Pre-conditioning of the tumor microenvironment with oncolytic reovirus converts CD3-bispecific antibody treatment into effective immunotherapy

Authors: Christianne Groeneveldt[1], Priscilla Kinderman[1], Diana J. M. van den Wollenberg[2], Ruben L. van den Oever[1], Jim Middelburg[1], Dana A. M. Mustafa[3], Rob C. Hoeben[2], Sjoerd H. van der Burg[1], Thorbald van Hall[1†*] and Nadine van Montfoort[1†*]

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Table S1. List of antibodies used for flow cytometric analysis.

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Supplementary Methods

Cell culture

All cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 in Iscove's Modified Dulbecco's medium (IMDM; Invitrogen) supplemented with 8% fetal calf serum (FCS; Bodinco, Alkmaar, The Netherlands), 2mM L-glutamine (Gibco), 100 µg/mL penicillin and 100 µg/mL streptomycin (Gibco). The tumor cell line TC-1 was additionally cultured in the presence of 400 µg/ml Geneticin (G418; Life Technologies), 1% nonessential amino acids (Life Technologies), and 1 mM sodium pyruvate (Life Technologies). Cell lines were assured to be free of *Mycoplasma* by regular PCR analysis. Authentication of the cell lines was done by Short Tandem Repeat (STR) profiling (IDEXX BioAnalytics, Ludwigsburg, Germany) and cells of low passage number were used for all experiments.

In vivo αPD-L1 treatment

Mice were treated on indicated days with intraperitoneal injections of 200 µg PD-L1-blocking antibody (clone 10F.9G2; GoInVivo™ Purified anti-mouse CD274 Antibody; BioLegend).

CsCl purification of reovirus stock

For purification, a freeze-thaw lysate containing reovirus particles was incubated with 0,1% Triton (Sigma-Aldrich, Zwijndrecht, the Netherlands) and 25 units/ml Benzonase (Santa Cruz, Bio-Connect B.V. Huissen, the Netherlands) for 15 min on ice followed by 15 min at 37 °C. After two extractions with Halotec CL10 (FenS B.V. Goes, the Netherlands) to remove cellular debris, the cleared lysate was loaded onto a discontinuous CsCl gradient (1.45 and 1.2 g/cm^3 in phosphate-buffered saline (PBS)). After centrifugation in a SW28 rotor (Beckman Coulter, Woerden, the Netherlands) at 69000 × g for 14 hours at 4 °C, the

lower band containing the infectious particles was harvested and desalted in an Amicon Ultra 100K device according to the manufacturer's protocol (Millipore, Merck Chemicals BV, Amsterdam, the Netherlands). The CsCl-purified reoviruses were recovered in reovirus storage buffer (RSB: 10mM Tris-HCl; pH 7.5, 150mM NaCl, 10mM $MgCl_2$ • 6 H2O), aliquoted and stored at 4 °C until use.

In vitro viability assays

The oncolytic capacity of reovirus was assessed using a colorimetric assay to determine metabolic activity. In short, KPC3 and B16.F10 cells were seeded in a concentration of 5000 (KPC3) and 2500 (B16.F10) cells/well and left to attach overnight. The next day, cells were infected with designated MOIs of reovirus. Cell viability was assessed after 48 hours using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (Promega). 20 µL/well of CellTiter 96® AQ_{ueous} One Solution Reagent was added for two hours. The ability of CD3xTRP1 bsAbs to induce specific killing was assessed using a colorimetric method for quantifying cellular cytotoxicity. In short, KPC3, KPC3.TRP1 and B16.F10 cells were irradiated at 6000 RAD and plated at a concentration of 30.000 cells/well. Splenocytes were isolated from a naïve C57BL/6J mice and B cells were removed by passaging through nylon wool before use. Splenocytes were added in an E/T ratio of 5:1 and then CD3xTRP1 or CD3xFluorescein bsAbs (CD3xcntrl) were added in a concentration of 1 µg/mL. 48 hours after incubation, 20 µL of Triton-X100 was added to wells containing tumor cells alone to serve as a positive control. 50 µL of supernatant was harvested of all conditions and incubated for 30 minutes with 50 µL of lactate dehydrogenase reaction mix (Pierce LDH Cytotoxicity Assay Kit, ThermoFisher Scientific). Absorbance was measured at 490 using a SpectraMax iD3 multi-mode plate reader (Molecular Devices). Viability was normalized to

the viability of non-infected conditions, and % of cytotoxicity was calculated using the positive control as 100 % cytotoxicity. All conditions were performed in triplicate.

