

We claim:

1. A method of generating antigen-binding proteins against a foreign antigen of interest, comprising:

(a) making a genetically modified non-human animal that is a mouse or a rat with reduced tolerance of a foreign antigen of interest, comprising:

(i) introducing into a mouse or rat one-cell stage embryo or a mouse or rat embryonic stem (ES) cell:

(I) a Cas9 protein or a nucleic acid encoding the Cas9 protein;

(II) a first guide RNA or a DNA encoding the first guide RNA, wherein the first guide RNA hybridizes to a first guide RNA recognition sequence within a target genomic locus, wherein the target genomic locus comprises all or part of a gene encoding a self-antigen homologous to, orthologous to, or sharing an epitope of interest with the foreign antigen of interest; and

(III) a second guide RNA or a DNA encoding the second guide RNA, wherein the second guide RNA hybridizes to a second guide RNA recognition sequence within the target genomic locus;

(ii) identifying a genetically modified mouse or rat one-cell stage embryo or a genetically modified mouse or rat ES cell with a biallelic modification at the target genomic locus, wherein the biallelic modification comprises a biallelic deletion of all or part of the gene encoding the self-antigen in a pair of corresponding first and second chromosomes, and wherein expression of the self-antigen is eliminated; and

(iii) producing a genetically modified F0 generation mouse or rat from the genetically modified mouse or rat one-cell stage embryo or the genetically modified mouse or rat ES cell, wherein the target genomic locus is modified in the pair of corresponding first and second chromosomes in the genetically modified F0 generation mouse or rat such that expression of the self-antigen is eliminated;

(b) immunizing the genetically modified F0 generation mouse or rat produced in step (a) with the foreign antigen of interest; and

(c) maintaining the genetically modified F0 generation mouse or rat under conditions to initiate an immune response to the foreign antigen of interest, wherein the genetically modified F0 generation mouse or rat produces antigen-binding proteins against the foreign antigen of interest.

2. The method of claim 1, wherein the Cas9 protein has double-strand-break-inducing activity, and the Cas9 protein and the guide RNAs create paired double-strand breaks at different sites within the target genomic locus.

3. The method of claim 1 or 2, wherein:

(I) the cell or embryo in step (a)(i) is the mouse or rat one-cell stage embryo, and the producing the genetically modified F0 generation mouse or rat in step (a)(iii) comprises implanting the genetically modified mouse or rat one-cell stage embryo into a mouse or rat surrogate mother to produce the genetically modified F0 generation mouse or rat in which the target genomic locus is modified in the pair of corresponding first and second chromosomes such that expression of the self-antigen is eliminated, or

(II) the cell or embryo in step (a)(i) is the mouse or rat ES cell, wherein the producing the genetically modified F0 generation mouse or rat in step (a)(iii) comprises introducing the genetically modified mouse or rat ES cell into a mouse or rat host embryo, and gestating the mouse or rat host embryo into a mouse or rat surrogate mother to produce the genetically modified F0 generation mouse or rat in which the target genomic locus is modified in the pair of corresponding first and second chromosomes such that expression of the self-antigen is eliminated.

4. The method of any one of claims 1-3, wherein the cell or embryo in step (a)(i) is the mouse or rat ES cell.

5. The method of any one of claims 1-4, further comprising:

(d) making a hybridoma from B cells isolated from the immunized, genetically modified F0 generation mouse or rat.

6. The method of any one of claims 1-5, further comprising:

(d) obtaining from the immunized, genetically modified F0 generation mouse or rat a first nucleic acid sequence encoding an immunoglobulin heavy chain variable domain of one of the antigen-binding proteins against the foreign antigen of interest and/or a second nucleic acid sequence encoding an immunoglobulin light chain variable domain of one of the antigen-binding proteins against the foreign antigen of interest.

7. The method of claim 6, wherein the first nucleic acid sequence and/or the second nucleic acid sequence are obtained from a lymphocyte of the genetically modified F0 generation mouse or rat or from a hybridoma produced from the lymphocyte, or

wherein the genetically modified F0 generation mouse or rat comprises a humanized immunoglobulin locus, and wherein the first nucleic acid sequence encodes a human immunoglobulin heavy chain variable domain, and the second nucleic acid sequence encodes a human immunoglobulin light chain variable domain.

