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(54) Title: RNA APTAMERS AND USES THEREOF

(57) Abstract: Described herein and RNA aptamers that specifically bind to tumor-infiltrating myeloid cells and uses thereof.

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RNA APTAMERS AND USES THEREOF**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of priority to U.S. Provisional Application No. 62/815,142, filed March 7, 2019, the disclosure of which is incorporated herein by reference in its entirety.

STATEMENT ON U.S. GOVERNMENT INTEREST

[0002] This invention was made with government support under BC084337 awarded by The United States Department of Defense. The government has certain rights in the invention

**INCORPORATION BY REFERENCE OF MATERIALS SUBMITTED
ELECTRONICALLY**

[0003] This application contains, as a separate part of the disclosure, a Sequence Listing in computer readable form (Filename: 53952A_Seqlisting.txt; Size: 13,241 bytes; Created: March 2, 2020), which is incorporated by reference in its entirety.

BACKGROUND

[0004] Chemotherapy using different cytotoxic agents is the standard of care treatment for most human malignancies. However, these treatments are associated with moderate to serious toxicities that decrease the overall quality-of-life for patients with cancer and are often dose limiting and therefore responsible for modest therapeutic efficacy (1, 2). Drug delivery nanoplateforms aimed at concentrating the chemotherapeutic agent(s) at the tumor site(s) are gaining interest as modalities to decrease systemic toxicity and increase efficacy (2-4). However, most nanoplateforms rely either on physical properties of tumor vasculature, such as the enhanced permeability and retention (EPR) effect (5), or on the presence of markers characterizing the neoplastic cells. The EPR effect allows the preferential targeting of primary tumors and established metastases but is poorly effective against not-yet vascularized micro-metastases (6). Targeting an appropriate tumor-associated antigen allows drug delivery to disseminated neoplastic cells but may result in tumor editing (7, 8) rather than eradication, because of the genetic instability of neoplastic cells (9). An alternative and burgeoning strategy is the delivery of therapeutic agents to the genetically stable tumor stromal cells that characterize primary tumor, metastases, and pre-metastatic niches (10). Myeloid cells in the tumor microenvironment are an interesting target because they account for a large proportion

of the tumor mass, they promote cancer cell survival and metastases (11-16), provide immune protection, and are recruited early during tumor progression in the primary tumor and in the pre-metastatic niches (17). Importantly, tumor-infiltrating myeloid cells (TIMC, comprising Myeloid Derived Suppressor Cells -MDSC, Tumor Associated Macrophages -TAM, neutrophils, and monocytes) express a peculiar activated, pro-tumoral phenotype that differentiates them from their systemic counterpart (18-22). Since markers expressed on TIMC but not on their circulating counterparts remain to be fully defined, we used an unsupervised approach to generate RNA aptamers against known and unknown TIMC-specific epitopes (23).

[0005] RNA aptamers penetrate deeply into the tissues, are non-immunogenic, and are easy to manufacture and engineer for improved pharmacokinetic and stability. Furthermore, these reagents can be selected to be rapidly internalized in the target cells (24), thus allowing specific drug (*e.g.*, RNA therapeutics or small molecules) absorption in the desired cells. Finally, the combinatorial use of aptamers that each bind a different epitope in the target cell should increase the overall specificity and maximize drug delivery in the chosen tissue.

BRIEF DESCRIPTION OF THE FIGURES

[0006] Figure 1. Selection of polyclonal aptamers specific for tumor infiltrating myeloid cells. HT-Cell SELEX was performed on MSC2 cells treated with IL4 or left untreated as a proxy of tumor infiltrating and splenic myeloid cells, respectively. A) FACS analysis of IL4-treated MSC2 stained with the Cy3-labelled RNA aptamers from cycle 0, as control, or cycle 11. B) FACS analysis of MSC2 or IL4 treated MSC2 with Cy3-labelled polyclonal aptamers from the indicated library. C) Single cell suspensions from the spleen or the tumor of mice bearing the CT26 colon carcinoma (0.5 cm in diameter) were stained with the polyclonal aptamer library and counterstained with antibodies against CD11b, Gr1, F4/80, CD11c, CD19, CD49b, CD4, and CD8. Data derived from one experiment representative of one another.

[0007] Figure 2. Identification of monoclonal aptamers specific for tumor infiltrating myeloid cells. A) Single cell suspensions from spleens and tumors of mice pooled from 3 mice bearing the 4T1 tumor (0.5 cm in diameter) were labelled with antibodies against CD11b, Ly6C, Ly6G and the 15 Cy3-labelled monoclonal aptamers identified by APTANI. Data derived from n=5 biological replica and derived from n=2 independent experiments. Aptamers 3, 6, 11, and 14 were chosen for further analysis and are in bold. * = P<0.001 by

one-way ANOVA and post-hoc Holm-Sidak comparisons versus irrelevant aptamer. Only background staining was observed in the CD11b⁻ cells. B) Secondary structure of aptamers 3, 6, 11, and 14. Binding motifs identified by APTANI are underlined in black. Fluorinated nucleotides are highlighted in gray. C) An equimolar mixture of the aptamers increases the specificity for tumor infiltrating myeloid cells. Pooled single cell suspension of tumor or spleen from 4T1 bearing mice were labeled with aptamers 3, 6, 11, 14, or equimolar mixtures of each aptamer and analyzed by FACS. Data derived from n=3 independent experiments.

[0008] Figure 3. The selected RNA aptamers preferentially recognized human TIMC over the circulating myeloid cells from patients with recurrent HNSCC. A) Single cell suspension from the blood and the tumor of patients (n=3) with recurrent HNSCC was stained with AF647-labelled aptamers, anti-CD33, anti-CD14, anti-CD124 antibodies, and zombie vital dye and analyzed by flow cytometry. B) Image cytometry: Paraffin embedded tumor specimens from patients (n=5) with HNSCC undergoing salvage surgery were stained with an equimolar mixture of Cy3-labelled aptamer, FITC-anti-CD33 antibody, and DAPI and analyzed by cell profiler and FCS-express V6 after gating on the “tumor area” or “healthy tissue area” and either on CD33⁺ or CD33⁻ cells. Aptamer MFI was normalized on the MFI of the all the cells in the corresponding region of interest. Approximately 10⁶ and 10⁵ cells were analyzed in the “tumor area” or “healthy tissue area” respectively. Significant paired T test are reported.

[0009] Figure 4. Characteristics of aptamers that preferentially recognize tumor infiltrating myeloid cells. A) Affinity of the aptamers for IL4 treated MSC2 cells was determined by FACS. Putative targets (ANXA4 and VIM) were identified via aptamer-based immunoprecipitation, SDS page, and mass spec analysis. Kds against ligands was determined by FACS against recombinant protein conjugated to epoxy beads. B) Epoxy beads conjugated with ANXA4 or irrelevant protein were stained with Cy3-labelled aptamer 3 or irrelevant aptamer and analyzed by FACS. C) Epoxy beads conjugated with vimentin or irrelevant protein were stained with Cy3-labelled aptamer 11 or irrelevant aptamer and analyzed by FACS. D) ANXA4 competitive assay. 5x10⁵ IL4 treated MSC2 were stained with aptamer 3 in the presence or in the absence of recombinant ANXA4. E) MCS2 cells were transfected via the 4PD nanoparticle with shRNA against vimentin or with a scrambled shRNA. 4 days later cells were stained with Cy3-labelled aptamer 11 and DAPI. Pa= One-way ANOVA p value. Data derived from n=2 independent experiments.

[0010] Figure 5. Aptamers target preferentially tumor stroma *in vivo*. A) Aptamers target tumors *in vivo*. Mice bearing the 4T1-luciferase mammary carcinoma were injected intravenously with an equimolar mixture of biotinylated aptamers 3, 6, 11, and 14 conjugated with AF750 streptavidin. B) Bio-distribution was evaluated by IVIS 2h later. C) Time course analysis of aptamer bio-distribution performed by IVIS. D) Mice (n=5) bearing the 4T1 mammary carcinoma were injected i.v. with aptamers 3, 6, 11, and 14 loaded on AF-647 streptavidin. After two hours, the indicated organs were harvested, counterstained with antibodies against CD11b, CD19, CD49b, CD11c, and CD3. E, F) Mice (n=5) were treated as in D, and aptamer distribution was evaluated by flow cytometry at different time points after counterstaining the single cell suspensions with antibodies against CD11b, F4/80, Ly6C, and Ly6G. * = one-way Anova $p < 0.001$.

[0011] Figure 6. TIMC specific aptamers increase doxorubicin concentration at the tumor site. A) TIMC specific aptamers were conjugated to doxorubicin by extending the 3' end with a GC rich tail. B) Fluorescence spectra of doxorubicin solution (1.5 μM) with increasing molar ratios of the TIMC aptamer equimolar mixture (from top to bottom: 0, 0.008, 0.016, 0.032, 0.062, 0.125, 0.25, and 0.5 equivalent). Inset: A Hill plot for the aptamer titration ($K_d = 0.16$ equivalent; 6.2 dox molecules/aptamer). C) BALB/c mice (n=5) were challenged with the 4T1 mammary carcinoma orthotopically (4T1_o; primary tumor: breast, metastases: lung) or intravenously (4T1_{iv}; metastasis in the lung and in the liver). An additional group of mice was challenged orthotopically with the non-metastatic, 4T1 derived, cell line 67NR (only primary tumor). 10 days later, mice were treated intravenously with doxorubicin (0.35 mg/kg) conjugated with TIMC specific aptamer. Doxorubicin bio-distribution was evaluated 24 h later. Data derived from one experiment representative of another one. D) Bio-distribution of free doxorubicin or doxorubicin given via TIMC specific or control aptamer. Mice (n=5) bearing the 4T1 mammary carcinoma (0.5 cm in diameter) in the third mammary gland were given i.v. free doxorubicin (Dox high 3.5 mg/kg), TIMC-aptamer conjugated doxorubicin (TIMC apt; 0.35mg/kg) or irrelevant aptamer (Irr. Apt) conjugated doxorubicin. 24h later doxorubicin in the tissues was quantified by spectrometry after acid alcohol extraction. Data derived from 1 experiments representative of another one. * = $p < 0.05$ in multiple pairwise comparison vs dox high group (Holm-Sidak method).

[0012] Figure 7. TIMC-specific aptamers increase doxorubicin therapeutic index. A) Mice bearing the 4T1 tumor in the third mammary gland were treated i.v. with free doxorubicin (3.5 mg/kg or 0.35 mg/kg), with Doxil (0.35 mg/kg), or doxorubicin conjugated to TIMC-

specific aptamer mix (0.35 mg/kg). Additional controls included irrelevant aptamers conjugated with doxorubicin, unconjugated TIMC specific aptamers, and untreated mice. Treatment was repeated 2 and 6 days later. Mice were euthanized when tumor index reached 1.2 cm². Log-rank and post-hoc multiple comparison analysis (Holm-Sidak method) is reported. Weight loss as measure of toxicity is reported. C) BALB/c mice were challenged with the 4T1wt breast cancer or with the doxorubicin resistant variant dox-resistant 4T1. When tumor reached 0.5 cm in diameter, mice treated with TIMC specific aptamers or left untreated. Survival was monitored.

[0013] Figure 8. Aptamer effectively deliver CCR1 and 5 siRNA to tumor infiltrating myeloid cells and delay tumor progression. A) Structure of aptamer /siRNA chimera. B) Balb/c mice were challenged orthotopically with the 4T1 mammary carcinoma and treated intravenously with aptamer loaded with scrambled siRNAs (black bar) or with siRNA against CCR1 and CCR5 (30 picomoles, empty bar) 5, 7, 9,12,14,16 and 19 days after challenge. qRT-PCR for CCR1 and CCR5 mRNA was performed 6 days after the last treatment on tumor infiltrating myeloid cells. C) Tumor size 25 days after challenge.

[0014] Figure 9. Affinity of aptamers #3, #6, #11 and #14 for IL4 treated MSC2 or their putative ligand. A) 10⁵ IL4-treated MSC2 were stained with different concentration of the indicated, Cy3 labelled aptamers. Binding was evaluated by FACS. B) Different concentration of Cy3 labelled aptamer #3 and aptamer #4 were used to stain epoxybeads conjugated with recombinant ANXA4 or recombinant VIM, respectively. The Cy3 labelled aptamer against IL4Ra was used as a irrelevant control. Binding was evaluated by FACS. Data derived from three independent experiments.

[0015] Figure 10. Cluster analysis of the 15 aptamers chosen with APTANI. The variable regions of the 15 monoclonal aptamers chosen with APTANI were aligned with clustal-Ω and neighbor-joining tree generated with Jailview using default parameters.

[0016] Figure 11. Sequence, frequency, and motifs of 15 aptamers selected by APTANI based on an enrichment of at least 0.1% and the presence of at least three motifs or of at least one motif with a frequency higher than 0.1%.

[0017] Figure 12. Oligonucleotides used in the Examples.

[0018] Figure 13. List of mouse and human antibodies used in the Examples.

DETAILED DESCRIPTION

[0019] There present disclosure is based, in part, on the discovery of RNA aptamers that specifically recognize tumor infiltrating myeloid cells (TIMC) across different preclinical models and, more importantly, in humans.