Cell preparation and flow cytometry

Tumors were minced in small pieces and additionally incubated with Liberase TL (Roche) for 15 minutes at 37 °C. The reaction was stopped by the addition of medium and the mixture was gently dissociated into a single-cell suspension over a cell strainer. Single-suspensions of splenocytes were resuspended in lysis buffer to remove all red blood cells before use. Cells were incubated with Zombie AquaTM Fixable Viability Dye (Biolegend) in PBS at room temperature followed by incubation with 2.4G2 FcR blocking antibodies (clone 2.4G2; BD Biosciences) in FACS buffer (PBS, 0.5% BSA and 1% NaAz) before surface marker staining **(Table S1)**. If applicable, cells were fixed and stained for transcription factors and nuclear proteins using the Foxp3 / Transcription Factor Staining Buffer Set (eBiosciences) according to manufacturers' instructions. TRP1 expression on KPC3.TRP1 tumor cells was measured using the αTRP1 primary antibody (clone: TA99) followed by a secondary Alexa Fluor 647-labeled anti-mouse IgG (BioLegend). HER2 expression on BT474 tumor cells was measured using the anti-erbB-2 (Her-2/neu) primary antibody (clone: 4D5-8) followed by a secondary PE-labeled anti-rabbit IgG (BioLegend). The frequency of sigma 3^+ cells was determined as a method of quantifying the infection efficiency of reovirus. Cells were harvested 48 hours after infection and fixed with Fixation Buffer (BioLegend) according to the manufacturer's instructions. Afterward, cells were washed with Permeabilization Wash Buffer (BioLegend) and stained with 4F2 hybridoma supernatant (dilution 1:500), recognizing the sigma 3 protein of reovirus T3D (Developmental Studies Hybridoma Bank) followed by a secondary Alexa Fluor 647-labeled anti-mouse IgG (BioLegend). After completion of staining protocols, samples were fixed in 1% paraformaldehyde and acquired using a BD LSRFortessa™ X20

cell analyzer (BD Biosciences) within 24 hours. Flow cytometry data was analyzed using FlowJo™ Software Version 10 (Becton, Dickinson and Company).

Ex vivo analysis of TIL specificity

To determine the specificity of T cells in the tumor and spleen, KPC3-bearing mice were treated with the standard regimen reovirus as described above. Single-cell suspensions of individual tumors and spleens, collected at seven days after the last reovirus injection, were co-cultured with irradiated (6000 RAD) target cells. The irrelevant tumor cell line TC-1 was used as a target to facilitate reovirus replication and was previously described.[1] PMA (20 ng/mL) and ionomycin (1 µg/mL) were used as positive control. After 1 hour of co-incubation, BD GolgiPlug™ (BD Biosciences) was added in a 1:1000 dilution. After an additional 5 hours, cells were washed and stained for surface markers. Afterward, cells were fixed, permeabilized, and stained for intracellular markers using the Foxp3/Transcription Factor Staining Buffer Set (eBiosciences) according to manufacturers' instructions. After completion of the staining protocol, samples were fixed, measured, and analyzed as described above.

RNA isolation

From *in vitro* samples, total RNA was isolated from cell pellets using the NucleoSpin® RNA Kit (Macherey-Nagel™) according to the manufacturer's instructions. For *in vivo* samples, a representative snap-frozen proportion (10-30 mg) of each tumor or organ was disrupted using a stain-less bead and the TissueLyser LT (Qiagen). Total RNA of *in vivo* samples was using the ReliaPrep™ RNA Tissue Miniprep System (Promega) according to manufacturer's protocol. RNA quality and integrity were determined using the Experion™ Automated Electrophoresis System (Bio-Rad).

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147 *RT-qPCR analysis*

148 For S4 analysis, 150 ng of RNA was used to generate cDNA with primer S4EndR
149 (GATGAATGAAGCCTGTCCCACGTCA) and GoScript™ Reverse Transcriptase
150 (Promega). For assessing the transcription levels of host genes *Ifit-1, Ifit-3, Oas1b, Ddx58,*
151 *Cxcl10, Ccl5,* and *β2M*, 500 ng of RNA was used to generate cDNA using the High-Capacity
152 RNA-to-cDNA^{TM} Kit (ThermoFisher Scientific) according to the manufacturer's protocol.
153 Subsequent qPCR analysis was performed using the Bio-Rad iQ^{TM} SYBR® Green Supermix
154 (Bio-Rad) and the primer sets are displayed in **(Table S2).** The expression of host genes was
155 normalized to reference genes *Mzt2, Ptp4a2, and Ubc* using the Bio-Rad CFX Manager 3.1
156 Software (Bio-Rad). All primers were quality controlled by assessing the slope, efficiency,
157 and R^2 value of dilution series using cDNA that was synthesized from murine reference
158 RNA. All samples were measured in technical duplicates or triplicates. The used PCR
159 program consisted of the following steps: (1) 3 min at 95 °C; (2) 40 cycles of 10 s at 96 °C
160 followed by 30 s at 60 °C and plate read; (3) 10 s at 95 °C; (4) Melt curve 65–95 °C with an
161 increment of 0.2 °C every 10 s, and plate read.

