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**Supplemental Figure S9.**

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**Supplemental Figure Legend**

**Figure S1. Activity of anti-FRα clones in an indirect cytotox assay.**

Serial dilutions (in triplicate) of anti-FR- antibodies in culture medium that contained the maximal non-toxic concentration of anti-murine Fab-DM4 were added to cells (1x103/well to reach a total volume of 200 L/well). The plates were incubated for 5 days at 37⁰C, 6% CO2. Cell viability was determined by the WST-8 assay (Donjindo Molecular Technologies, Inc.) in accordance with the manufacturer’s protocol. IC50 of clones vs. number of clones are plotted.

**Figure S2.** **The antibody-dependent cell-mediated cytotoxicity of M9346A antibody against Igrov-1 cells *in vitro*.**

The activity was measured with Igrov-1 target cells and purified human natural killer cells by lactate dehydrogenase (LDH) release (Cytotoxicity Detection Kit Roche) after 4-hour incubation at 37⁰C. Ratio of the target cells to natural killer cells was 1:4. The percentage of specific lysis was determined as % lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) x 100, where spontaneous release is LDH release of natural killer cells alone, and maximum release is LDH release of the target cells incubated with 1% Triton X-100.

**Figure S3. Activity of the M9346A antibody against KB xenograft tumors.**

Animals with established tumors of about 130 mm3 were intravenously treated with single injection of the M9346Aantibody at 5 mg/kg x1 (opened circles) on day 6 post inoculation or at 10 mg/kg qw x 3 (filled circles) on day 6, 13 and 20 post inoculation, or PBS (opened triangles). Mean tumor volumes in mm3 versus time (in days) after cell inoculation plotted.

**Figure S4. The bystander cytotoxic activity of IMGN853.**

A. Cytotoxicity of IMGN853 for FR-positive FR-300-19 cells and FR-negative 300-19 cells was measured to choose a concentration of the conjugate, cytotoxic for the former, but non-cytotoxic for the latter, to be used in bystander cytotoxic activity experiments. The cells were exposed to the conjugate for four days, and the cell survival was determined by WST-8 assay. The percent of surviving cells is plotted against IMGN853 molar concentration. The dotted line indicates the chosen concentration of IMGN853. The experiment was repeated three times, each experiment was run in triplicate, and representative results are shown.

B. The effect of the presence of FR-positive FR -300-19 cells on the survival of FR-negative 300-19 cells following their exposure to IMGN853. The two cell populations were plated either separately or together in culture medium containing 5 nM IMGN853, incubated for 4 days at 37⁰C in a humidified incubator with 5% CO2, and the cell survival was determined by WST-8 assay. The experiment was repeated three times, each experiment was run in triplicate, and representative results are shown.

**Figure 5. In vitro cytotoxicity of IMGN853 against KB cells (short exposure)**

KB cells were plated on 96-well flat-bottom plates at a density of 1x103 cells/well and cultured overnight. Dilutions of the conjugate in the culture medium supplemented or not with 2 µM M9346A were added to cells; the plates were then incubated at 37oC for four hours, after which the medium was replaced by fresh culture medium containing no test-agents, and cultured for an additional five day period at 37⁰C, 6% CO2. Cell viability was determined by the WST-8 assay and IC50 were generated using a sigmoidal dose-response (variable slope) nonlinear regression curve fit (GraphPad Software Inc). Log conjugate concentration *vs*. percent survival plotted.

**Figure S6. *In vitro* cytotoxicity of IMGN853, M9346A-SMCC-DM1 and non-targeting human IgG1-SMCC-DM1 against KB cells (continuous exposure).**

In vitro cytotoxicity was measured by WST-8 assay after a 5-day exposure of KB cells to IMGN853 (with or without blocking concentration of M9346A), M9346A-SMCC-DM1 or nonbinding huIgG1-SMCC-DM1.

**Figure S7. Catabolites of IMGN853 formed in KB cells *in vitro*.**

KB cells were exposed to 3[H]-IMGN853 for 30 min at 37⁰C, washed extensively, and incubated in culture medium for 22 h at 37⁰C. Protein-free cell and media extracts (processed conjugate) were analyzed by high pressure liquid chromatography C18 column from Vydak and Phenomenex, respectively. The effluent from the column was collected in 0.5 mL fractions, mixed with scintillation fluid, and monitored for tritium using a liquid scintillation counter. The counts per minute of tritium were then plotted vs. the elution time.

**Figure S8. Comparison of 3[H]-M9346A antibody and 3[H]-IMGN853 conjugate processing.**

KB cells were exposed to a saturating concentration of 3[H]-M9346A or 3[H]-IMGN853, incubated at 37⁰C for 22 h. The percentage of processed (proteolytically degraded) antibody was calculated from the amount of protein-free cell and media extract relative to the overall amount of antibody or conjugate (processed + unprocessed) bound to the cells.

