

METHODS OF CANCER TREATMENT WITH ANTAGONISTS AGAINST PD-1 AND PD-L1 IN COMBINATION WITH RADIATION THERAPY

DESCRIPTION

Sequence Listing

[000] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on June 16, 2015, is named B7H1-275WO1_SL.txt and is 103,313 bytes in size.

Field

[001] Methods of treatment for cancer.

Background

[002] Radiation therapy (RT) remains the most important non-surgical treatment in the management of solid malignancies with around 50-60% of all cancer patients receiving this treatment. The inclusion of RT in treatment regimens reduces disease recurrence and improves overall survival in the majority of common cancers (1-3). Although effective, many patients suffer from local recurrence and metastatic disease.

[003] In addition to the direct cytoreductive effect of RT, emerging evidence suggests that the generation of anti-tumor immune responses may play an important role in the effectiveness of this treatment (4, 5). RT can lead to expression of ecto-calreticulin on tumor cells as well as the release of several damage-associated molecular patterns (DAMPs) including High Mobility Group Box 1 (HMGB1) and ATP which can lead to recruitment and activation of antigen presenting cells (APCs) and priming of tumor antigen-specific T cell responses (6-10). Despite this immune-escape frequently occurs, with tumor recurrence remaining the leading

cause of mortality in patients receiving RT (11). The identification and inhibition of key drivers of immunosuppression may augment anti-tumor immune responses with the potential to improve patient outcome.

[004] New insights into treatment failure and more effective RT combination approaches are therefore urgently required.

[005] The programmed death 1 (PD-1) / programmed death ligand 1 (PD-L1) axis is involved in the maintenance of peripheral tolerance and the modulation of acute inflammatory responses through inhibition of T cell function and through apoptosis of activated T cells (12, 13). In addition to binding PD-1, PD-L1 can also suppress T cell function through interaction with CD80 (14). Expression of PD-L1 is inducible and thought to respond to local inflammatory milieu, particularly type I and II interferon (IFN) (12, 15, 16). Although barely detectable in most normal tissues, expression of PD-L1 has been described in multiple malignancies (reviewed in (17)). Importantly, recent clinical studies with either PD-1 or PD-L1 targeting monoclonal antibodies (mAb) have demonstrated encouraging responses in patients with advanced disease (18-21).

[006] A dosing strategy for combining radiation therapy and a PD-1 and/or PD-L1 antagonist is desired to create maximal benefit for patients suffering from cancer.

SUMMARY

[007] In accordance with the description, a method of treating cancer in a patient comprises

- a. administering at least one dose of radiation therapy; and
- b. administering at least one PD-1 and/or PD-L1 antagonist,

wherein at least one PD-1 and/or PD-L1 antagonist is administered on the same day as a dose of radiation therapy or up to and including 4 days later.

[008] In another mode, the at least one PD-1 and/or PD-L1 antagonist is an at least one PD-1 and/or PD-L1 antibody or functional part thereof.

[009] In one aspect, the radiation therapy is fractionated radiation therapy. In one mode, the fractionated radiation therapy comprises from 2 to 7 fractions. In another mode, the fractionated radiation therapy comprises 5 fractions.

[010] In one embodiment, the radiation therapy fractions are administered in sequential days. In another embodiment, the radiation therapy fractions are administered on day 1, day 2, day 3, day 4, and day 5. In one embodiment, the radiation therapy comprises about 10 Gy in 5 fractions.

[011] In one aspect, the at least one PD-1 and/or PD-L1 antagonist is administered on at least day 1 and/or on day 5. In one mode, the at least one PD-1 and/or PD-L1 antagonist is administered multiple times. For example, the at least one PD-1 and/or PD-L1 antagonist may be administered 3 times a week.

[012] In one embodiment, the anti-PD-1 and/or PD-L1 antibody or functional part thereof is MEDI4736.

[013] In another embodiment, the anti-PD-1 and/or anti-PD-L1 antibody or functional part thereof is pembrolizumab, nivolumab, BMS-936558, AMP-224, or MPDL3280A.

[014] In one aspect, the cancer is melanoma, colorectal cancer, or breast cancer. In another aspect, more than one treatment cycle is performed. In a further aspect, from 2-8 treatment cycles are performed. In one mode, treatment cycles are weekly or every other week.

[015] Additional objects and advantages will be set forth in part in the description which follows, and in part will be obvious from the description, or may be learned by practice.

The objects and advantages will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims.

[016] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the claims.

[017] The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate one (several) embodiment(s) and together with the description, serve to explain the principles described herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[018] Figures 1A-F illustrate that blockade of the PD-1 / PD-L1 axis potentiates the activity of fractionated radiation therapy. *A* and *B*, median fluorescence intensity (*A*) and representative histograms (*B*) of PD-L1 expression on CT26 cells isolated from tumors 1, 3 or 7 days after treatment with about 10 Gy in 5 daily fractions of 2 Gy. *C* and *D*, CT26 tumor bearing mice received about 10 Gy RT delivered in 5 daily fractions of 2 Gy either alone or in combination with either α PD-1 (*C*) or α PD-L1 (*D*) mAb dosed at 10 mg/kg 3qw for up to 3 weeks. *E*, tumor volumes 10 days after the start of therapy in 4T1 tumor bearing mice that received 20 Gy RT delivered in 5 daily fractions of 4 Gy either alone or in combination with α PD-L1 mAb. *F*, 4434 tumor bearing mice received about 10 Gy RT delivered in 5 daily fractions of 2 Gy either alone or in combination with α PD-L1 mAb. Experimental groups contained at least 7 mice and are representative of at least 2 independent experiments. *A*, *E* and *F* show mean $\pm$ SEM. *, $P < 0.05$ **, $P < 0.01$, Mann-Whitney test. *C* and *D*, *, denotes significance when compared to control mice. +, denotes significance when compared to monotherapy. ***/+++, $P < 0.001$, Log-rank (Mantel-Cox) test.

[019] Figures 2A-C demonstrate that therapeutic activity of fractionated RT and α PD-L1 mAb combination is dependent on the activity of CD8⁺ T-lymphocytes. A, tumor volume on day of therapy, 7 and 11 days post combination therapy with 5 fractions of 2 Gy and α PD-L1 mAb. Immune cell subsets (either CD8, CD4 or NK cell) were depleted 1 day prior to therapy with depletion maintained for 2 weeks. ***, $P > 0.001$, *, $P > 0.01$, *, $P > 0.05$, Mann-Whitney test. B, Survival curve. ***/+++, $P < 0.001$, Log-rank (Mantel-Cox) test. Data representative of 10 mice per cohort. C, representative density plots of peripheral blood confirming depletion of immune cell subsets.

[020] Figures 3A-C show that fractionated RT and α PD-L1 mAb combination generates protective immunological memory. A, Survival curve of LTS mice following contralateral rechallenge with 5×10^5 CT26 cells. * $P < 0.05$ compared to control mice (log-rank; Mantel-Cox test). B, representative dot blot of IFN γ production by CD8⁺ T cells isolated from either tumor naïve, or LTS mice originally treated with RT and α PD-L1 mAb. C, frequency of IFN γ ⁺ CD8⁺ T cells isolated from either tumor naïve, or LTS mice originally treated with RT and α PD-L1 mAb following co-culture with either H2-Ld restricted peptides (AH1 (SPSYVYHQF) (SEQ ID NO: 91); a defined CT26 tumor-associated antigen or β -galactosidase (TPHPARIGL) (SEQ ID NO: 92); control peptide of prokaryotic origin) or 50 Gy irradiated CT26 cells for 5 days, followed by priming with 50 Gy irradiated CT26 cells. * $P < 0.05$ (Mann-Whitney test). Data representative of 2 independent experiments.

[021] Figures 4A-C illustrate that fractionated RT increases tumor cell PD-L1 expression *in vivo* and is dependent on CD8⁺ T cells. A, Expression of PD-L1 on CT26 cells post treatment with RT (2.5-10Gy) *in vitro*. B and C, Representative contour plots (B) and median fluorescence intensity (C) of PD-L1 expression on CT26 cells (gated as CD45⁺ cells) isolated

from tumors 3 days after receiving about 10 Gy in 5 daily fractions of 2Gy in combination with either CD8, CD4 or NK cell depleting antibodies. * $P < 0.05$ (Mann-Whitney test). Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

[022] Figures 5A-E show that CD8⁺ T cell production of IFN γ is responsible for the upregulation of PD-L1 expression on tumor cells following fractionated RT. *A*, Expression of PD-L1 on wild-type CT26 tumor cells (WT), or cells transduced with either a non-targeting control (NTC) or IFN γ R1 ShRNA following co-culture for 24 hours with either 20ng/ml IFN γ , TNF α or a combination of both cytokines. *B*, representative density plots showing expression of IFN γ and TNF α by CD8⁺ T cells following treatment with either PBS or phorbol 12-myristate 13-acetate (PMA) and ionomycin, *C*, Frequency of PD-L1 positive CT26 tumor cells (WT, NTC ShRNA or IFN γ R1 ShRNA) following 24 hour co-culture with either PBS or PMA/ionomycin activated splenocytes. n/s = $P > 0.05$, *** $P < 0.001$ ** $P < 0.01$ (2-tailed Student *t* test). *D* and *E*, representative contour plots (*D*) and median fluorescence intensity (*E*) of PD-L1 expression on CT26 tumor cells following treatment with RT (about 10 Gy in 5 fractions) in the presence of an α IFN γ blocking mAb (or isotype control) *in vivo*. ** $P < 0.01$, (Mann-Whitney test). Experimental groups contained at least 5 mice.

[023] Figures 6A-D demonstrate that dosing schedule impacts outcomes with RT potentiation only observed with concurrent but not sequential α PD-L1 mAb therapy. *A*, Schema for dose-scheduling studies. Mice received fractionated dose RT (as about 10 Gy in 5 daily fractions of 2 Gy) alone or in combination with α PD-L1 mAb starting either on day 1 of RT cycle (schedule A), day 5 of RT cycle (schedule B) or 7 days post the last dose of RT (Schedule C). *B*, Survival curves of therapy. ++, $P < 0.01$ compared to monotherapy (log-rank; Mantel-Cox test). *C* and *D*, Expression of PD-1 on CD4⁺ (*C*) and CD8⁺ (*D*) T cells 24 hours and 7 days after

the last dose of RT. * $P < 0.05$ (Mann-Whitney test). Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

[024] Figure 7 shows blockade of both PD-1 and PD-L1 does not further enhance efficacy in combination with fractionated RT in CT26-tumor bearing mice. A) Survival curve following fractionated dose RT (as 10 Gy in 5 daily fractions of 2 Gy) alone or in combination with α PD-1, α PD-L1 or a combination of both mAb dosed 3qw for 3 weeks. n/s, $P > 0.05$, log-rank; Mantel-Cox test. Experimental groups contained at least 5 mice and are representative of at least 2 independent experiments.

