SUPPLEMENTAL MATERIALS AND METHODS

Generation of sdAbs

An alpaca (*Vicugna pacos*) was immunized according to a six-week alternating schedule of weekly injections of recombinant human monomeric fusion MMR protein (rhMMR) or recombinant mouse MMR protein (rmMMR). Peripheral blood lymphocyte mRNA was converted to cDNA, from which sdAb-coding sequences were amplified and ligated into the pMECS phagemid vector. Using M13K07 helper phages, the sdAb library was expressed on phages in order to obtain an anti-human/mouse MMR library. Enrichment for specific sdAb-phages was performed by 4 consecutive rounds of *in vitro* selection on rhMMR or alternatingly on rhMMR or rmMMR coated wells of Nunc Maxisorp flat bottom microtiter plates (Thermo Fisher scientific). Clones were randomly selected from all rounds of panning and screened for binding on both antigens using ELISA. Selected clones were sequenced and recloned into the pHEN6c vector to encode a C-terminal hexahistidine (His6) tag. For production, both the pMECS-sdAb and pHEN6c-sdAb plasmids were transformed into a non-suppressor E. coli WK6 strain resulting in the production of sdAbs with a HA-His6 tag or His6 tag respectively.

Surface Plasmon Resonance

Affinity analysis was performed using a BIAcore T200 (GE Healthcare) with HEPES-buffered saline running buffer (10 mM HEPES with 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20 at pH 7.4). rhMMR or rmMMR was immobilized on a CM5 chip in acetate buffer 50 mM (pH 5.0), until 2000 RU. Another channel on the same chip was activated/deactivated in an identical way and served as a negative control. The MMR sdAbs were used as analytes in 11 different concentrations, ranging from 1 to 500 nM, at a flow rate of 10 µl/min. Glycine–HCl 50 mM (pH 2.0) was used for regeneration. The kinetic and equilibrium parameters (k_d, k_a and K_D) values were calculated from the combined sensogram of all concentrations using BIAcore T200 evaluation software 1.0 (GE Healthcare).

Flow Cytometry

Single cell suspensions from 3LL-R tumors containing mMMR$^+$ macrophages were prepared as described previously.[3] The His6 tagged anti-MMR sdAbs or irrelevant control sdAb BcII10 (10 µg/ml) were incubated 1h on ice with 10^6 tumor cells per tube, washed twice with ice-cold MACS buffer and incubated with 5 µg/ml FITC-conjugated anti-His6 tag mouse IgG1 (R&D Systems). Stained cells were washed once more with ice-cold MACS buffer before analysis. The immature human dendritic cells (iDCs) expressing hMMR were a kind gift from Karine Breckpot (Vrije Universiteit Brussel, Jette, Belgium). A total of 10^7 cells were thawed on ice and incubated for 1h with RPMI 1640 medium supplemented with 500 U/ml IL-4 (Invitrogen) and 1000 U/ml GM-CSF (Gentaur). 10% rabbit serum was added to prevent aspecific Fc mediated binding. After 30 min HA tagged anti-MMR or irrelevant sdAb (10 µg/ml) were added to $2x10^5$ cells per tube. 1h later the cells were washed twice with ice-cold HBSS buffer supplemented with 1% rabbit serum and incubated with 0.5µg/ml Alexa Fluor 488 conjugated mouse anti-HA IgG1 (Invitrogen). Stained cells were washed once more with ice-cold MACS buffer and analysed on a FACS Canto II (BD Biosciences). The data were processed with FlowJo software (Tree Star, Inc., Ashland, OR 97520, USA). The irrelevant sdAb BcII10 used in this study binds to beta lactamase of 'Bacillus cereus 569/H'.