[0020] Systemic chemotherapy remains one of the most important treatments for cancer therapy, but it comes with systemic side effects, such as cardiotoxicity and profound neutropenia that impose limits on the use of this therapeutic option (38). Additionally, chemotherapy induces long-term morbidity that decreases the quality of life of cancer survivors.

[0021] Described herein is a new mode by which chemotherapeutic or other therapeutic agents (e.g., RNA therapeutics) can be concentrated in the tumor and in the metastatic sites by physically targeting the pro-tumoral activated phenotype of tumor- infiltrating myeloid cells. Myeloid cells are the most abundant innate immune cells present in the stroma of several types of mouse and human cancer (39-41). The presence of myeloid cells in human tumors correlates with increased vascular density, higher metastatic spread, and a worse clinical outcome, and their presence is necessary for tumor progression (42, 43). At the tumor site, myeloid cells acquire unique antigenic profiles and functional characteristics that differentiate them from their systemically circulating cell counterparts, and are necessary for their immune suppressive, tolerogenic, and pro-tumoral roles (20, 44, 45). Although, many approaches have been and are being tested to inhibit the mechanisms that myeloid cells employ to promote tumor progression or their interaction with the neoplastic cells (46-48), to our knowledge no reagent able to discriminate between tumor infiltrating and circulating myeloid cells is yet available. Furthermore, although aptamers and peptides able to bind both tumor-associated and circulating MDSC in mice has been isolated (49-51) and employed for MDSC depletion (50, 51) or to improve Doxil delivery (49), these reagents did not recognize preferentially tumor infiltrating myeloid cells. Indeed, when used for Doxil delivery an important accumulation of Dox in the heart and in the spleen and liver was noted (49).

[0022] Four RNA aptamers (aptamer 3, 6, 11, and 14, Fig.2) were isolated that were specific for the tumor-infiltrating myeloid cells but not for their circulating counterparts regardless of the used tumor model or mouse strain. Interestingly, aptamer 3 and aptamer 11 have ANXA4 and Vimentin as their ligands, respectively. Vimentin plays a key role in monocyte differentiation and in the production of reactive oxygen species and, under pro-inflammatory stimuli, translocates to the membrane of activated macrophages (52-56). Indeed, vimentin is present in the tumor stroma of different cancers, but it is poorly expressed

in cultured tumor cells (54-56). Functionally, this protein has been involved in epithelial-mesenchymal transition and metastasis (57). ANXA4 is a calcium-dependent, phospholipid-binding protein that promotes membrane fusion and exocytosis (58). It is overexpressed in activated MDSCs, in TIMC, and activated M2 macrophages (44, 59). Although localized in the cytoplasm in resting macrophages, ANXA4 translocates to the membrane during activation (60, 61). Both proteins are expressed in human malignancies and their expression correlates with a worse prognosis in renal, breast and ovarian cancer according to the TCGA data and the Protein Atlas. Thus, both identified targets support the specificity of the aptamers for TIMC activated phenotypes.

[0023] Aptamers described herein cross-react with and recognize preferentially human MDSC in the tumor but not in the blood of patients with HNSCC suggesting their possible use for MDSCs detection or as tumor targeting agents in human malignancies. Indeed, the combinatorial use of these four aptamers offers an efficient delivery of the chemotherapeutic agent, e.g., doxorubicin, or RNA therapeutic agent (e.g., siRNA against CCR1 and CCR5, in our proof-of-principle experiments, to the primary and metastatic tumor site with virtually no drug accumulation in other tissues. In a therapeutic setting our approach is far superior to free doxorubicin or Doxil (34, 35) (today's gold standard for doxorubicin targeted delivery) and results in tumor regression in approximately 40% of treated mice with no observed systemic toxicity.

[0024] Taken together, the data provided herein indicates that it is possible to potentiate the therapeutic index of chemotherapeutic agents by targeting the surface protein profile of tumor-infiltrating myeloid cells, allowing the bystander release of the drug within the tumor microenvironment. While combinatorial use of aptamers described herein is not required (i.e., the use of a single aptamer is contemplated), the combinatorial use of several different aptamers as a means of chemotherapeutic agent delivery, each targeting different TIMC specific epitopes, significantly increases treatment specificity, improve efficacy, and reduce the toxicity of chemotherapeutic agents as compared with the systemic delivery of these agents in current, clinically available treatment modalities.

[0025] The disclosure provides an RNA aptamer is conjugated to a therapeutic, wherein the aptamer specifically binds to a target expressed on a tumor-infiltrating myeloid cell. In various aspects, the therapeutic is a nucleic acid molecule (e.g., DNA, RNA, shRNA, siRNA or miRNA). In various aspects, the therapeutic is a chemotherapeutic agent (e.g., doxorubicin). The target expressed on the tumor-infiltrating myeloid cell is optionally

annexin or vimentin. The disclosure further provides a method of delivering a therapeutic to a tumor-infiltrating myeloid cell comprising contacting the cell with the aptamer. The disclosure also provides a method for detecting the presence of a tumor-infiltrating myeloid cell in a biological sample, comprising contacting the sample with the aptamer conjugated to a detectable label. The disclosure additionally provides a method of treating cancer in a subject in need thereof, comprising administering the aptamer to the subject. The subject is optionally suffering from breast cancer, colon cancer, or renal cancer.

EXAMPLES

[0026] *Materials and Methods*

[0027] *Study Design and rigor:* The goal of this study was to identify RNA aptamers able to discriminate myeloid cells infiltrating the tumor from those in the periphery. Aptamers were selected by unsupervised Cell-SELEX, bioinformatics analysis, and empirical testing by flow cytometry and immune fluorescence analysis. Each experiment has been performed at least twice by two independent experimentalists and, if possible, the same phenomenon was evaluated using two independent techniques to eliminate assay specific artifacts. Flow cytometry was performed on a daily-calibrated flow cytometer, using titrated and validated antibody control, vital dye, automatic compensation using single cell color, and FMO or control aptamer (cycle 0 or irrelevant aptamer) as negative controls. For *in vivo* experiments, mice were randomized before treatment and tumor measurement was taken by an experimentalist blind to the treatment. Unless otherwise specified, mice were euthanized when tumor reached ~1.2 cm of diameter, or if they lost more than 20% of initial body weight or showed clinical signs of treatment related toxicity (i.e., lethargic mice, ruffled coat etc.). Data are cumulative and derived from 2 to 3 replicate experiments each with 3-5 mice per group. Group size was determined by power analysis using the effect size from pilot experiments. Outliers are always included in the data. In one experiment, two mice were removed and euthanized for study unrelated reasons (i.e., wounds from fights).

[0028] *Cell lines and recombinant proteins:* 4T1(62), 4T1HAThy1.1luciferase(63), TS/A(64), MSC2(26), CT26(65), Renca(66), MCA203(67), and 67NR(68) cell lines were previously described. All cell lines were maintained in complete media (RPMI (Gibco), supplemented with HEPES (10 mM), streptomycin (150 U/mL), penicillin (200 U/mL), 10% heat-inactivated FBS (Invitrogen), and Beta-mercaptol (20μM)).

[0029] DOXR-4T1 was selected from the parental cell line by culturing the cells with increased dosage of DOX. The resulting DOXR-4T1 cell line was maintained in complete media supplemented with doxorubicin (3.5 µg/ml).

[0030] MSC2 were treated with IL4 as previously described (26) by incubating 5×10^5 cells in a T75 flask (Falcon) for 4 days with rmIL4 (100ng/ml, Peprotech) in 20 ml of complete media. Annexin A4 and vimentin recombinant proteins were purchased from myBiosource.

[0031] *Microarray gene expression profiling of MSC2 and CD11b⁺ cells:* After four days of culture with or without IL4, MSC2 were washed with PBS prior to RNA extraction. For each chip, 2.5 µg of total RNA, Trizol extracted and cleaned with RNeasy Qiagen columns, were amplified to biotinylated complementary RNA (cRNA) as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. All the pre-hybridization quality controls were performed with the Agilent 2100 bioanalyzer (Agilent Technologies). RNA from 5 biological replicates of MSC2 and MSC2 cells treated with IL4 was then hybridized on Affymetrix mouse genome expression MG-U74Av2 arrays. Microarray probe fluorescence signals were converted to log₂ expression values using the Robust Multiarray Average procedure of the *affy* Bioconductor package. Fluorescence intensities were background-adjusted and normalized using quantile normalization, and expression values were calculated using median polish summarization and the custom chip definition file for the mouse array MG-U74Av2 based on Entrez genes (mgu74av2_Mm_ENTREZG version 21.0.0; brainarray.mbi.med.umich.edu/Brainarray/Database/CustomCDF/21.0.0/entrezg.asp) for a total of 8,124 custom probe sets. Microarray data are available from Gene Expression Omnibus GSE110774.

[0032] CD11b⁺ cells were magnetically isolated from single suspension of the spleen or the tumor mice challenged 9 days before with C26GM. Purity was higher than 95% as per flow cytometry analysis. For each chip, 2.5 µg of total RNA, Trizol extracted and cleaned with RNeasy Qiagen columns, were amplified to biotinylated cRNA as described in the Affymetrix GeneChip® Expression Analysis Technical Manual. All the pre-hybridization quality controls were performed with the Agilent 2100 bioanalyzer (Agilent Technologies). RNA from 6 biological replicates of CD11b⁺ cells from the tumor and from the spleen was then hybridized on Affymetrix mouse genome expression MOE4302 arrays. Microarray probe fluorescence signals were converted to log₂ expression values using the Robust Multiarray Average procedure of the *affy* Bioconductor package. Fluorescence intensities were background-adjusted and normalized using quantile normalization, and expression

values were calculated using median polish summarization and the custom chip definition file for the mouse array MOE4302 based on Entrez genes (mouse4302_Mm_ENTREZG version 21.0.0; brainarray.mbi.med.umich.edu/Brainarray/Database/CustomCDF/21.0.0/entrezg.asp) for a total of 18,139 custom probe sets. Microarray data are available from Gene Expression Omnibus GSE110774. MSC2 and CD11b dataset were then merged using 7,860 common Entrez gene identifiers and the merged dataset batch-corrected using the *ComBat* function of Bioconductor *sva* package. To identify genes overexpressed in MSC2 treated with IL4 and CD11b⁺ cells from the tumor, we compared the expression levels of IL4-treated MSC2 with those of untreated cells (and of CD11b⁺ cells from tumor with those of cells from spleen) using Significance Analysis of Microarray (SAM) algorithm coded in the *samr* R package. In SAM, we estimated the percentage of false-positive predictions (i.e., false discovery rate, FDR) with 100 permutations and selected as differentially expressed those probe sets with an FDR q-value ≤ 0.05 and an absolute fold change ≥ 2 . Global unsupervised clustering was performed using the function *hclust* of R *stats* package with Pearson correlation as distance metric and average agglomeration method. Statistical significance of the clustering has been computed in terms of approximately unbiased p-value using the *cluster.bootstrap* function of the R *pvclust* package. Gene expression heatmaps have been generated using the function *heatmap.2* of R *gplots* package after row-wise standardization of the expression values. All data analyses were performed in R version 3.3.3 using Bioconductor libraries and R statistical packages.

[0033] *Aptamer selection:* The cDNA random library (previously described by the Sullenger group (69), was synthesized by Eurofins technology and amplified by PCR using recombinant Taq (Invitrogen)) with the SRlong and the SF primers (Figure 12) with the following cycling condition: 95°C 5', 3x (94°C 30", 52°C 20", 72°C 25"), 20 x (94°C 30", 54°C 20", 72°C 25"), 72°C 5'. Amplified DNA was purified using the PCR purification kit (Qiagen) and transcribed *in vitro* by Durascribe T7 RNA synthesis kit (Epicentre, Madison, WI, USA) or APT-GET T7 transcription kit. The resulting 2'Fluoro-RNA aptamers were purified using the RNeasy kit (Qiagen). In the initial round of selection, 27 pmoles of RNA were resuspended in 450 μ l PBS (pH 7.4, Life technologies, MO, USA), heated at 65°C for 5' and then cooled at room temperature (RT) for at least 10'. The RNA aptamer library was first incubated at RT in PBS (1 ml) for 10' on a rotator with 2×10^6 MSC2 cells as negative selector. ryCells were then spun down (514 g, 10'), and supernatant filtered (0.2 μ m supor membrane, Pall Corporation) to remove residual cells and to recover unbound aptamers.