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163 *Immunohistochemistry*

164 Formaldehyde-fixed, paraffin-embedded tissue sections were stained for reoviral protein
165 sigma 3 or murine CD3. Formalin-fixed tumor pieces were embedded in paraffin and then
166 sectioned randomly at 5 µm and placed on Superfrost® Plus slides (VWR). Sections were
167 dried overnight at 37 °C and stored at 4 °C until staining. Slides were deparaffinized and
168 endogenous peroxidase was blocked with 0,3% hydrogen peroxidase (VWR) in methanol for
169 20 minutes. After rehydration, antigen retrieval was performed by boiling slides for 10
170 minutes in 0,01M sodium citrate (Merck). Non-specific binding was blocked using

SuperBlock™ (ThermoFisher Scientific) before overnight incubation at 4 °C with rabbit anti-mouse CD3ε D7A6E™ XP® mAb (1:200; Cell Signaling Technology), rat anti-mouse CD8a (clone 4SM15, 1:1600; eBioscience™) or 4F2 hybridoma supernatant which recognizes the sigma 3 protein of reovirus (1:150; Developmental Studies Hybridoma Bank). Hereafter, samples were incubated for 30 min at RT with biotinylated goat anti-rabbit, rabbit anti-rat, or goat anti-mouse secondary antibodies (1:200; Agilent), followed by incubation with avidin-biotin complex (VECTASTAIN® Elite® ABC HRP Kit; Vector Laboratories). Peroxidase activity was detected using the 2-component liquid DAB+ system (Agilent) according to the manufacturer's instructions for 5 min. Slides were counterstained in hematoxylin (Sigma Aldrich), dehydrated, and mounted using Entellan (Sigma Aldrich). Control sections were processed in parallel, but without incubation with primary antibody. No labeling was observed in the control sections.

References

1. van Montfoort N, Borst L, Korrer MJ, et al. NKG2A Blockade Potentiates CD8 T Cell Immunity Induced by Cancer Vaccines. *Cell* 2018;175(7):1744-55.e15. doi: 10.1016/j.cell.2018.10.028 [published Online First: 2018/12/07]

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203 **Supplementary Figure Legends**

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205 **Figure S1. CD3xTRP1 bsAb treatment is not effective in a therapeutic setting in the**
206 **KPC3.TRP1 model.** Hematoxylin and eosin (H&E) staining **(A)** or CD3
207 immunohistochemical staining **(B)** of representative untreated KPC3 tumor at a size of 1000
208 mm^3. Arrows indicate $CD3^+$ cells. Scale bar equals 100 µm. **C)** *In vivo* treatment schedule.
209 Mice (n=8/group) with established KPC3.TRP1 tumors were treated i.p. with 12.5 µg
210 CD3xTRP1 on indicated days, after which tumor growth was monitored. **D)** Individual tumor
211 growth curves of mice treated with PBS or CD3xTRP1 BsAb. Dashed red vertical lines
212 indicate timing of injection with CD3xTRP1. **E)** Percentages of cytotoxicity of KPC3.TRP1
213 or KPC3 cells after *in vitro* co-culture with naïve T cells and CD3xTRP1 or CD3xcntrl bsAbs
214 (CD3xFluorescein; bAb0161, Absolute Antibody). Data represents mean ± SEM of
215 triplicates. BsAb, bispecific antibody.

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217 **Figure S2. Transcriptomic changes after treatment with replication-competent reovirus.**
218 **A)** Volcano plots showing the differentially expressed genes analyzed by NanoString at
219 various timepoints after treatment with replication-competent reovirus or day 3 after
220 treatment with UVi, normalized versus PBS (n=3-4/group). Horizontal dashed line indicates
221 FDR p-value of 0.1. **B)** Changes in signature scores on indicated days after reovirus

treatment. All scores are normalized for average score of PBS. Data are presented as mean ± SEM. UVi, UV-inactivated reovirus.

Figure S3. Gating strategy for flow cytometric analyses of the lymphoid and myeloid cell compartment in the tumor after reovirus treatment. Cells of the lymphoid and myeloid compartment were gated according to visualized strategy. Specific antibodies used for flow cytometry can be found in Table S1. Data was analyzed by FlowJo™ software.