8. The method of any one of claims 1-7, wherein:

(I) the antigen-binding proteins produced by the genetically modified F0 generation mouse or rat against the foreign antigen of interest have a higher titer than antigen-binding proteins produced by a control mouse or rat that is wild type at the target genomic locus following immunization of the control mouse or rat with the foreign antigen of interest; and/or

(II) a more diverse repertoire of antigen-binding proteins against the foreign antigen of interest is produced by the genetically modified F0 generation mouse or rat following immunization of the genetically modified F0 generation mouse or rat with the foreign antigen of interest compared with antigen-binding proteins produced by a control mouse or rat that is wild type at the target genomic locus following immunization of the control mouse or rat with the foreign antigen of interest; and/or

(III) the antigen-binding proteins produced by the genetically modified F0 generation mouse or rat against the foreign antigen of interest use a greater diversity of heavy chain V gene segments and/or light chain V gene segments compared with antigen-binding

proteins produced by a control mouse or rat that is wild type at the target genomic locus following immunization of the control mouse or rat with the foreign antigen of interest; and/or

(IV) some of the antigen-binding proteins produced by the genetically modified F0 generation mouse or rat against the foreign antigen of interest cross-react with the self-antigen.

9. The method of any one of claims 1-8, wherein the first guide RNA recognition sequence is 5' of the second guide RNA recognition sequence in the target genomic locus, and

wherein step (a)(ii) comprises performing a retention assay to determine the copy number is two for a region 5' and within 1 kb of the first guide RNA recognition sequence and/or for a region 3' and within 1 kb of the second guide RNA recognition sequence.

10. The method of any one of claims 1-9, wherein the foreign antigen of interest comprises all of or part of a human protein.

11. The method of any one of claims 1-10, wherein the target genomic locus is modified to comprise a replacement of one or more nucleotides.

12. The method of any one of claims 1-11, wherein the biallelic deletion is a precise deletion without random insertions and deletions (indels), or wherein the biallelic deletion is between 0.1 kb to 200 kb.

13. The method of any one of claims 1-12, wherein:

(I) the first guide RNA recognition sequence comprises the start codon for the gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the start codon, and the second guide RNA recognition sequence comprises the stop codon for the gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the stop codon; or

(II) the first and second guide RNA recognition sequences are different, and each of the first and second guide RNA recognition sequences comprises the start codon for the gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the start codon, and wherein the modification comprises a biallelic disruption of the start codon of the gene encoding the self-antigen.

14. The method of any one of claims 1-13, wherein the introducing step (a)(i) further comprises introducing into the mouse or rat ES cell or the mouse or rat one-cell stage embryo:

(IV) a third guide RNA or a DNA encoding the third guide RNA, wherein the third guide RNA hybridizes to a third guide RNA recognition sequence within the target genomic locus; and

(V) a fourth guide RNA or a DNA encoding the fourth guide RNA, wherein the fourth guide RNA hybridizes to a fourth guide RNA recognition sequence within the target genomic locus.

15. The method of any one of claims 1-14, wherein:

(I) step (a)(i) comprises introducing a DNA encoding the Cas9 protein, the DNA encoding the first guide RNA, and the DNA encoding the second guide RNA, or

(II) step (a)(i) comprises introducing an RNA encoding the Cas9 protein, the first guide RNA, and the second guide RNA.

16. The method of any one of claims 1-15, wherein an exogenous repair template is not introduced in step (a)(i).

17. The method of any one of claims 1-15, wherein the introducing step (a)(i) further comprises introducing into the mouse or rat ES cell or the mouse or rat one-cell stage embryo:

(IV) an exogenous repair template comprising a 5' homology arm that hybridizes to a 5' target sequence at the target genomic locus and a 3' homology arm that

hybridizes to a 3' target sequence at the target genomic locus, provided that if the cell or embryo in step (a)(i) is the mouse or rat one-cell stage embryo, the exogenous repair template is no more than 5 kb in length.

18. The method of claim 17, wherein the deleted nucleic acid sequence consists of the nucleic acid sequence between the 5' and 3' target sequences.

19. The method of claim 17 or 18, wherein the exogenous repair template further comprises a nucleic acid insert flanked by the 5' homology arm and the 3' homology arm, and

wherein the nucleic acid insert is homologous or orthologous to the target genomic locus, and the nucleic acid insert replaces the deleted nucleic acid sequence.

20. The method of any one of claims 17-19, wherein the exogenous repair template is between 50 nucleotides to 1 kb in length, is between 80 nucleotides to 200 nucleotides in length, or is a single-stranded oligodeoxynucleotide.

