

SOLUBLE NOTCH LIGANDS AND METHODS FOR GENERATING CELLS OF THE T CELL LINEAGE THEREWITH

Related application

- 5 [0001] This application claims benefit of United States Provisional Patent Application serial no. 63/592,684 filed October 24, 2023, incorporated herein by reference in its entirety.

Incorporation of sequence listing

- 10 [0002] A computer readable form of the Sequence Listing "22768-P72784PC00_SequenceListing.xml" (16,702 bytes), created on October 23, 2024, is herein incorporated by reference.

Field

- [0001] The present disclosure relates to soluble multispecific ligands comprising a Notch ligand and a functional protein partner. The present disclosure also relates to
15 methods of differentiating cells of the T cell lineage, compositions, kits, and methods of treatment comprising the same.

Background

- [0002] The Notch activation pathway is involved in cell fate decisions throughout evolution. Notch receptors play a fundamentally determinant role in T cell development.
20 Activation of Notch receptors on hematopoietic stem and/or progenitor cells (HSPC) leads to inhibition of B cell and erythromyeloid lineage differentiation and drives T lineage induction, commitment, differentiation and survival (1). Unlike all blood cells, T cells are generated outside of the bone marrow within the thymus, a primary lymphoid organ (2). T cells are critical mediators of adaptive immunity and can be harnessed as therapeutic
25 agents against pathogens and in cancer immunotherapy. The inventors previously developed Notch ligands, like Delta-like 4 (DLL4), conjugated to μ beads for generating cells of the T cell lineage in suspension (WO 2019/159597 A1, Zuniga-Pflucker et al., herein incorporated by reference in its entirety) (3). However, use of DLL4- μ beads requires a step of treating the cells to separate them from the DLL4- μ beads.
- 30 [0003] Soluble and biologic Notch ligands with the ability to effectively turn on the Notch signaling pathway using a cell-free system that does not employ bead/particle- or plate-bound molecules are desired.

Summary

[0004] The inventors have developed a cell-free, bead-free system for generating cells of the T cell lineage from mouse or human hematopoietic stem/progenitor cells (HSPCs). Non-bead/particle- or -plate-bound versions of Notch ligands in complex with a functional protein partner (FPP) allow for the effective activation of Notch signaling and the generation of T-lineage cells, including progenitor T cells and mature T cells.

[0005] Accordingly, herein provided is a method of generating a cell of the T cell lineage comprising (a) culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand comprising a Notch ligand operably linked to a functional protein partner (FPP) that binds a surface molecule on the stem cells or progenitor cells, and (b) isolating a cell of the T cell lineage.

[0006] In an embodiment, the stem cells or progenitor cells with the multispecific ligand are cultured in suspension.

[0007] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-1 (DLL1) or of Delta-like-4 (DLL4). In another embodiment, the Notch ligand further comprises a fragment crystallizable (Fc) region of human IgG (Notch ligand-Fc).

[0008] In an embodiment, the FPP comprises stem cell factor (SCF), interleukin-7 (IL-7), FMS-related tyrosine kinase 3 ligand (Flt3-L), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), or P-Selectin. In another embodiment, the FPP further comprises an Fc region of human IgG (FPP-Fc).

[0009] In an embodiment, the multispecific ligand further comprises a linker between the Notch ligand and the FPP. In another embodiment, the multispecific ligand comprises i) the Notch ligand-Fc, ii) the linker, and iii) the FPP. In yet another embodiment, the multispecific ligand comprises the Notch ligand-Fc and the FPP-Fc.

[0010] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[0011] In an embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 1 or a functional variant thereof. In another embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3 or a functional variant thereof.

[0012] In an embodiment, the Notch ligand is fused to the FPP. In another embodiment, the Notch ligand is conjugated to the FPP. In yet another embodiment, the

Notch ligand and the FPP are both biotinylated and are both conjugated to a same streptavidin molecule.

[0013] In an embodiment, the stem cells are selected from hematopoietic stem/progenitor cells (HSPCs), embryonic stem cells or induced pluripotent stem cells. In
5 another embodiment, the stem cells are CD34⁺ HSPCs.

[0014] In an embodiment, the cell of the T cell lineage is a progenitor T (proT) cell. In another embodiment, the stem cells or progenitor cells are human cells and the proT cell has the phenotype CD34⁺CD7⁺ or CD7⁺CD5⁺CD1a⁻. In yet another embodiment, the stem cells or progenitor cells are mouse cells and the proT cell has the phenotype CD25⁺ or
10 CD25⁺CD90⁺.

[0015] In an embodiment, the cell of the T-cell lineage is a CD4⁺CD8⁺ double positive cell, CD4⁺CD8⁺CD3⁺ double positive cell or CD8⁺CD3⁺ or CD4⁺CD3⁺ single positive cell.

[0016] In some embodiments, the stem cells or progenitor cells are cultured in
15 stromal cell-free media.

[0017] Also provided herein is a cell of the T cell lineage, wherein the cell is generated by a method disclosed herein.

[0018] In an embodiment, the cell is a progenitor T cell, CD4⁺CD8⁺ double positive cell, CD4⁺CD8⁺CD3⁺ double positive cell or CD8⁺CD3⁺ or CD4⁺CD3⁺ single positive cell.

20 [0019] Also provided herein is a soluble multispecific ligand comprising i) a Notch ligand operably linked to ii) a functional protein partner (FPP) that binds a surface molecule on stem cells or progenitor cells.

[0020] In an embodiment, the Notch ligand comprises an extracellular domain of DLL1 or of DLL4. In another embodiment, the Notch ligand further comprises an Fc region
25 of human IgG (Notch ligand-Fc).

[0021] In an embodiment, the FPP comprises SCF, IL-7, FLT3-L, ICAM-1, VCAM-1, or P-Selectin. In another embodiment, the FPP further comprises an Fc region of human IgG (FPP-Fc).

[0022] In an embodiment, the multispecific ligand further comprises a linker between
30 the Notch ligand and the FPP.

[0023] In an embodiment, the multispecific ligand comprises i) the Notch ligand-Fc, ii) the linker, and iii) the FPP. In another embodiment, the multispecific ligand comprises the Notch ligand-Fc and the FPP-Fc.

[0024] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[0025] In an embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 1 or a functional variant thereof. In another embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3 or a functional variant thereof.

[0026] In an embodiment, the Notch ligand is fused to the FPP. In another embodiment, the Notch ligand is conjugated to the FPP. In yet another embodiment, the Notch ligand and the FPP are both biotinylated and are both conjugated to a same streptavidin molecule.

[0027] Also provided herein is a use of a multispecific ligand disclosed herein for generating a cell of the T cell lineage.

[0028] Also provided herein is a kit comprising (i) a soluble multispecific ligand comprising (a) a Notch ligand operably linked to (b) an FPP that binds surface molecules on stem cells or progenitor cells, and (ii) instructions for use of the soluble multispecific ligand for generating a cell of the T cell lineage.

[0029] Further provided herein is a kit comprising (i) a soluble multispecific ligand comprising (a) a Notch ligand operably linked to (b) an FPP that binds surface molecules on stem cells or progenitor cells, and (ii) culture medium.

[0030] In an embodiment;

- the Notch ligand comprises DLL1 or DLL4; and/or
- the FPP comprises SCF, IL-7, FLT3-L, ICAM-1, VCAM-1, or P-Selectin.

[0031] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[0032] Also provided herein is a method of treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T-cell lineage are obtained by the methods described herein.

[0033] Further provided herein is a use of an effective amount of the cells of a T cell lineage for treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the methods described herein.

[0034] Also provided herein is a use of an effective amount of the cells of a T cell lineage in the manufacture of a medicament for treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the methods described herein.

[0035] In yet a further aspect of the disclosure is an effective amount of the cells of a T cell lineage for use for treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the methods described herein.

[0036] In an embodiment, the cells of the T cell lineage are progenitor T cells. In another embodiment, the cells of the T cell lineage are mature T cells.

[0037] These and other features and advantages of the present disclosure will become apparent from the following detailed description taken together with the accompanying drawings. It should be understood, however, that the detailed description and specific examples, while indicating preferred implementations of the present disclosure, are given by way of illustration only, since various changes and modifications within the spirit and scope of the disclosure will become apparent to those of skill in the art from this detailed description.

Brief Description of the Drawings

[0038] For a better understanding of the various embodiments described herein, and to show more clearly how these various embodiments may be carried into effect, reference will be made, by way of example, to the accompanying drawings which show at least one example embodiment, and which are now described. The drawings are not intended to limit the scope of the teachings described herein.

[0039] **FIG. 1** shows a schematic of the evolutionarily-conserved Notch signaling pathway as involved in T cell induction at the molecular level in an exemplary embodiment of the disclosure. Notch signaling occurs when Notch receptors on hematopoietic progenitor cells (e.g., the “thymic seeding progenitor cell” in this figure) engage their ligand on the thymic epithelial cells (TEC). This interaction is followed by the endocytosis of Notch ligand, in this case, Delta-like 4 (DLL4) by TECs, which has the effect of physically pulling

on Notch extracellular domain. This pull exposes a peptide sequence in Notch that is vulnerable to a disintegrin and metalloproteinase (ADAM) cleavage. This cleavage is followed by a subsequent protease activity by k-secretase, resulting in the release of the active, Notch intracellular domain (NICD) into the cytoplasm. NICD has a nuclear
5 localization signal that allows it to enter the nucleus, bind RBPJ and with its coactivators, including Mastermind-like (MAML) form a complex that targets expression of T cell specific genes.

[0040] **FIGs. 2A-D** show DLL4 and functional protein partner (FPP) construct designs in an exemplary embodiment of the disclosure, with stem cell factor (SCF) provided
10 as an example of an FPP. **FIG. 2A** depicts a schematic of a DLL4-SCF construct, with the extracellular domain of DLL4 connected to the secreted SCF by a flexible glycine-serine (GS) linker. **FIG. 2B** depicts a DLL4-Fc-SCF construct composed of the extracellular domain of human DLL4, the Fc region of human IgG (Fc), a combination of flexible and rigid linkers and secreted SCF. DLL4-Fc-SCF is the construct whose product is shown in
15 **FIGs. 4A-C** and **FIGs. 5A-D**. **FIG. 2C** depicts a schematic of a DLL4-Fc and SCF-Fc constructs covalently linked at the C-termini (DLL4/SCF-Fc-linked) and therefore the N-termini of each DLL4-Fc and SCF-Fc face in opposite directions. The Streptavidin DLL4-Fc/FPP-Fc construct shown in **FIG. 2D** includes a BirA recognition sequence (AviTag™) at the C-termini of each DLL4-Fc and SCF-Fc, to which a biotin moiety may be conjugated by
20 the enzyme BirA. One possible outcome of stoichiometric binding of biotinylated DLL4-Fc and SCF-Fc to streptavidin is shown, forming a complex composed of two DLL4-Fc and two SCF-Fc molecules.

[0041] **FIG. 3** shows a schematic of the mechanism of action of a DLL4-Fc and FPP-Fc fusion construct in an exemplary embodiment of the disclosure. In this example, the
25 FPP is stem cell factor (SCF), which binds and activates Kit, the stem cell factor receptor.

[0042] **FIGs. 4A-C** show the expression and function of a DLL4-Fc-L-SCF construct in an exemplary embodiment of the disclosure. **FIG. 4A** shows the immunoblot analysis of purified DLL4-Fc (50ng) compared to DLL4-Fc-L-SCF (25 µL supernatant) as detected by anti-human immunoglobulin G (hIgG) conjugated to peroxidase. **FIG. 4B** shows the cellular
30 binding of 32D-Kit cells (upper panel) and 3T3-N1Cluc cells (lower panel) to DLL4-Fc-SCF as analyzed by flow cytometry. "DLL4-Fc-L-SCF + hSCF" represents replicates wherein monomeric SCF was added in addition to DLL4-Fc-L-SCF. "DLL4-Fc-L-SCF + DLL4-histag" represents replicates wherein DLL4-his was added in addition to DLL4-Fc-L-SCF.

“hlgG + 2° Ab” represents replicates wherein human immunoglobulin and secondary antibodies were used, but not DLL4-Fc-SCF. “2° Ab” represents replicates wherein only secondary antibodies were used. **FIG. 4C** shows the result of Notch activity in control 3T3-N1CLuc cells (control left column), 3T3-N1CLuc cells bound to DLL4-Fc-L-SCF (second column from left), 3T3-N1CLuc cells bound to DLL4-Fc-L-SCF incubated with 32D-Kit cells (third column from left), and 3T3-N1CLuc cells bound to DLL4-Fc-L-SCF incubated with 32D-Kit cells that had been pre-incubated with DLL4-Fc-L-SCF (right column), demonstrating a 2-fold increase in Notch signaling in the presence of all three of 32D-Kit cells, 3T3-N1CLuc cells, and DLL4-Fc-L-SCF, which was reduced by ~40% with competitive binding.

