

CLAIMS

What is claimed is:

1. A method, the method comprising:
intratumorally injecting an mRNA nanoparticle comprising
 - (i) a first mRNA encoding a tumor-specific antigen;
 - (ii) a second mRNA encoding an immunomodulatory agent; and
 - (iii) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.
2. The method of claim 1, wherein the tumor-specific antigen comprises a viral antigen.
3. The method of any of claims 1-2, wherein the viral antigen is associated with Human papillomavirus (HPV), Kaposi's sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV), Merkel cell polyomavirus, Human cytomegalovirus (CMV), or any combination thereof.
4. The method of claim 1, wherein the tumor-specific antigen comprises a neo-epitope.
5. The method of claim 4, wherein the tumor-specific antigen comprises a patient-specific antigen.
6. The method of claim 4, wherein the tumor-specific antigen comprises a plurality of patient-specific antigens.
7. The method of claim 1, wherein the tumor-specific antigen comprises a shared tumor antigen.
8. The method of any of claims 1-7, wherein the delivery vehicle molecule comprises an amino-lipidated peptoid.
9. The method of any of claims 1-8, wherein the mRNA nanoparticle further comprises a third mRNA encoding a second immunomodulatory agent.
10. The method of any of claims 1-9, wherein the mRNA nanoparticle further comprises an immunomodulatory siRNA.
11. The method of any of claims 1-10, wherein the second mRNA encoding the immunomodulatory agent encodes: a checkpoint inhibitor, an immunosuppression antagonist, a pro-inflammatory agent, or any combination thereof.

12. The method of any of claims 1-11, wherein the second mRNA encoding the immunomodulatory agent encodes a pro-inflammatory cytokine.
13. The method of claim 12, wherein the pro-inflammatory cytokine is one of: (IL), IL-1, IL-2, IL-12, IL-17, IL-18, IFN- γ , and TNF- α .
- 5 14. The method of claim 12, wherein the pro-inflammatory cytokine is interleukin-12 (IL-12).
15. The method of any of claims 1-14, wherein the second mRNA encoding the immunomodulatory agent encodes a tumor necrosis factor superfamily member 14 (TNFSF14).
- 10 16. The method of any of claims 1-14, wherein the second mRNA encoding the immunomodulatory agent encodes anti-CTLA-4.
17. The method of any of claims 1-14, wherein the second mRNA encoding the immunomodulatory agent encodes a TGF-beta antagonist.
18. The method of any of claims 1-17, wherein the mRNA nanoparticle further includes an immunostimulator.
- 15 19. The method of any of claims 1-18, wherein the mRNA nanoparticle further includes an immunostimulator that comprises a CpG oligodeoxynucleotides.
20. The method of any of claims 1-19, further comprising repeating the intratumoral injection one or more times with a wait between injections, wherein the wait is between about 1 and about 14 days.
- 20 21. The method of any of claims 1-20, wherein the first mRNA encoding the tumor-specific antigen and the second mRNA encoding the immunomodulatory agent are on a single mRNA strand.
22. The method of any of claims 1-21, wherein the method is a method of treating a patient having Lymphoma.
- 25 23. The method of any of claims 1-22, wherein the method is a method of treating a patient having cervical cancer.
24. A method, the method comprising:

intratumorally injecting an mRNA nanoparticle into a patient in need thereof, the mRNA nanoparticle comprising

- (i) a first mRNA encoding an antigen specific to cervical cancer;
- (ii) a second mRNA encoding an immunomodulatory agent comprising: a pro-inflammatory cytokine; and
- (iii) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.

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25. An mRNA treatment nanoparticle, the nanoparticle comprising:

- (i) a first mRNA encoding a tumor-specific antigen;
- (ii) a second mRNA encoding an immunomodulatory agent; and
- (iii) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.

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26. An mRNA treatment nanoparticle, the nanoparticle comprising:

- (i) a first mRNA encoding a tumor-specific antigen;
- (ii) a second mRNA encoding an immunomodulatory agent that is at least about 80% identical to the nucleotide sequence of SEQ ID NO: 5; and
- (iii) a delivery vehicle molecule comprising an amino-lipidated peptoid delivery vehicle, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.

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27. An mRNA treatment nanoparticle, the nanoparticle comprising:

- (i) a first mRNA encoding an antigen specific to cervical cancer;
- (ii) a second mRNA encoding an immunomodulatory agent comprising: a pro-inflammatory cytokine; and
- (iii) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.

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28. The mRNA treatment nanoparticle of claims 25 or 27, wherein the delivery vehicle molecule comprises an amino-lipidated peptoid.

29. The mRNA treatment nanoparticle of claims 25-27, wherein the first mRNA encodes a patient-specific antigen.

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30. The mRNA treatment nanoparticle of claim 29, wherein the first mRNA encodes a plurality of patient-specific antigens.