[025] Figure 8A-B show combination therapy with fractionated RT and either α PD-1 or α PD-L1 mAb is well tolerated in mice. , CT26 tumor bearing mice received 10 Gy RT delivered in 5 daily fractions of 2 Gy either alone or in combination with α PD-L1 mAb dosed at 10 mg/kg 3qw either for 3 weeks or 1 week. Experimental groups contained at least 7 mice and are representative of at least 2 independent experiments. n/s, $P > 0.05$, Mann-Whitney test.

[026] Figure 9A-C illustrate that treatment of tumor cells with α PD-1 or α PD-L1 mAb does not sensitise to radiation-induced cell death *in vitro*. Clonogenic survival curves for CT26 cells (A), 4T1 cells (B) and 4434 cells (C) treated with RT (2.5-10Gy) in the presence absence of 2 μ g/ml α PD1 or α PD-L1.

[027] Figures 10A-C show that the dosing schedule impacts outcome. A) tumour volumes following fractionated dose RT (as 10 Gy in 5 daily fractions of 2 Gy) alone or in combination with α PD-L1 mAb starting either on day 1 of RT cycle (schedule A), day 5 of RT cycle (schedule B) or 7 days post the last dose of RT (Schedule C). B and C) tumour volumes of RT –treated mice demonstrating equivalent tumor volumes across different dosing schedules.

n/s, $P>0.05$ (Mann-Whitney test). Experimental groups contained at least 5 mice and are representative of at 2 independent experiments.

[028] Figure 11 shows that acute versus chronic administration of α PD-L1 mAb does not impact efficacy of combination therapy. A, CT26 tumor bearing mice received 10 Gy RT delivered in 5 daily fractions of 2 Gy either alone or in combination with α PD-L1 mAb dosed at 10 mg/kg 3qw either for 3 weeks or 1 week. Experimental groups contained at least 7 mice and are representative of at least 2 independent experiments. n/s, $P>0.05$, log-rank; Mantel-Cox test.

DESCRIPTION OF THE SEQUENCES

[029] Table 1 provides a listing of certain sequences referenced in present embodiments. CDRs have been indicated in bold and underlining.

Table 1		
Description	Sequence	SEQ ID NO
2.7A4 VH	EVQLVESGGGLVKPGGSLRLSCAASGFTFS <u>TYSMN</u> WVRQAPGKGLEWVS <u>SISSSGDYIYYADSVKGR</u> RFTISRDNAKNSLFLQMNSLKAEDTAVY YCARD <u>DLVTSMVAFDY</u> WGQGTLLTVSS	1
2.7A4 VL	TC <u>SGDALPQKYVF</u> WYQQKSGQAPVLVIY <u>EDSKRPS</u> GIPERFSGSSSGTMTATLTISGAQVEDEADYYC <u>YSTDRSGNHRV</u> FGGGTRLTVL	2
2.9D10 VH	EVQLVESGGGLVQPGGSLRLSCAASGFTFS <u>SYWMS</u> WVRQAPGKGLEWVAN <u>NIKQDGGEQYYVDSVKGR</u> RFTISRDNAKNSLYLQMNSLRAEDTAVY YCARD <u>DWNYGYDMDV</u> WGQGTLLTVSS	3
2.9D10 VL	EIVLTQSPGTLSSLSPGERATLSC <u>RASQSVSSNYLA</u> WFQQKPGQAPRL LIF <u>GTSSRAT</u> GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC <u>QQYGS</u> <u>SIFT</u> FGPGTKVDIK	4
2.14H9 VH	EVQLVESGGGLVQPGGSLRLSCAASGFTFS <u>RYWMS</u> WVRQAPGKGLEWVAN <u>NIKQDGGSEKYYVDSVKGR</u> RFTISRDNAKNSLYLQMNSLRAEDTAVY YCARD <u>EGGWFGELAFDY</u> WGQGTLLTVSS	5
2.14H9 VL	EIVLTQSPGTLSSLSPGERATLSC <u>RASQRVSSSYLA</u> WYQQKPGQAPRL LIY <u>DASSRAT</u> GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC <u>QQYGS</u> <u>LPWT</u> FGQGTEVEIK	6
2.20A8 VH	EVQLLES GGGLVQPGGSLRLSCAASGFTFS <u>NYAMS</u> WVRQAPGKGLEWVS <u>AIRSGGSTYYADSVKGR</u> RFTISRDN SKNTLYLQMNSLRAEDTAVY YCAK <u>DLHYDSSGYLDY</u> WGQGTLLTVSS	7

2.20A8 VL	DIQMTQSPSSVSASVGDRVITITCRASQGIRSWLAWYQQKPGKAPKLL IY <u>AI</u>SR<u>LQ</u>SGVPSRFSGSGSGTDFTLTISLQPEDFATYYC<u>QQ</u>ANSF <u>PLT</u>FGGGTKVEIK	8
3.15G8 VH	EVQLVESGGGLVQPGGSLRLSCAASGFTFS <u>SY</u>WMSWVRQAPGKGLEW VAN <u>NIKQDGG</u>KEY<u>YVD</u>SVKGR RFTISRDNAKNSLFLQMNSLRAEDTAVY YCAR <u>VQ</u>LYSDY FDYWGQGTLVTVSS	9
3.15G8 VL	DIQMTQSPSSVSASVGDRVITITCRASQGISSWLAWYQQKSGKAPKLL IY <u>AASGLQ</u> SGVPSRFSGSGSGTDFTLTISLQPEDLATYYC <u>QQ</u>SHSL <u>PPT</u> FGGGTKVEIK	10
3.18G1 VH	EVQLLESGGDLVQPGGSLRLSCAASGFTFN <u>SY</u>AMS WVRQAPGKGLEW VS <u>TISGSGGFTFSADSVKGR</u> RFTISRDNKNTLFLQMNSLRVEDSAVY SCAK <u>VLVGFNNGCWDY</u> WGQGTLVTVSS	11
3.18G1 VL	SYVLTQPPSVSVAPGQTARITC <u>GGNNIGSKSVH</u> WYQQKPGQAPVLV Y <u>DDSDRPS</u> SGIPERFSGSNSGNTATLTISRVEAGDEADYYC <u>QVWDSSN</u> <u>DHVV</u> FGGGTKLTVL	12
2.7A4 OPT VH	EVQLVESGGGLVKPGGSLRLSCAASGFTFS <u>TY</u>SMNWVRQAPGKGLEW VS <u>SISSSGDYIYYADSVKGR</u> RFTISRDNAKNSLYLQMNSLRAEDTAVY YCAR <u>DLVTSMVAFDY</u> WGQGTLVTVSS	13
2.7A4 OPT VL	SYELTQPPSVSVSPGQTARITC <u>SGDALPQKYVF</u> WYQQKSGQAPVLVI Y <u>EDSKRPS</u> SGIPERFSGSSSGTMTATLTISGAQVEDEADYYC <u>YSTDRSG</u> <u>NHRV</u> FGGGTKLTVL	14
2.14H9 OPT VH	EVQLVESGGGLVQPGGSLRLSCAASGFTFS <u>RY</u>WMSWVRQAPGKGLEW VAN <u>NIKQD</u>GSEK<u>YVD</u>SVKGR RFTISRDNAKNSLYLQMNSLRAEDTAVY YCAR <u>EGGWFGELAFDY</u> WGQGTLVTVSS	15
2.14H9 OPT VL	EIVLTQSPGTLISLSPGERATLSC <u>RASQ</u>RVSSSYLAWYQQKPGQAPRL LIY <u>DASSRAT</u> GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC <u>QQYGS</u> <u>LPWT</u> FGGGTKVEIK	16
1E12 VH (anti-PD- L1)	EVKLQESGPS LVKPSQTLSTCSVT <u>GYSIT</u> <u>SDYWN</u> WIRKF PGNKLEYVGY <u>ISYTGSTYYN</u> <u>PSLKS</u> RISIT RDTSKNQYYL QLNSVTSEDATYYCARY <u>GG</u> <u>WLSPF</u> FDYWGQ GTTLTVSS	17
1E12 VL (anti-PD- L1)	DIVMTQSHKL MSTSVGDRVS ITC <u>KASQDVG</u> <u>TAVA</u> WYQQKP GQSPKLLIYW <u>ASTRHT</u> GVPD RFTGSGSGTD FTLTISNVQS EDLADYFC <u>QQ</u> <u>DSSYPLT</u> FGA GTKVELK	18
1F4 VH (anti-PD- L1)	EVQLQESGPG LVAPSQSLSTCTVS <u>GFSLT</u> <u>TYSIN</u> WIRQP PGKGLEWLG <u>V</u> <u>MWAGG</u>TNSN <u>SVLKS</u> RLIIS KDNSKSQVFL KMNSLQTDDETARYYCARY <u>YG</u> <u>NSPYYA</u> IDYW GQGTSTVTVSS	19
1F4 VL (anti-PD- L1)	DIVTTQSHKL MSTSVGDRVS ITC <u>KASQDVG</u> <u>TAVA</u> WYQQKP GQSPKLLIYW <u>ASTRHT</u> GVPD RFTGSGSGTD FTLTISNVQS EDLADYFC <u>QQ</u> <u>DSSYPLT</u> FGA GTKVELK	20
2G11 VH (anti-PD- L1)	EVKLQESGPS LVKPSQTLSTCSVT <u>GYSII</u> <u>SDYWN</u> WIRKF PGNKLEYLG <u>Y</u> <u>ISYTGSTYYN</u> <u>PSLKS</u> RISIT RDTSKNQYYL	21