[0043] **FIGs. 5A-D** show the schematic, production and cell surface binding of multispecific ligand DLL4-Fc-SCF variants in an exemplary embodiment of the disclosure. **FIG. 5A** shows the DLL4-FcSCF variants generated, which include DLL4-Fc linked to SCF using a linker that contains rigid and flexible linker repeats (-L-) or only flexible linker repeats (-GS33-). **FIG. 5B** shows the expression constructs were generated, expressed on HEK293T cells, and purified samples were treated with reducing agent (R) or were kept in nonreducing conditions (N-R). Cell lysate proteins were separated by electrophoresis and stained with Coomassie Blue dye, with a MW marker to show size and purity. **FIG. 5C** shows flow cytometry analysis of the human Kit-expressing AML-derived Kasumi-1 cell line, binding to DLL4-Fc-L-SCF and DLL4-Fc-GS33-SCF proteins and detected by anti-human immunoglobulin G (hlgG) conjugated to APC, in a dose-dependent manner, with binding saturation at 3-10nM DL4-Fc-SCF. **FIG. 5D** shows flow cytometry analysis of the human NOTCH1-expressing 3T3-N1Cluc reporter cell line binding to DLL4(max)-Fc-GS33-SCF proteins and detected by anti-human immunoglobulin G (hlgG) conjugated to APC, in a dose-dependent manner.

[0044] **FIGs. 6A-B** demonstrate that multispecific ligand DLL4-Fc-SCF in plate- or cell-bound condition is able to activate NOTCH signaling in an exemplary embodiment of the disclosure. **FIG. 6A** shows a schematic of newly generated 32D-Kit-N1Cluc cells as Unbound or Cell-bound with bifunctional FPP proteins (right panel). Left Panel shows the interaction of unbound 32D-Kit-N1Cluc cells with plate-bound DL4-Fc-SCF molecules. **FIG. 6B** shows the Notch reporter Luciferase activity for two bifunctional variants DLL4-Fc-L-SCF and DLL4-Fc-GS33-SCF. Background luciferase measurements were subtracted from the activity of each sample.

[0045] **FIGs. 7A-C** show that soluble multispecific ligand DLL4-Fc-SCF activates NOTCH signaling and promotes T-lineage differentiation in human CD34⁺ hematopoietic stem/progenitor cells in an exemplary embodiment of the disclosure. **FIG. 7A** shows cells expressing both Notch and Kit receptors, depicted with bifunctional DLL4-Fc-SCF proteins in free form in media or bound to cells either through DLL4 (to Notch) or SCF (to Kit). **FIG. 7B** shows Luciferase activity based on Notch signaling for 32D-Kit-N1Cluc cells in the presence of DLL4-Fc-L-SCF at different concentrations. Background Luciferase measurements, without added bifunctional proteins, were subtracted from the activity of each sample. **FIG. 7C** shows flow cytometric analysis of cell surface expression of CD7, T-cell lineage marker, in cord blood-derived CD34⁺ cells incubated with plate-bound DLL4-Fc-L-SCF or two variants of DLL4-Fc-L-SCF and DLL4-Fc-GS33-SCF (1 nM). As a control, DLL4-Fc-SCF was not added in any form, but SCF was included in the media to promote cell survival (left panel, no bifunctional protein + hSCF).

[0046] **FIGs. 8A-D** show that multispecific ligand DLL4-Fc-SCF in soluble form mediates Notch activation leading to T cell differentiation and survival in an exemplary embodiment of the disclosure. **FIG. 8A** shows Kasumi-1 cells bearing only Kit on their surface and CD34⁺ hematopoietic progenitor cells, expressing both Notch-1 and Kit. DL4-Fc-SCF molecules are shown mediating the interaction between the two cell types. **FIG. 8B** shows flow cytometry results of early T cell markers, CD34, CD7 and CD5 in Kasumi-1 cells and CD34⁺ CB incubated (9:1 ratio) with plate-bound DLL4-Fc, soluble DL4-Fc-SCF (1nM) or soluble Fc-SCF (1nM) with daily addition of extra soluble protein at 1 nM for soluble conditions. Lower panel histograms were electronically pre-gated on CD7⁺ cells. Primary cells were electronically pre-gated based on forward and side scatter. **FIG. 8C** shows RNA transcripts levels of the Notch target gene DELTEX1 determined by QRT-PCR of harvested cells on D5 in cultures with CB-derived CD34⁺ cells incubated with DLL4-Fc plate-bound (PB) and soluble DLL4-Fc-L-SCF (1 nM) and Fc-SCF (1 nM). The results were indexed to housekeeping gene GAPDH (error bars indicate Standard deviation, n=3). **FIG. 8D** shows the total number of T-lineage cells based on expression of CD7 in CB-derived CD34⁺ cells incubated with different concentrations of DL4-Fc-SCF (0, 0.1, 0.5 and 2.5 nM) on D7 of culture.

[0047] Further aspects and features of the example embodiments described herein will appear from the following description taken together with the accompanying drawings.

Detailed Description of the Disclosure

[0048] Unless otherwise indicated, the definitions and embodiments described in this and other sections are intended to be applicable to all embodiments and aspects of the present disclosure herein described for which they are suitable as would be understood by
5 a person skilled in the art.

[0049] All features disclosed in the specification, including the claims, abstract, and drawings, and all the steps in any method or process disclosed, may be combined in any combination, except combinations where at least some of such features and/or steps are mutually exclusive. Each feature disclosed in the specification, including the claims,
10 abstract, and drawings, can be replaced by alternative features serving the same, equivalent, or similar purpose, unless expressly stated otherwise.

I. Definitions

[0050] In understanding the scope of the present disclosure, the term "comprising" and its derivatives, as used herein, are intended to be open ended terms that specify the presence of the stated features, elements, components, groups, integers, and/or steps, but
15 do not exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The foregoing also applies to words having similar meanings such as the terms, "including", "having" and their derivatives.

[0051] The term "consisting" and its derivatives, as used herein, are intended to be closed ended terms that specify the presence of stated features, elements, components, groups, integers, and/or steps, and also exclude the presence of other unstated features, elements, components, groups, integers and/or steps. The term "consisting essentially of", as used herein, is intended to specify the presence of the stated features, elements, components, groups, integers, and/or steps as well as those that do not materially affect
20 the basic and novel characteristic(s) of features, elements, components, groups, integers, and/or steps.

[0052] Further, terms of degree such as "substantially", "about" and "approximately" as used herein mean a reasonable amount of deviation of the modified term such that the end result is not significantly changed. These terms of degree should be construed as
30 including a deviation of at least $\pm 5\%$ of the modified term if this deviation would not negate the meaning of the word it modifies.

[0053] More specifically, the term "about" means plus or minus 0.1 to 50%, 5-50%, or 10-40%, 10-20%, 10%-15%, optionally 5-10%, further optionally about 5% of the number to which reference is being made.

[0054] As used in this specification and the appended claims, the singular forms "a",
5 "an" and "the" include plural references unless the content clearly dictates otherwise. Thus, for example, a composition containing "a compound" includes a mixture of two or more compounds.

[0055] The phrase "and/or," as used herein in the specification and in the claims,
10 should be understood to mean "either or both" of the elements so conjoined, i.e., elements that are conjunctively present in some cases and disjunctively present in other cases. Multiple elements listed with "and/or" should be construed in the same fashion, i.e., "one or more" of the elements so conjoined. Other elements may optionally be present other than the elements specifically identified by the "and/or" clause, whether related or unrelated to those elements specifically identified.

15 [0056] As used herein, "or" should be understood to have the same meaning as "and/or" as defined above. For example, when separating items in a list, "or" or "and/or" shall be interpreted as being inclusive, i.e., the inclusion of at least one, but also including more than one, of a number or list of elements, and, optionally, additional unlisted items. Only terms clearly indicated to the contrary, such as "only one of" or "exactly one of" or,
20 when used in the claims, "consisting of" will refer to the inclusion of exactly one element of a number or list of elements. In general, the term "or" as used herein shall only be interpreted as indicating exclusive alternatives (i.e., "one or the other but not both") when preceded by terms of exclusivity, such as "either," "one of," "only one of," or "exactly one of."

25 [0057] As used herein, the phrase "at least one," in reference to a list of one or more elements, should be understood to mean at least one element selected from anyone or more of the elements in the list of elements, but not necessarily including at least one of each and every element specifically listed within the list of elements and not excluding any combinations of elements in the list of elements. This definition also allows that elements
30 may optionally be present other than the elements specifically identified within the list of elements to which the phrase "at least one" refers, whether related or unrelated to those elements specifically identified.

[0058] The recitation of numerical ranges by endpoints herein includes all numbers and fractions subsumed within that range (e.g., 1 to 5 includes 1, 1.5, 2, 2.75, 3, 3.90, 4, and 5). It is also to be understood that all numbers and fractions thereof are presumed to be modified by the term "about."

5 [0059] It should also be understood that, in certain methods described herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited unless the context indicates otherwise.

[0060] Further, the definitions and embodiments described in particular sections are
10 intended to be applicable to other embodiments herein described for which they are suitable as would be understood by a person skilled in the art. For example, in the following passages, different aspects are defined in more detail. Each aspect so defined may be combined with any other aspect or aspects unless clearly indicated to the contrary. In particular, any feature indicated as being preferred or advantageous may be combined with
15 any other feature or features indicated as being preferred or advantageous.

[0061] Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present disclosure, examples of methods and materials are now described.

II. Methods of Generating Cells

20 [0062] The inventors have herein demonstrated Notch receptor activation in cells cultured with a soluble multispecific ligand that functions similarly to the physiological membrane-bound Notch ligand DLL1 or DLL4. When bound to Notch, full-length DLL1 or DLL4 is endocytosed resulting in a physical pulling action, or mechanosensing, on Notch receptor to expose the S2 cleavage site, naturally cleaved by ADAM-proteases (5).
25 Cleavage of this site leads to the subsequent S3 cleavage within the Notch transmembrane domain by gamma-secretase protein complex, releasing the activated Notch intracellular domain into the cytoplasm from where it enters into the nucleus, interacts with the DNA binding protein RBPJ and activates a cascade of cell-type-dependent genetic programs that lead to differentiation, for example the differentiation of stem cells or progenitor cells
30 into cells of the T cell lineage. In contrast to membrane-bound DLL1 or DLL4, the binding of the soluble extracellular domain of DLL1 or DLL4 protein to Notch receptors fails to activate them, as binding-alone induces no pulling action.

[0063] The multispecific ligands disclosed herein interact with Notch receptors at one end through soluble Notch ligands, for example the soluble extracellular domain of DLL1 or DLL4, and tether the Notch receptor to the cell membrane at another end of the multispecific ligand through a functional protein partner (FPP). This “Notch – multispecific
5 ligand – cell membrane” complex can induce a physical pulling on the Notch receptor similar to the physiological action of full length DLL1 or DLL4, causing Notch to become cleaved, and thereby induce the Notch-dependent differentiation of stem cells or progenitor cells into cells of the T cell lineage.

[0064] As described above, the inventors have developed a cell-free, bead-free
10 system for generating cells of the T-cell lineage from mouse or human hematopoietic stem/progenitor cells (HSPCs). Non-plate-bound soluble multispecific ligands comprising Notch ligands (for example DLL4-) and FPPs allow for the effective activation of Notch signaling and generation of T-lineage cells including progenitor T cells and mature T cells.

[0065] Accordingly, in one aspect is a method of generating a cell of the T cell
15 lineage comprising:

(a) culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand herein disclosed comprising i) a Notch ligand operably linked to ii) a functional protein partner (FPP) that binds a surface molecule on the stem cells or progenitor cells; and

20 (b) isolating a cell of the T cell lineage.

[0066] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[0067] In an embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID NO:1 or a functional variant thereof. In another embodiment, the
25 multispecific ligand comprises the amino acid sequence of SEQ ID NO:2 or SEQ ID NO: 3 or a functional variant thereof.

[0068] The term “cell” as used herein refers to a single cell or a plurality of cells.

[0069] The term “cells of the T cell lineage” refers to cells that show at least one phenotypic characteristic of a T cell or a precursor or progenitor thereof that distinguishes
30 the cells from other lymphoid cells, and cells of the erythroid or myeloid lineages. Such phenotypic characteristics can include expression of one or more proteins specific for T-

lineage on cells or a precursor or progenitor thereof, or a physiological, morphological, functional, or immunological feature specific for a T cell.

[0070] Cells of the T cell lineage may be (a) progenitor or precursor cells committed to the T cell lineage ("progenitor T cells" or "proT cells", as described herein); (b) CD25+ immature and inactivated T cells; (c) cells that have undergone CD4 or CD8 lineage commitment (e.g. CD4+CD8^{lo}TCR^{int} cells); (d) characterized by TCR gene rearrangement; (e) precursor thymocytes that are CD4+CD8+ double positive (DP); (f) CD4-CD8+ or CD4+CD8- and optionally TCR^{hi}; (g) CD3+CD90+; (h) single positive (SP) cells that are CD4-CD8+ or CD4+CD8- and TCR^{hi}; (i) TCR-αβ⁺ and/or TCR-γδ⁺; (j) characterized by expression of any of multiple Vβ chains (e.g. Vβ-3, -6, and 17a); or (k) mature and functional or activated T cells which may be characterized as TCR/CD3^{hi}, CD4-CD8+ or CD4+CD8-.

[0071] In one embodiment, a cell of the T cell lineage is a "progenitor T cell" or "proT cell". The term "progenitor T cell" or "proT cell" as used herein means a T cell that is capable of maturing into a mature T cell or lymphocyte.