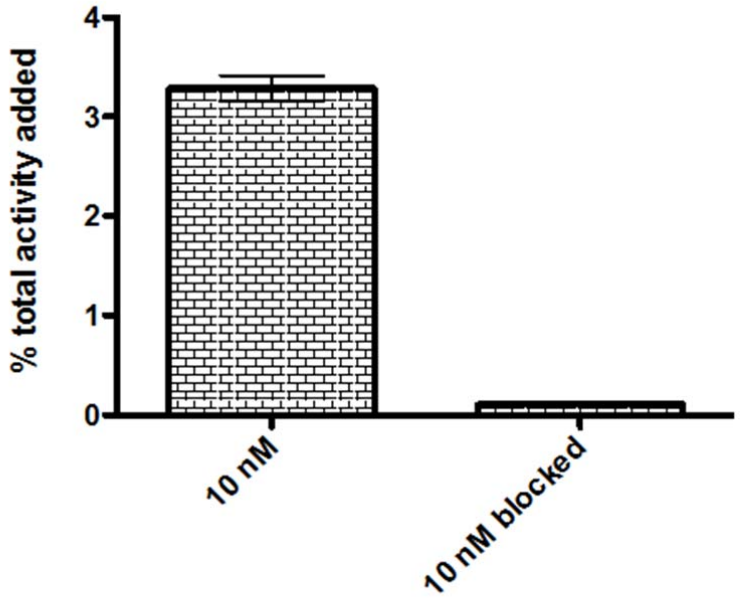
In Vitro Binding Study

To test sdAb functionality after radiolabeling, binding of the ^{99m}Tc-labeled lead compound (sdAb 3.49) to rmMMR protein (2 μg/ml) immobilized overnight in 0.1M $NaHCO_3$ buffer pH 8.8 was determined. The plate was blocked for 1 h at 37°C using a 2% bovine serum albumin solution in PBS. Three washing steps with PBS-0.1%/Tween-80 were performed and total binding was assessed by adding 10 nM of the labeled sdAb to the well plate for 1 h at 37°C. By co-incubation with a 500-fold excess of cold sdAb, non-specific binding was assessed. After incubation, the wells were washed three times with cold PBS-0.1 %/Tween-80 to remove unbound radioactivity. Bound activity was removed with 1 M NaOH for 10 min. Solutions were transferred into counting tubes and radioactivity was measured with an automatic gamma counter (Cobra II Inspector 5003, Canberra-Packard).

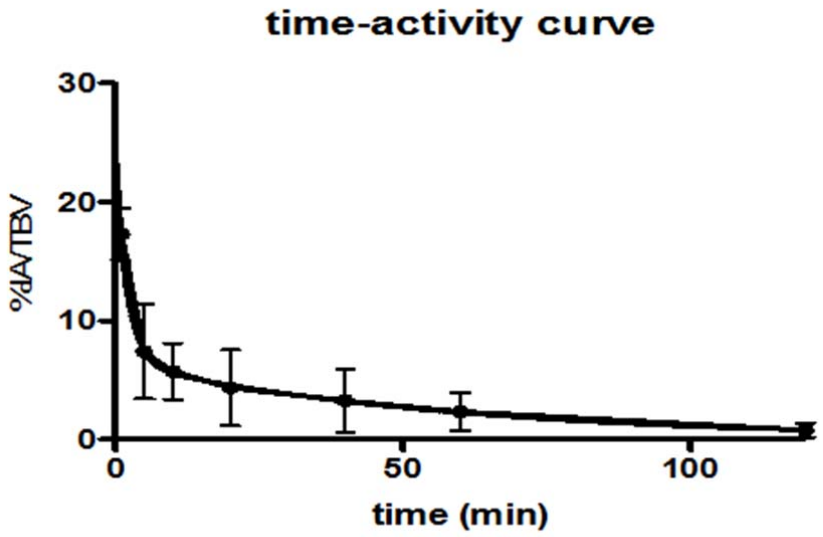
Supplemental Table 1: Conjugation Efficiency of [^{18}F]-SFB to anti-MMR sdAb in function of reaction parameters

	sdAb (mg/ml)	Temp. (°C)	Yield (%) 20 min	60 min
pH 8.3	*1*	*RT*	*9.5*	*12.4*
pH 8.4-8.5	*0.2*	*RT*	*11.3*	*12.3*
	0.4	*RT*	*12.5*	*12.2*
	1	*RT*	*24.8*	*25.3*
	1	*40°C*	*18.4*	*15.5*
pH 8.7	*1*	*RT*	*14.5*	*12.6*

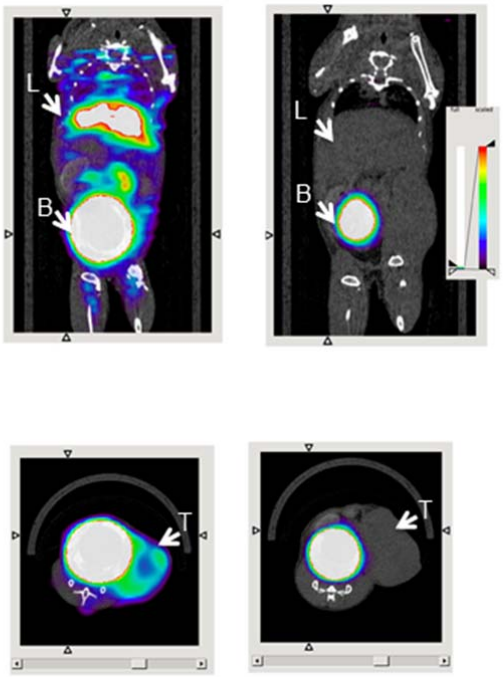
RT = room temperature



Supplemental Figure 1: Specific binding of ^{99m}Tc-anti-MMR 3.49 sdAb to rmMMR protein was observed (left) and efficiently blocked with 500-fold excess of cold anti-MMR 3.49 sdAb (right). Data are presented as mean ± SD (n = 6).



Supplemental Figure 2: Blood samples were collected at different time points after intravenous injection of [^{18}F]FB-anti-MMR 3.49 sdAb in WT mice and a blood clearance curve obtained. Half-lives were determined using bi-exponential nonlinear regression fit (GraphPad Prism).



Supplemental Figure 3: Transverse and coronal PET/CT images of WT (left) versus MMR deficient (right) 3LL-R tumor bearing mice scanned 3h post injection of [^{18}F]FB-anti MMR 3.49. [^{18}F]FB-anti-MMR 3.49 signals obtained by PET are encoded in color scale, CT-image in grey scale. Arrows point at tumor (T), kidney (K) and bladder (B). Autoradiography performed on slices from 3LL-R tumors grown in WT (left) versus MMR deficient (right) mice.