Unbound aptamers were incubated with IL4 treated MSC2 (0.5×10^6) as positive selector in PBS with yeast RNA (10 ug/ml, Ambion) for 10' at RT on a rotator. Cells were spun down (514 g, 10') and washed 3 times (3' wash) with 1ml of PBS with MgCl_2 (0.5 mM MgCl_2 , 1 mM CaCl_2). Total RNA was then isolated with Trizol (Invitrogen) and reverse transcribed in cDNA with SuperScript® III Reverse Transcriptase kit following manufacturer's instructions (Invitrogen) and SR oligonucleotide. The resulting cDNA was amplified by PCR (95°C, 5', 3x (94°C 30", 52°C 20", 72°C 25"), 15 x (94°C 30", 54°C 20", 72°C 25"), 72°C 5) using the SF and SRshort primers (Figure 12). Amplified DNA was purified with the PCR purification kit (Qiagen), transcribed using the DurascribeT7 kit and the resulting RNA aptamers were purified with RNeasy mini kit (Qiagen) and used for the next cycle of selection. To increase stringency, starting at the sixth round of selection, incubation time with the IL4-treated MSC2 was reduced to 5', 5 washes were performed. Additionally, a reduced number of cells (3×10^5 in cycle 6 and 7, 2.5×10^5 in cycle 8-10, and 2×10^5 in cycle 11) was used as positive selector to promote competition between aptamers with the same specificity and possibly isolate the most affine. Monoclonal aptamers were either synthesized by PCR and T7RNA polymerase using the DNA primers described in Figure 12 or synthesized with a biotin on the 5' end by Boston Open Labs.

[0034] *Flow cytometry:* Aptamers were labeled with Cy3 using the Silencer siRNA Labeling Kit - Cy3 (Ambion) and 37 pmoles were used to label 5×10^6 cells unless otherwise specified. In some experiments biotinylated aptamers were labelled with streptavidin conjugated with Alexa Fluor 647 or Alexa Fluor 750 and purified from unbound aptamer by Ultra-4 centrifugal filters, 50,000 kDa (Millipore).

[0035] Antibodies used are summarized in Figure 13. Dead cells were excluded by the analysis by using 4',6'-diamidino-2-phenylindole (DAPI; Sigma) or Live/Dead dye (Invitrogen). Samples were read on a LSR2 equipped with 405nm, 488nm, 532nm, and 635nm lasers (BD Bioscience). Data were analyzed using FCS6 express software (Denovo-Software).

[0036] *Immunofluorescence microscopy and Image cytometry:* Fresh frozen tissue and tissues microarrays (AMSBIO) were fixed in 10% neutral-buffered formalin (BDH) for 15' at RT, incubated with dextran sulfate sodium/PBS (1:2 m/V - Pharmacia biotech) for 30' and washed with PBS. Tissues were then stained with Cy3-labelled aptamer (10ug/ml) in PBS for 30', masked with 2% BSA, and counterstained with antibodies and/or DAPI.

[0037] Formalin-fixed tissues were deparaffinized with xylene (2 x 10' incubations) and rehydrated first with xylene/ethanol (1:1 v:v, 10' at RT) and then subsequentially incubated for 10' with the following ETOH solution: 100%, 100%, 95%, 90%, and 75%. Antigen retrieval was performed with Citrate buffer (pH6 Thermo-Fisher) by microwaving the tissue for 30 minutes at 100W. Tissues incubated in PBS O/N at RT, incubated with dextran sulfate sodium/PBS (1:2 m/V - Pharmacia biotech) for 30' washed with PBS, and then stained with Cy3-labelled aptamer (10ug/ml) in PBS for 30', masked with 2% BSA, and counterstained with antibodies and/or DAPI.

[0038] Whole stained tissue sections were scanned at 20X with the Olympus VS120 equipped with DAPI CUBE 455 nm (12.941 ms), a FITC CUBE 518 nm (410.271 ms) and a TRITC CUBE 580 nm (592.6 ms). Images were exported with OlyVIA software as single channel with a resolution of 5x, evaluated with ImageJ (fiji.sc/), tiled at 600x600 pixels with ImageSlicer (www.coolutils.com/) and processed with cell-profiler (www.cellprofiler.com) as follow: Nuclei were identified using the blue (DAPI) channel as primary object using the three classes Otsu Adaptive threshold method (correction factor of 1 threshold 0.1–1.0) with diameter between 2 and 10 pixels. Object declumping was based on shape and the size with default smoothing and distance parameters. Cells as secondary objects were segmented using fluorescence from the 3 merged channels by the three classes Otsu Adaptive threshold propagation method (threshold correction factor 0.0–1.0; regularization factor=0.02). Cytoplasm as tertiary object was identified as the area included in the cells (secondary object) but not in the nuclei (primary object). The resulting cpout files were analyzed with FCS Express 6 plus (www.denovosoftware.com) by evaluating aptamers' fluorescence intensity in the CD33+ or CD33- cells in the tumor and in the surrounding healthy tissue identified on serial H&E section by an experience pathologist.

[0039] *HNSCC specimens*: Specimens and blood from patients (median age 59 years, range 49-72) with recurrent stage 3 or 4 HNSCC of the oral or oropharynx cavity undergoing salvage surgery were collected at the time of surgery or biopsy under a clinical protocol approved by the University of Miami.

[0040] *shRNA transfection*: 2×10^5 MSC2 were transfected with 1 μ g of vimentin specific shRNA(29) (GCG CAA GAU AGA UUU GGA AUA UUC AAG AGA UAU UCC AAA UCU AUC UUG CGC UU – SEQ ID NO: 47) or scrambled shRNA using the 4PD MDSCs transfection kit (Kerafast) and following manufacturer instruction(30). Transfected cells were

treated for 30' with mytomicin C (10 µg/ml) washed twice and incubated in a 6 well plate for 4 days (37°C, 5% CO₂) with IL4 (100 ng/ml, Peprotech).

[0041] *Preparation of polyclonal library for HT-sequencing:* cDNA of cycles 1, 6, 10, and 11 were tagged using two sequential PCRs. The first PCR reactions were performed in 100 µl of water containing 1X PCR buffer, MgCl₂ solution (1.5 mM), dNTPs (200 µM each), DNA template (5 ng/µl) recombinant Taq polymerase (5 U, Invitrogen) and the PFA and PRA primers (supplementary Table 2) corresponding to each cycle described above. The reactions were performed in the GS482 thermocycler (G-STORM) using the following program: 95°C 5', 5x (95°C 1', 56°C 30'', 72°C 30''), 72°C 10'. PCR was purified via gel extraction using the QIAquick Gel Extraction Kit (QIAGEN) following manufacturer instructions. The second PCR was performed using the same condition described above but using the UFB and PRB primers (Supplementary Table 2) and the following program: 95°C 5', 6x (95°C 30'', 65°C 30'', 72°C 30''), 72°C 10'. Products were purified by gel extraction, quality and quantity evaluated via bio-analyzer (Agilent). Library quantitation and pooling took place at the Hussman Institute for Human Genomics-Center for Genome Technology using the KAPA Library Quantification Kit for Illumina platforms (part# KK4854). 10-13 pM of pooled samples were loaded on the Illumina cBot for cluster generation according to manufacturer's recommendations. Sequencing was performed on an Illumina HiSeq 2000/2500 (HCS 2.0.12.0) using the reagents provided in the Illumina TruSeq PE Cluster Kit v3 and the TruSeq SBS Kit-HS (200 cycle) kit. Data processing was done using HiSeq's Real Time Analysis (RTA) from Casava software. Base calling files were transformed into zipped FASTQ files containing raw reads with base qualities. These raw read files were then filtered by Illumina's internal filter resulting in 2 FASTQ files (1 per read) containing all pass-filter reads. FASTQ files were used as input to APTANI(27).

[0042] *Bioinformatics selection of aptamers using APTANI:* Aptamers were selected using APTANI, a computational method for the identification of target-specific aptamers from HT-SELEX data and secondary structure information (27). Briefly, APTANI first calculates the relative enrichment of each individual aptamer sequence produced by the HT-SELEX process and then, for each aptamer with an enrichment higher than a pre-selected threshold, predicts all secondary structures in a specific energy range and extracts the motifs represented in these structures. As a result, APTANI returns a list of aptamers, ranked by their abundance and by the presence of structural motifs. Here APTANI was used with default parameters on HT-SELEX data from cycles 1, 6, 10, and 11 and set the enrichment and motif frequency

thresholds to 0.01% and 0.05%, respectively. Since at each run APTANI randomly samples 20% of the library to reduce the computational load, each analysis was replicated $n=5$ times and pooled the results to fully cover the entire library.

[0043] *Mice:* All animal experiments were approved by the Division of Veterinary Resources and the Institutional Animal Care & Use Committee of the University of Miami. 8-10 weeks old BALB/c and C57Bl/6J mice were purchased from Jackson Laboratories and maintain in the pathogen free animal facilities at the University of Miami on a chlorophyll free diet. Mice were allowed to acclimate for at least one week before experiments, ear-tagged, and randomized after tumor inoculation.

[0044] *Aptamer-Doxorubicin treatment:* Aptamer-doxorubicin was prepared by making DNA template with the appropriate aptamer followed by a GC-rich primer sequence downstream (GC rich Sul3', Figure 12. From this template, RNA was transcribed and purified as described above. Doxorubicin (Sigma) was mixed with the RNA sequence as needed. Doxil (ALZA corporation) was purchased through the University of Miami pharmacy.

[0045] BALB/c mice were injected IV with the 4T1 luciferase thy1.1 tumor cell line. Treatment was started when tumors reached 5 mm of diameter and repeated 2 and 6 days later. Tumor growth and mice weight was reported. Mice were evaluated at least 3 times a week and humanely euthanized when tumor size index reached 150 mm^2 or if they lose $>20\%$ of initial weight due to chemotherapy or tumor growth in compliance with the IACUC policy and animal protocol. Data are expressed as tumor size index defined as the product of the main diameter with the perpendicular one.

[0046] *IVIS analysis:* Isofluorane-anesthetized, tumor-bearing mice were analyzed by the In Vivo Imaging System (Xenogen IVIS Spectrum - Perkin Elmer) 2h after i.v. injection of 5' biotinylated aptamers conjugated with Alexa Fluor-750 (AF750) streptavidin (13.6 pmol/g) with an imaging stage heated at 37°C . 15' before imaging mice were injected intraperitoneal with D-luciferine (150 $\mu\text{g/g}$). AF750 fluorescence was read at 800 nm after excitation at 748 nm. Raw signal was subject to spectral unmixing to remove background fluorescence signal and AF750 fluorescence was quantified with the living Image v4.3 software (Perkin Elmer).

[0047] *Detection of doxorubicin in tissue:* The fluorescent properties of doxorubicin were used to quantify doxorubicin in tumor, liver, spleen, and lung as previously described(70). Briefly, tissues were harvested, weighed, and homogenized in acid alcohol (0.3N HCl

solution, 70% EtOH), and doxorubicin was extracted over night at 4°C. Homogenate was spun down and supernatant samples were quantified by fluorometry (SpectraMax M5, Molecular Devices) using an excitation wavelength of 470 nm and measuring the intensity of emission at 590 nm and plotted on a standard curve of doxorubicin serially diluted in acid alcohol. Results were normalized on tissue weight.

[0048] *Statistical analyses:* All values depicted represent mean $\pm$ standard deviation of biological replica unless otherwise indicated in the figure legend. Statistical calculations were performed by a person blinded to the treatment group using Sigmaplot 12.5 (Systat software). Statistical tests (one way ANOVA followed by Holm Sidak test for multiple pairwise comparison or student T test) were applied as indicated in the figure legends in a two-sided, unpaired fashion after normality was evaluated by the Shapiro-Wilk test. The variance was similar between experimental groups in each experiment unless otherwise stated. *In vivo* experiments included cohorts of the size indicated in each figure legend but at least 6 mice per group. *In vitro* analyses and *in vivo* experiments were repeated two to five times to ensure reproducible conclusions; the exact number of repetitions is stated in each figure legend. Log-rank test was used for survival analysis followed by all pairwise multiple comparison procedures (Holm-Sidak method). Data from multiple experiments were cumulated unless otherwise indicated in the figure legends. No experimental data point was excluded from analysis. Sample size was chosen by power analysis using effect size determined by pilot experiments or prior experience of the authors.

Example 1 - Identification of aptamers specific for tumor-infiltrating myeloid cells

[0049] To identify TIMC-specific aptamers, an unsupervised, high-throughput (HT) sequencing-cell SELEX (systematic evolution of ligands by exponential enrichment) (25) was performed using the MDSC-derived cell line MSC2 (26), followed by an empirical selection of monoclonal aptamer binding TIMC but not the splenic counterpart.

[0050] MSC2 cells were considered a suitable TIMC surrogate for the initial aptamer's screening since these cells are available without requiring any artifact-causing manipulation of tumor-infiltrating cells, and they acquire the suppressive activity only after treatment with IL4 (26). Furthermore, as confirmed by a genome-wide transcriptional analysis, MSC2 cells express genes that are shared with TIMC. Indeed, MSC2 cells treated with IL4 are transcriptionally similar to TIMC, both in terms of genome-wide expression levels and over-

expressed genes, while untreated MSC2 share the gene expression profiles of splenic CD11b⁺ cells.

[0051] Aptamers were selected through 11 cycles of Cell-SELEX using untreated MSC2 as negative selector and IL4-treated MSC2 as positive selector, i.e. as TIMC surrogate. The resulting polyclonal aptamer library displayed a specificity increasing with the selection cycle (Figure 1A) only for IL4-treated MSC2, thus proving the overall efficacy of the SELEX enrichment (Figures 1A and 1B). Moreover, when tested on single cell suspensions obtained from either the tumor or the spleen of CT26 tumor-bearing mice, the aptamer library could discriminate TIMC from the splenic counterpart by preferentially recognizing MDSCs and macrophages from the tumor but not from the spleen of the same tumor-bearing animals or tumor-free mice (Figure 1C).