[0072] In one embodiment, the progenitor T cell is a human progenitor T cell. Phenotypes of human progenitor T cells include CD34+CD7+ and CD7+CD5+CD1a-. In another embodiment, the progenitor T cell is a mouse progenitor T cell. Phenotypes of mouse progenitor T cells include CD25+. In another embodiment, the mouse proT cells have the phenotype CD25+ or CD25+CD90+

[0073] In another embodiment, a cell of the T cell lineage is a double positive (DP) cell characterized by CD4+CD8+ or CD4+CD8+CD3+ double positive. In another embodiment, a cell of the T cell lineage is a single positive (SP) cell characterized by CD4-CD8+, CD4+CD8- or CD4-CD8+CD3+, CD4+CD8-CD3+.

[0074] According to the methods described here, cells of the T cell lineage are generated by culturing a sample comprising stem cells or progenitor cells. Stem or progenitor cells may be obtained from any suitable source, including, without limitation, umbilical cord blood, embryos, embryonic tissue, fetal tissue, bone marrow and blood. In one embodiment, the stem or progenitor cell is a hematopoietic stem or progenitor cell (HSPC). In another embodiment, the stem cell is an embryonic stem cell. In a further embodiment, the stem cell is an induced pluripotent stem cell (iPSC). For therapeutic applications, the stem cells or progenitor cells used to generate the cells of the T cell lineage may be obtained from the patient to be treated. In yet another embodiment, the stem cells are CD34⁺CD38^{-/lo} HSPCs. In a further embodiment, the stem cells are CD34⁺.

[0075] The term “hematopoietic stem/progenitor cell”, “hematopoietic stem or progenitor cell” or “HSPC” as used herein refers to undifferentiated hematopoietic cells that are capable of differentiation to other cell types, including cells of the T cell lineage.

5 [0076] The term “embryonic stem cell” or “ES cell” as used herein refers to undifferentiated embryonic stem cells that have the ability to integrate into and become part of the germ line of a developing embryo.

[0077] The term “induced pluripotent stem cell” or “iPSC” as used herein refers to cells derived from skin or blood cells that have been reprogrammed back into an embryonic-like pluripotent state.

10 [0078] Typically, a sample containing stem or progenitor cells is first depleted of non-stem cells or mature cells. Negative and positive selection methods known in the art may be used for enrichment of the stem or progenitor cells. For example, cells can be sorted based on cell surface antigens using a fluorescence activated cell sorter, or magnetic beads which bind cells with certain cell surface antigens. Negative selection columns can
15 be used to remove cells expressing lineage specific surface antigens.

[0079] In an embodiment, a sample containing stem or progenitor cells is separated into lineage-negative (Lin^-) and lineage positive (Lin^+) fractions. The Lin^- fraction can be sorted for CD34^+ cells.

[0080] The progenitor cells or stem cells are cultured under suitable conditions as
20 described herein to generate cells of the T cell lineage. In some embodiments, the cells are cultured in the presence of one or more soluble multispecific ligands herein disclosed for a sufficient time to form cells of the T cell lineage.

[0081] One advantage of the methods described herein is that they allow the cells of the T cell lineage to be cultured in suspension. In an embodiment, the progenitor cells
25 or stem cells are cultured in suspension with a soluble multispecific ligand herein disclosed. In a suspension culture, cells grow free-floating in a culture medium. In contrast, in an adherent culture, cells grow as monolayers on an artificial substrate.

[0082] In another embodiment, the progenitor cells or stem cells are cultured in suspension in a bioreactor, optionally a closed or a closed, automated bioreactor, with a
30 soluble multispecific ligand herein disclosed. Various bioreactors are known in the art and can include batch, fed batch or continuous bioreactors. An example of a continuous bioreactor is a continuous stirred-tank reactor model.

[0083] Various concentrations of progenitor cells or stem cells in the culture are contemplated. For example, the concentration of progenitor cells or stem cells in the culture may be anywhere from 1 to millions of cells per ml of media.

[0084] One or more positive cytokines that promote commitment and differentiation of cells of the T cell lineage may also be added to the culture. The cytokines may be human in origin, or may be derived from other species. The concentration of a cytokine in a culture is typically about 1-100ng/ml. The following are representative examples of cytokines that may be employed in the present disclosure: all members of the Flt-3-ligand, and interleukin-7 (IL-7) and Stem Cell Factor. In one embodiment, the cytokines used herein are Flt-3-ligand and IL-7 and Stem Cell Factor. The cytokines may be used in combination with equal molar or greater amounts of a glycosaminoglycan such as heparin sulfate. The cytokines are commercially available or can be produced by recombinant DNA techniques and purified to various degrees. Some of the cytokines may be purified from culture media of cell lines by standard biochemical techniques.

[0085] The progenitor cells and stem cells may be cultured in culture medium comprising conditioned medium, non-conditioned medium, or embryonic stem cell medium. Examples of suitable conditioned medium include IMDM, DMEM, or α MEM, conditioned with embryonic fibroblast cells (e.g., human embryonic fibroblast cells or mouse embryonic fibroblast cells), or equivalent medium. Examples of suitable non-conditioned medium include Iscove's Modified Dulbecco's Medium (IMDM), DMEM, or α MEM, or equivalent medium. The culture medium may comprise serum (e.g., bovine serum, fetal bovine serum, calf bovine serum, horse serum, human serum, or an artificial serum substitute) or it may be serum free. Other examples of media useful in the present methods includes StemCell Technologies media (StemSpanTMSFEM II) or any other commercially available equivalent media. In an embodiment, the stem cells or progenitor cells are cultured in stromal cell-free media.

[0086] In one embodiment, the culture conditions entail culturing the progenitor cells or stem cells for a sufficient period of time so that cells in the preparation form proT cells. In another embodiment, the culture conditions entail culturing the progenitor cells or stem cells for a sufficient period of time so that cells in the preparation form mature T cells, for example mature SP T cells. It will be appreciated that the cells may be maintained for the appropriate amount of time required to achieve the desired cellular composition. Optionally, the progenitor cells or stem cells are cultured for at least 6, 8, 10, 12, 14, 21, 28, 35 or 42

days. In one example, the progenitor cells or stem cells are cultured with the soluble multispecific ligand described herein for 4 to 21 days, 6 to 18 days or 7 to 14 days to generate proT cells. In another example, the progenitor cells or stem cells are cultured with the multispecific ligand described herein for at least 21, 28, 35 or 42 days to mature T cells.

5 [0087] The methods of the present disclosure allow the generation of large numbers of cells of the T cell lineage. In particular, in one embodiment, following 14 or more days of culture greater than 5-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 75-fold, 100-fold, 125-fold, 150-fold, 175-fold or 200-fold cell expansion over the initial starting number of stem cells or progenitor cells.

10 [0088] The term "isolated" as used herein means that the progenitor cell has been separated or purified from cellular or biological material found with the cells in their native environment. It thus distinguishes the cells from how they exist in nature.

III. Soluble Multispecific Ligands Comprising a Notch Ligand

[0089] The present inventors have also developed novel soluble multispecific
15 ligands. As used herein, the term "soluble multispecific ligand" refers to a soluble ligand comprising both a Notch ligand and a functional protein partner (FPP) for use in a cell culture.

[0090] Accordingly, another aspect of the present disclosure is a soluble multispecific ligand comprising i) a Notch ligand operably linked to ii) a functional protein
20 partner (FPP) that binds a surface molecule on stem cells or progenitor cells.

[0091] The term "operably linked" as used herein refers to an arrangement of two or more components, wherein the components so described are in a relationship permitting them to function in a coordinated manner. For example, a Notch ligand and an FPP are operably linked when they are joined together, for example they are conjugated to each
25 other or are fused to each other, in such an arrangement that each of the Notch ligand and the FPP can bind their respective binding partners.

[0092] The term "Notch ligand" as used herein refers to a ligand capable of binding to a Notch receptor polypeptide present in the membrane of a number of different mammalian cells including hematopoietic stem/progenitor cells. The Notch receptors that
30 have been identified in human cells include Notch-1, Notch-2, Notch-3 and Notch-4. Notch ligands typically have a diagnostic DSL domain (D-Delta, S-Serrate, and L-Lag2) comprising 20-22 amino acids at the amino terminus and between 3 to 8 EGF repeats on

the extracellular surface. Notch ligands can also include an anti-Notch antibody or Notch engager (an aptamer specific for Notch).

[0093] A Notch ligand is selected that promotes and maintains differentiation and proliferation of cells of the T cell lineage. The Notch ligand is optionally human in origin, or
5 may be derived from other species, including mammalian species such as rodent, dog, cat, pig, sheep, cow, goat and primates.

[0094] Particular examples of Notch ligands include the Delta family. The Delta family includes Delta-1 (e.g., Genbank Accession No. AF003522, Homo sapiens), Delta-3 (e.g., Genbank Accession No. AF084576, Rattus norvegicus), Delta-like 1 (DLL1; e.g.,
10 Genbank Accession No. NM_005618 and NP_005609, Homo sapiens; Genbank Accession No. X80903, 148324, M. musculus), Delta-like 3 (e.g., Genbank Accession No. NM_053666, N_446118, Rattus norvegicus), Delta-4 (e.g., Genbank Accession No. AF273454, BAB18580, Mus musculus; Genbank Accession No. AF279305, AAF81912, Homo sapiens), and Delta-like 4 (DLL4; e.g., Genbank Accession. No. Q9NR61,
15 AAF76427, AF253468, NM_019074, Homo sapiens; Genbank Accession No. NM 019454, mus musculus). Notch ligands are commercially available or can be produced by recombinant DNA techniques and purified to various degrees. In one embodiment, the Notch ligand comprises the extracellular domain of DLL1 or DLL4. In another embodiment, the Notch ligand comprises the extracellular domain of DLL1. In yet another embodiment,
20 the Notch ligand comprises the extracellular domain of DLL4.

[0095] In one embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) with an amino acid sequence as shown in SEQ ID NO: 6, or a functional variant thereof.

[0096] As used herein, the term "functional variant" includes modifications of the
25 amino acid and/or nucleic acid sequences disclosed herein that perform substantially the same function as the amino acid and/or nucleic acid molecules disclosed herein in substantially the same way. In one embodiment, the functional variant amino acid sequences comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 98% or at least 99% sequence
30 identity to the sequences disclosed herein.

[0097] In some embodiments, the functional variant comprises a DLL4 sequence with one or more mutations relative to SEQ ID NO: 6. In some embodiments, the DLL4 sequence comprises at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least

7, at least 8, at least 9, at least 10, at least 11, or at least 12 mutations relative to SEQ ID NO: 6. In some embodiments, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) with an amino acid sequence as shown in SEQ ID NO: 7.

[0098] In one embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) with an amino acid sequence having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 98%, or at least 99% sequence identity to SEQ ID NO: 6. In another embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) with an amino acid sequence having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 98%, or at least 99% sequence identity to SEQ ID NO: 7.

[0099] As used herein, the term "amino acid" includes all of the naturally occurring amino acids as well as modified L-amino acids. The atoms of the amino acid can, for example, include different isotopes. For example, the amino acids can comprise deuterium substituted for hydrogen, nitrogen-15 substituted for nitrogen-14, and carbon-13 substituted for carbon-12 and other similar changes.

[00100]

[00101]

[00102] The term "functional protein partner" or "FPP" as used herein refers to any ligand that binds a surface receptor on stem cells or progenitor cells. The FPP, when operably linked to a Notch ligand in a multispecific ligand, allows the multispecific ligand to be tethered to a cell at two ends, through Notch binding at one end and through binding with the FPP's binding partner at another end, for example in a suspension culture. This tethering can induce "pulling" on the Notch receptor (mechanosensing) and initiate the Notch signaling cascade. The multispecific ligand can be tethered at two ends to the same cell or to two different cells. The FPP can be a polypeptide or protein, or a natural or synthetic polymer, a biological material, or combination thereof. The FPP can be a cytokine, for example a chemokine, integrin, or growth factor. The FPP also can be a ligand to a receptor that is internalized once bound, for example through endocytosis, and can include, for example, stem cell factor (SCF), interleukin-7 (IL-7), or FMS-related tyrosine kinase 3 ligand (FLT3-L). SCF can be from any organism, optionally: human GenBank Accession No. M59964; Mus musculus Genbank Accession No. NM_013598. IL-7 can be from any organism, optionally: Mus musculus Genbank Accession No. NM_008371; H. sapiens GenBank Accession No. NM_001199886. FLT3-L can be from any organism and optionally:

H. sapiens GenBank Accession No. NM_001204503; Mus musculus Genbank Accession No. NR_175302.

[00103] The FPP can further be a ligand to a cell surface molecule that is not internalized upon binding, and wherein the mechanosensing is achieved through normal movements of cells in culture. For example, the FPP can be an adhesion molecule such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), or P-Selectin. ICAM-1 can be from any organism and optionally: H. sapiens GenBank Accession No. NM_000201; Mus musculus Genbank Accession No. NM_010493. VCAM-1 can be from any organism and optionally: H. sapiens GenBank Accession No. NM_001078; Mus musculus Genbank Accession No. NM_011693. P-Selectin can be from any organism and optionally: H. sapiens GenBank Accession No. NM_003005; Mus musculus Genbank Accession No. NM_011347. In one embodiment, the FPP comprises SCF, IL-7, FLT3-L, ICAM-1, VCAM-1, or P-Selectin. In one embodiment, the FPP comprises SCF. In another embodiment, the FPP comprises IL-7. In another embodiment, the FPP comprises FLT3-L. In another embodiment, the FPP comprises ICAM-1. In another embodiment, the FPP comprises VCAM-1. In yet another embodiment, the FPP comprises P-Selectin.