**Figure S9.** **IMGN853 activity against KB xenograft tumors.**

Mice with established tumors of about 130 mm3 were intravenously treated with injections of IMGN853 at 50 µg of conjugated maytansinoid per kg (equivalent to 2.8 mg/kg antibody) on day 6 post inoculation (single dose) or on day 7, 14 and 21 post inoculation (multiple injections, qw x 3). Vehicle control PBS was injected on day 6 post inoculation. Mean tumor volumes in mm3 versus time (in days) after cell inoculation plotted.

**Supplemental Table S1.**

Maytansinoid per antibody ratio (MAR) and doses of conjugates (expressed in µg DM/kg and in mg total conjugate/kg) used in *in vivo* studies reported in Figures 1, 2 and 3.

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Experiment presented in Figure 1. Comparative in vivo activities of SMCC-DM1 conjugates of five humanized anti-FRα antibodies | | | | | |
|  | M9346A-SMCC-DM1 | huFR1-48-SMCC-DM1 | huFR1-49-SMCC-DM1 | huFR1-57-SMCC-DM | huFR1-65-SMCC-DM1 |
| µg DM1/kg | 200 | 200 | 200 | 200 | 200 |
| mg antibody/kg | 11 | 8 | 11 | 12 | 10 |
| MAR | 3.6 | 5.0 | 3.6 | 3.3 | 3.8 |

|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Experiments presented in Figure 2. Activity of M9346A-DM noncleavable and cleavable conjugates *in vivo* | | | | | | | | | | | | | | | |
| Xeno-graft | M9346A-SPP-DM1 | | | M9346A-SPDB-DM4 | | | M9346A-sulfo-SPDB-DM4 | | | M9346A-SMCC-DM1 | | | huIgG1-SPDB-DM4 | | |
| MAR | µg DM/kg | mg  Ab/kg | MAR | µg DM/kg | mg  Ab/kg | MAR | µg  DM/kg | mg  Ab/kg | MAR | µg DM/kg | mg  Ab/kg | MAR | µg  DM/kg | mg  Ab/kg |
| KB | 4.3 | 54 | 2.5 | 4.4 | 59 | 2.5 | 3.8 | 51 | 2.5 | 3.9 | 50 | 2.5 | 3.2 | 82 | 5.0 |
| Igrov-1 | 3.6 | 50 | 2.8 | 3.9 | 50 | 2.4 | 3.9 | 50 | 2.4 | NA\* | | | NA\* | | |
| Ovcar-3 | 3.6 | 50 | 2.8 | 3.9 | 50 | 2.4 | 3.9 | 50 | 2.4 |
| \*NA – not analyzed | | | | | | | | | | | | | | | |

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Experiments presented in Figure 3 IMGN853 activity against xenograft models derived from cell lines and a patient tumor | | | | | | |
| Xenograft | IMGN853 | | | | huIgG1-sulfo-SPDB-DM4 | |
| MAR | Dose 1, µg DM4/kg (mg Ab/kg) | Dose 2, µg DM4/kg (mg Ab/kg) | Dose 3, µg DM4/kg (mg Ab/kg) | MAR | Dose µg DM4/kg (mg Ab/kg) |
| Ovcar-3 | 3.9 | 25 (1.2) | 50 (2.4) | 100 (4.9) | NA | NA |
| Igrov-1 | 3.9 | 25 (1.2) | 50 (2.4) | 100 (4.9) | NA | NA |
| OV-90 | 3.9 | 25 (1.2) | 50 (2.4) | 100 (4.9) | NA | NA |
| SKOV-3 | 3.9 | 25 (1.2) | 50 (2.4) | 100 (4.9) | NA | NA |
| NCI-H2110 | 3.3 | 25 (1.4) | 50 (2.9) | 100 (5.7) | 3.1 | 100 (6.0) |
| LXFA-737 | 3.3 | 18 (1.0) | 44 (2.5) | 88 (5) | 3.5 | 90 (5.0) |
| NA – not analyzed | | | | | | |

**Supplemental Table S2A. Results of statistical analysis of the data reported in Fig. 1.**

**P-values using 2-tailed t-test for comparison of treatments versus PBS at day 20 post inoculation.**

|  |  |
| --- | --- |
| Comparator groups  (conjugate *vs* PBS) | P-value |
| FR-1-48/PBS | 0.002\* |
| FR-1-49/PBS | 0.0004\* |
| FR-1-57/PBS | 0.0005\* |
| FR-1-65/PBS | 0.003\* |
| M9346A/PBS | 0.0003\* |

\* Difference between the comparator groups is statistically significant

**Supplemental Table S2B. Results of statistical analysis of the data reported in Fig. 1.**

**P-values using 2-tailed t-test for comparison of treatments versus PBS at day 20 post inoculation.**

|  |  |
| --- | --- |
| Comparator groups  (pairs of conjugates) | P-value |
| M9346A/FR-1-48 | 0.02\* |
| M9346A/FR-1-49 | 0.003\* |
| M9346A/FR-1-57 | 0.04\* |
| M9346A/FR-1-65 | 0.04\* |

