specificity of the receptor for its cognate peptide. Ideally, most mutations introduced to enhance the affinity of a TCR would be restricted to the CDR3 region for this reason. However, current methodologies are limited in the capacity to generate CDR3 diversity, because site-directed mutagenesis is constrained by the original length of the CDR3 region.

Given the difficulty of isolating high affinity T cells that recognize relevant tumor associated antigens, there is a continuing need for alternative methods for generating enhanced affinity TCRs.

BRIEF SUMMARY

In one aspect, the present disclosure provides a method for generating an enhanced affinity TCR comprising: a) contacting hematopoietic progenitor cells with stromal cells and a peptide antigen, under conditions and for a time sufficient to induce differentiation of the hematopoietic progenitor cells into DN TCR$\alpha\beta^+$ thymocytes, wherein the hematopoietic progenitor cells comprise a non-endogenous nucleic acid sequence encoding a TCRα chain from a parent TCR specific for the peptide antigen, and wherein the stromal cells comprise a non-endogenous nucleic acid sequence encoding Delta-like-1 or Delta-like-4 and a nucleic acid sequence encoding an MHC molecule; b) isolating nucleic acid sequences encoding the various TCRβ chains from the DN TCR$\alpha\beta^+$ thymocytes and introducing the nucleic acid sequences encoding the TCRβ chains into cells that are capable of expressing a TCR on the cell surface and comprising the nucleic acid sequence encoding the TCRα chain from step a); and identifying enhanced affinity TCR (*e.g.*, by detecting or selecting high affinity TCRαβ candidate by an MHC tetramer assay, and then measuring binding affinity as compared to a parent TCRαβ).

In further aspects, enhanced affinity TCRs generated by methods disclosed herein are provided, which may be cell-bound or in soluble form, and may further be codon optimized to enhance expression in T cells.

In further aspects, the present disclosure refers to a fusion protein comprising an enhanced affinity TCR generated by the method disclosed herein and a cytotoxic or detectable component.

In further aspects, the present disclosure refers to a pharmaceutical composition, comprising an enhanced affinity TCR generated by the method as disclosed herein, and a pharmaceutically acceptable carrier, diluent, or excipient.

In further aspects, the present disclosure refers to a use of an enhanced affinity TCR generated by the method as disclosed herein in the manufacture of a

medicament for treating a disease in a subject, wherein the disease is selected from the group consisting of viral infection, bacterial infection, cancer, and autoimmune disease.

In further aspects, the present disclosure refers to an enhanced affinity TCR generated by the method as disclosed herein for use in therapy.

In still further examples, enhanced affinity TCRs of the present disclosure may be used to treat a disease (such as cancer, infectious disease, or autoimmune disease) in a subject by administering a composition comprising the enhanced affinity TCRs. In further examples, enhanced affinity TCRs of the instant disclosure may be used in diagnostic methods or imaging methods, including these methods used in relation to the indications or conditions identified herein.

These and other examples of the present disclosure will become apparent upon reference to the following detailed description and attached drawings. All references disclosed herein are hereby incorporated by reference in their entirety as if each was incorporated individually.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURES 1A-D: Thymocytes from OT-1 transgenic mice were sorted for $TCR\beta^-TCR\gamma\delta^-CD4^-CD8^-CD117^+CD44^+$ DN1 and DN2 progenitor cells and cultured on OP9-DL1 cells expressing MHC Class I H-2Kb molecule for 20 days in the presence of various concentrations of ovalbumin SIINFEKL peptide (SEQ ID NO:1) as indicated. **(A, B, C)** Cultures were analyzed by flow cytometry at the timepoints indicated. **(D)** Total cellularity of each culture was determined on day 20 of culture.

FIGURE 2: $CD69^-$ DP thymocytes that have not yet gone through positive selection sorted from B6 or OT-1 transgenic mice were cultured on OP9-DL1 cells expressing MHC Class I H-2Kb molecule in the presence of ovalbumin SIINFEKL peptide (SEQ ID NO:1).

FIGURES 3A-C: B6 thymocytes were sorted for $CD4^-CD8^-CD117^+CD44^+$ DN1 and DN2 progenitor cells and transduced with the TCRα chain of the affinity enhanced WT1 specific TCR 3D clone, and cultured on OP9-DL1 cells expressing MHC Class I H-2Db molecule in the presence or absence of 1μM of WT1 peptide RMFPNAPYL (SEQ ID NO:2). **(A)** On day 16 of culture, transduced ($hCD2^+$) and untransduced ($hCD2^-$) cells were analyzed by flow cytometry. **(B)** On day 21 of OP9-DL1 culture in the presence of 1μM WT1 peptide RMFPNAPYL (SEQ ID NO:2), DN $TCR\alpha\beta^+$ cells were sorted according to the scheme indicated. **(C)** Sorted cells were lysed, DNA was isolated, and PCR was performed using a Vb10-specific forward primer and a Cb2-specific reverse primer. The Vb10 PCR product was then directionally TOPO-cloned into vector pENTR/D-TOPO, transferred to the retroviral

Claims

What is claimed is:

1. A method for generating an enhanced affinity T cell receptor (TCR), comprising:

a. contacting hematopoietic progenitor cells with stromal cells and a peptide antigen, under conditions and for a time sufficient to induce differentiation of hematopoietic progenitor cells into DN TCR$\alpha\beta^+$ thymocytes,

b. isolating nucleic acid sequences encoding the various TCRβ chains from the DN TCR$\alpha\beta^+$ thymocytes and introducing the nucleic acid sequences encoding the TCRβ chains into cells that are capable of expressing a TCR on the cell surface and comprise the nucleic acid sequence encoding the TCRα chain from step (a); and

c. identifying enhanced affinity TCR,

wherein the hematopoietic progenitor cells comprise a non-endogenous nucleic acid sequence encoding a TCRα chain from a parent TCR specific for the peptide antigen, and

wherein the stromal cells comprise a non-endogenous nucleic acid sequence encoding Delta-like-1 or Delta-like-4 and a nucleic acid sequence encoding an MHC molecule.