[00104] In one embodiment, the FPP is or comprises an SCF with an amino acid sequence as shown in SEQ ID NO: 8 or a functional variant thereof. In one embodiment, the FPP comprises an SCF with an amino acid sequence having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 98%, or at least 99% sequence identity to SEQ ID NO: 8.

[00105] In an embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[00106] The FPP can provide additional benefits aside from providing a structural tether. For example, when SCF binds Kit (CD117), Kit becomes activated and is endocytosed, resulting in cell survival and proliferation.

[00107] The term "Notch ligand" includes homologues of the known Notch ligands that may be identified by standard techniques. Likewise, the term FPP includes homologues of the FPP that may be identified by standard techniques. "Homologue" refers to a gene product that exhibits sequence homology, either amino acid or nucleic acid sequence homology, to any one of the known Notch ligands or FPPs. A Notch ligand or FPP may be at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 98 or 99% identical

at the amino acid level to a corresponding Notch ligand or FPP. The terms "Notch ligand" and "FPP" also include fragments thereof that retaining binding affinity for their respective binding partners.

[00108] In one embodiment, homologues of Notch ligands comprise a DSL domain at the N-terminus and have between 3 to 8 EGF-like repeats on the extracellular surface. Suitable homologues will also be capable of binding to a Notch receptor.

[00109] Binding to a Notch receptor or to a binding partner of an FPP may be determined by a variety of methods known in the art including in vitro binding assays.

[00110] The term "Notch ligand" also includes a mutant or variant of a known Notch ligand. Likewise, the term "FPP" also includes a mutant or variant of a known FPP. The term "mutant" refers to a polypeptide having a primary amino acid sequence, which differs from the wild type sequence by one or more amino acid additions, substitutions or deletions. In some embodiments, the mutant has at least 90% sequence identity with the wild type sequence. In some embodiments, the mutant has 20 mutations or less over the whole wild-type sequence. In some embodiments the mutant has 10 mutations or less, in some embodiments 5 mutations or less over the whole wild-type sequence.

[00111] Optionally, the Notch ligand and/or the FPP comprises at least one protein tag. A protein tag is a peptide sequence appended to a protein of interest such as a Notch ligand. The protein tag may be directly or indirectly linked to the protein of interest. Various protein tags are known in the art and can be used for a number of purposes. In one embodiment, the Notch ligand further comprises a fragment crystallizable (Fc) tag (Notch ligand-Fc). In another embodiment, the FPP further comprises an Fc tag (FPP-Fc). In a further embodiment, the multispecific ligand comprises Notch ligand-Fc and FPP-Fc. As used herein, the term "Fc" refers to the Fc domain or region of IgG. In an embodiment, the Fc tag comprises the Fc region of human IgG. Proteins fused to the Fc region of human IgG not only are more resistant to degradation but are also in dimerized form. In another embodiment, the tag is a His tag. In a further embodiment, the tag is a molecule that facilitates oligomerization of the Notch ligand. For example, a small domain of COMP (cartilage oligomeric matrix protein) can be fused to the Notch ligand (for example, DLL4) to form DLL4 pentamers. Ferritin can be used in a similar manner to form DLL4 multimers.

[00112] In an embodiment, the Fc tag is human IgG1 with an amino acid sequence as shown in SEQ ID NO: 12 or a functional variant thereof.

[00113] The Notch ligand and the FPP can be operably linked by any method known in the art, including through fusion or through conjugation, optionally using a linker. For example, the Notch ligand and the FPP can be fused in a single continuous peptide chain, optionally with a peptide linker between the Notch ligand and the FPP. Suitable peptide linkers can provide extra flexibility to the multispecific ligand to allow greater simultaneous binding of the Notch ligand and the FPP to their respective binding partners. The linker itself can be flexible or rigid or a combination of flexible and rigid. In another example, the Notch ligand and the FPP are conjugated to each other, for example using chemical methods such as carboxyl terminal cross linking. In yet another example, the Notch ligand and the FPP are conjugated to each other using self-ligating peptides, such as Spy-Tag/Spy-Catcher. In an embodiment, Notch ligand-Fc comprises Spy-Tag, and FPP-Fc comprises Spy-Catcher for covalent linkage of the C-termini of Notch ligand-Fc and FPP-Fc, optionally at different ratios, such as 2:1, 1:1, or 1:2 (Notch ligand-Fc:FPP-Fc). In an embodiment, are hole-knob Fc complexes for which only one Fc arm has Spy-Tag or Spy-Catcher. This avoids multiple, unrestricted ligation of the Notch ligand with the FPP. In still another example, the Notch ligand and the FPP are each biotinylated at their C-terminus, either chemically or enzymatically, and are then conjugated to a streptavidin molecule.

[00114] In an embodiment, the linker is GTGGGG (SEQ ID NO: 9). In an embodiment, the linker is

20 GGTGGGGGTGGGGAEEAAKEAAAKEAAAKEAAKALEAGGGGSAEEAAKEAAAKEAAAKEAAKALEAGTGGGGGTGGGG (SEQ ID NO: 10). In an embodiment, the linker is GGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGG (SEQ ID NO: 11). In some embodiments, the multispecific ligand comprises at least one linker. In some embodiments, the multispecific ligand comprises at least two linkers. In an embodiment, the linkers

25 comprise SEQ ID NO: 9 and 10. In some embodiments, the linkers comprise SEQ ID NO: 9 and 11.

[00115] The term "fused" or "translationally fused" as used herein means fusion of nucleic acids encoding two or more peptides or polypeptides into a single messenger RNA that when translated, produces a single fused protein comprising the two or more peptides or polypeptides.

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[00116] The term "conjugated" as used herein refers to the joining of two or more components (such as a Notch ligand, FPP, Fc region, and/or linker) by a covalent bond or weaker non-covalent interactions. Conjugation can occur passively, for example through

protein-protein interactions. Conjugation can also be achieved actively, for example through a specific chemical reaction. A multispecific ligand can be both fused and conjugated, for example a Notch ligand-Fc-FPP polypeptide wherein each of the components of Notch ligand-Fc-FPP are translationally fused to each other, but which becomes conjugated to another fusion Notch ligand-Fc-FPP polypeptide through dimerization (due to the Fc region) *in vitro*.

[00117] In an embodiment, the multispecific ligand comprises Notch ligand-Fc, a linker, and an FPP. In another embodiment, the multispecific ligand comprises a biotinylated Notch ligand and a biotinylated FPP, both conjugated to a same streptavidin molecule.

[00118] In an embodiment, the multispecific ligand comprises a leader sequence as shown in SEQ ID NO: 13.

[00119] In an embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 1 or a functional variant thereof. In another embodiment, the multispecific ligand comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3 or a functional variant thereof.

[00120] Another aspect of the disclosure is a protein or peptide comprising the amino acid sequence of SEQ ID No:1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 6, SEQ ID No: 7 or SEQ ID No: 8 or a functional variant thereof. In one embodiment, a protein or peptide is provided comprising an amino acid sequence having at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 98%, or at least 99% sequence identity SEQ ID No:1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 6, SEQ ID No: 7 or SEQ ID No: 8.

[00121] Another aspect of the disclosure is a nucleic acid encoding the multispecific ligand described herein. A further aspect of the disclosure is a nucleic acid encoding encoding a protein or peptide comprising the amino acid sequence of SEQ ID No:1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 6, SEQ ID No: 7 or SEQ ID No: 8 or a functional variant thereof.

[00122] In yet another aspect of the disclosure is one or more nucleic acids encoding the multispecific ligand described herein or SEQ ID No:1, SEQ ID No: 2, SEQ ID No: 3, SEQ ID No: 6, SEQ ID No: 7 or SEQ ID No: 8 or a functional variant thereof.

[00123] As used herein, the term “nucleic acid sequence” refers to a sequence of nucleoside or nucleotide monomers consisting of naturally occurring bases, sugars and intersugar (backbone) linkages. The term also includes modified or substituted sequences comprising non-naturally occurring monomers or portions thereof. The nucleic acid sequences of the present application may be deoxyribonucleic acid sequences (DNA) or ribonucleic acid sequences (RNA) and may include naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The sequences may also contain modified bases. Examples of such modified bases include aza and deaza adenine, guanine, cytosine, thymidine and uracil, and xanthine and hypoxanthine. The nucleic acid can be either double stranded or single stranded and represents the sense or antisense strand. Further, the term "nucleic acid" includes the complementary nucleic acid sequences as well as codon optimized or synonymous codon equivalents. The nucleic acid may be a mRNA.

[00124] In an embodiment, the multispecific ligand comprises a conservatively substitute amino acid sequence of SEQ ID No: 1. In another embodiment, the multispecific ligand comprises a conservatively substituted amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3.

[00125] As used herein, “conservative amino acid substitution” means one in which one amino acid residue is replaced with another amino acid residue without abolishing the protein's desired properties. Suitable conservative amino acid substitutions can be made by substituting amino acids with similar hydrophobicity, polarity, and R-group size for one another. Examples of conservative amino acid substitution include:

Conservative Substitutions	
Type of Amino Acid	Substitutable Amino Acids
Hydrophilic	Ala, Pro, Gly, Glu, Asp, Gln, Asn, Ser, Thr
Sulphydryl	Cys
Aliphatic	Val, Ile, Leu, Met
Basic	Lys, Arg, His
Aromatic	Phe, Tyr, Trp

[00126] In one embodiment, the present disclosure includes functional variants to the nucleic acid sequences disclosed herein. The functional variants include nucleotide sequences that hybridize to the nucleic acid sequences set out above, under at least moderately stringent hybridization conditions, optionally stringent hybridization conditions.

[00127] By “at least moderately stringent hybridization conditions” it is meant that conditions are selected which promote selective hybridization between two complementary nucleic acid molecules in solution. The term “at least moderately stringent hybridization conditions” encompasses stringent hybridization conditions and moderately stringent hybridization conditions. Hybridization may occur to all or a portion of a nucleic acid sequence molecule. The hybridizing portion is typically at least 15 (e.g. 20, 25, 30, 40 or 50) nucleotides in length. Those skilled in the art will recognize that the stability of a nucleic acid duplex, or hybrids, is determined by the T_m , which in sodium containing buffers is a function of the sodium ion concentration and temperature ($T_m = 81.5^{\circ}\text{C} - 16.6 (\text{Log}_{10} [\text{Na}^+] + 0.41(\%(\text{G}+\text{C}) - 600/\text{L}))$, or similar equation). Accordingly, the parameters in the wash conditions that determine hybrid stability are sodium ion concentration and temperature. In order to identify molecules that are similar, but not identical, to a known nucleic acid molecule a 1% mismatch may be assumed to result in about a 1°C decrease in T_m , for example if nucleic acid molecules are sought that have a >95% identity, the final wash temperature will be reduced by about 5°C . Based on these considerations those skilled in the art will be able to readily select appropriate hybridization conditions. In some embodiments, stringent hybridization conditions are selected. By way of example the following conditions may be employed to achieve stringent hybridization: hybridization at 5x sodium chloride/sodium citrate (SSC)/5x Denhardt's solution/1.0% SDS at $T_m - 5^{\circ}\text{C}$ based on the above equation, followed by a wash of 0.2x SSC/0.1% SDS at 60°C . Moderately stringent hybridization conditions include a washing step in 3x SSC at 42°C . It is understood, however, that equivalent stringencies may be achieved using alternative buffers, salts and temperatures. Additional guidance regarding hybridization conditions may be found in: Current Protocols in Molecular Biology, John Wiley & Sons, N.Y., 2002, and in: Sambrook et al., Molecular Cloning: a Laboratory Manual, Cold Spring Harbor Laboratory Press, 2001.

[00128] In another embodiment, the functional variant nucleic acid sequences comprise sequences having at least 50%, or at least 60%, or at least 70%, or at least 80%, or at least 90%, or at least 95%, or at least 98%, or at least 99% sequence identity to the sequences disclosed herein.

[00129] As used herein, the term “sequence identity” refers to the percentage of sequence identity between two polypeptide sequences or two nucleic acid sequences. To determine the percent identity of two amino acid sequences or of two nucleic acid

sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino acid or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % identity = number of identical overlapping positions/total number of positions times 100%). In one embodiment, the two sequences are the same length. The determination of percent identity between two sequences can also be accomplished using a mathematical algorithm. A, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. U.S.A. 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990, J. Mol. Biol. 215:403. BLAST nucleotide searches can be performed with the NBLAST nucleotide program parameters set, e.g., for score=100, word length=12 to obtain nucleotide sequences homologous to a nucleic acid molecules of the present application. BLAST protein searches can be performed with the XBLAST program parameters set, e.g., to score=50, word length=3 to obtain amino acid sequences homologous to a protein molecule described herein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-BLAST can be used to perform an iterated search which detects distant relationships between molecules (Id.). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., of XBLAST and NBLAST) can be used (see, e.g., the NCBI website). Another non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, 1988, CABIOS 4:11-17. Such an algorithm is incorporated in the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

[00130] In another embodiment, the functional variant nucleic acid sequences comprise degenerate codon substitutions or codon-optimized nucleic acid sequences. The term “degenerate codon substitution” as used herein refers to variant nucleic acid sequences in which the second and/or third base of a codon is substituted with a different
5 base that does not result in a change in the amino acid sequence encoded therein. The term “codon-optimized” as used herein refers to a variant nucleic acid molecule comprising one or more degenerate codon substitutions that reflect the codon usage bias of a particular organism.