31. The mRNA treatment nanoparticle of any of claims 25-26, wherein the first mRNA encodes a shared tumor antigen.
32. The mRNA treatment nanoparticle of claim 25, wherein the second mRNA encodes: a checkpoint inhibitor, an immunosuppression antagonist, a pro-inflammatory agent, or any combination thereof.
33. The mRNA treatment nanoparticle of claim 25, wherein the second mRNA encodes an anti-CTLA-4.
34. The mRNA treatment nanoparticle of any of claim 25, wherein the second mRNA encodes an anti-CTLA-4 that is at least about 80% identical to the nucleotide sequence of SEQ ID No: 1 or SEQ ID NO: 3.
35. The mRNA treatment nanoparticle of any of claims 25-27, further comprising a third mRNA that encodes a tumor necrosis factor superfamily member 14 (TNFSF14).
36. The mRNA treatment nanoparticle of any of claims 25-27, further comprising a third mRNA that encodes a TGF-beta antagonist that is at least about 80% identical to the nucleotide sequence of SEQ ID NO: 7 or SEQ ID NO: 9.
37. The mRNA treatment nanoparticle of claim 25, wherein the second mRNA encodes an interleukin-12 that is at least about 80% identical to the nucleotide sequence of SEQ ID NO: 5.
38. The mRNA treatment nanoparticle of any of claims 25-27, wherein the mRNA treatment nanoparticle further includes an immunostimulator.
39. The mRNA treatment nanoparticle of claim 38, wherein the immunostimulator comprises a CpG oligodeoxynucleotide.
40. The mRNA treatment nanoparticle of claim 25, wherein respective open reading frames (ORFs) of the tumor-specific antigen and the immunomodulatory agent are part of a single mRNA strand.
41. The mRNA treatment nanoparticle of claim 25, wherein respective open reading frames (ORFs) of the tumor-specific antigen and the immunomodulatory agent are part of different mRNA strands.
42. A method, the method comprising:

performing *in vitro* transcription of a first mRNA encoding a tumor-specific antigen from a template in a microfluidic path device;
purifying the first mRNA;
performing *in vitro* transcription of a second mRNA encoding a first
5 immunomodulatory agent in the microfluidic path device;
purifying the second mRNAs in the microfluidic path device; and
encapsulating together the first mRNA and the second mRNA with a delivery vehicle molecule in the microfluidic path device to form an mRNA treatment nanoparticle.

10 43. A method, the method comprising:
performing *in vitro* transcription of a first mRNA encoding a tumor-specific antigen from a template in a first chamber of a microfluidic path device;
purifying the first mRNA;
performing *in vitro* transcription of a second mRNA encoding a first
15 immunomodulatory agent in a second chamber of the microfluidic path device;
purifying the second mRNAs in the microfluidic path device;
performing *in vitro* transcription of a third mRNA encoding a second immunomodulatory agent in a third chamber of the microfluidic path device;
purifying the third mRNAs in the microfluidic path device; and
20 encapsulating together the first mRNA, the second mRNA and the third mRNA with a delivery vehicle molecule in the microfluidic path device to form an mRNA treatment nanoparticle.

44. The method of any of claims 42-43, wherein each of the purifying the first mRNA, the purifying the second mRNA and the purifying the third mRNA further comprises the
25 purifying in one or more reactors on the microfluidic path device.

45. The method of claim 44, wherein the purifying in the one or more reactors comprises removing double stranded mRNA using cellulose within the one or more reactors.

46. The method of claim any of claims 42-45, wherein the microfluidic path device comprises a closed-path system to automatically and continuously perform *in vitro*
30 transcription of each of the first mRNA and the second mRNA, purify the first mRNA and the second mRNA, and combine the first mRNA and the second mRNA with the delivery vehicle molecule.

47. The method of claim 46, wherein the closed-path system pneumatically controls performing *in vitro* transcription of first and second mRNA, purifying the first and second mRNA, and combining the first and second mRNA with the delivery vehicle molecule.
- 5 48. The method of claim 46, wherein the closed-path system pneumatically controls:
performing *in vitro* transcription of first mRNA and the second mRNA,
purifying the first mRNA and the second mRNA, and
combining the first mRNA and the second mRNA with the delivery vehicle
molecule, by deflecting one or more membranes within the microfluidic path
10 device.
49. The method of claim 46, wherein the closed-path system automatically and continuously:
performs *in vitro* transcription of the therapeutic mRNA,
purifies the therapeutic mRNA, and
combines the mRNA with a delivery vehicle,
15 collectively in less than about 5 days.
50. The method of any of claims 42-49, wherein the method is performed at a site of care.
51. The method of any of claims 42-50, wherein combining the mRNA with the delivery vehicle further comprises dialyzing the mRNA treatment nanoparticle in the microfluidic path device.
- 20 52. The method of any of claims 42-50, further comprising concentrating the mRNA treatment nanoparticle on the microfluidic path device.
53. The method of any of claims 42-52, wherein the delivery vehicle molecule comprises an amphipathic molecule.
54. The method of claim 53, wherein the amphipathic molecule comprises an amino-
25 lipidated peptoid.
55. The method of any of claims 42-54, wherein the first mRNA encoding the tumor-specific antigen comprises an mRNA encoding a patient-specific antigen.
56. The method of claim 55, wherein the first mRNA encodes a plurality of patient-specific antigens.