L1)	QLNSVTTEDT ATYYCARRGG <u>WLLPF</u> DYWGQ GTTLTVSS	
2G11 VL (anti-PD-L1)	DIVMTQSPSS LAVSVGEKVS MGCKSSQSL <u>YSSNQKNSLA</u> WYQQKPGQSP KLLIDWASTR <u>ESGVPDRFTG</u> SGSGTDFTLT ISSVKAEDLA VYYCQYYGY <u>PLTF</u> GAGTKL ELK	22
3B6 VH (anti-PD-L1)	EVKLQESGPS LVKPGASVKL SCKASGYTFT <u>SYDIN</u> WVKQR PGQGLEWIGW <u>IFPRDNNTKY</u> <u>NENFKG</u> KATL TVDTSSTAY MELHSLTSED SAVYFCTKEN <u>WVGDF</u> DYWGQ GTTLTLSS	23
3B6 VL (anti-PD-L1)	DIVMTQSPAI MSASPGEKVT MTCSSASSSIR YMHWYQQKPG TSPKRWISDT <u>SKLTS</u> GVPAR FSGSGSGTSY ALTISSMEAE DAATYYC <u>HQR</u> <u>SSYPWTF</u> GGG TKLEIK	24
3D10 VH (anti-PD-L1)	EVQLQQSGPD LVTPGASVRI SCQASGYTFP <u>DYYMN</u> WVKQS HGKSLEWIGD <u>IDPNYGGTTY</u> <u>NQKFKG</u> KAIL TVDRSSSTAY MELRSLTSED SAVYYCARGA <u>LTDWGQ</u> GTSL TVSS	25
3D10 VL (anti-PD-L1)	QIVLSQSPAI LSASPGEKVT MTCRASSSVS <u>YIYWF</u> QQKPG SSPKPWIYAT <u>FNLAS</u> GVPAR FSGSGSGTSY SLTISRVE DAATYYCQOW <u>SNNPLT</u> FGAG TKLEIK	26
IE3 VH (anti-PD-1)	EVQLQQSGPV LVKPGASVKM SCKASGYTFT <u>DYYMN</u> WVKQS HGKSLEWIGN <u>INPYNGGTTY</u> <u>NQKFKG</u> KATL TVDKSSRTAY MEINSLTSED SAVYYCARGR <u>IYDGS</u> LDYWG QGTALTSS	27
IE3 VL (anti-PD-1)	DIQMTQFPSS LCASQGGKVT VTC <u>KASQDIN</u> <u>NYMAWY</u> QHQP GKGPRLLIHY <u>TSTLLS</u> GIPS RFGSGSGGRD YSFSISNLEP EDIATYYC <u>LQ</u> <u>YDNLWT</u> FGG TKLEIK	28
1E8 VH (anti-PD-1)	QVQLQQSGAE LAKPGASVRL SCKASGYTFT <u>NYWMH</u> WVKQR PGQGLEWIGH <u>INPSSGFTTY</u> <u>NQNFKD</u> KATL TADKSSNTAY MQLSSLTYED SAVYFCARE <u>D</u> <u>YVDY</u> WGQGT TLTSS	29
1E8 VL (anti-PD-1)	DIVMTQSQKF MSTSVGDRVS VTC <u>KASQSV</u> D <u>TNVAWY</u> QQKP GQSPKALIFS <u>ASYRYS</u> GVPD RFTGSGSGTD FTLTINSVQS EDLAEIFC <u>QQ</u> <u>YNSYPY</u> TFGS GTKLEIK	30
1H3VH (anti-PD-1)	EVQLVESGGG LVKPGGSLKL SCAAS <u>GFTFS</u> <u>DYGMH</u> WVRQA PEKGLEWVAY <u>ISSGSYTIYY</u> <u>TDTVKG</u> RFTI SRDNAKNTLF LQMTSLRSED TAMYYCARRG <u>YGSFYEYYFD</u> YWGQGTTLTV SS	31
1H3 VL (anti-PD-1)	QIVLTQSPAL MSASPGEKVT MTC <u>SASSSVS</u> <u>YMYWY</u> QQKPR SSPKPWIY <u>LT</u> <u>SNLAS</u> GVPAR FSGSGSGTSY SLTISSMEAE DAATYYCQOW <u>SSNPFT</u> FGSG TKLEIK	32
Humanized Anti-Human B7-H1 Heavy Chain HC1_1	QVQLVQSGAEVKKPGASVKVSCKASGYTFPDYIMNWVRQAPGQGLEW MGDIDPNYGGTNYAQKFQGRVTMTSDTSISTAYMELSLRSDDTAVY YCARGALTDWGQGTMTVSS	33

Humanized Anti-Human B7-H1 Heavy Chain HC1_2	QVQLVQSGAEVKKPGASVKVSCKASGYTFPDYYMNWVRQAPGQSLEW MGDIDPNYGGTNYNQKFQGRVTMTTRDTSISTAYMELSRLSDDTAVY YCARGALTDWGQGTMTVTVSS	34
Humanized Anti-Human B7-H1 Heavy Chain HC1_3	EVQLVQSGAEVKKPGASVKVSCKASGYTFPDYYMNWVRQAPGQSLEW MGDIDPNYGGTNYNQKFQGRVTMTVDRSSSTAYMELSRLSDDTAVY YCARGALTDWGQGTMTVTVSS	35
Humanized Anti-Human B7-H1 Heavy Chain HC2_1	EVQLVESGGGLVQPGRSLRLSCTASGYTFPDYYMNWVRQAPGKGLEW VGIDIDPNYGGTTYAASVKGRFTISVDRSKSIAYLQMSSLKTEDTAVY YCTRGALTDWGQGTMTVTVSS	36
Humanized Anti-Human B7-H1 Heavy Chain HC2_2	EVQLVESGGGLVQPGRSLRLSCTASGYTFPDYYMNWVRQAPG KGLEWVGIDIDPNYGGTTYNASVKGRFTISVDRSKSIAYLQMSS LKTEDTAVYYCARGALTDWGQGTMTVTVSS	37
Humanized Anti-Human B7-H1 Heavy Chain HC2_3	EVQLVESGGGLVQPGRSLRLSCTASGYTFPDYYMNWVRQAPGKGLEW VGIDIDPNYGGTTYNQSVKGRFTISVDRSKSIAYLQMSSLKTEDTAVY YCARGALTDWGQGTMTVTVSS	38
Humanized Anti-Human B7-H1 Light Chain LC1_1	EIVLTQSPATLSLSPGERATLSCRASSSVSYIYWFQQKPGQAPRLLI YAAFNRATGIPARFSGSGSGTDYTLTISSELEPEDFAVYYCQQWSNNP LTFGQGTKVEIK	39
Humanized Anti-Human B7-H1 Light Chain LC1_2	EIVLTQSPATLSLSPGERATLSCRASSSVSYIYWFQQKPGQSPRPLI YAAFNRATGIPARFSGSGSGTDYTLTISSELEPEDFAVYYCQQWSNNP LTFGQGTKVEIK	40
Humanized Anti-Human B7-H1 Light Chain LC1_3	QIVLTQSPATLSLSPGERATLSCRASSSVSYIYWFQQKPGQSPRPLI YATFNLASGIPARFSGSGSGTSYTLTISRLEPEDFAVYYCQQWSNNP LTFGQGTKVEIK	41
Humanized	DIQLTQSPSSLSASVGDRVTITCRASSGVSYIYWFQQKPGKAPKLLI	42

Anti-Human B7-H1 Light Chain LC2_1	YAAFNLASGVPSRFSGSGSGTEYTLTISSLQPEDFATYYCQQWSNNP LTFGQGTKVEIK	
Humanized Anti-Human B7-H1 Light Chain LC2_2	DIQLTQSPSSLSASVGDRVTITCRASSGVSYIYWFQQKPGKAPKPLI YAAFNLASGVPSRFSGSGSGTEYTLTISSLQPEDFATYYCQQWSNNP LTFGQGTKVEIK	43
Humanized Anti-Human B7-H1 Light Chain LC2_3	DIQLTQSPSILSASVGDRVTITCRASSSVSYIYWFQQKPGKAPKPLI YATFNLASGVPSRFSGSGSGTSYTLTISSLQPEDFATYYCQQWSNNP LTFGQGTKVEIK	44
Humanized Anti- Human PD- 1 Heavy Chain HC1_1	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYGMHWVRQAPGKGLEW VSYISSGSSTIYYADSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVY YCARRGYGSFYEYYFDYWGQGTITVTVSS	45
Humanized Anti- Human PD- 1 Heavy Chain HC1_2	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYGMHWVRQAPGKGLEW VAYISSGSYTIYYADSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVY YCARRGYGSFYEYYFDYWGQGTITVTVSS	46
Humanized Anti- Human PD- 1 Heavy Chain HC1_3	EVQLVESGGGLVQPGGSLRLSCAASGFTFSDYGMHWVRQAPGKGLEW VAYISSGSYTIYSADSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVY YCARRGYGSFYEYYFDYWGQGTITVTVSS*	47
Humanized Anti- Human PD- 1 Heavy Chain HC2_1	QVQLVQSGAEVKKPGASVKVSCKASGFTFSDYGMHWVRQAPGQRLEW MGYISSGSSTIYYSQKFQGRVTITRDNSASTLYMELSSLRSED TAVY YCARRGYGSFYEYYFDYWGQGTITLVSS	48
Humanized Anti- Human PD- 1 Heavy Chain HC2_2	EVQLVQSGAEVKKPGASVKVSCAASGFTFSDYGMHWVRQAPGQRLEW MGYISSGSYTIYYSQKFQGRVTITRDNSASTLYMELSSLRSED TAVY YCARRGYGSFYEYYFDYWGQGTITLVSS	49
Humanized Anti- Human PD- 1 Heavy Chain HC2_3	EVQLVQSGAEVKKPGASVKVSCAASGFTFSDYGMHWVRQAPGQRLEW VAYISSGSYTIYYSQKFQGRVTITRDNSASTLYMELSSLRSED TAVY YCARRGYGSFYEYYFDYWGQGTITLVSS	50