21. The method of any one of claims 17-19, wherein the cell or embryo in step (a)(i) is the mouse or rat ES cell, and wherein:

(a) the exogenous repair template is a large targeting vector (LTVEC) that is at least 10 kb in length; or

(b) the exogenous repair template is an LTVEC, wherein the sum total of the 5' and 3' homology arms of the LTVEC is at least 10 kb in length.

22. The method of any one of claims 1-21, wherein the mouse or rat comprises a humanized immunoglobulin locus.

23. The method of any one of claims 1-22, wherein the non-human animal is the mouse, and wherein the mouse strain comprises a BALB/c strain.

24. The method of claim 23, wherein the mouse strain comprises a mix of BALB/c, C57BL/6, and 129 strains.

25. The method of claim 24, wherein the mouse strain is 50% BALB/c, 25% C57BL/6, and 25% 129.

26. The method of any one of claims 23-25, wherein the MHC haplotype of the mouse is MHC^{b/d}.

27. The method of any one of claims 23-26, wherein the mouse comprises in its germline human unrearranged variable region gene segments inserted at an endogenous mouse immunoglobulin locus, wherein the human unrearranged variable region gene segments are heavy chain gene segments, and the mouse immunoglobulin locus is a heavy chain locus, and/or wherein the human unrearranged variable region gene segments are kappa or lambda light chain segments, and the mouse immunoglobulin locus is a light chain locus.

28. The method of claim 27, wherein the mouse comprises in its germline human unrearranged variable region gene segments operably linked to a mouse constant region gene, wherein the mouse lacks a human constant region gene, and wherein the mouse constant region gene is at an endogenous mouse immunoglobulin locus.

29. The method of claim 27 or 28, wherein the mouse comprises:

(I) a hybrid heavy chain locus comprising an insertion of human immunoglobulin heavy chain V, D, and J gene segments, wherein the human heavy chain immunoglobulin V, D, and J gene segments are operably linked to a mouse immunoglobulin heavy chain gene, wherein the mouse immunoglobulin heavy chain gene is at an endogenous mouse immunoglobulin locus; and

(II) a hybrid light chain locus comprising an insertion of human immunoglobulin light chain V and J gene segments, wherein the human V and J gene segments are operably linked to a mouse immunoglobulin light chain constant region gene sequence;

wherein (I) rearranges to form a hybrid heavy chain sequence comprising a human variable region operably linked to a mouse constant region, and (II) rearranges to form a hybrid light chain sequence comprising a human variable region operably linked to a mouse constant region, and wherein the mouse is incapable of forming an antibody that comprises a human variable region and a human constant region.

30. The method of any one of claims 23-28, wherein the mouse comprises in its germline a humanized immunoglobulin light chain variable locus comprising no more than one or no more than two rearranged human light chain V/J sequences operably linked to a mouse light chain constant region, and wherein the mouse further comprises a humanized immunoglobulin heavy chain variable locus comprising at least one unrearranged human V, at least one unrearranged human D, and at least one unrearranged human J segment operably linked to a mouse heavy chain constant region gene.

31. The method of claim 30, wherein the mouse comprises a humanized heavy chain immunoglobulin variable locus and a humanized light chain immunoglobulin variable locus, wherein the mouse expresses a single light chain.

32. The method of claim 30, wherein the mouse comprises:

(a) a single rearranged human immunoglobulin light chain variable region (V_L/J_L) that encodes a human V_L domain of an immunoglobulin light chain, wherein the single rearranged human V_L/J_L region is selected from a human $V\kappa 1-39/J\kappa 5$ gene segment or a human $V\kappa 3-20/J\kappa 1$ gene segment; and

(b) a replacement of endogenous heavy chain variable (V_H) gene segments with one or more human V_H gene segments, wherein the human V_H gene segments are operably linked to an endogenous heavy chain constant (C_H) region gene, and the human V_H gene segments are capable of rearranging and forming a human/mouse chimeric heavy chain gene.

