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(54) Titre : HETERODIMERES DANS L'IMMUNITE DE L'INTERLEUKINE 12B (P40) DE TYPE ANTIGENE LYMPHOCYTAIRE CD5 (CD5L)
(54) Title: LYMPHOCYTE ANTIGEN CD5-LIKE (CD5L)-INTERLEUKIN 12B (P40) HETERODIMERS IN IMMUNITY

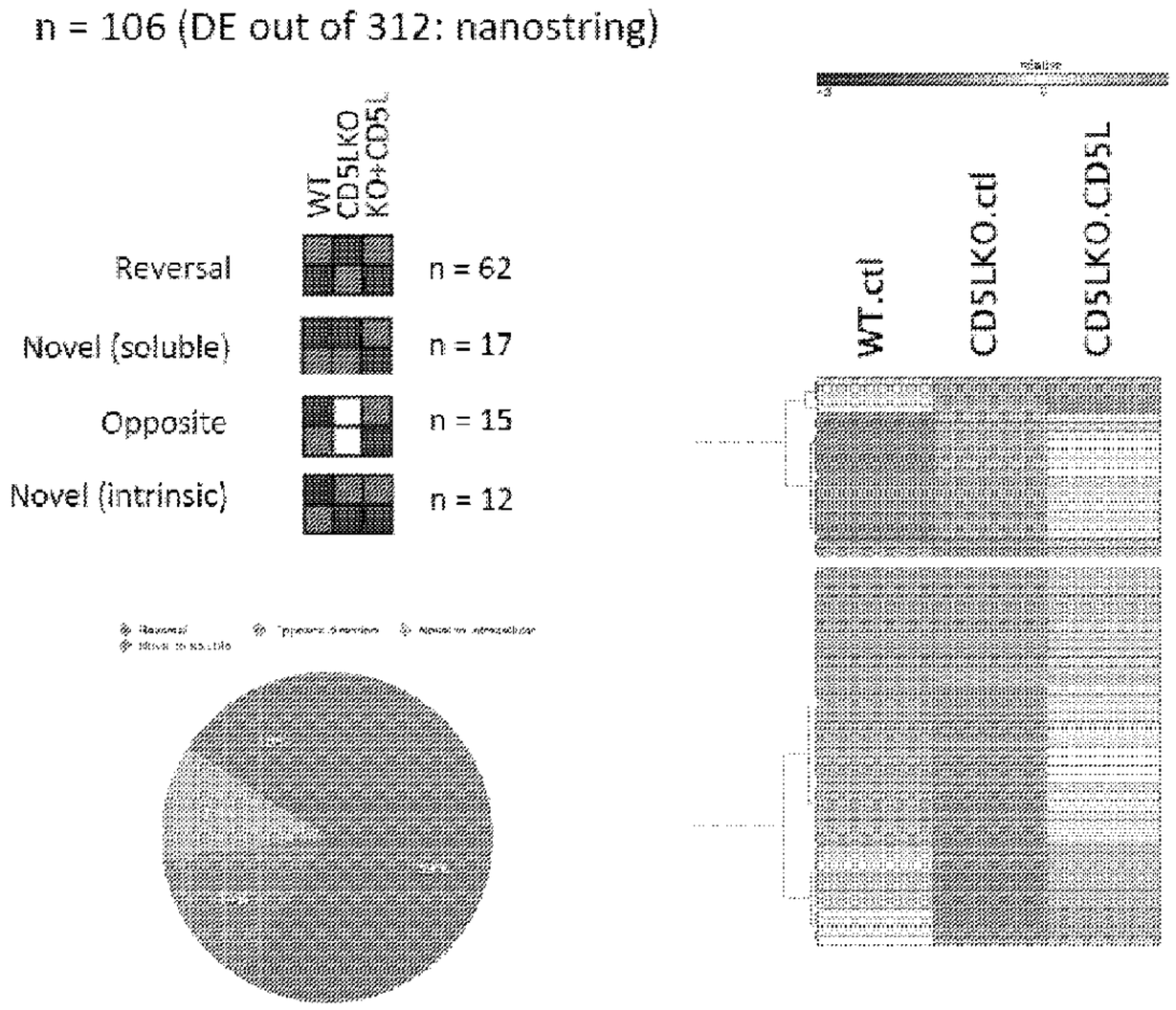

FIG. 1

(57) Abrégé/Abstract:
Described herein are methods for suppressing an immune response in a subject, e.g., a subject with an autoimmune disease, by administering to the subject a therapeutically effective amount of recombinant CD5L, CD5L homodimers and/or CD5L:p40 heterodimers, or nucleic acids encoding any of these. Also described are methods for enhancing an immune response in a subject, e.g., a subject with cancer, infection, or an immune deficiency, by administering to the subject a therapeutically effective amount of an antibody or antigen-binding fragment thereof that binds specifically to CD5L, D5L homodimers and/or CD5L:p40 heterodimers, and inhibits their binding to the IL-23 receptor, or inhibits formation of the CD5L homodimer and/or CD5L:p40 heterodimer, or inhibitory nucleic acids that target CD5L and/or p40.

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(54) Title: LYMPHOCYTE ANTIGEN CD5-LIKE (CD5L)-INTERLEUKIN 12B (P40) HETERODIMERS IN IMMUNITY

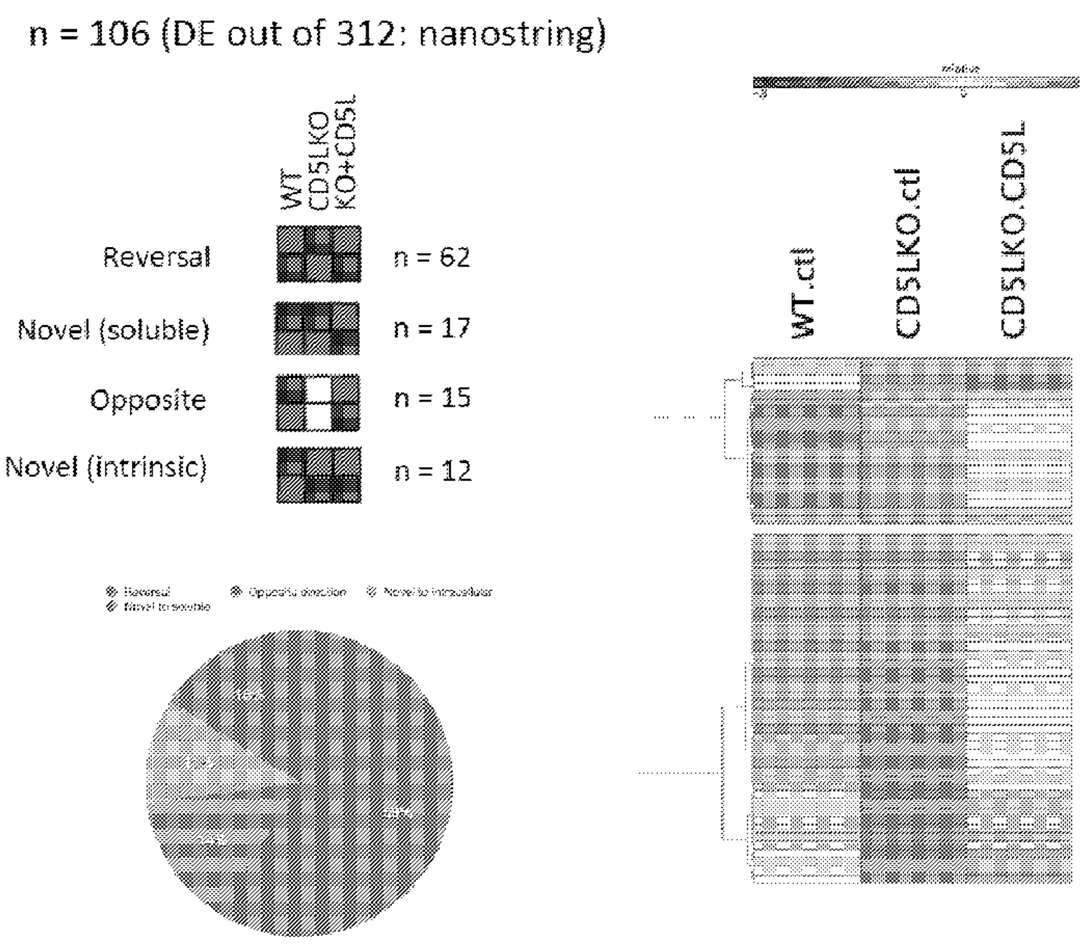

FIG. 1

(57) Abstract: Described herein are methods for suppressing an immune response in a subject, e.g., a subject with an autoimmune disease, by administering to the subject a therapeutically effective amount of recombinant CD5L, CD5L homodimers and/or CD5L:p40 heterodimers, or nucleic acids encoding any of these. Also described are methods for enhancing an immune response in a subject, e.g., a subject with cancer, infection, or an immune deficiency, by administering to the subject a therapeutically effective amount of an antibody or antigen-binding fragment thereof that binds specifically to CD5L, D5L homodimers and/or CD5L:p40 heterodimers, and inhibits their binding to the IL-23 receptor, or inhibits formation of the CD5L homodimer and/or CD5L:p40 heterodimer, or inhibitory nucleic acids that target CD5L and/or p40.

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Lymphocyte Antigen CD5-Like (CD5L)-Interleukin 12B (p40) Heterodimers in Immunity

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant Nos. P01 AI056299, P01 AI039671, P01 AI073748, and 5P01 AI045757 awarded by the National Institutes of Health. The Government has certain rights in the invention.

TECHNICAL FIELD

Described herein are methods for suppressing or enhancing an immune response in a subject.

BACKGROUND

The cytokine environment influences immune cell differentiation, function and plasticity. IL-23 has been identified as key player in inflammatory diseases, contributing largely to mucosal inflammation. It was discovered as a susceptibility gene in GWAS and is widely implicated in autoimmune diseases and cancer such as melanoma and colorectal carcinoma (Burkett et al., 2015; Cho and Feldman, 2015; Teng et al., 2015; Wang and Karin, 2015).

SUMMARY

The present invention is based, at least in part, on the discovery that CD5L and p40 form heterodimers *in vivo,* and that these heterodimers modulate the immune response. CD5L exists as a monomer, and is also able to form dimers; both forms may also serve as immunomodulators. Some embodiments comprise methods for modulating an immune response or suppressing an immune response (e.g., an inflammatory immune response) in a subject, the method comprising administering to the subject a therapeutically effective amount of recombinant soluble CD5L, a CD5L:CD5L homodimer, a CD5L:p40 heterodimer, or one or more nucleic acids encoding the same. In some embodiments, the subject has an autoimmune disease, e.g., Multiple Sclerosis

(MS), Irritable Bowel Disease (IBD), Crohn's disease, spondyloarthritides, Systemic Lupus Erythematosus (SLE), Vitiligo, rheumatoid arthritis, psoriasis, Sjögren's syndrome, or diabetes. In some embodiments, the subject has an inflammation-related cancer, e.g., colorectal cancer, carcinogen-induced skin papilloma, fibrosarcoma, or mammary carcinomas.

Some embodiments comprise methods of suppressing an immune response in a subject, the method comprising administering to the subject a therapeutically effective amount of one or more of: a recombinant soluble CD5L and/or a nucleic acid encoding CD5L; a recombinant soluble CD5L:CD5L homodimer and/or a nucleic acid encoding a CD5L homodimer; and a recombinant soluble CD5L:p40 heterodimer and/or nucleic acids encoding CD5L and p40. In some embodiments the subject has an autoimmune disease, such as Multiple Sclerosis (MS), Irritable Bowel Disease (IBD), Crohn's disease, spondyloarthritides, Systemic Lupus Erythematosus (SLE), Vitiligo, rheumatoid arthritis, psoriasis, Sjögren's syndrome, or diabetes. In some embodiments, subject has an inflammation-related cancer, such as colorectal cancer, carcinogen-induced skin papilloma, fibrosarcoma, or mammary carcinomas.

Some embodiments comprise administering the CD4L:p40 heterodimer. Some embodiments comprise administering the CD5L:CD5L homodimer.

Some embodiments relate to methods of enhancing an immune response in a subject, the method comprising administering to the subject a therapeutically effective amount of an agent that: (a) inhibits CD5L, a CD5L:CD5L homodimer, and/or a CD5L:p40 heterodimer from binding to an IL-23 receptor; and/or (b) inhibits formation of the CD5L:CD5L homodimer and/or the CD5L:p40 heterodimer. In some embodiments, the agent comprises an antibody, or an antigen binding fragment thereof, that binds to one or more of the CD5L, the CD5L homodimer, or the CD5L:p40 heterodimer. In some embodiments, the agent comprises inhibitory nucleic acids that target the CD5L and/or the p40.

In some embodiments, the subject has cancer that is not inflammation related. Some embodiments comprise administering an anti-cancer immunotherapy to the subject,

such as checkpoint inhibitors, PD-1/PDL-1, anti-cancer vaccines, adoptive T cell therapy, and/or combination of two or more thereof.

In embodiments that comprise administering inhibitory nucleic acids, the nucleic acids can include small interfering RNAs (e.g., shRNA), antisense oligonucleutides (*e.g.* antisense RNAs), and/or CRISPR-Cas.

In some embodiments, the subject has an immune deficiency, e.g., a primary or secondary immune deficiency. In some embodiments, the subject has an infection with a pathogen, e.g., viral, bacterial, or fungal pathogen.

Some embodiments comprised methods of modulating $CD8^+$ T cell exhaustion in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent that: (a) inhibits CD5L, a CD5L:CD5L homodimer, and/or a CD5L:p40 heterodimer from binding to an IL-23 receptor; and/or (b) inhibits formation of the CD5L:CD5L homodimer and/or the CD5L:p40 heterodimer. In some embodiments, said administering reduces $CD8^+$ T cell exhaustion. In some embodiments the subject has cancer, such as a non-inflammatory cancer.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Figure 1. Soluble CD5L can regulate T cell function, largely reversing CD5L deficiency-induced gene expression pattern in T cells. WT or CD5L-/- naïve T cells were sorted and activated under Th0 condition and treated with either PBS or soluble CD5L (50nM). RNA was extracted at 96h and analyzed using nanostring platform using Th17 codesets of 312 genes (only those showing a difference between any of the tested conditions were included in further analysis).

Figure 2. Soluble CD5L (CD5Lm) and CD5L/p40 premix can have unique functions on T cells. Similar to Figure 1. Th0 cells were incubated with soluble CD5L, CD5L/p40 mixture (premixed for 4 hours), p40 or control PBS.

Figures 3A-C. The impact of soluble CD5L or CD5L/p40 can be dependent on IL-23R expression Similar to Figure 1. CD5L-/- or CD5L-/- IL-23R-/- Th0 cells were incubated with soluble CD5L, CD5L/p40 mixture (premixed for 4 hours), p40 or control PBS.

Figures 4A-G. CD5L regulates ILC function at steady state and during inflammation. A-D. Naïve 6-month old mice that are either wildtype or $CD5L^{-/-}$ were sacrificed and cells from tissues as indicated are analyzed by flow cytometry or quantitative real time PCR. (A) IL-23R.$GFP^{+/-}$ reporter mice that are otherwise wildtype or $CD5L^{-/-}$ were used and cells were stained directly ex vivo; (B-C) Cells were incubated with IL-7 or IL-7/CD5L overnight and restimulated with PMA/ionomycine in the presence of brefaldin A for four hours. Cells were subsequently stained and analyzed by flow cytometry; (D) Cells were analyzed directly ex vivo by flow cytometry or sorted, RNA-extracted and analyzed by real time qPCR; E-G. 6-8 wk old WT or $CD5L^{-/-}$ IL-$17^{Cre}Rosa26^{Td\text{-}tomato}$ mice were treated with 2.5% DSS in drinking water for 6 days followed by 5 days of regular water. Mice were then sacrificed and cells isolated from respective tissues for PMA/ionomycine restimulation and flow cytometry analysis.

Figure 5. CD5L and CD5L:p40 regulate $CD11c^{+}$ DC function. $CD11c^{+}$ cells were enriched and sorted from spleen of WT, CD36-/- and IL-23R-/- naïve mice. CD11c+ cells were stimulated with 100ng/ml LPS in the presence of either control, sCD5L, p40 or CD5L:p40 at 5uM. Cells were harvested at 24 hours.

Figures 6A-D. $CD5L^{-/-}$ mice have more severe colitis in response to DSS-induced injury. 6-8 wk old WT or $CD5L^{-/-}$ mice were treated with 2.5% DSS in drinking water for 7 days followed by 7 days of regular water. Weight (A), colitis score (B) and colon length (C) and representative histology (D) were shown.

Figures 7A-D. Recombinant CD5L can bind to Th1 and Th17p (pathogenic Th17) cells and alleviate diseases severity of EAE and DSS induced colitis. Recombinant CD5L was generated with a His tag. A) Th0, Th1 (IL-12) and Th17p (IL-1b, IL-6, IL-23) are differentiated from naïve CD4 T cells *in vitro* for 4 days and cells were harvested for staining with recombinant CD5L followed by anti-His APC antibodies and flow cytometry analysis. B) Wildtype (WT) mice were immunized with MOG/CFA followed by PT injection to induce EAE. Mice at peak of disease (score = 3) were injected with either PBS (solid circles) or recombinant CD5L (empty circles, CD5Lm) intraperitoneally daily for five consecutive days and mice were followed for disease progression. C) WT mice were induced with colitis with 2.5%DSS in drinking water for a consecutive of 6 days followed by normal water for 8 days. Mice were given either control (PBS) or recombinant CD5L (CD5Lm) intraperitoneally on day 4, 6 and 8. Colon length and colitis score are recorded on day 14.

Figures 8A-B. (A) Recombinant CD5L and CD5L:p40 (genetically linked) were custom ordered from Biolegend. CD5L monomer formed a homodimer and CD5L:CD5L homodimer, which was further purified and was used in subsequent experiments to test its function separately; (B) Serum was collected kinetically from WT and *Cd5l*$^{-/-}$ mice with DSS-induced colitis (2% DSS in drinking water for 6 days followed by 7 days of normal water) and the level of CD5L:p40 was measured using an ELISA developed in house using anti-p40 antibody for capturing, biotinylated anti-CD5L antibody for detection and recombinant CD5L:p40 as a positive control.

Figures 9A-B. Figure 9A sets forth results of a screening assay showing that TLR ligands can induce secretion of CD5L:p40. Figure 9B sets forth flow cytometry experiments showing that IL-27 induces expression of CD5L.

Figures 10A-D. Figure 10A sets forth results of FACS experiments showing that CD5L homodimers and CD5L:p40 heterodimers inhibit IL-17 expression in pathogenic

Th17 cells; Figure 10(B) shows results of an serum ELISA measurements showing that both forms of CD5L inhibit IL-17 expression; Figures 10C and D show cell signatures for pathogenic Th17 cells treated with CD5L homodimers and CD5L:p40 heterodimers, respectively.

Figures 11A-B. Figure 11A shows inhibited IL-27 expression in pathogenic Th17 cells treated with CD5L homodimers and CD5L:p40 heterodimers, as measured by ELISA and qPCR; Figure 11B shows that IFNg expression in Th1 cells is inhibited by CD5L:CD5L homodimer and CD5L:p40 heterodimer, as measured by flow cytometry analysis.

Figures 12A-B. Figures 12A and B show heat maps and GSEA analysis for Th17 cells and Th1 cells, respectively, following treatment with CD5L homodimers and CD5L:p40 heterodimers.

Figures 13A-B. Figure 13A compares EAE disease severity measurements in wildtype mice and CD5L knockout mice; Figure 13B compares CD5L expression levels in Th17 and macrophage cells in the spleen and CNS.

Figures 14A-B. Figure 14A shows a construct used to generate CD5L conditional knockout mice; Figure 14B shows that mice CD5L deletion mice were produced in myeloid lineage cells, T cells, and IL-17 producing cells.

Figure 15A-B. Figure 15A sets forth a plot demonstrating tumor growth in $CD5L^{flox/flox}Lymz^{Cre+}$ mice injected with colon carcinoma; Figure 15B sets forth pictures showing tumor size in $CD5L^{flox/flox}$ mice and CD5L knockout mice 19 days after tumor injection.

Figure 16. Figure 16 depicts the lipodome of wildtype and *cd5l*$^{-/-}$ Th17 cells differentiated under pathogenic and non-pathogenic conditions.

Figure 17. Figure 17 is a plot showing that metabolic transcriptome expression covaries with Th17 cell pathogenicity.

Figures 18A-D. Figure 18 sets forth plots showing suppression of tumor progression in $CD5L^{-/-}$ mice injected with MC38 (Figure 19A) and MC38-OVA (Figure 19B) colon carcinoma; Figure 18C and D set forth flow cytometry diagrams assessing

tumor infiltrating lymphocytes and cytokines, respectively, in $CD5L^{-/-}$ mice and control mice.

Figures 19A-B. Figure 19 sets forth graphs showing CD5L:CD5L homodimer expression (Figure 19A) and CD5L:p40 heterodimer expression (Figure 19B) in serum during tumor progression, as measured using ELISA assays.

Figure 20. Figure 20 sets forth a heat map showing differentially expressed genes in CD5L:CD5L and CD5L:p40 experiments as compared to the control (differentially expressed genes are defined by $p<0.5$ as compared to control).

Figures 21A-B. Figures 21A-B set forth data showing the impact of CD5L:p40 and CD5L:CD5L on Tregs *in vivo* in DSS-induced colitis; Figure 21A shows frequency of Foxp3+ CD4 T cells in cells from mesenteric lymph node (mLN), peyer's patches (pp), lamina propria of colon (LP), and intraepithelial lymphocytes (IEL); Figure 21B sets forth data showing that CD5L:p40 decreased ILC3 in lamina propria cells.

Figures 22A-B. Figure 22A sets forth data showing serum concentrations of CD5L:p40 and CD5L:CD5L in mice immunized with CD5L:p40 and CD5L:CD5L, respectively; Figure 22B sets forth data showing pools of antibodies specific to either CD5L:p40 or CD5L:CD5L, and which were obtained from mice immunized with CD5L:p40 and CD5L:CD5L, respectively.

Figures 23A-D. Figure 23 demonstrates homology between mice and human protein sequences for CD5L (Figure 23A), p19 (Figure 23B), p40 (Figure 23C), and p35 (Figure 23D).

DETAILED DESCRIPTION

Interleukin 23 (IL-23) is formed of a heterodimer by p19 and p40. p40, also known as interleukin 12B, can form heterodimers with two other cytokines: p35 to make IL-12 and potentially CD5 Antigen Like protein (CD5L) (also known as apoptosis inhibitor of macrophage (AIM), SP-a, and Api6) to make CD5L:p40. It has not previously been demonstrated that the CD5L:p40 dimer has any function. Th17-cell intrinsic CD5L can regulate Th17 cell pathogenicity and regulate IL-23R expression (see WO2015130968). CD5L is a secreted protein and it may form a heterodimer with p40

(Abdi et al., 2014). Applicants tested the hypothesis that soluble CD5L, as a monomer, homodimer, or heterodimer with p40, can function as a cytokine regulating T cell function. Surprisingly, Applicants found that soluble CD5L, CD5L:CD5L homodimer, and CD5L:p40 heterodimer share a distinct ability to regulate T cell function. Not to be bound by theory, CD5L, either as a monomer, homodimer, or a heterodimer, is suspected to interfere with the pathogenic and non-pathogenic program of Th17 cells. Such findings have therapeutic implications with respect to neuroinflammation, autoimmune disorders, inflammatory cancers, and non-inflammatory cancers and disorders, *inter alia*.

CD5L function is largely dependent not on CD36, the known receptor for CD5L, but IL-23R expression on T cells. Further, CD5L:p40 appears to be less dependent on IL-23R and may require a different receptor for signaling. Moreover, CD5L can regulate not only T cells, but also other IL-23R expressing cells such as innate lymphoid cells and dendritic cells. CD5L plays a critical role in protecting host from acute inflammation and potentially tumor progression.

CD5L Proteins and CD5L:p40 Heterodimers

In some embodiments, the methods described herein can include the administration of soluble CD5L, CD5L:CD5L homodimers, or CD5L:p40 heterodimers. The homodimers include CD5L complexed to another CD5L, preferably complexed together in a homodimeric form. The heterodimers include p40 protein and CD5L protein, preferably complexed together in a heterodimeric form. The protein sequences will preferably be chosen based on the species of the recipient; thus, for example, human p40 and/or human CD5L can be used to treat a human subject. The sequences of human p40 and CD5L are as follows:

Human p40 (interleukin-12 subunit beta) precursor

```
  1 mchqqlvisw fslvflaspl vaiwelkkdv yvveldwypd apgemvvltc dtpeedgitw
 61 tldqssevlg sgktltiqvk efgdagqytc hkggevlshs llllhkkedg iwstdilkdq
121 kepknktflr ceaknysgrf tcwwlttist dltfsvkssr gssdpqgvtc gaatlsaerv
181 rgdnkeyeys vecqedsacp aaeeslpiev mvdavhklky enytssffir diikpdppkn
241 lqlkplknsr qvevsweypd twstphsyfs ltfcvqvqgk skrekkdrvf tdktsatvic
301 rknasisvra qdryysssws ewasvpcs  (SEQ ID NO:1)
```

In some embodiments, amino acids 23-328 of SEQ ID NO:1 (leaving off the signal sequence) are used. An exemplary mRNA sequence encoding p40 is accessible in GenBank at No. NM_002187.2.

CD5 molecule-like (CD5L)

```
  1 mallfslila ictrpgflas psgvrlvggl hrcegrveve qkgqwgtvcd dgwdikdvav
 61 lcrelgcgaa sgtpsgilye ppaekeqkvl iqsvsctgte dtlaqceqee vydcshdeda
121 gascenpess fspvpegvrl adgpghckgr vevkhqnqwy tvcqtgwslr aakvvcrqlg
181 cgravltqkr cnkhaygrkp iwlsqmscsg reatlqdcps gpwgkntcnh dedtwveced
241 pfdlrlvggd nlcsgrlevl hkgvwgsvcd dnwgekedqv vckqlgcgks lspsfrdrkc
301 ygpgvgriwl dnvrcsgeeq sleqcqhrfw gfhdcthqed vavicsg  (SEQ ID NO:2)
```

In some embodiments, amino acids 20-347 of SEQ ID NO:2 (leaving off the signal sequence) are used. An exemplary mRNA sequence encoding CD5L is accessible in GenBank at No. NM_005894.2.

Methods for making recombinant proteins are well known in the art, including *in vitro* translation and expression in a suitable host cell from nucleic acid encoding the variant protein. A number of methods are known in the art for producing proteins. For example, the proteins can be produced in and purified from yeast, *E. coli*, insect cell lines, plants, transgenic animals, or cultured mammalian cells; see, e.g., Palomares et al., "Production of Recombinant Proteins: Challenges and Solutions," Methods Mol Biol. 2004;267:15-52. In some embodiments, recombinant p40 and CD5L proteins are obtained and mixed in roughly equimolar amounts of p40 with CD5L and incubated, e.g., at 37°C. Immunoprecipitation and purification can be used to confirm formation of heterodimers, as can size exclusion chromatography or other purification methods, to obtain a substantially pure population of heterodimers. In some embodiments, p40 and CD5L are simply mixed together under conditions sufficient for heterodimerization, and optionally purified to obtain a substantially pure composition of heterodimers; alternatively, the heterodimers can be cross-linked and then purified. In some embodiments, an agent such as TLR9 can be used to increase heterodimer formation, *e.g.*, *in vitro* or *in vivo*.

In some embodiments, the methods include administering nucleic acids encoding a p40 and/or CD5L polypeptides or active fragment thereof. In some embodiments, the nucleic acids are incorporated into a gene construct to be used as a part of a gene therapy or cell therapy protocol. In some embodiments, the methods include targeted expression vectors for transfection and expression of polynucleotides that encode p40 and/or CD5L polypeptides as described herein, in particular cell types, especially in T cells.

Expression constructs of such components can be administered in any effective carrier, e.g., any formulation or composition capable of effectively delivering the component gene to cells *in vivo*. Approaches include insertion of the gene in viral vectors, including recombinant retroviruses, adenovirus, adeno-associated virus, lentivirus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors transfect cells directly; plasmid DNA can be delivered naked or with the help of, for example, cationic liposomes (lipofectamine) or derivatized conjugates (e.g., antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $CaPO_4$ precipitation carried out in vivo.

A preferred approach for *in vivo* introduction of nucleic acid into a cell is by use of a viral vector containing nucleic acid, e.g., a cDNA. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed efficiently in cells that have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors can be used as a recombinant gene delivery system for the transfer of exogenous genes in vivo, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are characterized for use in gene transfer for gene therapy purposes (for a review see Miller, Blood 76:271 (1990)). A replication defective

retrovirus can be packaged into virions, which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells in vitro or in vivo with such viruses can be found in Ausubel, et al., eds., Current Protocols in Molecular Biology, Greene Publishing Associates, (1989), Sections 9.10-9.14, and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ΨCrip, ΨCre, Ψ2 and ΨAm. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see for example Eglitis, et al. (1985) Science 230:1395-1398; Danos and Mulligan (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464; Wilson et al. (1988) Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano et al. (1990) Proc. Natl. Acad. Sci. USA 87:6141-6145; Huber et al. (1991) Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry et al. (1991) Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury et al. (1991) Science 254:1802-1805; van Beusechem et al. (1992) Proc. Natl. Acad. Sci. USA 89:7640-7644; Kay et al. (1992) Human Gene Therapy 3:641-647; Dai et al. (1992) Proc. Natl. Acad. Sci. USA 89:10892-10895; Hwu et al. (1993) J. Immunol. 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

Another viral gene delivery system useful in the present methods utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated, such that it encodes and expresses a gene product of interest but is inactivated in terms of its ability to replicate in a normal lytic viral life cycle. See, for example, Berkner et al., BioTechniques 6:616 (1988); Rosenfeld et al., Science 252:431-434 (1991); and Rosenfeld et al., Cell 68:143-155 (1992). Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, or Ad7 etc.) are known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances, in that they are not capable of infecting non-dividing cells and can be used to infect a wide variety of cell types, including epithelial

cells (Rosenfeld et al., (1992) supra). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis *in situ*, where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., supra; Haj-Ahmand and Graham, J. Virol. 57:267 (1986)).

Yet another viral vector system useful for delivery of nucleic acids is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review see Muzyczka et al., Curr. Topics in Micro. and Immunol.158:97-129 (1992)). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al., Am. J. Respir. Cell. Mol. Biol. 7:349-356 (1992); Samulski et al., J. Virol. 63:3822-3828 (1989); and McLaughlin et al., J. Virol. 62:1963-1973 (1989)). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., Mol. Cell. Biol. 5:3251-3260 (1985) can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., Proc. Natl. Acad. Sci. USA 81:6466-6470 (1984); Tratschin et al., Mol. Cell. Biol. 4:2072-2081 (1985); Wondisford et al., Mol. Endocrinol. 2:32-39 (1988); Tratschin et al., J. Virol. 51:611-619 (1984); and Flotte et al., J. Biol. Chem. 268:3781-3790 (1993)).

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a nucleic acid compound described herein (e.g., nucleic acids encoding p40 and/or CD5L polypeptides) in the tissue of a subject. Typically non-viral methods of gene transfer rely on the normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In

some embodiments, non-viral gene delivery systems can rely on endocytic pathways for the uptake of the subject gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes. Other embodiments include plasmid injection systems such as are described in Meuli et al., J. Invest. Dermatol. 116(1):131-135 (2001); Cohen et al., Gene Ther. 7(22):1896-905 (2000); or Tam et al., Gene Ther. 7(21):1867-74 (2000).

In some embodiments, genes encoding p40 and/or CD5L polypeptides are entrapped in liposomes bearing positive charges on their surface (e.g., lipofectins), which can be tagged with antibodies against cell surface antigens of the target tissue (*see, e.g.*, Mizuno et al., No Shinkei Geka 20:547-551 (1992); PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075)).

In clinical settings, the gene delivery systems for the therapeutic gene can be introduced into a subject by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g., by intravenous injection, and specific transduction of the protein in the target cells will occur predominantly from specificity of transfection, provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the receptor gene, or a combination thereof. In other embodiments, initial delivery of the recombinant gene is more limited, with introduction into the subject being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g., Chen et al., PNAS USA 91: 3054-3057 (1994)).

The pharmaceutical preparation of the gene therapy construct can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is embedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can comprise one or more cells, which produce the gene delivery system.

Pharmaceutical Compositions

The methods described herein include the manufacture and use of pharmaceutical compositions, which include an agent described herein as active ingredient(s). Also included are the pharmaceutical compositions themselves.

Pharmaceutical compositions typically include a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" includes saline, solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, and combinations of two or more thereof, compatible with pharmaceutical administration. Supplementary active compounds can also be incorporated into the compositions.

Pharmaceutical compositions are typically formulated to be compatible with the intended route of administration. Examples of routes of administration that are especially useful in the present methods include parenteral (e.g., intravenous), intrathecal, oral, and nasal or intranasal (e.g., by administration as drops or inhalation) administration. In some embodiments, such as for compounds that don't cross the blood brain barrier, delivery directly into the CNS or CSF can be used, e.g., using implanted intrathecal pumps (see, e,g., Borrini et al., Archives of Physical Medicine and Rehabilitation 2014;95:1032-8; Penn et al., N. Eng. J. Med. 320:1517-21 (1989); and Rezai et al., Pain Physician 2013; 16:415-417) or nanoparticles, e.g., gold nanoparticles (e.g., glucose-coated gold nanoparticles, see, e.g., Gromnicova et al. (2013) PLoS ONE 8(12): e81043). Methods of formulating and delivering suitable pharmaceutical compositions are known in the art, see, e.g., the books in the series Drugs and the Pharmaceutical Sciences: a Series of Textbooks and Monographs (Dekker, NY); and Allen et al., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Lippincott Williams & Wilkins; 8th edition (2004).

Pharmaceutical compositions suitable for injectable use can include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all

cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent that delays absorption, for example, aluminum monostearate and gelatin.

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

For oral administration, the compositions can be formulated with an inert diluent or an edible carrier. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules, e.g., gelatin capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills,

capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds can be delivered in the form of an aerosol spray from a pressured container or dispenser that contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer. Such methods include those described in U.S. Patent No. 6,468,798.

Therapeutic compounds that are or include nucleic acids can be administered by any method suitable for administration of nucleic acid agents, such as a DNA vaccine. These methods include gene guns, bio injectors, and skin patches as well as needle-free methods such as the micro-particle DNA vaccine technology disclosed in U.S. Patent No. 6,194,389, and the mammalian transdermal needle-free vaccination with powder-form vaccine as disclosed in U.S. Patent No. 6,168,587. Additionally, intranasal delivery is possible, as described in, *inter alia,* Hamajima et al., Clin. Immunol. Immunopathol., 88(2), 205-10 (1998).

Liposomes (e.g., as described in U.S. Patent No. 6,472,375) and microencapsulation can also be used to deliver a compound described herein. Biodegradable microparticle delivery systems can also be used (e.g., as described in U.S. Patent No. 6,471,996).

In one embodiment, the therapeutic compounds are prepared with carriers that will protect the therapeutic compounds against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques, or obtained commercially, e.g., from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to selected cells with monoclonal antibodies to cellular

antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

The pharmaceutical compositions can be included in a container, pack, or dispenser, e.g., single-dose dispenser together with instructions for administration. The container, pack, or dispenser can also be included as part of a kit that can include, for example, sufficient single-dose dispensers for one day, one week, or one month of treatment.

Dosage

Dosage, toxicity and therapeutic efficacy of the compounds can be determined, e.g., by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in

humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

An "effective amount" is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This amount can be the same or different from a prophylactically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages. A therapeutically effective amount of a composition depends on the composition selected. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the compositions described herein can include a single treatment or a series of treatments.

Methods of Treatment – Decreasing Immune Responses

Without being bound by theory, CD5L monomers, homodimers and heterodimers with p40 are believed to regulate T cells and alter immune function, and can promote suppression of pathogenic Th17 and Th1 phenotypes. Agonists of CD5L, CD5L:CD5L homodimers, and/or CD5L:p40 heterodimers (e.g., CD5L:p40 heterodimer polypeptides), can be administered to treat conditions associated with overactive inflammation or immunity, e.g., autoimmune diseases, e.g., in which pathogenic T cells are present at increased levels and/or have increased activity, such as multiple sclerosis (MS). Autoimmune conditions that may benefit from treatment using the compositions and methods described herein include, but are not limited to, for example, MS, Addison's Disease, alopecia, ankylosing spondylitis, antiphospholipid syndrome, autoimmune hemolytic anemia, autoimmune hepatitis, autoimmune oophoritis, Bechet's disease, bullous pemphigoid, celiac disease, chronic fatigue immune dysfunction syndrome

(CFIDS), chronic inflammatory demyelinating polyneuropathy, Churg-Strauss syndrome, cicatricial pemphigoid, cold agglutinin disease, CREST Syndrome, Crohn's disease, diabetes (e.g., type I), dysautonomia, endometriosis, eosinophilia-myalgia syndrome, essential mixed cryoglobulinemia, fibromyalgia, syndrome/fibromyositis, Graves' disease, Guillain Barré syndrome, Hashimoto's thyroiditis, idiopathic pulmonary fibrosis, idiopathic thrombocytopenia purpura (ITP), inflammatory bowel disease (IBD), lichen planus, lupus, Ménière's disease, mixed connective tissue disease (MCTD), multiple sclerosis, myasthenia gravis, pemphigus, pernicious anemia, polyarteritis nodosa, polychondritis, polymyalgia rheumatica, polymyositis and dermatomyositis, primary agammaglobulinemia, primary biliary cirrhosis, psoriasis, Raynaud's phenomenon, Reiter's syndrome, rheumatic fever, rheumatoid arthritis, sarcoidosis, scleroderma, Sjögren's syndrome, spondyloarthropathy (spondyloarthritides), stiff-man syndrome, Takayasu arteritis, temporal arteritis/giant cell arteritis, autoimmune thyroid disease, ulcerative colitis, autoimmune uveitis, autoimmune vasculitis, vitiligo, and Wegener's granulomatosis. In some embodiments, the autoimmune disease is MS, IBD, Crohn's disease, spondyloarthritides, Systemic Lupus Erythematosus, Vitiligo, rheumatoid arthritis, psoriasis, Sjögren's syndrome, or diabetes, e.g., Type I diabetes, all of which have been linked to Th17 cell dysfunction (see, e.g., Korn et al., Annu Rev Immunol. 2009;27:485–517Dong, Cell Research (2014) 24:901–903; Zambrano-Zaragoza et al., Int J Inflam. 2014; 2014: 651503; Waite and Skokos, International Journal of Inflammation; Volume 2012 (2012), Article ID 819467, 10 pages, dx.doi.org/10.1155/2012/819467; Han et al., Frontiers of Medicine 9(1):10-19 (2015).

Some embodiments include treatment of autoimmune diseases, such as multiple sclerosis (MS) or IBD, using CD5L monomers, CD5L homodimers and/or CD5L:p40 heterodimers. In some embodiments, once it has been determined that a person has an autoimmune disease, e.g., MS or IBD, then a treatment comprising administration of a therapeutically effective amount of CD5L monomers, CD5L homodimers and/or CD5L:p40 heterodimers can be administered.

Generally, the methods include administering a therapeutically effective amount of CD5L monomers, CD5L homodimers and/or CD5L:p40 heterodimers as described

herein, to a subject who is in need of, or who has been determined to be in need of, such treatment. As used in this context, to "treat" means to ameliorate or reduce the severity of at least one symptom of a disease or condition. For instance, a treatment can result in a reduction in one or more symptoms of an autoimmune disease, e.g., for MS, e.g., depression and fatigue, bladder dysfunction, spasticity, pain, ataxia, and intention tremor. A therapeutically effective amount can be an amount sufficient to prevent the onset of an acute episode or to shorten the duration of an acute episode, or to decrease the severity of one or more symptoms, e.g., heat sensitivity, internuclear ophthalmoplegia, optic neuritis, and Lhermitte symptom. In some embodiments, a therapeutically effective amount is an amount sufficient to prevent the appearance of, delay or prevent the growth (i.e., increase in size) of, or promote the healing of a demyelinated lesion in one or more of the brain, optic nerves, and spinal cord of the subject, e.g., as demonstrated on MRI.

Alternatively or in addition, the methods can be used to treat other conditions associated with hyperimmune responses, e.g., cancers associated with inflammation such as colorectal cancers. In certain inflammation-related cancers the IL-23 pathway has been shown to promote tumorigenesis (e.g., in colorectal cancer, carcinogen-induced skin papilloma, fibrosarcomas, mammary carcinomas and certain cancer metastasis; these studies have suggested that IL-23 and Thl7 cells play a role in some cancers, such as, by way of non-limiting example, colorectal cancers. See e.g., Ye J, Livergood RS, Peng G. "The role and regulation of human Thl7 ceils in tumor immunity." Am J Pathol 2013 Jan;182(l): 10-20. doi: 10.1016/j .ajpath.2012.08.041. Epub 2012 Nov 14). In such cancer types, CD5L and CD5L:p40 and agents that promote their function can have anti-tumor effects. (Teng et al., 2015 Nat Med 21; Wang and Karin, Clin Exp Rheumatol 2015; 33). Thus CD5L monomers, CD5L homodimers and/or CD5L:p40 heterodimers, or nucleic acids encoding CD5L monomers, CD5L homodimers and/or CD5L:p40 heterodimers, can be used to treat or reduce risk of developing these cancers.

Standard Treatments for Autoimmune Disease

In some embodiments, a treatment, e.g., comprising CD5L, CD5L:CD5L homodimers, or CD5L:p40 heterodimers, is administered in combination with a standard

treatment for an autoimmune disease. For example, in the case of MS, treatment can include administration of corticosteroid therapy, interferon beta-1b, Glatiramer acetate, mitoxantrone, Fingolimod, teriflunomide, dimethyl fumarate, natalizumab, cannabis, or a combination thereof. In some embodiments, the treatment described herein is administered in combination with a treatment for one or more symptoms of MS, e.g., depression and/or fatigue, bladder dysfunction, spasticity, pain, ataxia, and intention tremor. Such treatments can include pharmacological agents, exercise, and/or appropriate orthotics. Additional information on the diagnosis and treatment of MS can be found at the National MS Society website, on the world wide web at nationalmssociety.org.

Methods of Treatment – Enhancing Immune Responses

As shown herein and noted above, CD5L, CD5L:CD5L, and/or CD5L:p40 can regulate T cells and alter immune function. Methods that decrease the levels or activity of this CD5L, the CD5L homodimer, and/or the CD5L:p40 heterodimer can also be used to increase immune responses, e.g., to treat: subjects who have cancers that would benefit from immunotherapy (e.g., cancers that are not inflammation related); subjects who have a primary or secondary immune deficiency; or subjects who have an infection with a pathogen, e.g., viral, bacterial, or fungal pathogen.

Some embodiments comprised methods of modulating $CD8^+$ T cell exhaustion, *e.g.*, by administering a therapeutically effective amount of an agent that: (a) inhibits CD5L, a CD5L:CD5L homodimer, and/or a CD5L:p40 heterodimer from binding to an IL-23 receptor; and/or (b) inhibits formation of the CD5L:CD5L homodimer and/or the CD5L:p40 heterodimer. Some embodiments comprise reducing $CD8^+$ T cell exhaustion or dysfunction. Some embodiments comprise increasing $CD8^+$ T cell activity.