[00131] Interaction of the Notch ligand with Notch and the operably linked FPP with
10 its binding partner is optionally in anti-parallel form, meaning that the N-termini of the Notch ligand and of the FPP are facing in opposite directions. In multispecific ligands wherein the Notch ligand and the FPP are fused, this can be achieved using a flexible peptide linker. In multispecific ligands wherein the Notch ligand and the FPP are conjugated, this can be achieved *in vitro* through any method known in the art, including those disclosed herein.

15 [00132] In another example, the multispecific ligand comprising a fusion of the Notch ligand and FPP comprises an FPP wherein its peptide backbone is reversed according to its amino acid (aa) sequence, mirroring its original sequence. In this example, the aa residue that was at the C-terminal of the FPP is now positioned at the N-terminus closest to the C-terminus of Notch ligand. Mirroring the aa sequence backbone has been shown to
20 preserve the ability of many proteins to interact with binding partners in the right alignment.

[00133] The Notch ligand and FPP optionally have small dissociation constants and high binding affinity for their respective binding partners such that this binding can lead to pulling on the Notch receptor. Suitable dissociation constants (K_D) can, for example, include less than or equal to 25 μM for Notch ligands and less than or equal to 5 nM for FPPs.

25 **IV. Kits**

[00134] Soluble multispecific ligands may be prepared and packaged in kits for use in generating cells of the T cell lineage.

[00135] Accordingly, also provided herein is a kit for producing cells of the T cell lineage comprising a soluble multispecific ligand comprising (a) a Notch ligand operably
30 linked to a (b) FPP that binds surface molecules on stem cells or progenitor cells. Optionally, the soluble multispecific ligand is contained in a preservative and/or buffer solution and the kit further comprises a device for dispensing the soluble multispecific

ligand such as a vial or syringe. In one embodiment, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[00136] In one embodiment, the kit further comprises culture medium for culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand.

5 Examples of culture medium include conditioned medium, non-conditioned medium, or embryonic stem cell medium. The culture medium may comprise serum (e.g., bovine serum, fetal bovine serum, calf bovine serum, horse serum, human serum, or an artificial serum substitute) or it may be serum free. Other examples of culture medium useful include Stem Cell Technologies media or any other commercially available equivalent media.

10 [00137] The medium optionally includes one or more cytokines that promote commitment and differentiation of cells of the T cell lineage. The cytokines may be human in origin, or may be derived from other species. The concentration of a cytokine in a culture is typically about 1-100ng/ml. The following are representative examples of cytokines that may be employed in the present disclosure: all members of the Flt-3-ligand, and interleukin-
15 7 (IL-7) and Stem Cell Factor. In one embodiment, the cytokines used herein are Flt-3-ligand and IL-7 and Stem Cell Factor. The cytokines may be used in combination with equal molar or greater amounts of a glycosaminoglycan such as heparin sulfate. The cytokines are commercially available or can be produced by recombinant DNA techniques and purified to various degrees. Some of the cytokines may be purified from culture media of
20 cell lines by standard biochemical techniques.

[00138] In one embodiment, the kit comprises one or more containers for the within-described reagents.

[00139] Printed instructions providing guidance in the use of the reagent(s) may also be included in the kit, in various embodiments. The term "instructions" or "instructions for
25 use" typically includes a tangible expression describing the reagent concentration or amount of the soluble multispecific ligand and/or at least one assay method parameter, such as the relative amounts of soluble multispecific ligand and cells to be mixed, culturing time periods, temperature, media conditions, and the like. For example, in one embodiment, the instructions describe a method comprising (a) culturing a sample
30 comprising stem cells or progenitor cells with a soluble multispecific ligand comprising a Notch ligand operably linked to an FPP and (b) isolating cells of the T cell lineage.

V. Cells of the T Cell Lineage

[00140] The disclosure further provides cells of the T cell lineage generated by the methods, systems and kits described herein, or mitotic or differentiated cells that are progeny of the cells.

- 5 [00141] In one embodiment, the disclosure provides a “progenitor T cell” or “proT cell” generated by the methods described herein. In another embodiment, the progenitor T cell is a human progenitor T cell, for example a human progenitor T cell characterized by CD34+CD7+ or CD7+CD5+CD1a-.

- [00142] In another embodiment, the progenitor T cell is a mouse progenitor T cell, for
10 example a mouse progenitor T cell characterized by CD25+.

[00143] The disclosure also provides double positive (DP) T-cell characterized by CD4+CD8+ or CD4+CD8+CD3+. The disclosure further provides a cell of the T-cell lineage that is a single positive (SP) cell characterized by CD4-CD8+, CD4+CD8- or CD8+CD3+, CD4CD3+.

- 15 [00144] In one embodiment, a cell of the T cell lineage generated by the methods described herein (for example, a progenitor T cell or a mature T cell) is engineered to be generated with a T cell receptor (TCR) or a chimeric antigen receptor (CAR) to confer specificity to tumor associated antigens (TAA). Cells engineered as such can be useful for treating conditions such as cancer.

- 20 [00145] In another aspect, the present disclosure provides a pharmaceutical composition comprising isolated cells of the T cell lineage generated by the methods described herein and a pharmaceutically acceptable diluent or carrier.

- [00146] Suitable diluents and carriers are described, for example, in Remington's Pharmaceutical Sciences. On this basis, the compositions include, albeit not exclusively,
25 solutions of the proT cells in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids.

- [00147] Pharmaceutical compositions include, without limitation, lyophilized powders or aqueous or non-aqueous sterile injectable solutions or suspensions, which may further
30 contain antioxidants, buffers, bacteriostats and solutes that render the compositions substantially compatible with the tissues or the blood of an intended recipient. Other components that may be present in such compositions include water, surfactants (such as

Tween™), alcohols, polyols, glycerin and vegetable oils, for example. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules, tablets, or concentrated solutions or suspensions. The composition may be supplied, for example but not by way of limitation, as a lyophilized powder which is reconstituted with
5 sterile water or saline prior to administration to the patient.

[00148] Suitable pharmaceutically acceptable carriers include essentially chemically inert and nontoxic compositions that do not interfere with the effectiveness of the biological activity of the pharmaceutical composition. Examples of suitable pharmaceutical carriers include, but are not limited to, water, saline solutions, glycerol solutions, ethanol, N-(1(2,3-
10 dioleoyloxy)propyl)N,N,N-trimethylammonium chloride (DOTMA), diolesylphosphatidyl-ethanolamine (DOPE), and liposomes. Such compositions should contain a therapeutically effective amount of the compound, together with a suitable amount of carrier so as to provide the form for direct administration to the patient.

[00149] The compositions can be administered for example, by parenteral,
15 intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol or oral administration. For parenteral administration, solutions of the pro-T cells described herein can be prepared in water suitably mixed with a surfactant such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene
20 glycols, DMSO and mixtures thereof with or without alcohol, and in oils. Under ordinary conditions of storage and use, these preparations contain a preservative to prevent the growth of microorganisms. A person skilled in the art would know how to prepare suitable formulations.

[00150] In one embodiment, the cells of the T cell lineage are present in an amount
25 effective for treating a disease state in a subject need thereof. In one embodiment the cell of the T cell lineage is present in an amount effective to enhance hematopoietic progenitor cell engraftment in a subject in need thereof. Optionally, the composition further comprises cells of the T cell lineage, or tissue for transplantation. In one embodiment the tissue comprises a thymus. In another embodiment the tissue comprises an organ.

30 **VI. Therapeutic Applications**

[00151] The ability to generate *in vitro*-derived human progenitor T cells and to test their safety in human/mouse immune engraftment models, opens avenues for cellular based approaches for treating immune-related disorders of the T lineage (6, 7). T cells are

the major effector arm of the adaptive immune system in recognizing and eliminating viral and bacterial pathogens. In certain rare blood cancers such as T cell acute lymphoblastic leukemia (T-ALL), T cells proliferate crowding out healthy immune cells and perturbing normal immune function (8, 9). Although chemotherapy can often impart therapeutic benefits in cancer patients, it often can lead to immuno-deficiency and susceptibility to opportunistic infections. Opportunistic infections also pose a serious concern in AIDS patients whose CD4⁺ T cells have been depleted following infection with HIV. While immunodeficiency remains a serious concern in HIV/AIDS and cancer, immune-hyperreactivity is equally problematic in autoimmune disease where T cells that lack proper regulatory control, make immune responses to self-tissue.

[00152] Accordingly, the present disclosure includes a method of treating a subject having a condition requiring an increase in the number of T cells comprising:

(i) generating cells of the T cell lineage comprising (a) culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand comprising I) a Notch ligand operably linked to II) an FPP that engages surface molecules on stem cells or progenitor cells, and (b) isolating cells of the T cell lineage, and

(ii) administering an effective amount of the cells of the T cell lineage to a subject in need thereof.

[00153] The present disclosure further includes a use of an effective amount of the cells of a T cell lineage for treating a subject having a condition requiring an increase in the number of T cells, wherein cells of the T cell lineage are obtained by the methods described herein.

[00154] Another aspect of the present disclosure is a use of an effective amount of the cells of a T cell lineage in the manufacture of a medicament for treating a subject having a condition requiring an increase in the number of T cells, the cells of the T cell lineage obtained by the methods described herein.

[00155] A further aspect of the disclosure is an effective amount of the cells of a T cell lineage for use for treating a subject having a condition requiring an increase in the number of T cells, the cells of the T cell lineage obtained by the methods described herein.

[00156] In one embodiment, the cell of the T cell lineage is a progenitor T cell.

[00157] In another embodiment, the cell of the T cell lineage is a mature T cell.

[00158] The disclosure also provides a use of cells of the T cell lineage, optionally progenitor T cells or mature T cells, generated by the methods described herein for treating a subject having a condition requiring an increase in the number of T cells.

- 5 [00159] As used herein, the phrase "effective amount" or "therapeutically effective amount" means an amount effective, at dosages and for periods of time necessary to achieve the desired result. Effective amounts may vary according to factors such as the disease state, age, sex, weight of the subject. The amount of a given cell preparation that will correspond to such an amount will vary depending upon various factors. Such as the
10 pharmaceutical formulation, the route of administration, the type of disease or disorder, the identity of the subject or host being treated, and the like, but can nevertheless be routinely determined by one skilled in the art. An "effective amount" will optionally be an amount effective for the cell of the T cell lineage to engraft the subject being treated.

- [00160] The term "treating" or "treatment" as used herein and as is well understood
15 in the art, means an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e., not worsening) state of disease, preventing spread of disease, delay or slowing of disease progression, amelioration or palliation of the disease state, diminishment
20 of the reoccurrence of disease, and remission (whether partial or total), whether detectable or undetectable. "Treating" and "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

[00161] The term "subject" as used herein means any member of the animal kingdom and is optionally a human.

- 25 [00162] A "condition requiring an increase in number of T cells" includes any condition wherein T cell levels are reduced as compared to a healthy animal, including, without limitation, immunodeficiency, cancer, genetic diseases, infectious diseases and autoimmunity.

- [00163] As set out above, the cells of the T-cell lineage described herein can be
30 engineered to express T cell receptors (TCRs) or a chimeric antigen receptor (CAR) that specifically recognize tumour associated antigens.

[00164] Accordingly, the present disclosure also includes a method of treating cancer in a subject comprising:

(i) generating cells of the T cell lineage comprising (a) culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand comprising I) a
5 Notch ligand operably linked to II) an FPP that engages surface molecules on stem cells or progenitor cells, and (b) isolating cells of the T cell lineage, and

(ii) administering an effective amount of the cells of the T cell lineage to a subject in need thereof,

wherein the cells of the T cell lineage are engineered with a T cell receptor
10 (TCR) or a chimeric antigen receptor (CAR) to confer specificity to a tumor-associated antigen.

[00165] In some embodiments, the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).

[00166] The disclosure also provides a use of cells of the T cell lineage, optionally
15 progenitor T cells or mature T cells, generated by the methods described herein for treating a subject with cancer, wherein the cells of the T cell lineage are engineered with a T cell receptor (TCR) or a chimeric antigen receptor (CAR) to confer specificity to a tumor-associated antigen.

Examples

20 [00167] The following non-limiting Examples are illustrative of the present disclosure:

Example 1 – Multispecific Ligands

[00168] Multispecific ligands that interact with Notch receptors at one end through a soluble Notch ligand, for example the soluble extracellular domain of DLL1 or DLL4, and
25 tether the Notch receptor to the cell membrane at another end of the multispecific ligand through a functional protein partner (FPP) are created. This “Notch – multispecific ligand – cell membrane” complex can induce a physical pulling on the Notch receptor similar to the physiological action of full length DLL1 or DLL4.

[00169] These multispecific ligands activate Notch receptors in cells cultured with
30 them. When bound to Notch, full-length DLL1 or DLL4 is endocytosed resulting in a physical pulling action, or mechanosensing, on Notch receptor to expose the S2 cleavage site, naturally cleaved by ADAM-proteases. Cleavage of this site leads to the subsequent S3

cleavage within the Notch transmembrane domain by gamma-secretase protein complex, releasing the activated Notch intracellular domain into the cytoplasm from where it enters into the nucleus, interacts with the DNA binding protein RBPJ and activates a cascade of cell-type-dependent genetic programs that lead to differentiation, for example the differentiation of stem cells or progenitor cells into cells of the T cell lineage (FIG. 1). In contrast to membrane-bound DLL1 or DLL4, the binding of the soluble extracellular domain of DLL1 or DLL4 protein to Notch receptors fails to activate them, as binding-alone induces no pulling action. The present multispecific ligands provide a structural tether that permits a pulling action on the Notch receptor and leads to cell differentiation.

