable benefit/risk ratio applicable to any medical treatment. It will be understood that the total usage of these cells will be decided by the attending physicians within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular subject will depend upon a variety of factors including the age, body weight, general health, sex and diet of the subject; the time of administration, route of administration, and survival rate of the cells employed; the duration of the treatment; drugs used in combination or coincidental with the administered cells; and like factors well known in the medical arts. For example, it is well known within the skill of the art to start doses of cells at levels lower than those required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is achieved.

In some embodiments, the Tregs cells are formulated by first harvesting them from their culture medium, and then washing and concentrating the cells in a medium and container system suitable for administration (a "pharmaceutically acceptable" carrier) in a treatment-effective amount. Typically, the population of Tregs cells of the present invention is administered to the subject in the form of pharmaceutical composition. The pharmaceutical composition may be produced by those of skill, employing accepted principles of treatment. Such principles are known in the art, and are set forth, for example, in Braunwald et al., eds., Harrison's Principles of Internal Medicine, 19th Ed., McGraw-Hill publisher, New York, N.Y. (2015), which is incorporated by reference herein. The pharmaceutical composition may be administered by any means that achieve their intended purpose. For example, administration may be by parenteral, subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, transdermal, or buccal routes. The pharmaceutical compositions may be administered parenterally by bolus injection or by gradual perfusion over time. The pharmaceutical compositions typically comprises suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which may facilitate processing of the active compounds into preparations which can be used pharmaceutically. The pharmaceutical compositions may contain from about 0.001 to about 99 percent, or from about 0.01 to about 95 percent of active compound(s), together with the excipient.

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

FIGURES:

Figure 1 depicts the different constructions tested by the inventors.

Figure 2 depicts the flow cytometry analysis at Day 5.

EXAMPLE:

We compared 6 different lentiviral constructs according to 4 criteria (vector titers, level of transduction of human CD4⁺ T cells, level of expression of *FOXP3* and *ΔLNGFR* genes, degree of correlation between both expression) (**Figure 1**) :

#91 : unidirectional, EFS-FOXP3, PGK- ΔLNGFR

#95 : unidirectional, PGK-FOXP3, EFS- ΔLNGFR

#101 : bidirectional, ΔLNGFR- EFS,PGK-FOXP3

#103 : bidirectional, ΔLNGFR- mCMV, EF1a-FOXP3

#151 : bicistronic, EF1a- Δ LNGFR-T2A-FOXP3

#155 : bicistronic, EF1a- FOXP3-T2A- Δ LNGFR

Table 1 below illustrates vector titer, transduction efficiency measured in vector copy number (VCN) per cell at day 12 of culture, and co-expression of FOXP3 and Δ LNGFR measured by flow cytometry indicated as % of CD4⁺ T cells at day 5. In some cases, Δ LNGFR⁺ cells were sorted at day 5, further cultured for 12 days and analyzed by flow cytometry at D12 (**Figure 2**).

Table 1:

Vector	Titer	VCN (D12)	% LNGFR+FOXP3+ (D5)	% LNGFR+FOXP3+ (D12) after sorting at D5
#91	1.49x10e9	4	13.2	ND
#95	ND	ND	9.1	ND
#101	4.1x10e9	4	69	88
#103	1.3x10e9	0.45	11.8	ND
#151	2.35x10e8	0.61	25.9	ND
#155	6.36x10e7	0.31	20.6	ND

Constructs #151 and #155 were excluded because of low titers. #95 and #103 were excluded because of low levels of co-expression of *FOXP3* and *Δ LNGFR* genes and low VCN for #103. #91 was excluded because of the low level of expression of FOXP3. To note, the bidirectional construct tested by Passerini and coll (Passerini et al., 2013) that we reproduce herein with the codon optimized version (#103) was not efficient in terms of correlation of expression of *FOXP3* and *Δ LNGFR* genes. The only constructs that fulfilled the 4 criteria defined above is the bidirectional designs including forward hFOXP3co under the control of the PGK promoter and reverse *Δ LNGFR*co under the control of EFS promoter (#101, pCCL. *Δ LNGFR*co.EFS.PGK.hFOXP3co).

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.