In some embodiments, the methods include administering an agent that specifically inhibits binding of the CD5L monomer, CD5L homodimer, and/or CD5L:p40 heterodimer to a cognate receptor (*e.g.*, the IL-23 receptor or the IL-12 receptor, beta 1 subunit), or that specifically inhibits formation of the CD5L homodimer or CD5L:p40 heterodimer. In some embodiments, the agent is an antibody, or an antigen binding fragment thereof, that binds to and inhibits the activity of the CD5L monomer, CD5L

homodimer, and/or CD5L:p40 heterodimer. In some embodiments, the agent is an antagonist of CD5L, CD5L:CD5L homodimer, or CD5L:p40 heterodimer. In some embodiments, the methods include inhibiting expression of CD5L and/or p40, for example using CRISPR or by administering inhibitory nucleic acids that inhibit expression of CD5L and/or p40.

As used in this context, to "treat" means to ameliorate or reduce the severity of at least one clinical parameter of the cancer. In some embodiments, the parameter is tumor size, tumor growth rate, recurrence, or metastasis, and an improvement would be a reduction in tumor size or no change in a normally fast growing tumor; a reduction or cessation of tumor growth; a reduction in, delayed, or no recurrence, or a reduction in, delayed, or no metastasis. Administration of a therapeutically effective amount of a compound described herein for the treatment of a cancer would result in one or more of a reduction in tumor size or no change in a normally fast growing tumor; a reduction or cessation of tumor growth; or a reduction in, delayed, or no metastasis. In some embodiments, e.g., a treatment designed to prevent recurrence of cancer, the treatment would be given after a localized tumor has been removed, e.g., surgically, or treated with radiation therapy or with targeted therapy with or without other therapies such as standard chemotherapy. Without wishing to be bound by theory, such a treatment may work by keeping micrometastases dormant, e.g., by preventing them from being released from dormancy.

As used herein, the term "hyperproliferative" refer to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. Hyperproliferative disease states may be categorized as pathologic, i.e., characterizing or constituting a disease state, or may be categorized as non-pathologic, i.e., a deviation from normal but not associated with a disease state. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. A "tumor" is an abnormal growth of hyperproliferative cells. "Cancer" refers to pathologic disease states, e.g., characterized by malignant tumor growth. The methods described herein can be used to treat cancer,

e.g., solid tumors of epithelial origin, e.g., as defined by the ICD-O (International Classification of Diseases – Oncology) code (revision 3), section (8010-8790), e.g., early stage cancer, is associated with the presence of a massive levels of satellite due to increase in transcription and processing of satellite repeats in epithelial cancer cells. Thus the methods can include the interference of satellite repeats in a sample comprising cells known or suspected of being tumor cells, e.g., cells from solid tumors of epithelial origin, e.g., pancreatic, lung, breast, prostate, renal, ovarian or colon/colorectal cancer cells.

Cancers of epithelial origin can include pancreatic cancer (e.g., pancreatic adenocarcinoma), lung cancer (e.g., non-small cell lung carcinoma or small cell lung carcinoma), prostate cancer, breast cancer, renal cancer, ovarian cancer, melanoma or colon cancer. Leukemia may include AML, CML or CLL and in some embodiments comprises cancerous MDSC. The methods can also be used to treat early preneoplastic cancers as a means to prevent the development of invasive cancer.

In some embodiments, CD5L, CD5L homodimer, and/or CD5L:p40 heterodimer may be used as a biomarker for cancer progression. For example, serum CD5L, CD5L homodimer, and/or CD5L:p40 concentration can be measured and compared against a control concentration. In some embodiments, serum CD5L, CD5L homodimer, and/or CD5L:p40 concentration in a subject is measured at multiple time points, and the change in concentration is used to indicate progression of the cancer.

Standard Treatments for Cancer

In some embodiments, the methods include administering a standard anti-cancer therapy to a subject. Cancer treatments include those known in the art, e.g., surgical resection with cold instruments or lasers, radiotherapy, phototherapy, biologic therapy (e.g., with tyrosine kinase inhibitors), radiofrequency ablation (RFA), radioembolisation (e.g., with 90^{Y} spheres), chemotherapy, and immunotherapy. Immunotherapies can also include administering one or more of: adoptive cell transfer (ACT) involving transfer of *ex vivo* expanded autologous or allogeneic tumor-reactive lymphocytes, e.g., dendritic cells or peptides with adjuvant; chimeric antigen receptors (CARs); cancer vaccines such

as DNA-based vaccines, cytokines (e.g., IL-2), cyclophosphamide, anti-interleukin-2R immunotoxins, Prostaglandin E2 Inhibitors (e.g., using SC-50) and/or checkpoint inhibitors including antibodies such as anti-CD137 (BMS-663513), anti-PD1 (e.g., Nivolumab, pembrolizumab/MK-3475, Pidilizumab (CT-011)), anti-PDL1 (e.g., BMS-936559, MPDL3280A), or anti-CTLA-4 (e.g., ipilumimab; see, e.g., Krüger et al., "Immune based therapies in cancer," Histol Histopathol. 2007 Jun;22(6):687-96; Eggermont et al., "Anti-CTLA-4 antibody adjuvant therapy in melanoma," Semin Oncol. 2010 Oct;37(5):455-9; Klinke DJ 2nd, "A multiscale systems perspective on cancer, immunotherapy, and Interleukin-12," Mol Cancer. 2010 Sep 15;9:242; Alexandrescu et al., "Immunotherapy for melanoma: current status and perspectives," J Immunother. 2010 Jul-Aug;33(6):570-90; Moschella et al., "Combination strategies for enhancing the efficacy of immunotherapy in cancer patients," Ann N Y Acad Sci. 2010 Apr;1194:169-78; Ganesan and Bakhshi, "Systemic therapy for melanoma," Natl Med J India. 2010 Jan-Feb;23(1):21-7; Golovina and Vonderheide, "Regulatory T cells: overcoming suppression of T-cell immunity," Cancer J. 2010 Jul-Aug;16(4):342-7. In some embodiments, the methods include administering a composition comprising tumor-pulsed dendritic cells, e.g., as described in WO2009/114547 and references cited therein. See also Shiao et al., Genes & Dev. 2011. 25: 2559-2572.

As mentioned above, adoptive cell transfer (ACT) can be used as an anti-cancer therapy. ATC can refer to the transfer of cells, most commonly immune-derived cells, back into the same patient or into a new recipient host with the goal of transferring the immunologic functionality and characteristics into the new host. If possible, use of autologous cells helps the recipient by minimizing graft versus host disease (GVHD) issues. The adoptive transfer of autologous tumor infiltrating lymphocytes (TIL) (Besser et al., (2010) Clin. Cancer Res 16 (9) 2646–55; Dudley et al., (2002) Science 298 (5594): 850–4; and Dudley et al., (2005) Journal of Clinical Oncology 23 (10): 2346–57.) or genetically re-directed peripheral blood mononuclear cells (Johnson et al., (2009) Blood 114 (3): 535–46; and Morgan et al., (2006) Science 314(5796) 126-9) has been used to successfully treat patients with advanced solid tumors, including melanoma and

colorectal carcinoma, as well as patients with CD19-expressing hematologic malignancies (Kalos et al., (2011) Science Translational Medicine 3 (95): 95ra73).

Aspects of the invention involve the adoptive transfer of immune system cells, such as T cells, specific for selected antigens, such as tumor associated antigens (see Maus et al., 2014, Adoptive Immunotherapy for Cancer or Viruses, Annual Review of Immunology, Vol. 32: 189-225; Rosenberg and Restifo, 2015, Adoptive cell transfer as personalized immunotherapy for human cancer, Science Vol. 348 no. 6230 pp. 62-68; Restifo et al., 2015, Adoptive immunotherapy for cancer: harnessing the T cell response. Nat. Rev. Immunol. 12(4): 269-281; and Jenson and Riddell, 2014, Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev. 257(1): 127–144). Various strategies may, for example, be employed to genetically modify T cells by altering the specificity of the T cell receptor (TCR), for example, by introducing new TCR α and β chains with selected peptide specificity (see U.S. Patent No. 8,697,854; PCT Patent Publications: WO2003020763, WO2004033685, WO2004044004, WO2005114215, WO2006000830, WO2008038002, WO2008039818, WO2004074322, WO2005113595, WO2006125962, WO2013166321, WO2013039889, WO2014018863, WO2014083173; U.S. Patent No. 8,088,379).

As an alternative to, or addition to, TCR modifications, chimeric antigen receptors (CARs) may be used in order to generate immunoresponsive cells, such as T cells, specific for selected targets, such as malignant cells, with a wide variety of receptor chimera constructs having been described (see U.S. Patent Nos. 5,843,728; 5,851,828; 5,912,170; 6,004,811; 6,284,240; 6,392,013; 6,410,014; 6,753,162; 8,211,422; and, PCT Publication WO9215322).

In general, CARs are comprised of an extracellular domain, a transmembrane domain, and an intracellular domain, wherein the extracellular domain comprises an antigen-binding domain that is specific for a predetermined target. While the antigen-binding domain of a CAR is often an antibody or antibody fragment (e.g., a single chain variable fragment, scFv), the binding domain is not particularly limited so long as it results in specific recognition of a target. For example, in some embodiments, the antigen-binding domain may comprise a receptor, such that the CAR is capable of

binding to the ligand of the receptor. Alternatively, the antigen-binding domain may comprise a ligand, such that the CAR is capable of binding the endogenous receptor of that ligand.

The antigen-binding domain of a CAR is generally separated from the transmembrane domain by a hinge or spacer. The spacer is also not particularly limited, and it is designed to provide the CAR with flexibility. For example, a spacer domain may comprise a portion of a human Fc domain, including a portion of the CH3 domain, or the hinge region of any immunoglobulin, such as IgA, IgD, IgE, IgG, or IgM, or variants thereof. Furthermore, the hinge region may be modified so as to prevent off-target binding by FcRs or other potential interfering objects. For example, the hinge may comprise an IgG4 Fc domain with or without a S228P, L235E, and/or N297Q mutation (according to Kabat numbering) in order to decrease binding to FcRs. Additional spacers/hinges include, but are not limited to, CD4, CD8, and CD28 hinge regions.

The transmembrane domain of a CAR may be derived either from a natural or from a synthetic source. Where the source is natural, the domain may be derived from any membrane-bound or transmembrane protein. Transmembrane regions of particular use in this disclosure may be derived from CD8, CD28, CD3, CD45, CD4, CD5, CDS, CD9, CD 16, CD22, CD33, CD37, CD64, CD80, CD86, CD 134, CD137, CD 154, TCR. Alternatively the transmembrane domain may be synthetic, in which case it will comprise predominantly hydrophobic residues such as leucine and valine. Preferably a triplet of phenylalanine, tryptophan and valine will be found at each end of a synthetic transmembrane domain. Optionally, a short oligo- or polypeptide linker, preferably between 2 and 10 amino acids in length may form the linkage between the transmembrane domain and the cytoplasmic signaling domain of the CAR. A glycine-serine doublet provides a particularly suitable linker.

Alternative CAR constructs may be characterized as belonging to successive generations. First-generation CARs typically consist of a single-chain variable fragment of an antibody specific for an antigen, for example comprising a V_L linked to a V_H of a specific antibody, linked by a flexible linker, for example by a CD8α hinge domain and a CD8α transmembrane domain, to the transmembrane and intracellular signaling domains

of either CD3ζ or FcRγ (scFv-CD3ζ or scFv-FcRγ; see U.S. Patent No. 7,741,465; U.S. Patent No. 5,912,172; U.S. Patent No. 5,906,936). Second-generation CARs incorporate the intracellular domains of one or more costimulatory molecules, such as CD28, OX40 (CD134), or 4-1BB (CD137) within the endodomain (for example scFv-CD28/OX40/4-1BB-CD3ζ; see U.S. Patent Nos. 8,911,993; 8,916,381; 8,975,071; 9,101,584; 9,102,760; 9,102,761). Third-generation CARs include a combination of costimulatory endodomains, such a CD3ζ-chain, CD97, GDI la-CD18, CD2, ICOS, CD27, CD2, CD7, LIGHT, LFA-1, NKG2C, B7-H3, CD30, CD40, PD-1, CD154, CDS, OX40, 4-1BB, or CD28 signaling domains (for example scFv-CD28-4-1BB-CD3ζ or scFv-CD28-OX40-CD3ζ; see U.S. Patent No. 8,906,682; U.S. Patent No. 8,399,645; U.S. Pat. No. 5,686,281; PCT Publication No. WO2014134165; PCT Publication No. WO2012079000). Alternatively, costimulation may be orchestrated by expressing CARs in antigen-specific T cells, chosen so as to be activated and expanded following engagement of their native αβTCR, for example by antigen on professional antigen-presenting cells, with attendant costimulation. In addition, additional engineered receptors may be provided on the immunoresponsive cells, for example to improve targeting of a T-cell attack and/or minimize side effects.

Alternatively, T-cells expressing CARs may be further modified to reduce or eliminate expression of endogenous TCRs in order to reduce off-target effects. Reduction or elimination of endogenous TCRs can reduce off-target effects and increase the effectiveness of the T cells (U.S. 9,181,527). T cells stably lacking expression of a functional TCR may be produced using a variety of approaches. T cells internalize, sort, and degrade the entire T cell receptor as a complex, with a half-life of about 10 hours in resting T cells and 3 hours in stimulated T cells (von Essen, M. et al. 2004. J. Immunol. 173:384-393). Proper functioning of the TCR complex requires the proper stoichiometric ratio of the proteins that compose the TCR complex. TCR function also requires two functioning TCR zeta proteins with ITAM motifs. The activation of the TCR upon engagement of its MHC-peptide ligand requires the engagement of several TCRs on the same T cell, which all must signal properly. Thus, if a TCR complex is destabilized with

proteins that do not associate properly or cannot signal optimally, the T cell will not become activated sufficiently to begin a cellular response.

Accordingly, in some embodiments, TCR expression may be eliminated using RNA interference (e.g., shRNA, siRNA, miRNA, etc.), CRISPR, or other methods that target the nucleic acids encoding specific TCRs (e.g., TCR-α and TCR-β) and/or CD3 chains in primary T cells. By blocking expression of one or more of these proteins, the T cell will no longer produce one or more of the key components of the TCR complex, thereby destabilizing the TCR complex and preventing cell surface expression of a functional TCR.

In some instances, CAR may also comprise a switch mechanism for controlling expression and/or activation of the CAR. For example, a CAR may comprise an extracellular, transmembrane, and intracellular domain, in which the extracellular domain comprises a target-specific binding element that comprises a label, binding domain, or tag that is specific for a molecule other than the target antigen that is expressed on or by a target cell. In such embodiments, the specificity of the CAR is provided by a second construct that comprises a target antigen binding domain (e.g., an scFv or a bispecific antibody that is specific for both the target antigen and the label or tag on the CAR) and a domain that is recognized by or binds to the label, binding domain, or tag on the CAR. See, e.g., WO 2013/044225, WO 2016/000304, WO 2015/057834, WO 2015/057852, WO 2016/070061, US 9,233,125, US 2016/0129109. In this way, a T-cell that expresses the CAR can be administered to a subject, but the CAR cannot bind its target antigen until the second composition comprising an antigen-specific binding domain is administered.

Alternative switch mechanisms include CARs that require multimerization in order to activate their signaling function (see, e.g., US 2015/0368342, US 2016/0175359, US 2015/0368360) and/or an exogenous signal, such as a small molecule drug (US 2016/0166613, Yung et al., Science, 2015), in order to elicit a T-cell response. Some CARs may also comprise a "suicide switch" to induce cell death of the CAR T-cells following treatment (Buddee et al., PLoS One, 2013) or to downregulate expression of the CAR following binding to the target antigen (WO 2016/011210).

Various techniques may be used to transform target immunoresponsive cells, such as protoplast fusion, lipofection, transfection or electroporation. A wide variety of vectors may be used, such as retroviral vectors, lentiviral vectors, adenoviral vectors, adeno-associated viral vectors, plasmids or transposons, such as a Sleeping Beauty transposon (see U.S. Patent Nos. 6,489,458; 7,148,203; 7,160,682; 7,985,739; 8,227,432), may be used to introduce CARs, for example using 2nd generation antigen-specific CARs signaling through CD3ζ and either CD28 or CD137. Viral vectors may for example include vectors based on HIV, SV40, EBV, HSV or BPV.

Cells that are targeted for transformation may for example include T cells, Natural Killer (NK) cells, cytotoxic T lymphocytes (CTL), regulatory T cells, human embryonic stem cells, tumor-infiltrating lymphocytes (TIL) or a pluripotent stem cell from which lymphoid cells may be differentiated. T cells expressing a desired CAR may for example be selected through co-culture with γ-irradiated activating and propagating cells (AaPC), which co-express the cancer antigen and co-stimulatory molecules. The engineered CAR T-cells may be expanded, for example by co-culture on AaPC in presence of soluble factors, such as IL-2 and IL-21. This expansion may for example be carried out so as to provide memory CAR+ T cells (which may for example be assayed by non-enzymatic digital array and/or multi-panel flow cytometry). In this way, CAR T cells may be provided that have specific cytotoxic activity against antigen-bearing tumors (optionally in conjunction with production of desired chemokines such as interferon-γ). CAR T cells of this kind may for example be used in animal models, for example to treat tumor xenografts.

Approaches such as the foregoing may be adapted to provide methods of treating and/or increasing survival of a subject having a disease, such as a neoplasia, for example by administering an effective amount of an immunoresponsive cell comprising an antigen recognizing receptor that binds a selected antigen, wherein the binding activates the immunoreponsive cell, thereby treating or preventing the disease (such as a neoplasia, a pathogen infection, an autoimmune disorder, or an allogeneic transplant reaction).

In some embodiments, the treatment can be administrated into patients undergoing an immunosuppressive treatment. The cells, or population of cells, may be

made resistant to at least one immunosuppressive agent due to the inactivation of a gene encoding a receptor for such immunosuppressive agent. Not being bound by a theory, the immunosuppressive treatment should help the selection and expansion of the immunoresponsive or T cells according to the invention within the patient.

The administration of the cells or population of cells according to the present invention may be carried out in any convenient manner, including by aerosol inhalation, injection, ingestion, transfusion, implantation or transplantation. The cells or population of cells may be administered to a patient subcutaneously, intradermally, intratumorally, intranodally, intramedullary, intramuscularly, intrathecally, by intravenous or intralymphatic injection, or intraperitoneally. In some embodiments, the disclosed CARs may be delivered or administered into a cavity formed by the resection of tumor tissue (i.e. intracavity delivery) or directly into a tumor prior to resection (i.e. intratumoral delivery). In one embodiment, the cell compositions of the present invention are preferably administered by intravenous injection.

The administration of the cells or population of cells can consist of the administration of 10^4- 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. Dosing in CAR T cell therapies may for example involve administration of from 10^6 to 10^9 cells/kg, with or without a course of lymphodepletion, for example with cyclophosphamide. The cells or population of cells can be administrated in one or more doses. In another embodiment, the effective amount of cells are administrated as a single dose. In another embodiment, the effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions are within the skill of one in the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

In another embodiment, the effective amount of cells or composition comprising those cells are administrated parenterally. The administration can be an intravenous administration. The administration can be directly done by injection within a tumor.

To guard against possible adverse reactions, engineered immunoresponsive cells may be equipped with a transgenic safety switch, in the form of a transgene that renders the cells vulnerable to exposure to a specific signal. For example, the herpes simplex viral thymidine kinase (TK) gene may be used in this way, for example by introduction into allogeneic T lymphocytes used as donor lymphocyte infusions following stem cell transplantation (Greco, et al., Improving the safety of cell therapy with the TK-suicide gene. Front. Pharmacol. 2015; 6: 95). In such cells, administration of a nucleoside prodrug such as ganciclovir or acyclovir causes cell death. Alternative safety switch constructs include inducible caspase 9, for example triggered by administration of a small-molecule dimerizer that brings together two nonfunctional icasp9 molecules to form the active enzyme. A wide variety of alternative approaches to implementing cellular proliferation controls have been described (see U.S. Patent Publication No. 20130071414; PCT Patent Publication WO2011146862; PCT Patent Publication WO2014011987; PCT Patent Publication WO2013040371; Zhou et al. BLOOD, 2014, 123/25:3895 – 3905; Di Stasi et al., The New England Journal of Medicine 2011; 365:1673-1683; Sadelain M, The New England Journal of Medicine 2011; 365:1735-173; Ramos et al., Stem Cells 28(6):1107-15 (2010)).

In a further refinement of adoptive therapies, genome editing may be used to tailor immunoresponsive cells to alternative implementations, for example providing edited CAR T cells (see Poirot et al., 2015, Multiplex genome edited T-cell manufacturing platform for "off-the-shelf" adoptive T-cell immunotherapies, Cancer Res 75 (18): 3853). For example, the CAR T cells can comprise a T cell with CD5L and/or p40 knockouts. Cells may be edited using any CRISPR system and method of use thereof as described herein. CRISPR systems may be delivered to an immune cell by any method described herein. In preferred embodiments, cells are edited *ex vivo* and transferred to a subject in need thereof. Immunoresponsive cells, CAR T cells or any cells used for adoptive cell transfer may be edited. Editing may be performed to eliminate

potential alloreactive T-cell receptors (TCR), disrupt the target of a chemotherapeutic agent, block an immune checkpoint, activate a T cell, and/or increase the differentiation and/or proliferation of functionally exhausted or dysfunctional CD8+ T-cells (see PCT Patent Publications: WO2013176915, WO2014059173, WO2014172606, WO2014184744, and WO2014191128). Editing may result in inactivation of a gene.

By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In a particular embodiment, the CRISPR system specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. The nucleic acid strand breaks caused are commonly repaired through the distinct mechanisms of homologous recombination or non-homologous end joining (NHEJ). However, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions (Indel) and can be used for the creation of specific gene knockouts. Cells in which a cleavage induced mutagenesis event has occurred can be identified and/or selected by well-known methods in the art.

T cell receptors (TCR) are cell surface receptors that participate in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, α and β, which assemble to form a heterodimer and associates with the CD3-transducing subunits to form the T cell receptor complex present on the cell surface. Each α and β chain of the TCR consists of an immunoglobulin-like N-terminal variable (V) and constant (C) region, a hydrophobic transmembrane domain, and a short cytoplasmic region. As for immunoglobulin molecules, the variable region of the α and β chains are generated by V(D)J recombination, creating a large diversity of antigen specificities within the population of T cells. However, in contrast to immunoglobulins that recognize intact antigen, T cells are activated by processed peptide fragments in association with an MHC molecule, introducing an extra dimension to antigen recognition by T cells, known as MHC restriction. Recognition of MHC disparities between the donor and recipient through the T cell receptor leads to T cell proliferation and the potential development of GVHD. The inactivation of TCRα or TCRβ can result in the elimination of the TCR from the surface of T cells preventing recognition of alloantigen and thus GVHD. However,

TCR disruption generally results in the elimination of the CD3 signaling component and alters the means of further T cell expansion.

Allogeneic cells are rapidly rejected by the host immune system. It has been demonstrated that, allogeneic leukocytes present in non-irradiated blood products will persist for no more than 5 to 6 days (Boni, Muranski et al. 2008 Blood 1;112(12):4746-54). Thus, to prevent rejection of allogeneic cells, the host's immune system usually has to be suppressed to some extent. However, in the case of adoptive cell transfer the use of immunosuppressive drugs also have a detrimental effect on the introduced therapeutic T cells. Therefore, to effectively use an adoptive immunotherapy approach in these conditions, the introduced cells would need to be resistant to the immunosuppressive treatment. Thus, in a particular embodiment, the present invention further comprises a step of modifying T cells to make them resistant to an immunosuppressive agent, preferably by inactivating at least one gene encoding a target for an immunosuppressive agent. An immunosuppressive agent is an agent that suppresses immune function by one of several mechanisms of action. An immunosuppressive agent can be, but is not limited to a calcineurin inhibitor, a target of rapamycin, an interleukin-2 receptor α-chain blocker, an inhibitor of inosine monophosphate dehydrogenase, an inhibitor of dihydrofolic acid reductase, a corticosteroid or an immunosuppressive antimetabolite. The present invention allows conferring immunosuppressive resistance to T cells for immunotherapy by inactivating the target of the immunosuppressive agent in T cells. As non-limiting examples, targets for an immunosuppressive agent can be a receptor for an immunosuppressive agent such as: CD52, glucocorticoid receptor (GR), a FKBP family gene member and a cyclophilin family gene member.

Immune checkpoints are inhibitory pathways that slow down or stop immune reactions and prevent excessive tissue damage from uncontrolled activity of immune cells. In certain embodiments, the immune checkpoint targeted is the programmed death-1 (PD-1 or CD279) gene (*PDCD1*). In other embodiments, the immune checkpoint targeted is cytotoxic T-lymphocyte-associated antigen (CTLA-4). In additional embodiments, the immune checkpoint targeted is another member of the CD28 and CTLA4 Ig superfamily such as BTLA, LAG3, ICOS, PDL1 or KIR. In further additional

embodiments, the immune checkpoint targeted is a member of the TNFR superfamily such as CD40, OX40, CD137, GITR, CD27 or TIM-3.

Additional immune checkpoints include Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) (Watson HA, et al., SHP-1: the next checkpoint target for cancer immunotherapy? Biochem Soc Trans. 2016 Apr 15;44(2):356-62). SHP-1 is a widely expressed inhibitory protein tyrosine phosphatase (PTP). In T-cells, it is a negative regulator of antigen-dependent activation and proliferation. It is a cytosolic protein, and therefore not amenable to antibody-mediated therapies, but its role in activation and proliferation makes it an attractive target for genetic manipulation in adoptive transfer strategies, such as chimeric antigen receptor (CAR) T cells. Immune checkpoints may also include T cell immunoreceptor with Ig and ITIM domains (TIGIT/Vstm3/WUCAM/VSIG9) and VISTA (Le Mercier I, et al., (2015) Beyond CTLA-4 and PD-1, the generation Z of negative checkpoint regulators. Front. Immunol. 6:418).

WO2014172606 relates to the use of MT1 and/or MT1 inhibitors to increase proliferation and/or activity of exhausted CD8+ T-cells and to decrease CD8+ T-cell exhaustion (e.g., decrease functionally exhausted or unresponsive CD8+ immune cells). In certain embodiments, metallothioneins are targeted by gene editing in adoptively transferred T cells.

In certain embodiments, targets of gene editing may be at least one targeted locus involved in the expression of an immune checkpoint protein. Such targets may include, but are not limited to CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, ICOS (CD278), PDL1, KIR, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244 (2B4), TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, VISTA, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, MT1, MT2, CD40, OX40, CD137, GITR, CD27, SHP-1, T-BET, RORC, or TIM-3. In preferred embodiments, the gene locus involved in the expression of PD-1 or CTLA-4 genes is targeted. In other preferred embodiments,

combinations of genes are targeted, such as but not limited to PD-1 and TIGIT. In preferred embodiments, the novel genes or gene combinations described herein are targeted or modulated.

In other embodiments, at least two genes are edited. Pairs of genes may include, but are not limited to PD1 and TCRα, PD1 and TCRβ, CTLA-4 and TCRα, CTLA-4 and TCRβ, LAG3 and TCRα, LAG3 and TCRβ, Tim3 and TCRα, Tim3 and TCRβ, BTLA and TCRα, BTLA and TCRβ, BY55 and TCRα, BY55 and TCRβ, TIGIT and TCRα, TIGIT and TCRβ, B7H5 and TCRα, B7H5 and TCRβ, LAIR1 and TCRα, LAIR1 and TCRβ, SIGLEC10 and TCRα, SIGLEC10 and TCRβ, 2B4 and TCRα, 2B4 and TCRβ.

Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and 7,572,631. T cells can be expanded *in vitro* or *in vivo*.

Immune cells may be obtained using any method known in the art. In one embodiment T cells that have infiltrated a tumor are isolated. T cells may be removed during surgery. T cells may be isolated after removal of tumor tissue by biopsy. T cells may be isolated by any means known in the art. In one embodiment the method may comprise obtaining a bulk population of T cells from a tumor sample by any suitable method known in the art. For example, a bulk population of T cells can be obtained from a tumor sample by dissociating the tumor sample into a cell suspension from which specific cell populations can be selected. Suitable methods of obtaining a bulk population of T cells may include, but are not limited to, any one or more of mechanically dissociating (e.g., mincing) the tumor, enzymatically dissociating (e.g., digesting) the tumor, and aspiration (e.g., as with a needle).

The bulk population of T cells obtained from a tumor sample may comprise any suitable type of T cell. Preferably, the bulk population of T cells obtained from a tumor sample comprises tumor infiltrating lymphocytes (TILs).

The tumor sample may be obtained from any mammal. Unless stated otherwise, as used herein, the term "mammal" refers to any mammal including, but not limited to,

mammals of the order Logomorpha, such as rabbits; the order Carnivora, including Felines (cats) and Canines (dogs); the order Artiodactyla, including Bovines (cows) and Swines (pigs); or of the order Perssodactyla, including Equines (horses). The mammals may be non-human primates, e.g., of the order Primates, Ceboids, or Simoids (monkeys) or of the order Anthropoids (humans and apes). In some embodiments, the mammal may be a mammal of the order Rodentia, such as mice and hamsters. Preferably, the mammal is a non-human primate or a human. An especially preferred mammal is the human.

T cells can be obtained from a number of sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, spleen tissue, and tumors. In certain embodiments of the present invention, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled artisan, such as Ficoll separation. In one preferred embodiment, cells from the circulating blood of an individual are obtained by apheresis or leukapheresis. The apheresis product typically contains lymphocytes, including T cells, monocytes, granulocytes, B cells, other nucleated white blood cells, red blood cells, and platelets. In one embodiment, the cells collected by apheresis may be washed to remove the plasma fraction and to place the cells in an appropriate buffer or media for subsequent processing steps. In one embodiment of the invention, the cells are washed with phosphate buffered saline (PBS). In an alternative embodiment, the wash solution lacks calcium and may lack magnesium or may lack many if not all divalent cations. Initial activation steps in the absence of calcium lead to magnified activation. As those of ordinary skill in the art would readily appreciate a washing step may be accomplished by methods known to those in the art, such as by using a semi-automated "flow-through" centrifuge (for example, the Cobe 2991 cell processor) according to the manufacturer's instructions. After washing, the cells may be resuspended in a variety of biocompatible buffers, such as, for example, Ca-free, Mg-free PBS. Alternatively, the undesirable components of the apheresis sample may be removed and the cells directly resuspended in culture media.

In another embodiment, T cells are isolated from peripheral blood lymphocytes by lysing the red blood cells and depleting the monocytes, for example, by centrifugation through a PERCOLL™ gradient. A specific subpopulation of T cells, such as CD28+,

CD4+, CDC, CD45RA+, and CD45RO+ T cells, can be further isolated by positive or negative selection techniques. For example, in one preferred embodiment, T cells are isolated by incubation with anti-CD3/anti-CD28 (i.e., 3×28)-conjugated beads, such as DYNABEADS® M-450 CD3/CD28 T, or XCYTE DYNABEADS™ for a time period sufficient for positive selection of the desired T cells. In one embodiment, the time period is about 30 minutes. In a further embodiment, the time period ranges from 30 minutes to 36 hours or longer and all integer values there between. In a further embodiment, the time period is at least 1, 2, 3, 4, 5, or 6 hours. In yet another preferred embodiment, the time period is 10 to 24 hours. In one preferred embodiment, the incubation time period is 24 hours. For isolation of T cells from patients with leukemia, use of longer incubation times, such as 24 hours, can increase cell yield. Longer incubation times may be used to isolate T cells in any situation where there are few T cells as compared to other cell types, such in isolating tumor infiltrating lymphocytes (TIL) from tumor tissue or from immunocompromised individuals. Further, use of longer incubation times can increase the efficiency of capture of CD8+ T cells.

Enrichment of a T cell population by negative selection can be accomplished with a combination of antibodies directed to surface markers unique to the negatively selected cells. A preferred method is cell sorting and/or selection via negative magnetic immunoadherence or flow cytometry that uses a cocktail of monoclonal antibodies directed to cell surface markers present on the cells negatively selected. For example, to enrich for CD4+ cells by negative selection, a monoclonal antibody cocktail typically includes antibodies to CD14, CD20, CD11b, CD16, HLA-DR, and CD8.

Further, monocyte populations (i.e., CD14+ cells) may be depleted from blood preparations by a variety of methodologies, including anti-CD14 coated beads or columns, or utilization of the phagocytotic activity of these cells to facilitate removal. Accordingly, in one embodiment, the invention uses paramagnetic particles of a size sufficient to be engulfed by phagocytotic monocytes. In certain embodiments, the paramagnetic particles are commercially available beads, for example, those produced by Life Technologies under the trade name Dynabeads™. In one embodiment, other non-specific cells are removed by coating the paramagnetic particles with "irrelevant"

proteins (e.g., serum proteins or antibodies). Irrelevant proteins and antibodies include those proteins and antibodies or fragments thereof that do not specifically target the T cells to be isolated. In certain embodiments the irrelevant beads include beads coated with sheep anti-mouse antibodies, goat anti-mouse antibodies, and human serum albumin.

In brief, such depletion of monocytes is performed by preincubating T cells isolated from whole blood, apheresed peripheral blood, or tumors with one or more varieties of irrelevant or non-antibody coupled paramagnetic particles at any amount that allows for removal of monocytes (approximately a 20:1 bead:cell ratio) for about 30 minutes to 2 hours at 22 to 37 degrees C., followed by magnetic removal of cells which have attached to or engulfed the paramagnetic particles. Such separation can be performed using standard methods available in the art. For example, any magnetic separation methodology may be used including a variety of which are commercially available, (e.g., DYNAL® Magnetic Particle Concentrator (DYNAL MPC®)). Assurance of requisite depletion can be monitored by a variety of methodologies known to those of ordinary skill in the art, including flow cytometric analysis of CD14 positive cells, before and after depletion.

For isolation of a desired population of cells by positive or negative selection, the concentration of cells and surface (e.g., particles such as beads) can be varied. In certain embodiments, it may be desirable to significantly decrease the volume in which beads and cells are mixed together (i.e., increase the concentration of cells), to ensure maximum contact of cells and beads. For example, in one embodiment, a concentration of 2 billion cells/ml is used. In one embodiment, a concentration of 1 billion cells/ml is used. In a further embodiment, greater than 100 million cells/ml is used. In a further embodiment, a concentration of cells of 10, 15, 20, 25, 30, 35, 40, 45, or 50 million cells/ml is used. In yet another embodiment, a concentration of cells from 75, 80, 85, 90, 95, or 100 million cells/ml is used. In further embodiments, concentrations of 125 or 150 million cells/ml can be used. Using high concentrations can result in increased cell yield, cell activation, and cell expansion. Further, use of high cell concentrations allows more efficient capture of cells that may weakly express target antigens of interest, such as CD28-negative T

cells, or from samples where there are many tumor cells present (i.e., leukemic blood, tumor tissue, etc). Such populations of cells may have therapeutic value and would be desirable to obtain. For example, using high concentration of cells allows more efficient selection of CD8+ T cells that normally have weaker CD28 expression.

In a related embodiment, it may be desirable to use lower concentrations of cells. By significantly diluting the mixture of T cells and surface (e.g., particles such as beads), interactions between the particles and cells is minimized. This selects for cells that express high amounts of desired antigens to be bound to the particles. For example, CD4+ T cells express higher levels of CD28 and are more efficiently captured than CD8+ T cells in dilute concentrations. In one embodiment, the concentration of cells used is 5×10^6/ml. In other embodiments, the concentration used can be from about 1×10^5/ml to 1×10^6/ml, and any integer value in between.

T cells can also be frozen. Wishing not to be bound by theory, the freeze and subsequent thaw step provides a more uniform product by removing granulocytes and to some extent monocytes in the cell population. After a washing step to remove plasma and platelets, the cells may be suspended in a freezing solution. While many freezing solutions and parameters are known in the art and will be useful in this context, one method involves using PBS containing 20% DMSO and 8% human serum albumin, or other suitable cell freezing media, the cells then are frozen to −80° C at a rate of 1° per minute and stored in the vapor phase of a liquid nitrogen storage tank. Other methods of controlled freezing may be used as well as uncontrolled freezing immediately at −20° C. or in liquid nitrogen.

T cells for use in the present invention may also be antigen-specific T cells. For example, tumor-specific T cells can be used. In certain embodiments, antigen-specific T cells can be isolated from a patient of interest, such as a patient afflicted with a cancer or an infectious disease. In one embodiment neoepitopes are determined for a subject and T cells specific to these antigens are isolated. Antigen-specific cells for use in expansion may also be generated in vitro using any number of methods known in the art, for example, as described in U.S. Patent Publication No. US 20040224402 entitled, Generation And Isolation of Antigen-Specific T Cells, or in U.S. Pat. Nos. 6,040,177.

Antigen-specific cells for use in the present invention may also be generated using any number of methods known in the art, for example, as described in Current Protocols in Immunology, or Current Protocols in Cell Biology, both published by John Wiley & Sons, Inc., Boston, Mass.

In a related embodiment, it may be desirable to sort or otherwise positively select (e.g. via magnetic selection) the antigen specific cells prior to or following one or two rounds of expansion. Sorting or positively selecting antigen-specific cells can be carried out using peptide-MHC tetramers (Altman, et al., *Science.* 1996 Oct. 4; 274(5284):94-6). In another embodiment the adaptable tetramer technology approach is used (Andersen et al., 2012 Nat Protoc. 7:891-902). Tetramers are limited by the need to utilize predicted binding peptides based on prior hypotheses, and the restriction to specific HLAs. Peptide-MHC tetramers can be generated using techniques known in the art and can be made with any MHC molecule of interest and any antigen of interest as described herein. Specific epitopes to be used in this context can be identified using numerous assays known in the art. For example, the ability of a polypeptide to bind to MHC class I may be evaluated indirectly by monitoring the ability to promote incorporation of ^{125}I labeled β2-microglobulin (β2m) into MHC class I/β2m/peptide heterotrimeric complexes (see Parker et al., J. Immunol. 152:163, 1994).

In one embodiment cells are directly labeled with an epitope-specific reagent for isolation by flow cytometry followed by characterization of phenotype and TCRs. In one T cells are isolated by contacting the T cell specific antibodies. Sorting of antigen-specific T cells, or generally any cells of the present invention, can be carried out using any of a variety of commercially available cell sorters, including, but not limited to, MoFlo sorter (DakoCytomation, Fort Collins, Colo.), FACSAria™, FACSArray™, FACSVantage™, BD™ LSR II, and FACSCalibur™ (BD Biosciences, San Jose, Calif.).

In a preferred embodiment, the method comprises selecting cells that also express CD3. The method may comprise specifically selecting the cells in any suitable manner. Preferably, the selecting is carried out using flow cytometry. The flow cytometry may be carried out using any suitable method known in the art. The flow cytometry may employ any suitable antibodies and stains. Preferably, the antibody is chosen such that it

specifically recognizes and binds to the particular biomarker being selected. For example, the specific selection of CD3, CD8, TIM-3, LAG-3, 4-1BB, or PD-1 may be carried out using anti-CD3, anti-CD8, anti-TIM-3, anti-LAG-3, anti-4-lBB, or anti-PD-1 antibodies, respectively. The antibody or antibodies may be conjugated to a bead (e.g., a magnetic bead) or to a fluorochrome. Preferably, the flow cytometry is fluorescence-activated cell sorting (FACS). TCRs expressed on T cells can be selected based on reactivity to autologous tumors. Additionally, T cells that are reactive to tumors can be selected for based on markers using the methods described in patent publication Nos. WO20141335567 and WO20141335568, herein incorporated by reference in their entirety. Additionally, activated T cells can be selected for based on surface expression of CD107a.

In one embodiment of the invention, the method further comprises expanding the numbers of T cells in the enriched cell population. Such methods are described in U.S. Patent No. 8,637,307 and is herein incorporated by reference in its entirety. The numbers of T cells may be increased at least about 3-fold (or 4-, 5-, 6-, 7-, 8-, or 9-fold), more preferably at least about 10-fold (or 20-, 30-, 40-, 50-, 60-, 70-, 80-, or 90-fold), more preferably at least about 100-fold, more preferably at least about 1,000 fold, or most preferably at least about 100,000-fold. The numbers of T cells may be expanded using any suitable method known in the art. Exemplary methods of expanding the numbers of cells are described in patent publication No. WO 2003057171, U.S. Patent No. 8,034,334, and U.S. Patent Application Publication No. 2012/0244133, each of which is incorporated herein by reference.

In one embodiment, *ex vivo* T cell expansion can be performed by isolation of T cells and subsequent stimulation or activation followed by further expansion. In one embodiment of the invention, the T cells may be stimulated or activated by a single agent. In another embodiment, T cells are stimulated or activated with two agents, one that induces a primary signal and a second that is a co-stimulatory signal. Ligands useful for stimulating a single signal or stimulating a primary signal and an accessory molecule that stimulates a second signal may be used in soluble form. Ligands may be attached to the surface of a cell, to an Engineered Multivalent Signaling Platform (EMSP), or

immobilized on a surface. In a preferred embodiment both primary and secondary agents are co-immobilized on a surface, for example a bead or a cell. In one embodiment, the molecule providing the primary activation signal may be a CD3 ligand, and the co-stimulatory molecule may be a CD28 ligand or 4-1BB ligand.

Antibodies to CD5L, CD5L:CD5L, or CD5L:p40 heterodimer

As already mentioned, some embodiments comprise methods that include administering an antibody or an antigen fragment thereof that binds to and inhibits the activity of CD5L monomer, CD5L homodimer, or the CD5L:p40 heterodimer, e.g., that specifically inhibits binding of the CD5L monomer, CD5L homodimer, or CD5L:p40 heterodimer to the IL-23 receptor, or that specifically inhibits formation of the CD5L homodimer or CD5L:p40 heterodimer.

The term "antibody" as used herein refers to an immunoglobulin molecule or an antigen-binding portion thereof. Examples of antigen-binding portions of immunoglobulin molecules include F(ab) and F(ab')2 fragments, which retain the ability to bind antigen. The antibody can be polyclonal, monoclonal, recombinant, chimeric, de-immunized or humanized, fully human, non-human, (e.g., murine), or single chain antibody. In some embodiments the antibody has effector function and can fix complement. In some embodiments, the antibody has reduced or no ability to bind an Fc receptor. For example, the antibody can be an isotype or subtype, fragment or other mutant, which does not support binding to an Fc receptor, e.g., it has a mutagenized or deleted Fc receptor binding region. Methods for making antibodies and fragments thereof are known in the art, see, e.g., Harlow et. al., editors, Antibodies: A Laboratory Manual (1988); Goding, Monoclonal Antibodies: Principles and Practice, (N.Y. Academic Press 1983); Howard and Kaser, Making and Using Antibodies: A Practical Handbook (CRC Press; 1st edition, Dec 13, 2006); Kontermann and Dübel, Antibody Engineering Volume 1 (Springer Protocols) (Springer; 2nd ed., May 21, 2010); Lo, Antibody Engineering: Methods and Protocols (Methods in Molecular Biology) (Humana Press; Nov 10, 2010); and Dübel, Handbook of Therapeutic Antibodies: Technologies,

Emerging Developments and Approved Therapeutics, (Wiley-VCH; 1 edition September 7, 2010).

