Supplementary Table 1. Comparison of ONCR vectors

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Virus Name** | **Transgene in UL3 – UL4 Locus** | **miR-T in 3’ UTR ICP4 Locus** | **miR-T in 3’ UTR ICP27 Locus** | **miR-T in 3’ UTR ICP34.5 Locus** | **miR-T in 3’ UTR UL8 Locus** | **US12** | **UL37** |
| ONCR-125 | None | miR-124-3p | None | None | None | First 6 aa deleted | None |
| ONCR-159 | None | miR-124-3p, miR-1-3p, miR-143-3p | miR-128-3p, miR-219a-5p, miR-122-5p | miR-219a-5p,miR-204-5p, miR-128-3p | miR-137-3p, miR-217-5p, miR-126-3p | Null mutant | Mutant |
| ONCR-177 | ipilimumab, FLT3L (EDC), CCL4, IL-12, anti-PD-1 nanobody | miR-124-3p, miR-1-3p, miR-143-3p | miR-128-3p, miR-219a-5p, miR-122-5p | miR-219a-5p,miR-204-5p, miR-128-3p | miR-137-3p, miR-217-5p, miR-126-3p | Null mutant | Mutant |
| mONCR-171 | Anti-mCTLA-4 antibody, mFLT3L (EDC), mCCL4, mIL-12, anti-PD-1 nanobody | miR-124-3p, miR-1-3p, miR-143-3p | miR-128-3p, miR-219a-5p, miR-122-5p | miR-219a-5p,miR-204-5p, miR-128-3p | miR-137-3p, miR-217-5p, miR-126-3p | Null mutant | Mutant |
| 3’ UTR, three prime untranslated region; aa, amino acids; miR, microRNA; miR-T, miR target elements.  |

**Supplementary Table 2.** ONCR-177 oncolytic activity

|  |  |  |  |
| --- | --- | --- | --- |
| **Cell Line** | **Indication** | **ONCR-159 IC50 MOI +/- SD** | **ONCR-177 IC50 MOI +/- SD** |
| A375 | Melanoma | 0.30 +/- 0.02 | 1.00 +/- 0.06 |
| SK-MEL-28 | Melanoma | 0.12 +/- 0.03 | 0.20 +/- 0.02 |
| SW-837 | CRC | 0.01 +/- 0.01 | 0.10 +/- 0.01 |
| COLO 205 | CRC | 0.05 +/- 0.02 | 0.08 +/- 0.01 |
| FaDu | SCCHN | 0.10 +/- 0.03 | 0.06 +/- 0.02 |
| SCC25 | SCCHN | 0.17 +/- 0.03 | 0.15 +/- 0.02 |
| All cells were plated at 10,000 cells per well and were infected with mock or serially diluted viral suspension starting from MOI 30. Seventy-two hours post-infection, cell viability was determined by addition of equal media volume of CellTiter-Glo® reagent as a readout for total ATP consumption and cell viability. IC50 was calculated with GraphPad PRISM and are a mean of 4 replicates. ATP, adenosine triphosphate; CRC, colorectal carcinoma; IC50, half maximal inhibitory concentration; MOI, multiplicity of infection; SCCHN, squamous cell carcinoma of the head and neck. |

Supplementary Table 3. Summary of mONCR-171 efficacyin mouse syngeneic tumor models