Humanized Anti-Human PD-1 Light Chain LC1_1	EIVLTQSPATLSLSPGERATLSCRASSSVSYMYWYQQKPGQAPRLLI YLASNRATGIPARFSGSGSGTDYTLTISSELEPEDFAVYYCQQWSSNP FTFGQGKLEIK	51																																													
Humanized Anti-Human PD-1 Light Chain LC1_2	QIVLTQSPATLSLSPGERATLSCSASSSVSYMYWYQQKPGQAPRLLI YLTSNRATGIPARFSGSGSGTDYTLTISSELEPEDFAVYYCQQWSSNP FTFGQGKLEIK	52																																													
Humanized Anti-Human PD-1 Light Chain LC2_1	DIQLTQSPSSLSASVGDRVTITCRASSSVSYMYWYQQKPGKAPKLLI YLASNLASGVPSRFSGSGSGTEYTLTISSELEPEDFATYYCQQWSSNP FTFGQGKLEIK	53																																													
Humanized Anti-Human PD-1 Light Chain LC2_2	QIQLTQSPSSLSASVGDRVTITCSASSSVSYMYWYQQKPGKAPKLLI YLTSNLASGVPSRFSGSGSGTEYTLTISSELEPEDFATYYCQQWSSNP FTFGQGKLEIK	54																																													
Certain Specific Combinations	<table> <thead> <tr> <th>Antibody</th><th>Light Chain</th><th>Heavy Chain</th></tr> </thead> <tbody> <tr><td>h1H3 Var 1</td><td>LC1_1</td><td>HC1_1</td></tr> <tr><td>h1H3 Var 2</td><td>LC1_1</td><td>HC1_2</td></tr> <tr><td>h1H3 Var 3</td><td>LC1_1</td><td>HC1_3</td></tr> <tr><td>h1H3 Var 4</td><td>LC1_2</td><td>HC1_1</td></tr> <tr><td>h1H3 Var 5</td><td>LC1_2</td><td>HC1_2</td></tr> <tr><td>h1H3 Var 6</td><td>LC1_2</td><td>HC1_3</td></tr> <tr><td>h1H3 Var 7</td><td>LC2_1</td><td>HC2_1</td></tr> <tr><td>h1H3 Var 8</td><td>LC2_1</td><td>HC2_2</td></tr> <tr><td>h1H3 Var 9</td><td>LC2_1</td><td>HC2_3</td></tr> <tr><td>h1H3 Var 10</td><td>LC2_2</td><td>HC2_1</td></tr> <tr><td>h1H3 Var 11</td><td>LC2_2</td><td>HC2_2</td></tr> <tr><td>h1H3 Var 12</td><td>LC2_2</td><td>HC2_2</td></tr> <tr><td>h1H3 Var 13</td><td>LC1_1</td><td>HC2_1</td></tr> <tr><td>h1H3 Var 14</td><td>LC2_1</td><td>HC1_1</td></tr> </tbody> </table>	Antibody	Light Chain	Heavy Chain	h1H3 Var 1	LC1_1	HC1_1	h1H3 Var 2	LC1_1	HC1_2	h1H3 Var 3	LC1_1	HC1_3	h1H3 Var 4	LC1_2	HC1_1	h1H3 Var 5	LC1_2	HC1_2	h1H3 Var 6	LC1_2	HC1_3	h1H3 Var 7	LC2_1	HC2_1	h1H3 Var 8	LC2_1	HC2_2	h1H3 Var 9	LC2_1	HC2_3	h1H3 Var 10	LC2_2	HC2_1	h1H3 Var 11	LC2_2	HC2_2	h1H3 Var 12	LC2_2	HC2_2	h1H3 Var 13	LC1_1	HC2_1	h1H3 Var 14	LC2_1	HC1_1	
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h1H3 Var 1	LC1_1	HC1_1																																													
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h1H3 Var 4	LC1_2	HC1_1																																													
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h1H3 Var 13	LC1_1	HC2_1																																													
h1H3 Var 14	LC2_1	HC1_1																																													
17D8 VH (#1)	QVQLVESGGDVVQPGGSLRLSCAASGVAFSNYGMHWVRQAPGKGLEW VAVIWDGSKNYADSVKGRFTISRDN SKNMLYLQMNSLR AEDTAMY YCARNDYWGQGT LVT VSS	55																																													

17D8 VL (#8)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI IYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNW PLTFGGGTKVEIK	56
2D3 VH (#2)	QVQLVESGGDVVQPGRSLRLSCAASGLTFTNYGFHWVRQAPGKGLEW VAVIWDGSKKYYADSVKGRFTISRDN SKNTLYLQMNNLRAEDTAVY YCATGDDYWGQGTLLTVSS	57
2D3 VL (#9)	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI IYDTSNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNW PLTFGGGTKVEIK	58
4H1 VH 3	QVYLVESGGGVVQPGRSLRLSCAASGFTFSNYGMHWVRQAPGKGLEW VALIWDGSKKYYADSVKGRFTISRDN SKNTLYLQMTSLRVEDTAVY YCASNVDPHWGQGTLLTVSS	59
4H1 VL 10	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI IYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQSSNW PRTFGQGTKVEIK	60
5C4 VH 4 BMS- 936558	QVQLVESGGGVVQPGRSLRLDCKASGITFSNSGMHWVRQAPGKGLEW VAVIWDGSKRYADSVKGRFTISRDN SKNTLFLQMNSLRAEDTAVY YCATNDDYWGQGTLLTVSS	61
5C4 VL 11 BMS- 936558	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI IYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQSSNW PRTFGQGTKVEIK	62
4A11 VH 5	QLQLQESGPGLVKPSETLSLTCTVSGGSLSRSSFFWGWIRQPPGKGL EWIGSIYYSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAV YYCVRDYLITGDEDDYWGQGTLLTVSS	63
4A11 VL 12	DIQMTQSPSSLSASVGRVSIITCRASQGIISSWLAWYQQKPEKAPKSL IYAASNLRSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQYYSY PRTFGQGTKVEIK	64
7D3 VH 6	QVQLVESGGGVVQPGRSLRLSCTTSGITFSSYGFHWVRQAPGKGLEW VAVIWDGSKKYYADSVKGRFTLSRDD SKNTLYLQMNSLRAEDTAVY YCVTGDDYWGQGTLLTVSS	65
7D3 VL 13	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLI IYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNW PLTFGGGTKVEIK	66
5F4 VH 7	QLQLQESGPGLVKPSETLSLTCSVSGGSLSRSSYFWGWIRQPPGKGL EWIASIFYSGETYFNPSLKSRVTISVDTSRNQFSLKLSSVTAADTAV YYCARDYDILITGDEDDYWGQGTLLTVSS	67
5F4 VL 14	DIQMTQSPSSLSASVGRVTITCRASQGIISSWLAWYQQKPEKAPKSL IYAASSLQSGVPSRFSGSGSGTDFTLTISLQPEDFATYYCQYYSY PRTFGQGTKVEIK	68
3G10 VH 15	QVQLVQSGAEVKKPGASVKVSCKASGYFTDYGFHWVRQAPGQGLEW MGWITAYNGNTNYAQKLQGRVTMTTDTSTSTVYMELRSLRSDDTAVY YCARDYFYGMDVWGQGTITVTVSS	69
3G10 VL 25	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLVWYQQKPGQAPRLI IYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNW	70

	PRTFGQGTKVEIK	
12A4 VH 16 BMS- 936559	QVQLVQSGAEVKKPGSSVKV SCKTSGDTFSTY AISWVRQAPGQGLEW MGGIIP IFGKAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVY FCARKFHFVSGSPFGMDVWGQGT TTVTVSS	71
12A4 VL 26 BMS-93655	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLL IYDASN RATGIPARFSGSGSGTDFTLT ISSLEPEDFAVYY	72
10A5 VH 17	QVQLVQSGAEVKKPGASVKV SCKASGYTFTSYDVHWVRQAPGQRLEW MGWLHADTGITKFSQKFQGRVTITRDT SASTAYMELSSLRSEDTAVY YCARERIQLWFDYWGQGT LTVTVSS	73
10A5 VL 27	DIQMTQSPSSLSASVGDRV TITCRASQGISSWLAWYQQKPEKAPKSL IYAASSLQSGVP SRFSGSGSGTDFTLT ISSLQPEDFATYYCQQYNSY PYTFGQGTKLEIK	74
5F8 VH 18	QVQLVQSGAEVKKPGSSVKV SCKVSGGIFSTYAINWVRQAPGQGLEW MGGIIP IFGTANHAQKFQGRVTITADESTSTAYMELSSLRSEDTAVY YCARDQGIAAALFDYWGQGT LTVTVSS	75
5F8 28	EIVLTQSPGTL SLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRL LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGS SPWTFGQGTKVEIK	76
10H10 VH 19	EVQLVESGGGLVQPGRSLRLSCAVSGFTFDDYVHWVRQAPGKGLEW VSGISGNSGNIGYADSVKGRFTISRDN AKNSLYLQMNSLRAEDTALY YCAVPFDYWGQGT LTVTVSS	77
10H10 29	DIQMTQSPSSLSASVGDRV TITCRASQGISSWLAWYQQKPEKAPKSL IYAASSLQSGVP SRFSGSGSGTDFTLT ISSLQPEDFATYYCQQYNSY PYTFGQGTKLEIK	78
1B12 VH 20	QVQLVQSGAEVKKPGSSVKV SCKTSGDTFSSY AISWVRQAPGQGLEW MGGIIP IFGRAHYAQKFQGRVTITADESTSTAYMELSSLRSEDTAVY FCARKFHFVSGSPFGMDVWGQGT TTVTVSS	79
1B12 30	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLL IYDASN RATGIPARFSGSGSGTDFTLT ISSLEPEDFAVYYCQQRSNW PTFGQGTKVEIK	80
7H1 VH 21	QVQLVQSGAEVKKPGSSVKV SCKTSGGT FSSY AISWVRQAPGQGLEW MGGIIP IFGKAHYAQKFQGRVTITADESTTTTAYMELSSLRSEDTAVY YCARKYDYVSGSPFGMDVWGQGT TTVTVSS	81
7H1 31	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLL IYDASN RATGIPARFSGSGSGTDFTLT ISSLEPEDFAVYYCQQRSNW PTFGQGTKVEIK*	82
11E6 VH 22	QVQLVQSGAEVKKPGSSVKV SCKASGGTFSSYAINWVRQAPGQGLEW MGGIIP IFGSANYAQKFQDRVTITADESTSAAYMELSSLRSEDTAVY YCARDSSGWSRY YMDVWGQGT TTVTVSS	83
11E6 32	EIVLTQSPGTL SLSPGERATLSCRASQSVSSSYLAWYQQKPGQAPRL LIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGS SPFGGGTKVEIK*	84
12B7 VH 23	QVQLVQSGAEVKEPGSSVKV SCKASGGTFNSY AISWVRQAPGQGLEW MGGIIP LFGIAHYAQKFQGRVTITADESTNTAYMDLSSLRSEDTAVY	85

