

MACROPHAGE-SPECIFIC PROMOTERS AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of U.S. Provisional Application Nos. 63/386,117, filed December 5, 2022, 63/459,988, filed April 17, 2023, 63/506,013, filed June 2, 2023, and 63/588,196, filed October 5, 2023, each of which is hereby incorporated by reference in their entirety for all purposes.

SEQUENCE LISTING

[0002] The instant application contains a Sequence Listing which has been submitted via EFS-Web and is hereby incorporated by reference in its entirety. Said XML copy, created on Month XX, 20XX, is named XXXXXUS_sequencelisting.xml, and is X,XXX,XXX bytes in size.

BACKGROUND

[0003] Cell-based therapy platforms provide promising avenues for treating a variety of diseases. Engineering of macrophages as cell therapies and drug delivery vehicles has become prominent as a potential immunotherapy. These engineered macrophages are typically genetically modified to express checkpoint inhibitors (*e.g.*, PD-1/PD-L1 binders, SIRP α or CD47 blockers, *etc.*), immunomodulatory cytokines (*e.g.*, interferons or interleukins), chimeric antigen receptors and/or other immune regulatory elements under control of a constitutive promoter. The constitutive expression of these engineered elements may not be desirable and may cause unwanted toxicities.

[0004] Given their promise, improvements in cell-based therapies are needed. An active area of exploration is engineering cell-based therapies to produce and/or secrete effector molecules such as cytokines, a process referred to as armoring, that enhance the cell-based therapy. Thus, additional methods of controlling and regulating the armoring of cell-based therapies, such as regulating production and/or secretion of payload effector molecules, are required.

SUMMARY

[0004a] In one aspect, provided here is an engineered macrophage-specific promoter system comprising: (i)(a) a regulatory element, wherein the regulatory element is derived from a promoter of a gene selected from the group consisting of CCL19, CCR7, CXCL11, GBP5, IDO1, UBD, and UNQ6494.1; and (b) a heterologous payload, optionally wherein

the heterologous payload is selected from the group consisting of transcriptions factors, cytokines, receptors, enzymes, chemokines, antibodies, fragments of antibodies, miRNAs, and shRNAs, wherein the regulatory element exhibits greater activity in an M1 macrophage compared to an M2 or MO macrophage, and wherein the regulatory element is or comprises an enhancer region that is derived from a promoter of a gene that is more highly expressed in M1 macrophage compared to M2 or MO macrophages, optionally wherein M2 macrophages are selected from the group consisting of M2a macrophages, M2b macrophages, and M2c macrophages, optionally wherein the regulatory element comprises a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 132 – 138 or (ii)(a) a regulatory element, wherein the regulatory element is derived from a promoter of a gene selected from the group consisting of CD28, SOCS3, PLXDC1, IL7R ZNF704, LNCAROD, MRC1, and ID3; and (b) a heterologous payload, optionally wherein the heterologous payload is selected from the group consisting of transcriptions factors, cytokines, receptors, enzymes, chemokines, antibodies, fragments of antibodies, miRNAs, and shRNAs, wherein the regulatory element exhibits greater activity in an M2 macrophage compared to an M1 or MO macrophage, and wherein the regulatory element is or comprises an enhancer region that is derived from a promoter of a gene that is more highly expressed in M2 macrophage compared to M1 or MO macrophages, optionally wherein M2 macrophages are selected from the group consisting of M2a macrophages, M2b macrophages, and M2c macrophages, optionally wherein the regulatory element comprises a sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO: 139 — 141, 392, 393, and 414 – 419.

[0004b] In another aspect, provided herein is an engineered macrophage-specific promoter comprising an ablation of at least one nucleotide motif, wherein the ablation increases specific activity of the engineered macrophage-specific promoter in M1 macrophages, as compared to activity of a corresponding macrophage-specific promoter lacking the ablation in M1 macrophages, optionally wherein the corresponding macrophage-specific promoter lacking the ablation in M1 macrophages is a wildtype macrophage promoter, and wherein the wildtype macrophage promoter comprises a sequence selected from the group consisting of SEQ ID NOs: 132-138, wherein the