Aarts-Riemens, T., M.E. Emmelot, L.F. Verdonck, and T. Mutis. 2008. Forced overexpression of either of the two common human Foxp3 isoforms can induce regulatory T cells from CD4(+)CD25(-) cells. *Eur J Immunol* 38:1381-1390.

Aiuti, A., R. Bacchetta, R. Seger, A. Villa, and M. Cavazzana-Calvo. 2012. Gene therapy for primary immunodeficiencies: Part 2. *Curr Opin Immunol* 24:585-591.

Aiuti, A., S. Vai, A. Mortellaro, G. Casorati, F. Ficara, G. Andolfi, G. Ferrari, A. Tabucchi, F. Carlucci, H.D. Ochs, L.D. Notarangelo, M.G. Roncarolo, and C. Bordignon. 2002. Immune reconstitution in ADA-SCID after PBL gene therapy and discontinuation of enzyme replacement. *Nat Med* 8:423-425.

Allan, S.E., A.N. Alstad, N. Merindol, N.K. Crellin, M. Amendola, R. Bacchetta, L. Naldini, M.G. Roncarolo, H. Soudeyans, and M.K. Levings. 2008. Generation of potent and stable human CD4+ T regulatory cells by activation-independent expression of FOXP3. *Mol Ther* 16:194-202.

Barzaghi, F., L. Passerini, and R. Bacchetta. 2012. Immune dysregulation, polyendocrinopathy, enteropathy, x-linked syndrome: a paradigm of immunodeficiency with autoimmunity. *Front Immunol* 3:211.

Bennett, C.L., J. Christie, F. Ramsdell, M.E. Brunkow, P.J. Ferguson, L. Whitesell, T.E. Kelly, F.T. Saulsbury, P.F. Chance, and H.D. Ochs. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet* 27:20-21.

Blaese, R.M., K.W. Culver, A.D. Miller, C.S. Carter, T. Fleisher, M. Clerici, G. Shearer, L. Chang, Y. Chiang, P. Tolstoshev, J.J. Greenblatt, S.A. Rosenberg, H. Klein, M. Berger, C.A. Mullen, W.J. Ramsey, L. Muul, R.A. Morgan, and W.F. Anderson. 1995. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. *Science* 270:475-480.

Bonino, C., G. Ferrari, S. Verzeletti, P. Servida, E. Zappone, L. Ruggieri, M. Ponzoni, S. Rossini, F. Mavilio, C. Traversari, C. Bordignon. 1997. HSV-TK Gene Transfer into Donor Lymphocytes for Control of Allogeneic Graft-Versus-Leukemia. *Science* 276: 1717-1724.

Bonini, C., M.K. Brenner, H.E. Heslop, and R.A. Morgan. 2011. Genetic modification of T cells. *Biol Blood Marrow Transplant* 17:S15-20.

Brunkow, M.E., E.W. Jeffery, K.A. Hjerrild, B. Paepers, L.B. Clark, S.A. Yasayko, J.E. Wilkinson, D. Galas, S.F. Ziegler, and F. Ramsdell. 2001. Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet* 27:68-73.

Ferraro, A., A.M. D'Alise, T. Raj, N. Asinovski, R. Phillips, A. Ergun, J.M. Replogle, A. Bernier, L. Laffel, B.E. Stranger, P.L. De Jager, D. Mathis, and C. Benoist. 2014. Interindividual variation in human T regulatory cells. *Proc Natl Acad Sci U S A* 111:E1111-1120.

5 Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky. 2003. Foxp3 programs the development and function of CD4⁺CD25⁺ regulatory T cells. *Nat Immunol* 4:330-336.

Fu, W., A. Ergun, T. Lu, J.A. Hill, S. Haxhinasto, M.S. Fassett, R. Gazit, S. Adoro, L. Glimcher, S. Chan, P. Kastner, D. Rossi, J.J. Collins, D. Mathis, and C. Benoist. 2012. A multiply redundant genetic switch 'locks in' the transcriptional signature of regulatory T cells.
10 *Nat Immunol* 13:972-980.

Hori, S., T. Nomura, and S. Sakaguchi. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299:1057-1061.

Horino, S., Y. Sasahara, M. Sato, H. Niizuma, S. Kumaki, D. Abukawa, A. Sato, M. Imaizumi, H. Kanegane, Y. Kamachi, S. Sasaki, K. Terui, E. Ito, I. Kobayashi, T. Ariga, S.
15 Tsuchiya, and S. Kure. 2014. Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome. *Pediatr Transplant* 18:E25-30.