Figure S4. Extended analysis of changes in immune cell composition in the tumor 5 days after reovirus treatment. A) CD3 and CD8 immunohistochemical staining of representative KPC3 tumors injected with PBS, UVi or replication-competent reovirus. Arrows indicate $CD3^+$ or $CD8^+$ cells. Scale bar equals 50 µm. **B)** Frequency of $CD45^+$ immune cells out of all live cells. **C)** Frequency of $CD3^+$, $CD8^+$ and $CD4^+$ T cells and NK1.1 cells out of $CD45^+$ immune cells in tumors after administration of reovirus or PBS. **D)** Frequency of $CD4^+$ $FoxP3^-$ (conventional $CD4^+$ T cells) and $CD4^+$ $FoxP3^+$ (regulatory T cells). **E)** Ratio between $CD8^+$ T cells and $CD4^+FoxP3^+$ cells (regulatory T cells) within the $CD45^+$ immune cell population in the tumor after treatment with reovirus or PBS. Statistical difference between groups is determined using a Mann Whitney U test. **F)** Activation status of intratumoral $CD8^+$ and $CD4^+$ T cells after reovirus or PBS treatment. **G)** Percentages of other immune cells within the $CD45^+$ population after treatment with reovirus or PBS. Significance of data visualized in B-D, F and G is determined using unpaired t tests. **H)** Presence of $CD3^+$ T cells and $Ly6G^+$ cells in tail blood of mice treated as indicated, without or with FTY720. Representative flow cytometry dot plot of one mouse per group is shown. **I)** Frequency of $CD45^+$ immune cells in de tumor. Data is representative for 2 independent experiments. **J)**

Presence of $IFNy^+$ $CD8^+$ cells in the spleen after ex vivo co-culture with indicated targets. All data are presented as mean ± SEM (n=5/group for A-I, n=8/group for J). In figure I, significance between groups is determined using an ordinary one-way ANOVA with Tukey's post-hoc test. In figure J, significance versus negative control is determined using an unpaired t test. Significance levels are indicated with asterisks, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. UVi, UV-inactivated reovirus.

Figure S5. Strategies to prevent immune escape after combined reovirus and CD3xTRP1 bsAb combination treatment. A) Individual tumor growth curves of experiment described in Figure 4B. Dashed vertical lines indicate timing of treatment with Reovirus (blue) or CD3xTRP1 (red). **B)** Individual tumor growth curves of groups receiving one round or two rounds of reovirus + CD3xTRP1 therapy (n=10/group). **C)** Relative changes in tumor volume of individual mice from the start of CD3xTRP1 bsAb treatment. Indicated is the number of mice with tumor regressions. **D)** Individual growth curves of KPC3.TRP1-bearing mice receiving indicated treatments. Grey lines indicate timing of treatment with αPD-L1. **E)** Average tumor growth curves. Differences in mean tumor volumes versus PBS treatment on day 22 is determined by ordinary one-way ANOVA with Dunnett's post-hoc test. **F)** Relative changes in tumor volume from start of CD3xTRP1 bsAb treatment. Indicated is the number of mice with tumor regressions. All data are presented as mean ± SEM. Significance level: *** $p < 0.001$. BsAb, bispecific antibody.

Figure S6. Analysis of mechanisms underlying escape to reovirus and CD3xTRP1 bsAb combination treatment. A) Representative images obtained from immunohistochemical CD3 staining (light brown) of tumors treated with PBS or reovirus + CD3xTRP1. Arrows indicate $CD3^+$ cells. Scale bars equal 100 µm. **B)** Flow cytometric analysis of the frequency of tumor-infiltrating T cells (TILs) in end stage tumor samples (n=6-8/group). Significance

versus PBS treatment is determined using an ordinary one-way ANOVA with Dunnett's post-hoc test. **C)** Expression of activation markers and checkpoint receptors on TILs. All data are presented as mean ± SEM with white dots indicating individual mice. **D)** Gating strategy to determine TRP1 expression by a 2-step flow cytometry protocol. A sample from the PBS group is used. Significance levels are indicated with asterisks, with * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. BsAb, bispecific antibody.