Inhibitory Nucleic Acids

Some embodiments comprise decreasing protein expression (*e.g.*, CD5L or p40 expression) with inhibitory nucleic acids. Inhibitory nucleic acids useful in the present methods and compositions include antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, siRNA compounds, single- or double-stranded RNA interference (RNAi) compounds such as siRNA compounds, modified bases/locked nucleic acids (LNAs), antagomirs, peptide nucleic acids (PNAs), ribozymes, and other oligomeric compounds or oligonucleotide mimetics which hybridize to at least a portion of the target nucleic acid and modulate its function. In some embodiments, the inhibitory nucleic acids include antisense RNA, antisense DNA, chimeric antisense oligonucleotides, antisense oligonucleotides comprising modified linkages, interference RNA (RNAi), short interfering RNA (siRNA); a micro, interfering RNA (miRNA); a small, temporal RNA (stRNA); or a short, hairpin RNA (shRNA); small RNA-induced gene activation (RNAa); small activating RNAs (saRNAs), or combinations thereof. See, e.g., WO 2010040112; Burnett and Rossi (2012) Chem Biol. 19 (1):60-71; and WO2015130968, which is incorporated herein by reference in its entirety.

In some embodiments, the inhibitory nucleic acids are 10 to 50, 13 to 50, or 13 to 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies oligonucleotides having antisense portions of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length, or any range there within. In some embodiments, the oligonucleotides are 15 nucleotides in length. In some embodiments, the antisense or oligonucleotide compounds of the invention are 12 or 13 to 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies inhibitory nucleic acids having antisense portions of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length, or any range there within.

In some embodiments, the inhibitory nucleic acids are chimeric oligonucleotides that contain two or more chemically distinct regions, each made up of at least one nucleotide. These oligonucleotides typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. Chimeric inhibitory nucleic acids of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures comprise, but are not limited to, US patent nos. 5,013,830; 5,149,797; 5, 220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; 5,700,922; 8,604,192; 8,697,663; 8,703,728; 8,796,437; 8,865,677; and 8,883,752 each of which is herein incorporated by reference.

In some embodiments, the inhibitory nucleic acid comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. In other preferred embodiments, RNA modifications include 2'-fluoro, 2'-amino and 2' O-methyl modifications on the ribose of pyrimidines, abasic residues or an inverted base at the 3' end of the RNA. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher Tm (i.e., higher target binding affinity) than; 2'-deoxyoligonucleotides against a given target.

A number of nucleotide and nucleoside modifications have been shown to make the oligonucleotide into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide; these modified oligos survive intact for a longer time than unmodified oligonucleotides. Specific examples of modified oligonucleotides include those comprising modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred

are oligonucleotides with phosphorothioate backbones and those with heteroatom backbones, particularly CH2 -NH-O-CH2, CH,~N(CH3)~O~CH2 (known as a methylene(methylimino) or MMI backbone], CH2 --O--N (CH3)-CH2, CH2 -N (CH3)-N (CH3)-CH2 and O-N (CH3)- CH2 -CH2 backbones, wherein the native phosphodiester backbone is represented as O- P-- O- CH,); amide backbones (De Mesmaeker (1995) Ace. Chem. Res. 28:366-374); morpholino backbone structures (Summerton and Weller, U.S. Pat. No. 5,034,506); peptide nucleic acid (PNA) backbone (wherein the phosphodiester backbone of the oligonucleotide is replaced with a polyamide backbone, the nucleotides being bound directly or indirectly to the aza nitrogen atoms of the polyamide backbone, Nielsen (1991) Science 254, 1497). Phosphorus-containing linkages include, but are not limited to, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates comprising 3'alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates comprising 3'-amino phosphoramidate and aminoalkylphosphoramidates, phosphonoacetate phosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'; see US patent nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5, 177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455, 233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563, 253; 5,571,799; 5,587,361; and 5,625,050.

Morpholino-based oligomeric compounds are described in Dwaine A. Braasch and David R. Corey (2002) Biochemistry 41(14), 4503-4510); Genesis, volume 30, issue 3, 2001; Heasman, (2002) Dev. Biol. 243, 209-214; Nasevicius (2000) Nat. Genet. 26, 216-220; Lacerra (2000) Proc. Natl. Acad. Sci. 97, 9591-9596; and U.S. Pat. No. 5,034,506, issued Jul. 23, 1991. Cyclohexenyl nucleic acid oligonucleotide mimetics are described in Wang (2000) Am. Chem. Soc. 122, 8595-8602.

Modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside

linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These comprise those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH2 component parts; see US patent nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264, 562; 5, 264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596, 086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623, 070; 5,663,312; 5,633,360; 5,677,437; 5,677,439; and 8,927,513 each of which is herein incorporated by reference.

One or more substituted sugar moieties can also be included, e.g., one of the following at the 2' position: OH, SH, SCH_3, F, OCN, OCH_3, OCH_3 $O(CH_2)n$ CH_3, $O(CH_2)n$ NH_2 or $O(CH_2)n$ CH_3 where n is from 1 to about 10; Ci to C10 lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF3 ; OCF3; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH3; SO2 CH3; ONO2; NO2; N3; NH2; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an oligonucleotide; or a group for improving the pharmacodynamic properties of an oligonucleotide and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-0-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl)] (Martin (1995) HeIv. Chim. Acta 78, 486). Other preferred modifications include 2'-methoxy (2'-0-CH_3), 2'-propoxy (2'-OCH_2 CH_2CH_3) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Inhibitory nucleic acids can also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-Me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2' deoxycytosine and often referred to in the art as 5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well as synthetic nucleobases, e.g., 2-aminoadenine, 2- (methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalklyamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5- hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N6 (6-aminohexyl)adenine, 2,6- diaminopurine; 5-ribosyluracil (Carlile (2014) Nature 515(7525): 143-6) . Kornberg, A., DNA Replication, W. H. Freeman & Co., San Francisco, 1980, pp75-77; Gebeyehu (1987) Nucl. Acids Res. 15:4513). A "universal" base known in the art, e.g., inosine, can also be included. 5-Me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2<0>C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

It is not necessary for all positions in a given oligonucleotide to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single oligonucleotide or even at within a single nucleoside within an oligonucleotide. In some embodiments, both the nucleobase and backbone may be modified to enhance stability and activity (El-Sagheer (2014) Chem Sci 5:253-259)

In some embodiments, both a sugar and an internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, for example, an aminoethylglycine backbone. The nucleobases are

retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds comprise, but are not limited to, US patent nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference . Further teaching of PNA compounds can be found in Nielsen (1991) Science 254, 1497-1500; and Shi (2015).

Inhibitory nucleic acids can also include one or more nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases comprise the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases comprise other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo-uracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8- thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5- bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylquanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3- deazaguanine and 3-deazaadenine.

Further, nucleobases comprise those disclosed in United States Patent No. 3,687,808, those disclosed in 'The Concise Encyclopedia of Polymer Science And Engineering', pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandle Chemie, International Edition', 1991, 30, page 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications', pages 289- 302, Crooke, S.T. and Lebleu, B. ea., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, comprising 2-aminopropyladenine, 5-propynyluracil and 5- propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2<0>C

(Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds, 'Antisense Research and Applications', CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Modified nucleobases are described in US patent nos. 3,687,808, as well as 4,845,205; 5,130,302; 5,134,066; 5,175, 273; 5, 367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,596,091; 5,614,617; 5,750,692, and 5,681,941, each of which is herein incorporated by reference.

In some embodiments, the inhibitory nucleic acids are chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or cellular uptake of the oligonucleotide. Such moieties comprise but are not limited to, lipid moieties such as a cholesterol moiety (Letsinger (1989) Proc. Natl. Acad. Sci. USA 86, 6553-6556), cholic acid (Manoharan (1994) Bioorg. Med. Chem. Let. 4, 1053-1060), a thioether, e.g., hexyl-S- tritylthiol (Manoharan (1992) Ann. N. Y. Acad. Sci. 660, 306-309; Manoharan (1993) Bioorg. Med. Chem. Let. 3, 2765-2770), a thiocholesterol (Oberhauser (1992) Nucl. Acids Res. 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Kabanov (1990) FEBS Lett. 259, 327-330; Svinarchuk (1993) Biochimie 75, 49- 54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1, 2-di-O-hexadecyl- rac-glycero-3-H-phosphonate (Manoharan (1995) Tetrahedron Lett. 36, 3651-3654; Shea (1990) Nucl. Acids Res.18, 3777-3783), a polyamine or a polyethylene glycol chain (Mancharan (1995) Nucleosides & Nucleotides 14, 969-973), or adamantane acetic acid (Manoharan (1995) Tetrahedron Lett. 36, 3651-3654), a palmityl moiety (Mishra (1995) Biochim. Biophys. Acta 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-t oxycholesterol moiety (Crooke (1996) J. Pharmacol. Exp. Ther. 277, 923-937). See also US patent nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552, 538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486, 603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762, 779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082, 830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5, 245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391, 723;

5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5, 565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599, 928; 5,688,941, 8,865,677; 8,877,917 each of which is herein incorporated by reference.

These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterols, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins, rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application No. PCT/US92/09196, filed Oct. 23, 1992, and U.S. Pat. No. 6,287,860, which are incorporated herein by reference. Conjugate moieties include, but are not limited to, lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-5-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac- glycerol or triethylammonium l,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxy cholesterol moiety. See, e.g., U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203,

5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

The inhibitory nucleic acids useful in the present methods are sufficiently complementary to the target lncRNA, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect. "Complementary" in this context refers to the capacity for pairing, through hydrogen bonding, between two sequences comprising naturally or non-naturally occurring bases or analogs thereof. For example, if a base at one position of an inhibitory nucleic acid is capable of hydrogen bonding with a base at the corresponding position of a lncRNA, then the bases are considered to be complementary to each other at that position. 100% complementarity is not required.

In some embodiments, the location on a target lncRNA to which an inhibitory nucleic acids hybridizes is defined as a target region to which a protein binding partner binds. These regions can be identified by reviewing the data submitted herewith in Appendix I and identifying regions that are enriched in the dataset; these regions are likely to include the protein binding sequences. Routine methods can be used to design an inhibitory nucleic acid that binds to this sequence with sufficient specificity. In some embodiments, the methods include using bioinformatics methods known in the art to identify regions of secondary structure, e.g., one, two, or more stem-loop structures, or pseudoknots, and selecting those regions to target with an inhibitory nucleic acid.

While the specific sequences of certain exemplary target segments are set forth herein, one of skill in the art will recognize that these serve to illustrate and describe particular embodiments within the scope of the present invention. Additional target segments are readily identifiable by one having ordinary skill in the art in view of this disclosure. Target segments 5-500 nucleotides in length comprising a stretch of at least five (5) consecutive nucleotides within the protein binding region, or immediately adjacent thereto, are considered to be suitable for targeting as well. Target segments can include sequences that comprise at least the 5 consecutive nucleotides from the 5 '-terminus of one of the protein binding regions (the remaining nucleotides being a consecutive stretch of the same RNA beginning immediately upstream of the 5'-terminus of the binding segment and continuing until the inhibitory nucleic acid contains about 5

to about 100 nucleotides). Similarly preferred target segments are represented by RNA sequences that comprise at least the 5 consecutive nucleotides from the 3 '-terminus of one of the illustrative preferred target segments (the remaining nucleotides being a consecutive stretch of the same lncRNA beginning immediately downstream of the 3'-terminus of the target segment and continuing until the inhibitory nucleic acid contains about 5 to about 100 nucleotides). One having skill in the art armed with the sequences provided herein will be able, without undue experimentation, to identify further preferred protein binding regions to target.

Once one or more target regions, segments or sites have been identified, inhibitory nucleic acid compounds are chosen that are sufficiently complementary to the target, i.e., that hybridize sufficiently well and with sufficient specificity (i.e., do not substantially bind to other non-target RNAs), to give the desired effect.

Making and Using Inhibitory Nucleic Acids

The inhibitory nucleic acids used to practice the methods described herein, whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed, generated recombinantly or synthetically by well-known chemical synthesis techniques, as described in, e.g., Adams (1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; Maier (2000) Org Lett 2(13):1819-1822; Egeland (2005) Nucleic Acids Res 33(14):e125; Krotz (2005) Pharm Dev Technol 10(2):283-90 U.S. Patent No. 4,458,066. Recombinant nucleic acid sequences can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including e.g. *in vitro* bacterial, fungal, mammalian, yeast, insect or plant cell expression systems.

Nucleic acid sequences of the invention can be inserted into delivery vectors and expressed from transcription units within the vectors. The recombinant vectors can be DNA plasmids or viral vectors. Generation of the vector construct can be accomplished

using any suitable genetic engineering techniques well known in the art, including, without limitation, the standard techniques of PCR, oligonucleotide synthesis, restriction endonuclease digestion or "seamless cloning", ligation, transformation, plasmid purification, and DNA sequencing, for example as described in Sambrook et al. "Molecular Cloning: A Laboratory Manual." (1989)), Coffin et al. (Retroviruses. (1997)) and "RNA Viruses: A Practical Approach" (Alan J. Cann, Ed., Oxford University Press, (2000)). "Seamless cloning" allows joining of multiple fragments of nucleic acids in a single, isothermal reaction (Gibson (2009) Nat Methods 6:343-345; Werner (2012) Bioeng Bugs 3:38-43; Sanjana (2012) Nat Protoc 7:171-192). As will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring nucleic acids of the invention into cells. The selection of an appropriate vector to deliver nucleic acids and optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue experimentation. Viral vectors comprise a nucleotide sequence having sequences for the production of recombinant virus in a packaging cell. Viral vectors expressing nucleic acids of the invention can be constructed based on viral backbones including, but not limited to, a retrovirus, lentivirus, adenovirus, adeno-associated virus, pox virus or alphavirus (Warnock (2011) Methods in Molecular Biology 737:1-25). The recombinant vectors capable of expressing the nucleic acids of the invention can be delivered as described herein, and persist in target cells (e.g., stable transformants).

This can be achieved, for example, by administering an inhibitory nucleic acid, e.g., antisense oligonucleotides complementary to p40 and/or CD5L. Other inhibitory nucleic acids for use in practicing the methods described herein and that are complementary to p40 and/or CD5L can be those which inhibit post-transcriptional processing of p40 or CD5L, such as inhibitors of mRNA translation (antisense), agents of RNA interference (RNAi), catalytically active RNA molecules (ribozymes), and RNAs that bind proteins and other molecular ligands (aptamers). Additional methods exist to inhibit endogenous microRNA (miRNA) activity through the use of antisense-miRNA oligonucleotides (antagomirs) and RNA competitive inhibitors or decoys (miRNA sponges).

For further disclosure regarding inhibitory nucleic acids, please see US2010/0317718 (antisense oligos); US2010/0249052 (double-stranded ribonucleic acid (dsRNA)); US2009/0181914 and US2010/0234451 (LNAs); US2007/0191294 (siRNA analogues); US2008/0249039 (modified siRNA); and WO2010/129746 and WO2010/040112 (inhibitory nucleic acids).

Antisense

In some embodiments, the inhibitory nucleic acids are antisense oligonucleotides. Antisense oligonucleotides are typically designed to block expression of a DNA or RNA target by binding to the target and halting expression at the level of transcription, translation, or splicing. Antisense oligonucleotides of the present invention are complementary nucleic acid sequences designed to hybridize under stringent conditions to p40 and/or CD5L. Thus, oligonucleotides are chosen that are sufficiently complementary to the target, i.e., that hybridize sufficiently well and with sufficient specificity, to give the desired effect, while striving to avoid significant off-target effects i.e. must not directly bind to, or directly significantly affect expression levels of, transcripts other than the intended target. The optimal length of the antisense oligonucleotide may very but it should be as short as possible while ensuring that its target sequence is unique in the transcriptome i.e. antisense oligonucleotides may be as short as 12-mers (Seth (2009) J Med Chem 52:10-13) to 18-22 nucleotides in length.

In the context of this invention, hybridization means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. Complementary, as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in

each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target.

It is understood in the art that a complementary nucleic acid sequence need not be 100% complementary to that of its target nucleic acid to be specifically hybridisable. A complementary nucleic acid sequence of the invention is specifically hybridisable when binding of the sequence to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the sequence to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed under suitable conditions of stringency. The antisense oligonucleotides useful in the methods described herein have at least 80% sequence complementarity to a target region within the target nucleic acid, e.g., 90%, 95%, or 100% sequence complementarity to the target region within p40 or CD5L (e.g., a target region comprising the seed sequence). Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul (1990) J. Mol. Biol. 215, 403-410; Zhang and Madden (1997) Genome Res. 7, 649-656). The specificity of an antisense oligonucleotide can also be determined routinely using BLAST program against the entire genome of a given species

For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least

about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 μg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 μg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art. For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular

Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, Hilario (2007) Methods Mol Biol 353:27-38.

Inhibitory nucleic acids for use in the methods described herein can include one or more modifications, e.g., be stabilized against nucleolytic degradation such as by the incorporation of a modification, e.g., a nucleotide modification. For example, inhibitory nucleic acids can include a phosphorothioate at least the first, second, or third internucleotide linkage at the 5' or 3' end of the nucleotide sequence. As another example, inhibitory nucleic acids can include a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethyloxyethyl (2'-O-DMAEOE), or 2'-O--N-methylacetamido (2'-O--NMA). As another example, the inhibitory nucleic acids can include at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides include a 2'-O-methyl modification.

Modifications

Chemical modifications, particularly the use of locked nucleic acids (LNAs) (Okiba (1997) Tetrahedron Lett 39:5401-5404; Singh (1998) Chem Commun 4:455-456), 2'-O-methoxyethyl (2'-O-MOE) (Martin (1995) Helv Chim Acta 78:486-504; You (2006) Nucleic Acids Res 34(8):e60; Owczarzy (2011) Biochem 50(43):9352-9367), constrained ethyl BNA (cET) (Murray (2012) Nucleic Acids Res 40: 6135-6143), and gapmer oligonucleotides, which contain 2-5 chemically modified nucleotides (LNA, 2'-O-MOE RNA or cET) at each terminus flanking a central 5-10 base "gap" of DNA (Monia (1993) J Biol Chem 268:14514-14522; Wahlestedt (2000) PNAS 97:5633-5638), improve antisense oligonucleotide binding affinity for the target RNA, which increases the steric block efficiency. Antisense oligos that hybridize to p40 or CD5L, can be identified through experimentation.

Techniques for the manipulation of inhibitory nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in

the scientific and patent literature, see, e.g., Sambrook et al., Molecular Cloning; A Laboratory Manual 3d ed. (2001); Current Protocols in Molecular Biology, Ausubel et al., eds. (John Wiley & Sons, Inc., New York 2010); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); Laboratory Techniques In Biochemistry And Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Modified bases/Locked Nucleic Acids (LNAs)

In some embodiments, the inhibitory nucleic acids are "locked," i.e., comprise nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom and the 4'-C atom (see, e.g., Kaupinnen (2005) Drug Disc. Today 2(3):287-290; Koshkin (1998) J. Am. Chem. Soc. 120(50):13252–13253). For additional modifications see US 20100004320, US 20090298916, and US 20090143326.

siRNA/shRNA

In some embodiments, the nucleic acid sequence that is complementary to p40 or CD5L can be an interfering RNA, including but not limited to a small interfering RNA ("siRNA") or a small hairpin RNA ("shRNA"). Methods for constructing interfering RNAs are well known in the art. For example, the interfering RNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure); the antisense strand comprises nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof (i.e., an undesired gene) and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, interfering RNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions are linked by means of nucleic acid based or non-nucleic acid-based linker(s). The interfering RNA can be a polynucleotide with a

duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The interfering can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siRNA molecule capable of mediating RNA interference. RNA interference may cause translational repression and degradation of target mRNAs with imperfect complementarity or sequence-specific cleavage of perfectly complementary mRNAs.

In some embodiments, the interfering RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region. Such an RNA molecule when expressed desirably forms a "hairpin" structure, and is referred to herein as an "shRNA." The loop region is generally between about 2 and about 10 nucleotides in length. In some embodiments, the loop region is from about 6 to about 9 nucleotides in length. In some embodiments, the sense region and the antisense region are between about 15 and about 20 nucleotides in length. Following post-transcriptional processing, the small hairpin RNA is converted into a siRNA by a cleavage event mediated by the enzyme Dicer, which is a member of the RNase III family. The siRNA is then capable of inhibiting the expression of a gene with which it shares homology. After the siRNA has cleaved its target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets (Brummelkamp (2002) Science 296:550-553; Lee (2002) Nature Biotechnol., 20, 500-505; Miyagishi and Taira (2002) Nature Biotechnol 20:497-500; Paddison (2002) Genes & Dev. 16:948-958; Paul (2002) Nature Biotechnol 20, 505-508; Sui (2002) Proc. Natl. Acad. Sd. USA

99(6), 5515-5520; Yu (2002) Proc Natl Acad Sci USA 99:6047-6052; Peer and Lieberman (2011) Gen Ther 18, 1127-1133).

The target RNA cleavage reaction guided by siRNAs is highly sequence specific. In general, siRNA containing a nucleotide sequences identical to a portion of the target nucleic acid are preferred for inhibition. However, 100% sequence identity between the siRNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence. For example, siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Alternatively, siRNA sequences with nucleotide analog substitutions or insertions can be effective for inhibition. In general the siRNAs must retain specificity for their target, i.e., must not directly bind to, or directly significantly affect expression levels of, transcripts other than the intended target. shRNAs that are constitutively expressed form promoters can ensure long-term gene silencing. Most methods commonly used for delivery of siRNAs rely on commonly used techniques for introducing an exogenous nucleic acid into a cell including calcium phosphate or calcium chloride precipitation, microinjection, DEAE-dextrin-mediated transfection, lipofection, commercially available cationic polymers and lipids and cell-penetrating peptides, electroporation or stable nucleic acid-lipid particles (SNALPs), all of which are routine in the art. siRNAs can also be conjugated to small molecules to direct binding to cell-surface receptors, such as cholesterol (Wolfrum (2007) Nat Biotechnol 25:1149-1157), alpha-tocopherol (Nishina (2008) Mol Ther 16:734-40), lithocholic acid or lauric acid (Lorenz (2004) Bioorg Med Chem Lett 14:4975-4977), polyconjugates (Rozema (2007) PNAS 104:12982-12987). A variation of conjugated siRNAs are aptamer-siRNA chimeras (McNamara (2006) Nat Biotechnol 24:1005-1015; Dassie (2009) Nat Biotechnol 27:839-849) and siRNA-fusion protein complexes, which is composed of a targeting peptide, such as an antibody fragment that recognizes a cell-surface receptor or ligand, linked to an RNA-binding peptide that can be complexed to siRNAs for targeted systemic siRNA delivery (Yao (2011) Sci Transl Med 4(130):130ra48.

Ribozymes

Trans-cleaving enzymatic nucleic acid molecules can also be used; they have shown promise as therapeutic agents for human disease (Usman & McSwiggen, (1995) Ann. Rep. Med. Chem. 30, 285-294; Christoffersen and Marr (1995) J. Med. Chem. 38, 2023-2037; Weng (2005) Mol Cancer Ther 4, 948-955; Armado (2004) Hum Gene Ther 15, 251-262; Macpherson (2005) J Gene Med 7,552-564; Muhlbacher (2010) Curr Opin Pharamacol 10(5):551-6). Enzymatic nucleic acid molecules can be designed to cleave specific p40 and/or CD5L targets within the background of cellular RNA. Such a cleavage event renders the p40 and/or CD5L non- functional.

In general, enzymatic nucleic acids with RNA cleaving activity act by first binding to a target RNA. Such binding occurs through the target binding portion of an enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

Several approaches such as *in vitro* selection (evolution) strategies (Orgel (1979) Proc. R. Soc. London B 205, 435) have been used to evolve new nucleic acid catalysts with improved properties, new functions and capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce (1989) Gene 82, 83-87; Beaudry (1992) Science 257, 635-641; Joyce (1992) Scientific American 267, 90-97; Breaker (1994) TIBTECH 12, 268; Bartel (1993) Science 261 :1411-1418; Szostak (1993) TIBS 17, 89-93; Kumar (1995) FASEB J. 9, 1183; Breaker (1996) Curr. Op. Biotech. 1, 442; Scherer (2003) Nat Biotechnol 21, 1457-1465; Berens (2015) Curr. Op. Biotech. 31, 10-15). Ribozymes can also be engineered to be allosterically activated by effector molecules (riboswitches, Liang (2011) Mol Cell 43, 915-926; Wieland (2010) Chem Biol 17, 236-242; US Patent No 8,440,810). The development of ribozymes that are optimal for catalytic activity would contribute

significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The most common ribozyme therapeutics are derived from either hammerhead or hairpin/paperclip motifs. The hammerhead ribozyme, for example, functions with a catalytic rate (kcat) of about 1 min-1 in the presence of saturating (10 rnM) concentrations of Mg2+ cofactor. An artificial "RNA ligase" ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of about 100 min-1. In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA cleavage with multiple turn-over rates that approach 100 min-1. Ribozymes can be delivered to target cells in RNA form or can be transcribed from vectors. Due to poor stability of fully-RNA ribozymes, ribozymes often require chemical modification, such as, 5'-PS backbone linkage, 2'-O-Me, 2'-deoxy-2'-C-allyl uridine, and terminal inverted 3'-3' deoxyabasic nucleotides (Kobayashi (2005) Cancer Chemother Pharmacol 56, 329-336).

CRISPR/Cas, TALENs, and Zinc Finger Nucleases (ZFNs)

As mentioned above, some embodiments comprise methods gene targeting and/or genome editing. Such methods are useful, *e.g.*, in the context of decreasing protein expression *in vivo* and/or modifying cells *in vitro* (*e.g.*, in the context of adoptive cell therapies). In some embodiments, genes are targeting and/or edited using DNA binding proteins.

In some embodiments, the methods described herein include the use of transcription activator effector-like nucleases (TALENs), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) Cas RNA-guided nucleases (RGNs), or zinc finger nucleases (ZFNs) to inhibit expression of CD5L and/or p40. In these methods, engineered nucleases are used to specifically target and disrupt expression of CD5L and/or p40. Methods for using CRISPR, TALENs, and ZFNs are well known in the art.

Gene Targeting and Genome Editing

As mentioned above, some embodiments comprise methods gene targeting and/or genome editing. Such methods are useful, *e.g.*, in the context of decreasing protein expression *in vivo* and/or modifying cells *in vitro* (*e.g.*, in the context of adoptive cell

therapies). In some embodiments, genes are targeting and/or edited using DNA binding proteins.

In certain embodiments, the DNA binding protein is a (endo)nuclease or a variant thereof having altered or modified activity (i.e. a modified nuclease, as described herein elsewhere). In certain embodiments, said nuclease is a targeted or site-specific or homing nuclease or a variant thereof having altered or modified activity. In certain embodiments, said nuclease or targeted/site-specific/homing nuclease is, comprises, consists essentially of, or consists of a (modified) CRISPR/Cas system or complex, a (modified) Cas protein, a (modified) zinc finger, a (modified) zinc finger nuclease (ZFN), a (modified) transcription factor-like effector (TALE), a (modified) transcription factor-like effector nuclease (TALEN), or a (modified) meganuclease. In certain embodiments, said (modified) nuclease or targeted/site-specific/homing nuclease is, comprises, consists essentially of, or consists of a (modified) RNA-guided nuclease. As used herein, the term "Cas" generally refers to a (modified) effector protein of the CRISPR/Cas system or complex, and can be without limitation a (modified) Cas9, or other enzymes such as Cpf1, The term "Cas" may be used herein interchangeably with the terms "CRISPR" protein, "CRISPR/Cas protein", "CRISPR effector", "CRISPR/Cas effector", "CRISPR enzyme", "CRISPR/Cas enzyme" and the like, unless otherwise apparent, such as by specific and exclusive reference to Cas9. It is to be understood that the term "CRISPR protein" may be used interchangeably with "CRISPR enzyme", irrespective of whether the CRISPR protein has altered, such as increased or decreased (or no) enzymatic activity, compared to the wild type CRISPR protein. Likewise, as used herein, in certain embodiments, where appropriate and which will be apparent to the skilled person, the term "nuclease" may refer to a modified nuclease wherein catalytic activity has been altered, such as having increased or decreased nuclease activity, or no nuclease activity at all, as well as nickase activity, as well as otherwise modified nuclease as defined herein elsewhere, unless otherwise apparent, such as by specific and exclusive reference to unmodified nuclease.

As used herein, the term "targeting" of a selected nucleic acid sequence means that a nuclease or nuclease complex is acting in a nucleotide sequence specific manner.

For instance, in the context of the CRISPR/Cas system, the guide RNA is capable of hybridizing with a selected nucleic acid sequence. As uses herein, "hybridization" or “hybridizing” refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson Crick base pairing, Hoogstein binding, or in any other sequence specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of PGR, or the cleavage of a polynucleotide by an enzyme. A sequence capable of hybridizing with a given sequence is referred to as the "complement" of the given sequence.

In certain embodiments, the DNA binding protein is a (modified) transcription activator-like effector nuclease (TALEN) system. Transcription activator-like effectors (TALEs) can be engineered to bind practically any desired DNA sequence. Exemplary methods of genome editing using the TALEN system can be found for example in Cermak T. Doyle EL. Christian M. Wang L. Zhang Y. Schmidt C, et al. Efficient design and assembly of custom TALEN and other TAL effector-based constructs for DNA targeting. Nucleic Acids Res. 2011;39:e82; Zhang F. Cong L. Lodato S. Kosuri S. Church GM. Arlotta P Efficient construction of sequence-specific TAL effectors for modulating mammalian transcription. Nat Biotechnol. 2011;29:149–153 and US Patent Nos. 8,450,471, 8,440,431 and 8,440,432, all of which are specifically incorporated by reference. By means of further guidance, and without limitation, naturally occurring TALEs or “wild type TALEs” are nucleic acid binding proteins secreted by numerous species of proteobacteria. TALE polypeptides contain a nucleic acid binding domain composed of tandem repeats of highly conserved monomer polypeptides that are predominantly 33, 34 or 35 amino acids in length and that differ from each other mainly in amino acid positions 12 and 13. In advantageous embodiments the nucleic acid is DNA. As used herein, the term “polypeptide monomers”, or “TALE monomers” will be used to refer to the highly conserved repetitive polypeptide sequences within the TALE nucleic acid binding domain and the term “repeat variable di-residues” or “RVD” will be

used to refer to the highly variable amino acids at positions 12 and 13 of the polypeptide monomers. As provided throughout the disclosure, the amino acid residues of the RVD are depicted using the IUPAC single letter code for amino acids. A general representation of a TALE monomer which is comprised within the DNA binding domain is X1-11-(X12X13)-X14-33 or 34 or 35, where the subscript indicates the amino acid position and X represents any amino acid. X12X13 indicate the RVDs. In some polypeptide monomers, the variable amino acid at position 13 is missing or absent and in such polypeptide monomers, the RVD consists of a single amino acid. In such cases the RVD may be alternatively represented as X*, where X represents X12 and (*) indicates that X13 is absent. The DNA binding domain comprises several repeats of TALE monomers and this may be represented as (X1-11-(X12X13)-X14-33 or 34 or 35)z, where in an advantageous embodiment, z is at least 5 to 40. In a further advantageous embodiment, z is at least 10 to 26. The TALE monomers have a nucleotide binding affinity that is determined by the identity of the amino acids in its RVD. For example, polypeptide monomers with an RVD of NI preferentially bind to adenine (A), polypeptide monomers with an RVD of NG preferentially bind to thymine (T), polypeptide monomers with an RVD of HD preferentially bind to cytosine (C) and polypeptide monomers with an RVD of NN preferentially bind to both adenine (A) and guanine (G). In yet another embodiment of the invention, polypeptide monomers with an RVD of IG preferentially bind to T. Thus, the number and order of the polypeptide monomer repeats in the nucleic acid binding domain of a TALE determines its nucleic acid target specificity. In still further embodiments of the invention, polypeptide monomers with an RVD of NS recognize all four base pairs and may bind to A, T, G or C. The structure and function of TALEs is further described in, for example, Moscou et al., Science 326:1501 (2009); Boch et al., Science 326:1509-1512 (2009); and Zhang et al., Nature Biotechnology 29:149-153 (2011), each of which is incorporated by reference in its entirety.

In certain embodiments, the nucleic acid modification is effected by a (modified) zinc-finger nuclease (ZFN) system. The ZFN system uses artificial restriction enzymes generated by fusing a zinc finger DNA-binding domain to a DNA-cleavage domain that can be engineered to target desired DNA sequences. Exemplary methods of genome

editing using ZFNs can be found for example in U.S. Patent Nos. 6,534,261, 6,607,882, 6,746,838, 6,794,136, 6,824,978, 6,866,997, 6,933,113, 6,979,539, 7,013,219, 7,030,215, 7,220,719, 7,241,573, 7,241,574, 7,585,849, 7,595,376, 6,903,185, and 6,479,626, all of which are specifically incorporated by reference. By means of further guidance, and without limitation, artificial zinc-finger (ZF) technology involves arrays of ZF modules to target new DNA-binding sites in the genome. Each finger module in a ZF array targets three DNA bases. A customized array of individual zinc finger domains is assembled into a ZF protein (ZFP). ZFPs can comprise a functional domain. The first synthetic zinc finger nucleases (ZFNs) were developed by fusing a ZF protein to the catalytic domain of the Type IIS restriction enzyme FokI. (Kim, Y. G. et al., 1994, Chimeric restriction endonuclease, Proc. Natl. Acad. Sci. U.S.A. 91, 883−887; Kim, Y. G. et al., 1996, Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. Proc. Natl. Acad. Sci. U.S.A. 93, 1156−1160). Increased cleavage specificity can be attained with decreased off target activity by use of paired ZFN heterodimers, each targeting different nucleotide sequences separated by a short spacer. (Doyon, Y. et al., 2011, Enhancing zinc-finger-nuclease activity with improved obligate heterodimeric architectures. Nat. Methods 8, 74−79). ZFPs can also be designed as transcription activators and repressors and have been used to target many genes in a wide variety of organisms.

In certain embodiments, the nucleic acid modification is effected by a (modified) meganuclease, which are endodeoxyribonucleases characterized by a large recognition site (double-stranded DNA sequences of 12 to 40 base pairs). Exemplary method for using meganucleases can be found in US Patent Nos: 8,163,514; 8,133,697; 8,021,867; 8,119,361; 8,119,381; 8,124,369; and 8,129,134, which are specifically incorporated by reference.

In certain embodiments, the nucleic acid modification is effected by a (modified) CRISPR/Cas complex or system. With respect to general information on CRISPR/Cas Systems, components thereof, and delivery of such components, including methods, materials, delivery vehicles, vectors, particles, and making and using thereof, including as to amounts and formulations, as well as Cas9CRISPR/Cas-expressing eukaryotic cells, Cas-9 CRISPR/Cas expressing eukaryotes, such as a mouse, reference is made to: US

Patents Nos. 8,999,641, 8,993,233, 8,697,359, 8,771,945, 8,795,965, 8,865,406, 8,871,445, 8,889,356, 8,889,418, 8,895,308, 8,906,616, 8,932,814, 8,945,839, 8,993,233 and 8,999,641; US Patent Publications US 2014-0310830 (US App. Ser. No. 14/105,031), US 2014-0287938 A1 (U.S. App. Ser. No. 14/213,991), US 2014-0273234 A1 (U.S. App. Ser. No. 14/293,674), US2014-0273232 A1 (U.S. App. Ser. No. 14/290,575), US 2014-0273231 (U.S. App. Ser. No. 14/259,420), US 2014-0256046 A1 (U.S. App. Ser. No. 14/226,274), US 2014-0248702 A1 (U.S. App. Ser. No. 14/258,458), US 2014-0242700 A1 (U.S. App. Ser. No. 14/222,930), US 2014-0242699 A1 (U.S. App. Ser. No. 14/183,512), US 2014-0242664 A1 (U.S. App. Ser. No. 14/104,990), US 2014-0234972 A1 (U.S. App. Ser. No. 14/183,471), US 2014-0227787 A1 (U.S. App. Ser. No. 14/256,912), US 2014-0189896 A1 (U.S. App. Ser. No. 14/105,035), US 2014-0186958 (U.S. App. Ser. No. 14/105,017), US 2014-0186919 A1 (U.S. App. Ser. No. 14/104,977), US 2014-0186843 A1 (U.S. App. Ser. No. 14/104,900), US 2014-0179770 A1 (U.S. App. Ser. No. 14/104,837) and US 2014-0179006 A1 (U.S. App. Ser. No. 14/183,486), US 2014-0170753 (US App Ser No 14/183,429); US 2015-0184139 (U.S. App. Ser. No. 14/324,960); 14/054,414 European Patent Applications EP 2 771 468 (EP13818570.7), EP 2 764 103 (EP13824232.6), and EP 2 784 162 (EP14170383.5); and PCT Patent Publications WO 2014/093661 (PCT/US2013/074743), WO 2014/093694 (PCT/US2013/074790), WO 2014/093595 (PCT/US2013/074611), WO 2014/093718 (PCT/US2013/074825), WO 2014/093709 (PCT/US2013/074812), WO 2014/093622 (PCT/US2013/074667), WO 2014/093635 (PCT/US2013/074691), WO 2014/093655 (PCT/US2013/074736), WO 2014/093712 (PCT/US2013/074819), WO 2014/093701 (PCT/US2013/074800), WO 2014/018423 (PCT/US2013/051418), WO 2014/204723 (PCT/US2014/041790), WO 2014/204724 (PCT/US2014/041800), WO 2014/204725 (PCT/US2014/041803), WO 2014/204726 (PCT/US2014/041804), WO 2014/204727 (PCT/US2014/041806), WO 2014/204728 (PCT/US2014/041808), WO 2014/204729 (PCT/US2014/041809), WO 2015/089351 (PCT/US2014/069897), WO 2015/089354 (PCT/US2014/069902), WO 2015/089364 (PCT/US2014/069925), WO 2015/089427 (PCT/US2014/070068), WO 2015/089462 (PCT/US2014/070127), WO 2015/089419 (PCT/US2014/070057), WO 2015/089465 (PCT/US2014/070135), WO 2015/089486

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Preferred DNA binding proteins are CRISPR/Cas enzymes or variants thereof. In certain embodiments, the CRISPR/Cas protein is a class 2 CRISPR/Cas protein. In certain embodiments, said CRISPR/Cas protein is a type II, type V, or type VI

CRISPR/Cas protein. The CRISPR/Cas system does not require the generation of customized proteins to target specific sequences but rather a single Cas protein can be programmed by an RNA guide (gRNA) to recognize a specific nucleic acid target, in other words the Cas enzyme protein can be recruited to a specific nucleic acid target locus (which may comprise or consist of RNA and/or DNA) of interest using said short RNA guide.

In general, the CRISPR/Cas or CRISPR system is as used herein foregoing documents refers collectively to elements involved in the expression of or directing the activity of CRISPR-associated ("Cas") proteins or genes, including sequences encoding a Cas protein and a guide RNA. In this context of the guide RNA this may include one or more of, a tracr (trans-activating CRISPR) sequence (e.g. tracrRNA or an active partial tracrRNA), a tracr-mate sequence (encompassing a "direct repeat" and a tracrRNA-processed partial direct repeat in the context of an endogenous CRISPR system), a guide sequence (also referred to as a "spacer" in the context of an endogenous CRISPR system), In general, a CRISPR system is characterized by elements that promote the formation of a CRISPR complex at the site of a target sequence. In the context of formation of a CRISPR complex, "target sequence" refers to a sequence to which a guide sequence is designed to have complementarity, where hybridization between a target DNA sequence and a guide sequence promotes the formation of a CRISPR complex.

In certain embodiments, the gRNA comprises a guide sequence fused to a tracr mate sequence (or direct repeat), and a tracr sequence In particular embodiments, the guide sequence fused to the tracr mate and the tracr sequence are provided or expressed as discrete RNA sequences. In preferred embodiments, the gRNA is a chimeric guide RNA or single guide RNA (sgRNA), comprising a guide sequence fused to the tracr mate which is itself linked to the tracr sequence. In particular embodiments, the CRISPR/Cas system or complex as described herein does not comprise and/or does not rely on the presence of a tracr sequence (e.g. if the Cas protein is Cpf1).

As used herein, the term "guide sequence" in the context of a CRISPR/Cas system, comprises any polynucleotide sequence having sufficient complementarity with a target nucleic acid sequence to hybridize with the target nucleic acid sequence and direct

sequence-specific binding of a nucleic acid-targeting complex to the target nucleic acid sequence. In some embodiments, the degree of complementarity, when optimally aligned using a suitable alignment algorithm, is about or more than about 50%, 60%, 75%, 80%, 85%, 90%, 95%, 97.5%, 99%, or more. Optimal alignment may be determined with the use of any suitable algorithm for aligning sequences, non-limiting example of which include the Smith-Waterman algorithm, the Needleman-Wunsch algorithm, algorithms based on the Burrows-Wheeler Transform (e.g., the Burrows Wheeler Aligner), ClustalW, Clustal X, BLAT, Novoalign (Novocraft Technologies; available at www.novocraft.com), ELAND (Illumina, San Diego, CA), SOAP (available at soap.genomics.org.cn), and Maq (available at maq.sourceforge.net). The ability of a guide sequence (within a nucleic acid-targeting guide RNA) to direct sequence-specific binding of a nucleic acid -targeting complex to a target nucleic acid sequence may be assessed by any suitable assay.

A guide sequence, and hence a nucleic acid-targeting guide RNA may be selected to target any target nucleic acid sequence. The target sequence may be DNA. The target sequence may be genomic DNA. The target sequence may be mitochondrial DNA.

In certain embodiments, the gRNA comprises a stem loop, preferably a single stem loop. In certain embodiments, the direct repeat sequence forms a stem loop, preferably a single stem loop. In certain embodiments, the spacer length of the guide RNA is from 15 to 35 nt. In certain embodiments, the spacer length of the guide RNA is at least 15 nucleotides. In certain embodiments, the spacer length is from 15 to 17 nt, e.g., 15, 16, or 17 nt, from 17 to 20 nt, e.g., 17, 18, 19, or 20 nt, from 20 to 24 nt, e.g., 20, 21, 22, 23, or 24 nt, from 23 to 25 nt, e.g., 23, 24, or 25 nt, from 24 to 27 nt, e.g., 24, 25, 26, or 27 nt, from 27-30 nt, e.g., 27, 28, 29, or 30 nt, from 30-35 nt, e.g., 30, 31, 32, 33, 34, or 35 nt, or 35 nt or longer. In particular embodiments, the CRISPR/Cas system requires a tracrRNA. The “tracrRNA” sequence or analogous terms includes any polynucleotide sequence that has sufficient complementarity with a crRNA sequence to hybridize. In some embodiments, the degree of complementarity between the tracrRNA sequence and crRNA sequence along the length of the shorter of the two when optimally aligned is about or more than about 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 97.5%,

99%, or higher. In some embodiments, the tracr sequence is about or more than about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 40, 50, or more nucleotides in length. In some embodiments, the tracr sequence and gRNA sequence are contained within a single transcript, such that hybridization between the two produces a transcript having a secondary structure, such as a hairpin. In an embodiment of the invention, the transcript or transcribed polynucleotide sequence has at least two or more hairpins. In preferred embodiments, the transcript has two, three, four or five hairpins. In a further embodiment of the invention, the transcript has at most five hairpins. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop may correspond to the tracr mate sequence, and the portion of the sequence 3' of the loop then corresponds to the tracr sequence. In a hairpin structure the portion of the sequence 5' of the final "N" and upstream of the loop may alternatively correspond to the tracr sequence, and the portion of the sequence 3' of the loop corresponds to the tracr mate sequence. In alternative embodiments, the CRISPR/Cas system does not require a tracrRNA, as is known by the skilled person.