engineered macrophage-specific promoter comprises: i) a motif within the nucleotide sequence of SEQ ID NO: 132, wherein the motif comprises a sequence selected from the group consisting of: position 63 to position 73 of SEQ ID NO: 132, position 80 to position 102 of SEQ ID NO: 132, position 141 to position 162 of SEQ ID NO: 132, position 212 to position 222 of SEQ ID NO: 132, position 229 to position 251 of SEQ ID NO: 132, position 307 to position 361 of SEQ ID NO: 132, position 365 to position 376 of SEQ ID NO: 132, position 559 to position 571 of SEQ ID NO: 132, position 617 to position 633 of SEQ ID NO: 132, position 782 to position 799 of SEQ ID NO: 132, position 852 to position 871 of SEQ ID NO: 132, position 886 to position 920 of SEQ ID NO: 132, position 933 to position 959 of SEQ ID NO: 132, position 1002 to position 1028 of SEQ ID NO: 132, position 1032 to position 1045 of SEQ ID NO: 132, position 1064 to position 1087 of SEQ ID NO: 132, position 1169 to position 1192 of SEQ ID NO: 132, position 1212 to position 1232 of SEQ ID NO: 132, position 1257 to position 1275 of SEQ ID NO: 132, position 1310 to position 1333 of SEQ ID NO: 132, position 1381 to position 1434 of SEQ ID NO: 132, position 1698 to position 1753 of SEQ ID NO: 132, position 1783 to position 1826 of SEQ ID NO: 132, position 1909 to position 1927 of SEQ ID NO: 132, position 1946 to position 1961 of SEQ ID NO: 132; and/or ii) a motif within the nucleotide sequence of SEQ ID NO: 136, wherein the motif comprises a sequence selected from the group consisting of: to position 133 to position 144 of SEQ ID NO: 136, position 200 to 217 of SEQ ID NO: 136, position 225 to position 247 of SEQ ID NO: 136, position 303 to position 325 of SEQ ID NO: 136, position 332 to position 342 of SEQ ID NO: 136, position 391 to position 413 of SEQ ID NO: 136, position 423 to position 460 of SEQ ID NO: 136, position 467 to position 477 of SEQ ID NO: 136, position 693 to position 717 of SEQ ID NO: 136, position 738 to position 761 of SEQ ID NO: 136, position 838 to position 861 of SEQ ID NO: 136, position 1229 to position 1246 of SEQ ID NO: 136, position 1286 to position 1309 of SEQ ID NO: 136, position 1413 to position 1431 of SEQ ID NO: 136, position 1456 to position 1473 of SEQ ID NO: 136, to position 1530 to position 1544 of SEQ ID NO: 136, position 1577 to position 1590 of SEQ ID NO: 136, position 1816 to position 1836 of SEQ ID NO: 136, position 1852 to position 1872 of SEQ ID NO: 136, and to position 1876 to position to position 1896 of SEQ ID NO: 136; and/or iii) a motif within the nucleotide sequence of SEQ ID NO: 137, wherein the motif comprises a sequence selected from the group consisting of: to position 43 to position 60, position 107 to position 120 of SEQ ID NO: 137, position 210 to position 230 of SEQ ID NO: 137, position 345 to

position 407 of SEQ ID NO: 137, position 427 to position 457 of SEQ ID NO: 137, position 468 to position 484 of SEQ ID NO: 137, position 560 to position 582, position 730 to position 746 of SEQ ID NO: 137, position 809 to position 820 of SEQ ID NO: 137, position 827 to position 837 of SEQ ID NO: 137, position 858 to position 878 of SEQ ID NO: 137, position 1291 to position 1302 of SEQ ID NO: 137, position 1321 to position 1341 of SEQ ID NO: 137, position 1435 to position 1463 of SEQ ID NO: 137, position 1530 to position 1541 of SEQ ID NO: 137, position 1707 to position 1718 of SEQ ID NO: 137, position 1834 to position 1863 of SEQ ID NO: 137, position 1870 to position 1882 of SEQ ID NO: 137, and to position 1913 to position 1929 of SEQ ID NO: 137, optionally wherein the ablation comprises a substitution or deletion of one or more nucleotides of the at least one nucleotide motif.

[0004c] In another aspect, provided herein is an engineered macrophage-specific promoter comprising at least one regulatory element, wherein the regulatory element exhibits greater activity in an M1 macrophage compared to an M2 or MO macrophage or exhibits greater activity in an M2 macrophage compared to an M1 or MO macrophage, optionally wherein the engineered macrophage-specific promoter comprises at least 2, at least 3, at least 4, or at least 5 regulatory elements, optionally wherein each of the regulatory elements are the same or different, optionally wherein M2 macrophages are selected from the group consisting of M2a macrophages, M2b macrophages, and M2c macrophages.