Example 2 - Identification of four monoclonal aptamers specific for tumor-infiltrating myeloid cells

[0052] Monoclonal aptamers that can be easily produced and manipulated were identified using a bioinformatics pipeline. Briefly, polyclonal aptamer libraries from cycles 1, 6, 10, and 11 were HT-sequenced and data analyzed with APTANI, a computational tool to identify target-specific aptamers from HT-SELEX and secondary structure information (27). The analysis on the library from cycle 11 resulted in 154 monoclonal aptamers that showed an enrichment in the library higher than 0.01% and contained 158 secondary RNA motifs with a motif frequency higher than 0.05% of the total number of motifs. From this set of aptamers, we focused on 15 sequences that had an enrichment of at least 0.1% and contained either a large number of different secondary RNA motifs (i.e., at least 3 motifs) or at least 1 motif with a frequency higher than 0.1%. Interestingly, frequency analysis among cycles shows that most of these aptamers emerged starting from cycle 6, when stringency was gradually increased (data not shown).

[0053] These 15 aptamers were tested by flow cytometry against single cell suspensions from tumor and spleen of mice challenged with the 4T1 tumor (Fig.2A). While 12 out of 15 aptamers recognized macrophages, granulocytic MDSCs (gMDSC), and/or monocytic MDSCs (mMDSCs) from the tumor with statistically significant accuracy (as compared to an irrelevant aptamer; $p < 0.001$; Fig.1D), only 6 of them (i.e., apt 3, 6, 11, 12, 14, 15) were specific for tumor infiltrating myeloid cells and did not recognize any subset of splenic MDSCs nor CD11b-cells ($p > 0.05$). Cluster and secondary structure analysis indicated that

aptamers 15 and 11 were similar to aptamers 14 and 12, respectively (Figure 10), thus only aptamer 3, 6, 11, and 14 (Figure 2B) were chosen for further experiments.

Example 4 - Aptamers 3, 6, 11 and 14 recognize tumor infiltrating myeloid cells from multiple mouse tumors

[0054] The binding properties of monoclonal RNA aptamers 3, 6, 11 and 14 were evaluated in other tumor models. Aptamers 3, 6, 11, or 14 single cell suspensions were stained from tumor, bone marrow, spleen, and lungs of BALB/c mice bearing the 4T1 mammary carcinoma, the CT26 colon carcinoma, or the RENCA renal carcinoma. Similar analysis was performed on single cell suspensions from C57Bl/6 mice bearing B16LU8 melanoma, MCA203 fibrosarcoma or E0771 mammary carcinoma. The selected aptamers were able to recognize TIMC from all the tumors in both mice strains, whereas no or low staining was observed in myeloid cells from either the spleen or bone marrow, nor in the T and B cells. This finding indicates that, in all analyzed tumor samples, aptamer 3, 6, 11 and 14 were able to recognize TIMC, regardless of the mouse strain or tumor type. Interestingly, in different tumors, the selected aptamers recognize different tumor-infiltrating myeloid subsets.

[0055] Specificity was further evaluated by immunofluorescence microscopy using 4T1 tumors and tissues arrays from naïve mice. While all the selected aptamers recognized cells in the tumors most of the tissues appear to be negative for the aptamers with a few exceptions: aptamer 3 recognized hepatocytes at low intensity, aptamer 6 showed binding for epithelial cells in the uterus and low binding for pancreatic acinar tissue, and aptamer 14 recognized intestinal villi and seems to show a nuclear staining of cerebellum, pancreatic acinar tissue, stomach and spleen.

[0056] Next, whether an equimolar mixture of aptamers 3, 6, 11 and 14 was more efficient in recognizing TIMC as compared to each individual aptamer was evaluated. FACS analysis on cells from the spleen and from the tumor of 4T1 bearing mice revealed a clear additive/synergistic effect when using a mixture of the four aptamers (Figure 2C).

[0057] The selected aptamers recognized human myeloid cells in the tumor but not in the blood of patients with Head and Neck Squamous Cell Carcinoma.

[0058] Having seen that the selected aptamers can discriminate between splenic and tumor infiltrating myeloid cells in mice, whether they could cross-react with human myeloid cells was tested and whether these aptamers could discriminate human TIMC from their

circulating counterparts. Briefly, single cell suspension from the tumor or the blood of patients with recurrent HNSCC were stained with antibodies against CD33, HLADR, CD11b, CD14, CD15, and IL4Ra, counterstained with PE conjugated monoclonal aptamers and vital dye, and analyzed by flow cytometry (Figure 3A). While no or low staining was detected in the PBMCs and in the non-myeloid cells, at least two aptamers recognized myeloid cells infiltrating the tumor from all the analyzed patients to different degrees. In particular, at the tumor site the selected aptamers recognize the majority of mMDSC and CD14⁻CD15⁻ macrophages/iMDSC and a subset of gMDSC (Figure 3). Interestingly, within each subset, the aptamers recognize mostly the IL4Ra⁺ cells that we have previously shown to be the suppressive myeloid cells and correlate with tumor recurrence in HNSCC (28).

[0059] Similar results were obtained by image cytometry. Briefly, paraffin-embedded tissue from patients with recurrent HNSCC were stained with an equimolar mixture of the 4 Cy3-labelled aptamers, and counter-stained with DAPI and anti-CD33 antibody. Images were acquired by a high-resolution scanner and data fed into cellprofiler and FCS express for final analysis. Aptamers showed a higher binding on the CD33⁺ cells infiltrating the tumor compared to the CD33⁻ cells in the tumor and the CD33⁺ cells present in the surrounding healthy tissue (Figure 3B). Taken together, these data indicate that the selected aptamers cross-react with human myeloid cells and recognize preferentially myeloid cells infiltrating HNSCC.

Example 4 - Annexin A4 and vimentin are the putative ligands for aptamer 3 and 11

[0060] Aptamer-based immunoprecipitation and mass spectrometry identified annexin A4 (ANXA4 isoform X1 P97495) and vimentin (VIM P20152) as putative targets for aptamer 3 and 11, respectively (Figure 4A). FACS analysis using beads loaded with relevant or irrelevant recombinant proteins confirmed the specificity of these two aptamers, for annexin and vimentin proteins and for IL4-treated MSC2 (Figures 4B and 4C). The specificity of aptamer 3 for ANXA4 and aptamer 11 for vimentin were tested by competitive experiments (Figure 4D) and with validated shRNA (29) (Figure 4E), respectively. No targets have been identified thus far for aptamers 6 and 14.

Example 5 - Aptamers specifically recognize tumor-associated myeloid cells in vivo

[0061] To evaluate whether the aptamers can accumulate at the tumor site *in vivo*, mice orthotopically implanted with the 4T1-luciferase mammary carcinoma or tumor-free mice were treated intravenously with a mixture of aptamer 3, 6, 11 and 14 conjugated with

Streptavidin- Alexa Fluor 750 or Alexa Fluor 647 (Figure 5A). These fluorochromes allow detection of aptamer biodistribution by either in vivo imaging system (IVIS) or flow cytometry, respectively.

[0062] IVIS analysis revealed that the selected aptamers rapidly accumulate at the tumor site and in the liver (Figures 5B and 5C) and, subsequently, in the bladder. Signal from the TIMC-specific aptamers was detectable in the tumor 8 days after injection, whereas fluorescence signal in other tissues disappeared more rapidly indicating a preferential retention of the aptamers for the tumor microenvironment. Flow cytometry analysis (Figures 5D-5F) confirmed the IVIS results and indicated that aptamers in the tumor microenvironment preferentially bind to CD11b⁺ myeloid cells (Figure 5D), and in particular to mMDSC and TAM. Interestingly, time course analysis revealed that a population of aptamer-positive gMDSCs appears at later time points in the tumor and in the spleen of treated mice, (Figures 5E-5F), supporting previous evidence that mMDSCs can differentiate into gMDSCs (30, 31).

Example 6 - TIMC-specific aptamers maximize the delivery of doxorubicin at the tumor site

[0063] Since TIMC-specific aptamers can target the tumor microenvironment, their drug delivery ability using doxorubicin (DOX) as chemotherapeutic agent was evaluated. Briefly, aptamers were extended in the 3' terminus with a GC rich tail that facilitate DOX intercalation (32, 33) and optimal loading conditions were evaluated by fluorescence spectroscopy (Figures 6A and 6B). To evaluate whether DOX-aptamer conjugates could target primary and metastatic lesions, an equimolar mixture of the four aptamers was loaded with DOX and injected intravenously into mice previously challenged with: i) 4T1 mammary carcinoma injected into the mammary gland (primary tumor in breast, metastasis in lung); ii) 4T1 administered intravenously (metastasis in the liver and lungs); or iii) the non-metastatic 4T1 variant 67NR injected into the mammary gland (no metastasis). Two hours after the injection, the bio-distribution of DOX-aptamer conjugates was evaluated by spectrophotometer analysis (Figure 6C). The aptamers were able to deliver DOX to the primary mammary tumor and the metastatic sites. In mice with the non-metastatic 67NR tumor, DOX was found only in the affected breast tissue. In mice with the metastatic 4T1 injected intravenously, DOX was found in the liver and lungs (sites of metastatic cancer), whereas in mice with the 4T1 injected into the mammary gland, DOX accumulates at the primary tumor site and in the lung, a primary metastatic site in this model. Additional

experiments indicated that DOX concentration at the tumor site of mice treated intravenously with 0.35 mg/kg ("low dose doxorubicin") of DOX-aptamer complexes was similar to those of mice treated with 3.5 mg/kg ("high dose") of free DOX, whereas in all other evaluated compartments the overall DOX concentration was significantly lower in the mice treated with DOX-aptamer complexes (Figure 6D). These results not only indicate that doxorubicin conjugation does not affect the aptamer specificity but also that TIMC-specific aptamers can deliver chemotherapeutic agents to the primary and metastatic sites.

Example 7 - TIMC-specific aptamers increase doxorubicin's therapeutic index

[0064] Next, the therapeutic efficacies of doxorubicin delivered by TIMC-specific aptamers was compared by PEGylated liposomes (Doxil, the first clinically available nanoparticle that became the gold standard for doxorubicin treatment (34, 35)), and as unconjugated molecules. Briefly, 4T1 bearing BALB/c mice were treated with unconjugated DOX at dose high, or low dose, or with low doses of DOX conjugated to the TIMC-specific aptamer, or with Doxil (low dose - 0.35 mg/kg). Treatment was repeated 2 and 6 days later. As additional controls, mice were treated with unconjugated aptamers, irrelevant aptamers conjugated with doxorubicin, or left untreated. No significant anti-tumor effect was observed in mice treated with either high doses of free Doxorubicin or Doxil (Figure 7A). Conversely, low doses of doxorubicin delivered via TIMC-specific aptamer significantly delayed tumor progression and resulted in 40% of treated mice being without a clinically detectable tumor 60 days after treatment (Fig.7A). Furthermore, while high doses of free doxorubicin resulted in high toxicity, as determined by significant body weight loss and 15% treatment related mortality, no toxicity was observed with DOX-loaded aptamer (Figure 7B).

[0065] Since depletion or inactivation of MDSCs can delay tumor progression (19) and doxorubicin has reportedly depleted (36) MDSCs or impaired their function (37), we asked whether the observed anti-tumor efficacy was due to the bystander release of doxorubicin in the tumor micro-environment or rather the depletion/inactivation of MDSCs. To this aim, we evaluated the efficacy of treatment using a doxorubicin resistant 4T1 cell line (DoxR-4T1) and the doxorubicin sensitive parental cell line. DOX-loaded aptamer treatment confirmed its efficacy on the doxorubicin sensitive parental cell line, whereas no therapeutic effect was observed in mice challenged with the doxorubicin resistant 4T1 tumor (Figure 7C).

Example 8 - Aptamer effectively deliver CCR1 and 5 siRNA to tumor infiltrating myeloid cells and delay tumor progression.

[0066] Balb/c mice were challenged orthotopically with the 4T1 mammary carcinoma and treated intravenously with a mixture of aptamers 3, 6, 11 and 14 each loaded with a) scrambled siRNAs (black bar) or with siRNAs against CCR1 and CCR5 (30 picomoles, empty bar) 5, 7, 9, 12, 14, 16 and 19 days after the challenge. qRT-PCR for CCR1 and CCR5 mRNA was performed 6 days after the last treatment on magnetically isolated tumor infiltrating myeloid cells from each group. Results show that the aptamers' mixture effectively deliver the siRNAs against CCR1 and CCR5 allowing the *in vivo* silencing of both genes whereas no effect were seen with the scrambled siRNAs controls (Figure 8B). Importantly, the aptamer mediated delivery of both siRNA into the tumor infiltrating myeloid cells induced a significant reduction of tumor size (Figure 8C). Taken together, these results indicate that aptamers can deliver therapeutic siRNAs *in vivo* into tumor infiltrating myeloid cells and affect tumor progression.