In certain embodiments, the guide RNA (capable of guiding Cas to a target locus) may comprise (1) a guide sequence capable of hybridizing to a target locus and (2) a tracr mate or direct repeat sequence (in 5' to 3' orientation, or alternatively in 3' to 5' orientation, depending on the type of Cas protein, as is known by the skilled person). In particular embodiments, the CRISPR/Cas protein is characterized in that it makes use of a guide RNA comprising a guide sequence capable of hybridizing to a target locus and a direct repeat sequence, and does not require a tracrRNA. In particular embodiments, where the CRISPR/Cas protein is characterized in that it makes use of a tracrRNA, the guide sequence, tracr mate, and tracr sequence may reside in a single RNA, i.e. an sgRNA (arranged in a 5' to 3' orientation or alternatively arranged in a 3' to 5' orientation), or the tracr RNA may be a different RNA than the RNA containing the guide and tracr mate sequence. In these embodiments, the tracr hybridizes to the tracr mate sequence and directs the CRISPR/Cas complex to the target sequence.

In particular embodiments, the DNA binding protein is a catalytically active protein. In these embodiments, the formation of a nucleic acid-targeting complex

(comprising a guide RNA hybridized to a target sequence results in modification (such as cleavage) of one or both DNA or RNA strands in or near (e.g., within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from) the target sequence. As used herein the term "sequence(s) associated with a target locus of interest" refers to sequences near the vicinity of the target sequence (e.g. within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50, or more base pairs from the target sequence, wherein the target sequence is comprised within a target locus of interest). The skilled person will be aware of specific cut sites for selected CRISPR/Cas systems, relative to the target sequence, which as is known in the art may be within the target sequence or alternatively 3' or 5' of the target sequence.

Accordingly, in particular embodiments, the DNA binding protein has nucleic acid cleavage activity. In some embodiments, the nuclease as described herein may direct cleavage of one or both nucleic acid (DNA, RNA, or hybrids, which may be single or double stranded) strands at the location of or near a target sequence, such as within the target sequence and/or within the complement of the target sequence or at sequences associated with the target sequence. In some embodiments, the nucleic acid-targeting effector protein may direct cleavage of one or both DNA or RNA strands within about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 50, 100, 200, 500, or more base pairs from the first or last nucleotide of a target sequence. In some embodiments, the cleavage may be blunt (e.g. for Cas9, such as SaCas9 or SpCas9). In some embodiments, the cleavage may be staggered (e.g. for Cpf1), i.e. generating sticky ends. In some embodiments, the cleavage is a staggered cut with a 5' overhang. In some embodiments, the cleavage is a staggered cut with a 5' overhang of 1 to 5 nucleotides, preferably of 4 or 5 nucleotides. In some embodiments, the cleavage site is upstream of the PAM. In some embodiments, the cleavage site is downstream of the PAM.

In certain embodiments, the target sequence should be associated with a PAM (protospacer adjacent motif) or PFS (protospacer flanking sequence or site); that is, a short sequence recognized by the CRISPR complex. The precise sequence and length requirements for the PAM differ depending on the CRISPR enzyme used, but PAMs are typically 2-5 base pair sequences adjacent the protospacer (that is, the target sequence). Examples of PAM sequences are given in the examples section below, and the skilled

person will be able to identify further PAM sequences for use with a given CRISPR enzyme. Further, engineering of the PAM Interacting (PI) domain may allow programing of PAM specificity, improve target site recognition fidelity, and increase the versatility of the Cas, e.g. Cas9, genome engineering platform. Cas proteins, such as Cas9 proteins may be engineered to alter their PAM specificity, for example as described in Kleinstiver BP et al. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature. 2015 Jul 23;523(7561):481-5. doi: 10.1038/nature14592. In some embodiments, the method comprises allowing a CRISPR complex to bind to the target polynucleotide to effect cleavage of said target polynucleotide thereby modifying the target polynucleotide, wherein the CRISPR complex comprises a CRISPR enzyme complexed with a guide sequence hybridized to a target sequence within said target polynucleotide, wherein said guide sequence is linked to a tracr mate sequence which in turn hybridizes to a tracr sequence. The skilled person will understand that other Cas proteins may be modified analogously.

In some embodiments, the nucleic acid-targeting effector protein may be mutated with respect to a corresponding wild-type enzyme such that the mutated nucleic acid-targeting effector protein lacks the ability to cleave one or both DNA strands of a target polynucleotide containing a target sequence. As a further example, two or more catalytic domains of a Cas protein (e.g. RuvC I, RuvC II, and RuvC III or the HNH domain of a Cas9 protein) may be mutated to produce a mutated Cas protein which cleaves only one DNA strand of a target sequence.

In particular embodiments, the nucleic acid-targeting effector protein may be mutated with respect to a corresponding wild-type enzyme such that the mutated nucleic acid-targeting effector protein lacks substantially all DNA cleavage activity. In some embodiments, a nucleic acid-targeting effector protein may be considered to substantially lack all DNA and/or RNA cleavage activity when the cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the nucleic acid cleavage activity of the non-mutated form of the enzyme; an example can be when the nucleic acid cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form.

As used herein, the term "modified" Cas generally refers to a Cas protein having one or more modifications or mutations (including point mutations, truncations, insertions, deletions, chimeras, fusion proteins, etc.) compared to the wild type Cas protein from which it is derived. By derived is meant that the derived enzyme is largely based, in the sense of having a high degree of sequence homology with, a wildtype enzyme, but that it has been mutated (modified) in some way as known in the art or as described herein.

As detailed above, in certain embodiments, the nuclease as referred to herein is modified. As used herein, the term "modified" refers to which may or may not have an altered functionality. By means of example, and in particular with reference to Cas proteins, modifications which do not result in an altered functionality include for instance codon optimization for expression into a particular host, or providing the nuclease with a particular marker (e.g. for visualization). Modifications with may result in altered functionality may also include mutations, including point mutations, insertions, deletions, truncations (including split nucleases), etc., as well as chimeric nucleases (e.g. comprising domains from different orthologues or homologues) or fusion proteins. Fusion proteins may without limitation include for instance fusions with heterologous domains or functional domains (e.g. localization signals, catalytic domains, etc.). Accordingly, in certain embodiments, the modified nuclease may be used as a generic nucleic acid binding protein with fusion to or being operably linked to a functional domain. In certain embodiments, various different modifications may be combined (e.g. a mutated nuclease which is catalytically inactive and which further is fused to a functional domain, such as for instance to induce DNA methylation or another nucleic acid modification, such as including without limitation a break (e.g. by a different nuclease (domain)), a mutation, a deletion, an insertion, a replacement, a ligation, a digestion, a break or a recombination). As used herein, "altered functionality" includes without limitation an altered specificity (e.g. altered target recognition, increased (e.g. "enhanced" Cas proteins) or decreased specificity, or altered PAM recognition), altered activity (e.g. increased or decreased catalytic activity, including catalytically inactive nucleases or nickases), and/or altered stability (e.g. fusions with destalilization domains).

Suitable heterologous domains include without limitation a nuclease, a ligase, a repair protein, a methyltransferase, (viral) integrase, a recombinase, a transposase, an argonaute, a cytidine deaminase, a retron, a group II intron, a phosphatase, a phosphorylase, a sulpfurylase, a kinase, a polymerase, an exonuclease, etc.. Examples of all these modifications are known in the art. It will be understood that a "modified" nuclease as referred to herein, and in particular a "modified" Cas or "modified" CRISPR/Cas system or complex preferably still has the capacity to interact with or bind to the polynucleic acid (e.g. in complex with the gRNA).

By means of further guidance and without limitation, in certain embodiments, the nuclease may be modified as detailed below. As already indicated, more than one of the indicated modifications may be combined. For instance, codon optimization may be combined with NLS or NES fusions, catalytically inactive nuclease modifications or nickase mutants may be combined with fusions to functional (heterologous) domains, etc.

In certain embodiments, the nuclease, and in particular the Cas proteins of prokaryotic origin, may be codon optimized for expression into a particular host (cell). An example of a codon optimized sequence, is in this instance a sequence optimized for expression in a eukaryote, e.g., humans (i.e. being optimized for expression in humans), or for another eukaryote, animal or mammal as herein discussed; see, e.g., SaCas9 human codon optimized sequence in WO 2014/093622 (PCT/US2013/074667). Whilst this is preferred, it will be appreciated that other examples are possible and codon optimization for a host species other than human, or for codon optimization for specific organs is known. In some embodiments, an enzyme coding sequence encoding a Cas is codon optimized for expression in particular cells, such as eukaryotic cells. The eukaryotic cells may be those of or derived from a particular organism, such as a mammal, including but not limited to human, or non-human eukaryote or animal or mammal as herein discussed, e.g., mouse, rat, rabbit, dog, livestock, or non-human mammal or primate. In some embodiments, processes for modifying the germ line genetic identity of human beings and/or processes for modifying the genetic identity of animals which are likely to cause them suffering without any substantial medical benefit to man or animal, and also animals resulting from such processes, may be excluded. In general, codon optimization

refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cells of interest by replacing at least one codon (e.g. about or more than about 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more codons) of the native sequence with codons that are more frequently or most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Various species exhibit particular bias for certain codons of a particular amino acid. Codon bias (differences in codon usage between organisms) often correlates with the efficiency of translation of messenger RNA (mRNA), which is in turn believed to be dependent on, among other things, the properties of the codons being translated and the availability of particular transfer RNA (tRNA) molecules. The predominance of selected tRNAs in a cell is generally a reflection of the codons used most frequently in peptide synthesis. Accordingly, genes can be tailored for optimal gene expression in a given organism based on codon optimization. Codon usage tables are readily available, for example, at the "Codon Usage Database" available at www.kazusa.orjp/codon/ and these tables can be adapted in a number of ways. See Nakamura, Y., et al. "Codon usage tabulated from the international DNA sequence databases: status for the year 2000" Nucl. Acids Res. 28:292 (2000). Computer algorithms for codon optimizing a particular sequence for expression in a particular host cell are also available, such as Gene Forge (Aptagen; Jacobus, PA), are also available. In some embodiments, one or more codons (e.g. 1, 2, 3, 4, 5, 10, 15, 20, 25, 50, or more, or all codons) in a sequence encoding a Cas correspond to the most frequently used codon for a particular amino acid. Codon optimization may be for expression into any desired host (cell), including mammalian, plant, algae, or yeast.

In certain embodiments, the nuclease, in particular the Cas protein, may comprise one or more modifications resulting in enhanced activity and/or specificity, such as including mutating residues that stabilize the targeted or non-targeted strand (e.g. eCas9; "Rationally engineered Cas9 nucleases with improved specificity", Slaymaker et al. (2016), Science, 351(6268):84-88, incorporated herewith in its entirety by reference). In certain embodiments, the altered or modified activity of the engineered CRISPR protein comprises increased targeting efficiency or decreased off-target binding. In certain embodiments, the altered activity of the engineered CRISPR protein comprises modified

cleavage activity. In certain embodiments, the altered activity comprises increased cleavage activity as to the target polynucleotide loci. In certain embodiments, the altered activity comprises decreased cleavage activity as to the target polynucleotide loci. In certain embodiments, the altered activity comprises decreased cleavage activity as to off-target polynucleotide loci. In certain embodiments, the altered or modified activity of the modified nuclease comprises altered helicase kinetics. In certain embodiments, the modified nuclease comprises a modification that alters association of the protein with the nucleic acid molecule comprising RNA (in the case of a Cas protein), or a strand of the target polynucleotide loci, or a strand of off-target polynucleotide loci. In an aspect of the invention, the engineered CRISPR protein comprises a modification that alters formation of the CRISPR complex. In certain embodiments, the altered activity comprises increased cleavage activity as to off-target polynucleotide loci. Accordingly, in certain embodiments, there is increased specificity for target polynucleotide loci as compared to off-target polynucleotide loci. In other embodiments, there is reduced specificity for target polynucleotide loci as compared to off-target polynucleotide loci. In certain embodiments, the mutations result in decreased off-target effects (e.g. cleavage or binding properties, activity, or kinetics), such as in case for Cas proteins for instance resulting in a lower tolerance for mismatches between target and gRNA. Other mutations may lead to increased off-target effects (e.g. cleavage or binding properties, activity, or kinetics). Other mutations may lead to increased or decreased on-target effects (e.g. cleavage or binding properties, activity, or kinetics). In certain embodiments, the mutations result in altered (e.g. increased or decreased) helicase activity, association or formation of the functional nuclease complex (e.g. CRISPR/Cas complex). In certain embodiments, the mutations result in an altered PAM recognition, i.e. a different PAM may be (in addition or in the alternative) be recognized, compared to the unmodified Cas protein (see e.g. “Engineered CRISPR-Cas9 nucleases with altered PAM specificities”, Kleinstiver et al. (2015), Nature, 523(7561):481-485, incorporated herein by reference in its entirety). Particularly preferred mutations include positively charged residues and/or (evolutionary) conserved residues, such as conserved positively charged residues, in

order to enhance specificity. In certain embodiments, such residues may be mutated to uncharged residues, such as alanine.

In certain embodiments, the nuclease, in particular the Cas protein, may comprise one or more modifications resulting in a nuclease that has reduced or no catalytic activity, or alternatively (in case of nucleases that target double stranded nucleic acids) resulting in a nuclease that only cleaves one strand, i.e. a nickase. By means of further guidance, and without limitation, for example, an aspartate-to-alanine substitution (D10A) in the RuvC I catalytic domain of Cas9 from S. pyogenes converts Cas9 from a nuclease that cleaves both strands to a nickase (cleaves a single strand). Other examples of mutations that render Cas9 a nickase include, without limitation, H840A, N854A, and N863A. As further guidance, where the enzyme is not SpCas9, mutations may be made at any or all residues corresponding to positions 10, 762, 840, 854, 863 and/or 986 of SpCas9 (which may be ascertained for instance by standard sequence comparison tools). In particular, any or all of the following mutations are preferred in SpCas9: D10A, E762A, H840A, N854A, N863A and/or D986A; as well as conservative substitution for any of the replacement amino acids is also envisaged. As a further example, two or more catalytic domains of Cas9 (RuvC I, RuvC II, and RuvC III or the HNH domain) may be mutated to produce a mutated Cas9 substantially lacking all DNA cleavage activity. In some embodiments, a D10A mutation is combined with one or more of H840A, N854A, or N863A mutations to produce a Cas9 enzyme substantially lacking all DNA cleavage activity. In some embodiments, a Cas is considered to substantially lack all DNA cleavage activity when the DNA cleavage activity of the mutated enzyme is about no more than 25%, 10%, 5%, 1%, 0.1%, 0.01%, or less of the DNA cleavage activity of the non-mutated form of the enzyme; an example can be when the DNA cleavage activity of the mutated form is nil or negligible as compared with the non-mutated form. Thus, the Cas may comprise one or more mutations and may be used as a generic DNA binding protein with or without fusion to a functional domain. The mutations may be artificially introduced mutations or gain- or loss-of-function mutations. The mutations may include but are not limited to mutations in one of the catalytic domains (e.g., D10 and H840) in the RuvC and HNH catalytic domains respectively; or the CRISPR enzyme can comprise

one or more mutations selected from the group consisting of D10A, E762A, H840A, N854A, N863A or D986A and/or one or more mutations in a RuvC1 or HNH domain of the Cas or has a mutation as otherwise as discussed herein.

In certain embodiments, the nuclease is a split nuclease (see e.g. "A split-Cas9 architecture for inducible genome editing and transcription modulation", Zetsche et al. (2015), Nat Biotechnol. 33(2):139-42, incorporated herein by reference in its entirety). In a split nuclease, the activity (which may be a modified activity, as described herein elsewhere), relies on the two halves of the split nuclease to be joined, i.e. each half of the split nuclease does not possess the required activity, until joined. As further guidance, and without limitation, with specific reference to Cas9, a split Cas9 may result from splitting the Cas9 at any one of the following split points, according or with reference to SpCas9: a split position between 202A/203S; a split position between 255F/256D; a split position between 310E/311I; a split position between 534R/535K; a split position between 572E/573C; a split position between 713S/714G; a split position between 1003L/104E; a split position between 1054G/1055E; a split position between 1114N/1115S; a split position between 1152K/1153S; a split position between 1245K/1246G; or a split between 1098 and 1099. Identifying potential split sides is most simply done with the help of a crystal structure. For Sp mutants, it should be readily apparent what the corresponding position for, for example, a sequence alignment. For non-Sp enzymes one can use the crystal structure of an ortholog if a relatively high degree of homology exists between the ortholog and the intended Cas9. Ideally, the split position should be located within a region or loop. Preferably, the split position occurs where an interruption of the amino acid sequence does not result in the partial or full destruction of a structural feature (e.g. alpha-helixes or beta-sheets). Unstructured regions (regions that did not show up in the crystal structure because these regions are not structured enough to be "frozen" in a crystal) are often preferred options. In certain embodiments, a functional domain may be provided on each of the split halves, thereby allowing the formation of homodimers or heterodimers. The functional domains may be (inducible) interact, thereby joining the split halves, and reconstituting (modified) nuclease activity. By means of example, an inducer energy source may inducibly allow

dimerization of the split halves, through appropriate fusion partners. An inducer energy source may be considered to be simply an inducer or a dimerizing agent. The term 'inducer energy source' is used herein throughout for consistency. The inducer energy source (or inducer) acts to reconstitute the Cas9. In some embodiments, the inducer energy source brings the two parts of the Cas9 together through the action of the two halves of the inducible dimer. The two halves of the inducible dimer therefore are brought tougher in the presence of the inducer energy source. The two halves of the dimer will not form into the dimer (dimerize) without the inducer energy source. Thus, the two halves of the inducible dimer cooperate with the inducer energy source to dimerize the dimer. This in turn reconstitutes the Cas9 by bringing the first and second parts of the Cas9 together. The CRISPR enzyme fusion constructs each comprise one part of the split Cas9. These are fused, preferably via a linker such as a GlySer linker described herein, to one of the two halves of the dimer. The two halves of the dimer may be substantially the same two monomers that together that form the homodimer, or they may be different monomers that together form the heterodimer. As such, the two monomers can be thought of as one half of the full dimer. The Cas9 is split in the sense that the two parts of the Cas9 enzyme substantially comprise a functioning Cas9. That Cas9 may function as a genome editing enzyme (when forming a complex with the target DNA and the guide), such as a nickase or a nuclease (cleaving both strands of the DNA), or it may be a deadCas9 which is essentially a DNA-binding protein with very little or no catalytic activity, due to typically two or more mutations in its catalytic domains as described herein further.

In certain embodiments, the nuclease may comprise one or more additional (heterologous) functional domains, i.e. the modified nuclease is a fusion protein comprising the nuclease itself and one or more additional domains, which may be fused C-terminally or N-terminally to the nuclease, or alternatively inserted at suitable and appropriate sited internally within the nuclease (preferably without perturbing its function, which may be an otherwise modified function, such as including reduced or absent catalytic activity, nickase activity, etc.). any type of functional domain may suitably be used, such as without limitation including functional domains having one or

more of the following activities: (DNA or RNA) methyltransferase activity, methylase activity, demethylase activity, DNA hydroxylmethylase domain, histone acetylase domain, histone deacetylases domain, transcription or translation activation activity, transcription or translation repression activity, transcription or translation release factor activity, histone modification activity, nuclease activity, single-strand RNA cleavage activity, double-strand RNA cleavage activity, single-strand DNA cleavage activity, double-strand DNA cleavage activity, nucleic acid binding activity, a protein acetyltransferase, a protein deacetylase, a protein methyltransferase, a protein deaminase, a protein kinase, a protein phosphatase, transposase domain, integrase domain, recombinase domain, resolvase domain, invertase domain, protease domain, repressor domain, activator domain, nuclear-localization signal domains, transcription-regulatory protein (or transcription complex recruiting) domain, cellular uptake activity associated domain, nucleic acid binding domain, antibody presentation domain, histone modifying enzymes, recruiter of histone modifying enzymes; inhibitor of histone modifying enzymes, histone methyltransferase, histone demethylase, histone kinase, histone phosphatase, histone ribosylase, histone deribosylase, histone ubiquitinase, histone deubiquitinase, histone biotinase, histone tail protease, HDACs, histone methyltransferases (HMTs), and histone acetyltransferase (HAT) inhibitors, as well as HDAC and HMT recruiting proteins, HDAC Effector Domains, HDAC Recruiter Effector Domains, Histone Methyltransferase (HMT) Effector Domains, Histone Methyltransferase (HMT) Recruiter Effector Domains, or Histone Acetyltransferase Inhibitor Effector Domains. In some embodiments, the functional domain is an epigenetic regulator; see, e.g., Zhang et al., US Patent No. 8,507,272 (incorporated herein by reference in its entirety). In some embodiments, the functional domain is a transcriptional activation domain, such as VP64, p65, MyoD1, HSF1, RTA, SET7/9 or a histone acetyltransferase. In some embodiments, the functional domain is a transcription repression domain, such as KRAB. In some embodiments, the transcription repression domain is SID, or concatemers of SID (eg SID4X), NuE, or NcoR. In some embodiments, the functional domain is an epigenetic modifying domain, such that an epigenetic modifying enzyme is provided. In some embodiments, the functional domain

is an activation domain, which may be the P65 activation domain. In some embodiments, the functional domain comprises nuclease activity. In one such embodiment, the functional domain may comprise Fok1. Mention is made of U.S. Pat. Pub. 2014/0356959, U.S. Pat. Pub. 2014/0342456, U.S. Pat. Pub. 2015/0031132, and Mali, P. et al., 2013, Science 339(6121):823-6, doi: 10.1126/science.1232033, published online 3 January 2013 and through the teachings herein the invention comprehends methods and materials of these documents applied in conjunction with the teachings herein. It is to be understood that also destabilization domains or localization domains as described herein elsewhere are encompassed by the generic term "functional domain". In certain embodiments, one or more functional domains are associated with the nuclease itself. In some embodiments, one or more functional domains are associated with an adaptor protein, for example as used with the modified guides of Konnerman et al. (Nature 517(7536): 583-588, 2015; incorporated herein by reference in its entirety), and hene form part of a Synergistic activator mediator (SAM) complex. The adaptor proteins may include but are not limited to orthogonal RNA-binding protein / aptamer combinations that exist within the diversity of bacteriophage coat proteins. A list of such coat proteins includes, but is not limited to: Qβ, F2, GA, fr, JP501, M12, R17, BZ13, JP34, JP500, KU1, M11, MX1, TW18, VK, SP, FI, ID2, NL95, TW19, AP205, ϕCb5, ϕCb8r, ϕCb12r, ϕCb23r, 7s and PRR1. These adaptor proteins or orthogonal RNA binding proteins can further recruit effector proteins or fusions which comprise one or more functional domains.

In certain embodiments, the nuclease, in particular the Cas protein, may comprise one or more modifications resulting in a destabilized nuclease when expressed in a host (cell). Such may be achieved by fusion of the nuclease with a destabilization domain (DD). Destabilizing domains have general utility to confer instability to a wide range of proteins; see, e.g., Miyazaki, J Am Chem Soc. Mar 7, 2012; 134(9): 3942–3945, incorporated herein by reference. CMP8 or 4-hydroxytamoxifen can be destabilizing domains. More generally, A temperature-sensitive mutant of mammalian DHFR (DHFRts), a destabilizing residue by the N-end rule, was found to be stable at a permissive temperature but unstable at 37 °C. The addition of methotrexate, a high-

affinity ligand for mammalian DHFR, to cells expressing DHFRts inhibited degradation of the protein partially. This was an important demonstration that a small molecule ligand can stabilize a protein otherwise targeted for degradation in cells. A rapamycin derivative was used to stabilize an unstable mutant of the FRB domain of mTOR (FRB*) and restore the function of the fused kinase, GSK-3β.6,7 This system demonstrated that ligand-dependent stability represented an attractive strategy to regulate the function of a specific protein in a complex biological environment. A system to control protein activity can involve the DD becoming functional when the ubiquitin complementation occurs by rapamycin induced dimerization of FK506-binding protein and FKBP12. Mutants of human FKBP12 or ecDHFR protein can be engineered to be metabolically unstable in the absence of their high-affinity ligands, Shield-1 or trimethoprim (TMP), respectively. These mutants are some of the possible destabilizing domains (DDs) useful in the practice of the invention and instability of a DD as a fusion with a CRISPR enzyme confers to the CRISPR protein degradation of the entire fusion protein by the proteasome. Shield-1 and TMP bind to and stabilize the DD in a dose-dependent manner. The estrogen receptor ligand binding domain (ERLBD, residues 305-549 of ERS1) can also be engineered as a destabilizing domain. Since the estrogen receptor signaling pathway is involved in a variety of diseases such as breast cancer, the pathway has been widely studied and numerous agonist and antagonists of estrogen receptor have been developed. Thus, compatible pairs of ERLBD and drugs are known. There are ligands that bind to mutant but not wild-type forms of the ERLBD. By using one of these mutant domains encoding three mutations (L384M, M421G, G521R)12, it is possible to regulate the stability of an ERLBD-derived DD using a ligand that does not perturb endogenous estrogen-sensitive networks. An additional mutation (Y537S) can be introduced to further destabilize the ERLBD and to configure it as a potential DD candidate. This tetra-mutant is an advantageous DD development. The mutant ERLBD can be fused to a CRISPR enzyme and its stability can be regulated or perturbed using a ligand, whereby the CRISPR enzyme has a DD. Another DD can be a 12-kDa (107-amino-acid) tag based on a mutated FKBP protein, stabilized by Shield1 ligand; see, e.g., Nature Methods 5, (2008). For instance a DD can be a modified FK506 binding protein 12 (FKBP12) that

binds to and is reversibly stabilized by a synthetic, biologically inert small molecule, Shield-1; see, e.g., Banaszynski LA, Chen LC, Maynard-Smith LA, Ooi AG, Wandless TJ. A rapid, reversible, and tunable method to regulate protein function in living cells using synthetic small molecules. Cell. 2006;126:995–1004; Banaszynski LA, Sellmyer MA, Contag CH, Wandless TJ, Thorne SH. Chemical control of protein stability and function in living mice. Nat Med. 2008;14:1123–1127; Maynard-Smith LA, Chen LC, Banaszynski LA, Ooi AG, Wandless TJ. A directed approach for engineering conditional protein stability using biologically silent small molecules. The Journal of biological chemistry. 2007;282:24866–24872; and Rodriguez, Chem Biol. Mar 23, 2012; 19(3): 391–398—all of which are incorporated herein by reference and may be employed in the practice of the invention in selected a DD to associate with a CRISPR enzyme in the practice of this invention. As can be seen, the knowledge in the art includes a number of DDs, and the DD can be associated with, e.g., fused to, advantageously with a linker, to a CRISPR enzyme, whereby the DD can be stabilized in the presence of a ligand and when there is the absence thereof the DD can become destabilized, whereby the CRISPR enzyme is entirely destabilized, or the DD can be stabilized in the absence of a ligand and when the ligand is present the DD can become destabilized; the DD allows the CRISPR enzyme and hence the CRISPR-Cas complex or system to be regulated or controlled—turned on or off so to speak, to thereby provide means for regulation or control of the system, e.g., in an in vivo or in vitro environment. For instance, when a protein of interest is expressed as a fusion with the DD tag, it is destabilized and rapidly degraded in the cell, e.g., by proteasomes. Thus, absence of stabilizing ligand leads to a D associated Cas being degraded. When a new DD is fused to a protein of interest, its instability is conferred to the protein of interest, resulting in the rapid degradation of the entire fusion protein. Peak activity for Cas is sometimes beneficial to reduce off-target effects. Thus, short bursts of high activity are preferred. The present invention is able to provide such peaks. In some senses the system is inducible. In some other senses, the system repressed in the absence of stabilizing ligand and de-repressed in the presence of stabilizing ligand. By means of example, and without limitation, in some embodiments, the DD is ER50. A corresponding stabilizing ligand for this DD is, in some embodiments, 4HT. As such, in

some embodiments, one of the at least one DDs is ER50 and a stabilizing ligand therefor is 4HT or CMP8. In some embodiments, the DD is DHFR50. A corresponding stabilizing ligand for this DD is, in some embodiments, TMP. As such, in some embodiments, one of the at least one DDs is DHFR50 and a stabilizing ligand therefor is TMP. In some embodiments, the DD is ER50. A corresponding stabilizing ligand for this DD is, in some embodiments, CMP8. CMP8 may therefore be an alternative stabilizing ligand to 4HT in the ER50 system. While it may be possible that CMP8 and 4HT can/should be used in a competitive matter, some cell types may be more susceptible to one or the other of these two ligands, and from this disclosure and the knowledge in the art the skilled person can use CMP8 and/or 4HT. More than one (the same or different) DD may be present, and may be fused for instance C-terminally, or N-terminally, or even internally at suitable locations. Having two or more DDs which are heterologous may be advantageous as it would provide a greater level of degradation control.

In some embodiments, the fusion protein as described herein may comprise a linker between the nuclease and the fusion partner (e.g. functional domain). In some embodiments, the linker is a GlySer linker. Attachment of a functional domain or fusion protein can be via a linker, e.g., a flexible glycine-serine (GlyGlyGlySer) or (GGGS)3 or a rigid alpha-helical linker such as (Ala(GluAlaAlaAlaLys)Ala). Linkers such as (GGGGS)3 are preferably used herein to separate protein or peptide domains. (GGGGS)3 is preferable because it is a relatively long linker (15 amino acids). The glycine residues are the most flexible and the serine residues enhance the chance that the linker is on the outside of the protein. (GGGGS)6 (GGGGS)9 or (GGGGS)12 may preferably be used as alternatives. Other preferred alternatives are (GGGGS)1, (GGGGS)2, (GGGGS)4, (GGGGS)5, (GGGGS)7, (GGGGS)8, (GGGGS)10, or (GGGGS)11. Alternative linkers are available, but highly flexible linkers are thought to work best to allow for maximum opportunity for the 2 parts of the Cas9 to come together and thus reconstitute Cas9 activity. One alternative is that the NLS of nucleoplasmin can be used as a linker. For example, a linker can also be used between the Cas9 and any functional domain. Again, a (GGGGS)3 linker may be used here (or the 6, 9, or 12 repeat versions therefore) or the NLS of nucleoplasmin can be used as a linker between Cas9 and the functional domain.

In some embodiments, the nuclease is fused to one or more localization signals, such as nuclear localization sequences (NLSs), such as about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs. In some embodiments, the nuclease comprises about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the amino-terminus, about or more than about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more NLSs at or near the carboxy-terminus, or a combination of these (e.g. zero or at least one or more NLS at the amino-terminus and zero or at one or more NLS at the carboxy terminus). When more than one NLS is present, each may be selected independently of the others, such that a single NLS may be present in more than one copy and/or in combination with one or more other NLSs present in one or more copies. In a preferred embodiment of the invention, the nuclease comprises at most 6 NLSs. In some embodiments, an NLS is considered near the N- or C-terminus when the nearest amino acid of the NLS is within about 1, 2, 3, 4, 5, 10, 15, 20, 25, 30, 40, 50, or more amino acids along the polypeptide chain from the N- or C-terminus. Non-limiting examples of NLSs include an NLS sequence derived from: the NLS of the SV40 virus large T-antigen, having the amino acid sequence PKKKRKV; the NLS from nucleoplasmin (e.g. the nucleoplasmin bipartite NLS with the sequence KRPAATKKAGQAKKKK); the c-myc NLS having the amino acid sequence PAAKRVKLD or RQRRNELKRSP; the hRNPA1 M9 NLS having the sequence NQSSNFGPMKGGNFGGRSSGPYGGGGQYFAKPRNQGGY; the sequence RMRIZFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from importin-alpha; the sequences VSRKRPRP and PPKKARED of the myoma T protein; the sequence POPKKKPL of human p53; the sequence SALIKKKKKMAP of mouse c-abl IV; the sequences DRLRR and PKQKKRK of the influenza virus NS1; the sequence RKLKKKIKKL of the Hepatitis virus delta antigen; the sequence REKKKFLKRR of the mouse Mx1 protein; the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase; and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid.

With particular reference to the CRISPR/Cas system as described herein, besides the Cas protein, in addition or in the alternative, the gRNA and/or tracr (where

applicable) and/or tracr mate (or direct repeat) may be modified. Suitable modifications include, without limitation dead guides, escorted guides, protected guides, or guides provided with aptamers, suitable for ligating to, binding or recruiting functional domains (see e.g. also elsewhere herein the reference to synergistic activator mediators (SAM)). Mention is also made of WO/2016/049258 (FUNCTIONAL SCREENING WITH OPTIMIZED FUNCTIONAL CRISPR-CAS SYSTEMS (SAM)), WO/2016/094867 (PROTECTED GUIDE RNAS (PGRNAS); WO/2016/094872 (DEAD GUIDES FOR CRISPR TRANSCRIPTION FACTORS); WO/2016/094874 (ESCORTED AND FUNCTIONALIZED GUIDES FOR CRISPR-CAS SYSTEMS); all incorporated herein by reference. In certain embodiments, the tracr sequence (where appropriate) and/or tracr mate sequence (direct repeat), may comprise one or more protein-interacting RNA aptamers. The one or more aptamers may be located in the tetraloop and/or stemloop 2 of the tracr sequence. The one or more aptamers may be capable of binding MS2 bacteriophage coat protein. In certain embodiments, the gRNA (or trace or tracr mate) is modified by truncations, and/or incorporation of one or more mismatches vis-à-vis the intended target sequence or sequence to hybridize with.

By means of further guidance, and without limitation, in certain embodiments, the gRNA is a dead gRNA (dgRNA), which are guide sequences which are modified in a manner which allows for formation of the CRISPR complex and successful binding to the target, while at the same time, not allowing for successful nuclease activity (i.e. without nuclease activity / without indel activity). These dead guides or dead guide sequences can be thought of as catalytically inactive or conformationally inactive with regard to nuclease activity. Several structural parameters allow for a proper framework to arrive at such dead guides. Dead guide sequences are shorter than respective guide sequences which result in active Cas-specific indel formation. Dead guides are 5%, 10%, 20%, 30%, 40%, 50%, shorter than respective guides directed to the same Cas protein leading to active Cas-specific indel formation. Guide RNA comprising a dead guide may be modified to further include elements in a manner which allow for activation or repression of gene activity, in particular protein adaptors (e.g. aptamers) as described herein elsewhere allowing for functional placement of gene effectors (e.g. activators or

repressors of gene activity). One example is the incorporation of aptamers, as explained herein and in the state of the art. By engineering the gRNA comprising a dead guide to incorporate protein-interacting aptamers (Konermann et al., "Genome-scale transcription activation by an engineered CRISPR-Cas9 complex," doi:10.1038/nature14136, incorporated herein by reference), one may assemble a synthetic transcription activation complex consisting of multiple distinct effector domains. Such may be modeled after natural transcription activation processes. For example, an aptamer, which selectively binds an effector (e.g. an activator or repressor; dimerized MS2 bacteriophage coat proteins as fusion proteins with an activator or repressor), or a protein which itself binds an effector (e.g. activator or repressor) may be appended to a dead gRNA tetraloop and/or a stem-loop 2. In the case of MS2, the fusion protein MS2-VP64 binds to the tetraloop and/or stem-loop 2 and in turn mediates transcriptional up-regulation, for example for Neurog2. Other transcriptional activators are, for example, VP64. P65, HSF1, and MyoD1. By mere example of this concept, replacement of the MS2 stem-loops with PP7-interacting stem-loops may be used to recruit repressive elements.

By means of further guidance, and without limitation, in certain embodiments, the gRNA is an escorted gRNA (egRNA). By "escorted" is meant that the CRISPR-Cas system or complex or guide is delivered to a selected time or place within a cell, so that activity of the CRISPR-Cas system or complex or guide is spatially or temporally controlled. For example, the activity and destination of the CRISPR-Cas system or complex or guide may be controlled by an escort RNA aptamer sequence that has binding affinity for an aptamer ligand, such as a cell surface protein or other localized cellular component. Alternatively, the escort aptamer may for example be responsive to an aptamer effector on or in the cell, such as a transient effector, such as an external energy source that is applied to the cell at a particular time. The escorted Cpf1 CRISPR-Cas systems or complexes have a gRNA with a functional structure designed to improve gRNA structure, architecture, stability, genetic expression, or any combination thereof. Such a structure can include an aptamer. Aptamers are biomolecules that can be designed or selected to bind tightly to other ligands, for example using a technique called systematic evolution of ligands by exponential enrichment (SELEX; Tuerk C, Gold L:

"Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase." Science 1990, 249:505-510). Nucleic acid aptamers can for example be selected from pools of random-sequence oligonucleotides, with high binding affinities and specificities for a wide range of biomedically relevant targets, suggesting a wide range of therapeutic utilities for aptamers (Keefe, Anthony D., Supriya Pai, and Andrew Ellington. "Aptamers as therapeutics." Nature Reviews Drug Discovery 9.7 (2010): 537-550). These characteristics also suggest a wide range of uses for aptamers as drug delivery vehicles (Levy-Nissenbaum, Etgar, et al. "Nanotechnology and aptamers: applications in drug delivery." Trends in biotechnology 26.8 (2008): 442-449; and, Hicke BJ, Stephens AW. "Escort aptamers: a delivery service for diagnosis and therapy." J Clin Invest 2000, 106:923-928.). Aptamers may also be constructed that function as molecular switches, responding to a que by changing properties, such as RNA aptamers that bind fluorophores to mimic the activity of green flourescent protein (Paige, Jeremy S., Karen Y. Wu, and Samie R. Jaffrey. "RNA mimics of green fluorescent protein." Science 333.6042 (2011): 642-646). It has also been suggested that aptamers may be used as components of targeted siRNA therapeutic delivery systems, for example targeting cell surface proteins (Zhou, Jiehua, and John J. Rossi. "Aptamer-targeted cell-specific RNA interference." Silence 1.1 (2010): 4).

By means of further guidance, and without limitation, in certain embodiments, the gRNA is a protected guide. Protected guides are designed to enhance the specificity of a Cas protein given individual guide RNAs through thermodynamic tuning of the binding specificity of the guide RNA to target nucleic acid. This is a general approach of introducing mismatches, elongation or truncation of the guide sequence to increase / decrease the number of complimentary bases vs. mismatched bases shared between a target and its potential off-target loci, in order to give thermodynamic advantage to targeted genomic loci over genomic off-targets. In certain embodiments, the guide sequence is modified by secondary structure to increase the specificity of the CRISPR-Cas system and whereby the secondary structure can protect against exonuclease activity and allow for 3' additions to the guide sequence. In certain embodiments, a "protector RNA" is hybridized to a guide sequence, wherein the "protector RNA" is an RNA strand

complementary to the 5' end of the guide RNA (gRNA), to thereby generate a partially double-stranded gRNA. In an embodiment of the invention, protecting the mismatched bases with a perfectly complementary protector sequence decreases the likelihood of target binding to the mismatched basepairs at the 3' end. In certain embodiments, additional sequences comprising an extented length may also be present. [0004] Guide RNA (gRNA) extensions matching the genomic target provide gRNA protection and enhance specificity. Extension of the gRNA with matching sequence distal to the end of the spacer seed for individual genomic targets is envisaged to provide enhanced specificity. Matching gRNA extensions that enhance specificity have been observed in cells without truncation. Prediction of gRNA structure accompanying these stable length extensions has shown that stable forms arise from protective states, where the extension forms a closed loop with the gRNA seed due to complimentary sequences in the spacer extension and the spacer seed. These results demonstrate that the protected guide concept also includes sequences matching the genomic target sequence distal of the 20mer spacer-binding region. Thermodynamic prediction can be used to predict completely matching or partially matching guide extensions that result in protected gRNA states. This extends the concept of protected gRNAs to interaction between X and Z, where X will generally be of length 17-20nt and Z is of length 1-30nt. Thermodynamic prediction can be used to determine the optimal extension state for Z, potentially introducing small numbers of mismatches in Z to promote the formation of protected conformations between X and Z. Throughout the present application, the terms "X" and seed length (SL) are used interchangeably with the term exposed length (EpL) which denotes the number of nucleotides available for target DNA to bind; the terms "Y" and protector length (PL) are used interchangeably to represent the length of the protector; and the terms "Z", "E", "E"" and EL are used interchangeably to correspond to the term extended length (ExL) which represents the number of nucleotides by which the target sequence is extended. An extension sequence which corresponds to the extended length (ExL) may optionally be attached directly to the guide sequence at the 3' end of the protected guide sequence. The extension sequence may be 2 to 12 nucleotides in length. Preferably ExL may be denoted as 0, 2, 4, 6, 8, 10 or 12 nucleotides in length.. In a preferred embodiment the ExL is

denoted as 0 or 4 nuleotides in length. In a more preferred embodiment the ExL is 4 nuleotides in length. The extension sequence may or may not be complementary to the target sequence. An extension sequence may further optionally be attached directly to the guide sequence at the 5' end of the protected guide sequence as well as to the 3' end of a protecting sequence. As a result, the extension sequence serves as a linking sequence between the protected sequence and the protecting sequence. Without wishing to be bound by theory, such a link may position the protecting sequence near the protected sequence for improved binding of the protecting sequence to the protected sequence. Addition of gRNA mismatches to the distal end of the gRNA can demonstrate enhanced specificity. The introduction of unprotected distal mismatches in Y or extension of the gRNA with distal mismatches (Z) can demonstrate enhanced specificity. This concept as mentioned is tied to X, Y, and Z components used in protected gRNAs. The unprotected mismatch concept may be further generalized to the concepts of X, Y, and Z described for protected guide RNAs.

In certain embodiments, any of the nucleases, including the modified nucleases as described herein, may be used in the methods, compositions, and kits according to the invention. In particular embodiments, nuclease activity of an unmodified nuclease may be compared with nuclease activity of any of the modified nucleases as described herein, e.g. to compare for instance off-target or on-target effects. Alternatively, nuclease activity (or a modified activity as described herein) of different modified nucleases may be compared, e.g. to compare for instance off-target or on-target effects.

Also provided herein are compositions for use in carrying out the methods of the invention. More particularly, non-naturally occurring or engineered compositions are provided which comprise one or more of the elements required to ensure genomic perturbation. In particular embodiments, the compositions comprise one or more of the (modified) DNA binding protein, and/or a guide RNA. In particular embodiments, the composition comprises a vector. In further particular embodiments, the vector comprises a polynucleotide encoding a gRNA. In particular embodiments, the vector comprises two or more guide RNAs. Said two or more guide RNAs may target a different target (so as to ensure multiplex targeting) or the same target, in which case the different guide RNAs

will target different sequences within the same target sequence. Where provided in a vector the different guide RNAs may be under common control of the same promotor, or may be each be under control of the same or different promoters.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials and Methods

The CD5L monomer, CD5L dimer and CD5L:p40 heterodimer generations were out-sourced to Biolegend under CDA. Briefly, to generate the CD5L:p40 heterodimer, Cd5l and Il12b (p40) were cloned into mammalian expression vector through a linker: P40-linker 2-3 (SGGG)- CD5L with His tag. Similarly, CD5L monomer and dimer were generated by cloning CD5L with His tag at C-terminus into a mammalian expression vector. The plasmids are expressed in mammalian cell line and secreted CD5L:p40, CD5L (monomer and dimer) were purified and confirmed by gel electrophoresis and HPLC.