2. The method of claim 1, wherein the TCRβ chain is isolated from the parent TCR.

3. The method of claim 1, wherein the hematopoietic progenitor cells comprise thymocyte progenitor cells or embryonic stem cells.

4. The method of claim 1, wherein the hematopoietic progenitor cells comprise hematopoietic stem cells derived from bone marrow or cord blood.

5. The method of claim 1, wherein a viral vector is used to introduce the non-endogenous nucleic acid sequence encoding the TCRα chain specific for the peptide antigen into the hematopoeitic progenitor cells.

6. The method of claim 5, wherein the viral vector is a retroviral vector.

7. The method of claim 5, wherein the viral vector is a lentiviral vector.

8. The method of claim 5, wherein the viral vector further comprises a gene marker for transduction.

9. The method of claim 8, wherein the gene marker for transduction comprises green fluorescent protein or the extracellular domain of human CD2.

10. The method of claim 1, wherein the stromal cells express Delta-like-1.

11. The method of claim 1, wherein the stromal cells are derived from OP9.

12. The method of claim 1, wherein the method further comprises selecting the cells that are capable of expressing a TCR on the cell surface resulting from step (b) with MHC-peptide tetramer staining.

13. The method of claim 12, wherein the cells that are capable of expressing a TCR on the cell surface resulting from step (b) are selected with MHC-peptide tetramer staining multiple times.

14. The method of claim 1, wherein a viral vector is used to introduce the nucleic acid sequences encoding the various TCRβ chains from step (b) into the cells that are capable of expressing TCR on the cell surface.

15. The method of claim 14, wherein the viral vector is a retroviral vector.

16. The method of claim 14, wherein the viral vector is a lentiviral vector.

17. The method of claim 14, wherein the viral vector further comprises a gene marker for transduction.

18. The method of claim 17, wherein the gene marker for transduction comprises green fluorescent protein.

19. The method of claim 1, wherein the cells that are capable of expressing a TCR on the cell surface are derived from TCRα^-/β^- 58 T cell hybridoma.

20. The method of claim 1, wherein the enhanced affinity TCR is a human TCR.

21. The method of claim 1, wherein the MHC molecule comprises a Class I MHC molecule or a Class II MHC molecule.

22. The method of claim 21, wherein the MHC molecule comprises HLA-A2 and human beta-2-microglobulin (β2M).

23. The method of claim 1, wherein the peptide antigen is selected from the group consisting of: a viral antigen, a bacterial antigen, a cancer antigen, and an autoimmune antigen.

24. The method of claim 23, wherein the peptide antigen is a WT1 peptide antigen or a mesothelin peptide antigen.

25. The method of claim 24, wherein the WT1 peptide antigen comprises an amino acid sequence RMFPNAPYL (SEQ ID NO:2).

26. The method of claim 24, wherein the mesothelin peptide antigen comprises an amino acid sequence GQKMNAQAI (SEQ ID NO:31).

27. The method of claim 1, wherein the peptide antigen is added to the hematopoietic progenitor cells and stromal cells in culture.

28. The method of claim 1, wherein the stromal cells comprise a nucleic acid sequence encoding the peptide antigen.

29. The method of claim 1, wherein isolating the nucleic acid sequences encoding the various TCRβ chains from the DN TCRαβ$^+$ thymocytes further comprises selecting TCRβ chains with the same V_β gene as the parent TCRβ chain prior to introducing the selected TCRβ chains into cells capable of expressing a TCR on the cell surface.

30. An enhanced affinity TCR generated by the method of claim 1.

31. A fusion protein comprising an enhanced affinity TCR generated by the method of claim 1 and a cytotoxic or detectable component.

32. An enhanced affinity TCR generated by the method of claim 23 or 24.

33. A pharmaceutical composition, comprising an enhanced affinity TCR generated by the method of claim 1, and a pharmaceutically acceptable carrier, diluent, or excipient.

34. Use of an enhanced affinity TCR generated by the method of claim 1 in the manufacture of a medicament for treating a disease in a subject, wherein the disease is selected from the group consisting of viral infection, bacterial infection, cancer, and autoimmune disease.

35. An enhanced affinity TCR generated by the method of claim 1 for use in therapy.

36. The use of claim 34, wherein the subject is human.

37. The use of claim 34, wherein the enhanced affinity TCR is to be administered to the subject as a soluble TCR.

38. The use of claim 34, wherein the subject is to be administered T cells comprising the enhanced affinity TCR.

39. The use of claim 38, wherein the T cells comprise regulatory T cells.

40. The use of claim 38, wherein the T cells comprise $CD8^+$ T cells or $CD4^+$ T cell.

41. The use of claim 38, wherein the T cells are autologous T cells.