	YCARKYSYVSGSPFGMDVWGQGTITVTVSS	
12B7 33	EIVLTQSPATLSLSPGERATLSCRASQSVSSYLAWYQQKPGQAPRLL IYDASNRAITGIPARFSGSGSGTDFTLTISSELEPEDFAVYYCQQRSNW PTFGQGTRLEIK	86
13G4 VH 24	EVQLVESGGGLVQPGRSLRLSCAASGITFDDYGMHWVRQAPGKGLEW VSGISWNRGRIEYADSVKGRFTISRDNKNSLYLQMNSLRAEDTALY YCAKGRFRYFDWFLDYWGQGTITVTVSS	87
13G4 34	AIQLTQSPSSLSASVGRVTITCRASQGIISSALAWYQQKPGKAPKLL IYDASSLESQVPSRFSGSGSGTDFTLTISSELEPEDFATYYCQQFNSY PFTFGPGTKVDIK	88
28-8 VH 35	METGLRWLLLVAVLKGVQCLSVESGGRLVTPGTPLTLTCTASGFTI TNYHMFWRQAPGKGLEWIGVITSSGIGSSSTYYATWAKGRFTISK TSTTVNLRIITPTTEDTATYFCARDYFTNTYYALDIWGPGLTVTVSS	89
28-8 VL 36	MDTRAPTQLLGLLLWLPGARCALVMTQTPSSTSTAVGGTVTIKCA SQSISVYLAWYQQKPGQPPKLLIYSASTLASGVPSRFKGSRSRGTEYT LTISGVQREDAATYYCLGSAGS	90

DESCRIPTION OF THE EMBODIMENTS

I. Methods of Treatment

[030] The present methods encompass treatment of cancer employing at least one dose or radiation therapy and at least one dose of at least one PD-1 and/or PD-L1 antagonist. For instance, a method of treating cancer in a patient may include administering at least one dose of radiation therapy and administering at least one PD-1 and/or PD-L1 antagonist, wherein the at least one PD-1 and/or PD-L1 antagonist is administered on the same day as a dose of radiation therapy or up to and including 4 days later.

[031] The method of treating cancer may be administered once (as a single treatment cycle) or it may be administered more than once (i.e., with multiple treatment cycles), such as on a weekly, every other week, every three weeks, or every month cycle. If the method is administered more than once, it may be administered 2, 3, 4, 5, 6, 7, or 8 times, or more.

[032] While not being bound by theory, in a range of established syngeneic tumor models we found that low doses of fractionated RT led to upregulation of tumor cell expression

of PD-L1 *in vivo*. We show that fractionated RT delivered in combination with α PD-1 or α PD-L1 mAbs generated effective anti-tumor CD8⁺ responses, which improved local tumor control and long-term survival, and protected against tumor rechallenge by the induction of a tumor antigen-specific memory immune response. CD8⁺ T cell production of IFN γ was found to be responsible for the upregulation of PD-L1 on tumor cells following fractionated RT.

Furthermore, scheduling of anti-PD-L1 mAb relative to the delivery of fractionated RT appeared to affect therapeutic outcome; with administration of the antagonist on the same day as RT or up to and including 4 days after the first dose of fractionated RT showing benefit over administration of the antagonist more than 7 days after the conclusion of radiation therapy. And, while not being bound by theory, upregulation of tumor cell PD-L1 expression in response to low doses of fractionated RT, as used routinely in the clinic, appears to be a mechanism of adaptive immunological resistance by tumor cells; with the PD-L1/PD-1 signalling axis therefore potentially contributing to RT treatment failure. Combination therapy with RT and blockade of the PD-1/ PD-L1 signalling axis has the potential to overcome this resistance but, based on our preclinical studies, dosage timing impacts the treatment results, providing important new insights for translation to the clinic.

A. PD-1 and/or PD-L1 Antagonists for Use in Methods of Treatment

[033] In one embodiment, the at least one PD-1 and/or PD-L1 antagonist for use the present methods is an anti-PD-1 and/or anti-PD-L1 antibody or functional part thereof.

[034] In one aspect, the at least one PD-1 and/or PD-L1 antagonist is administered on the same day as a dose of radiation therapy or up to and including 4 days later. For example, in one aspect, if radiation therapy is provided on day 1 of a treatment cycle, the at least one PD-1 and/or PD-L1 antagonist may also be administered on day 1 of the treatment cycle (i.e., the same

day as a dose of radiation therapy). In another aspect, if radiation therapy is provided on day 1 of a treatment cycle, the at least one PD-1 and/or PD-L1 antagonist is administered on day 5 (i.e., 4 days later). In a further aspect, the PD-1 and/or PD-L1 antagonist may be administered on day 1, day 2, day 3, day 4, day 5, day 6, and/or day 7 of a treatment cycle (including both single and multiple treatment schedules).

[035] In one embodiment, the at least one PD-1 and/or PD-L1 antagonist is administered multiple times in a treatment cycle. In another embodiment, the at least one PD-1 and/or PD-L1 antagonist is administered 2, 3, 4, 5 or more times in a treatment cycle. For example, the antagonist may be administered three times a week for a one-week treatment cycle, or three times a week for a treatment cycle with two or more weeks as described above.

[036] In one aspect, the antibody or functional part thereof is chosen from those disclosed in US Publication 2010/0028330, which is incorporated by reference for the teaching of these antibodies and functional parts thereof. In one embodiment, the antibody is MEDI4736.

[037] In another embodiment, the anti-PD-1 and/or anti-PD-L1 antibody is pembrolizumab, nivolumab, BMS-936558, AMP-224, or MPDL3280A.

[038] The antibodies or functional parts thereof are administered in therapeutically effective amounts. Generally, a therapeutically effective amount may vary with the subject's age, condition, and sex, as well as the severity of the medical condition of the subject. A therapeutically effective amount of an antibody or functional part thereof ranges from about 0.001 to about 30 mg/kg body weight, from about 0.01 to about 25 mg/kg body weight, from about 0.1 to about 20 mg/kg body weight, or from about 1 to about 10 mg/kg. The dosage may be adjusted, as necessary, to suit observed effects of the treatment. The appropriate dose is chosen based on clinical indications by a treating physician.

[039] The antibodies may be given as a bolus dose, to maximize the circulating levels of antibodies for the greatest length of time after the dose. Continuous infusion may also be used after the bolus dose.

[040] As used herein, the term antibody or functional part thereof is used in the broadest sense. It may be man-made such as monoclonal antibodies (mAbs) produced by conventional hybridoma technology, recombinant technology and/or a functional fragment thereof. It may include both intact immunoglobulin molecules for example a polyclonal antibody, a monoclonal antibody (mAb), a monospecific antibody, a bispecific antibody, a polyspecific antibody, a human antibody, a humanized antibody, an animal antibody (e.g. camelid antibody), chimeric antibodies, as well as portions, fragments, regions, peptides and derivatives thereof (provided by any known technique, such as, but not limited to, enzymatic cleavage, peptide synthesis, or recombinant techniques), such as, for example, immunoglobulin devoid of light chains, Fab, Fab', F(ab')₂, Fv, scFv, antibody fragment, diabody, Fd, CDR regions, or any portion or peptide sequence of the antibody that is capable of binding antigen or epitope. In one embodiment, the functional part is a single chain antibody, a single chain variable fragment (scFv), a Fab fragment, or a F(ab')₂ fragment.

[041] An antibody or functional part is said to be “capable of binding” a molecule if it is capable of specifically reacting with the molecule to thereby bind the molecule to the antibody. Antibody fragments or portions may lack the Fc fragment of intact antibody, clear more rapidly from the circulation, and may have less non-specific tissue binding than an intact antibody. Examples of antibody may be produced from intact antibodies using methods well known in the art, for example by proteolytic cleavage with enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')₂ fragments). Portions of antibodies may be made by any of the

above methods, or may be made by expressing a portion of the recombinant molecule. For example, the CDR region(s) of a recombinant antibody may be isolated and subcloned into an appropriate expression vector.

[042] In one embodiment, an antibody or functional part is a human antibody. The use of human antibodies for human therapy may diminish the chance of side effects due to an immunological reaction in a human individual against nonhuman sequences. In another embodiment, the antibody or functional part is humanized. In another embodiment, an antibody or functional part is a chimeric antibody. This way, sequences of interest, such as for instance a binding site of interest, can be included into an antibody or functional part.

[043] In one embodiment, the antibody may have an IgG, IgA, IgM, or IgE isotype. In one embodiment, the antibody is an IgG.

B. Radiation Therapy for Use in Methods of Treatment

[044] Radiation therapy, also known as high-dose ionizing irradiation, is a component of the present therapeutic approach.

[045] In one mode, the radiation therapy is fractionated radiation therapy. In one embodiment, the fractionated radiation therapy comprises from 2 to 7 fractions. In another embodiment, the fractionated radiation therapy comprises from 3 to 6 fractions. In another embodiment, the fractionated radiation therapy comprises from 4 to 5 fractions. In one mode, the fractionated radiation therapy comprises 2, 3, 4, 5, 6, or 7 fractions. In one embodiment, the fractionated radiation therapy comprises 5 fractions.

[046] In one mode, the radiation therapy fractions are administered in sequential days. In one mode, radiation therapy may include more than one dose on a day and/or doses on sequential days. In one mode, the radiation therapy fractions are administered on day 1, day 2,

day 3, day 4, and day 5. In another mode, the radiation therapy comprises about 10 Gy in 5 fractions (i.e., 2 Gy on each of 5 days).

[047] Other fractionation schedules may be employed including accelerated fractionation (treatment given in larger daily or weekly doses to reduce the number of weeks of treatment), hyperfractionation (smaller doses of radiation given more than once a day), or hypofractionation (larger doses given once a day or less often to reduce the number of treatments).

[048] The radiation therapy may be x-rays, gamma rays, or charged particles. The radiation therapy may be external-beam radiation therapy or internal radiation therapy (also called brachytherapy). Systemic radiation therapy, using radioactive substances, such as radioactive iodine, may also be employed.

[049] External-beam radiation therapy includes 3D conformational radiation therapy, intensity-modulated radiation therapy, image-guided radiation therapy, tomotherapy, stereotactic radiosurgery, proton therapy, or other charged particle beams.

C. Cancers for Treatment

[050] The present methods may be used to treat a variety of types of cancer. In one aspect, the method may be used to treat melanoma, colorectal cancer, or breast cancer. In one embodiment, the breast cancer is triple negative breast cancer.