[0004d] In another aspect, provided herein is a heterologous construct comprising i) the engineered macrophage-specific promoter system as described herein; or ii) the engineered macrophage-specific promoter as described herein operably linked to a heterologous payload, optionally wherein the heterologous payload is a polynucleotide comprising a nucleotide sequence encoding a polypeptide, optionally wherein the polypeptide comprises at least one effector molecule, optionally wherein the polypeptide comprises a first effector molecule and a second effector molecule, optionally wherein the engineered macrophage specific promoter comprises a regulatory element selected from: a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 420; and a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 427, optionally wherein the polynucleotide comprises a nucleotide sequence encoding the first effector molecule, a

linker nucleotide sequence, and a nucleotide sequence encoding the second effector, optionally wherein the linker nucleotide sequence encodes one or more 2A ribosome skipping elements, optionally wherein the one or more 2A ribosome skipping elements comprise elements that are each selected from the group consisting of: P2A, T2A, E2A, and F2A and optionally wherein the at least one effector molecule or each effector molecule is selected from a therapeutic class, wherein the therapeutic class is selected from the group consisting of: a cytokine, a chemokine, a homing molecule, a growth factor, a polynucleotide molecule, a co-activation molecule, a tumor microenvironment modifier, a receptor, a ligand, a transcription factor, an antibody, a peptide, and an enzyme, optionally wherein the transcription factor is a master regulator, optionally wherein the transcription factor is a master regulator of polarization to an M1 macrophage, optionally wherein the transcription factor is IRF7 or a derivative thereof, or p65/RelA or a derivative thereof, optionally wherein the transcription factor is a master regulator of polarization to an M2 macrophage, optionally wherein the at least one effector molecule or each effector molecule is or comprises a cytokine, chemokine, homing molecule, growth factor, a tumor microenvironment modifier, co-optionally wherein the cytokine is selected from the group consisting of: IL1-beta, IL2, IL4, IL6, IL7, IL10, IL12, an IL12p70 fusion protein, IL15, IL17A, IL18, IL21, IL22, Type I interferons, Interferon-gamma, and TNF-alpha, optionally wherein the cytokine is a master regulator of polarization to an M1 macrophage, optionally wherein the cytokine is IFNgamma, IFNalpha, TNF alpha, GM-CSF, IL-12, IL-12p70, IL-12p40, IL-12p35, IL-6, IL-23, IL-lalpha, IL-lbeta, or a derivative thereof, optionally wherein the cytokine is a master regulator of polarization to an M2 macrophage, optionally wherein the cytokine is IL-10, IL-4, IL-13, IL-21, TGF-beta, M-CSF, or a derivative thereof, optionally wherein the chemokine is selected from the group consisting of: CCL21a, CXCL10, CXCL11, CXCL13, a CXCL10-CXCL11 fusion protein, CCL19, CXCL9, and CXCL1, optionally wherein the homing molecule is selected from the group consisting of: anti-integrin alpha4, beta7; anti-MAdCAM; CCR9; CXCR4; SDF1; MMP-2; CXCR1; CXCR7; CCR2; CCR4; and GPR15, optionally wherein the growth factor is selected from the group consisting of: FLT3L and GM-CSF, optionally wherein the co-activation molecule is selected from the group consisting of: c-Jun, 4-1BBL and CD40L, optionally wherein the tumor microenvironment modifier is selected from the group consisting of: an adenosine deaminase, a TGFbeta inhibitor, an immune checkpoint inhibitor, a VEGF inhibitor, and an HPGE2, optionally wherein each of the first effector molecule and the

second effector molecule are from separate therapeutic classes, optionally wherein each effector molecule is a human-derived effector molecule, optionally wherein the cytokine is modified to comprise a membrane tethering domain, optionally wherein the membrane tethering domain is or comprises a transmembrane-intracellular domain and/or transmembrane domain of a protein selected from: PDGFR-beta, CD8, CD28, CD3zeta-chain, CD4, 4-1BB, OX40, ICOS, CTLA-4, PD-1, LAG-3, 2B4, LNGFR, NKG2D, EpoR, TNFR2, B7-1, and BTLA, or a functional portion thereof, optionally wherein the master regulator of polarization to an M1 macrophage is IRF7 or a derivative thereof, optionally wherein the derivative of IRF7 comprises IRF7 operably linked to a degron domain, optionally wherein the degron domain is selected from: a PEST domain, HCV NS4 degron, GRR (residues 352-408 of human p105), DRR (residues 210-295 of yeast Cdc34), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B), RPB (four copies of residues 1688-1702 of yeast RPB), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein), NS2 (three copies of residues 79-93 of influenza A virus NS protein), ODC (residues 106-142 of ornithine decarboxylase), Nek2A, mouse ODC (residues 422-461), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COPT E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS (SEQ ID NO: 687) phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, a PCNA binding PIP box, and derivatives thereof, optionally wherein the degron domain is a PEST domain, optionally wherein the PEST comprises the amino acid sequence SEQ ID NO: 501 or a derivative thereof.