\* Difference between the comparator groups is statistically significant

**Supplemental Table S3. Results of the statistical analysis of the data reported in Fig. 2C.**

**P-values using 2-tailed t-test for comparison of treatments versus PBS at day 32 post inoculation.**

|  |  |
| --- | --- |
| Comparator groups | P-value |
| Sulfo-SPDB/PBS | 0.00002\* |
| Sulfo-SPDB /SPP | 0.01\* |
| Sulfo-SPDB /SPDB | 0.04\* |

\* Difference between the comparator groups is statistically significant

**Supplemental Table S4. Results of the statistical analysis of the data reported in Fig. 2D.**

**P-values using 2-tailed t-test for comparison of treatments versus PBS at day 50 post inoculation.**

|  |  |
| --- | --- |
| Comparator groups | P-value |
| Sulfo-SPDB/PBS | 0.003\* |
| Sulfo-SPDB /SPP | 0.02\* |
| Sulfo-SPDB /SPDB | 0.08\*\* |

\* Difference between the comparator groups is statistically significant

\*\* Difference between the comparator groups is statistically insignificant

**Supplemental Table S5. Results of the statistical analysis of the data reported in Fig. 2E.**

**P-values using 2-tailed t-test for comparison of treatments versus PBS at day 17 post inoculation.**

|  |  |
| --- | --- |
| Comparator groups | P-value |
| Sulfo-SPDB/PBS | 0.0008\* |
| Sulfo-SPDB /SPP | 0.1\*\* |
| Sulfo-SPDB /SPDB | 0.6\*\* |

\* Difference between the comparator groups is statistically significant

\*\* Difference between the comparator groups is statistically insignificant

**Supplemental Table S6. FRα expression by IHC in patient-derived samples**

| **Indication** | **Histological**  **subtype** | **Sample number (% positive)** | | | | |
| --- | --- | --- | --- | --- | --- | --- |
| **Total** | **3 heterogeneous and homogeneous** | **≥2 heterogeneous and homogeneous\*** | **≥1 heterogeneous and homogeneous\*\*** | **Any positivity** |
| **Ovarian tumor** | **Serous** | **129 (100)** | **44 (34)** | **92 (71)** | **92 (71)** | **100 (78)** |
| **Endometrioid** | **35 (100)** | **15 (43)** | **18 (51)** | **20 (57)** | **26 (74)** |
| **Clear Cell** | **5 (100)** | **1 (20)** | **1 (20)** | **1 (20)** | **3 (60)** |
| **Mucinous** | **29 (100)** | **2 (7)** | **4 (14)** | **5 (17)** | **5 (17)** |
| **Other** | **19 (100)** | **0 (0)** | **2 (11)** | **2 (11)** | **2 (11)** |
| **NSCLC** | **Adenocarcinoma** | **74 (100)** | **19 (26)** | **44 (59)** | **47 (64)** | **52 (70)** |
| **Adenosquamous** | **9 (100)** | **0 (0)** | **3 (33)** | **3 (33)** | **5 (56)** |
| **Large Cell** | **7 (100)** | **0 (0)** | **1 (14)** | **1 (14)** | **3 (43)** |
| **Squamous** | **74 (100)** | **1 (1)** | **4 (5)** | **6 (8)** | **12 (16)** |
| **Other** | **10 (100)** | **0 (0)** | **1 (10)** | **1 (10)** | **1 (10)** |

\*Includes samples with the staining intensity of 2 and 3

\*\*Includes samples with the staining intensity of 1, 2 and 3

**Supplemental material**

**3[H]-M9346A Processing by Tumor Cells**

A known number of cells at a concentration of approximately 4 x 105 cell/mL in culture medium were exposed to 25 nM of 3[H]-M9346A for 30 min at 37⁰C, then extensively washed with PBS to remove any unbound conjugate, resuspended in fresh medium, and incubated for 22 h at 37⁰C with 6% CO2. Then the cells and medium were harvested, and extracted with (1:3 v/v) acetone to separate soluble (protein-free) 3[H]-species from protein-associated 3[H]-species, and kept at -80⁰C for a minimum of 1 h to precipitate protein. The supernatants containing the protein-free 3H catabolites (processed) were dried using an evacuated centrifuge. The dried extracts (processed antibody) and the pellets (unprocessed antibody) were solubilized using Solvable (PerkinElmer) as described by the manufacturer, and radioactivity was determined by liquid scintillation counting. ABC values for cell lines were determined from the CPM associated with processed and unprocessed samples. The pmoles antibody processed per million cells was determined from the CPM of the protein-free extract. The counting efficiency of the scintillation spectrophotometer was 0.6. The specific antibody radioactivity, which was used in these calculations, was determined separately. The percentage of processed antibody after 37⁰C incubation was determined from the equation: CPMprocessed / (CPMprocessed + CPMunprocessed) x 100%.