10 Multispecific ligands wherein the FPP is stem cell factor (SCF)

[00170] In one example, the FPP of the multispecific ligand is stem cell factor (SCF), the Notch ligand is the extracellular domain of DLL1 or DLL4, and the Notch ligand and the SCF are covalently linked to each other, each preforming a different function. At one end, the DLL1 or DLL4 domain of the multispecific ligand binds Notch receptors present on a hematopoietic stem/progenitor cell (HSPC) surface. At the other end, SCF binds its receptor Kit (CD117), a tyrosine kinase receptor, on another or the same HSPC. When bound to SCF, Kit becomes activated and endocytosed, then SCF linked to DLL1 or DLL4 protein interacting with its receptor, Notch, leads to pulling action on Notch and mechanosensing. Therefore, the DLL1/4-SCF protein complex provides the dual function of Kit activation, resulting in survival and proliferation, as well as the pulling action and activation of the Notch receptor, leading to T cell lineage fate determination.

Other multispecific ligands wherein the bound FPP leads to internalization of its bound receptor

[00171] An assorted number of FPPs other than SCF are linked to the extracellular domain of Notch ligands DLL1 or DLL4 to form a series of multispecific ligands that exerts the pulling action on Notch receptors. These include FLT3-L and IL-7, both of which are required for T cell development and their receptors are both expressed by HPSCs. Flt3 and IL-7 receptor (IL-7R) are also internalized upon binding to their respective ligands, similar to Kit and SCF, and therefore provide similar molecular dynamics of pulling on Notch receptors, leading to their activation.

Other multispecific ligands wherein the bound FPP does not lead to internalization of its bound receptor

[00172] Other candidates for FPPs are not necessarily endocytosed but still mediate Notch activation by simply holding the multispecific ligand in place, akin to plate-bound DLL4 which is capable of activating Notch receptor without endocytosis. Binding of DLL1- or DLL4-FPP to Notch at one end and the interaction of FPP to its partner at another end, in combination with the normal movement of cells in culture, creates the force on Notch receptor necessary to induce a conformational change (mechanosensing) and start the activation cascade. Examples of non-endocytosed FPPs include ICAM-1, VCAM-1, and P-Selectin, which are adhesion molecules required for normal thymic development of T cells. The binding of ICAM-1 or VCAM-1 to integrins or P-Selectin to P-Selectin glycoprotein ligand 1 (PSGL-1) and to CD34, on HSPCs does not result in internalization, but DLL1-ICAM1, DLL4-ICAM1, DLL1-VCAM1, DLL4-VCAM1, DLL1-PSelectin, and DLL4-PSelectin effectively pull-on Notch receptor to activate it.

Multispecific ligand structure

[00173] The extracellular domain of DLL1 or DLL4 protein is linked to a functional protein partner (FPP) using a variety of methods. There are several factors that can influence Notch activation, as described below:

[00174] 1) Mode of linkage: DLL1/4-FPP complexes are produced such that DLL1/4 is linked to an FPP through different methods, namely i) in one continuous single peptide chain (FIG. 2A, B), ii) through biotinylation of DLL1/4 and FPPs at the C-terminus and subsequent binding of up to 4 subunits to a single streptavidin molecule (FIG. 2D), iii) linkage at the C-terminus of DLL1/4 and FPPs through a) chemical methods such as carboxyl terminal cross linking, or b) self-ligating peptides such as Spy-Tag/ Spy-Catcher (e.g., FIG. 2C). In cases ii and iii, DLL1/4 and FPPs are generated separately with different tags for protein purification purposes. Using the purification tags allows the linkage of two molecules through biotin/streptavidin interactions or covalent bonds through cross-linking. The protein complex is taken through two separate affinity purification columns that bind tagged DLL1/4 (e.g., with 6xHis tag with Ni beads) followed by tagged FPPs (e.g., with Myc-tag/anti-Myc Ab column or strep-tag ii/streptavidin bead column). Only entities that contain both tags (i.e., both DLL1/4 and FPP) are purified to ensure bifunctional nature of the product.

[00175] 2) Orientation: Interaction of DLL1/4 with Notch and the FPPs with their binding partners are in anti-parallel form, meaning that the N-termini of the two interacting molecules are facing in opposite direction. To engage Notch on one cell and FPP's binding

partner on another cell DLL1 or DLL4 and FPP face in opposite directions to each other. This can be achieved as described in “mode of linkage” (above) where the two proteins are generated separately *in vitro* and are subsequently linked to each other through the different ways described above. As an alternative, DLL1/4-FPP fusion proteins are created as single peptides. In these fusion proteins, a linker is provided between DLL1/4 and FPP that allow for greater flexibility to realign the FPP to interact with its binding partner in the correct orientation.

[00176] 3) Valency /Stoichiometry: Valency is a major determinant of the efficiency of DLL1/4-FPP to activate Notch receptor. Proteins fused to the Fc region of human IgG, not only are more resistant to degradation but are also in dimerized form. DLL1/4-Fc and FPP-Fc, such as SCF-Fc fusion protein dimers have previously been shown to be functional in activating Notch and Kit, respectively (FIG. 3) (10). A variety of DLL1/4-FPP complex proteins are created that incorporate Fc, including: i) single peptide DLL1/4-Fc-FPP fusion proteins with flexible and rigid linkers between Fc and FPP regions, and ii) DLL1/4Fc-SpyTag and FPP-Fc-SpyCatcher for covalent linkage of C-termini of the fusion proteins at different ratios (2 DLL1/4-Fc: 1 FPP-Fc, 1:1 or 1:2). As such, hole and knob Fc complexes are created for which only one Fc arm has the SpyTag or Spy-Catcher to avoid multiple, unrestricted ligation of DLL1/4-Fc with FPP-Fc complexes. iii) Biotinylated DLL1/4-Fc and FPP-Fc protein at the C-termini for binding to Streptavidin to form multimeric complexes with a maximum valency of 4.

[00177] 4) Differential dissociation constant: The strength of interaction of DLL1/4 with Notch and FPPs with their binding partners (such as SCF with Kit), influences the activation of Notch receptors (11, 13). As such, variants of DLL1/4-FPPs are created that modulate the binding sites to their partners in order to maximize the activation of Notch signaling.

25 **Example 2 – Validating Multispecific Ligands**

Materials and Methods:

Hematopoietic stem cell sources

[00178] Human umbilical cord blood (UCB) samples were obtained by syringe extraction and collected in a blood-pack unit containing citrate phosphate dextrose anti-coagulant (Baxter Healthcare, Deerfield, Illinois) from consenting mothers following delivery in accordance to approved guidelines established by the Research Ethics Board of Sunnybrook Health Sciences Centre. Within 24 hours of collection, UCB mononuclear cells were isolated using Ficoll density centrifugation, and pre-enriched for lineage negative

(Lin⁻) CD34⁺ cells using the EasySep Human CD34 Positive Selection Kit (Stemcell Technologies, Vancouver, B.C.) according to the manufacturer's instructions. To isolate human HSPCs, Lin⁻ cells were stained with anti-human CD38-APC and anti-human CD34-PE mAbs and sorted for CD34⁺CD38^{-/lo} cells using BD Biosciences FACSARIA sorter (BD, San Jose, CA). Sorted human HSPCs were greater than 99% pure as determined by post-sort analysis. Some CD34⁺ cells derived from cord blood and all CD34⁺ cells derived from mobilized peripheral blood (mPB) were purchased from Stemcell Technologies. For mPB, volunteers were treated with a combination of G-CSF (maximum of 10 µg/kg/day of G-CSF for 3 - 5 days prior to collection) and Plerixafor (maximum of 0.24 mg/kg 1 day prior to collection).

DLL1/4-FPP production

[00179] Delta-like-4 is genetically engineered into a pDLL4-SCF plasmid construct by fusing the coding sequence of the extracellular domain of human DLL4 (amino acid residues 1-529) with a flexible and rigid linker and the coding sequence of Stem Cell Factor (SCF) and the 6x His tag for purification purposes. The resultant fusion protein has a structure as shown in FIG. 2A, wherein SCF is the FPP. This construct is inserted into pCDNA3.4 (Thermo-Fisher) mammalian expression plasmid. The resulting plasmid is transfected into Expi293F cells using standard transfection methods and selected cells with stably integrated plasmid is based on their resistance to 1 mg/mL of Geneticin added to the standard DMEM [supplemented with 10%(v/v) FBS, 2 mM Glutamax, Penicillin (100 U/ml) /Streptomycin (100 mg/ml) (all products of Thermo Fisher Scientific, Rockford, IL), 2 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MI)]. Cells are expanded and transferred to grow in Freestyle 293 expression media (Thermo Fisher Scientific). DLL4-SCF fusion protein secreted into the media is purified using Ni bead column attached to the ÄKTAprime plus (GE Healthcare) automated chromatography system.

DLL1/4-Fc-FPP production

[00180] Delta-like-4 was genetically engineered into a pDLL4-Fc-SCF plasmid construct by fusing the coding sequence of the extracellular domain of human DLL4 (amino acid residues 1-529) to the Fc portion of human IgG1 (including the hinge region), with a flexible and rigid linker and the coding sequence of Stem Cell Factor (SEQ ID NO: 1). The resultant fusion protein has a structure as shown in FIG. 2B, wherein SCF is the FPP. This construct was inserted into pCDNA3.4 (Thermo-Fisher) mammalian expression plasmid. The resulting plasmid was transfected into Expi293F cells using standard transfection

methods and selected cells with stably integrated plasmid was based on their resistance to 1 mg/mL of Geneticin added to the standard DMEM [supplemented with 10%(v/v) FBS, 2 mM Glutamax, Penicillin (100 U/ml) /Streptomycin (100 mg/ml) (all products of Thermo Fisher Scientific, Rockford, IL), 2 mM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MI)].

5 Cells were expanded and transferred to grow in Freestyle 293 expression media (Thermo Fisher Scientific). DLL4-Fc-SCF fusion protein secreted into the media was purified using HiTrap Protein G affinity column (GE Healthcare Life Sciences, Marlborough, MA) attached to the ÄKTAprime plus (GE Healthcare) automated chromatography system.

DLL4-Fc-SCF variants.

10 [00181] Variants of DLL4-Fc-SCF were generated with changes to the linker, removing rigid linkers and extending the flexible GSSS sequence to 33 repeats (SEQ ID NOs: 2 and 3). Amino acid variants were encoded for the DLL4 protein to enhance binding to NOTCH receptor (DLL4(max); SEQ ID NO: 3). Variants with DLL4 extracellular domain situated at the C-terminus and SCF at the N-terminus with intervening Fc and linker were
15 also created. Based on work in (11, 13), mutations in both DLL4 and in SCF were introduced to enhance binding to their respective receptors. Variants of DLL4-Fc-SCF sequences were provided to National Research Council (Montreal) where codon sequence optimization and production took place.

[00182] The DLL4-Fc-SCF variants are as shown in Table 1:

20 **Table 1.**

Construct	Sequence	SEQ ID NOs
DLL4-Fc-L-SCF (SEQ ID NO: 1)	<u>MHSSALLCCLVLLTGVR</u> SGVFQLQLQEFINERGVLASGRPCEPGC RTFFRVCLKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDSSGGGR NPLQLPFNFTWPGTFSLI IEAWHAPGDDLPEALPPDALISKIAIQ GSLAVGQNWLLDEQTSTLTRLRYSYRVICSDNYYGDNCSRLCKKRN DHFGHYVCQPDGNLSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAE CLCRPGWQGRLCNECIPHNGCRHGTCSTPWQCTCDEGWGGLFCDQD LNYCTHHSPCKNGATCSNSGQRSYTCTCRPGYTGVDCELELSECD NPCRNNGSCKDQEDGYHCLCPPGYGLHCEHSTLSCADSPCFNGGS CRERNQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGP SRMCRCPGFTGTYCELHVSDCARNPCAHHGTCHDLENGLMCTCPA GFSGRRCVVRTSIDACASSPCFNATCYTDLSTDTFVCNCPYGFVG	<u>Leader Sequence</u> <u>(SEQ ID NO: 13)</u> DLL4 Extracellular Domain (SEQ ID NO: 6) <u>Linker</u> <u>(SEQ ID</u>

	SRCEFPVGLPGTGGGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGGGTGGGGTGGGGAEEAAKEAAAKEAAAKEAAAK ALEAGGGGSAEAAAKEAAAKEAAAKEAAAKALEAGTGGGGTGGGG EGICRNRVTNNVKDVTKLVANLPKDYMITLKYVPGMDVLP SHCWIS EMVQLSDSLTDLLDKFSNISEGLSNYSIIDKLVNIVDDLVECVKE NSSKDLKKSFKSPEPRLFTPEEFFRIFNRSIDAFKDFVVASETSDC VVSSTLSPEKDSRVSVTKPFMLPPVA	Nos: 9, 10) Human Fc-1 (SEQ ID NO: 12) SCF Extracellular Domain (SEQ ID NO: 8)
DLL4-Fc- GS33-SCF (SEQ ID NO: 2)	<u>MHSSALLCCLVLLTGVRAS</u>GVFQLQLQEFINERGLASGRPCEPGC RTFFRVCLKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGGR NPLQLPFNFTWPGTFSLLIEAWHAPGDDLREALPPDALISKIAIQ GS LAVGQNWLLDEQTSTLTRLSYRVICSDNYYGDNCSRLCKKR DHFGHYVCQPDGNLSCLPGWTGEYCQQPICLSGCHEQNGYCSKPAE CLCRPGWQGRCLCNECIPHNGCRHGTCSTPWQCTCDEGWGGLFCDQD LN YCTHHS PCKNGATCSNSGQRSYTCTCRPGYTGVDCLELSECD NPCRNGGSCKDQEDGYHCLCPPGYGLHCEHSTLSCADSPCFNGGS CRERNQGANYACECPPNFTGSNCEKKVDRCTSNPCANGGQCLNRGP SRMCRCPGFTGT YCELHVSDCARNPCA HGGTCHDLENGLMCTCPA GFSGRRCEVRTSIDACASSPCFN RATCYTDLSTDTFVCNCPYGFVG SRCEFPVGLPGTGGGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDT LMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQ PREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHN HYTQKSLSLSPGGGGSGGGSGGGSGGGSGGGSGGGSGGGSGGGGE GICRNRVTNNVKDVTKLVANLPKDYMITLKYVPGMDVLP SHCWISE MVVQLSDSLTDLLDKFSNISEGLSNYSIIDKLVNIVDDLVECVKEN SSKDLKKSFKSPEPRLFTPEEFFRIFNRSIDAFKDFVVASETSDCV VVSSTLSPEKDSRVSVTKPFMLPPVA	<u>Leader Sequence (SEQ ID NO: 13)</u> DLL4 Extracellular Domain (SEQ ID NO: 6) <u>Linker (SEQ ID NO: 9, 11)</u> Human Fc-1 (SEQ ID NO: 12) SCF Extracellular Domain (SEQ ID NO: 8)
DLL4(Max)- Fc-GS33- SCF	<u>MHSSALLCCLVLLTGVRAS</u>SSVFQLQLQEFINERGLASGRPCEPG CRTFFRVCLKHFQAVVSPGPCTFGTVSTPVLGTNSFAVRDDSSGGG RNPLQLPLNFTWPGTFSLLIEAWHAPGDDLREALPPDALISKFAI QGSLAVGQNWLLDEQTSTLTRLSYRVICSDNYYGDNCSRLCKKR NDYFGHYVCQPDGNPSCPLPGWTGEYCQQPICLSGCHEQNGYCSKPA	<u>Leader Sequence (SEQ ID NO: 13)</u> DLL4 Extracellular

[00186] Acute Myeloid Leukemia-derived Kasumi-1 cells were selected for experiments because of their high expression of human KIT for binding the FPP end of DLL4-Fc-SCF, and their non-adherent nature serve as a good model for primary HSPCs. 3T3-N1Cluc cells are a mouse fibroblast-derived adherent Notch reporter cell line used for binding to DLL4 constructs, as well as Notch activation assays (3). 32D-Kit cells were transduced with viral particles that carried MIGR-1-hNOTCH1 construct. GFP+ cells were sorted 5 days after transfection and transfected with the plasmid pGL4.17-6xN1Rep. The latter plasmid contained a DNA Notch-responsive element (6x RBPJ consensus binding sites) that drives the expression of luciferase gene in response to Notch activation, also utilized in 3T3-N1Cluc cells. In addition, cells stably transfected with the plasmid were selected for by resistance to neomycin. The pool of 32D-Kit cells expressing hNotch1 and resistant to neomycin were named 32D-kit-N1Cluc.

3T3-N1CLuc and 32D-kit-N1Cluc Luciferase Assay to Measure Notch Activation

[00187] DLL4-Fc-SCF was added to 3×10^4 3T3-N1CLuc cells or to 1×10^5 32D-kit-N1Cluc in a standard tissue culture (TC)-treated flat-bottom 96-well plate and incubated overnight in α MEM supplemented with 5% FBS. Cells were lysed and assayed for luciferase activity using the Firefly Luciferase Assay Kit 2.0 (Biotium, Fremont, CA) according to the manufacturer's instructions. Briefly, growth medium was removed and cells were washed with PBS prior to adding lysis buffer. Cells were lysed by freezing at -80°C for 10 min followed by thawing before transferring lysates to a 96-well flat-bottom opaque polystyrene plate (Corning). D-luciferin was prepared and added to each well by automatic dispenser and analyzed using Synergy H1Platereader (BioTek Instruments Inc., Winooski, VT). Human IgG and DLL4-Fc plate-bound controls were prepared the previous day by adsorbing 50 μ L/well of protein (20 μ g/mL) to flat-bottom 96-well plates overnight at 4°C prior to washing and seeding of 3T3-N1CLuc cells the following day.

[00188] For cell-bound to multispecific ligand, 32D-kit-N1Cluc cells were incubated with 10nM DLL4-Fc-SCF on ice for 15 minutes followed by a wash with cold PBS. Cell-bound DLL4-Fc-SCF were then incubated with unbound cells at a 1:1 ratio and placed in the cell culture incubator at 37°C. After overnight incubation, the cells were lysed and assayed for luciferase activity.

[00189] The effects of soluble multispecific ligand, DLL4-Fc-SCF, was measured through luciferase activity. 32D-kit-N1Cluc cells were incubated in DMEM supplemented with 10%(v/v) FBS, 2 mM Glutamax, Penicillin (100 U/ml) /Streptomycin (100 mg/ml) (all

products of Thermo Fisher Scientific, Rockford, IL), 2 mM 2-mercaptoethanol and different concentrations of DLL4-Fc-SCF. After overnight incubation, the cells were lysed and assayed for luciferase activity.

[00190] The luciferase assay to measure Notch activation with DLL4-SCF, 5 DLL4/SCF-Fc-linked, or streptavidin DLL4-Fc/SCF-Fc is performed as described above for DLL4-Fc-SCF.

Multispecific Ligand Cultures with HSPCs

[00191] Different concentrations of DLL4-Fc-SCF are added to 3×10^3 mouse Lin⁻ Sca-1⁺ Kit⁺ HSCs (sorted from the bone marrow of C57BL6 mice by flow cytometry) incubated 10 in single well of a round-bottom 96-well plate in 200 μ L IMDM [supplemented with 20% BIT (STEMCELL Technologies), 1% Glutamax (Thermo), 50 ng/mL SCF, 10 ng/mL Flt3L and 10 ng/mL IL-7 (R&D Systems, Minneapolis, MN)]. Human IgG and DLL4-Fc plate-bound controls are prepared the previous day by adsorbing 50 μ L/well of protein (20 μ g/mL) to 15 flat-bottom 96-well plates overnight at 4°C prior to washing and plating of cells the following day. On day 7 of co-culture, cells are harvested and stained with antibodies against mouse CD45, CD25, CD44, CD90, CD11b, and CD19, followed by analysis on LSR II cytometer (BD Biosciences).

[00192] For human HSPCs, CB-derived CD34⁺ cells were either enriched by magnetic sorting (Stemcell Technologies, Vancouver, BC) or CB-derived and mPB-derived CD34⁺ 20 cells were purchased from Stemcell Technologies. CD34⁺ cells were either in a mix culture with Kasumi-1 cells or cultured by themselves. For mixed cultures Kasumi-1 cells were gamma-irradiated at 12.5 Gy prior to incubation with CD34⁺ cells. 9×10^4 irradiated Kasumi-1 cells were incubated with 1×10^4 CB-derived CD34⁺ cells in media containing DLL4-Fc-SCF at 1nM. Cultures were supplemented with additional 1nM DLL4-Fc-SCF every day for 25 6 days. Cultures were stained with antibodies against CD34, CD5, CD1a and CD7 to assay for T-lineage progression analyzed using A5 Symphony (BD) flow cytometer.

[00193] 3×10^4 to 1×10^5 CB-derived cells were cultured with DLL4-Fc-SCF (0.1-10nM) per well of a 96 round-bottom well plate in 200 μ L StemSpanTMSFEM II (Stemcell Technologies) supplemented with StemSpanTM T Cell Progenitor Expansion Supplement 30 (Stemcell Technologies) for up to 7 days. The media was supplemented with extra DLL4-Fc-SCF once every 2 days at the same concentration. On various days up to day 7 of culture, cells were counted to determine cell expansion and stained with antibodies against

CD34, CD5, CD1a and CD7 to assay for T-lineage progression. To assay for development beyond proT cell stage, cells are stained with antibodies against CD4, CD8 and CD3 at later time points. To determine Notch activity in primary HSP/C cells, cultured cells were lysed and examined for Notch target gene, DELTEX RNA transcript using QRT-PCR with the following primers: hDtx1F: CCAGGACACAGGCACCAT (SEQ ID NO: 4); hDtx1R: CTGCTTCTCGTAGGCGTTCT (SEQ ID NO: 5).

[00194] DLL4-SCF, DLL4/SCF-Fc-linked, and streptavidin DLL4-Fc/SCF-Fc cultures with HSPCs are prepared and assayed as described above for DLL4-Fc-SCF.

Results

10 *Expression of Multispecific Ligand Construct*

[00195] Previous work from the inventors' laboratory showed that a DLL4 fusion protein, DLL4-Fc, when immobilized to the surface of a standard TC-treated well/plate, could induce Notch signaling in mouse and human HSPCs that was sufficient to induce the development of T-lineage cells (12). However, this 2D format is limited in scalability and capacity to deliver strong and persistent Notch signals to promote T cell development.

[00196] Accordingly, the present inventors investigated whether operably linking DLL4-Fc to SCF creates a dimeric platform that could effectively engage Notch receptors and function in suspension with HSPCs and therefore be amenable to scale-up.

[00197] The DLL4-Fc-SCF construct (as depicted in FIG. 2B) was transfected into Expi293F cells and following 7 days of incubation, a sample of the culture supernatant (supe) was harvested. FIG. 4A shows the immunoblot analysis of purified DLL4-Fc (50 ng) compared to DLL4-Fc-L-SCF (25 μ L supernatant) as detected by anti-human immunoglobulin G (hIgG) conjugated to peroxidase. To test the ability of DLL4-Fc-L-SCF to bind to Kit and Notch1, 100 μ L of supernatant was incubated for 20 min on ice with 32D-Kit (4) (Fig. 4B, upper panel) and 3T3-N1CLuc (3) (Fig. 4B; lower panel) cells. 32D-Kit cells express Kit, the receptor for SCF and 3T3-N1CLuc cells express human Notch1, the receptor for DLL4.

[00198] To determine binding specificity, monomeric SCF (5 μ g/mL) was added to the 32D-Kit/DLL4-Fc-L-SCF sample (FIG. 4B, upper panel) and DLL4-His (10 μ g/mL) was added to the 3T3-N1CLuc/DLL4-Fc-L-SCF sample (Fig. 4B, lower panel). In addition, to determine non-specific binding with Fc domain of DLL4-Fc-L-SCF, 32D-Kit and 3T3-N1CLuc cell were incubated with hIgG (5 μ g/mL). Cell surface binding was detected by

anti-hlgG-APC using flow cytometry. The results demonstrate specific binding by DLL4-Fc-LSCF to Notch1 and Kit bearing cells, respectively.

[00199] Whether DLL4-Fc-L-SCF leads to the activation of Notch signaling was then tested. Fig. 4C shows the results of Notch activity in control 3T3-N1CLuc (control, 1st column) and 3T3-N1CLuc cells bound to DLL4-Fc-L-SCF, following similar binding procedure as described for the flow cytometry assay, above. To measure Notch activity in 3T3-N1CLuc cells bound to DLL4-Fc-L-SCF, the cells were incubated with or without 32D-Kit cells before they were plated into 96 well plates and incubated at 37°C for 18 hours. In one condition, no 32D-Kit cells were added to 3T3-N1CLuc cells (2nd column). When 32D-Kit cells were added to DLL4-Fc-L-SCF-coated 3T3-N1CLuc cells, it resulted in an increase (≥2 fold) in Notch activity (3rd column). For the 4th column, 32D-Kit cells were pre-incubated with DLL4-Fc-L-SCF, saturating SCF binding sites and blocking their interaction with DLL4-Fc-L-SCF bound to 3T3-N1CLuc cells. This competitive inhibition resulted in decreased Notch activation. The results show specific activation of Notch signaling induced by DLL4-Fc-L-SCF in 3T3-N1CLuc cells interacting with 32D-Kit cells.

[00200] Variants of multispecific ligand, DLL4-Fc-SCF, with different linkers and mutations that predicted enhancement of their affinity to Notch and Kit were generated. No clear differences were observed in binding or the activity by the different variants. They all bound both Kasumi-1 cells that express KIT only (FIG. 5C) and 3T3-N1CLuc that only express hNotch1 (FIG. 5D), reaching saturation levels at similar concentrations of 3-10 nM.

[00201] When multispecific ligand, DL4-Fc-SCF, was cell-bound to 32D-Kit-N1CLuc cells in a saturating manner, and then was mixed with unbound cells, it resulted in luciferase activity that was higher than the standard plate-bound activity (FIG. 6). This demonstrated that DL4-Fc-SCF could function as the tether between Notch on one cell and Kit receptor on the other, mediating the activation of Notch.