| **Model/****Study** | **Treatment** | **Dose (PFU)** | **Right (Injected) Tumor**  | **Left (Contralateral) Tumor** |
| --- | --- | --- | --- | --- |
| **% RR (PR;CR)** | **% TGI** | ***P*** | **% RR (PR;CR)** | **% TGI** | ***P***  |
| A20/dose escalation | PBS | --- | 10 (0;1) | --- | --- | 10 (0;1) | --- | --- |
| mONCR-171 | 3x103 | 0 (0;0) | 27 | 0.01 | 0 (0;0) | 20 | 0.02 |
| 3x104 | 40 (2;2) | 55 | <0.0001 | 40 (2;2) | 31 | 0.04 |
| 3x105 | 70 (6;1) | 81 | <0.0001 | 20 (2;0) | 57 | ≤0.01 |
| 3x106 | 90 (8;1) | 93 | <0.0001 | 70 (6;1) | 85 | ≤0.0001 |
| 3x107 | 100 (10;0) | 89 | <0.0001 | 40 (1;3) | 86 | ≤0.0001 |
| MC38/dose escalation | PBS | --- | 0 (0;0) | --- | -- | 0 (0;0) | --- | --- |
| mONCR-171 | 3x104 | 0 (0;0) | 29 | 0.32 | 0 (0;0) | 34 | 0.55 |
| 3x105 | 10 (0;1) | 50 | 0.0001 | 0 (0;0) | 25 | 0.06 |
| 3x106 | 30 (0;3) | 80 | <0.0001 | 10 (0;1) | 43 | 0.005 |
| 3x107 | 70 (0;7) | 94 | <0.0001 | 50 (0;5) | 74 | ≤0.0001 |
| A20/efficacy | PBS | --- | 10 (1;0) | --- | <0.0001 | 10 (1;0) | --- | ≤0.0001 |
| ONCR-159 | 3x105 | 70 (3;4) | 86 | 0.0006 | 10 (0;1) | 16 | ≤0.0001 |
| mONCR-171 | 3x105 | 100 (2;8) | 98 | --- | 80 (4;4) | 71 | --- |
| MC38/efficacy | PBS | --- | 0 (0;0) | --- | <0.001 | 0 (0;0) | --- | ≤0.01 |
| ONCR-159 | 3x106 | 30 (2;1) | 71 | ≤0.01 | 0 (0;0) | 43 | 0.08 |
| mONCR-171 | 3x106 | 50 (5;1) | 88 | --- | 0 (0;0) | 65 | --- |
| CT26/ efficacy | PBS | --- | 0 (1;0) | --- | ≤0.0001 | 0 (0;0) | --- | ≤0.0001 |
| ONCR-159 | 6x106 | 33 (1;3) | 78 | 0.07 | 0 (0;0) | 31 | ≤0.0001 |
| mONCR-171 | 6x106 | 50 (0;6) | 88 | --- | 42 (0;5) | 59 | --- |
| B16F10N1/efficacy | PBS | --- | 0 (0;0) | --- | ≤0.0001 | 0 (0;0) | --- | 0.002 |
| ONCR-159 | 5x106 | 38 (2;1) | 91 | ≤0.0001 | 0 (0;0) | 43 | 0.18 |
| mONCR-171 | 5x106 | 100 (2;6) | 98 | --- | 0 (0;0) | 66 | --- |
| TGI is relative to PBS control, calculated on Day 14 (A20 dose escalation), Day 20 (MC38 dose escalation), Day 17 (A20 and MC38 efficacy), Day 15 (CT26 efficacy), Day 11 (B16F10N1 efficacy). Animals (*n* = 10-12 per group) were dosed IT on Days 1, 4, and 7, with Day 1 being considered the first day of dosing. A mixed linear model was used to evaluate statistical differences across treatment groups. *P* values are relative to mONCR-171, except where indicated. CR, complete regression is defined as no palpable tumor remaining; IT, intra-tumoral; PBS, phosphate buffer saline; PR, partial regression is defined as a smaller tumor volume at the end of the study compared to Day 1; RR, response rate (PR+CR); TGI, tumor growth inhibition. |

Supplementary Table 4. Summary of *in vivo* anti-tumor activity of mONCR-171 and anti-PD-1 combination therapy in the MC38 tumor model