[0004e] In another aspect, provided herein is use of a therapeutically effective dose of the vector as described herein, the dual expression vector as described herein, the immunoresponsive cell as described herein, or the pharmaceutical composition as described herein in the manufacture of a medicament for treating cancer.

[0004f] In another aspect, provided herein is the vector as described herein, the dual expression vector as described herein, the immunoresponsive cell as described herein, or the pharmaceutical composition as described herein for use in therapy.

[0005] This disclosure provides polarization-state specific promoters which enable the controlled expression of payloads only when macrophages encounter a given polarization cue. These polarization-state specific promoters not only provide selective payload expression but can also be used to prevent macrophage polarization plasticity.

in M2 macrophages; or (iii) an engineered macrophage-specific promoter comprising at least one regulatory element, wherein the regulatory element exhibits greater activity in an M2 macrophage compared to an M1 or M0 macrophage; and (b) a heterologous payload encoding a master regulator of polarization to an M1 macrophage, wherein the regulatory element or engineered macrophage-specific promoter of (a) is operably linked to the heterologous payload and configured to induce expression of the heterologous payload. In some embodiments, the master regulator of polarization to an M1 macrophage is a cytokine. In some embodiments, the cytokine is IFN γ , IFN α , TNF α , GM-CSF, IL-12, IL-12p70, IL-12p40, IL-12p35, IL-6, IL-23, IL-1 α , IL-1 β , or a derivative thereof. In some embodiments, the master regulator of polarization to an M1 macrophage is a transcription factor selected from IRF7 or a derivative thereof, or p65/RelA or a derivative thereof. In some embodiments, the master regulator of polarization to an M1 macrophage is IRF7 or a derivative thereof. In some embodiments, the derivative of IRF7 comprises IRF7 operably linked to a degron domain. In some embodiments, the degron domain is selected from: a PEST domain, HCV NS4 degron, GRR (residues 352-408 of human p105), DRR (residues 210-295 of yeast Cdc34), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B), RPB (four copies of residues 1688-1702 of yeast RPB), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein), NS2 (three copies of residues 79-93 of influenza A virus NS protein), ODC (residues 106-142 of ornithine decarboxylase), Nek2A, mouse ODC (residues 422-461), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COP1 E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS (SEQ ID NO: 687) phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, a PCNA binding PIP box, and derivatives thereof. In some embodiments, the degron domain is a PEST domain, optionally wherein the PEST comprises the amino acid sequence SEQ ID NO: 501 or a derivative thereof. In some embodiments, the engineered macrophage specific promoter comprises a regulatory element selected from: (i) a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% sequence identity to SEQ ID NO: 420 ; and (ii) a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least

domain. In some embodiments, the degron domain is selected from: a PEST domain, HCV NS4 degron, GRR (residues 352-408 of human p105), DRR (residues 210-295 of yeast Cdc34), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B), RPB (four copies of residues 1688-1702 of yeast RPB), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein), NS2 (three copies of residues 79-93 of influenza A virus NS protein), ODC (residues 106-142 of ornithine decarboxylase), Nek2A, mouse ODC (residues 422-461), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COP1 E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS (SEQ ID NO: 687) phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, a PCNA binding PIP box, and derivatives thereof. In some embodiments, the degron domain is a PEST domain. In some embodiments, the PEST comprises the amino acid sequence SEQ ID NO: 501 or a derivative thereof.