Kalos, M., and C.H. June. 2013. Adoptive T cell transfer for cancer immunotherapy in the era of synthetic biology. *Immunity* 39:49-60.

20 Kasow, K.A., V.M. Morales-Tirado, D. Wichlan, S.A. Shurtleff, A. Abraham, D.A. Persons, and J.M. Riberdy. 2011. Therapeutic in vivo selection of thymic-derived natural T regulatory cells following non-myeloablative hematopoietic stem cell transplant for IPEX. *Clin Immunol* 141:169-176.

Khattari, R., T. Cox, S.A. Yasayko, and F. Ramsdell. 2003. An essential role for Scurfin
25 in CD4⁺CD25⁺ T regulatory cells. *Nat Immunol* 4:337-342.

Li, W., L. Wang, H. Katoh, R. Liu, P. Zheng, and Y. Liu. 2011. Identification of a tumor suppressor relay between the FOXP3 and the Hippo pathways in breast and prostate cancers. *Cancer Res* 71:2162-2171.

Mottet, C., H.H. Uhlig, and F. Powrie. 2003. Cutting edge: cure of colitis by
30 CD4⁺CD25⁺ regulatory T cells. *J Immunol* 170:3939-3943.

Muul, L.M., L.M. Tuschong, S.L. Soenen, G.J. Jagadeesh, W.J. Ramsey, Z. Long, C.S. Carter, E.K. Garabedian, M. Alleyne, M. Brown, W. Bernstein, S.H. Schurman, T.A. Fleisher, S.F. Leitman, C.E. Dunbar, R.M. Blaese, and F. Candotti. 2003. Persistence and expression of

the adenosine deaminase gene for 12 years and immune reaction to gene transfer components: long-term results of the first clinical gene therapy trial. *Blood* 101:2563-2569.

Passerini, L., E. Rossi Mel, C. Sartirana, G. Fousteri, A. Bondanza, L. Naldini, M.G. Roncarolo, and R. Bacchetta. 2013. CD4(+) T cells from IPEX patients convert into functional and stable regulatory T cells by FOXP3 gene transfer. *Sci Transl Med* 5:215ra174.

Provani, E., P. Genovese, A. Lombardo, Z. Magnani, P.Q. Liu, A. Reik, V. Chu, D.E. Paschon, L. Zhang, J. Kuball, B. Camisa, A. Bondanza, G. Casorati, M. Ponzoni, F. Ciceri, C. Bordignon, P.D. Greenberg, M.C. Holmes, P.D. Gregory, L. Naldini, and C. Bonini. 2012. Editing T cell specificity towards leukemia by zinc finger nucleases and lentiviral gene transfer. *Nat Med* 18:807-815.

Sawant, D.V., and D.A. Vignali. 2014. Once a Treg, always a Treg? *Immunol Rev* 259:173-191.

Seidel, M.G., G. Fritsch, T. Lion, B. Jurgens, A. Heitger, R. Bacchetta, A. Lawitschka, C. Peters, H. Gadner, and S. Matthes-Martin. 2009. Selective engraftment of donor CD4+25high FOXP3-positive T cells in IPEX syndrome after nonmyeloablative hematopoietic stem cell transplantation. *Blood* 113:5689-5691.

Tang, Q., K.J. Henriksen, M. Bi, E.B. Finger, G. Szot, J. Ye, E.L. Masteller, H. McDevitt, M. Bonyhadi, and J.A. Bluestone. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199:1455-1465.

Thornton, A.M., and E.M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188:287-296.

Trzonkowski, P., M. Bieniaszewska, J. Juscinska, A. Dobyszek, A. Krzystyniak, N. Marek, J. Mysliwska, and A. Hellmann. 2009. First-in-man clinical results of the treatment of patients with graft versus host disease with human ex vivo expanded CD4+CD25+CD127- T regulatory cells. *Clin Immunol* 133:22-26.

Wieckiewicz, J., R. Goto, and K.J. Wood. 2010. T regulatory cells and the control of alloimmunity: from characterisation to clinical application. *Curr Opin Immunol* 22:662-668.