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What is claimed is:

1. An RNA aptamer conjugated to a therapeutic, wherein the aptamer specifically binds to a target expressed on a tumor-infiltrating myeloid cell.
2. The aptamer of claim 1, wherein the therapeutic is a nucleic acid molecule.
3. The aptamer of claim 1, wherein the nucleic acid molecule is DNA, RNA, shRNA, siRNA or miRNA.
4. The aptamer of claim 1, wherein the therapeutic is a chemotherapeutic agent.
5. The aptamer of claim 4, wherein the chemotherapeutic agent is doxorubicin.
6. The aptamer of any one of claims 1-5, wherein the target expressed on a tumor-infiltrating myeloid cell is annexin or vimentin.
7. A method of delivering a therapeutic to a tumor-infiltrating myeloid cell comprising contacting the cell with the aptamer of any one of claims 1-6.
8. A method of detecting the presence of a tumor-infiltrating myeloid cell in a biological sample, comprising contacting the sample with the aptamer of any one of claims 1-6 conjugated to a detectable label.
9. A method of treating cancer in a subject in need thereof, comprising administering the aptamer of any one of claims 4-6 to the subject.
10. The method of claim 10, wherein the subject is suffering from breast cancer, colon cancer, renal cancer, head and neck cancer or melanoma.

Figure 1

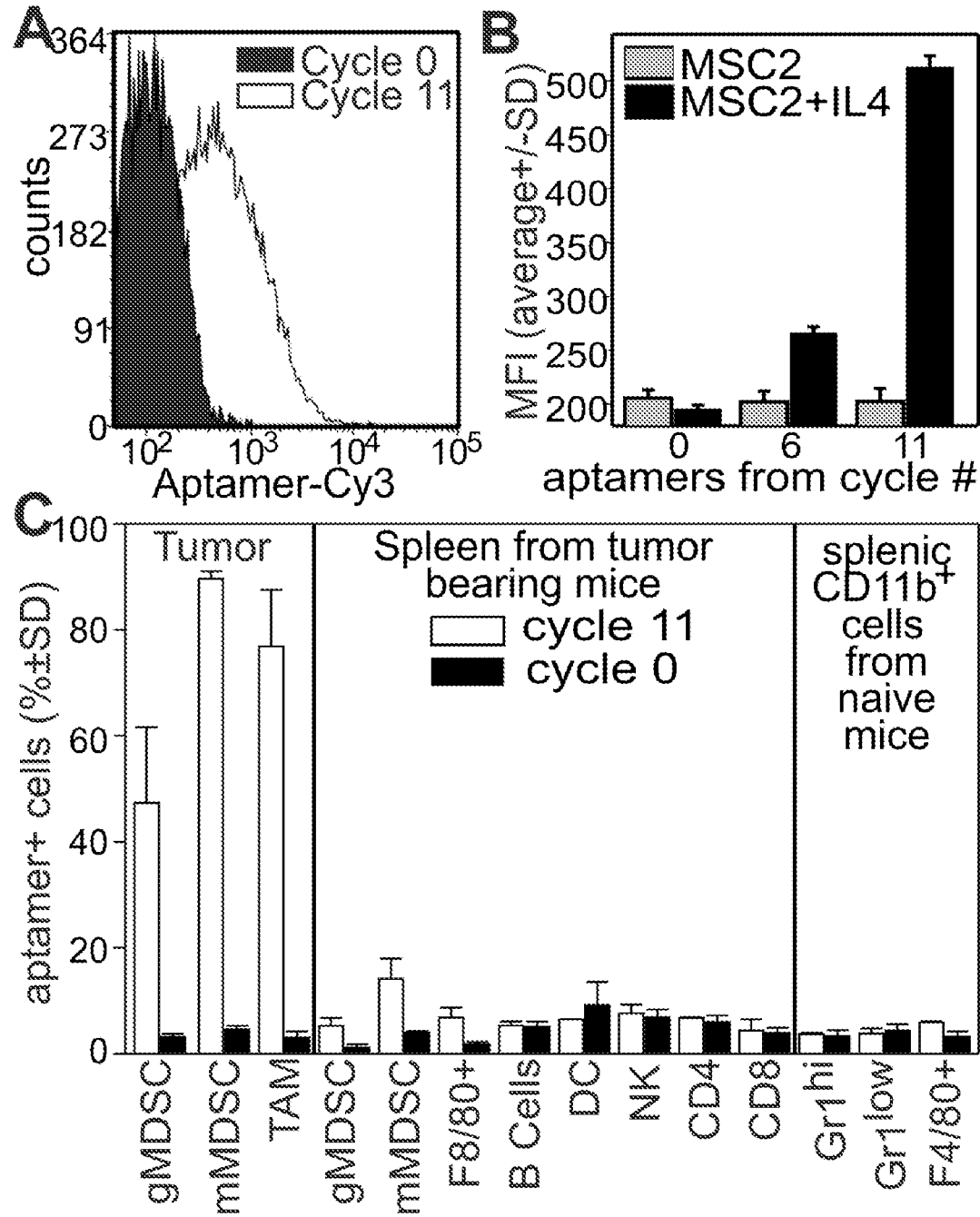


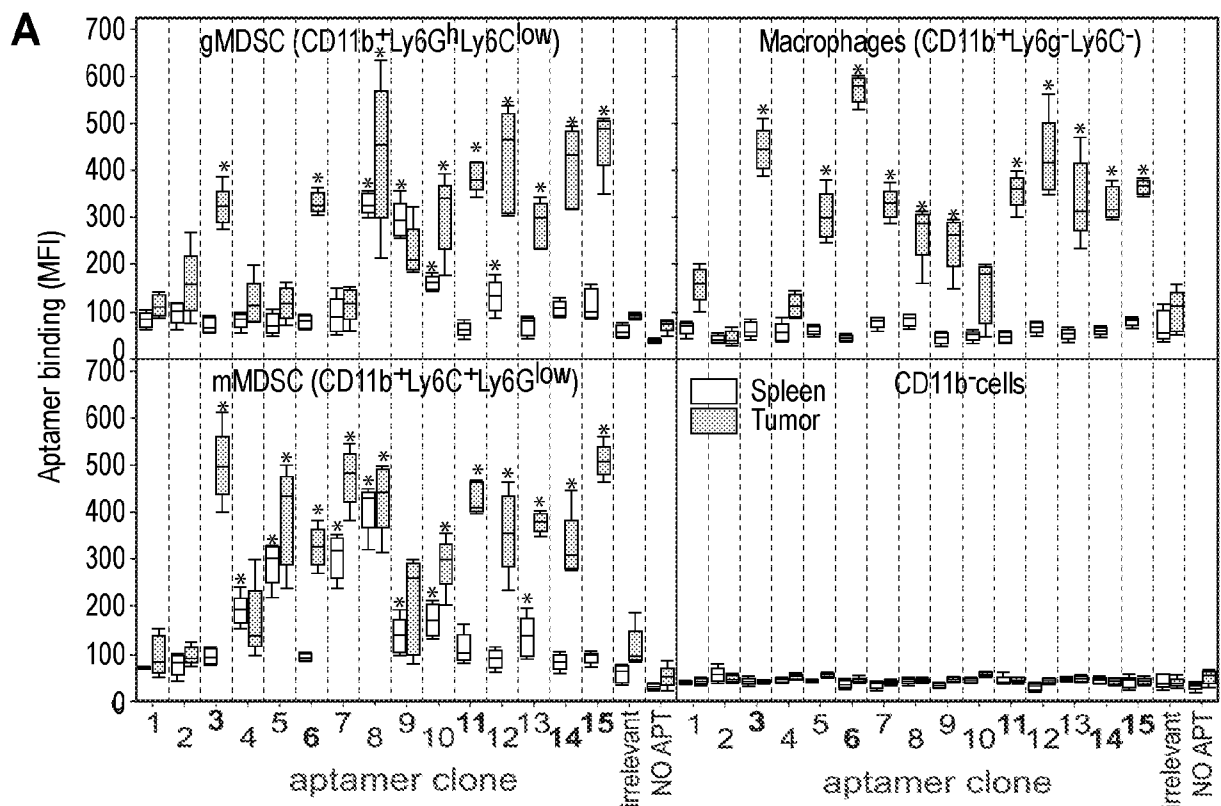
Figure 2

Figure 2 continued B

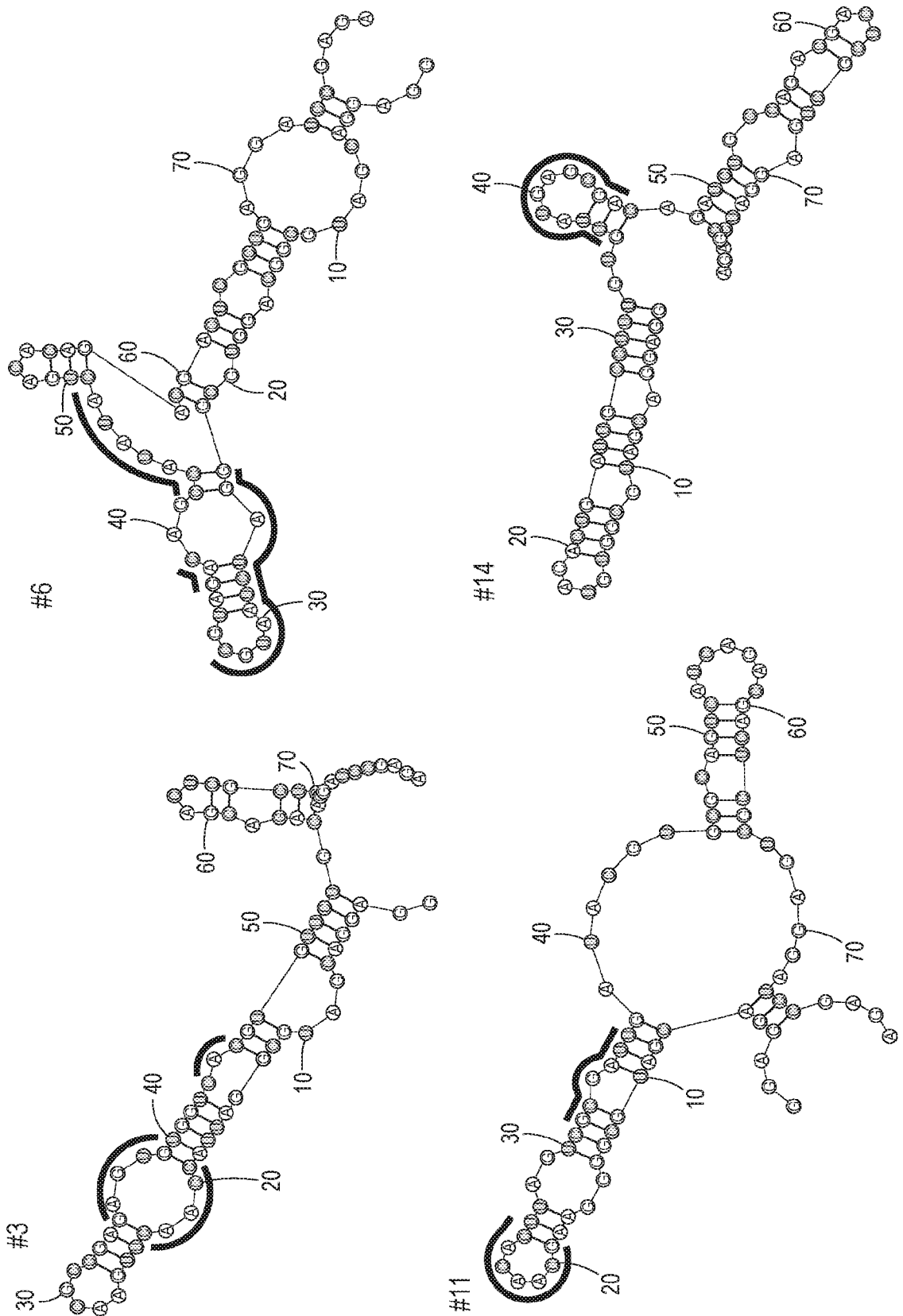


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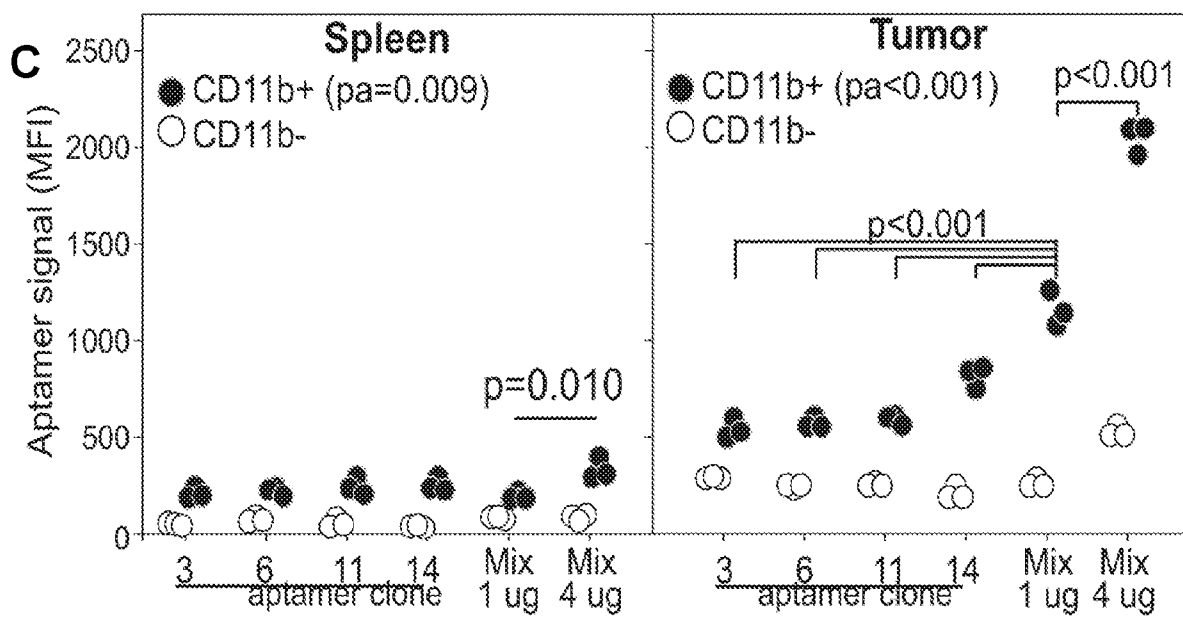


