

CLAIMS

1. An *in vitro* method for culturing regulatory T-cells (Tregs), the method comprising:
 - (a) contacting a population of Tregs with an mTOR inhibitor prior to activation;
 - (b) activating the Tregs within the population; and
 - (c) culturing the population of Tregs from step (b) in the presence of an mTOR inhibitor.
2. The method of claim 1, further comprising a step of isolating a population of Tregs prior to step (a), particularly a population of CD4+CD25+ Tregs.
3. The method of claim 1 or 2, wherein the mTOR inhibitor used in step (a) and/or step (c) is rapamycin or a rapalog, and/or wherein the mTOR inhibitor used in step (a) is the same as the mTOR inhibitor used in step (c).
4. The method of any preceding claim, wherein:
 - (i) the method does not include a step of removing the mTOR inhibitor used in step (a) prior to step (b) or step (c);
 - (ii) the method does not include a step of washing the Tregs prior to step (b) or step (c); and/or
 - (iii) the mTOR inhibitor used in step (a) is present during step (b) and step (c) and particularly additional mTOR inhibitor is not added for the purpose of step (c).
5. The method of any preceding claim, wherein:
 - (i) the initial concentration of the mTOR inhibitor used in step (a) and/or step (c) is from about 30 nM to about 500 nM;
 - (ii) the Tregs are contacted with the mTOR inhibitor in step (a) for at least about 15 minutes, for example for about 30 minutes to about 90 minutes prior to step (b); and/or
 - (iii) the Tregs are cultured in the presence of the mTOR inhibitor in step (c) for at least about 6 hours, for example from about 12 hours to about 72 hours, for example from about 12 hours to about 60 hours, for example from about 36 hours to about 60 hours, for example about 48 hours.

6. The method of any preceding claim, wherein the Tregs are activated by contacting them with a TCR/CD3 activator and/or a TCR co-stimulator activator, optionally wherein the TCR/CD3 activator is an anti-CD3 antibody or CD3-binding fragment thereof and/or wherein the TCR co-stimulator activator is an anti-CD28 antibody or CD28-binding fragment thereof.
7. The method of any preceding claim, wherein step (c) occurs concurrently with or immediately after step (b).
8. The method of any preceding claim, further comprising a step (d) of reducing the concentration of the mTOR inhibitor in contact with the population of Tregs or removing the mTOR inhibitor from contact with the population of Tregs; and a step (e) of further culturing or expanding the population of Tregs in the presence of the reduced concentration of the mTOR inhibitor, or in the absence of any mTOR inhibitor; wherein steps (d) and (e) are carried out after step (c).
9. The method of claim 8, wherein:
 - (i) the concentration of mTOR inhibitor is reduced to a concentration equal to or less than about 25 nM;
 - (ii) wherein the concentration of the mTOR inhibitor is reduced or the mTOR inhibitor is removed at least about 6 hours, for example from about 12 hours to about 72 hours, for example from about 12 hours to about 60 hours, for example from about 36 hours to about 60 hours after the start of step (c); and/or
 - (iii) the population of Tregs is cultured or expanded for at least about 6 days, for example from about 8 days to about 36 days, for example from about 10 days to about 14 days after the concentration of the mTOR inhibitor is reduced or the mTOR inhibitor is removed.
10. The method of any preceding claim, further comprising a step of introducing a heterologous nucleic acid into the Tregs, for example by transducing with a viral vector comprising the heterologous nucleic acid, optionally wherein the heterologous nucleic acid encodes a chimeric antigen receptor (CAR) and/or a FOXP3 polypeptide and/or a safety switch and/or a polypeptide that increases persistence of the cells.

11. The method of any preceding claim, further comprising:

(i) the steps of (d) reducing the concentration of the mTOR inhibitor in contact with the population of Tregs, (e) introducing a heterologous nucleic acid into the Tregs, and (f) further culturing or expanding the population of Tregs in the presence of the reduced concentration of an mTOR inhibitor, wherein steps (d), (e) and (f) are carried out after step (c), step (e) is carried out before step (f), and step (e) is carried out before, at the same time or after step (d); or

(ii) the steps of (d) removing the mTOR inhibitor from contact with the population of Tregs, (e) introducing a heterologous nucleic acid into the Tregs, and (f) further culturing or expanding the population of Tregs in the absence of an mTOR inhibitor, wherein steps (d), (e) and (f) are carried out after step (c), step (e) is carried out before step (f), and step (e) is carried out after step (d).

12. The method of claim 11(i), wherein the concentration of the mTOR inhibitor is reduced to a concentration equal to or less than about 25 nM simultaneously with introducing the heterologous nucleic acid into the Tregs within the population.

13. The method of any of claims 10 to 12, wherein:

(i) the heterologous nucleic acid is introduced into the Tregs at least about 6 hours, for example from about 12 hours to about 72 hours, for example from about 12 hours to about 60 hours, for example from about 36 hours to about 60 hours after the start of step (b) and/or the start of step (c); and/or

(ii) wherein the population of Tregs are cultured or expanded for at least about 6 days, for example from about 8 days to about 36 days, for example from about 10 days to about 14 days after the heterologous nucleic acid is introduced into the Tregs.

14. The method of any preceding claim, wherein the population of Tregs are harvested and optionally cryopreserved from about 8 days to about 36 days after the start of step (b), for example from about 8 days to about 22 days after the start of step (b).

15. A product obtained by and/or obtainable by the method of any of claims 1 to 14.