[051] In one embodiment, the cancer is cancer is adrenocortical tumors, adrenal cancer, anal cancer, bile duct cancer, bladder cancer, bone cancer, brain cancer, breast cancer, central nervous system cancer, cervical cancer, chest cancer, colon cancer, colorectal cancer, endometrial cancer, epidermoid carcinoma, esophageal cancer, eye cancer, glioblastoma, glioma, gallbladder cancer, gastrointestinal carcinoid tumors, gastrointestinal stromal tumor, gestational

trophoblastic disease, head and neck, Hodgkin disease, Kaposi sarcoma, kidney cancer, laryngeal and hypopharyngeal cancer, leukemia, liver cancer (such as hepatocellular carcinoma), lung cancer (including non-small cell, small cell, and lung carcinoid tumors), lymph node cancer, lymphoma, lymphoma of the skin, melanoma, mesothelioma, mouth cancer, multiple myeloma, nasal cavity and paranasal sinus cancer, nasopharyngeal cancer, neuroblastoma, non-Hodgkin lymphoma, oral cavity and oropharyngeal cancer, osteosarcoma, ovarian cancer, pancreatic cancer, penile cancer, pituitary cancer, prostate cancer, pediatric malignancies, rectal cancer, retinoblastoma, rhabdomyosarcoma, salivary gland, sarcoma, skin cancer, small intestine cancer, stomach cancer, testicular cancer, thymus cancer, throat cancer, thyroid cancer, uterine cancer, vaginal cancer, or vulvar cancers.

[052] Reference will now be made in detail to the present exemplary embodiments, examples of which are illustrated in the accompanying drawings. Wherever possible, the same reference numbers will be used throughout the drawings to refer to the same or like parts. Other embodiments will be apparent to those skilled in the art from consideration of the specification and practice disclosed herein. The embodiments are further explained in the following examples. These examples do not limit the scope of the claims, but merely serve to clarify certain embodiments. It is intended that the specification and examples be considered as exemplary only, with a true scope and spirit being indicated by the following claims.

EXAMPLES

Example 1. Methods

A) Mice and cell lines

[053] BALB/c and C57Bl/6 mice were obtained from Harlan, U.K.. All animal experiments were approved by a local ethical committee and performed under a United Kingdom Home Office license. CT26 murine colon carcinoma cells (ATCC) and 4434 cells isolated from

BRafV600E p16^{-/-} mice (Richard Marias, Cancer Research UK, Manchester Institute) were maintained in DMEM, and 4T1 triple negative breast cancers (ATCC) maintained in RPMI-1640, supplemented with 10 % FCS, 1 % L-glutamine (Invitrogen, U.K.). All cell lines were routinely screened to confirm the absence of *Mycoplasma* contamination.

B) Tumor therapy

[054] Mice were inoculated sub-cutaneously (s.c.) with either 5x10⁵ CT26, 1x10⁵ 4T1 or 5x10⁶ 4434 cells. Irradiations were performed 7-10 days after inoculation (when tumors were at least 100 mm³) using a Pantak HF-320 320 kV x-ray unit (Gulmay Medical, U.K.). The machine was operated at 300 kV, 9.2 mA, with filtration fitted in the x-ray beam to give a radiation quality of 2.3 mm Cu half-value layer. Mice were positioned at a distance of 350 mm from the x-ray focus, where the dose rate was 0.80 Gy/min. Administration of αPD-1 (clone RMPI-14), αPD-L1 (clone 10F.9G2) (both Biolegend) or isotype control mAb (IgG2a and IgG2b respectively) commenced on day 1 of the fractionated RT cycle (unless otherwise stated) and was administered intra-peritoneally (i.p.) 3qw for up to 3 weeks at a dose of 10 mg/kg in a dose volume of 100 μl/10 g in PBS. For cellular and cytokine depletion experiments mice received either αCD8 mAb; clone YTS169 (a gift from M. Glennie, Southampton University), αCD4 mAb; clone GK1.5 (Biolegend), αAsialo-GM1 (Wako Chemicals) or αIFNγ; clone XMG1.2 (BioXcell). Peripheral blood was sampled during therapy to confirm cellular depletion. For tumor rechallenge experiments, long-term surviving (LTS) mice were implanted contralaterally with tumor cells a minimum of 100 days after previous tumor implantation. Additional control mice were also implanted to confirm tumor growth. Experimental groups contained at least 5 mice / group and are representative at least 2 independent experiments.

C) Measurement of cytokine production by CD8⁺ T cells isolated from long-term surviving mice

[055] For *in vitro* stimulation 3.5×10^6 splenocytes from either LTS or control mice were cultured for 5 days in RPMI-1640 supplemented with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 % L-glutamine, 50 µM 2-ME and 10 IU/ml human recombinant IL-2 in the presence of either 1×10^6 tumor cells irradiated with 50 Gy or 1 µmol/ml of the H2-Ld restricted peptides SPSYVYHQF (SEQ ID NO: 91) (AH1) / TPHPARIGL (SEQ ID NO: 92) (β-galactosidase) (Anaspec, U.K.). Experimental groups contained 3-5 mice and are representative of 2 independent experiments. After 5 days in culture, cells were restimulated at a 1:1 ratio with 50 Gy irradiated tumor cells for 16 hours in the presence of 3 µg/ml Brefeldin A (BD Pharmingen, U.K.) and 100 IU/ml human recombinant IL-2 (Chiron, NL). For FACS analysis, cells were washed and incubated with rat anti-CD16/32 (eBioscience, U.K.) to block non-specific binding and then stained with a FITC conjugated anti-CD8α mAb (eBioscience, U.K.). Cells were then fixed/permeabilized and stained for expression of IFNγ using an APC conjugated mAb (eBioscience, U.K.).

D) Tumor and immune cell phenotyping by flow cytometry

[056] To obtain single cell suspensions, tumors were processed using a gentleMacs dissociator and a murine tumor dissociation kit (Miltenyi Biotec, U.K.). For analysis, non-specific binding was blocked as described above and expression of CD4, CD8 (BD Biosciences, UK), CD45, NKP46, PD-1 and PD-L1 examined by multi-parameter flow cytometry (all eBioscience unless otherwise stated).

E) *In vitro* co-cultures

[057] Tumor cells were cultured in the presence of 20ng/ml IFNγ and/or TNFα for 24 hours prior to evaluation of PD-L1 expression by flow cytometry as described above. For co-

culture assay either resting or activated splenocytes (treated with PBS or phorbol 12-Myristate 13-Acetate and Ionomycin cell stimulation cocktail respectively, (eBioscience, UK)) were co-cultured at a 1:1 ratio with tumor cells and tumor cell expression of PD-L1 determined as described above. Silencing of IFN γ R1 expression was achieved by lentiviral transduction of cells with ShRNA (cells were also transduced with non-targeting ShRNA as controls) (Thermo Scientific, UK). Measurement of splenocyte cytokine production (IFN γ and TNF α) was measured by intracellular flow cytometry as described above.

Example 2. Blockade of PD-1 or PD-L1 enhances the therapeutic efficacy of RT

[058] Radiation therapy has been shown to modulate the immunogenicity of tumor cells but is rarely able to generate durable therapeutic responses that lead to systemic anti-tumor immunity alone. We show that low doses of local fractionated dose RT delivered as about 10 Gy in 5 fractions leads to increased tumor cell expression of PD-L1 with elevated expression evident 1, 3 and 5 days after the last dose of RT when compared to time-matched, non-treated (NT) mice (Figure 1A and B). This RT-mediated increase in tumor cell PD-L1 expression peaks at 72 hours after the last dose of RT and although it declines significantly by 7 days post RT (when compared to expression at days 1 and 3; $P < 0.05$ and $P < 0.01$ respectively, Mann-Whitney test) remains elevated when compared to NT mice ($P < 0.05$, Mann-Whitney test).

[059] Given these observations we hypothesized that the immune response generated following RT may be limited through the PD-1/PD-L1 axis. Local RT delivered as about 10 Gy in 5 daily fractions was found to significantly improve survival in mice bearing established CT26 tumors when compared to NT controls (Figure 1C and D; $P < 0.05$ Log-rank; Mantel-Cox test). Our data demonstrate that this RT-mediated local tumor control can be substantially improved through combination with either α PD-L1 mAb or α PD-1 mAb (Figure 1C and D; $P < 0.001$ Log-rank; Mantel-Cox test). Combined therapy led to a synergistic anti-tumor response which was

curative in 66% and 80% of mice that received RT in combination with α PD-L1 mAb or α PD-1 mAb respectively. In contrast to combination with RT, monotherapy with either α PD-L1 mAb or α PD-1 mAb did not significantly improve survival (Figure 1C and D; $P > 0.05$ Log-rank; Mantel-Cox test). Further, no significant benefit was observed when mice received RT in combination with both α PD-1 and α PD-L1 mAb vs. combination with either mAb alone (Figure 7).

[060] Blockade of PD-L1 also improved response to RT in mice bearing established 4T1 tumors where combined therapy significantly reduced tumor burden by 38% when compared to RT alone (Figure 1E; 10 days post start of therapy; $184.3 \pm 13.5 \text{ mm}^2$ vs. $292.8 \pm 14.3 \text{ mm}^2$ respectively, $P < 0.01$ Mann-Whitney test) and significantly improved survival ($P < 0.001$ Log-rank; Mantel-Cox test; data not shown). Similar results were also observed in mice bearing established 4434 melanomas (Figure 1F). Combination therapy with local RT and mAbs targeting either PD-1 or PD-L1 was well tolerated in both BALB/c and C57Bl/6 mice (Figure 8A and B). These preclinical data clearly demonstrate the potential to improve outcome following low dose fractionated RT of established solid tumors through blockade of the PD-1/PD-L1 axis.

Example 3. NK cells contribute to local tumor control following combination therapy but long-term survival is dependent on CD8⁺ T cells

[061] We next investigated the mechanisms underlying the long-term tumor control observed following combination RT and α PD-L1 mAb therapy. Initially colony forming assays were used to confirm that α PD-1 and α PD-L1 mAb were not acting as radiation sensitizers through direct interaction with tumor cells (Figure 9A-C). Using depleting antibodies, we next explored the role of effector T cells and NK cells in mediating anti-tumor activity following RT/ α PD-L1 mAb combination therapy. Our data demonstrate that as early as 7 days after the start of a 5 day fractionated RT cycle (with α PD-L1 mAb therapy commencing on day 1 of the

RT cycle) reduced tumor burden is evident in mice following combination therapy when compared to NT mice ($207.5 \pm 29.2 \text{ mm}^2$ vs. $409.4 \pm 86.88 \text{ mm}^2$ respectively; $P = 0.067$, Mann-Whitney U test) (Figure 2A). However, this statistical trend to reduced volume was lost following depletion of either CD8⁺ T cells or NK cells, where tumor volumes were not significantly different from those in NT cohorts ($P = 0.52$ and $P = 0.70$ respectively, Mann-Whitney U test) but significantly larger than mice that received combination therapy without immune cell depletion ($P < 0.01$; combined therapy vs. CD8 depletion, and $P < 0.05$; combined therapy vs. NK cell depletion, Mann-Whitney U test). By day 11 post treatment, combination therapy significantly reduces tumor burden when compared to NT controls ($P < 0.001$, Mann-Whitney U test). Whilst the depletion of either CD8⁺ T cells or NK cells at this time point reduces the efficacy of combination therapy ($P < 0.001$ and $P < 0.05$ respectively, Mann-Whitney U test), the relative contribution of CD8⁺ T cells and NK cells becomes more evident with significantly reduced tumor control in CD8 vs. NK cell depleted mice ($P < 0.05$, Mann-Whitney U test). Our data also reveal that the depletion of CD4⁺ T cells improved local tumor control following combination therapy ($153.2 \pm 27.0 \text{ mm}^2$ vs. $72.7 \pm 17.3 \text{ mm}^2$ respectively; $P < 0.05$, Mann-Whitney U test).