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Treatment** | **Dose** | **Right (Injected) Tumor** | **Left (Contralateral) Tumor** |  |
| **% RR (PR;CR)** | **% TGI** | ***P*** | **% RR (PR;CR)** | **% TGI** | ***P*** | **# Animals, Day 26c** |
| PBS | --- | 0 (0;0) | --- | --- | 10 (1;0) | --- | --- | 1 |
| IgG2a Iso | 200 g | 0 (0;0) | --- | ns | 0 (0;0) | --- | ns | 0 |
| mONCR-171 | 3x106 PFU  | 70 (4;3) | 92 | <0.0001 | 20 (2;0) | 64 | <0.0001 | 9 |
| Anti-PD-1 mAb | 200 g | 20 (1;1) | 53 | <0.0001 | 20 (0;2) | 55 | <0.0001 | 3 |
| mONCR-171anti-PD-1 mAb | 3x106 PFU 200 g | 80 (2;6) | 96 | <0.00010.09a<0.0001b | 40 (0;4) | 88 | <0.00010.002a0.003b | 10 |
| aRelative to mONCR-171; bRelative to anti-PD-1; cNumber of animals remaining at the end of the study on Day 26. Dosing was performed on Days 1, 4, 7, with Day 1 being considered the first day of dosing. Route of administration was IT for PBS and mONCR-171 treatments and IP for IgG2a Iso and anti-PD-1 mAb treatments. *n* = 10 mice/treatment group. TGI is relative to PBS control calculated on Day 18. A mixed linear model was used to evaluate statistical differences across treatment groups. *P* values are in reference to PBS control (for mONCR-171) or IgG2a (for anti-mouse PD-1), except where noted. CR, complete regression; IP, intra-peritoneal; Iso, isotype; IT, intra-tumoral; mAB, monoclonal antibody; ns, non-significant; PBS, phosphate buffer saline; PFU, plaque-forming unit; PR, partial regression defined as a smaller tumor volume at the end of the study compared to Day 1; RR, response rate (PR+CR); TGI, tumor growth inhibition. |

**Supplementary Figures**

**Supplementary Figure 1.** ONCR-177 retains sensitivity to acyclovir

**A,** Dose-dependent inhibition of ONCR-177 plaque formation by acyclovir was confirmed with an IC50 concentration of 0.068 µg/mL +/- 0.012. **B,** Acyclovir inhibition of ONCR-177 transgene expression. Conc, concentration; IC50, half maximal inhibitory concentration.

Supplementary Figure 2. CT26 re-challenge

**A,** Group mean ± SEM longitudinal tumor growth curves after challenge (of naïve BALB/c mice) or re-challenge (of BALB/c mice previously cured of CT26 tumors by mONCR-171 treatment) of 1x106 CT26 cells. **B,** Survival analysis of animals challenged or re-challenged with CT26 tumor cells. Mice were humanely euthanized once the combined tumor burden reached the endpoint value of >2000 mm3. *n* = 5 mice/re-challenge group, *n* = 4 mice/challenge group. Differences in survival curves were analyzed using a log-rank test. SEM, standard error of mean.

**Supplementary Figure 3.** mONCR-171 treatment results in the expansion of functional tumor antigen specific T cells in the injected and contralateral tumor

C57BL/6 mice bearing bilateral MC38 tumors (mean starting tumor volume of 125 mm3, 100-165 mm3 range) were administered two IT doses, on Days 1 and 4, of PBS or 3x106 PFU of mONCR-171. Tumors were harvested and disaggregated on Day 6, followed by *in vitro* stimulation with 1 g/ml tumor specific peptides for 5 hours in presence of Golgi plug before surface and intracellular staining. Statistics were calculated with a 2-way ANOVA in GraphPad Prism. \* *P* ≤ 0.05 and \*\* *P* ≤ 0.01.

**Supplementary Figure 4.** Transcriptional analysis after mONCR-171 and anti-PD-1 combination therapy

Transcriptional analysis (Nanostring platform using the PanCancer IO 360 Gene Expression Panel) of MC38 tumors 8 days after the indicated treatments are shown as box and whiskers plots, with individual replicate values, depicting the gene signature scores indicative of T cell infiltration ‘T Cell Score’ or T cell/NK cell cytotoxic activity ‘Cytotoxic Score’. *n* = 5 mice/treatment group. Group comparisons were performed with an unpaired two-tailed t test; \* *P* < 0.05, \*\*\* *P* < 0.001, \*\*\*\* *P* < 0.0001.

**Supplementary Figure 1**

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**Supplementary Figure 2**

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**Supplementary Figure 3**



Supplementary Figure 4