<u>CD5L sequence cloned:</u>

1 (maplfnlmla ilsifvgscf s)*esptkvqlv ggahrcegrv evehngqwgt vcddgwdrrd
61 vavvcrelnc gaviqtprga syqppaseqr vliqgvdcng tedtlaqcel nydvfdcshe
121 edagaqcenp dsdllfiped vrlvdgpghc qgrvevlhqs qwstvckagw nlqvskvvcr
181 qlgcgrallt ygscnkstqg kgpiwmgkms csgqeanlrs cllsrlennc thgedtwmec
241 edpfelklvg gdtpcsgrle vlhkgswgsv cddnwgeked qvvckqlgcg kslhpspktr
301 kiygpgagri wlddvncsgk eqslefcrhr lwgyhdcthk edvevictdf dv

*the signaling peptide was not included to better guide protein secretion in the expression system

<u>p40/il12b sequence cloned</u>

1 mcpqkltisw faivllvspl mamwelekdv yvvevdwtpd apgetvnltc dtpeedditw
61 tsdqrhgvig sgktltitvk efldagqytc hkggetlshs hlllhkkeng iwsteilknf

121 knktflkcea pnysgrftcs wlvqrnmdlk fnikssssp dsravtcgma slsaekvtld
181 qrdyekysvs cqedvtcpta eetlpielal earqqnkyen ystsffirdi ikpdppknlq
241 mkplknsqve vsweypdsws tphsyfslkf fvriqrkkek mketeegcnq kgaflvekts
301 tevqckggnv cvqaqdryyn sscskwacvp crvrs

Recombinant protein CD5L monomer and homodimer was purified from the supernanant of 293E cells transfected with a CD5L expression vector. Recombinant mCD5L:p40 was recovered from the supernatant of 293E cells transfected with the CD5L:p40 expression vector. After harvesting transfected 293E cells by centrifugation, the protein was affinity purified from the supernatant using Ni Sepharose 6 Fast Flow resin (GE Healthcare). After binding the protein to resin, the resin was washed with 20mM Tris, 0.3M NaCl, pH 8.0 and the protein eluted using 20mM Tris, 0.3M NaCl, 0.4M Imidazole, pH 8.0. The protein was further polished by a Superdex S200 sizing exclusion column (GE Healthcare) in buffer 10mM NaHPO4, 0.15M NaCl, pH 7.2. The S200 profile of the mCD5L:p40 showed a single peak. The S200 profile of the mCD5L transfection showed two overlapping peaks, corresponding to the homo-dimer fraction first and then monomer fraction

Example 1. Soluble CD5L and CD5L/p40 can regulate T cell function and have overlapping as well as distinct roles

CD5L can be secreted by macrophages (Miyazaki et al., 1999) and given its T-cell intrinsic role, we tested the hypothesis that soluble CD5L can regulate T cell function directly *in vitro*. Although Abdi et al. reported that CD5L can form a heterodimer with p40, no specific function was attributed to this potential cytokine (Abdi et al., 2014). We hypothesized that both soluble CD5L and CD5L:p40 heterodimer can regulate T cell function directly.

To this end, we used recombinant CD5L monomer either alone or with recombinant p40 monomer and analyzed the transcriptome of activated CD4 T cells, either WT or $CD5L^{-/-}$, co-incubated with these soluble factors. First, we analyzed the effect of soluble CD5L alone. We reasoned that if soluble CD5L (sCD5L) functions

similarly to that of T-cell intrinsic CD5L, the addition of sCD5L can reverse the effects of CD5L deficiency on T cells. Indeed, we showed that sCD5L reversed the expression profile of majority of genes differentially regulated by any of the conditions tested (**Figure 1A**). To exclude inference from T cell endogenous CD5L expression, we focused on the impact of sCD5L on *Cd5l*$^{-/-}$ T cells. Of interest, sCD5L also regulated expression profile of genes that were not changed comparing WT and *Cd5l*$^{-/-}$ T cells or opposed the T-cell intrinsic function of CD5L (**Figure 1A**), suggesting potential novel role of the soluble CD5L.

Next, we performed pathway analysis of genes regulated by soluble CD5L and found sCD5L regulated gene profile contains both a regulatory and an inflammatory component. First, we observed that in sCD5L treated T cells there was a significant enrichment of signature genes of regulatory T cells from four different datasets using MSigDB (**Table 1**). Interestingly the key transcription factor of Treg, Foxp3, was downregulated by sCD5L (**Table 1**). This is consistent with sCD5L also promoting factors (such as *Il4*, *Il9*) that have been implicated in destabilizing Foxp3 expression antagonizing retinoic acid (**Table 1** and (Hill et al., 2008)). These data suggest that soluble CD5L may promote a regulatory program but independent of Foxp3 expression and maybe an inducer of Th9 response. In addition to the regulatory component, we found that sCD5L regulated genes are significantly enriched for genes induced by IL-6/IL-1B but downregulated by IL-6/IL-1B/IL-23, suggesting soluble CD5L may antagonize IL-23 function (**Table 1**).

Table 1. Pathway analysis of soluble CD5L-dependent regulation of T cells.

A. Reversal/Novel (soluble) UP

Enriched pathways	genes
Treg (4 independent datasets) (FDR q-value 1.63 e -8)	(PDL2, LIF, SOCS2, IKZF4, ICOS, PROCR,NFIL3, CD200, TGM2, PRNP, CD70,XBP1,ATF4, LAD1,KLF9, CD83, Runx2, IRF8, IFNg etc)
RA treated memory CD4 (FDR q-value 9.58 e-10)	IER3, IL4, RAB33A, FZD7, NFIL3, SLAMF7, TNFSF9, FAIM3, IL9, Foxp3
IL-6/IL-1B	IL-22, GJA1, EGR2, IL1RN, CD200, ITGA3
IL-4	IL-4

B. Reversal/Novel (soluble) DOWN

Enriched pathways	genes
-IL-6/IL-1B/IL-23	GMFG, MGLL, FRMD4B, MINA

Soluble CD5L induces both a regulatory and proinflammatory program including Il9 response. Differentially regulated genes were investigated using Msigdb and selected significant enrichment are listed in A and B showing those upregulated and downregulated by soluble CD5L respectively. Red and Green indicates directionality: Red pathway means soluble CD5L treatment goes with, green pathway means goes against such pathways (In the above tables, the "Treg," "IL-6/IL-1B," and "IL-4" rows are red pathways, and the "RA treated treated memory CD4" and "IL-6/IL-1B/IL-23" rows are green pathways).

Finally, we compared the effect of sCD5L to that of sCD5L:p40 and found these two cytokines to regulate the expression profile of both similar and distinct set of genes (**Figure 2**). Thus, these data collectively suggest sCD5L and sCD5L:p40 are novel cytokines that can regulate T cell function.

Example 2. T cell regulation by sCD5L and CD5L:p40 depends on IL-23R signaling

As sCD5L and CD5L:p40 can regulate gene expression in T cells, we investigated what receptor(s) might be responsible for their function. CD5L was reported to interact

with CD36, a scavenger receptor, and thus can be internalized into adipocytes (Kurokawa et al., 2010). We investigated whether CD36 is required for signaling of sCD5L in T cells. We showed that His-tagged sCD5L can stain WT and $CD36^{-/-}$ T cells equally well even at lower concentrations (**Figure 3A** and data not shown). While this data is consistent with lower expression of CD36 on T cells compared to macrophage (ImmGen database), it also raises the question whether the sCD5L can bind to a different receptor on T cells.

CD5L can form a heterodimer with p40 and p40 can bind to either p19 or p35. We hypothesized that if sCD5L binds to a surface receptor it may be co-regulated/dependent on receptors for the other two cytokines: that is IL-12RB1, IL-12RB2 or IL-23R. We tested whether sCD5L can stain *Il12rb1*$^{-/-}$, *Il12rb2*$^{-/-}$ or *Il23r*$^{-/-}$ T cells as compared to WT (**Figure 3A** and data not shown). Interestingly, the binding of sCD5L is abolished on *Il23r*$^{-/-}$ T cells and partially reduced on *Il12rb1*$^{-/-}$, *Il12rb2*$^{-/-}$ T cells. These findings suggest that CD5L may interact with a receptor that depends on IL-23R signaling.

Next, we asked the question whether the function of sCD5L is also affected by the absence of IL-23R on T cells. To this end, we crossed *Cd5l*$^{-/-}$ mice with *Il23r*$^{-/-}$ mice and found that in the absence of IL-23R, the expression of 89% of genes (84 out of 94 based on nanostring set) regulated by sCD5L were no longer affected (**Figure 3B**). The effect of CD5L:p40 heterodimer could also be partially dependent on IL-23R expression (**Figure 3C**). Thus sCD5L and CD5L:p40 may interact with different receptors on T cells.

Example 3. CD5L regulates not only T cells but also restrains proinflammatory function of innate lymphoid cells (ILC) and is expressed by ILC in naïve mouse

The discovery that soluble CD5L can regulate T cell function directly and that its impact may dependent on IL-23R expression prompted us to study whether CD5L can regulate other cells that may also express IL-23R. To this end, we investigated the impact of CD5L on two such populations that express IL-23R: innate lymphoid cells (ILC) and dendritic cells (DC).

First, we analyzed the percent and function of ILC in naïve 6-month old WT versus *Cd5l*$^{-/-}$ mice. We observed that IL-23R expression on ILC from lamina propria is significantly increased in the absence of CD5L (**Figure 4A**). This is accompanied with higher proportion of ILCs producing IL-17 and Tbet, but lower percent of IL-22 producers (**Figure 4BC**). We further demonstrated that the reduced IL-22 expression and increased Tbet expression by ILC can be reverted by soluble CD5L *ex vivo* (**Figure 4C**). These data suggest that CD5L can regulate ILC function at steady state. Of interest, we observed that ILC isolated from both mLN and lamina propria from naïve mice can express CD5L (**Figure 4D**).

Next, we asked whether CD5L influence ILC during inflammation. As CD5L regulates IL-17 and IL-17 production is associated with ILC3, we crossed *Cd5l*$^{-/-}$ mice with fate mapping reporter mice *Il17a*^{Cre}*Rosa26*$^{Td\text{-}tomato}$ to better track ILC3 that has ever transcribed sufficient IL-17 to turn on the Cre. Using the DSS-induced acute colitis model, we showed that there is similar percent of Rosa26^{+} ILC comparing 8-wk old *WT.Il17a*^{Cre}*Rosa26*$^{Td\text{-}tomato}$ and *Cd5l*$^{-/-}$*Il17a*^{Cre}*Rosa26*$^{Td\text{-}tomato}$ mice at day 11 since DSS treatment (**Figure 4F**), suggesting CD5L does not influence the differentiation of ILCs initially. Consistently, the percent of ILC that expresses Rorgt is not significantly altered (**Figure 4E**). In contrast to the Rosa26 expression, ILC from *WT.Il17a*^{Cre}*Rosa26*$^{Td\text{-}tomato}$ make little IL-17 and turned on IL-10 expression in striking contrast to those from *Cd5l*$^{-/-}$ *Il17a*^{Cre}*Rosa26*$^{Td\text{-}tomato}$ mice which continue to produce much higher expression of IL-17 and are IL-10 negative (**Figure 4G**). Thus CD5L can restrain proinflammatory function of ILC during acute inflammation.

Example 4. CD5L:p40 promotes regulatory programs in CD11c+ cells in an IL-23R but not CD36 dependent manner

It has been reported that CD5L can induce autophagy in the human macrophage cell line, THP, limiting TNFa and IL-1B expression and promoting IL-10 expression (Sanjurjo et al., 2015). The authors propose CD36 is the major recipient of CD5L in these cells. As we discovered that sCD5L (and CD5L:p40 heterodimer) could regulate T cells

through an IL-23R-dependent alternative receptor, we tested the hypothesis that CD5L and CD5L:p40 may regulate myeloid cells in an IL-23R dependent pathway.

To test this hypothesis, we isolated WT, $CD36^{-/-}$ and IL-$23R^{-/-}$ $CD11c^{+}$ cells from spleen of naïve mice and stimulated the cells with LPS in the presence of sCD5L, p40 or CD5L:p40. We showed that sCD4L, p40 and CD5L:p40 can all induce IL-10 expression from $CD11c^{+}$ cells, however the effect of CD5L:p40 is dependent on IL-23R whereas the effect of sCD5L is dependent on CD36 (**Figure 5**).

Example 5. CD5L plays a protective role in acute colitis and cancer

To test the function of CD5L and CD5L:p40 *in vivo*, we tested several disease models. $CD5L^{-/-}$ mice were treated with 2% DSS in drinking water for 6 days followed by normal water. Weight loss was reported as a percentage of initial weight in Figure 6A. Colitis score and colon length were determined on day 14, and are shown in Figures 6B and C, respectively. Colon histology on day 14 is shown in Figure 6D. This data demonstrates that CD5L influenced tumor progression in a B16 melanoma model.

Example 6. CD5L ameliorates autoimmune diseases (including MS), acute colitis, and cancer

To show that CD5L:p40 can ameliorate disease, we therapeutically treat mouse models of multiple sclerosis (EAE), colitis (e.g., DSS-induced injury model which is a mouse model for ulcerative colitis and T-cell dependent colitis model) or cancer (e.g., mice with inflammation-induced cancers, or human cancer xenografted onto mice) with recombinant CD5L:p40, or antibodies or antigen-binding fragments thereof or that bind to the heterodimers.

Example 7. Recombinant CD5L binds to T cells and suppresses EAE and DSS-induced colitis.

Experiments were conducted to assess whether soluble CD5L could regulate effector T cells. In particular, soluble CD5L was directly evaluated using recombinant CD5L with a His-tag. Th0, Th1 (IL-12), and TH17p (IL-1b, IL-6, IL-23) cells were differentiated from naïve CD4 T cells *in vitro* for 4 days, and cells were harvested for

staining with recombinant CD5L followed by anti-His APC antibodies and flow cytometry analysis. Flow cytometry data showed that CD5L can bind to both Th1 and pathogenic Th17 cells (Th17p) and to a lesser extent Th0 cells (**Figure 7A**). The binding of CD5L on T cells was shown to not require CD36, but to be dependent on IL-23R (*e.g.*, loss of IL-23R abrogated CD5L binding to T cells).

In vivo therapeutic experiments were conducted by immunizing wildtype mice with MOG/CFA following by PT injection to induce EAE. Mice at peak of disease (score = 3 in Figure 7B) were injected with either PBS (solid circles) or recombinant CD5L (empty circles) intraperitoneally daily for 5 consecutive days and mice were measured for disease progression. As shown in Figure 7B, soluble CD5L was shown to have a therapeutic effect on EAE.

In a separate experiment, wildtype mice were induced with colitis via 2.5% DS in drinking water for 6 consecutive days, followed by normal water for 8 days. Mice were given either a control (PBS) or recombinant CD5L (CD5Lm) intraperitoneally on day 4, 6, and 8. Colon length and colitis score were recorded on day 14. As shown in Figure 7C, recombinant CD5L was sufficient in alleviating colitis disease severity.

Example 8. Endogeneous CD5L forms a heterodimer (CD5L:p40) and is inducible during an acute inflammation.

CD5L can bind to p40, the subunit shared by the cytokines IL-12 and IL-23, and form a heterodimer *in vitro*. This raises the intriguing possibility that CD5L can generate different soluble mediators with potentially distinct functions. To determine whether CD5L:p40 heterodimer can be detected *in vivo* in biological settings, recombinant CD5L:p40 (Figure 8A) was generated and used to optimize an ELISA that allowed the detection of endogenous CD5L:p40 heterodimer.

Serum was collected kinetically from wildtype and *Cd5l*$^{-/-}$ mice with DSS-induced colitis (2% DSS in drinking water for 6 days followed by 7 days of normal water) and the level of CD5L:p40 was measured using an ELISA assay. In the ELISA assay anti-IL-12 p40 was used to capture the heterodimer and enzyme linked anti-CD5L

was used to detect the heterodimer. Data from this assay showed that natural CD5L:p40 heterodimer was induced during the course of DSS-induced colitis in serum (Figure 8B).

Example 9. IL-27 and TLR9 induce CD5L dimerization.

Preliminary screens were conducted to determine what signals could induce CD5L homodimer and CD5L:p40 heterodimer. In particular, bone marrow derived dendritic cells were stimulated with TLR ligands for 24 hours and the supernatant was analyzed for CD5L:p40 secretion by ELISA. The screens showed that TLR9 can induce the secretion of CD5L:p40 (Figure 9A). To determine the signals that could induce CD5L on T cells, CD5L expression in Th0, Th1, Th2, Th17 and Tr1 cells was analyzed, and the data showed that the immunosuppressive cytokine IL-27 can indeed induce CD5L (Figure 9B and data not shown).

Example 10. CD5L homo/heterodimer inhibits IL-17 production and the pathogenic Th17 cell signature.

To determine the function of CD5L homo/heterodimers on Th17 cells directly, pathogenic Th17 cells (IL-1b+IL-6+IL-23) were treated with either PBS (control), CD5L homodimers or CD5L:p40 heterodimers. IL-17 expression of T cells was measuring by FACS (Figure 10A), and IL-17 production in serum was measured by ELISA (Figure 10B). These experiments showed that both forms of CD5L inhibited IL-17 expression (Figures 10A-B).

To test whether recombinant CD5L can regulate the transcriptome of Th17 cells and particularly the pathogenic signature, the RNA expression of control and treated cells was studied with a custom-code set of 337 genes, and analyzed against signature genes of pathogenic Th17 cells (*e.g.*. *il23r*, *il22*, *il1r1*, *csf2*) with GSEA, using the nanostring platform. The signature of pathogenic Th17 cells was significantly reduced by both CD5L:CD5L and CD5L:p40 as compared to a control (Figure 10 C (FDR q = 0.031, NOM p = 0.000, NES = -1.66) and 10D (FDR q = 0.031, NOM p = 0.000, NES = -1.47), respectively).

Example 11. CD5L suppresses IL-17 and IFNg expression from pathogenic Th17 cells and Th1 cells, respectively.

Pathogenic Th17 cells and Th1 cells were differentiated from naïve CD4 cells ($CD44^{low}CD62L^{+}CD25\text{-}CD4^{+}$) from wildtype mice with IL-1b, IL-6, and IL-23 (Th17) or IL-12 (Th1) in the presence of a control, CD5L homodimer, or CD5L:p40 heterodimer for 48 hrs (Th17) or 72 hours (Th1). IL-27 expression in Th17 cells was measured by ELISA in supernatant (Figure 11A, left side) and by qPCR from RNA purified from cells (Figure 11A, right side). IFNg expression in Th1 cells was measured by intracellular staining followed by flow cytometry analysis (Figure 11B). The results showed that CD5L suppresses IL-17 and IFNg production in pathogenic T cells.

To assess pathogenic T cell signatures, RNA was extracted from both Th17 and Th1 cells after 48 hours of differentiation. Extracted RNA was analyzed with a custom codeset of 337 genes using the nanostring platform (four replicates for each conditions were measured). The Spearman coefficient was used for clustering. A heat map of differentially expressed genes as compared to control (defined by $p < 0.05$) is shown in Figure 12A for Th17 cells and Figure 12B for Th1 cells (left panels). GSEA analysis against the pathogenic signatures are shown in the right panels of Figures 12A and B.

Example 12. Endogenous CD5L promotes EAE resolution and is expressed by both non-pathogenic Th17 cells and CD11b+ cells during EAE development.

To determine which cells express CD5L during EAE, *cd5l*$^{-/-}$ mice were immunized with MOG/CFA to induce EAE and followed for clinical scores. Th17 cells (IL-17.GFP+CD4+) and CD11b+ myeloid cells were sorted from both spleen and CNS of mice at peak disease (score = 3). Mice with global CD5L deficiency showed more severe and sustained EAE compared to controls (Figure 13A), indicated that CD5L contributes to EAE resolution.

To assess CD5L expression in EAE, IL-17 GFP reported mice were immunized with MOG/CFA to induce EAE. Mice were sacrificed at peak of disease (score = 3). Th17 cells were sorted based on CD4+GFP+ and macrophage were sorted based on CD11b+ from both the spleen and CNS of the mice. RNA was purified from sorted cells

and qPCR was used to measured CD5L expression. The experiments showed that CD5L was preferentially expressed by Th17 cells in the spleen and by macrophage cells in the CNS (Figure 13B).

Example 13. Generation of CD5L conditional knockout mouse; role in tumor immunity.

To study the cellular source of CD5L during EAE development, CD5L flox/flox mice (CD5Lfl/fl) were generated by crossing FLPo mice and mice that were heterozygous with the construct shown in Figure 14A (purchased from EUCOMM/KOMP). The CD5L flox/flox mice were bred to homozygosity and crossed with CD4-Cre, IL-17-Cre and LysM-Cre for conditional deletion of the Cre-loxP system. Representative genotyping results for CD5L flox/flox mice are shown in Figure 14B. $CD5L^{fl/fl}$ mice were successfully crossed with LysMCre, CD4Cre and IL-17Cre mice to specifically delete CD5L in myeloid lineage cells, T cells and IL-17- producing cells respectively.

$CD5L^{flox/flox}Lymz^{Cre+}$ (CD5L CKO) and $CD5L^{flox/flox}$ mice were injected with 1 x 10^6 MC38 colon carcinoma subcutaneously on the right flank. Tumor size was measured up to 19 days post-injection, and is plotted in Figure 15A. Pictures of mice sacrificed on day 19 post tumor cell injection are shown in Figure 15B.

Example 14. CD5L and IL-23 alter lipidome of Th17 cells in correlation with T cell function and EAE.

Th17 cells were differentiated from naïve cells under pathogenic and non-pathogenic conditions and harvested for LC/MS at 96 hours. The lipidome of wildtype and *Cd5l*$^{-/-}$ Th17 cells was analyzed. A striking correlation of the lipidome of Th17 cells to their function and ability to induce EAE was found (Figure 16). In fact, Th17 cell function could be changed based on alterations of the Th17 cell lipidome.

Example 15. Gene expression profile of metabolic pathways correlates with Th17 cell pathogenicity.

To determine whether metabolic genes are differentially expressed at the transcriptome level in Th17 cells with different functional state, the metabolic transcriptome in single cell RNAseq data was analyzed. The analysis showed metabolic transcriptome expression covariance with Th17 cell pathogenicity (Figure 17).

Example 16. CD5L plays a critical role in tumor immunity, regulating T cell exhaustion

Littermate controls of $CD5L^{+/-}$ and $CD5L^{-/-}$ mice were grafted with 1 x 10^6 MC38 or MC38-OVA colon carcinoma subcutaneously on the right flank, and then tumor progression was followed. Tumor size progression for MC38 and MC38-OVA experiments are shown Figures 18A and B, respectively. Tumor infiltrating lymphocytes were isolated from MC38 on day 30 and analyzed, and the results are shown in Figure 19C. Tumor infiltrating lymphocytes were isolated from MC38-OVA on day 14 and inculcated with OVA peptide or no peptide (control) for 20 hours. Brefaldin A and monensin was added in the last 4 hours and cytokines were measured instracellularly by flow cytometry (see Figure 19D). These results demonstrate that CD5L deficiency inhibits T cell dysfunction and promotes tumor suppression.

Example 17: Link between CD5L:p40 heterodimer and tumor progression.

Litter mate controls of wildtype, $CD5L^{+/+}$ and $CD5L^{-/-}$ mice were injected with 1 x 10^6 MC38 colon carcinoma subcutaneously on the right flank, and CD5L:CD5L and CD5L:p40 were measured in serum during tumor progression. Serum was obtained and measured for (a) CD5L:p40 heterodimer using sandwich ELISA captured by anti-IL-12p40 antibody and detected with biotinylated anti-CD5L antibody and (b) CD5L:CD5L homodimer using sandwich ELISA captured and detected by anti-CD5L antibodies. Results are shown in Figures 19A-B.

Example 18: CD5L Suppresses pathogenic T cell signatures

Pathogenic Th17 cells and Th1 cells were differentiated from naïve CD4 T cells ($CD44^{low}CD62L^{+}CD25\text{-}CD4^{+}$) from wildtype mice with IL-1b, IL-6 and IL-23 (Th17) in the presence of control, CD5L homodimer, or CD5L:p40 heterodimer for 48 hours. RNA were extracted and subjected to RNAseq using NextSeq. A heat map prepared from this data (Figure 20; four replicates from each condition is shown; spearman coefficient was used for clustering) shows that the presence of CD5L:CD5L results in expression of different signature genes than does the presence of CD5L:p40. The heat map shows differentially expressed genes in the CD5L:CD5L and CD5L:p40 experiments as compared to the control (differentially expressed genes are defined by $p<0.5$ as compared to control). This data demonstrates that both CD5L:CD5L and CD5L:p40 can suppress pathogenic T cell signatures, but that the suppression via CD5L:CD5L and CD5L:p40 is associated with expression of distinct cell signatures.

Example 19: *In vivo* effect of CD5L:p40

To assess *in vivo* efficacy of CD5L dimers, wildtype mice were treated with 2% DSS in drinking water for 5 days, followed by normal water for 6 days. Mice were injected with PBS, recombinant CD5L:CD5L, or recombinant CD5L:p40 intraperitoneally on days 4, 6, and 8. Cells from mesenteric lymph nodes (mLN), peyer's patches (pp), lamina propria of colon (LP), and intraepithelial lymphocytes (IEL) were isolated, stained, and analyzed directly with flow cytometry on day 11. The frequency of Foxp3+ CD4 T cells in various cell types is shown in Figure 21A. The frequency of ILC3 as defined by CD45+Lineage-Thy1.2+CD127+Rorγt is shown in Figure 21B. This data demonstrates that CD5L:p40 increased Tregs *in vivo* in DSS-induced colitis.

Example 20: Generation of anti-CD5L:CD5L homodimer and anti-CD5L:p40 heterodimer antibodies

CD5L-/- mice were immunized with either recombinant CD5L:CD5L (labeled "714" in Figure 22A) or recombinant CD5L:p40 ("711", "712") for antibody generation. Serum samples were taken from each mouse before spleen infusion and tested for their

ability to bind to either CD5L:p40 or CD5L:CD5L in a sandwich ELISA assay (Figure 20A). B cells from the spleen of immunized mice were fused to generate pools of clones that were allowed to expand. Serum from the pools were tested in the same ELISA assay. Polyclonal antibody pools that have preferential specificity to either CD5L:p40 or CD5L:CD5L were observed (Figure 22B).

It is contemplated that human antibodies CD5L:CD5L and CD5L:p40 can be prepared based on the degree of homology between mouse and human CD5L and p40 (Figures 23A and C). Also shown are homology between mouse and human protein sequences in p19 and p35 (Figure 23B and D), which can form a dimer with p40.

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

WHAT IS CLAIMED IS:

1. A method of suppressing an immune response in a subject, the method comprising administering to the subject a therapeutically effective amount of:

(i) a recombinant soluble CD5L:p40 heterodimer and/or nucleic acids encoding CD5L and p40;

(ii) a recombinant soluble CD5L:CD5L homodimer and/or a nucleic acid encoding a CD5L homodimer; and/or

(iii) a recombinant soluble CD5L and/or a nucleic acid encoding CD5L.

2. The method of claim 1, wherein the subject has an autoimmune disease.

3. The method of claim 2, wherein the autoimmune disease is Multiple Sclerosis (MS), Irritable Bowel Disease (IBD), Crohn's disease, spondyloarthritides, Systemic Lupus Erythematosus (SLE), Vitiligo, rheumatoid arthritis, psoriasis, Sjögren's syndrome, or diabetes.

4. The method of claim 1, wherein the subject has an inflammation-related cancer.

5. The method of claim 4, wherein the inflammation-related cancer is colorectal cancer, carcinogen-induced skin papilloma, fibrosarcoma, or mammary carcinomas.

6. The method of claim 1, comprising administering the CD5L:p40 heterodimer.

7. The method of claim 1, comprising administering the CD5L:CD5L homodimer.

8. A method of enhancing an immune response in a subject, the method comprising administering to the subject a therapeutically effective amount of an agent that: (a) inhibits a CD5L:p40 heterodimer, a CD5L:CD5L homodimer, and/or CD5L from binding to an IL-23 receptor; and/or (b) inhibits formation of the CD5L:p40 heterodimer and/or the CD5L:CD5L homodimer.

9. The method of claim 8, wherein the agent comprises an antibody, or an antigen binding fragment thereof, that binds to one or more of the CD5L:p40 heterodimer, the CD5L homodimer, and the CD5L.

10. The method of claim 8, wherein the agent comprises inhibitory nucleic acids that target the CD5L and/or the p40.

11. The method of claim 8, wherein the subject has cancer that is not inflammation related.

12. The method of claim 11, further comprising administering an anti-cancer immunotherapy to the subject.

13. The method of claim 12, wherein the anti-cancer immunotherapy is selected from the group consisting of checkpoint inhibitors, PD-1/PDL-1, anti-cancer vaccines, adoptive T cell therapy, and combinations of two or more thereof.

14. The method of claim 10, wherein the inhibitory nucleic acids are small interfering RNAs (e.g., shRNA), antisense oligonucleotides, and/or CRISPR-Cas.

15. The method of claim 8, wherein the subject has an immune deficiency, e.g., a primary or secondary immune deficiency.

16. The method of claim 8, wherein the subject has an infection with a pathogen, e.g., viral, bacterial, or fungal pathogen.

17. A method of modulating $CD8^+$ T cell exhaustion in a subject in need thereof, the method comprising administering to the subject a therapeutically effective amount of an agent that: (a) inhibits a CD5L:p40 heterodimer, a CD5L:CD5L homodimer, and/or CD5L from binding to an IL-23 receptor; and/or (b) inhibits formation of the CD5L:p40 heterodimer and/or the CD5L:CD5L homodimer.

18. The method of claim 17, wherein said administering reduces $CD8^+$ T cell exhaustion.

19. The method of claim 17, wherein the subject has cancer.

20. The method of claim 19, wherein the cancer is a non-inflammatory cancer.

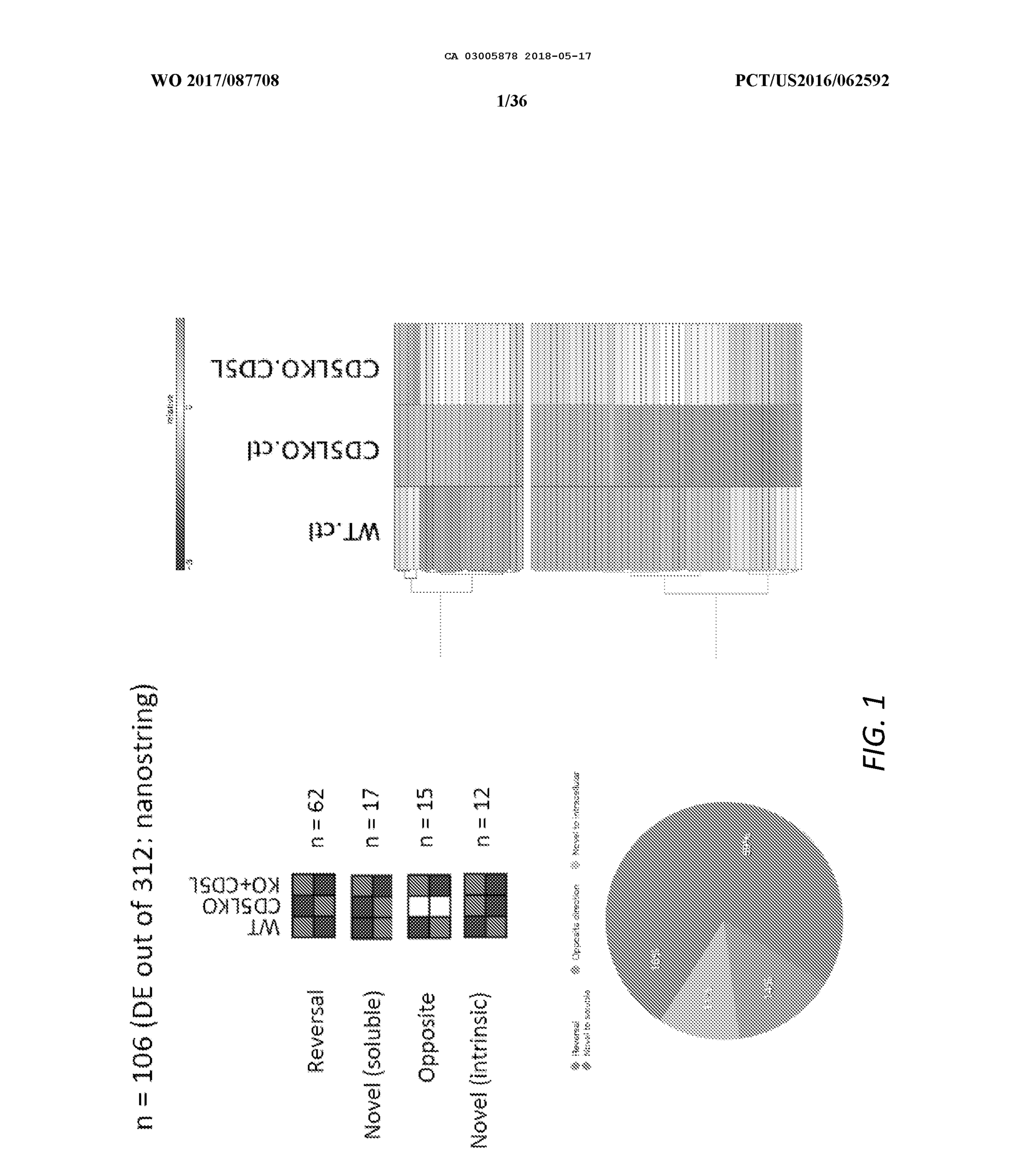

WT.ctl
CD5LKO.ctl
CD5LKO.CD5L
relative
n = 106 (DE out of 312; nanostring)
WT
CD5LKO
KO+CD5L
Reversal
n = 62
Novel (soluble)
n = 17
Opposite
n = 15
Novel (intrinsic)
n = 12
Reversal
Novel to soluble
Opposite direction
Novel to intracellular
Soluble
Intrinsic

FIG. 1

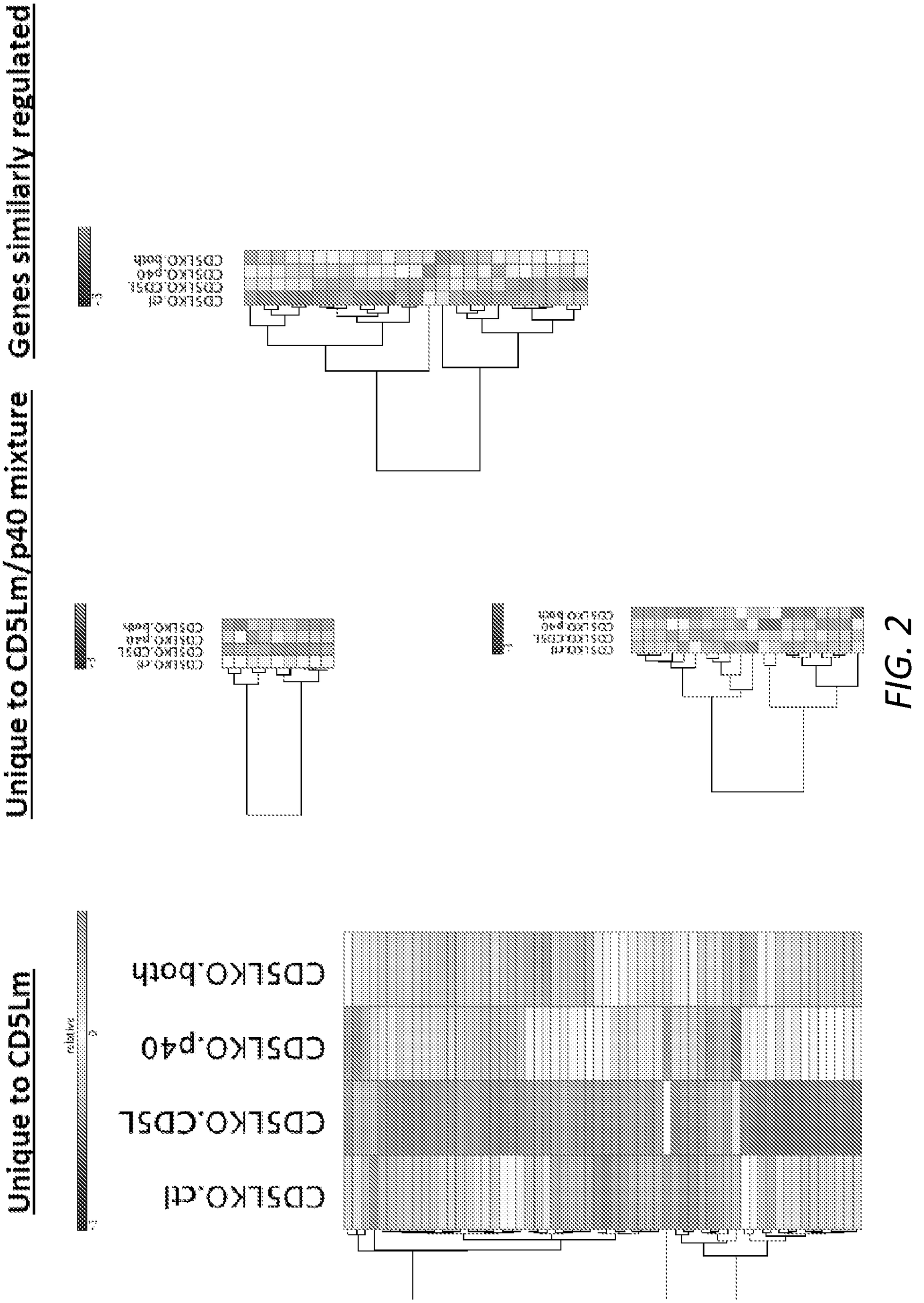

Genes similarly regulated
Lm/p40 mixture
CD5LKO.p40
CD5LKO.CD5L
CD5LKO.ctl
Unique to CD5Lm
Unique to CD5Lm
CD5LKO.cti
CD5LKO.CD5L
CD5LKO.p40
CD5LKO.both
relative

FIG. 2

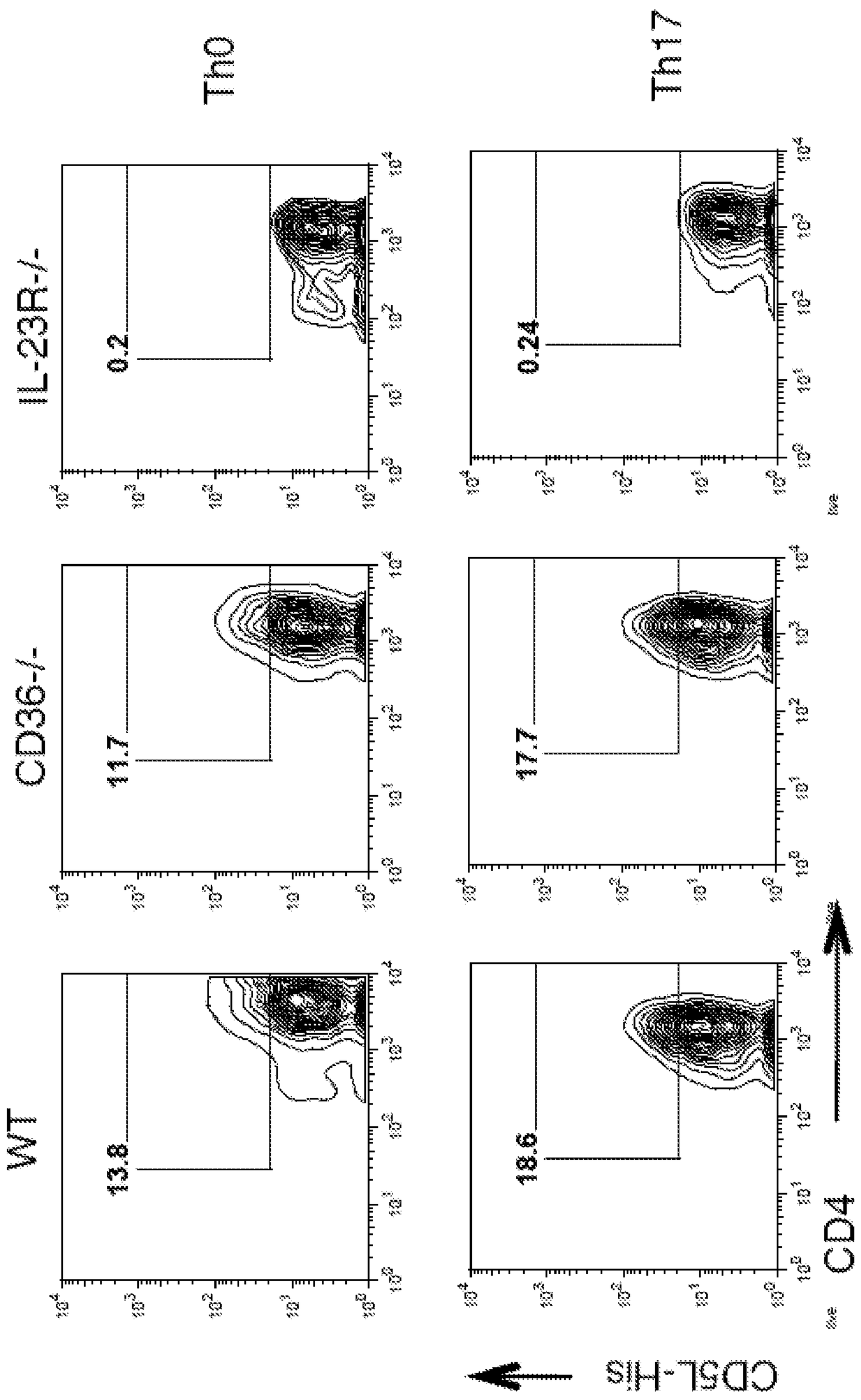


FIG. 3A

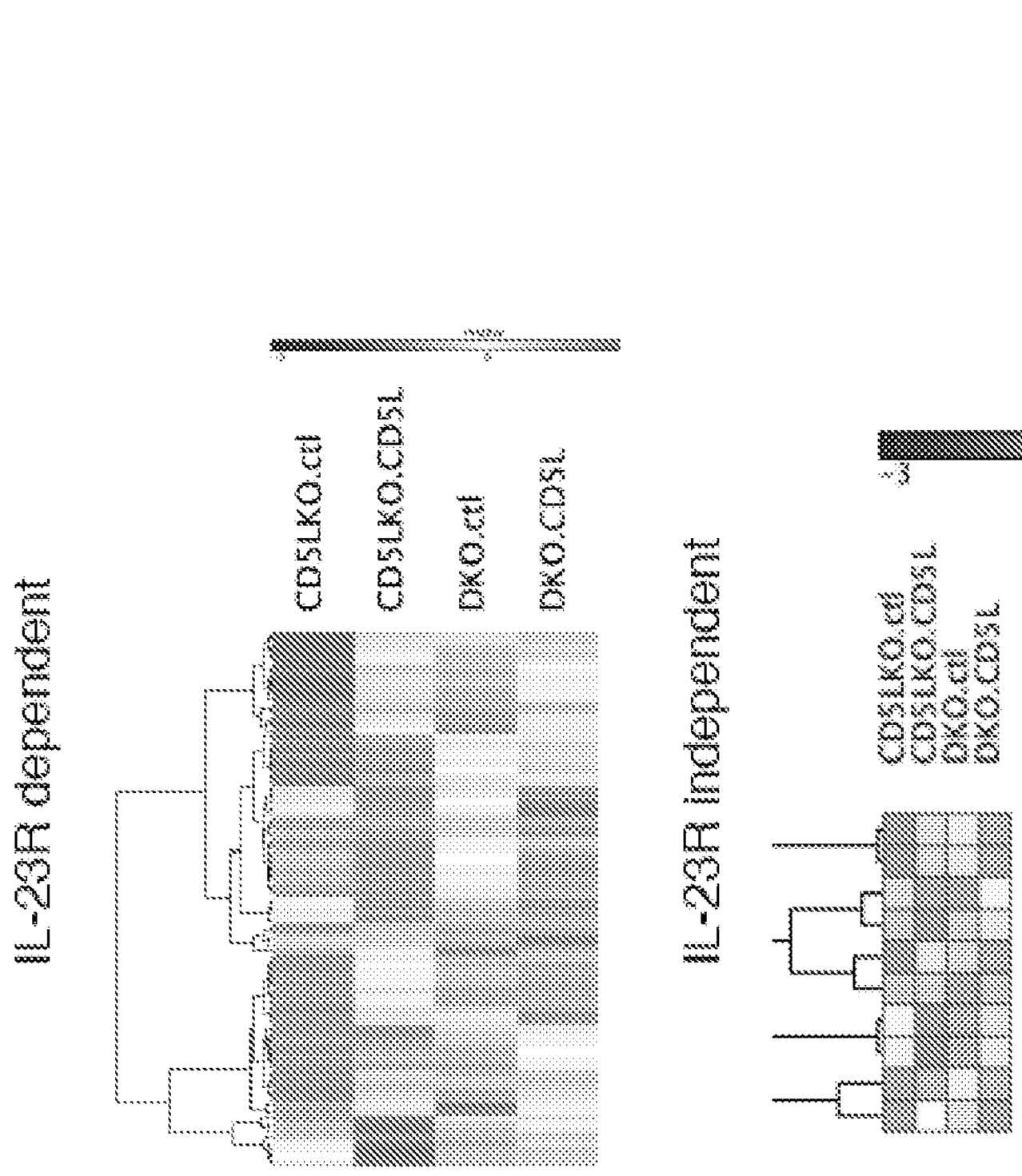

IL-23R dependent
CD51KO.ctl
CD51KO.CD51L
DKO.ctl
DKO.CD51L
IL-23R independent
CD51KO.ctl
CD51KO.CD51L
DKO.ctl
DKO.CD51L