Figure S7. Characterization of reovirus efficacy in B16.F10. A) TRP1 expression percentages and intensities on B16.F10 and KPC3.TRP1 cells, as analysed by flow cytometry using a 2-step protocol. **B)** Number of reovirus S4 copies in B16.F10 cells after reovirus infection. B16.F10 cells (62.500/well) were infected with increasing MOIs of reovirus, or PBS (Mock) or UVi (equal number of viral particles as MOI 100) as controls. Samples (n=3) were harvested 24 hours after infection and the number of viral S4 copies was determined by RT-qPCR. **C)** Frequency of Sigma 3-positive B16.F10 cells 48 hours after infection with increasing MOIs of reovirus (blue histograms), or PBS or UVi as controls (grey histograms). **D)** Analysis of oncolytic activity of reovirus. B16.F10 cells (2500/well) were plated and infected with reovirus or controls. Metabolic activity was determined 48 hours after infection. **E)** S4 copy numbers in tumors harvested 5 days after reovirus treatment (n=3-4/group). **F)** Flow cytometric analysis of the frequency of tumor-infiltrated T cells (TILs) in B16.F10 tumor samples, 7 days after reovirus treatment (n=3-4/group). All data are presented as means ± SEM and individual values. In figures E-F, significance versus PBS treatment is determined using an unpaired t test. Significance levels are indicated with asterisks, with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. UVi, UV-inactivated reovirus. MOI, multiplicity of infection.

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303 **Supplementary Tables**

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305 **Table S1. List of antibodies used for flow cytometric analysis.**

	Marker	Clone	Fluorochrome	Supplier
Lymphoid panel	**CD45.2**	104	APC-Cy7	eBioscience
	CD3	145-2C11	PE-CF594	BD Biosciences
	CD8α	53-6.7	Alexa Fluor 700	eBioscience
	CD4	RM4-5	BV605	BioLegend
	CD44	IM-7	BV785	BioLegend
	CD62L	MEL-14	BV421	BioLegend
	NK1.1	Pk136	BV650	BD Biosciences
	PD-1	RMP1-30	FITC	eBioscience
	TIM-3	RMT3-23	APC	BioLegend
	NKG2A	16A11	PE	eBioscience
	CD43	1b11	PE-Cy5	BioLegend
	KLRG-1	2F1	PE-Cy7	eBioscience
Myeloid panel	**CD45.2**	104	FITC	BioLegend
	CD19	eBio1D3	PE	eBioscience
	CD11b	M1/70	PE-Cy7	BioLegend
	Ly6G	1A8	BV785	BioLegend
	F4/80	BM8	PE-Cy5	BioLegend
	CD11c	N418	APC-Cy7	BioLegend
Treg panel	**CD45.2**	104	FITC	BioLegend
	CD3	145-2C11	PE-CF594	BD Biosciences
	CD8α	53-6.7	Alexa Fluor 700	eBioscience
	CD4	RM4-5	BV605	BioLegend
	FoxP3	FJK-16s	PE	eBioscience
	Ki67	B56	BV711	BD Biosciences
Intracellular T cell activation panel	**CD45.2**	104	APC-Cy7	eBioscience
	CD3	145-2C11	PE-CF594	BD Biosciences
	CD8α	53-6.7	Alexa Fluor 700	eBioscience

INFγ	XMG1.2	APC	BioLegend

Table S2. List of primers used for RT-qPCR analysis.

Gene	Forward	Reverse
S4Q	5'-CGCTTTTGAAGGTCGTGTATCA-3'	5'-CTGGCTGTGCTGAGATTGTTTT-3'
Ifit-1	5'-CTGGACAAGGTGGAGAAGGT-3'	5'-AGGGTTTTCTGGCTCCACTT-3'
Ifit-3	5'-GTGCAACCAGGTCGAACATT-3'	5'- AGGTGACCAGTCGACGAATT-3'
Oas1b	5'-AGCATGAGAGACGTTGTGGA-3'	5'-GCGTAGAATTGTTGGTTAGGCT-3'
Ddx58	5'-AAGGCCACAGTTGATCCAAA-3'	5'-TTGGCCAGTTTTCCTTGTCG-3'
Cxcl10	5'-ACGAACTTAACCACCATCT-3'	5'-TAAACTTTAACTACCCATTGATACATA-3'
Ccl5	5'-ATTGCTTGCTCTAGTCCTA-3'	5'-ATGCTGATTTCTTGGGTTT-3'
β2M	5'-CTCGGTGACCCTGGTCTTT-3'	5'-CCGTTCTTCAGCATTTGGAT-3'
Mzt2	5'-TCGGTGCCCATATCTCTGTC-3'	5'-CTGCTTCGGGAGTTGCTTTT-3'
Ptp4a2	5'-AGCCCCTGTGGAGATCTCTT-3'	5'-AGCATCACAAACTCGAACCA-3'
Ubc	5'-GCCCAGTGTTACCACCAAGA-3'	5'-CCCATCACACCCAAGAACA-3'

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