Wildin, R.S., F. Ramsdell, J. Peake, F. Faravelli, J.L. Casanova, N. Buist, E. Levy-Lahad, M. Mazzella, O. Goulet, L. Perroni, F.D. Bricarelli, G. Byrne, M. McEuen, S. Proll, M. Appleby, and M.E. Brunkow. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27:18-20.

Yagi, H., T. Nomura, K. Nakamura, S. Yamazaki, T. Kitawaki, S. Hori, M. Maeda, M. Onodera, T. Uchiyama, S. Fujii, and S. Sakaguchi. 2004. Crucial role of FOXP3 in the

development and function of human CD25+CD4+ regulatory T cells. Int Immunol 16:1643-1656.

CLAIMS:

1. A recombinant nucleic acid molecule comprising a bidirectional EFS-PGK promoter operably linked to a first transgene in one direction and to a second transgene in the opposite direction wherein the first transgene that is under the control of the EFS portion of the bidirectional promoter encodes for a protein that is not constitutively expressed by a T cell and the second transgene that is under the control of the PGK portion of the bidirectional promoter encodes for a transcription factor.
2. The recombinant nucleic acid molecule of claim 1 wherein the first portion that derives from the EFS promoter comprises a nucleic sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:3.
3. The recombinant nucleic acid molecule of claim 1 wherein the second portion that derives from the PGK promoter comprises a nucleic sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:2.
4. The recombinant nucleic acid molecule of claim 1 wherein the first portion that derives from the EFS promoter and the second portion derives from the PGK promoter are separated by a spacer sequence.
5. The recombinant nucleic acid molecule of claim 4 wherein the spacer sequence comprises a nucleic sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:4.
6. The recombinant nucleic acid molecule of claim 1 wherein the bidirectional promoter comprises a nucleic acid sequence having at least 80% of identity with the sequence as set forth in SEQ ID NO:5.
7. The recombinant nucleic acid molecule of claim 1 wherein the sequences of the transgenes are codon-optimized.
8. The recombinant nucleic acid molecule of claim 1 wherein the first transgene that is under the control of the EFS portion of the bidirectional promoter encodes for a low-affinity nerve growth factor receptor (LNGFR), in a particular a low-affinity nerve growth factor receptor truncated of its intracytoplasmic part (Δ LNGFR).

9. The recombinant nucleic acid molecule of claim 1 wherein the second transgene that is under the control of the PGK portion of the bidirectional promoter encodes for FoxP3.
10. The recombinant nucleic acid molecule of claim 1 which comprises:
- i) a first nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID: 8 (which corresponds to the nucleic acid sequence encoding for Δ LNGFR (i.e. SEQ ID NO:6) a in reverse orientation),
 - ii) a second nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:5 (which corresponds to the bidirectional promoter) and
 - iii) a third nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:7 (which corresponds to the nucleic acid sequence encoding for FoxP3).
11. The recombinant acid molecule of claim 1 which comprises a nucleic acid sequence having at least 80% of identity with the nucleic acid sequence as set forth in SEQ ID NO:11.
12. A lentiviral vector which comprises the recombinant acid molecule of claim 1.
13. Use of the lentiviral vector of claim 12 for obtaining a population of Treg cells that express LNGFR at their cell surface
14. A method of producing a population of Treg cells, which comprises the step of transfecting or transducing a population of T cells *in vitro* or *ex vivo* with the lentiviral vector of claim 12.
15. A population of Treg cells obtainable by the method of claim 14.
16. A method of treating an autoimmune disease in a subject in need thereof comprising administering to the subject a therapeutically effective amount of the population of Treg cells of claim 15.
17. The method of claim 16 wherein the autoimmune disease is IPEX syndrome.
18. A nucleic acid sequence as set forth in SEQ ID NO:7.