[00202] In soluble form, DLL4-Fc-SCF showed Notch induction when incubated with 32D-kit-N1CLuc cells as reported by luciferase assay (FIG. 7B) and QPCR evidence for increased expression of Deltex, a Notch target (FIG 8C). Soluble DLL4-Fc-SCF was also able to induce HSPC to T lineage differentiation when incubated with either CB-derived CD34⁺ cells alone (FIG 7C) or when CD34⁺ cells were mixed with irradiated Kasumi cells (FIG 8B). In addition, increasing concentration of soluble DLL4-Fc-SCF in culture, resulted in higher survival and/or proliferation of CB-derived CD34⁺ cells (FIG. 8D).

[00203] DLL4-SCF, DLL4/SCF-Fc-linked, and streptavidin DLL4-Fc/SCF-Fc multispecific ligands are validated as described above for DLL4-Fc-SCF and also induce increased Notch signaling.

Summary

5 [00204] Here, the development of a cell-free, bead-free, soluble, and biologic Notch ligand-based system for the generation of T cells from both mouse and human HSPCs is described. Non-plate-bound or suspension Notch ligands that are entirely biologic, such as DLL4-SCF, DLL4-Fc-SCF, DLL4/SCF-Fc-linked, and streptavidin DLL4-Fc/SCF-Fc ligands, represent a unique strategy to allow for the effective generation T-lineage cells,
10 which can be readily achieved in large-scale bioreactor-based suspension cultures, and potentially overcome the developmental roadblocks, plus the inefficiency and scalability drawbacks, associated with plate-bound approaches. Further, they do not necessitate a dissociation step to separate the cells from μbeads.

[00205] Previous studies have not demonstrated the generation of human T-lineage
15 cells beyond the immature proT cell stage in a cell-free, matrix-free support system. Here, it is shown that the suspension Notch ligand culture system described herein allows for the emergence of mature SP T cells, showing that the Notch signaling achieved with the soluble, and biologic Notch ligand described herein could overcome the developmental roadblock for T cell maturation seen with plate-bound approaches.

20 [00206] While the present disclosure has been described with reference to examples, it is to be understood that the scope of the claims should not be limited by the embodiments set forth in the examples, but should be given the broadest interpretation consistent with the description as a whole.

[00207] All publications, patents and patent applications are herein incorporated by
25 reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety. Where a term in the present description is found to be defined differently in a document incorporated herein by reference, the definition provided herein is to serve as the definition for the term.

CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

1. Petrie, H. T., and J. C. Zuniga-Pflucker. 2007. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu Rev Immunol* 25: 649-679.
- 5 2. Shah, D. K., and J. C. Zuniga-Pflucker. 2014. An overview of the intrathymic intricacies of T cell development. *J Immunol* 192: 4017-4023.
3. Trotman-Grant, A. C., M. Mohtashami, J. De Sousa Casal, E. C. Martinez, D. Lee, S. Teichman, P. M. Brauer, J. Han, M. K. Anderson, and J. C. Zuniga-Pflucker. 2021. DL4-mubeads induce T cell lineage differentiation from stem cells in a stromal cell-free system. *Nat Commun* 12: 5023.
- 10 4. Hu, Q., M. Trevisan, Y. Xu, W. Dong, S. A. Berger, S. D. Lyman, and M. D. Minden. 1995. c-KIT expression enhances the leukemogenic potential of 32D cells. *J Clin Invest* 95: 2530-2538.
- 15 5. Shah, D. K., and J. C. Zuniga-Pflucker. 2012. Notch receptor-ligand interactions during T cell development, a ligand endocytosis-driven mechanism. *Curr Top Microbiol Immunol* 360: 19-46.
6. Legrand, N., K. Weijer, and H. Spits. 2006. Experimental models to study development and function of the human immune system in vivo. *J Immunol* 176: 2053-2058.
- 20 7. van den Brink, M. R., O. Alpdogan, and R. L. Boyd. 2004. Strategies to enhance T-cell reconstitution in immunocompromised patients. *Nat Rev Immunol* 4: 856-867.
8. Ferrando, A. A., D. S. Neuberg, J. Staunton, M. L. Loh, C. Huard, S. C. Raimondi, F. G. Behm, C. H. Pui, J. R. Downing, D. G. Gilliland, E. S. Lander, T. R. Golub, and A. T. Look. 2002. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell* 1: 75-87.
- 25 9. Weng, A. P., A. A. Ferrando, W. Lee, J. P. t. Morris, L. B. Silverman, C. Sanchez-Irizarry, S. C. Blacklow, A. T. Look, and J. C. Aster. 2004. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306: 269-271.
- 30 10. Erben, U., E. Thiel, and M. Notter. 1999. Differential effects of a stem cell factor-immunoglobulin fusion protein on malignant and normal hematopoietic cells. *Cancer Res* 59: 2924-2930.
- 35 11. Gonzalez-Perez, D., S. Das, D. Antfolk, H. S. Ahsan, E. Medina, C. E. Dundes, R. T. Jokhai, E. D. Egan, S. C. Blacklow, K. M. Loh, P. C. Rodriguez, and V. C. Luca. 2023. Affinity-matured DLL4 ligands as broad-spectrum modulators of Notch signaling. *Nat Chem Biol* 19: 9-17.
12. Shukla, S., M. A. Langley, J. Singh, J. M. Edgar, M. Mohtashami, J. C. Zuniga-Pflucker, and P. W. Zandstra. 2017. Progenitor T-cell differentiation from hematopoietic stem cells using Delta-like-4 and VCAM-1. *Nat Methods* 14: 531-538.
- 40 13. Ho CCM, Chhabra A, Starkl P, Schnorr PJ, Wilmes S, Moraga I, Kwon HS, Gaudenzio N, Sibilano R, Wehrman TS, Gakovic M, Sockolosky JT, Tiffany MR,

Ring AM, Piehler J, Weissman IL, Galli SJ, Shizuru JA, Garcia KC. Decoupling the Functional Pleiotropy of Stem Cell Factor by Tuning c-Kit Signaling. *Cell*. 2017 Mar 9;168(6):1041-1052.e18. doi: 10.1016/j.cell.2017.02.011. PMID: 28283060; PMCID: PMC5526607.

Claims:

1. A method of generating a cell of the T cell lineage comprising (a) culturing a sample comprising stem cells or progenitor cells with a soluble multispecific ligand comprising a
5 Notch ligand operably linked to a functional protein partner (FPP) that binds a surface molecule on the stem cells or progenitor cells, and (b) isolating a cell of the T cell lineage.
2. The method of claim 1, wherein the stem cells or progenitor cells with the multispecific ligand are cultured in suspension.
3. The method of claim 1 or 2, wherein the Notch ligand comprises an extracellular
10 domain of Delta-like-1 (DLL1) or of Delta-like-4 (DLL4).
4. The method of any one of claims 1 to 3, wherein the FPP comprises stem cell factor (SCF), interleukin-7 (IL-7), FMS-related tyrosine kinase 3 ligand (Flt3-L), intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), or P-Selectin.
5. The method of any one of claims 1 to 4, wherein the Notch ligand further comprises
15 a fragment crystallizable (Fc) region of human IgG (Notch ligand-Fc).
6. The method of any one of claim 1 to 5, wherein the FPP further comprises an Fc region of human IgG (FPP-Fc).
7. The method of any one of claims 1 to 6, wherein the multispecific ligand further comprises a linker between the Notch ligand and the FPP.
- 20 8. The method of claim 7, wherein the multispecific ligand comprises i) the Notch ligand-Fc, ii) the linker, and iii) the FPP.
9. The method of claim 8, wherein the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).
10. The method of claim 9, wherein the multispecific ligand comprises the amino acid
25 sequence of SEQ ID No: 1 or a functional variant thereof.
11. The method of claim 9, wherein the multispecific ligand comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3, or a functional variant thereof.

12. The method of any one of claims 6 to 9, wherein the multispecific ligand comprises the Notch ligand-Fc and the FPP-Fc.
13. The method of any one of claims 1 to 9, wherein the Notch ligand is fused to the
5 FPP.
14. The method of any one of claims 1 to 9, wherein the Notch ligand is conjugated to the FPP.
15. The method of claim 14, wherein the Notch ligand and the FPP are both biotinylated and are both conjugated to a same streptavidin molecule.
- 10 16. The method of any one of claims 1 to 15, wherein the stem cells are selected from hematopoietic stem/progenitor cells (HSPCs), embryonic stem cells or induced pluripotent stem cells.
17. The method of any one of claims 1 to 16, wherein the stem cells are CD34⁺ HSPCs.
18. The method of any one of claims 1 to 17, wherein the cell of the T cell lineage is a
15 progenitor T (proT) cell.
19. The method according to claim 18 wherein the stem cells or progenitor cells are human cells and the proT cell has the phenotype CD34⁺CD7⁺ or CD7⁺CD5⁺CD1a⁻.
20. The method according to claim 18, wherein the stem cells or progenitor cells are mouse cells and the proT cell has the phenotype CD25⁺ or CD25⁺CD90⁺.
- 20 21. The method according to any one of claims 1 to 17, wherein the cell of the T-cell lineage is a CD4⁺CD8⁺ double positive cell, CD4⁺CD8⁺CD3⁺ double positive cell or CD8⁺CD3⁺ or CD4⁺CD3⁺ single positive cell.
22. The method of any one of claims 1 to 21, wherein the stem cells or progenitor cells are cultured in stromal cell-free media.
- 25 23. A cell of the T cell lineage, wherein the cell is generated by the method of any one of claims 1 to 22.

24. The cell of claim 23, wherein the cell is a progenitor T cell, CD4+CD8+ double positive cell, CD4+CD8+CD3+ double positive cell or CD8+CD3+ or CD4+CD3+ single positive cell.
25. A soluble multispecific ligand comprising i) a Notch ligand operably linked to ii) a functional protein partner (FPP) that binds a surface molecule on stem cells or progenitor cells.
26. The multispecific ligand of claim 25, wherein the Notch ligand comprises an extracellular domain of DLL1 or of DLL4.
27. The multispecific ligand of claim 25 or 26, wherein the FPP comprises SCF, IL-7, FLT3-L, ICAM-1, VCAM-1, or P-Selectin.
28. The multispecific ligand of any one of claims 25 to 27, wherein the Notch ligand further comprises a fragment crystallizable (Fc) region of human IgG (Notch ligand-Fc).
29. The multispecific ligand of any one of claims 25 to 28, wherein the FPP further comprises an Fc region of human IgG (FPP-Fc).
30. The multispecific ligand of any one of claims 25 to 29, wherein the multispecific ligand further comprises a linker between the Notch ligand and the FPP.
31. The multispecific ligand of claim 30, wherein the multispecific ligand comprises i) the Notch ligand-Fc, ii) the linker, and iii) the FPP.
32. The multispecific ligand of claim 31, wherein the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).
33. The multispecific ligand of claim 32, wherein the multispecific ligand comprises the amino acid sequence of SEQ ID No: 1 or a functional variant thereof.
34. The multispecific ligand of claim 32, wherein the multispecific ligand comprises the amino acid sequence of SEQ ID No: 2 or SEQ ID No: 3 or a functional variant thereof.

35. The multispecific ligand of any one of claims 29 to 32, wherein the multispecific ligand comprises the Notch ligand-Fc and the FPP-Fc.
36. The multispecific ligand of any one of claims 25 to 32, wherein the Notch ligand is fused to the FPP.
- 5 37. The multispecific ligand of any one of claims 25 to 32, wherein the Notch ligand is conjugated to the FPP.
38. The multispecific ligand of claim 37, wherein the Notch ligand and the FPP are both biotinylated and are both conjugated to a same streptavidin molecule.
39. A use of the multispecific ligand of any one of claims 25 to 38 for generating a cell
10 of the T cell lineage.
40. A kit comprising (i) a soluble multispecific ligand comprising (a) a Notch ligand operably linked to (b) an FPP that binds surface molecules on stem cells or progenitor cells, and (ii) instructions for use of the soluble multispecific ligand for generating a cell of the T cell lineage.
- 15 41. A kit comprising (i) a soluble multispecific ligand comprising (a) a Notch ligand operably linked to (b) an FPP that binds surface molecules on stem cells or progenitor cells, and (ii) culture medium.
42. The kit of claim 41 or 42, wherein;
- the Notch ligand comprises DLL1 or DLL4; and/or
 - 20 – the FPP comprises SCF, IL-7, FLT3-L, ICAM-1, VCAM-1, or P-Selectin.
43. The kit of claim 41 or 42, wherein the Notch ligand comprises an extracellular domain of Delta-like-4 (DLL4) and the FPP comprises stem cell factor (SCF).
44. A use of cells of the T cell lineage for treating a subject having a condition requiring
25 an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the method of any one of claims 1-22.

45. A use of cells of the T cell lineage in the manufacture of a medicament for treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the method of any one of claims 1-22.
46. The use of claim 44 or 45, wherein the cells of the T cell lineage are progenitor T cells.
47. The use of claim 44 or 45, wherein the cells of the T cell lineage are mature T cells.
48. Cells of the T cell lineage for use for treating a subject having a condition requiring an increase in the number of T cells, wherein the cells of the T cell lineage are obtained by the method of any one of claims 1-22
49. The cells for use of claim 48, wherein the cells of the T cell lineage are progenitor T cells.
50. The cells for use of claim 48, wherein the cells of the T cell lineage are mature T cells.