FIG. 3B

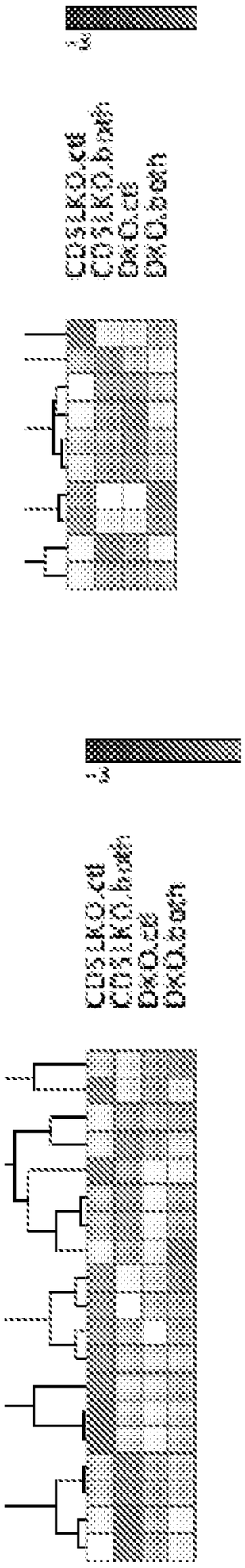

IL-23R independent
CD51KO.cd
CD51KO.both
DxO.cd
DxO.both
4
IL-23R dependent
CD51KO.cd
CD51KO.both
DxO.cd
DxO.both
4

FIG. 3C

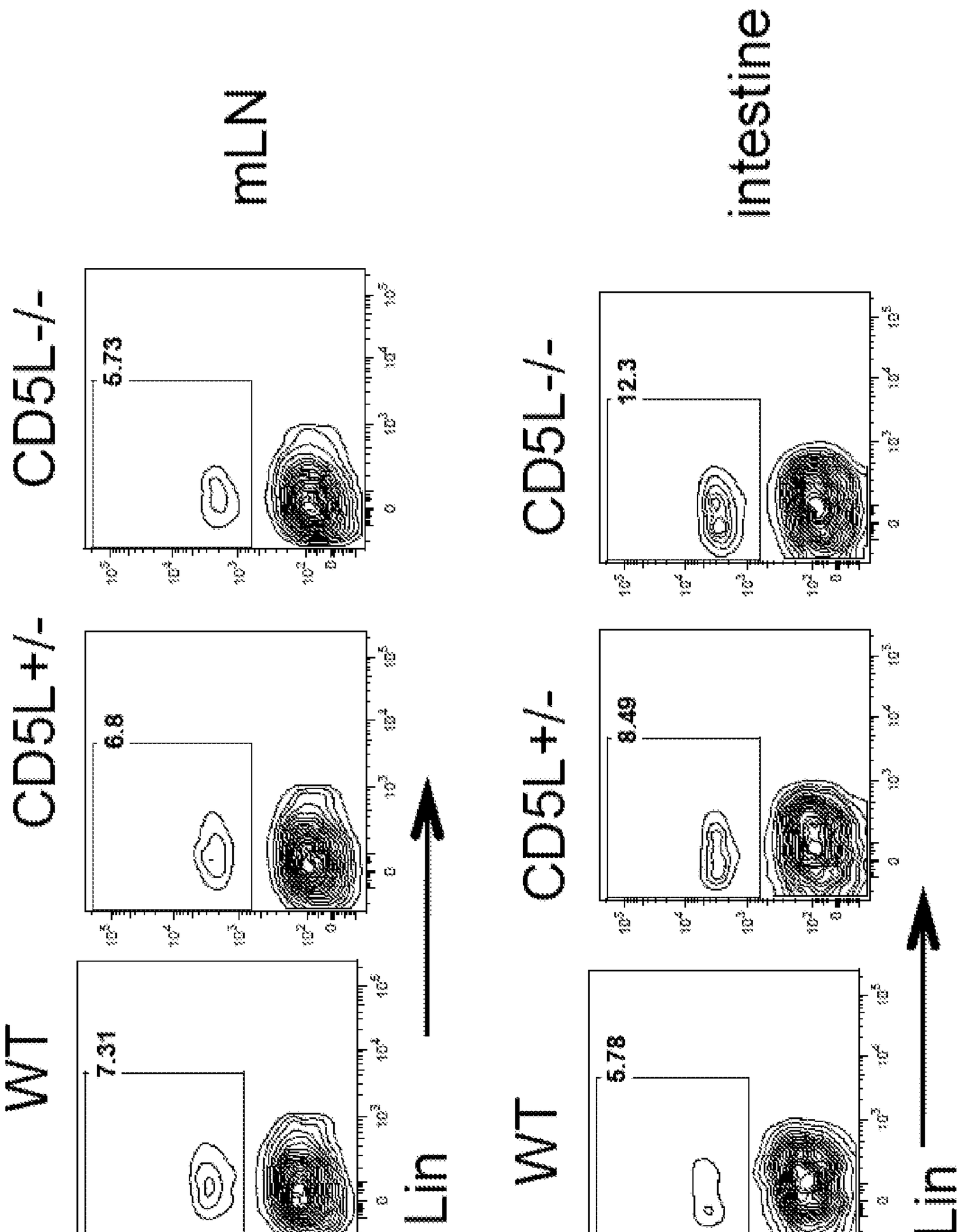

CD5L-/-
5.73
mesentery
CD5L-/-
12.3
intestine
CD5L+/-
6.8
CD5L+/-
8.49
WT
7.31
WT
5.78
Lin
Lin
IL-23R GFP
IL-23R GFP

FIG. 4A

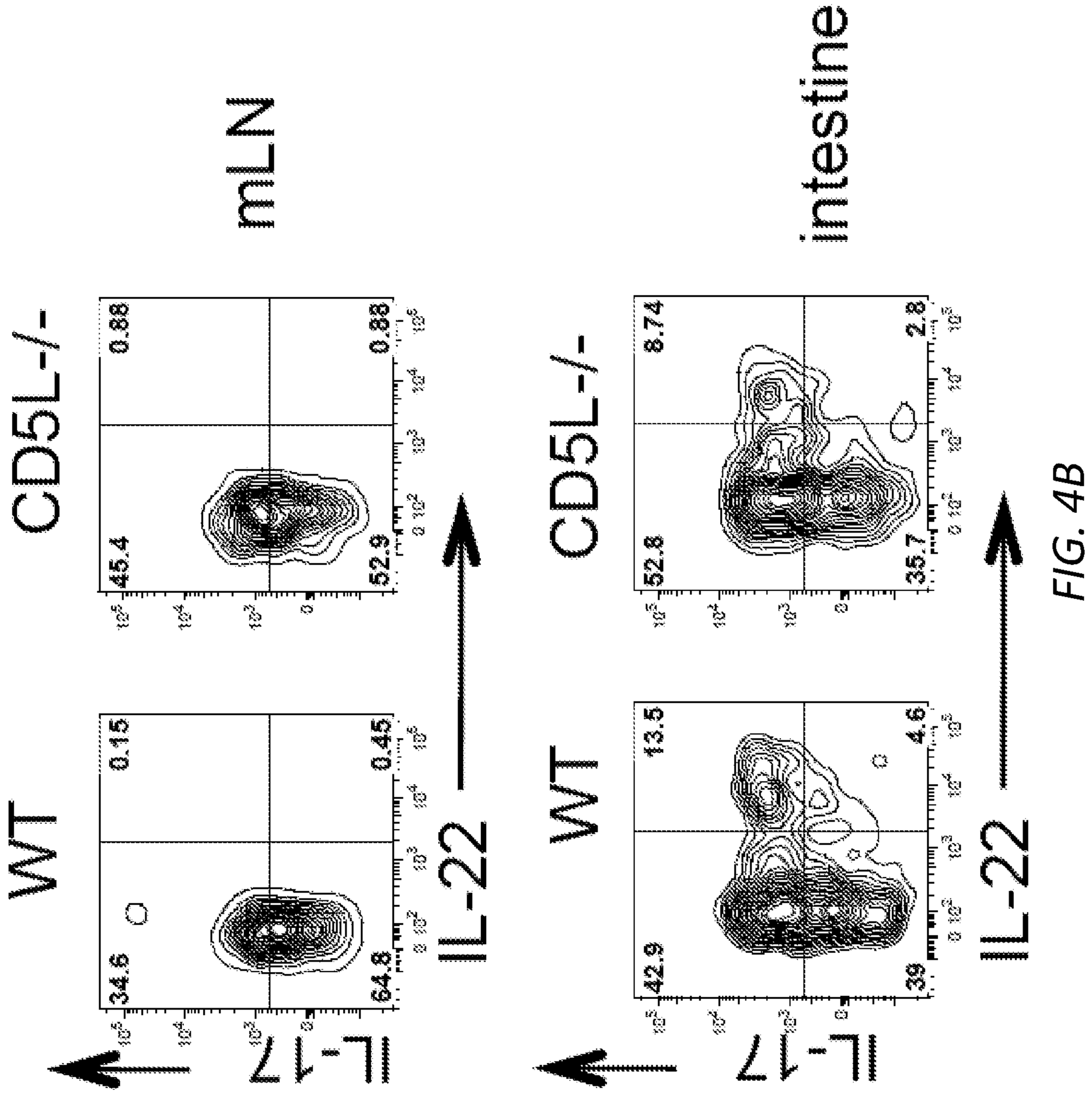

CD5L-/-
mLN
0.88
45.4
52.9
0.88
IL-22
IL-17
WT
0.15
34.6
64.8
0.45
CD5L-/-
intestine
8.74
52.8
35.7
2.8
IL-22
IL-17
WT
13.5
42.9
39
4.6

FIG. 4B

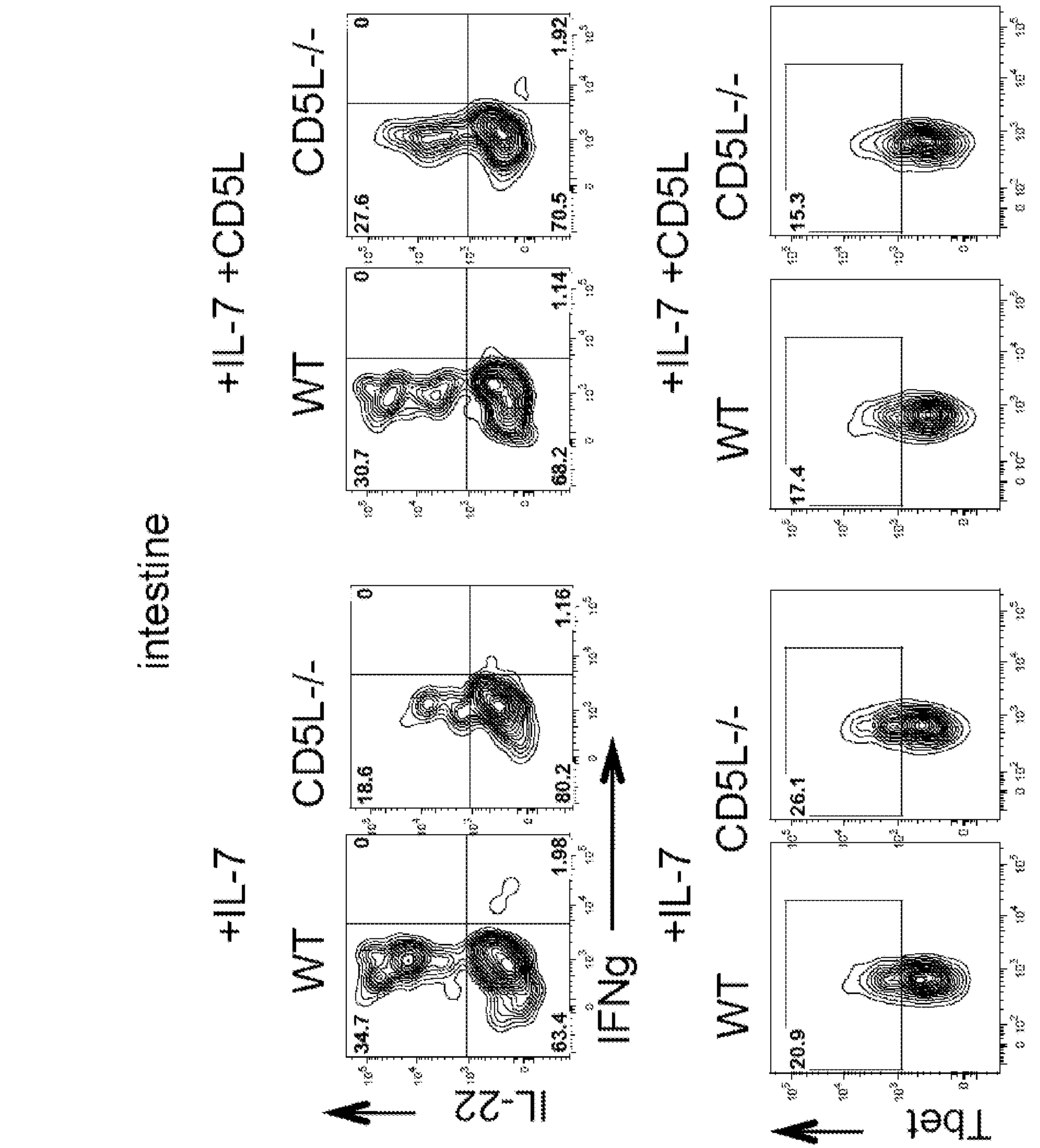
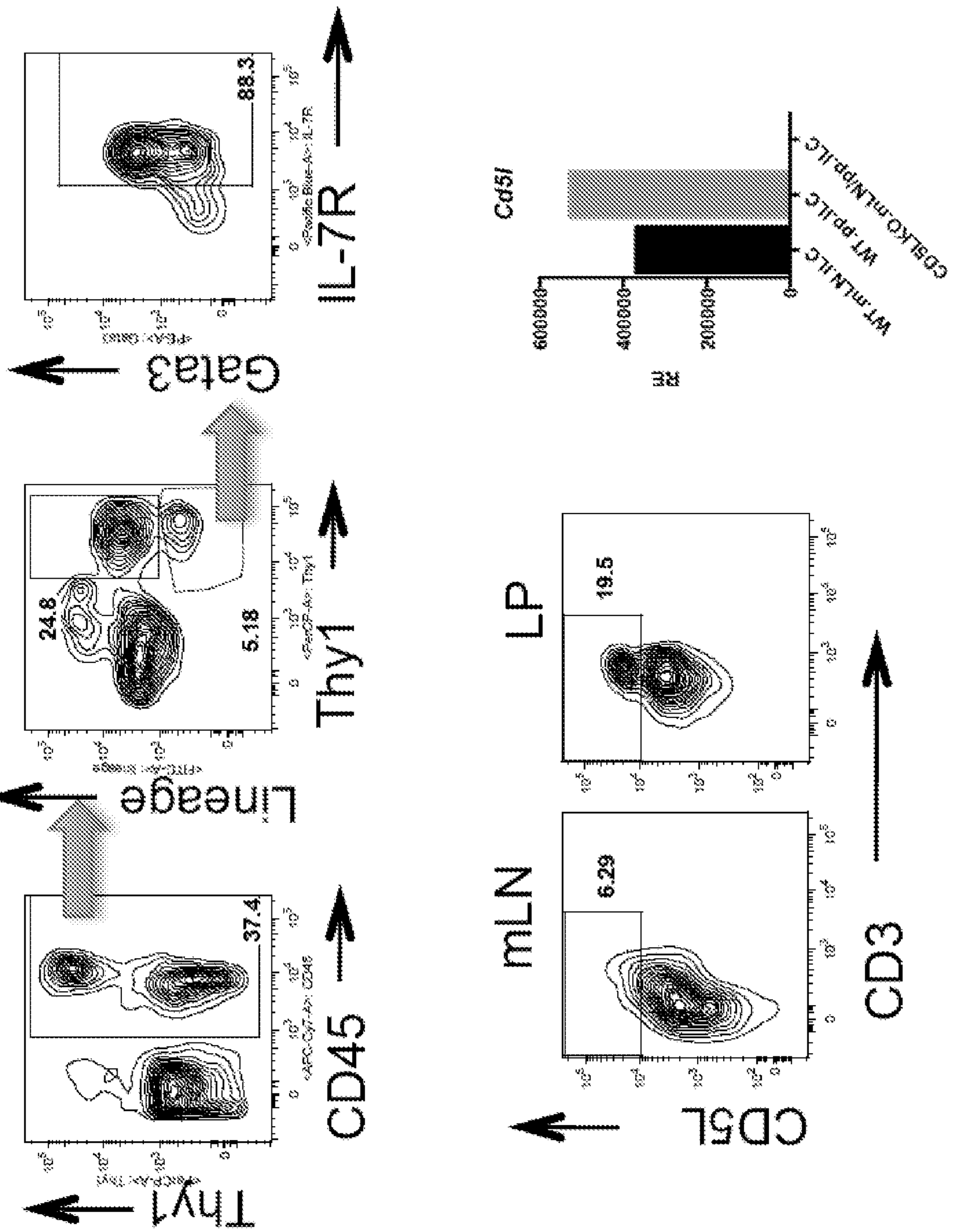

+IL-7 +CD5L
CD5L-/-
WT
0
1.92
27.6
70.5
30.7
1.14
63.2
+IL-7 +CD5L
CD5L-/-
WT
15.3
17.4
intestine
+IL-7
CD5L-/-
WT
0
1.16
18.6
80.2
0
1.98
34.7
63.4
+IL-7
CD5L-/-
WT
26.1
20.9
IFNg
IL-22
Tbet

FIG. 4C


ILC gating strategy
Lineage
Thy1
CD45
Gata3
IL-7R
Cd5l
WT_mLN_ILC
WT_pp_ILC
Cd5lKO_mLN/pp_ILC
mLN
LP
CD5L
CD3
FIG. 4D

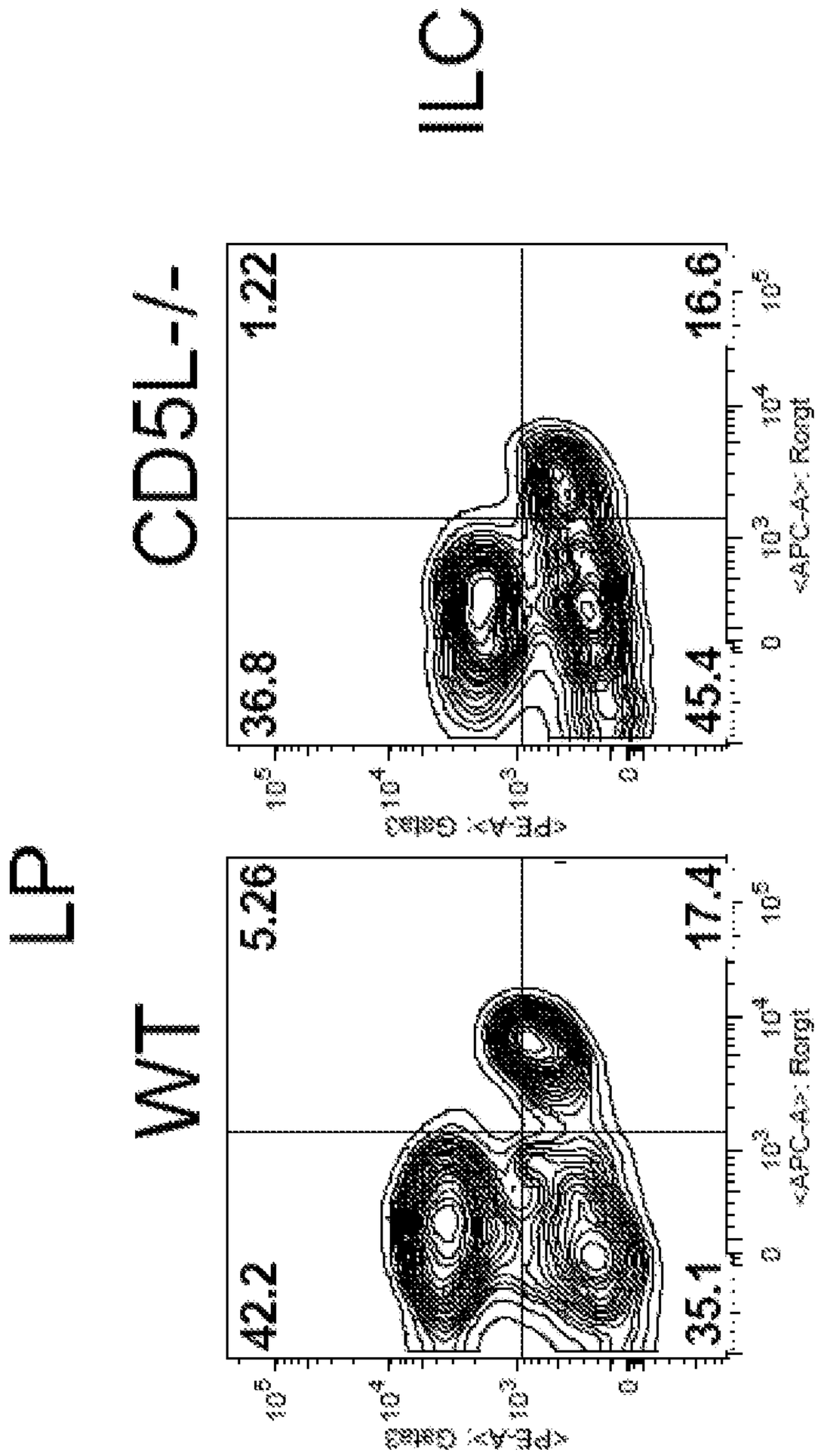

IL
CD5L-/-
1.22
36.8
16.6
45.4
<APC-A>: Rorgt
<PE-A>: Gata3
LP
WT
5.26
42.2
17.4
35.1
Rorgt
Gata3

FIG. 4E

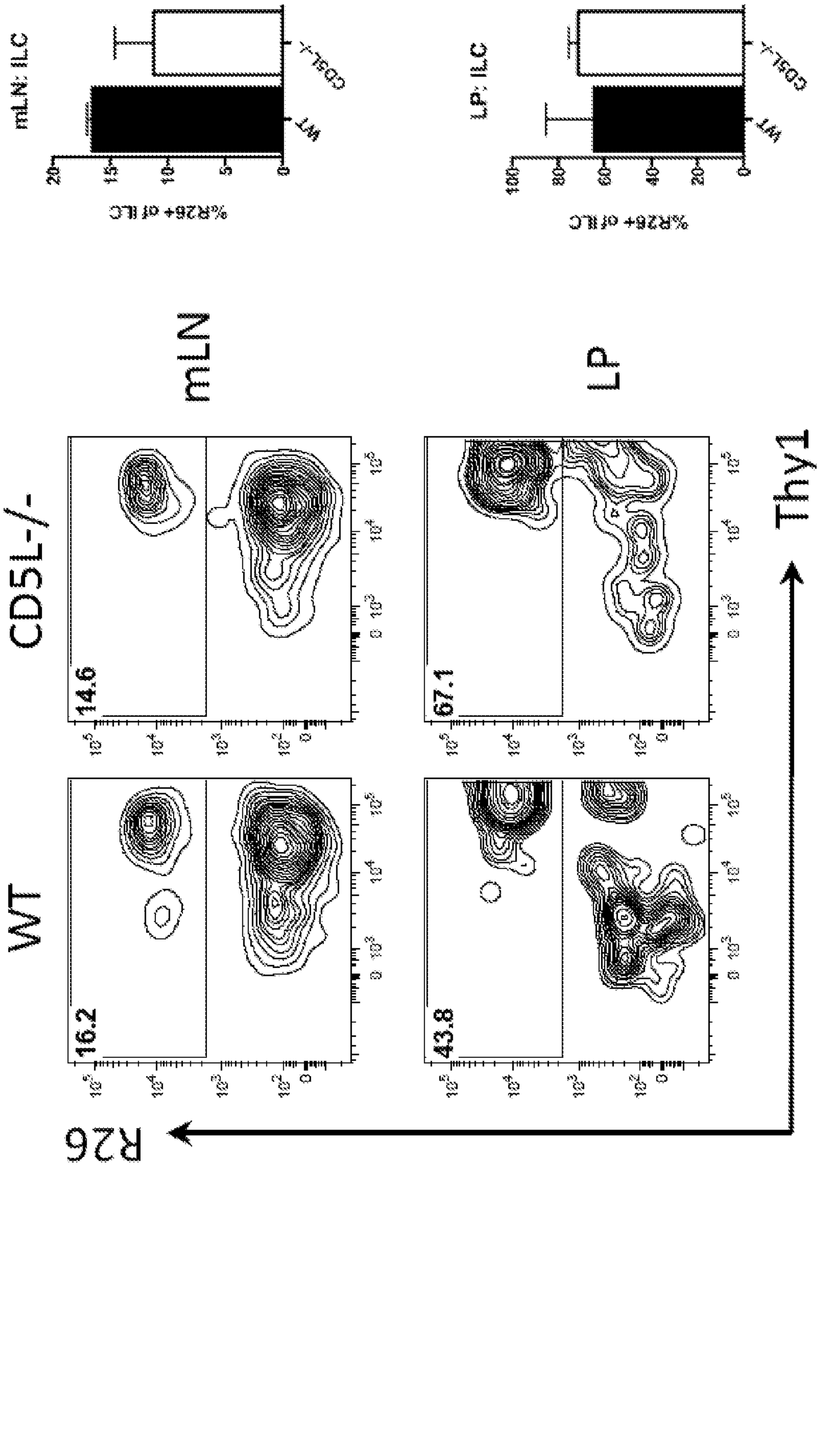

mLN: ILC
LP: ILC
CD5L-/-
WT
%R26+ of ILC
mLN
LP
ILC+
CD5L-/-
WT
14.6
67.1
16.2
43.8
Thy1
R26

FIG. 4F

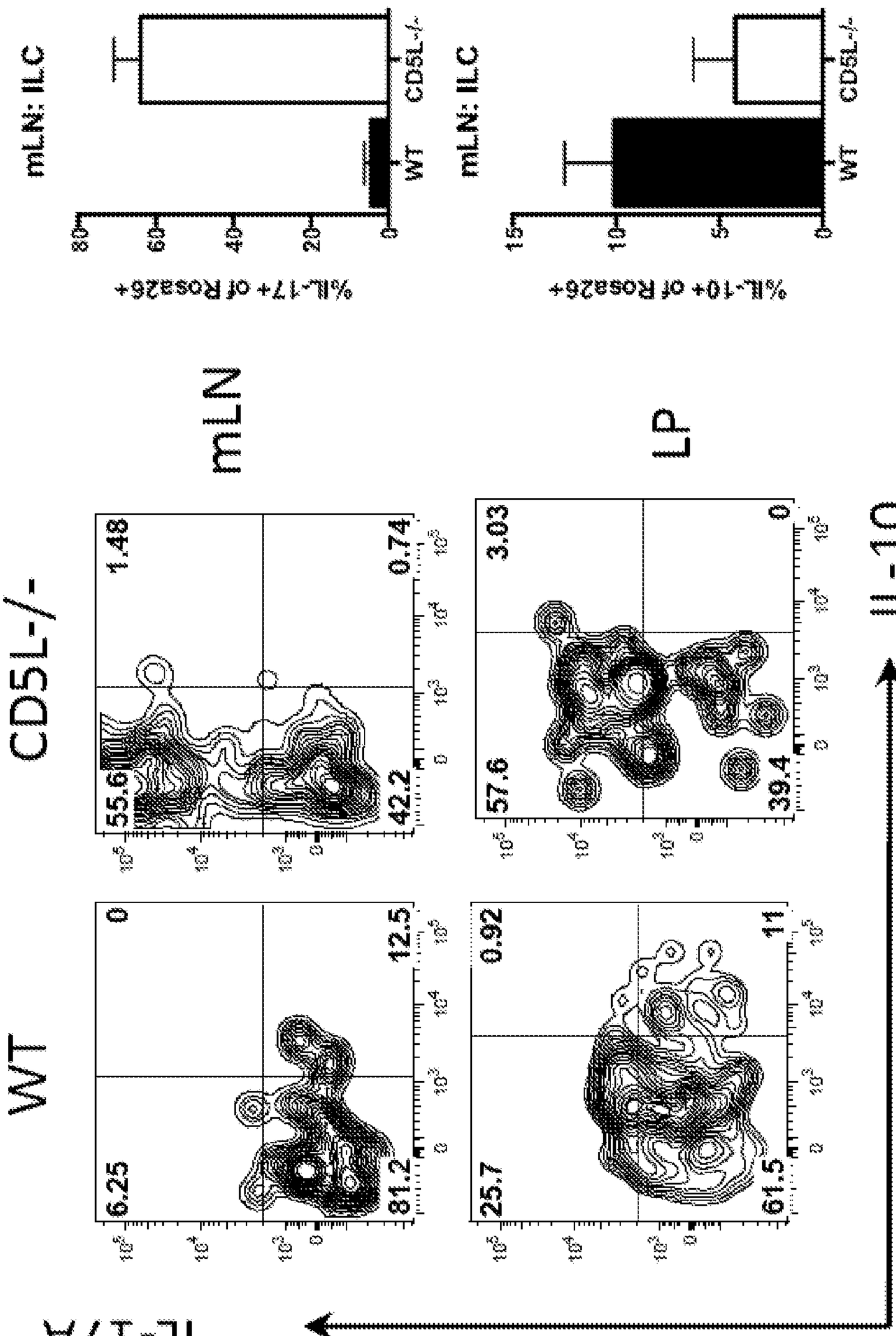

mLN: ILC
CD5L-/-
WT
%IL-17+ of Rosa26+
mLN: ILC
CD5L-/-
WT
%IL-10+ of Rosa26+
IL-17Cre.Rosa26 Td-tomato+
WT
CD5L-/-
mLN
LP
6.25
81.2
12.5
0
1.48
42.2
55.6
0.74
25.7
61.5
11
0.92
57.6
39.4
3.03
0
IL-10
IL-17A