[062] In contrast to early tumor control following combination RT/ α PD-L1 mAb therapy, long term survival (LTS) was not impacted by the depletion of NK cells (70% LTS mice following treatment with RT/ α PD-L1 vs. 77.8% following combination therapy with NK cell depletion) (Figure 2B). The depletion of CD4⁺ T lymphocytes increased the frequency of LTS mice from 70% to 87.5% (combination vs. combination + CD4 depletion) but this did not achieve significance ($P > 0.05$ Log-rank; Mantel-Cox test). However, the depletion of CD8⁺ T cells completely abrogated the therapeutic efficacy of combination RT/ α PD-L1 mAb therapy (P

< 0.001 Log-rank; Mantel-Cox test). Depletion of immune cell populations was confirmed by flow cytometry on peripheral blood samples (Figure. 2C). These data suggest that whilst NK cells may exert some local tumor control this does not impact overall survival and while CD4⁺ T cells are dispensable or even capable of suppressing responses, CD8⁺ T cells appear to mediate effective long term tumor control following treatment with RT and α PD-L1 mAb.

Example 4. Treatment with α PD-L1 mAb and RT generates protective tumor antigen-specific memory T cell responses

[063] We next investigated whether immunological memory was generated following treatment with RT and α PD-L1 mAb. We show that LTS mice originally treated with RT and α PD-L1 mAb were able to completely reject tumors following contralateral rechallenge (Figure 3A). To quantify this memory response splenocytes were harvested from LTS mice that had greater than 100 days of disease free survival and the capacity of CD8⁺ T cells to produce IFN γ following co-culture with peptide derived from a CT26 tumor associated antigen (AH1: SPSYVYHQF (SEQ ID NO: 91)), control peptide (β -galactosidase: TPHARIGL (SEQ ID NO: 93)) or with irradiated CT26 cells was assessed (Figure 3B and C). Our data reveal that LTS mice were endowed with a significantly greater frequency of IFN γ -producing CD8⁺ T lymphocytes following co-culture with AH1 peptide than naïve mice (6.6 % $\pm$ 0.8 vs 2.3 % $\pm$ 0.2 respectively; P < 0.05, Mann-Whitney test). A similar response was observed following co-culture of splenocytes with CT26 cells. Comparison of peptide and tumor cell co-cultures revealed that the frequency of memory CD8⁺ T cells was ~3 fold lower in LTS mice following co-culture with tumor cells than with AH1 peptide and may reflect tumor cell-mediated suppression of T cell activation. Taken together these data demonstrate that RT when combined with blockade of the PD-1 / PD-L1 axis in mouse models can generate protective immunological memory in long-term survivors.

Example 5. Fractionated RT leads to CD8⁺ T cell dependent adaptive upregulation of tumor cell PD-L1 expression

[064] Initially we confirmed that treatment of tumor cells with a range of RT doses *in vitro* did not have any direct impact on expression of PD-L1 (Figure 4A). To identify which cellular populations within the tumor microenvironment were responsible for modulating tumor cell expression of PD-L1 mice received a fractionated RT cycle in combination with CD8, CD4 or NK cell depleting antibodies. Our data reveal that the depletion of CD8⁺ T cells but not NK cells completely abrogates the RT-mediated upregulation of PD-L1 on tumor cells (Figure 4B and C). Interestingly, the depletion of CD4⁺ T cells was found to further augment RT-mediated upregulation of PD-L1 on tumor cells (~ 2 fold compared to treatment with RT alone).

Example 6. Adaptive upregulation of PD-L1 by tumor cells following fractionated RT is IFN γ dependent

[065] Given the clinical correlation between IFN γ in the tumor microenvironment and PD-L1 expression (16) and the impact of TNF α on this response (22) we evaluated the impact of these cytokines on PD-L1 in our cell lines. Co-culture of tumor cells with recombinant IFN γ leads to a significant 20 fold increase in cell surface expression of PD-L1 *in vitro* (Figure 5A). Furthermore, whilst addition of recombinant TNF α alone has no impact on tumor cell expression of PD-L1, a cocktail of both IFN γ and TNF α can further augment tumor cell PD-L1 expression (~2 fold) when compared to expression with IFN γ alone (Figure 5A). We show that silencing of IFN- γ -receptor 1 (IFN γ R1) on CT26 tumor cells using ShRNA completely abrogates the upregulation of PD-L1 following co-culture with either recombinant IFN γ or both IFN γ and TNF α (Figure 5A).

[066] To establish whether activated immune cells could lead to an adaptive change in tumor cell expression of PD-L1 through the production of IFN γ and TNF α , WT CT26 cells and those transduced with either non-targeting ShRNA (NTC ShRNA) and IFN γ R1 ShRNA were co-

cultured with isolated resting (PBS-treated) and activated (phorbol 12-myristate 13-acetate (PMA) and ionomycin-treated) splenocytes (Figure 5B and C). Importantly, these experiments demonstrate that activated immune cells can elevate tumor cell PD-L1 expression at physiologically relevant concentrations of IFN γ and TNF α , in an IFN γ R1-dependent manner.

[067] To confirm the role of IFN γ on tumor cell expression of PD-L1 in response to RT *in vivo*, mice received RT in combination with α IFN γ blocking antibody (or isotype control). IFN γ blockade reduced tumor cell expression of PD-L1 by 2.6 fold in NT mice to the level observed on CT26 cells cultured *in vitro*; suggesting adaptive upregulation of PD-L1 occurs following implantation of tumor (Figure 5D and E). However, the significant upregulation of PD-L1 observed following RT (2.4 fold compared to NT control) was completely abrogated by IFN γ blockade confirming this as the driver of adaptive tumor cell expression of PD-L1 following RT treatment.

Example 7. Concomitant scheduling of α PD-L1 mAb and RT generates an effective anti-tumor immune response

[068] We evaluated 3 distinct combination schedules where mice bearing established CT26 tumors received a fractionated RT cycle of about 10 Gy in 5 fractions with administration of α PD-L1 mAb commencing either on day 1 of the fractionated RT cycle (schedule A), day 5 of the cycle (schedule B) or 7 days after completion of RT (schedule C) (Figure 6A). No significant difference in overall survival was found between schedule A and B with LTS of 60% and 57 respectively ($P > 0.05$ Log-rank; Mantel-Cox test) (Figure 6B and Figure 10A). In contrast, sequential treatment with RT followed 7 days later by α PD-L1 mAb (Schedule C) was completely ineffective at enhancing overall survival when compared to RT alone (median survival of 30 days vs. 35 days respectively; $P > 0.05$ Log-rank; Mantel-Cox test) despite similar tumor burden across groups (Figure 10B and C).

[069] Analysis of tumor infiltrating CD4⁺ and CD8⁺ T cells 24 hours after the last dose of RT reveal increased expression of PD-1 when compared to time-matched NT controls ($P < 0.05$, Mann-Whitney test) (Figure 6C). In contrast, by 7 days post RT no change in PD-1 expression was evident on CD4⁺ T cells and was found to be significantly decreased on CD8⁺ T cells when compared to time-matched NT controls ($P < 0.05$, Mann-Whitney test) (Figure 6D). Expression of PD-1 was consistently found to be higher on tumor infiltrating CD8⁺ than CD4⁺ T cells (Figure 6C and D). We next compared activity following an acute vs. extended α PD-L1 mAb dosing protocol where mice received either 3 doses of mAb concurrent with RT or the same with dosing extended (3qw) for an additional 2 weeks. No additional benefit was observed with extended α PD-L1 mAb administration (Figure 11).

[070] Taken together these data demonstrate that treatment with low dose fractionated RT leads to an acute increase in PD-1 expression on T cells and that sequential therapy where blockade of the PD-1/PD-L1 signalling axis is delayed until completion of an RT cycle may be ineffective potentially due to deletion or anergy of tumor-reactive CD8⁺ T cells.

Example 8. Discussion of Preceding Examples

[071] Low doses of fractionated RT leads to upregulation of PD-L1 expression on tumor cells secondary to CD8⁺ T cell production of IFN γ . In mouse models of melanoma, colorectal and breast cancer we demonstrate that the activity of RT can be enhanced through combination with α PD-L1 mAb, leading to the generation of immunological memory in long-term surviving mice which is capable of protecting against tumor recurrence. Furthermore, our data reveal that dose scheduling may affect outcomes, with concurrent but not sequential therapy effective at improving local tumor control and survival.

[072] This is the first preclinical study to demonstrate that fractionated RT leads to increased tumor cell expression of PD-L1 through CD8⁺ T cell production of IFN γ . Here, tumor

cell expression of PD-L1 may act as a biomarker of a local anti-tumor response, suggesting that local RT may be sufficient to prime CD8⁺ T cell responses. However, whilst treatment with RT alone was unable to generate durable anti-cancer immunity, activity was found to be enhanced through mAb-mediated blockade of either PD-1 or PD-L1 suggesting that signalling through this axis may limit the immune response to RT. We saw no significant differences in overall survival between RT delivered in combination with either α PD-1 or α PD-L1, or a combination of both mAb. Given this observation it seems likely that the activity of these mAbs when delivered in combination with RT is through blockade of PD-1/PD-L1 signalling, and not mediated through the PD-L1/CD80 or PD-1/PD-L2 axes, although further experiments are needed to confirm this.