33. The method of claim 30, wherein the mouse expresses a population of antibodies, and the mouse's germline includes only a single immunoglobulin kappa light chain variable region gene that is a rearranged human germline kappa light chain variable region gene, wherein the mouse is either heterozygous for the single immunoglobulin kappa light chain variable region gene in that it contains only one copy, or is homozygous for the single immunoglobulin kappa light chain variable region gene in that it contains two copies, the mouse being characterized by active affinity maturation so that:

(a) each immunoglobulin kappa light chain of the population comprises a light chain variable domain that is encoded by the rearranged human germline kappa light chain variable region gene, or by a somatically mutated variant thereof;

(b) the population includes antibodies comprising the immunoglobulin kappa light chains whose light chain variable domain is encoded by the rearranged human germline kappa light chain variable region gene and antibodies comprising the immunoglobulin kappa light chains whose light chain variable domain is encoded by the somatically mutated variants thereof; and

(c) the mouse generates a diverse collection of somatically mutated high affinity heavy chains that successfully pair with the immunoglobulin kappa light chains to form the antibodies of the population.

34. The method of claim 30, wherein the mouse is heterozygous or homozygous in its germline for:

(a) an insertion at an endogenous mouse κ immunoglobulin light chain variable region locus of a rearranged $V\kappa/J\kappa$ sequence comprising:

(i) a single human germline $V\kappa$ sequence, which single human germline $V\kappa$ sequence is present in SEQ ID NO: 148 or SEQ ID NO: 149; and

(ii) a single human germline $J\kappa$ sequence, wherein the rearranged $V\kappa/J\kappa$ sequence is operably linked to the endogenous mouse κ constant region; and

(b) an insertion at an endogenous mouse immunoglobulin heavy chain variable region locus of a plurality of human immunoglobulin heavy chain variable region gene

segments, wherein the human immunoglobulin heavy chain variable region gene segments are operably linked to an endogenous mouse immunoglobulin heavy chain constant region, and the human immunoglobulin heavy chain variable region gene segments are capable of rearranging and forming a rearranged human/mouse chimeric immunoglobulin heavy chain gene.

35. The method of claim 27-34, wherein the mouse comprises a modification of an immunoglobulin heavy chain locus, wherein the modification reduces or eliminates endogenous ADAM6 function,

wherein the mouse comprises an ectopic nucleic acid sequence encoding a mouse ADAM6 protein, an ortholog thereof, a homolog thereof, or a fragment thereof, wherein the ADAM6 protein, ortholog thereof, homolog thereof, or fragment thereof is functional in a male mouse, and

wherein the ectopic nucleic acid sequence encoding the mouse ADAM6 protein, ortholog thereof, homolog thereof, or fragment thereof is present at the human heavy chain variable region locus.

36. The method of any one of claims 1-35, wherein the non-human animal is the mouse, wherein the mouse is at least partially derived from a BALB/c strain and comprises a humanized immunoglobulin locus,

wherein the foreign antigen of interest is all or part of a human protein that is orthologous to the self-antigen, and wherein:

(I) the first guide RNA recognition sequence comprises the start codon for the gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the start codon and the second guide RNA recognition sequence comprises the stop codon for the gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the stop codon, whereby expression of the self-antigen is eliminated; or

(II) the first and second guide RNA recognition sequences are different, and each of the first and second guide RNA recognition sequences comprise the start codon for the

gene encoding the self-antigen or is within 10, 20, 30, 40, 50, 100, 200, 300, 400, 500, or 1,000 nucleotides of the start codon, and the modification comprises biallelic disruption of the start codon for the gene encoding the self-antigen, whereby expression of the self-antigen is eliminated.

37. The method of claim 36, wherein the mouse comprises:

(a) an ectopic nucleic acid sequence encoding a mouse ADAM6 protein, an ortholog thereof, a homolog thereof, or a fragment thereof, wherein the ADAM6 protein, ortholog thereof, homolog thereof, or fragment thereof is functional in a male mouse;

(b) a hybrid heavy chain locus comprising an insertion of human immunoglobulin heavy chain V, D, and J gene segments, wherein the human heavy chain immunoglobulin V, D, and J gene segments are operably linked to a mouse immunoglobulin heavy chain gene, wherein the mouse immunoglobulin heavy chain gene is at an endogenous mouse immunoglobulin locus; and

(c) a hybrid light chain locus comprising an insertion of human immunoglobulin light chain V and J gene segments, wherein the human V and J gene segments are operably linked to a mouse immunoglobulin light chain constant region gene sequence;

wherein (b) rearranges to form a hybrid heavy chain sequence comprising a human variable region operably linked to a mouse constant region, and (c) rearranges to form a hybrid light chain sequence comprising a human variable region operably linked to a mouse constant region, and wherein the mouse is incapable of forming an antibody that comprises a human variable region and a human constant region.