[00514] The present disclosure provides a heterologous construct for inducing a macrophage to transition from an M1 state to an M2 state, comprising: either a regulatory element derived from a promoter of a gene that is more highly expressed in M1 macrophage compared to M2 or M0 macrophages, as provided herein, or an engineered macrophage-specific promoter as provided herein; and a heterologous payload encoding a master regulator of polarization to an M2 macrophage, wherein the regulatory element or engineered macrophage-specific promoter of (a) is operably linked to the heterologous payload and configured to induce expression of the heterologous payload. In some embodiments, the master regulator of polarization to an M2 macrophage is IL-10, IL-4, IL-13, IL-21, TGF-beta, M-CSF, or a derivative thereof. In some embodiments, the master regulator of polarization to an M2 macrophage is IL-10. In some embodiments, the M2 state is an M2c state, an M2a state, or an M2b state. In some embodiments, (a) is a regulatory element derived from a CCL19 promoter. In some embodiments, (a) comprises the nucleotide sequence of SEQ ID NO: 132.

[00515] The present disclosure provides a heterologous construct for stabilizing a macrophage in an M1 polarization state, comprising: either a regulatory element derived from a promoter of a gene that is more highly expressed in M1 macrophage compared to M2 or

[00532] FIG. 7 depicts fluorescence level of a fluorescent reporter regulated by of selected native M2 macrophage promoters. The x-axis shows the reporter expression level from cells transduced with that promoter-reporter pair normalized to cells transduced with no virus (NV). The y-axis shows the activity in M2c polarized cells divided by either M0 (circles) or M1 (triangles).

[00533] FIG. 8 is a schematic depicting engineered enhancers with length of up to 100 bp and made of arrays of transcription factor (TF) binding sites selected based on motif enrichment analysis.

[00534] FIG. 9 is a schematic of the primer binding sites for MPRA based next-generation sequencing analysis of our integrated promoter library constructs. Open arrows indicate primer binding sites used in Next generation sequencing (NGS).

[00535] FIG. 10 depicts schematically the general protocol of screening of engineered promoter sequences.

[00536] FIGs. 11A-C depicts engineered promoters identified from the promoter library that are selective for M1 vs M0 polarization state (FIG. 11A), M2c vs M0 polarization state (FIG. 11B), and M1 vs M2c polarization state (FIG. 11C).

[00537] FIGs. 12A-C depicts heat maps of engineered promoters identified from the promoter library that are selective for M1 vs M0 polarization state (FIG. 12A), M2c vs M0 polarization state (FIG. 12B), M1 vs M2c polarization state (FIG. 12C) and indicates distinct patterns of motif enrichment.

[00538] FIG. 13 (SEQ ID NO: 297-390 and 502-686) provides selected hits from the SB07479 targeted M1 library screening.

[00539] FIG. 14 depicts macrophage polarization to M1 or M2 states, and phenotype plasticity of macrophages between M1 and M2 states.

[00540] FIG. 15 depicts exemplary promoter system designs for keeping macrophages in a stable M2 state or directing M1 macrophages from an M1 phenotype to M2 phenotype.

[00541] FIG. 16 depicts exemplary promoter system designs for keeping macrophages in a stable M1 state or directing M2 macrophages from an M2 phenotype to M1 phenotype.

[00542] FIG. 17 depicts polarization state selective activity and promoter strength as analyzed by flow cytometry. Color scale indicates the promoter strength (normalized to the EFS constitutive promoter). The x-axis shows M1/M0 state selectivity, and the y-axis shows M1/ M2c state selectivity. SB07683 is a constitutive control, SB09385 includes the original native IDO1 promoter sequence (has the same IDO1 promoter sequence as SB05125), and SB09386- SB09405 include the promoter ablation variants of the IDO1 promoter (see Tables 1 and 2).

can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, [GS]₄GG [SEQ ID NO: 490]), A(EAAAK)₃A (SEQ ID NO: 491), and Whitlow linkers (*e.g.*, a “KEGS(SEQ ID NO: 688)” linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 492), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 493), and linkers described in more detail in U.S. Pat. No. 5,990,275 herein incorporated by reference). Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art.

[00658] In some embodiments, a linker is a cleavable linker, *e.g.*, comprising a protease cleavage site. In some embodiments, the cell membrane tethering domain can be connected to a protease cleavage site by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of cell membrane tethering domain or protease cleavage site.

[00659] In some embodiments, the cell-membrane tethering domain is oriented such that the effector molecule and optionally the protease cleavage site are extracellularly exposed following insertion into, or association with, the cell membrane. In some embodiments wherein the linker comprises a protease cleavage site, the effector molecule and the protease cleavage site are extracellularly exposed such that the protease cleavage site is capable of being cleaved by its respective protease and releasing (“secreting”) the effector molecule into the extracellular space.