[073] Our preclinical data reveal that the depletion of NK cells reduced local tumor control at early time points (up to 11 days post completion of RT) but did not impact on overall survival. In addition, the depletion of NK cells did not impact tumor cell expression of PD-L1 following RT suggesting their limited contribution to local IFN γ production. In contrast, whilst depletion of CD4⁺ T cells did not impact survival following combination therapy, substantial increases in tumor cell expression of PD-L1 post RT was observed. The depletion of CD4⁺ T cells impacts both helper T cell and Treg populations. Although further studies are needed to delineate the relative contributions of these sub-populations of CD4⁺ T cells we can speculate that CD4⁺ helper T cells may be dispensable for the generation of effective CD8⁺ T cell responses post RT/PD-L1 mAb therapy in our model and that either helper T cells, or more likely Treg, may be actively suppressing IFN γ production in the local tumor milieu. Therefore, the therapeutic potential to enhance anti-tumor responses by Treg depletion may be at least in part attenuated through enhanced tumor cell expression of PD-L1.

[074] PD-L1 expression can be modulated by a number of cytokines including type-1 and 2 IFN, TNF α and TGF β (16, 22, 30-32). We show that tumor cell expression of PD-L1 can be enhanced following co-culture with IFN γ and that addition of TNF α can further augment this response. However, blockade of IFN γ R1 or *in vivo* depletion of IFN γ demonstrates dependence on IFN γ mediated signalling for upregulation of PD-L1, with TNF α alone incapable of modulating tumor cell PD-L1 expression. Interestingly, *in vivo* depletion of IFN γ reduces PD-L1 expression on syngeneic tumor cells, matching the expression profile of tumor cells *in vitro*. This suggests that the local tumor microenvironment in the absence of therapeutic intervention may foster immunologically driven tumor adaptation which may support tumor development. Similarly, in human melanocytic lesions expression of PD-L1 has been shown to co-localize with areas of CD8⁺ T cell infiltration and is postulated to represent an adaptive mechanism of immune escape (16). However, in our preclinical mouse models monotherapy with mAbs targeting PD-1/PD-L1 only demonstrates modest activity, suggesting that targeting this axis alone may be unlikely to bring about durable anti-tumor immune responses in all settings and underscores the requirement for a combinatorial approach.

[075] In contrast to the use of a single 12 Gy dose (30), our results demonstrate that upregulation of PD-L1 occurs at much lower biologically effective doses involving delivery of fractionated RT using about 10 Gy in 5 daily fractions. This is an important finding given that this dose per fraction is more commonly used in routine clinical practice. Several studies have assessed the impact of RT dose fractionation; comparing single ablative doses, hypofractionated and fractionated RT, on the generation of anti-tumor immune responses and on the tumor microenvironment (4, 5, 25, 26, 33, 34). However, the results of these studies are equivocal and

optimal RT delivery and the impact of RT dose fractionation on tumor cell expression of PD-L1 requires further investigation.

[076] Previously, no research has addressed the impact of sequencing on the activity of RT and α PD-L1 mAb therapy. This is especially pertinent given the predisposition to adopting a sequenced combination regimen in the clinic as a strategy to maximize tolerability. We show that only blockade of PD-L1 at the time of RT delivery can enhance the therapeutic response in mouse models, with therapy 7 days after completion of RT no better than treatment with RT alone. In addition, our data reveal that PD-L1 expression is elevated by 24 hours after completion of a fractionated RT cycle and remains elevated for at least 7 days post completion. Moreover, we found elevated PD-1 expression on tumor infiltrating CD4⁺ and CD8⁺ T cells 24 hours post RT when compared to control cohorts. Expression of PD-1 was found to be consistently higher on CD8⁺ vs. CD4⁺ T cells. Taken together these data suggest that local fractionated RT can prime anti-tumor CD8⁺ T cell responses but that these may be attenuated by signalling through the PD-1/PD-L1 axis and early inhibition of this axis may generate durable effective anti-tumor responses. Further, the requirement for chronic vs. acute blockade of the PD-1/PD-L1 axis when combined with RT is unclear. We observed no difference in survival when α PD-L1 mAb was administered 3qw over 1 week concurrent with RT delivery vs. an extended schedule of up to 3 weeks. These data suggest that combination approaches with RT may permit a reduction in the duration of α PD-1/PD-L1 mAb therapy required to achieve therapeutic anti-tumor immune responses. Together these data have important implications for clinical trial design.

[077] In summary, this study demonstrates that treatment with fractionated RT leads to upregulation of tumor cell expression of PD-L1 and that blockade of the PD-1/PD-L1 axis can

enhance the immune response to fractionated RT in multiple syngeneic mouse models of cancer. Our data suggest that dose scheduling of α PD-L1 mAb with RT may generate therapeutic immune responses with the capacity to reduce tumor burden and improve survival. This therapeutic combination may be a promising approach for the treatment of many solid malignancies where RT is commonly used and translation to early phase clinical trial is clearly warranted.

Example 9. Treatment Protocols

[078] Patient A has colorectal cancer. In week 1, patient A receives an effective amount of radiation therapy in five fractions. In week 1, patient A also receives a therapeutic dose of MEDI4736 on day 1, day 3, and day 5. Patient A repeats this schedule for weeks 2, 3, 4, and 5.

[079] Patient B has breast cancer. In week 1, patient B receives an effective amount of radiation therapy. In week 1, patient B also receives a therapeutic dose of MEDI4736 on day 5. Patient repeats this schedule for weeks 3, 5, and 7.

Example 10. Certain Embodiments

[080] The following items provide certain embodiments disclosed herein.

[081] Item 1. A method of treating cancer in a patient comprising

- a. administering at least one dose of radiation therapy; and
- b. administering at least one PD-1 and/or PD-L1 antagonist,

wherein at least one PD-1 and/or PD-L1 antagonist is administered on the same day as a dose of radiation therapy or up to and including 4 days later.

[082] Item 2. The method of item 1, wherein the at least one PD-1 and/or PD-L1 antagonist is an at least one PD-1 and/or PD-L1 antibody or functional part thereof.

[083] Item 3. The method of any one of items 1-2, wherein the dose of radiation therapy is about 11 Gy or lower.

[084] Item 4. The method of item 3, wherein the dose of radiation therapy is about 10 Gy or lower.

[085] Item 5. The method of any one of items 1-4, wherein the radiation therapy is fractionated radiation therapy.

[086] Item 6. The method of item 5, wherein the fractionated radiation therapy comprises from 2 to 7 fractions.

[087] Item 7. The method of item 6, wherein the fractionated radiation therapy comprises 5 fractions.

[088] Item 8. The method of any one of items 5-7, wherein the radiation therapy fractions are administered in sequential days.

[089] Item 9. The method of any one of items 5-8, wherein the radiation therapy fractions are administered on day 1, day 2, day 3, day 4, and day 5.

[090] Item 10. The method of any one of items 7-9, wherein the radiation therapy comprises about 10 Gy in 5 fractions.

[091] Item 11. The method of any one of items 1-10, wherein the at least one PD-1 and/or PD-L1 antagonist is administered on day 1.

[092] Item 12. The method of any one of items 1-11, wherein the at least one PD-1 and/or PD-L1 antagonist is administered on day 5.

[093] Item 13. The method of any one of items 1-12, wherein the at least one PD-1 and/or PD-L1 antagonist is administered multiple times.

[094] Item 14. The method of any one of items 1-13, wherein the at least one PD-1 and/or PD-L1 antagonist is administered 3 times a week.

[095] Item 15. The method of any one of items 1-14, wherein the anti-PD-1 and/or PD-L1 antibody or functional part thereof is MEDI4736.

[096] Item 16. The method of any one of items 1-15, wherein the anti-PD-1 and/or anti-PD-L1 antibody or functional part thereof is pembrolizumab, nivolumab, BMS-936558, AMP-224, or MPDL3280A.

[097] Item 17. The method of any one of items 1-16, wherein the cancer is melanoma, colorectal cancer, or breast cancer.

[098] Item 18. The method of any one of items 1-17, wherein more than one treatment cycle is performed.

[099] Item 19. The method of item 18, wherein from 2-8 treatment cycles are performed.

[0100] Item 20. The method of any one of items 18-19, wherein the treatment cycles are weekly.

[0101] Item 21. The method of any one of items 18-19, wherein the treatment cycles are every other week.

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EQUIVALENTS

[0135] The foregoing written specification is considered to be sufficient to enable one skilled in the art to practice the embodiments. The foregoing description and Examples detail certain embodiments and describes the best mode contemplated by the inventors. It will be appreciated, however, that no matter how detailed the foregoing may appear in text, the embodiments may be practiced in many ways and the claims include any equivalents thereof.

[0136] As used herein, the term about refers to a numeric value, including, for example, whole numbers, fractions, and percentages, whether or not explicitly indicated. The term about generally refers to a range of numerical values (e.g., $\pm 5\text{-}10\%$ of the recited value) that one of ordinary skill in the art would consider equivalent to the recited value (e.g., having the same function or result). In some instances, the term about may include numerical values that are rounded to the nearest significant figure.

WHAT IS CLAIMED IS:

1. Use of at least one PD-1 and/or PD-L1 antibody in the manufacture of a medicament for treating cancer in a patient, wherein the medicament is to be used in a treatment cycle wherein:
 - (a) the patient is to be administered with fractionated radiation therapy,
wherein the fractionated radiation therapy comprises 5 radiation therapy fractions, and a dose of each radiation therapy fraction is about 2 Gy,
wherein the total dose of fractionated radiation therapy is about 10 Gy or lower;
and
 - (b) the medicament is to be administered on the same day as a radiation therapy fraction or up to and including 4 days later, and wherein the medicament is to be administered 3 times a week and,
wherein the treatment cycle is to be performed every other week.
2. The use according to claim 1, wherein the radiation therapy fractions are to be administered on sequential days.
3. The use according to claim 2, wherein the radiation therapy fractions are to be administered on day 1, day 2, day 3, day 4, and day 5.
4. The use according to claim 1, wherein the medicament is to be administered on day 1 of the fractionated radiation therapy.
5. The use according to claim 1, wherein the medicament is to be administered on day 5 of the fractionated radiation therapy.
6. The use according to claim 1, wherein the anti-PD-1 and/or PD-L1 antibody is durvalumab (MEDI4736).
7. The use according to claim 1, wherein the anti-PD-1 and/or anti-PD-L1 antibody is pembrolizumab, nivolumab (BMS-936558), or atezolizumab (MPDL3280A).
8. The use according to claim 1, wherein the cancer is melanoma, colorectal cancer or breast cancer.
9. The use according to claim 1, wherein more than one treatment cycle is to be performed.

10. The use according to claim 9, wherein from 2-8 treatment cycles are to be performed.