Figure 3

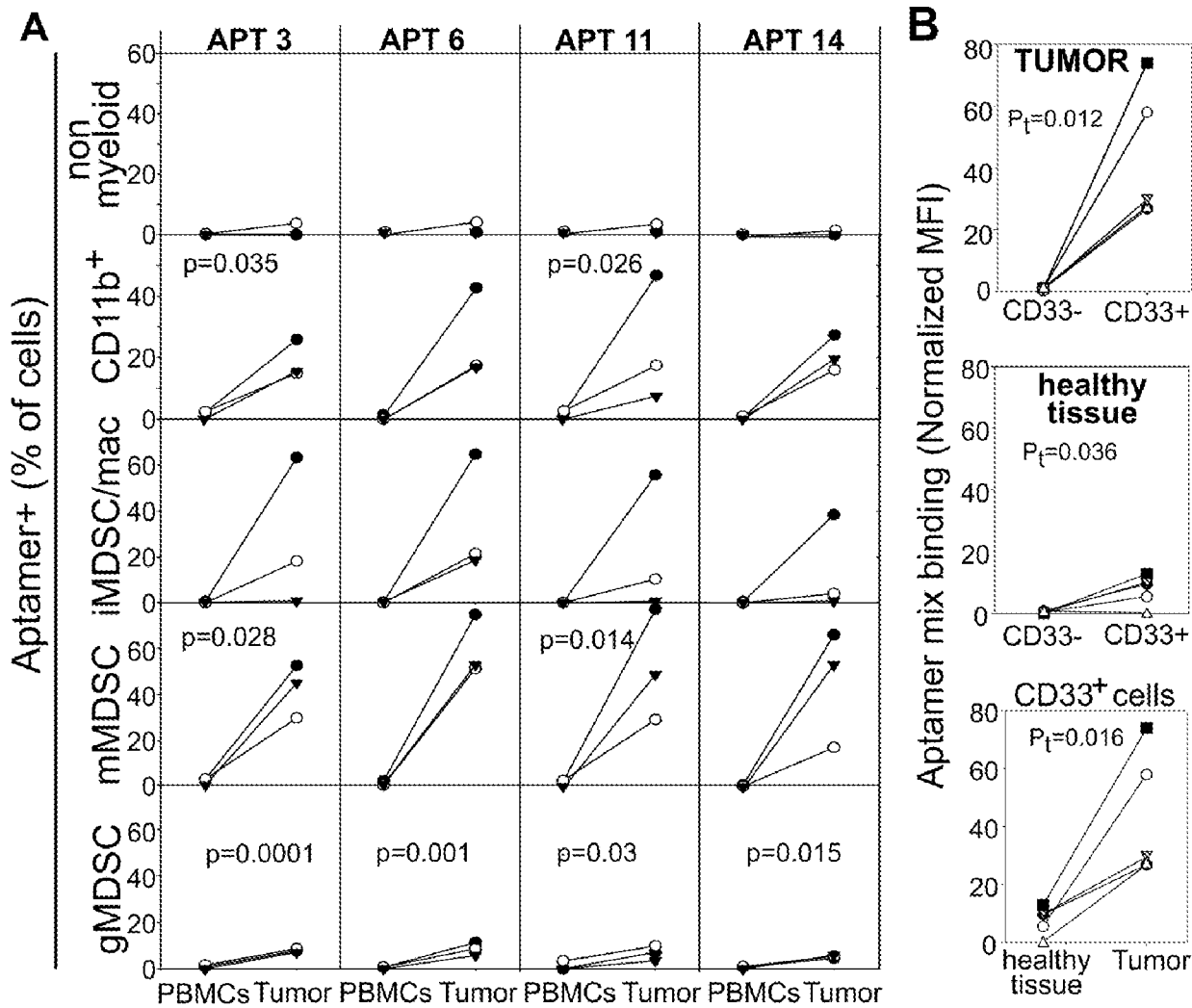


Figure 4**A**

Apt.	Kd vs IL4+MSC2	Putative target	Mascot mass score	% of target covered	Kd vs target
# 3	8.8×10^{-8}	ANX IV	652	37.36%	5.5×10^{-7}
# 6	8.8×10^{-8}	N/A			
# 11	3.4×10^{-7}	VIM	1589	67.52%	7.7×10^{-7}
# 14	1.9×10^{-7}	N/A			

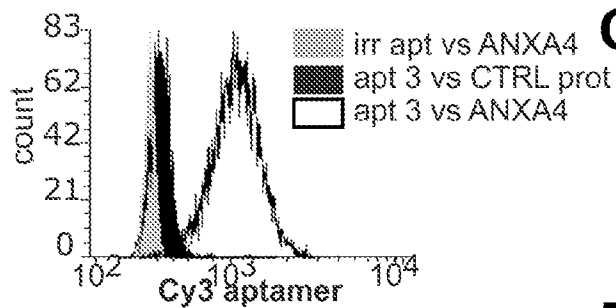
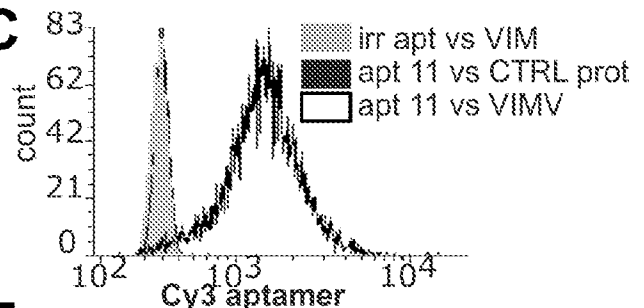
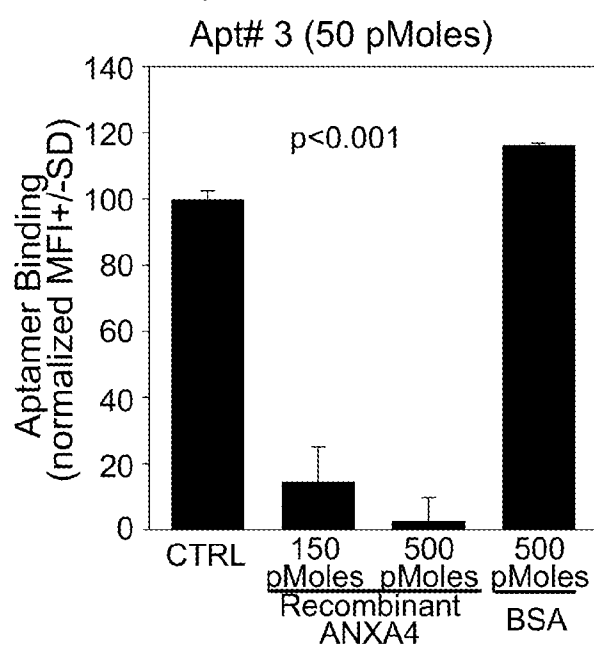
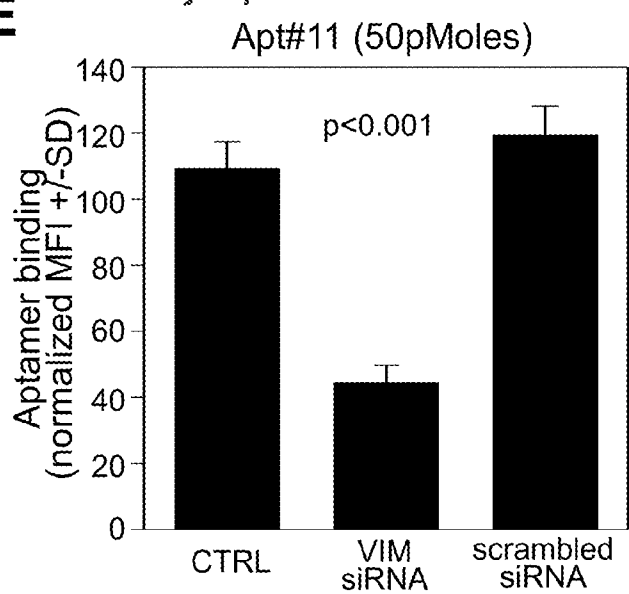
B**C****D****E**