38. The method of claim 36, wherein the mouse is heterozygous or homozygous in its germline for:

(a) an ectopic nucleic acid sequence encoding a mouse ADAM6 protein, an ortholog thereof, a homolog thereof, or a fragment thereof, wherein the ADAM6 protein, ortholog thereof, homolog thereof, or fragment thereof is functional in a male mouse;

(b) an insertion at an endogenous mouse κ immunoglobulin light chain variable region locus of a rearranged V κ /J κ sequence comprising:

(i) a single human germline V κ sequence, which single human germline V κ sequence is present in SEQ ID NO: 148 or SEQ ID NO: 149; and

(ii) a single human germline J κ sequence, wherein the rearranged V κ /J κ sequence is operably linked to the endogenous mouse κ constant region; and

(c) an insertion at an endogenous mouse immunoglobulin heavy chain variable region locus of a plurality of human immunoglobulin heavy chain variable region gene segments, wherein the human immunoglobulin heavy chain variable region gene segments are operably linked to an endogenous mouse immunoglobulin heavy chain constant region, and the human immunoglobulin heavy chain variable region gene segments are capable of rearranging and forming a rearranged human/mouse chimeric immunoglobulin heavy chain gene.

39. The method of any one of claims 1-38, wherein the mouse or rat ES cell is a hybrid cell or the mouse or rat one-cell stage embryo is a hybrid one-cell stage embryo, and wherein the method further comprises:

(a') comparing the sequence of the pair of corresponding first and second chromosomes within the target genomic locus, and selecting a target region within the target genomic locus prior to the introducing step (a) based on the target region having a higher percentage of sequence identity between the pair of corresponding first and second chromosomes relative to all or part of the remainder of the target genomic locus, wherein the target region comprises:

the first guide RNA recognition sequence and at least 10 bp, 20 bp, 30 bp, 40 bp, 50 bp, 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, 2 kb, 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, or 10 kb of flanking sequence on the 5' side, the 3' side, or each side of the first guide RNA recognition sequence, and/or

the second guide RNA recognition sequence and at least 10 bp, 20 bp, 30 bp, 40 bp, 50 bp, 100 bp, 200 bp, 300 bp, 400 bp, 500 bp, 600 bp, 700 bp, 800 bp, 900 bp, 1 kb, 2

kb, 3 kb, 4 kb, 5 kb, 6 kb, 7 kb, 8 kb, 9 kb, or 10 kb of flanking sequence on the 5' side, the 3' side, or each side of the second guide RNA recognition sequence.

40. The method of claim 39, wherein the target region has a higher percentage of sequence identity between the pair of corresponding first and second relative to the remainder of the target genomic locus, or

wherein the target region has at least 99.9% sequence identity between the pair of corresponding first and second chromosomes, and the remainder of the target genomic locus has no more than 99.8% sequence identity between the pair of corresponding first and second chromosomes.

41. A method of making a genetically modified non-human animal that is a mouse or rat with reduced tolerance of a foreign antigen of interest, comprising:

(a) introducing into a mouse or rat one-cell stage embryo or a mouse or rat embryonic stem (ES) cell:

(i) a Cas9 protein or a nucleic acid encoding the Cas9 protein;

(ii) a first guide RNA or a DNA encoding the first guide RNA,

wherein the first guide RNA hybridizes to a first guide RNA recognition sequence within a target genomic locus, wherein the target genomic locus comprises all or part of a gene encoding a self-antigen homologous to, orthologous to, or sharing an epitope of interest with the foreign antigen of interest; and

(iii) a second guide RNA or a DNA encoding the second guide RNA, wherein the second guide RNA hybridizes to a second guide RNA recognition sequence within the target genomic locus;

(b) identifying a genetically modified mouse or rat one-cell stage embryo or a genetically modified mouse or rat ES cell with a biallelic modification at the target genomic locus, wherein the biallelic modification comprises a biallelic deletion of all or part of the gene encoding the self-antigen in a pair of corresponding first and second chromosomes, and wherein expression of the self-antigen is eliminated; and

(c) producing a genetically modified F0 generation mouse or rat from the genetically modified mouse or rat one-cell stage embryo or the genetically modified mouse or rat ES cell, wherein the target genomic locus is modified in the pair of corresponding first and second chromosomes in the genetically modified F0 generation mouse or rat such that expression of the self-antigen is eliminated.