[00660] In some embodiments a transmembrane domain comprises a B7-1 transmembrane domain. In some embodiments a B7-1 transmembrane domain comprises an amino acid sequence as set forth in SEQ ID NO: 481. In some embodiments, an effector molecule is operably linked to a transmembrane domain via a linker. In some embodiments, a linker comprises an amino acid sequence as set forth in SEQ ID NO: 478.

[00661] In some embodiments, a tethered effector molecule is a tethered cytokine. In some embodiments, a tethered cytokine is a tethered IL-10 cytokine. In some embodiments, a tethered IL-10 cytokine comprises an amino acid sequence having at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or greater sequence identity to SEQ ID NO: 467. In some embodiments, a tethered IL-10 cytokine comprises an amino acid sequence as set forth in SEQ ID NO: 467.

[00662] In some embodiments, a tethered cytokine is a tethered IFN γ cytokine. In some embodiments, a tethered IFN γ cytokine comprises an amino acid sequence having at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least

[00671] In some embodiments, each effector molecule is a human-derived effector molecule.

[00672] In some embodiments, an effector molecule is operably linked to a degron domain. The degron domain can be any amino acid sequence motif capable of directing regulated degradation, such as regulated degradation through a ubiquitin-mediated pathway. Exemplary degron domains can include, e.g., a PEST domain, HCV NS4 degron, GRR (residues 352-408 of human p105), DRR (residues 210-295 of yeast Cdc34), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B), RPB (four copies of residues 1688-1702 of yeast RPB), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein), NS2 (three copies of residues 79-93 of influenza A virus NS protein), ODC (residues 106-142 of ornithine decarboxylase), Nek2A, mouse ODC (residues 422-461), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COP1 E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS (SEQ ID NO: 687) phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, a PCNA binding PIP box, and derivatives thereof. Exemplary degron domains are described in International Patent Application Pub. No. WO2022155500A1 and WO2022/266396A1, which are hereby incorporated by reference.

[00673] In some embodiments, the degron domain is a PEST domain. PEST domains comprise sequences enriched with Pro, Glu, Ser, and Thr. Exemplary PEST domains are described in Cancer Res., 64 (2004), pp. 8821-8825; Journal of Biological Chemistry 274.43 (1999): 30874-30881; International Patent Application Pub. No. WO2022155500A1; and WO2022/266396A1, which are hereby incorporated by reference.

[00674] In some embodiments, the PEST domain comprises the amino acid sequence SEQ ID NO: 501 or a derivative thereof (e.g., a functional fragment thereof). In some embodiments, the derivative of SEQ ID NO: 501 comprises a sequence at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 501.

[00675] In some embodiments, engineered nucleic acids are configured to produce at least one reporter molecule. In some embodiments, a reporter molecule is a fluorescent protein

Embodiment 589: The heterologous construct of embodiment 587, wherein the membrane tethering domain is or comprises a transmembrane domain of B7-1 protein, or a functional portion thereof.

Embodiment 590: The heterologous construct of any one of embodiments 587-589, wherein the cytokine is IFN γ .

Embodiment 591: The heterologous construct of any one of embodiments 587-590, wherein the cytokine and the tethering domain are linked by a linker.

Embodiment 592: The heterologous construct of embodiment 524, wherein the master regulator of polarization to an M1 macrophage is IRF7 or a derivative thereof.

Embodiment 593: The heterologous construct of embodiment 592, wherein the derivative of IRF7 comprises IRF7 operably linked to a degron domain.

Embodiment 594: The heterologous construct of embodiment 593, wherein the degron domain is selected from: a PEST domain, HCV NS4 degron, GRR (residues 352-408 of human p105), DRR (residues 210-295 of yeast Cdc34), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B), RPB (four copies of residues 1688-1702 of yeast RPB), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein), NS2 (three copies of residues 79-93 of influenza A virus NS protein), ODC (residues 106-142 of ornithine decarboxylase), Nek2A, mouse ODC (residues 422-461), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COP1 E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS (SEQ ID NO: 687) phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, a PCNA binding PIP box, and derivatives thereof.

Embodiment 595: The heterologous construct of embodiment 594, wherein the degron domain is a PEST domain, optionally wherein the PEST comprises the amino acid sequence SEQ ID NO: 501 or a derivative thereof.