Figure 5

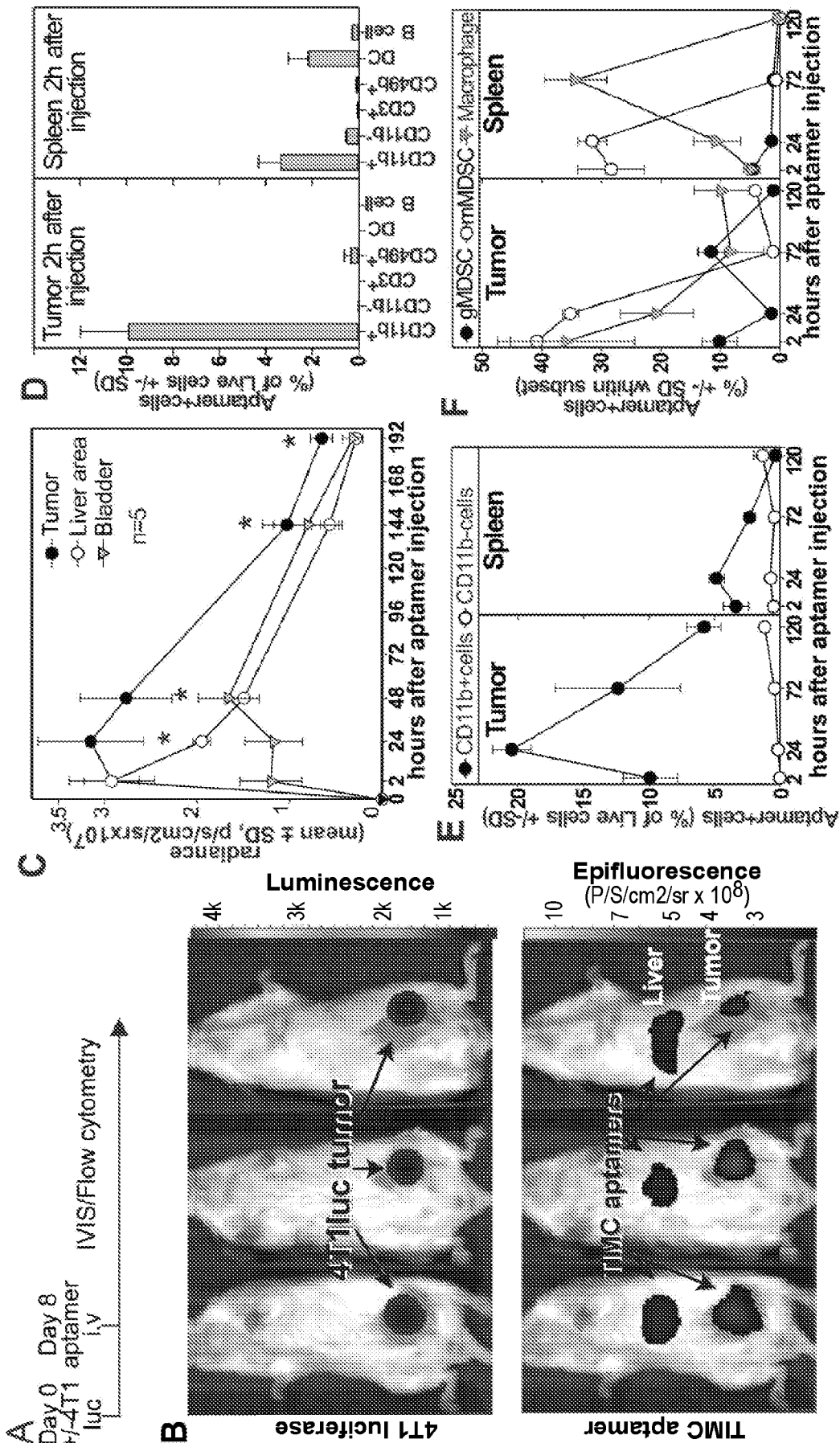


Figure 6

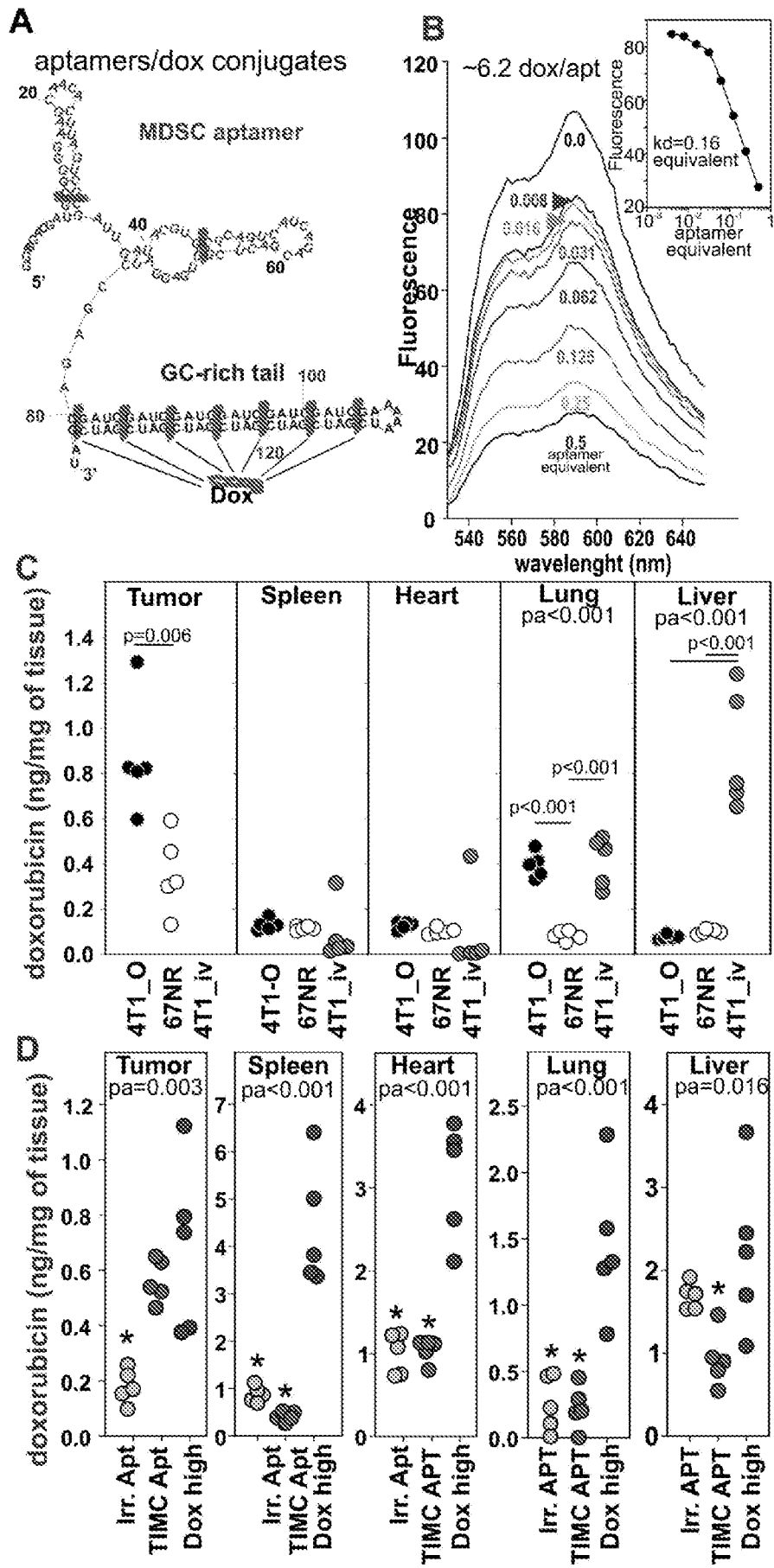


Figure 7

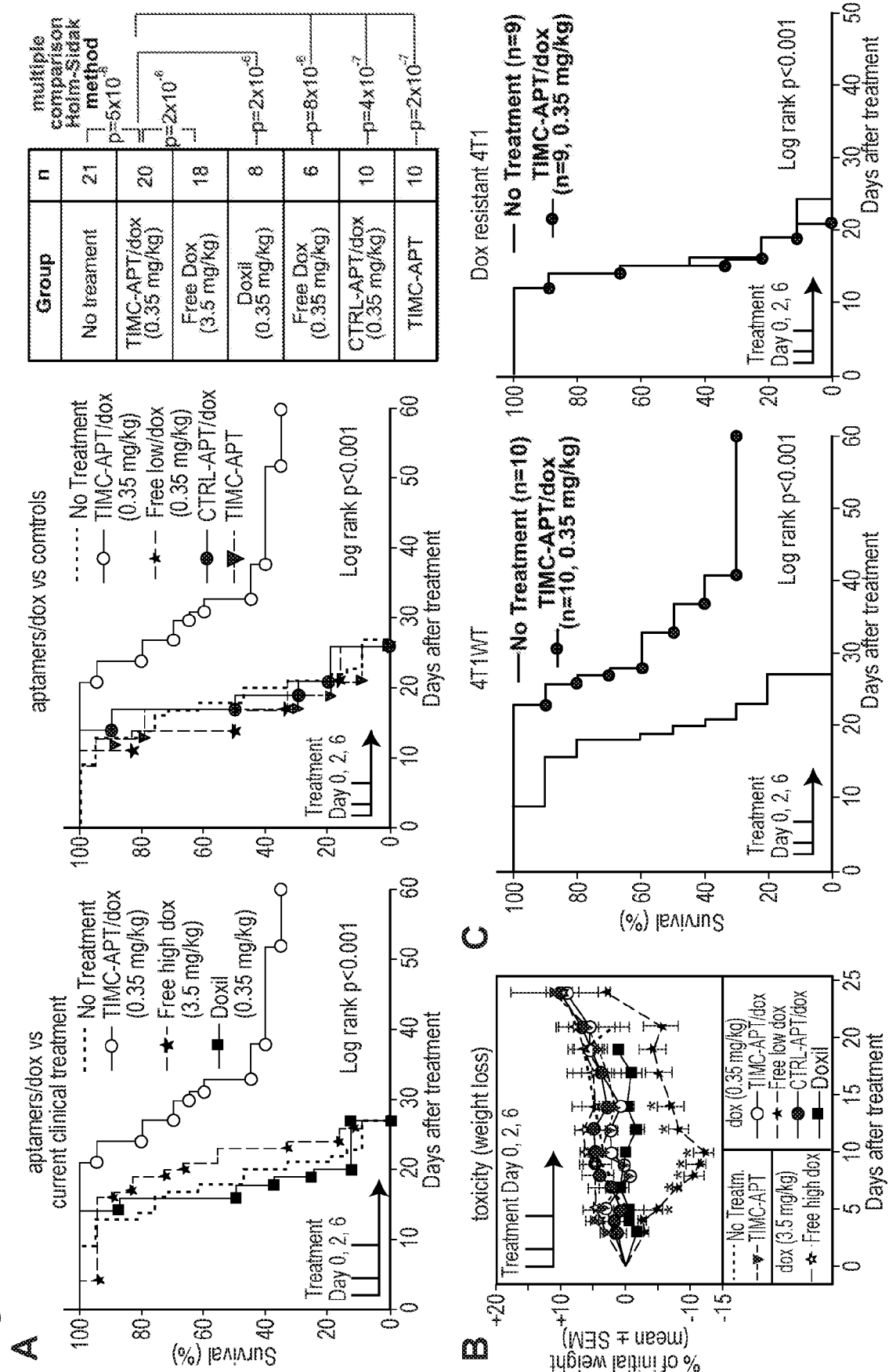
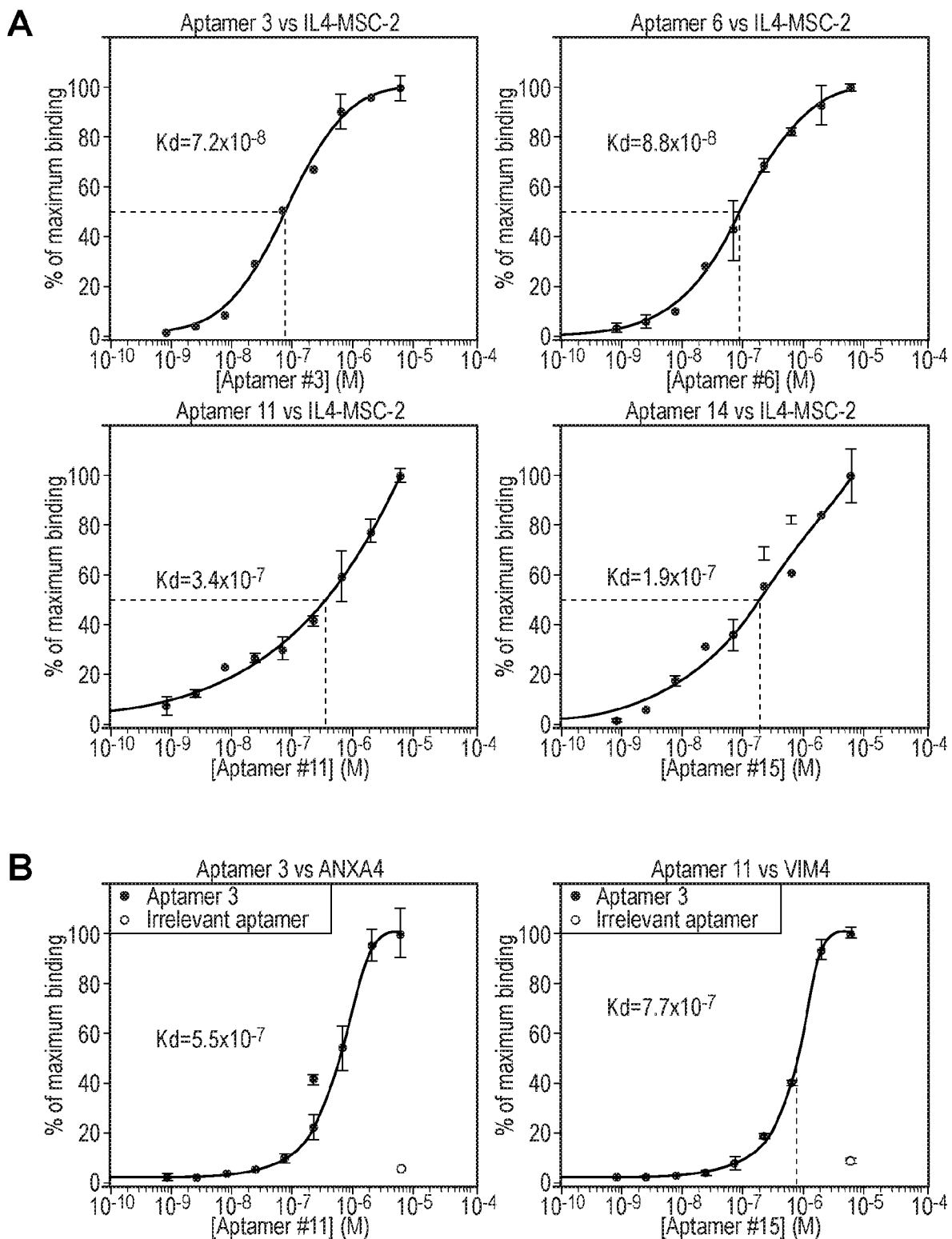