FIG. 4G

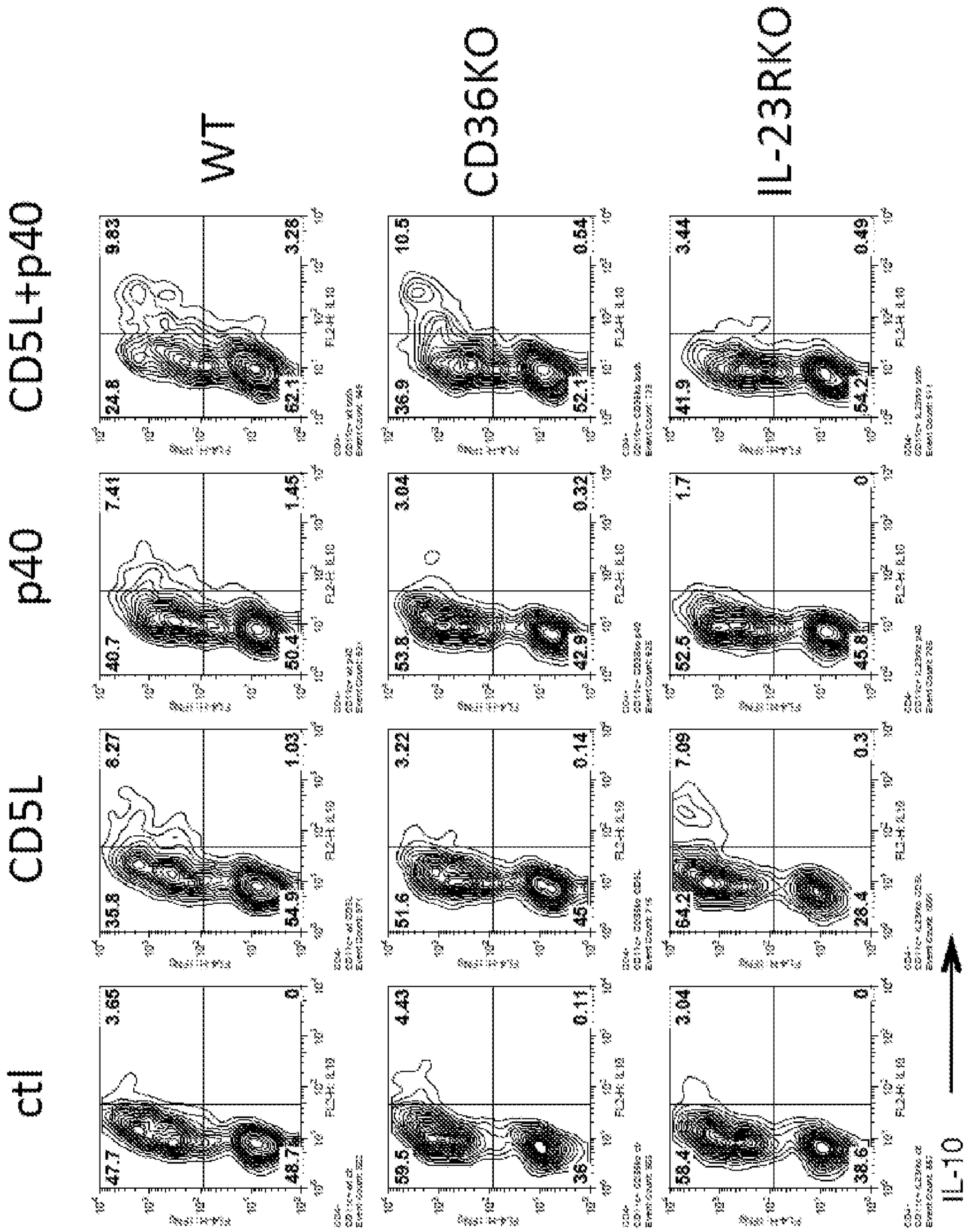


FIG. 5

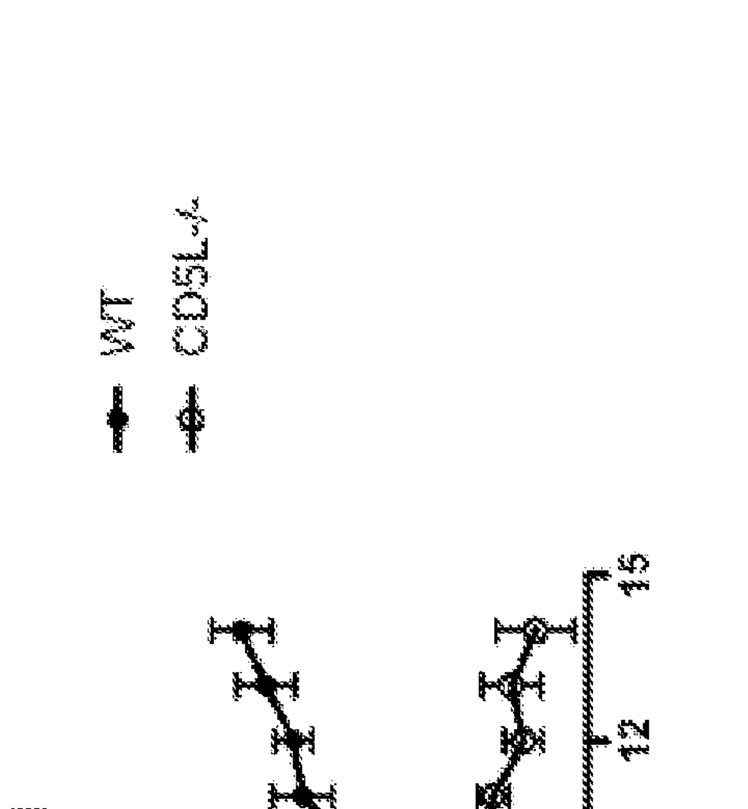


FIG. 6A

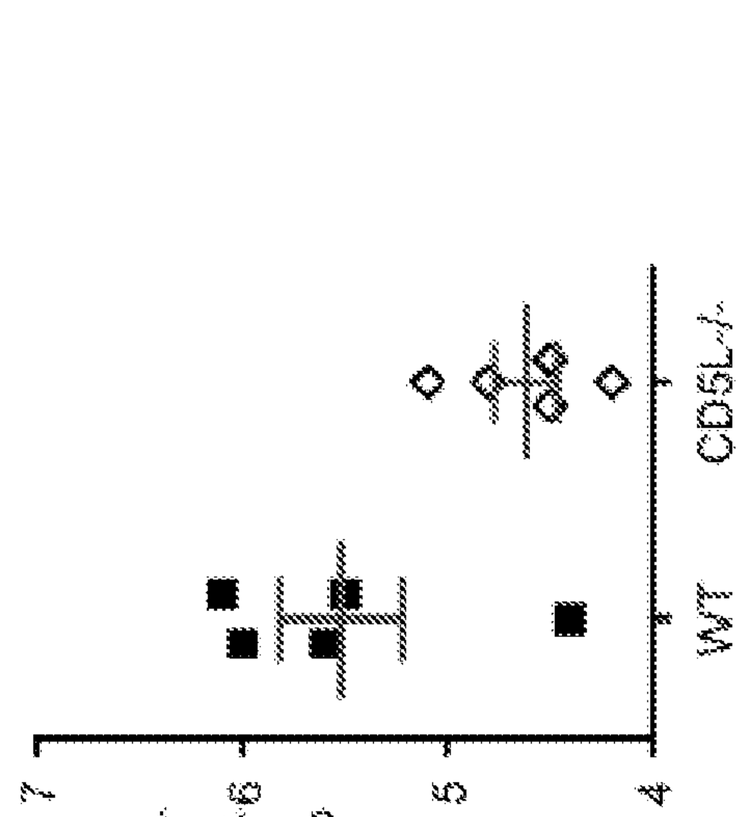


FIG. 6C

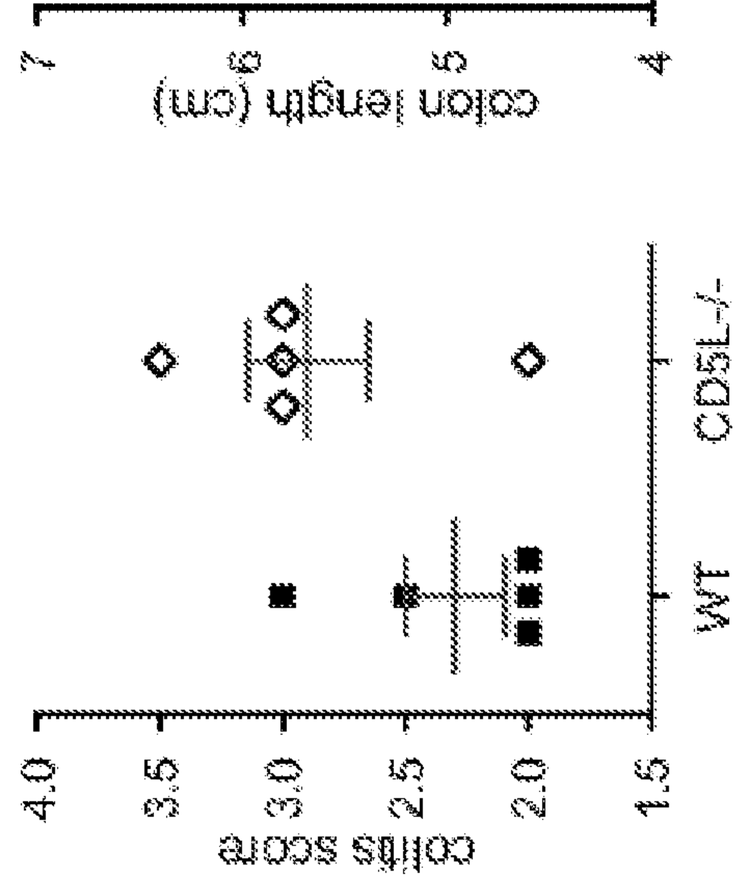


FIG. 6B

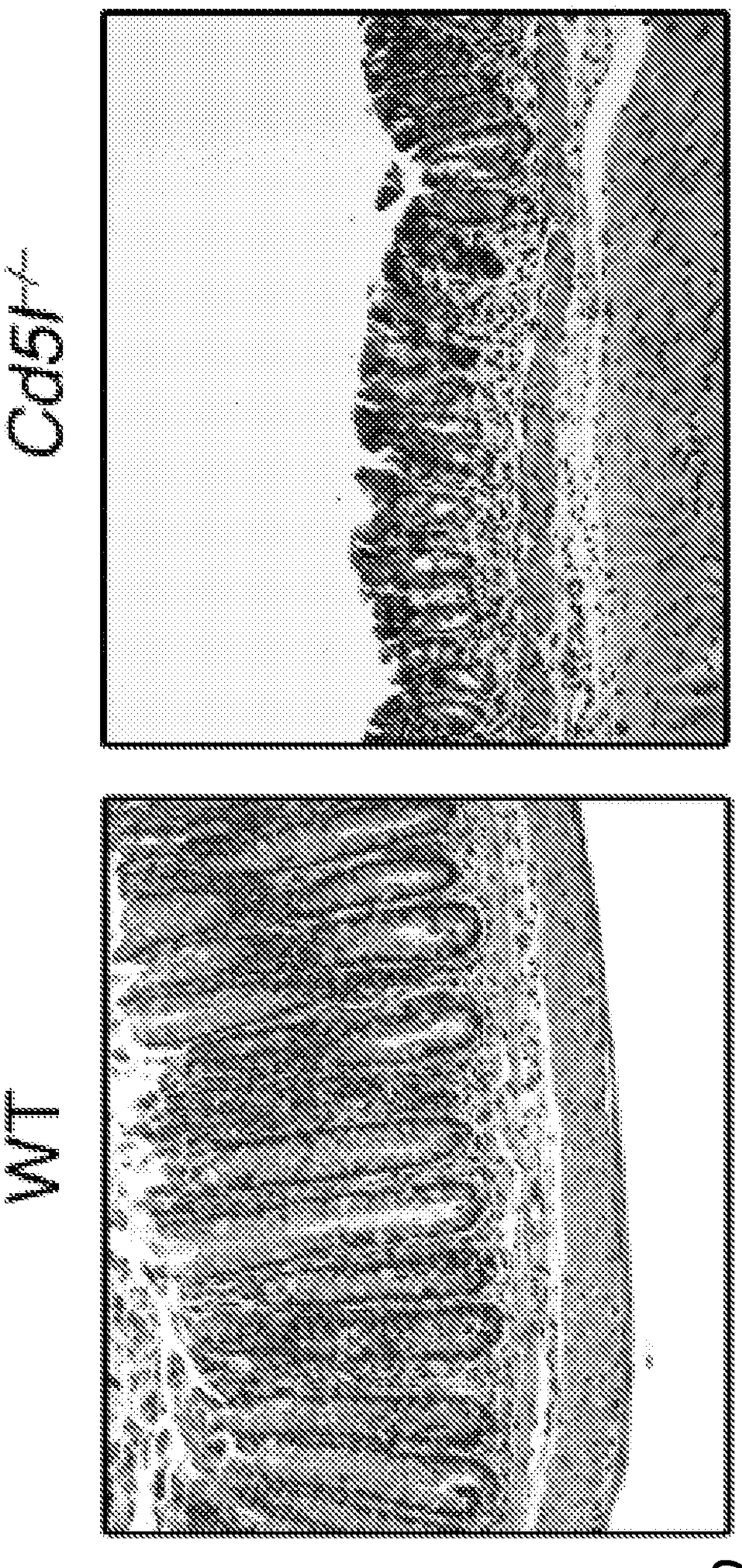


FIG. 6D

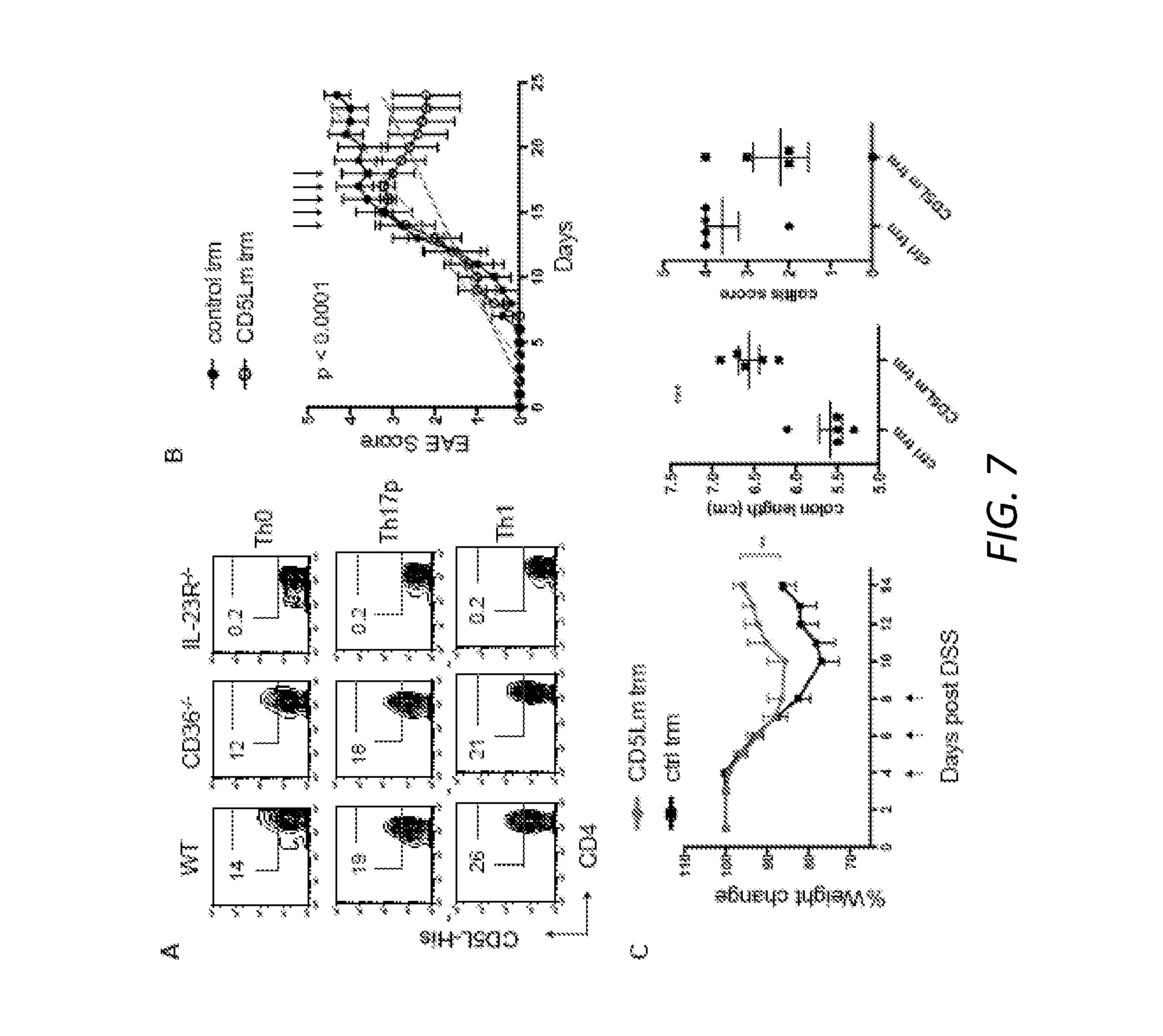

A
WT
CD36-/-
IL-23R-/-
Th0
Th17p
Th1
CD4
CD5L-His
14
12
0.2
19
18
0.2
26
21
0.2
B
control tm
CD5Lm tm
p < 0.0001
EAE score
Days
C
CD5Lm tm
ctrl tm
% weight change
Days post DSS
colon length (cm)
ctrl tm
CD5Lm tm

FIG. 7

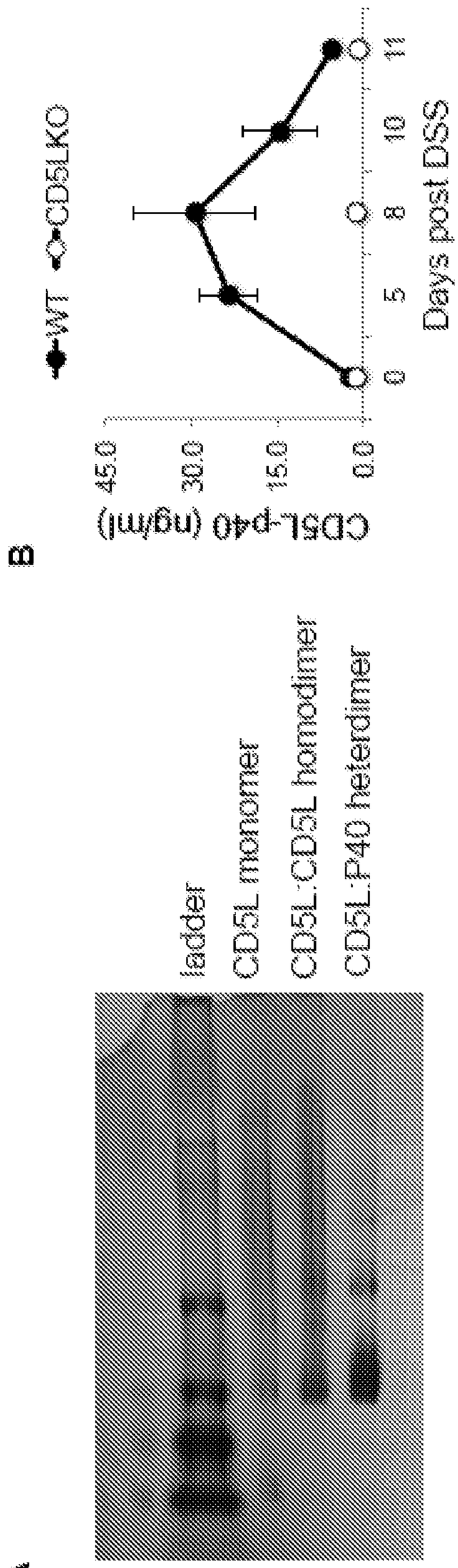

a
CD5L-p40 (ng/ml)
45.0
30.0
15.0
0.0
0
5
8
10
11
Days post DSS
WT
CD5LKO
b
ladder
CD5L monomer
CD5L-CD5L homodimer
CD5L-P40 heterdimer

FIG. 8

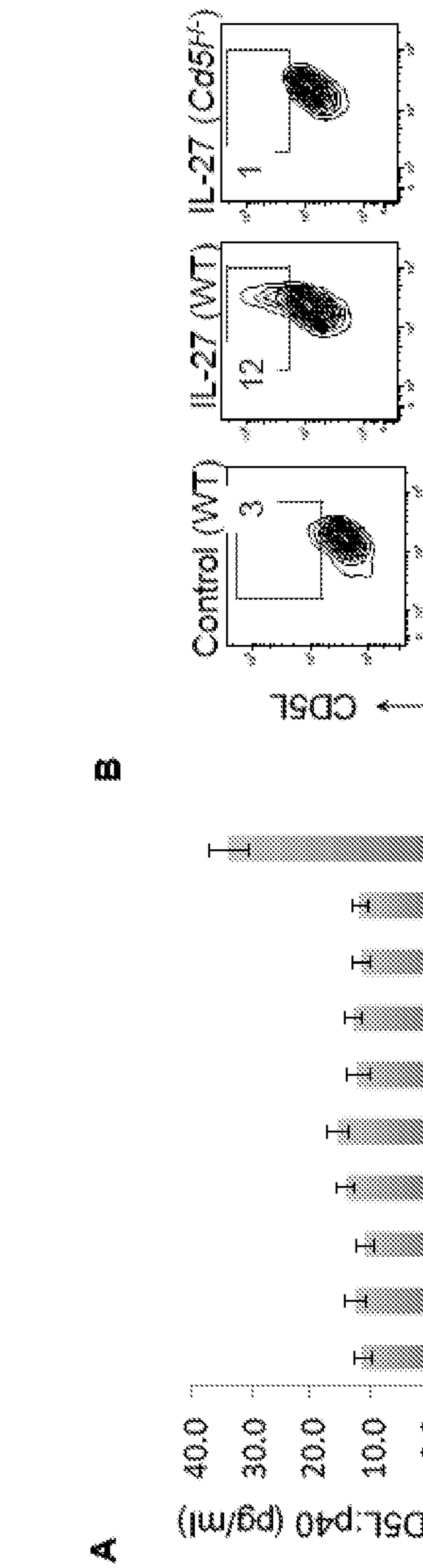

IL-27 (Cd5l-)
1
CD5L
CD4
Control (WT)
3
IL-27 (WT)
12
CD5L:p40 (pg/ml)
40.0
30.0
20.0
10.0
0.0
ctl
TLR1/2
TLR2
TLR3H
TLR3L
TLR4
TLR5
TLR6
TLR7
TLR9

FIG. 9

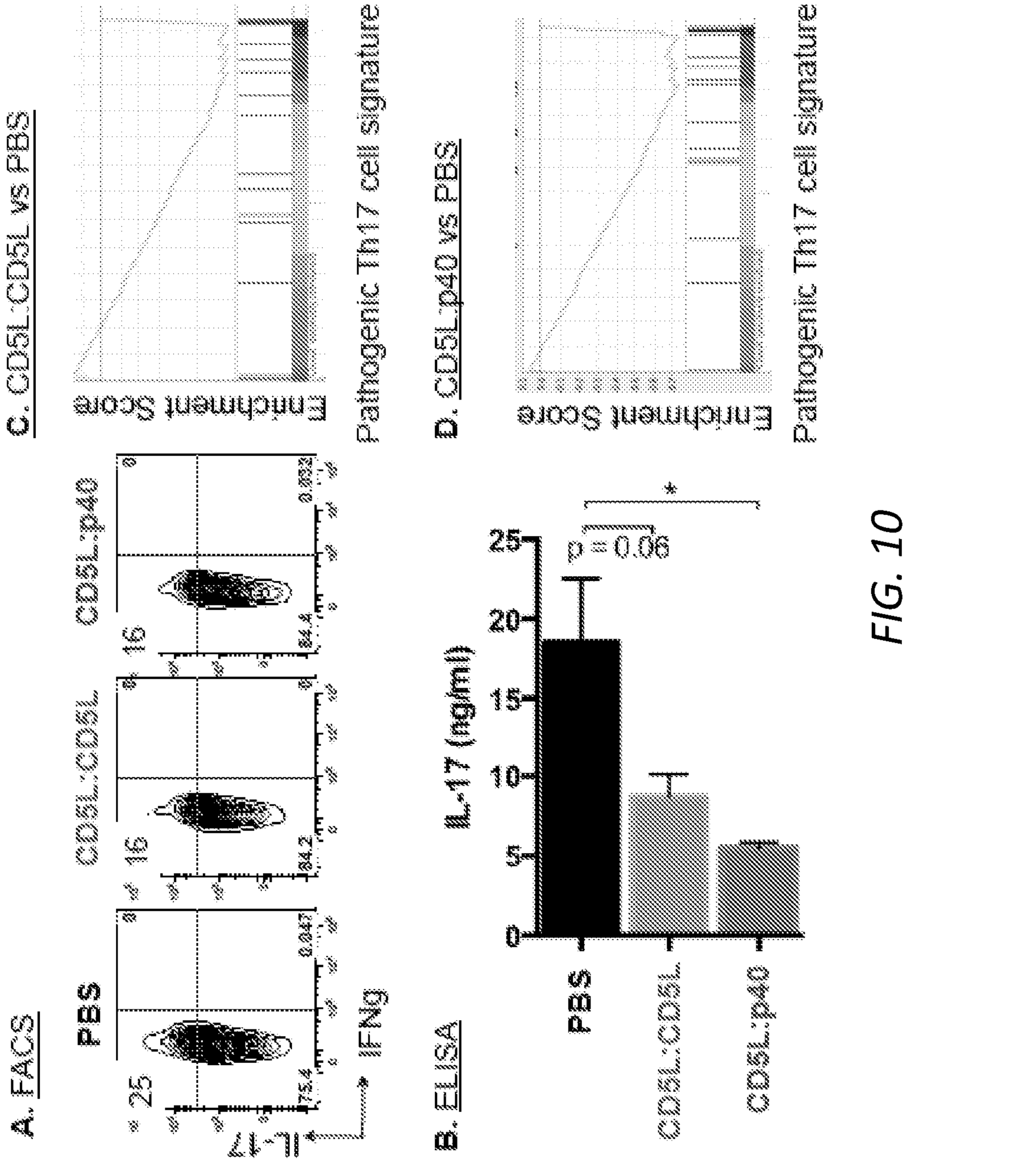

A. FACS
PBS
CD5L:CD5L
CD5L:p40
IL-17
IFNg
B. ELISA
IL-17 (ng/ml)
p = 0.06
*
PBS
CD5L:CD5L
CD5L:p40
C. CD5L:CD5L vs PBS
Enrichment Score
Pathogenic Th17 cell signature
D. CD5L:p40 vs PBS
Enrichment Score
Pathogenic Th17 cell signature

FIG. 10

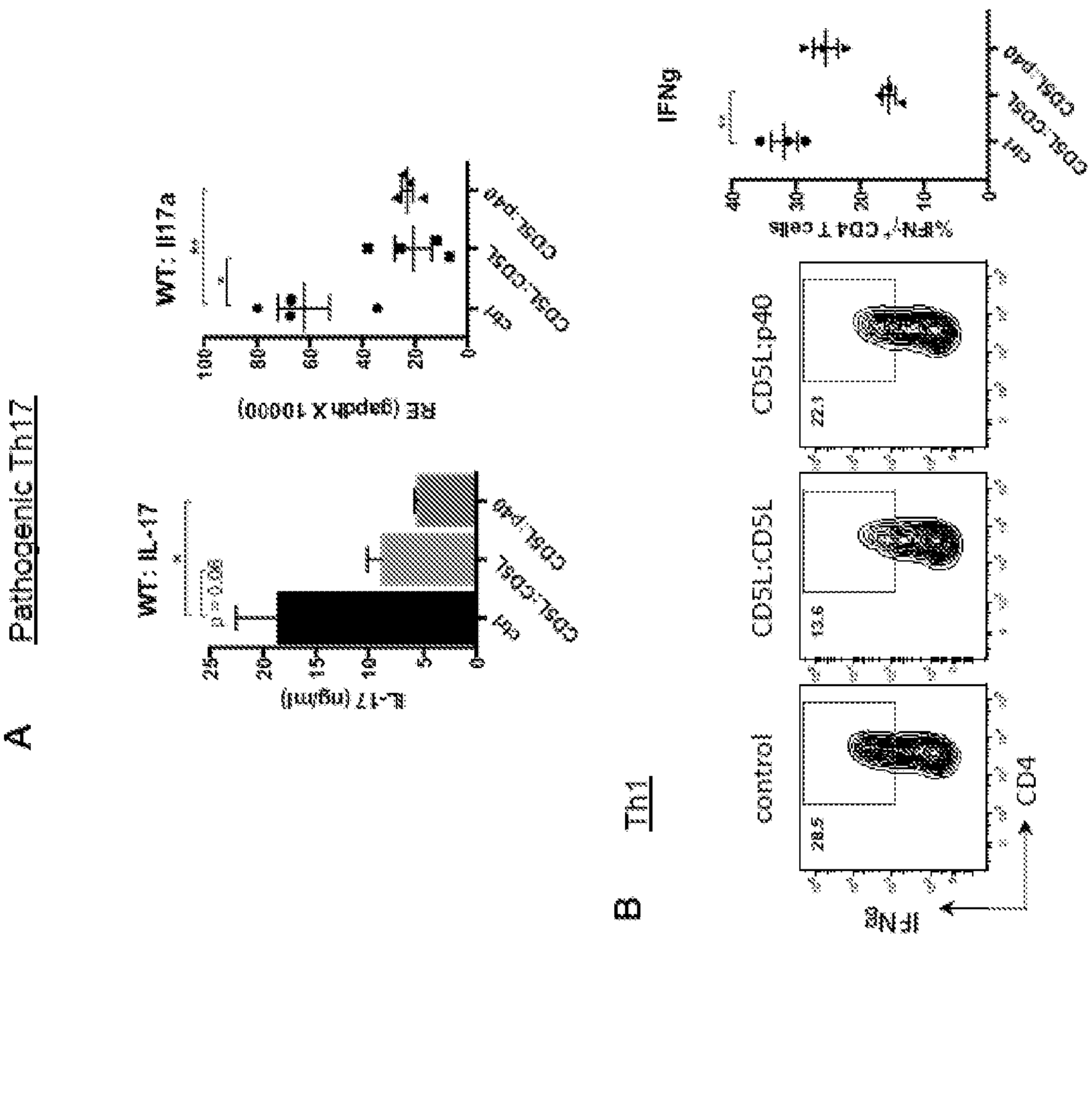

A
Pathogenic Th17
WT: Il17a
RE (gapdh X 10000)
100
80
60
40
20
0
**
ctrl
CD5L-CD5L
CD5L-p40
WT: IL-17
IL-17 (ng/ml)
25
20
15
10
5
0
*
p = 0.06
ctrl
CD5L-CD5L
CD5L-p40
B
Th1
IFNg
%IFN$^+$ CD4 T cells
40
30
20
10
0
**
ctrl
CD5L-CD5L
CD5L-p40
CD5L:p40
22.1
control
CD5L:CD5L
28.5
13.6
IFN$_{gc}$
CD4

FIG. 11

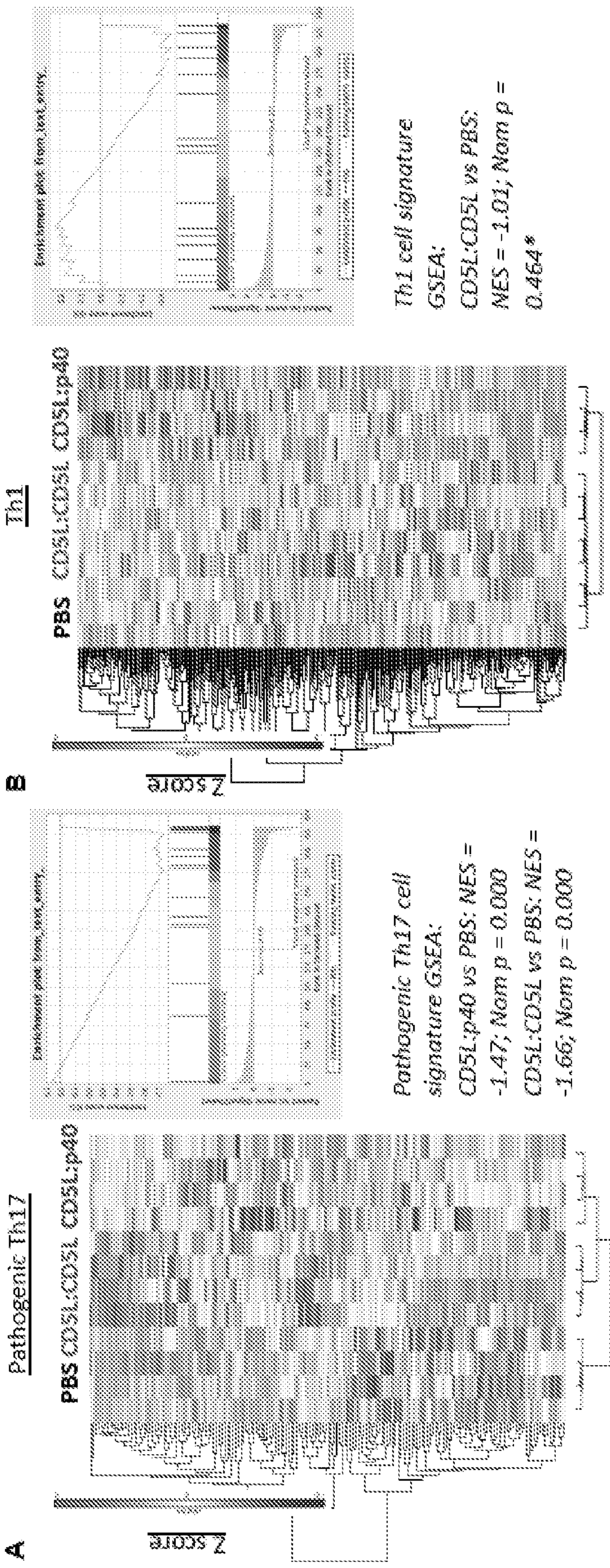

B
Th1
PBS
CD5L:CD5L
CD5L:p40
Z score
Enrichment plot: Th1_test_entry
Enrichment score (ES)
Ranked list metric (PreRanked)
Th1 cell signature GSEA: CD5L:CD5L vs PBS: NES = -1.01; Nom p = 0.464*
A
Pathogenic Th17
PBS
CD5L:CD5L
CD5L:p40
Z score
Enrichment plot: Pathogenic_Th17_test_entry
Enrichment score (ES)
Ranked list metric (PreRanked)
Pathogenic Th17 cell signature GSEA: CD5L:p40 vs PBS: NES = -1.47; Nom p = 0.000
CD5L:CD5L vs PBS: NES = -1.66; Nom p = 0.000

FIG. 12

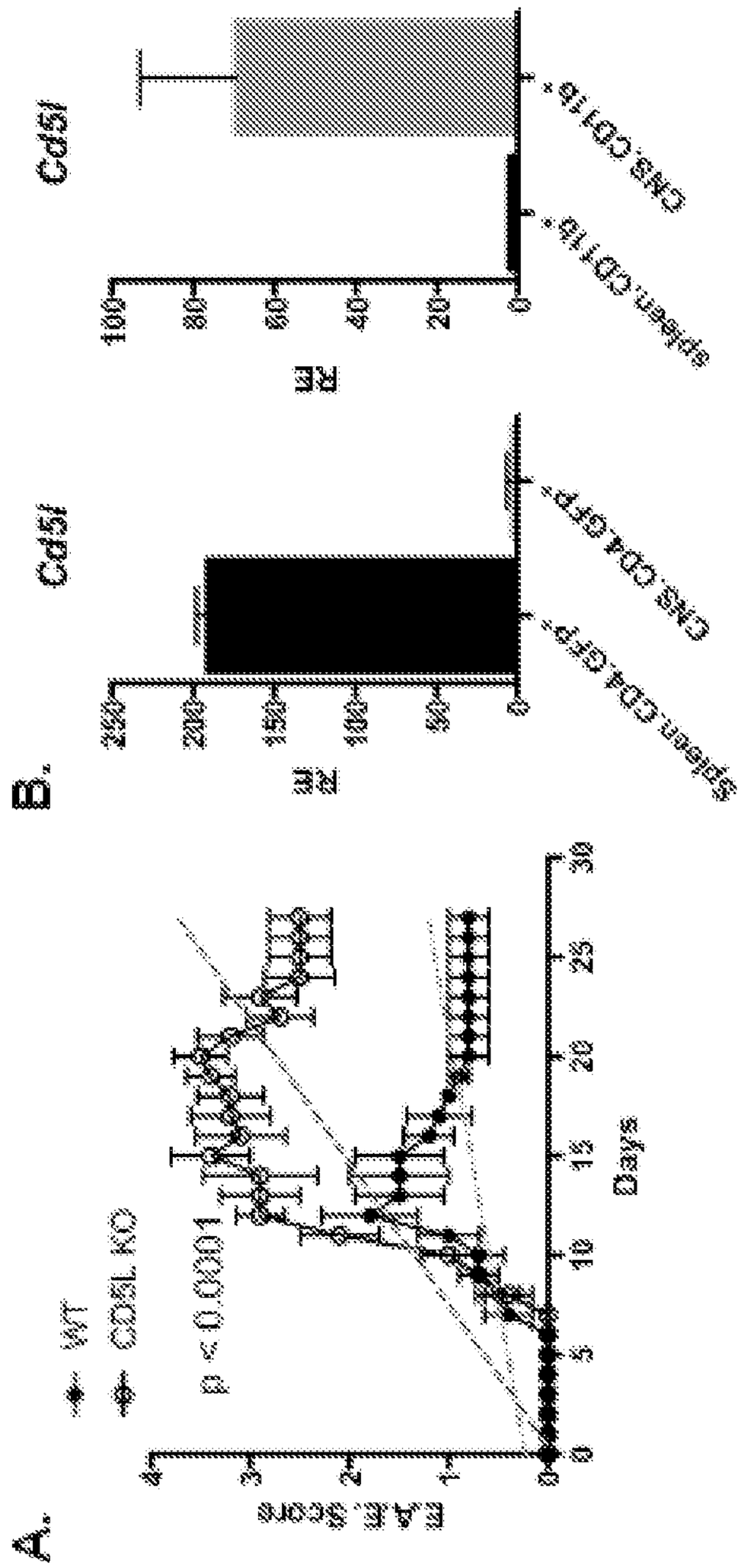
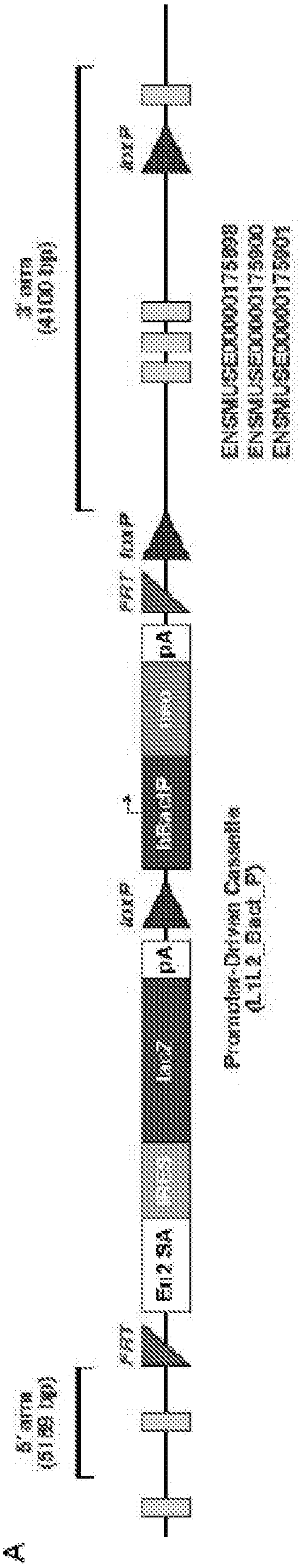

B.
Cd5l
RFI
320
200
150
100
50
0
Spleen_CD4_GFP+
CMS_CD4_GFP+
Cd5l
RFI
100
80
60
40
20
0
Spleen_CD71b+
CMS_CD71b+
WT
CD5L KO
p < 0.0001
EAE_Score
0
1
2
3
4
0
5
10
15
20
25
30
Days

FIG. 13

A


5' arm
FRT
En2 SA
lacZ
pA
loxP
hBactP
pA
FRT
loxP
Promoter-Driven Cassette
(L1L2_Bact_P)
3' arm
loxP

B

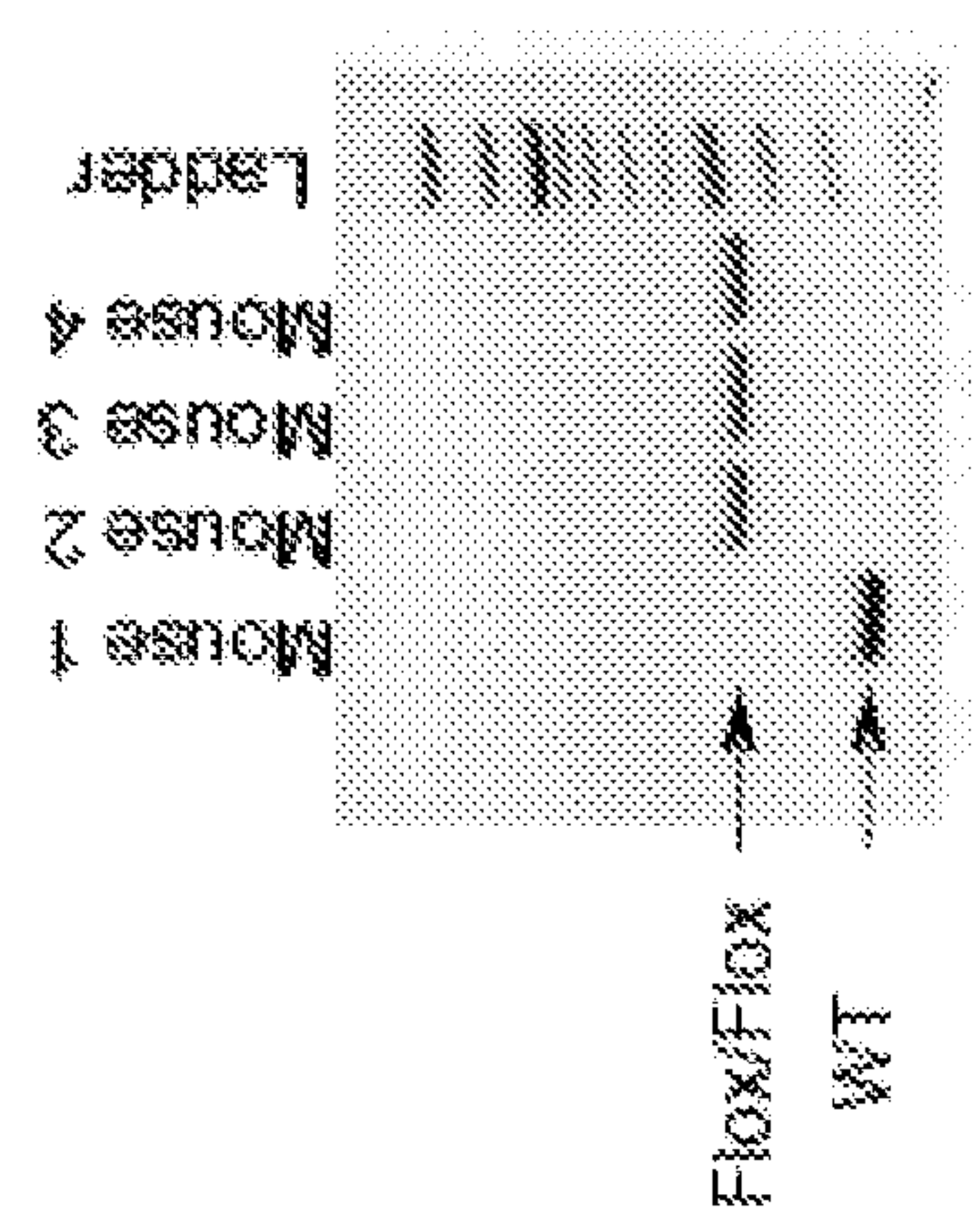

Ladder
Mouse 4
Mouse 3
Mouse 2
Mouse 1
Flox/Flox
WT

FIG. 14

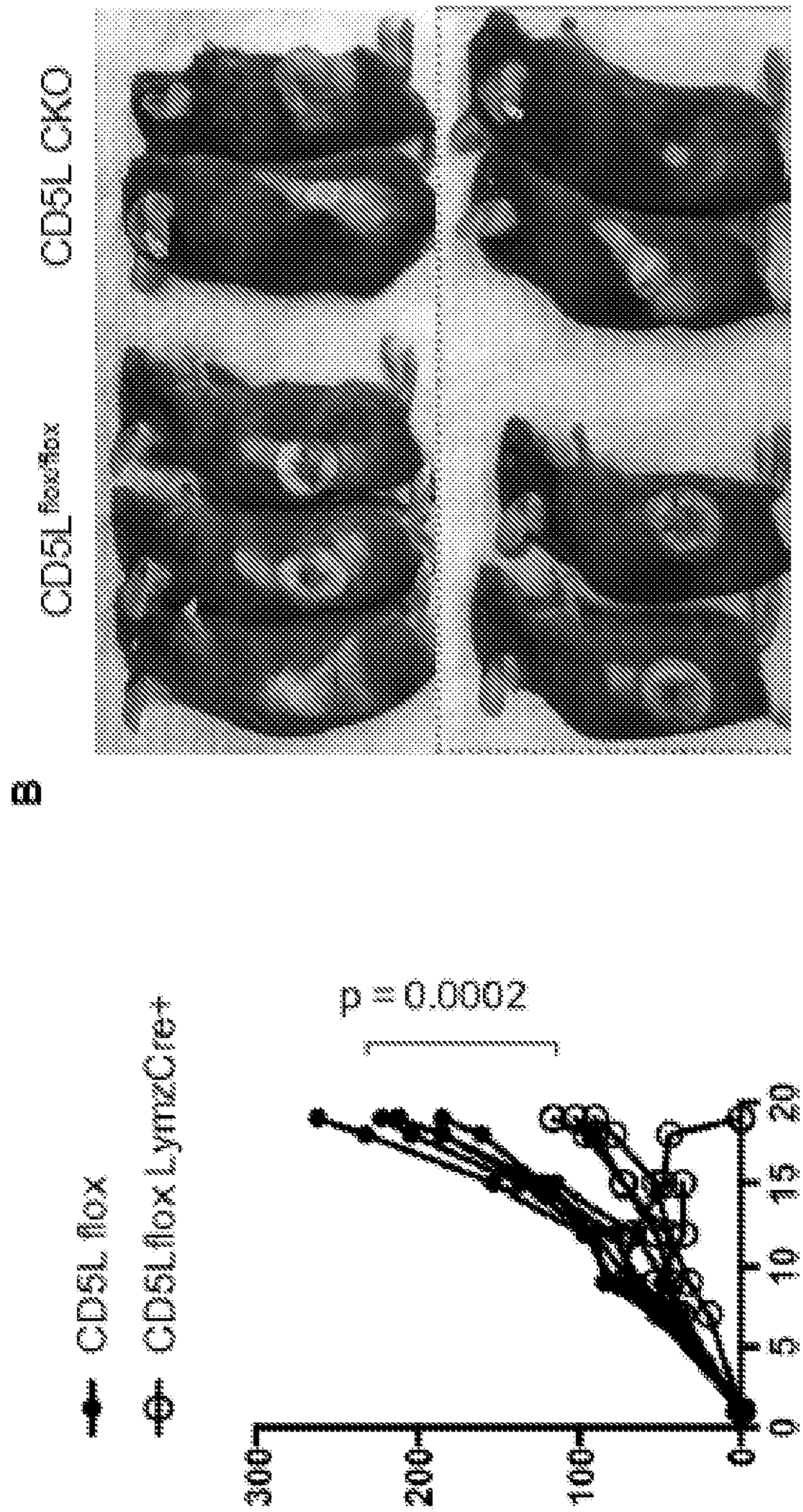

CD5L flox/flox
CD5L CKO
B
CD5L flox
CD5Lflox LysmCre+
p = 0.0002
0
5
10
15
20
days
300
200
100
0
Tumor size (mm^2)
A

FIG. 15

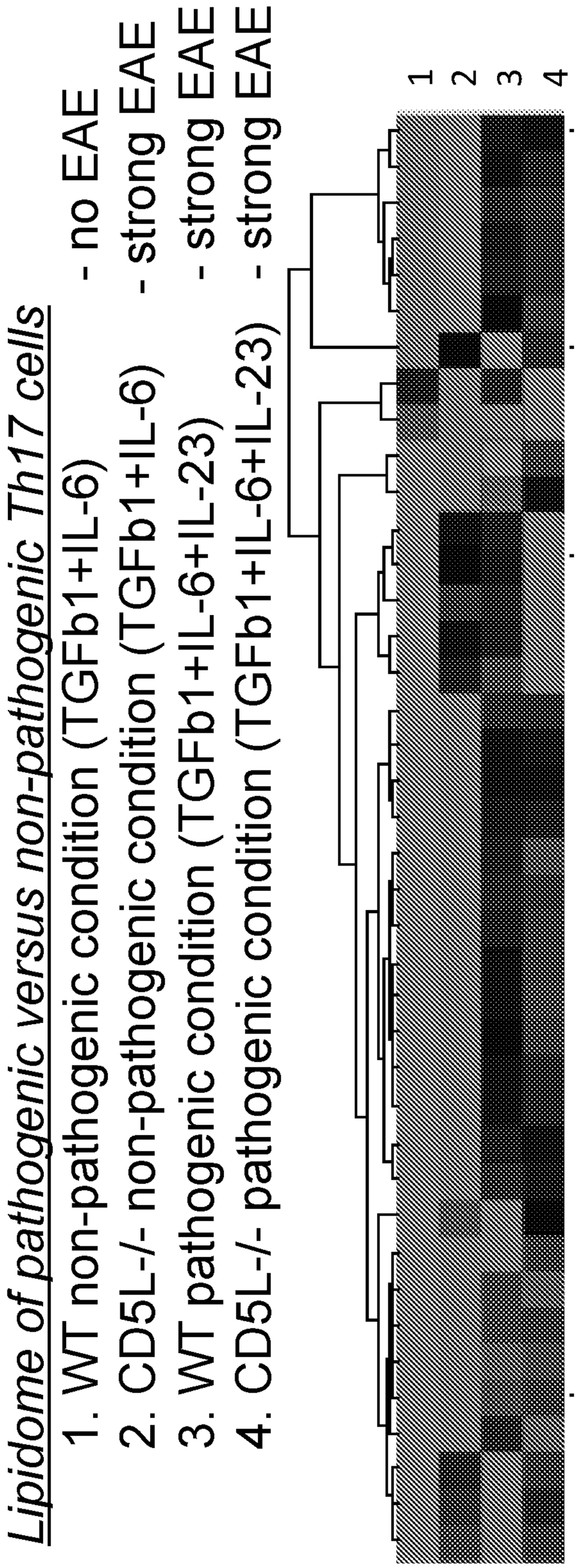

Lipidome of pathogenic versus non-pathogenic Th17 cells
1. WT non-pathogenic condition (TGFb1+IL-6) - no EAE
2. CD5L-/- non-pathogenic condition (TGFb1+IL-6) - strong EAE
3. WT pathogenic condition (TGFb1+IL-6+IL-23) - strong EAE
4. CD5L-/- pathogenic condition (TGFb1+IL-6+IL-23) - strong EAE
1
2
3
4

FIG. 16

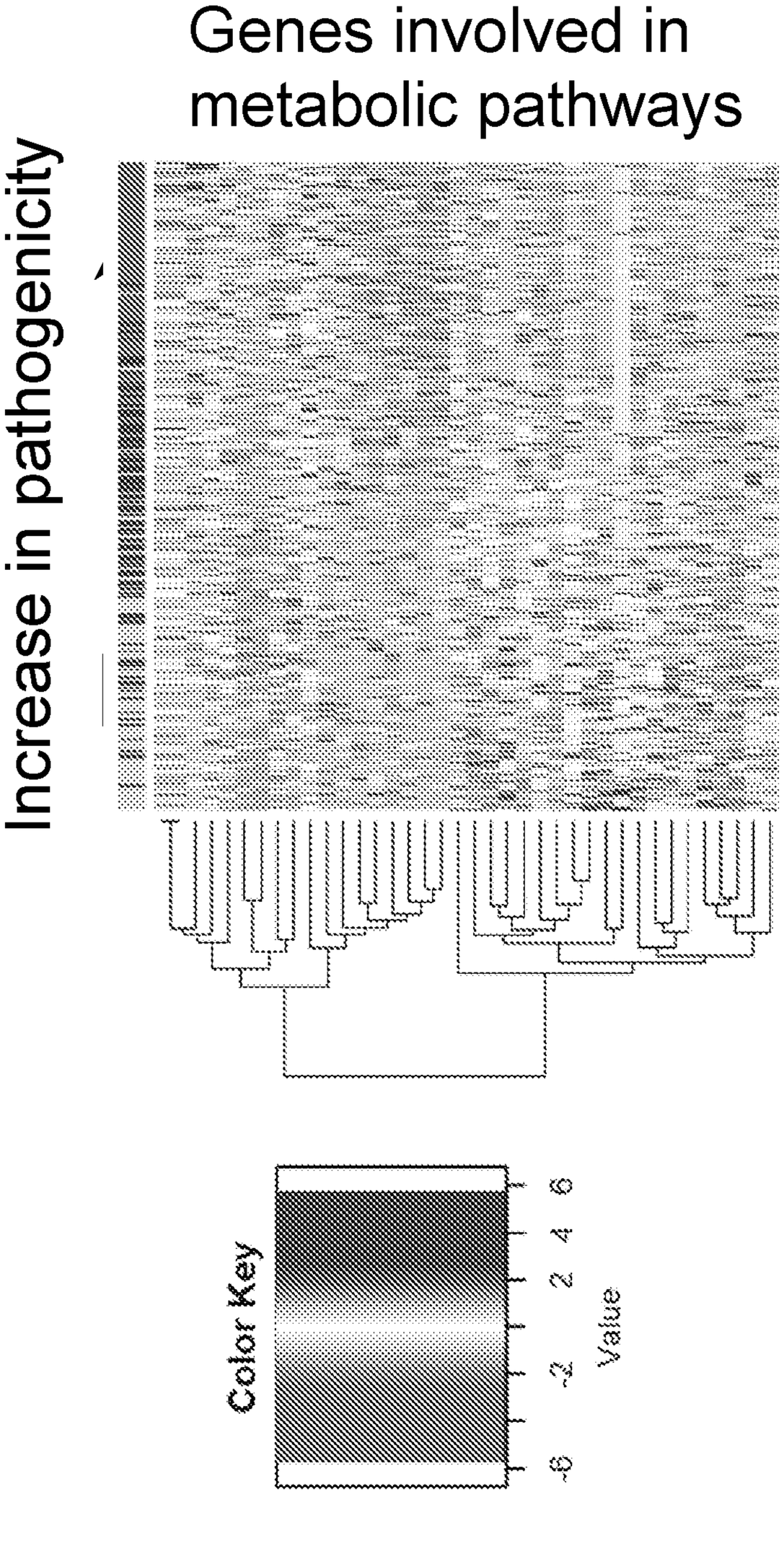

Genes involved in
metabolic pathways
Increase in pathogenicity
Color Key
Value
-6
-2
2
4
6