57. The method of any of claims 42-56, wherein the second mRNA encoding the immunomodulatory agent encodes: a checkpoint inhibitor, an immunosuppression antagonist, a pro-inflammatory agent, or any combination thereof.
58. The method of any of claims 42-57, wherein the second mRNA encoding an immunomodulatory agent encodes anti-CTLA-4.
59. The method of any of claims 42-57, wherein the second mRNA encoding an immunomodulatory agent encodes a TGF-beta antagonist.
60. The method of any of claims 42-57, wherein the second mRNA encoding an immunomodulatory agent encodes a single chain interleukin-12 (IL-12).
61. The method of any of claims 42-57, wherein combining the first mRNA and the second mRNA comprises adding an immunostimulator in the microfluidic path device.
62. The method of claim 61, wherein the immunostimulator comprises a CpG oligodeoxynucleotides.
63. A microfluidic path device comprising:
- an elastic layer sandwiched between a first surface and a second surface;
 - a template *in vitro transcription* (IVT) chamber formed between the first surface and the second surface, wherein a portion of the elastic layer divides the template IVT chamber into a fluid-contacting side in the second surface and a pressure-receiving side in the first surface, wherein the fluid-contacting side of the template IVT chamber is in fluid communication with a source of an mRNA template encoding a tumor-specific antigen;
 - a first reactor in fluid communication with the fluid-contacting side of the template IVT chamber, the first reactor including a double-stranded mRNA binding material within the first reactor to remove double stranded mRNA;
 - a mixing assembly in fluid communication with the first reactor, with a source of a first immunomodulator mRNA, and with a delivery vehicle port to couple to a source of a delivery vehicle molecule to encapsulate together the first mRNA and the second mRNA with the delivery vehicle molecule to form an mRNA treatment nanoparticle; and
 - a plurality of pressure channels each extending from a one or more pressure ports, through the first surface and the elastic layer, into the second surface and back through the elastic layer into the first surface, wherein each pressure channel of

the plurality of pressure channels fluidly connects with the pressure-receiving side of the template IVT chamber, further wherein the volume of the fluid-contacting side of the template IVT chamber may be adjusted by applying pressure from the one or more of the pressure ports to drive fluid through the microfluidic path device to form the mRNA treatment nanoparticle.

64. A microfluidic path device comprising:

- an elastic layer sandwiched between a first surface and a second surface;
- a template in vitro transcription (IVT) chamber formed between the first surface and the second surface, wherein a portion of the elastic layer divides the template IVT chamber into a fluid-contacting side in the second surface and a pressure-receiving side in the first surface, wherein the fluid-contacting side of the template IVT chamber is in fluid communication with a source of a first mRNA template encoding a tumor-specific antigen;
- a first reactor in fluid communication with the fluid-contacting side of the template IVT chamber, the first reactor including a double-stranded mRNA binding material within the first reactor to remove double stranded mRNA;
- a first immunomodulator IVT chamber formed between the first surface and the second surface, wherein a portion of the elastic layer divides the first immunomodulator IVT chamber into a fluid-contacting side in the second surface and a pressure-receiving side in the first surface, wherein the fluid-contacting side of the first immunomodulator IVT chamber is in fluid communication with a source of a first immunomodulator template encoding a first immunomodulator;
- a second reactor in fluid communication with the fluid-contacting side of the first immunomodulator IVT chamber, the second reactor including a double-stranded mRNA binding material within the first reactor to remove double stranded mRNA;
- a first mixing chamber in fluid communication with the first reactor and the second reactor;
- a second mixing chamber in fluid communication with an output of the first mixing chamber and a delivery vehicle port to couple to a source of a delivery vehicle molecule to encapsulate together the first mRNA and the second mRNA with the delivery vehicle molecule to form an mRNA treatment nanoparticle; and
- a plurality of pressure channels each extending from a one or more pressure ports, through the first surface and the elastic layer, into the second surface and back

through the elastic layer into the first surface, wherein each pressure channel of the plurality of pressure channels fluidly connects with the pressure-receiving side of the template IVT chamber and the first immunomodulator IVT chamber, further wherein the volumes of the fluid-contacting side of the template IVT chamber and the fluid-contacting side of the first immunomodulator IVT chamber may be adjusted by applying pressure from the one or more of the pressure ports.

65. A method, the method comprising:

injecting an mRNA nanoparticle comprising

- (iv) a first mRNA encoding a tumor-specific antigen;
- (v) a second mRNA encoding an immunomodulatory agent; and
- (vi) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.

66. A method, the method comprising:

injecting an mRNA nanoparticle into a patient in need thereof, the mRNA nanoparticle comprising

- (iv) a first mRNA encoding an antigen specific to cervical cancer;
- (v) a second mRNA encoding an immunomodulatory agent comprising: a pro-inflammatory cytokine; and
- (vi) a delivery vehicle molecule, wherein the first mRNA and the second mRNA are encapsulated together by the delivery vehicle molecule.