Figure 8

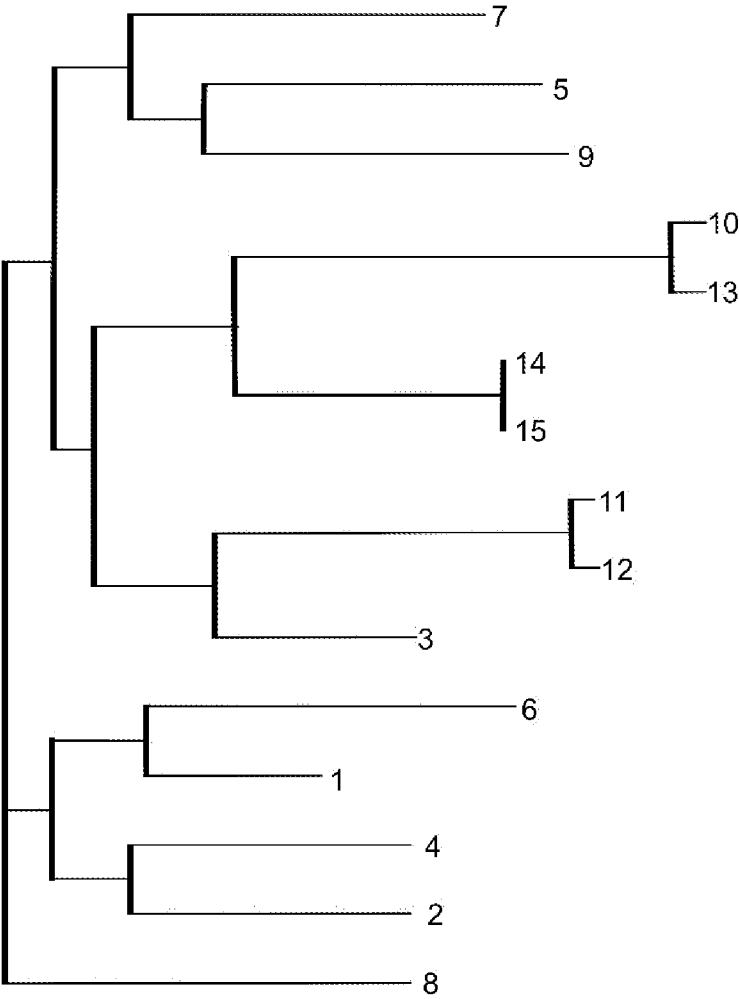


Figure 9

Figure 10

SEQ ID NO	#	Aptamer sequence	Apt. frequency (%)	Motif (SEQ ID NO)	Motif frequency (%)
1	1	5'GGAGGACGAUGCGGCCUAGUACACAAGAUC UGACACCUCGAUACAGAUAGAGGCAGACGAC UCGCUGAGGAUCCGAGA3'	0.06	ACAGAU GACACCUCGAUAC (SEQ ID NO: 48) GAU AU	0.13 0.1 2.38
2	2	5'GGAGGACGAUGCGGCCUAGUACAAAAGCCU GAUCUCUGUGAGCAGACACUAGAACAGACGAC UCGCUGAGGAUCCGAGA3'	0.06	GAGCAGAC	0.29
3	3	5'GGAGGACGAUGCGGAUUAACCAACUUGAACG CCGAGAGUGUGGUCACGUGUUCUGCAGACGA CUCGCUGAGGAUCCGAGA3'	0.12	GAACGCC CCAAC GAGUG CCAAC UG	0.48 1.33 0.69
4	4	5'GGAGGACGAUGCGGCAACAAACUAAUCAGAC ACGAGACAGAGAGAUAGAUACAGACGACU CGCUGAGGAUCCGAGA3'	0.36	CAGACACG	0.14
5	5	5'GGAGGACGAUGCGGCCGGAGGCAGUCACUA AUCUUCACUUCUCUCAGACAUAGCGCAGACGAC UCGCUGAGGAUCCGAGA3'	0.34	CGGAG	1.8
6	6	5'GGAGGACGAUGCGGCAGGUGCGGGAUUCUAA UGCGUAGACAGCCAUUAUACUGACACAGACGAC UCGCUGAGGAUCCGAGA3'	0.4	GC GAC CGUAGACAG UGCGGGA GCCAUUAC UAGACAG	9.5 0.18 0.11 0.1 0.1
7	7	5'GGAGGACGAUGCGGACGACGUAUACUGACC ACGAUAUGUCAGAUUCGGUCCUACAGACGA CUCGCUGAGGAUCCGAGA3'	0.05	CCACGAUAUG (SEQ ID NO: 49)	0.54
8	8	5'GGAGGACGAUGCGGCAUACACACUUGACUC UAGAGAACGAGCAUCUAGCGGUGUCCAGACGA CUCGCUGAGGAUCCGAGA3'	0.07	AUACACACUU (SEQ ID NO: 50) CUAGAGAACG (SEQ ID NO: 51)	0.11 0.3
9	9	5'GGAGGACGAUGCGGGUGACUAGGCAAGCAC AAAACUGUCGCUAUGACAGAUUCUGUCAGACG ACUCGCUGAGGAUCCGAGA3'	0.09	CGCUCAUG AGAUCU UGACUAGG UAGGCAA AAAACU CA UCG	1.57 0.26 0.15 0.12 0.1 0.57
10	10	5'GGAGGACGAUGCGGACGGAGGAUAGUUGCU AAUCGAGCGCUGCCGACGCUCCAGACGACUC GCUAGGAUCCGAGA3'	3.61	GGAG CGAC GGAGG CGAGC GAU GC UUG CG GGAG CC	13.43 3.61 33.35 18.87 0.78
11	11	5'GGAGGACGAUGCGGGGAAGCAACACUUAU CGCGAUUGAUACGUGCGCAGUACAGACGAC UCGCUGAGGAUCCGAGA3'	1.01	GCAACAC	1.49
12	12	5'GGAGGACGAUGCGGGGAAGCAACACUUAU CGCGAUUGAUACGUGCGCAGUACAGACGACGA CUCGCUGAGGAUCCGAGA3'	0.63	GAUACGUGCGC (SEQ ID NO: 52)	0.18
13	13	5'GGAGGACGAUGCGGACGGAGGAUAGUUGCU AAUCGAGCGCUGCGCACGCUCCAGACGACUC GCUAGGAUCCGAGA3'	0.78	GUUGC GCUGCGCAC UA UAA	1.42 0.16 4.56
14	14	5'GGAGGACGAUGCGGUGUACACUGAUUUGCCU UUGUGUUAUGAGCGACAGAUCCAGACGAC UCGCUGAGGAUCCGAGA3'	0.16	UAUGAGCG	0.8
15	15	5'GGAGGACGAUGCGGUGUACACUGAUUUGCCU CUGUGUUAUGAGCGACAGAUCCAGACGAC UCGCUGAGGAUCCGAGA3'	0.15	UUAUGAG UG CGA	0.79 12.27

Figure 11

SEQ ID	Sequence	Oligonucleotides
16 and 17	Sullenger library	5'-TCTCGGATCCTCAGCGAGTCGTCTG (40N) CCGCATCGTCCTCCCTA-3'
18	SUL3'	5'-TCTCGGATCCTCAGCGAGTCGTC-3'
19	SUL5'	5'-GGGGGAATTCTAATACGACTCACTATAGGGAGGACGATGCGG-3'
20	SUL3short	5'-TCTCGGATCCTCAGCGAGTC-3'
21	GC-rich-SUL3'	5'-ATC GAT CGA TCG ATC GAT CGA TCG ATC GAT TTT TCG ATC GAT CGA TCG ACT GAT CGA TCG TCTCGGATCCTCAGCGAGTCGTC-3'
22	Apt#1 oligo	5'-TAGGGAGGACGATGCGG CCT AGT ACA CAA GAT CTG ACA CCT CGA TAC AGA TAT GAG GCA GACGACTCGCTGAGGATCCGAGA-3'
23	Apt#2 oligo	5'-TAGGGAGGACGATGCGG CCT AGT ACA AAA GCC TGA TCT CTG TGA GCA GAC ACT AGA ACA GACGACTCGCTGAGGATCCGAGA-3'
24	Apt#3 oligo	5'-TAGGGAGGACGATGCGG ATT ACC AAC TTG AAC GCC GAG AGT GTG GTC ACG TGT TCT GCA GACGACTCGCTGAGGATCCGAGA-3'
25	Apt#4 oligo	5'-TAGGGAGGACGATGCGG CAA CAA ACT AAT CAG ACA CGA GAC AGA GAG ATA GAT CTG ACA GACGACTCGCTGAGGATCCGAGA-3'
26	Apt#5 oligo	5'-TAGGGAGGACGATGCGG CCG GAG GCA GTC ACT AAT CTT CAC TTC TCT CAG ACA TGC GCA GACGACTCGCTGAGGATCCGAGA-3'
27	Apt#6 oligo	5'-TAGGGAGGACGATGCGG CAG GTG CGG GAT CTA ATG CGT AGA CAG CCA TAT ACT GAC ACA GACGACTCGCTGAGGATCCGAGA-3'
28	Apt#7 oligo	5'-TAGGGAGGACGATGCGG ACG ACG TTT ACT GAC CAC GAT ATG TCA GAT TCG GTC CTC ATC A GACGACTCGCTGAGGATCCGAGA-3'
29	Apt#8 oligo	5'-TAGGGAGGACGATGCGG CAT ACA CAC TTG ACT CTA GAG AAC GAG CAT CTA GCG GTG TCC A GACGACTCGCTGAGGATCCGAGA-3'
30	Apt#9 oligo	5'-TAGGGAGGACGATGCGG GTG ACT AGG CAA GCA CAA AAC TGT CGC TCA TGA CAG ATC TGT CA GACGACTCGCTGAGGATCCGAGA-3'
31	Apt#10 oligo	5'-TAGGGAGGACGATGCGG ACG GAG GAT AGT TGC TAA TCG AGC GCT GCC GAC GCT CCA GACGACTCGCTGAGGATCCGAGA-3'
32	Apt#11 oligo	5'-TAGGGAGGACGATGCGG GGA AGC AAC ACT TAG TCG CGA TTG ATA CGT GCG CAG TCA TCAGACGACTCGCTGAGGATCCGAGA-3'
33	Apt#12 oligo	5'-TAGGGAGGACGATGCGG GGA AGC AAC ACT TAG TCG CGA TTG ATA CGT GCG CAG TCA GCA GACGACTCGCTGAGGATCCGAGA-3'
34	Apt#13 oligo	5'-TAGGGAGGACGATGCGG ACG GAG GAT AGT TGC TAA TCG AGC GCT GCG CAC GCT CCA GACGACTCGCTGAGGATCCGAGA-3'
35	Apt#14 oligo	5'-TAGGGAGGACGATGCGG TGT ACA CTG ATT GCC TTT GTG TTA TGA GCG ACA GAT CTG CCA GACGACTCGCTGAGGATCCGAGA-3'
36	Apt#15 oligo	5'-TAGGGAGGACGATGCGG TGT ACA CTG ATT GCC TCT GTG TTA TGA GCG ACA GAT CTG CCA GACGACTCGCTGAGGATCCGAGA-3'
Illumina oligonucleotide		
37	ILL-PF1 A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTAGTGAGGACGATGCGG
38	ILL-PF2 A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGCTGGAGGACGATGCGG
39	ILL-PF3 A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCGACGAGGACGATGCGG
40	ILL-PF4 A	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGATCAGAGGACGATGCGG
41	ILL-PFU B	AATGATACGCGACCAACGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT
42	ILL-PRU A	ATCTCGTATGCCGTCTTCTGCTTGCTCGGATCCTCAGCGAGTC
43	ILL-PR2 B	GATCGGAAGAGCACACGTCTGAACTCCAGTCACCGATGTATCTCGTAIGCCGTCTTCTGCTTG
44	ILL-PR1 B	GATCGGAAGAGCACACGTCTGAACTCCAGTCACATCACGATCTCGTATGCCGTCTTCTGCTTG
45	ILL-PR3 B	GATCGGAAGAGCACACGTCTGAACTCCAGTCACTGACCAATCTCGTAIGCCGTCTTCTGCT
46	ILL-PR4 B	GATCGGAAGAGCACACGTCTGAACTCCAGTCACGCCAATATCTCGTAIGCCGTCTTCTGCTTG

Figure 12

Mouse Antibody	Clone	Company
CD11b- PE CY7	M1/70	BD
F4/80 FITC	A3-1	AbD Serotec
LC6C PAC BLUE	AL-21	BD
LY6G APC CY7	1-A8	BD
IL4R -PE	mIL4R-M1	BD
Live/Dead fixable dead cell stain		Invitrogen
CD3 PERCP CY5.5	17A2	eBioscience
CD4 PE	GK1.5	BD
CD8 – PACIFIC BLUE	53-6.7	BD
CD3E percp	145-2c11	BD
CD11C PE CY7	HL3	BD
CD49B APC	DX5	eBioscience
CD19 APC-CY7	ID3	BD
CD11B APC	M1/70	BD
GRL PERCP 5.5	RB6-8C5	BD
F4/80 PE CY7	BM8	BD
DAPI		Sigma-Aldrich
CD25 PE	3C7	BD
FOXP3 APC	FJK-16S	eBioscience
CD3 PERCP	145-2C11	BD
CD4 APC CY7	GK1.5	BD
CD8 pac blue	53-6.7	BD
Alexafluor 647 Streptavidin		Biolegend
Alexafluor 750 Streptavidin		Biolegend
Human antibodies		
CD33-FITC	HIM3-4	BD
IL4Ra-PE	25463	R&D
CD14-APCH7	MφP9	BD
CD15-BV711	W6D3	BD
HLADR-v500	G46-6	BD
CD11b-BV605	ICRF44	BD

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/20799

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61P 35/00, A61K 45/06, A61K 31/00, A61K 41/00 (2020.01)

CPC - C12N 15/115, C12N 2310/16, C07K 16/2818, C12N 2310/351, C12N 2320/30, A61K 9/0019

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE LA FUENTE et al., RNA aptamers specific for tumor-infiltrating myeloid cells. [Abstract]. In: Proceedings of the AACR Special Conference on Tumor Immunology and Immunotherapy; 27-30 November 2018; AACR; Cancer Immunol Res, Vol. 8, Suppl. 4:Abstract nr PR16. Abstract	1-5
Y		6
Y	WILLIAMS et al., A Proinflammatory Role for Proteolytically Cleaved Annexin A1 in Neutrophil Transendothelial Migration. J Immunol. 1 September 2010, Vol.185, No.5, p 3057-3063. Abstract; p3059, col 1, para 2; p3062, col 1, para 2	6

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"D" document cited by the applicant in the international application

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

09 May 2020

Date of mailing of the international search report

12 JUN 2020

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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 20/20799

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 7-10
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.