FIG. 17

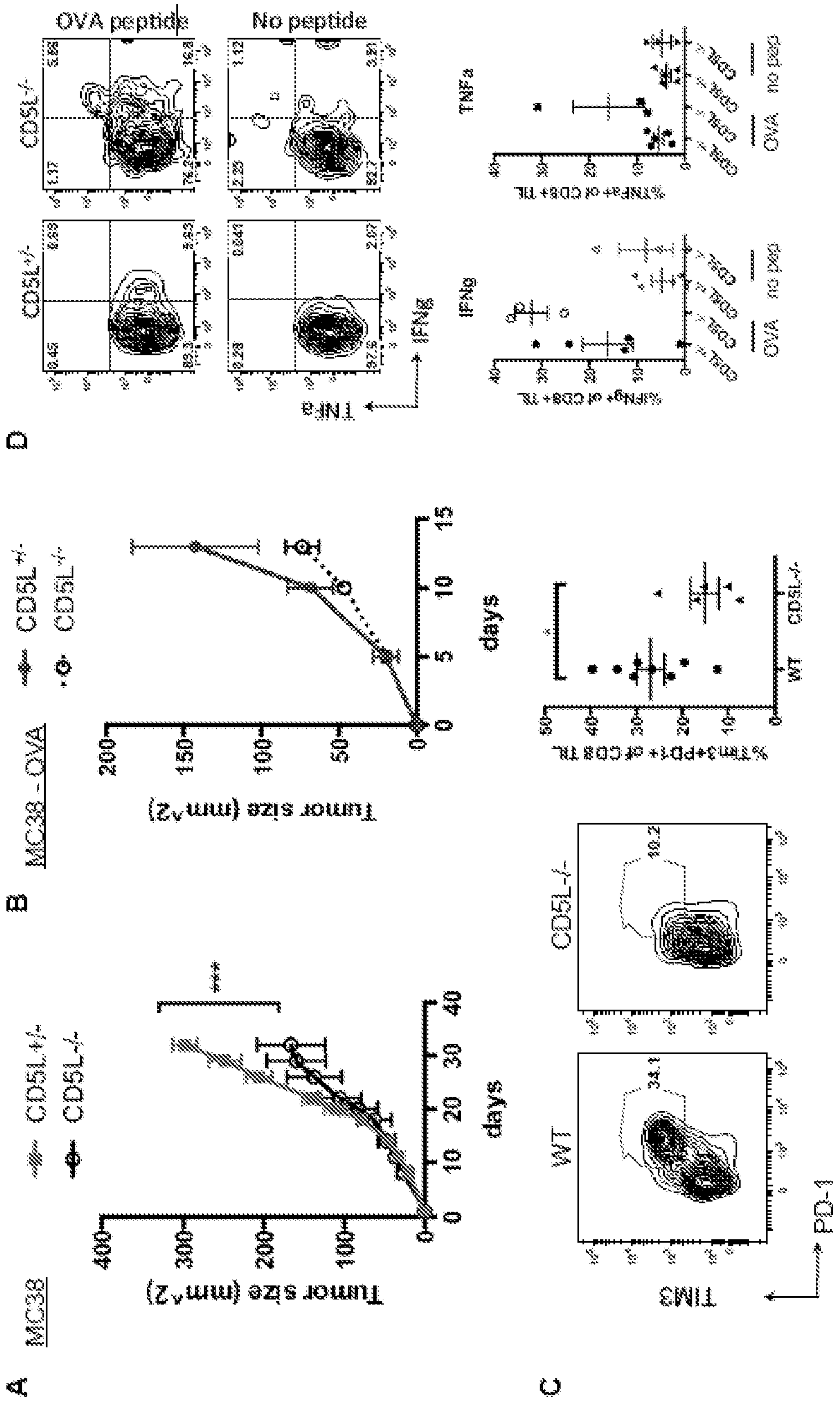

A
MC38
CD5L+/-
CD5L-/-
Tumor size (mm^2)
days
B
MC38 - OVA
CD5L +/-
CD5L -/-
Tumor size (mm^2)
days
C
WT
CD5L-/-
34.1
10.2
TIM3
PD-1
%Tim3+PD1+ of CD8 TIL
WT
CD5L-/-
D
OVA peptide
No peptide
CD5L+/-
CD5L-/-
TNFa
IFNg
%IFNg+ of CD8+ TIL
%TNFa+ of CD8+ TIL
OVA
no pep

FIG. 18

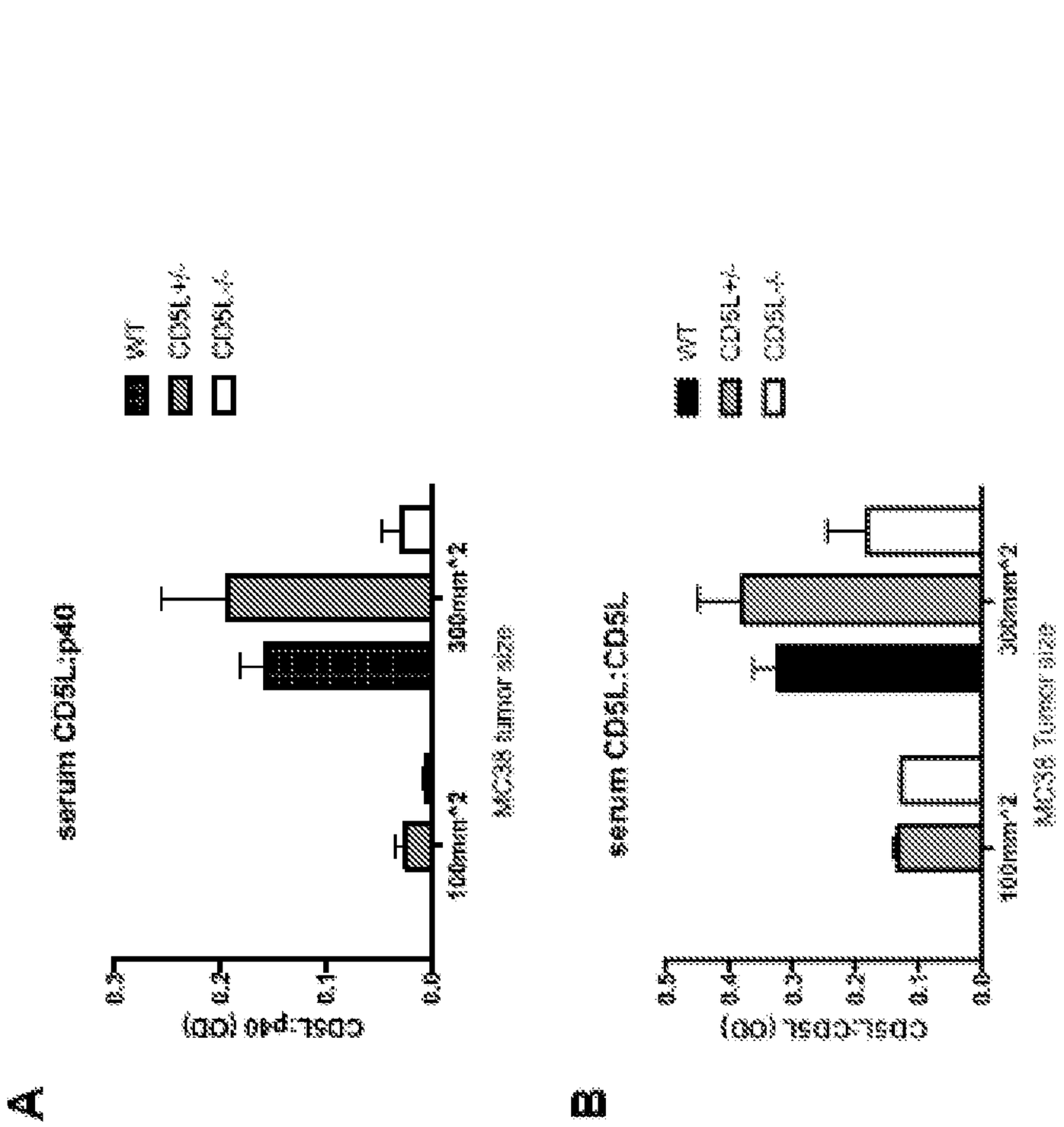

A
serum CD5L:p40
CD5L:p40 (OD)
0.3
0.2
0.1
0.0
100mm^2
300mm^2
MC38 tumor size
WT
CD5L+/-
CD5L-/-
B
serum CD5L:CD5L
CD5L:CD5L (OD)
0.5
0.4
0.3
0.2
0.1
0.0
100mm^2
300mm^2
MC38 Tumor size
WT
CD5L+/-
CD5L-/-

FIG. 19

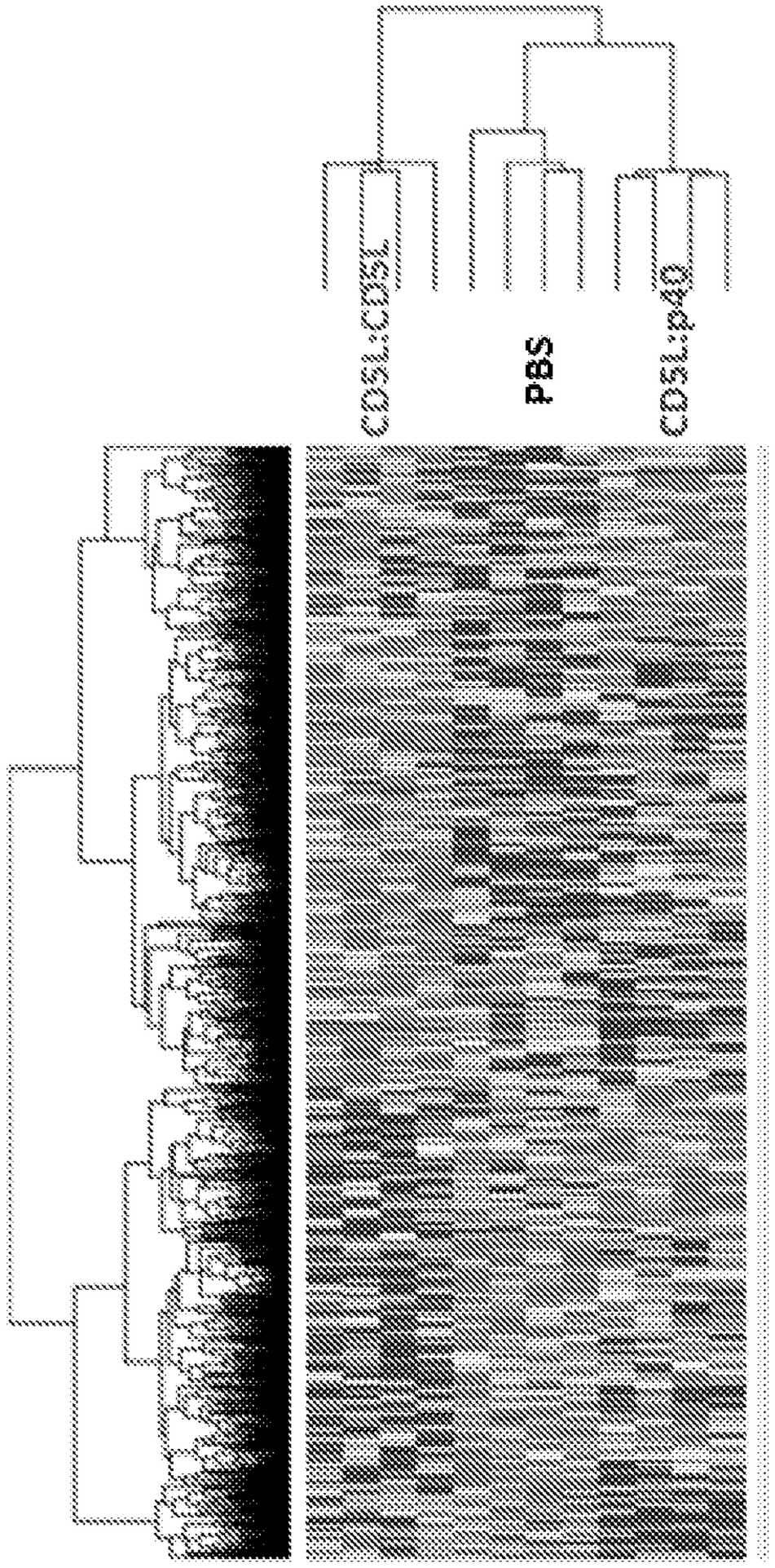


FIG. 20

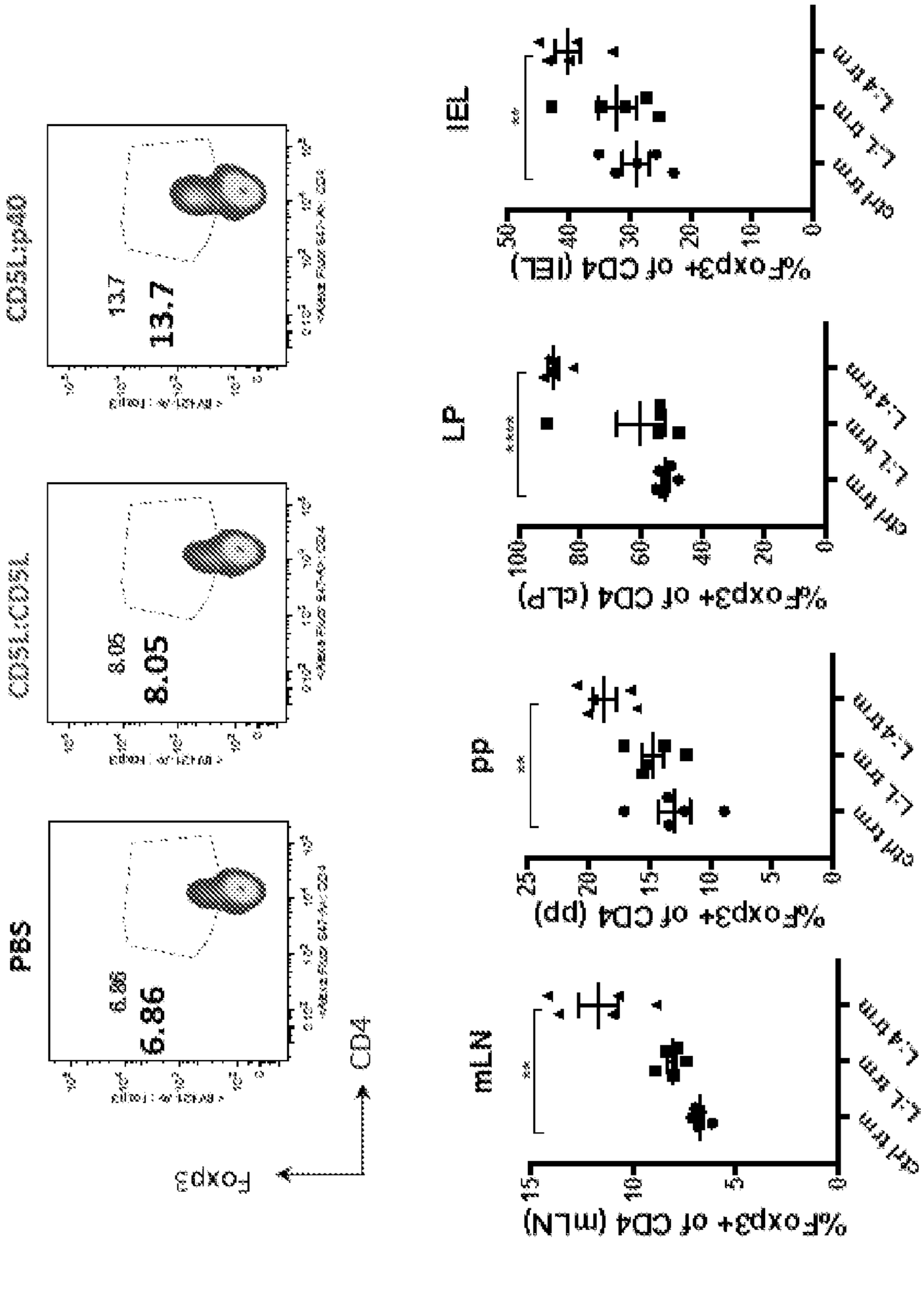

A
PBS
CD5L:p40
CD5L:CD5L
Foxp3
CD4
6.86
6.86
13.7
13.7
8.05
8.05
mLN
LP
IEL
pp
%Foxp3+ of CD4 (mLN)
%Foxp3+ of CD4 (cLP)
%Foxp3+ of CD4 (iEL)
%Foxp3+ of CD4 (pp)
cntl trm
L-L trm
L-4 trm

FIG. 21A

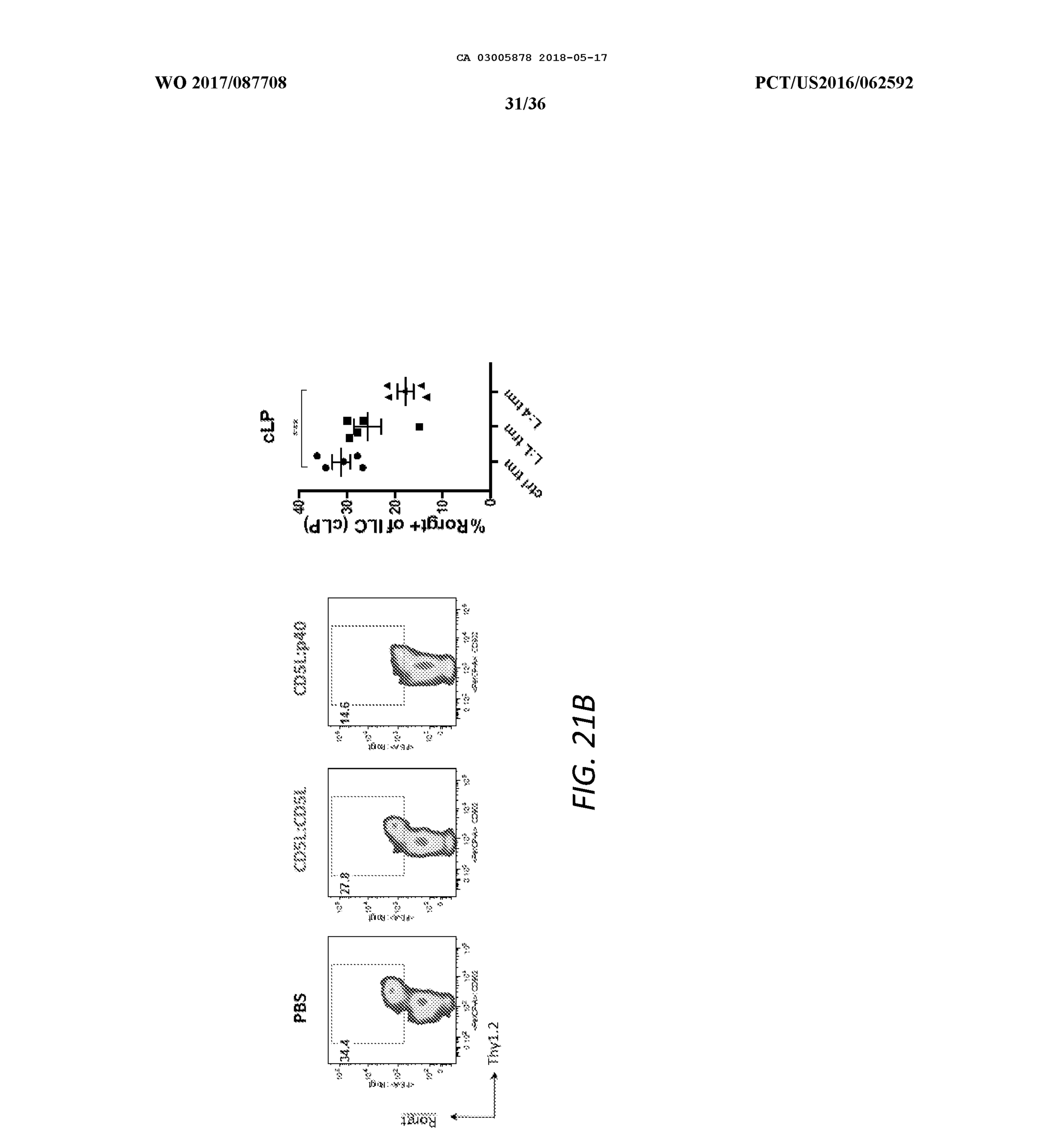

cLP
%Rorgt+ of ILC (cLP)
40
30
20
10
0
ctrl tm
L.L tm
L.A tm
***
CD3L.p40
14.6
CD3L.CD3L
27.8
PBS
34.4
Rorgt
Thy1.2

FIG. 21B

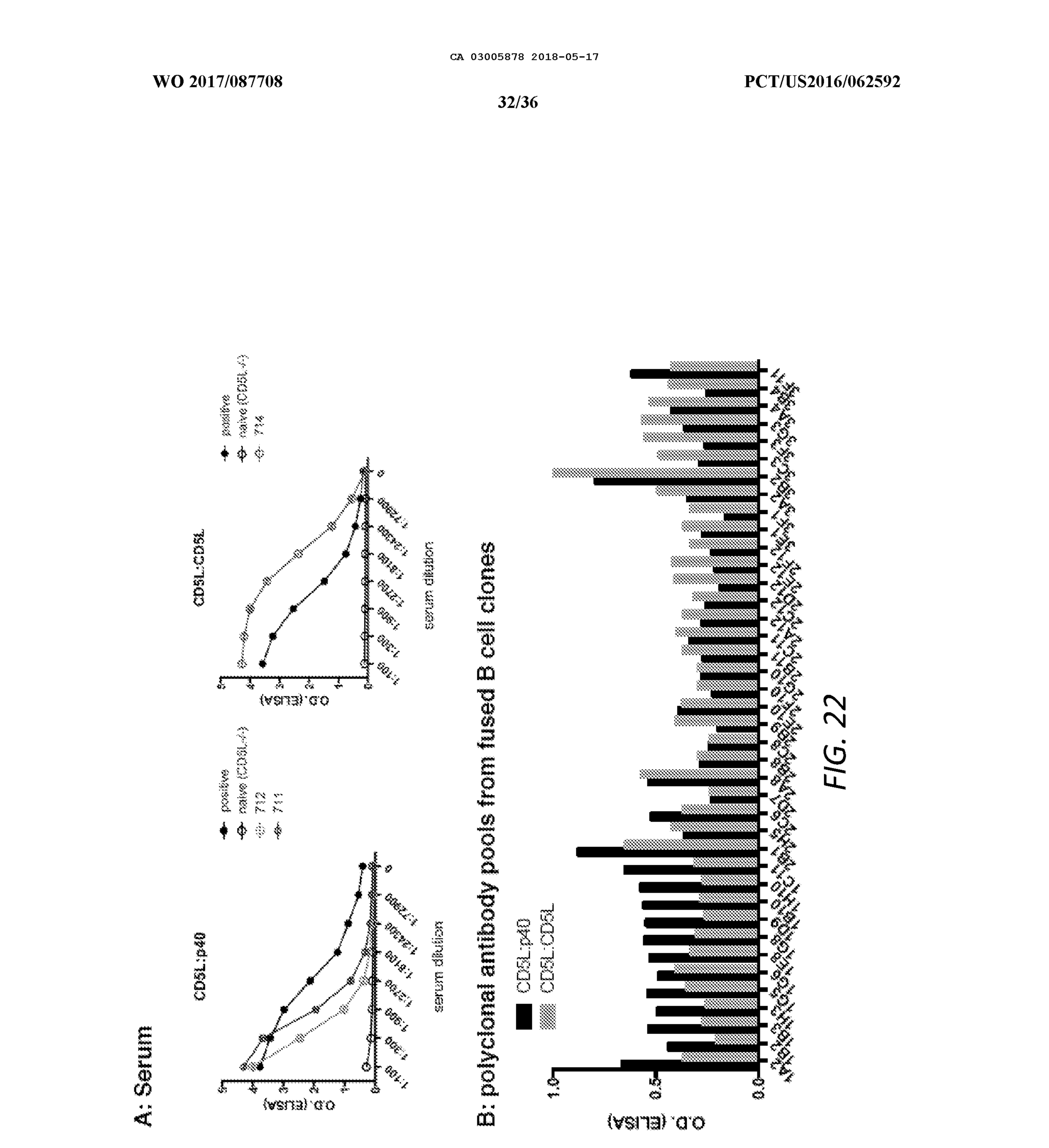

A
CD5L:p40
positive
naive (CD5L-/-)
712
711
O.D. (ELISA)
serum dilution
1:100
1:300
1:900
1:2700
1:8100
1:24300
1:72900
0
B
CD5L:p40
CD5L:CD5L
O.D. (ELISA)
1.0
0.5
0.0

FIG. 22

CD5L

```
Mouse    1    MAPLFNLMLAILSIFVGSCFSESPTKVQLVGGADRCEGRVEVRHNGQWGTVCDDGWDRRDVAVVCRELNCGAVIQTPRGA  80
Human    1    MALLFSLLIAICT~~~RPGFLASPSGVRLVGGLHRCEGRVEVEQKGQWGTVCDDGWDIKDVAVLCRELGCGAASGTPSGI  77

Mouse    81   SYQPPAS~EQRVLIQGVDCNGTEDTLAQCELNYDVFDCSHEEDAGAQCENPDSDLLFIPSDVRLVDGPGRCQGRVEVLHQ  159
Human    78   LYEPPAEKEQKVLIQSVSCTGTEDTLAQCELE~EVYDCSHDEDAGASCENPESSFSPVPEGVRLADGPGSCKGRVEVKHQ  156

Mouse    160  SQWSTVCKAGWNLQVSKVVCRQLGCGRALLTYGSCRKSTQGKGPIWMGKMSCSGQEANLRSCLLSRL~EENCTHGEDTWM  238
Human    157  NQWYTVCQTGWSLRAAKVVCRQLGCGRAVLTQKRCNKHAYGRKPIWLSQMSCSGREATLQDCPSGPWGKNTCNHDEDTWV  236

Mouse    239  KCEDPFELKLVGGDTPCSGRLEVLHKGSWGSVCDDNWGEKEDQVVCRQLGCGKSLHPSPKTKKIYGPGAGRIWLDDVNCS  318
Human    237  KCEDPFDLRLVGGDNLCSGRLEVLHRGVWGSVCDDNWGEKEDQVVCRQLGCGRSLSPSFRDKECYGPGVGRIWLDNVRCS  316

Mouse    319  GKEQSLEFCRARLWGYHDCTHKEDVEVICTDFDV  352
Human    317  GEEQSLEQCQHRFWGFHDCTHQEDVAVICS~~~V  347
```

FIG. 23A

IL-23a (p19)

```
Mouse   1    MLDCRAVISLWLLPWVTQGLAVPRSSSPDWAQCQQLSRNLCMLARNAHAPAGHNLLREEEDEETKHNVPRIQCEDGCDP  80
Human   1    MLGSRAVMLLLLLPWTAQGRAVPGGSSPAWTQCQQLSQKLCTLAWSAHPLVGHMDL-REEGDEETTNDVPHIQCGDGCDP  79

Mouse   81   QGLKDNSQFCLQRIRQGLAFYEKLLQSDIFTGEPALLPDSPMEQLRTSLLGLSQLLQPEDSPRETQQMPSLSSSQQWQRP  160
Human   80   QGLRDNSQFCLQRIHQGLIFYEKLLGSDIFTGEPSLLPDSPVGQLHASLLGLSQLLQPEGSHWETQQIPSLSPSQPWQRL  159

Mouse   161  LLRSKILRSLQAFLAIAARVFAHGAATLTEPLVPTA  196
Human   160  LLRFKILRSLQAFVAVAARVFAHGAATLS-P------  189
```

FIG. 23B

IL-12b (p40)

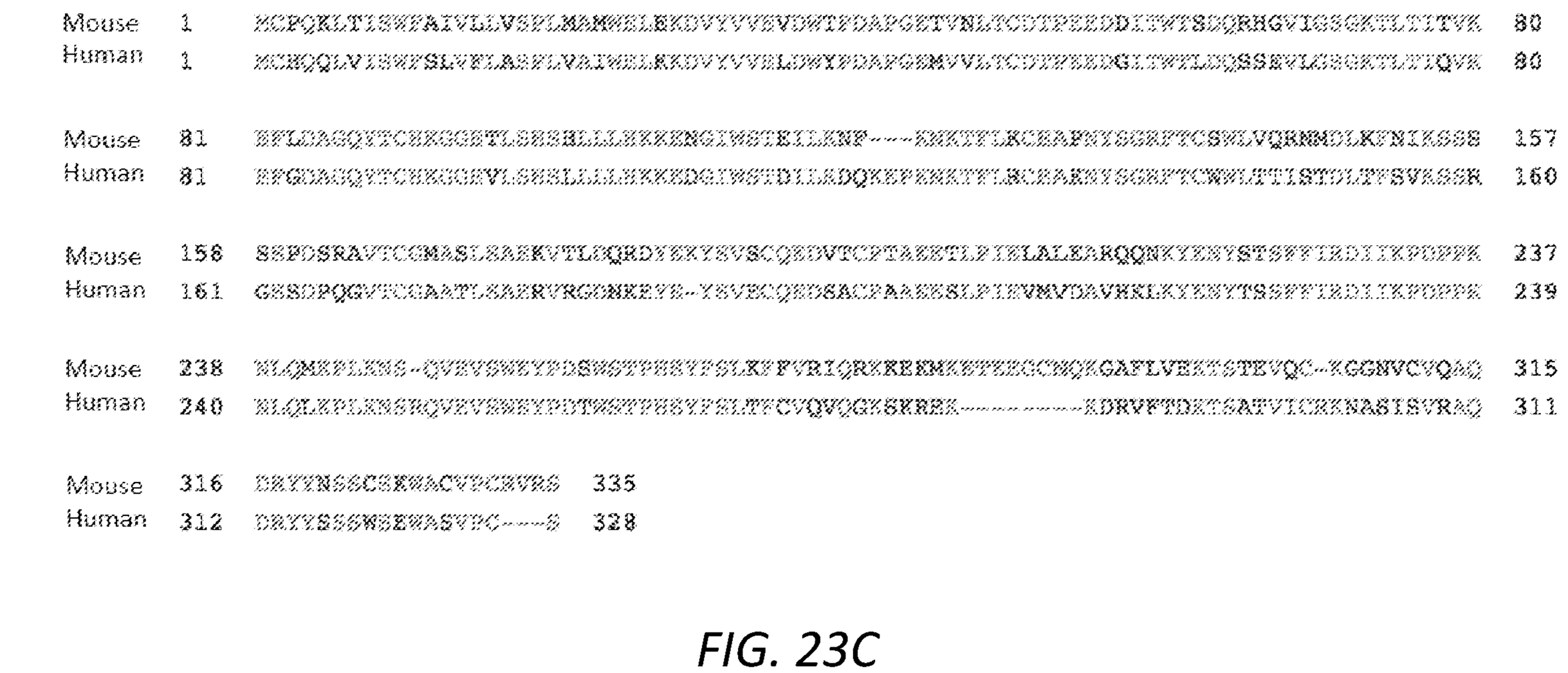


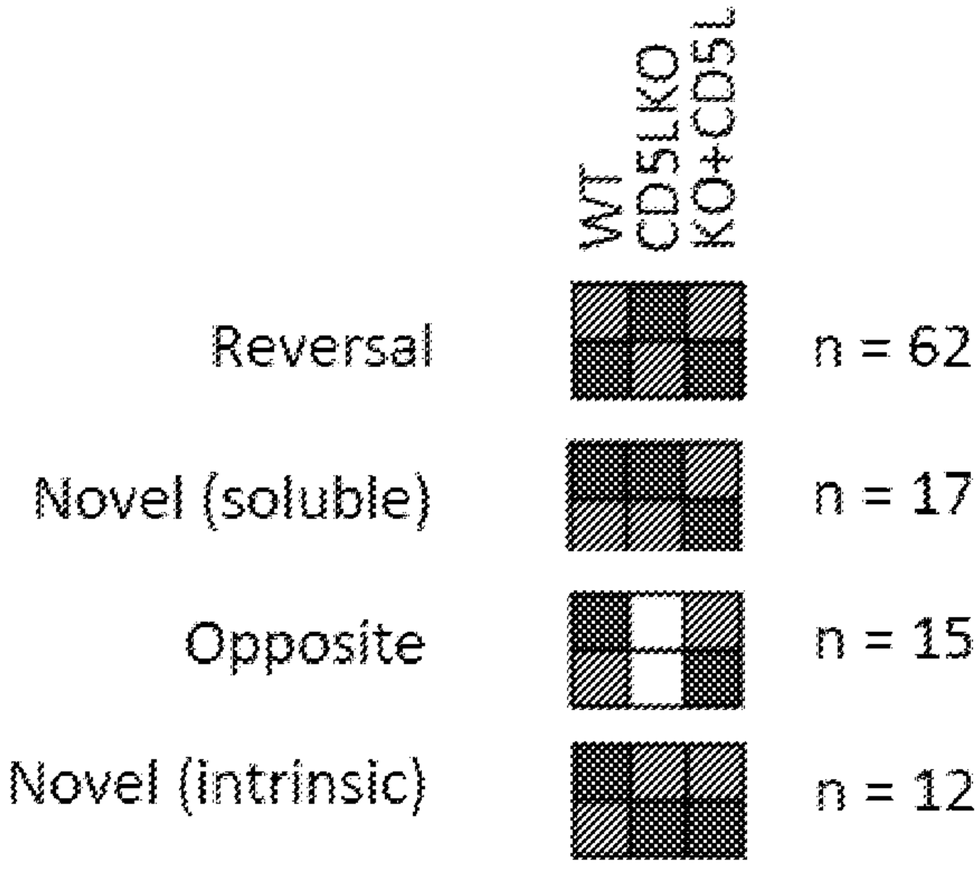
FIG. 23C

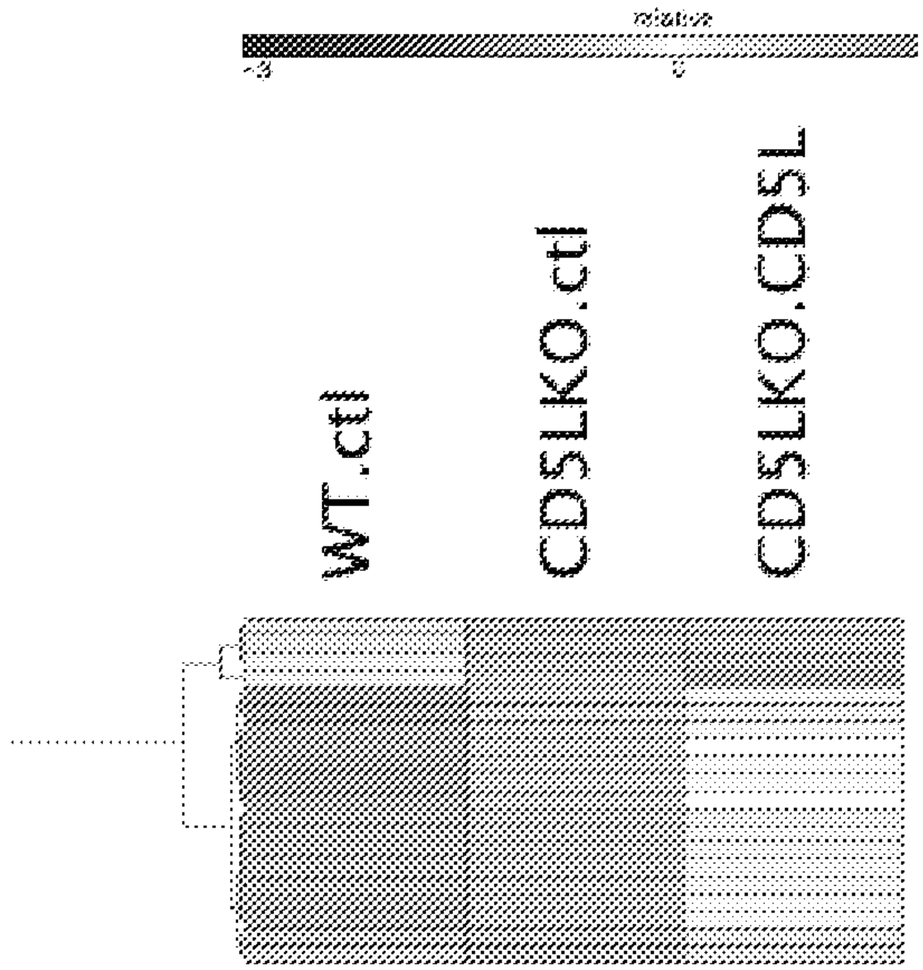
IL-12a (p35)

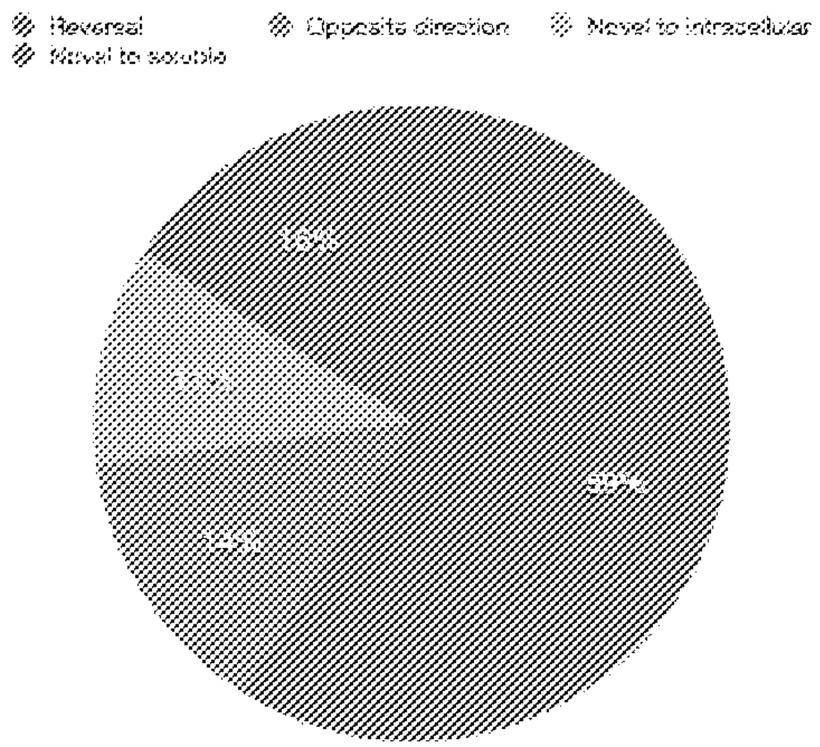
```
Mouse   1   ------------------------------------------MCQSRYLLFLATLVLLNHLTSAVIPVSGP------AKCLNQSQNLLK   42
Human   1   MWPPGSASQPPPSPAAATGLHPAARPVSLQCRLSMCPARSLLLVATLVLLDHLSLARNLPVATPDPGMFPCLHHSQNLLR   80

Mouse   43  TTDDMVKTAREKLKHYSCTAEDIDHEDITRDKTSTLEACLPLELHKNESCLATRETSSTTRGSCLPPQKTSLMMTLCLGS   122
Human   81  AVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLELTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSS   160

Mouse   123 IYEDLKMYQSEFQAINAALQNHNHQQIILDKGMLVAIDELMQSLNHNGETLRQKPPVGEADPYRVKMKLCILLHAFSTRV   202
Human   161 IYEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRA   240

Mouse   203 MTIDRVMNYLSS   215
Human   241 VTIDRVMSYLNA   253
```

FIG. 23D

n = 106 (DE out of 312: nanostring)







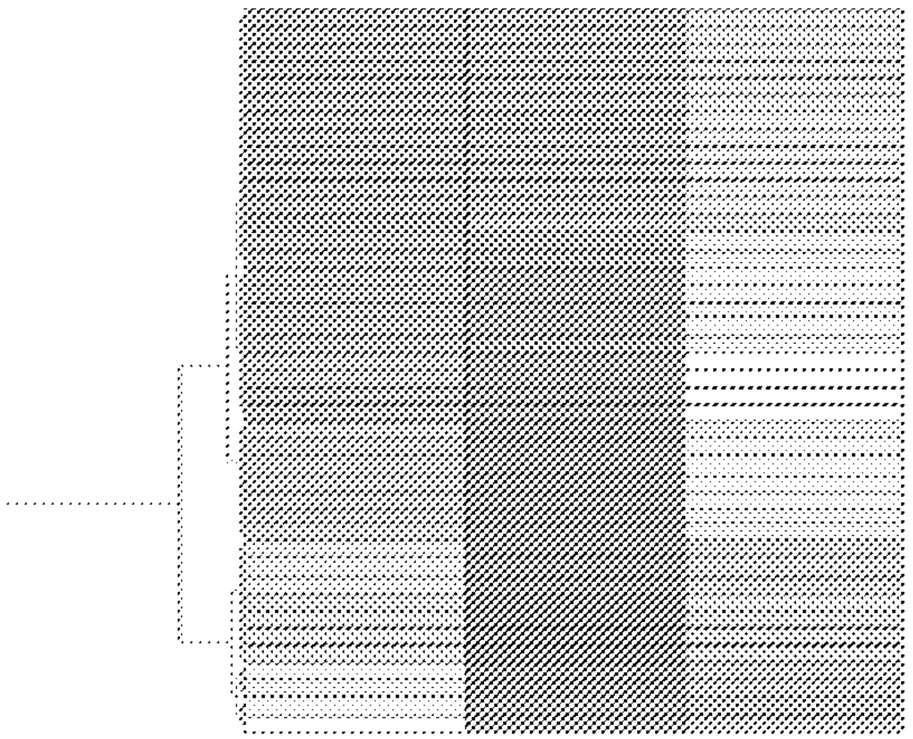


FIG. 1