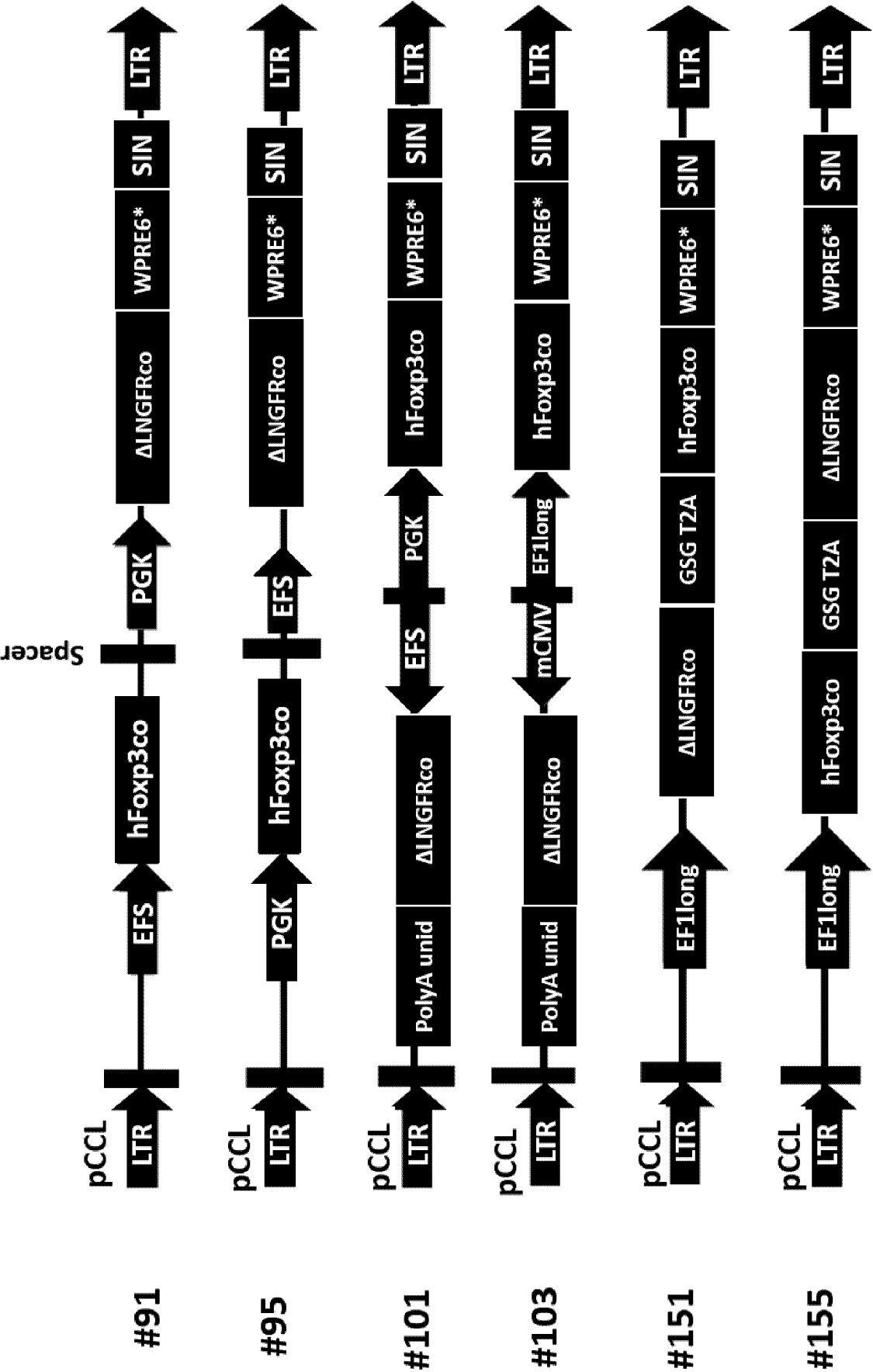


Figure 1

Dot plot analysis at Day 5

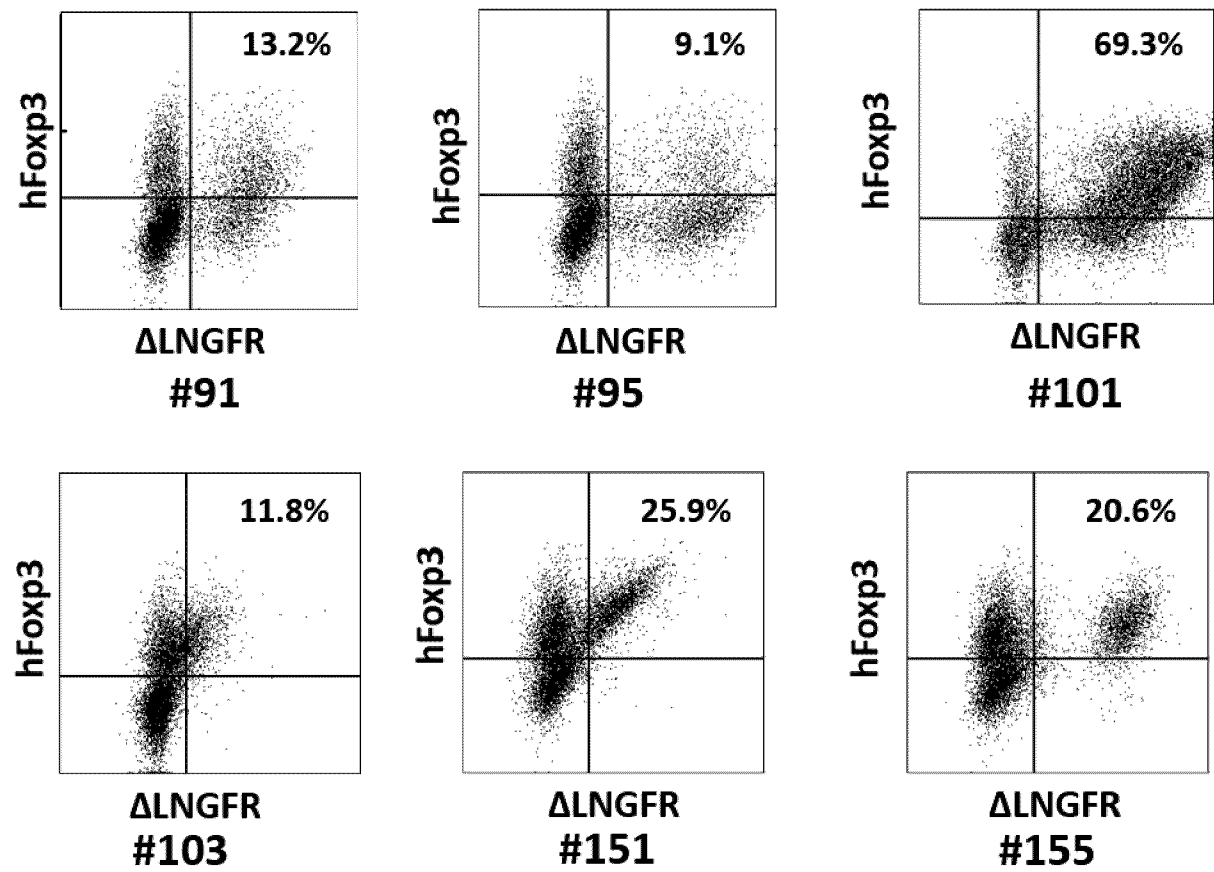


Figure 2

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/052731

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/85 C12N15/867
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 C07K

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, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SARAH E ALLAN ET AL: "Generation of Potent and Stable Human CD4+ T Regulatory Cells by Activation-independent Expression of FOXP3", MOLECULAR THERAPY, vol. 16, no. 1, 1 January 2008 (2008-01-01), pages 194-202, XP055133056, ISSN: 1525-0016, DOI: 10.1038/sj.mt.6300341 cited in the application page 195, left-hand column - page 199, right-hand column; figure 1a ----- -/--	1-18



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

25 February 2020

Date of mailing of the international search report

04/03/2020

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

Stoyanov, Borislav

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2020/052731

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M C GOLDING ET AL: "A bidirectional promoter architecture enhances lentiviral transgenesis in embryonic and extraembryonic stem cells", GENE THERAPY, vol. 18, no. 8, 10 March 2011 (2011-03-10), pages 817-826, XP055357962, ISSN: 0969-7128, DOI: 10.1038/gt.2011.26 figures 1, 4 -----	1-18
A	WO 2018/031762 A1 (UNIV DUKE [US]) 15 February 2018 (2018-02-15) claim 1; sequence 2712 -----	1-18

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2020/052731

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2018031762 A1	15-02-2018	EP 3497221 A1	19-06-2019
		US 2019194633 A1	27-06-2019
		WO 2018031762 A1	15-02-2018
