NANOBODY-BASED TARGETING OF THE MACROPHAGE MANNOSE RECEPTOR FOR EFFECTIVE IN VIVO IMAGING OF TUMOR-ASSOCIATED MACROPHAGES

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ABSTRACT

Tumor-associated macrophages (TAMs) are an important component of the tumor stroma and exert several tumor-promoting activities. Strongly pro-angiogenic TAMs that reside in hypoxic tumor areas highly express Macrophage Mannose Receptor (MMR, CD206). In this study, we targeted MMR+ TAMs using nanobodies, which are single-domain antigen-binding fragments derived from Camelidae heavy-chain antibodies. MMR-specific nanobodies stained TAMs in lung and breast tumor single-cell suspensions in vitro, and intravenous injection of 99mTc-labeled anti-MMR nanobodies successfully targeted tumor in vivo. Retention of the nanobody was receptor-specific and absent in MMR-deficient mice. Importantly, co-injection of excess unlabeled, bivalent anti-MMR nanobodies reduced nanobody accumulation in extra-tumoral organs to background levels, without compromising tumor uptake. Within tumors, the 99mTc-labeled nanobodies specifically labeled MMR+ TAMs, as CCR2-deficient mice that contain fewer TAMs showed significantly reduced tumor uptake. Anti-MMR nanobodies also accumulated in hypoxic regions, therefore targeting pro-angiogenic MMR+ TAMs. Together, our findings provide preclinical proof of concept that anti-MMR nanobodies can be used to selectively target and image TAM subpopulations in vivo.
INTRODUCTION

Tumors harbor dynamic microenvironments in which cancer cells are intimately associated with non-transformed host cells. The tumor-associated stroma is considered to play an important role during tumor growth, influencing phenomena such as angiogenesis, metastasis and immune suppression (1). As such, the stroma forms an attractive target for diagnostic and therapeutic applications (2).

Different myeloid cell types are important components of the tumor stroma (3, 4). Especially macrophages are often found to infiltrate tumors in high numbers (5-7). We previously characterized tumor-associated macrophages (TAMs) in different pre-clinical tumor models (8). Extensive gene and protein expression analysis led us to identify distinct TAM subsets, termed MHC IIhi and MHC IIlow TAMs. Interestingly, these subsets reside in different intratumoral microenvironments and differentially express molecules involved in inflammation, chemotaxis and angiogenesis. MHC IIlow TAMs are mainly located in hypoxic tumor areas and are strongly pro-angiogenic. In contrast, MHC IIhi TAMs are found in normoxic/perivascular regions and are significantly less pro-angiogenic. Besides MHC II, we identified several membrane markers that can distinguish between these TAM subpopulations. This included the Macrophage Mannose Receptor (MMR, CD206), an endocytic C-type lectin receptor known for its prominent expression on alternatively activated macrophages, which is consistently upregulated on MHC IIlow TAMs in all tumor models studied (8). These observations make MMR an attractive marker for targeting the MHC IIlow hypoxic TAM subset in vivo.

Antibody-based tumor targeting strategies are widely explored (9, 10). Antibodies can be used for tumor imaging or delivering therapeutics to tumor cells. However, limitations of conventional antibodies include a poor penetration of solid tumors and high Fc-mediated aspecific binding, highlighting the need for smaller and more specific binding units.
Nanobodies (Nbs) are the smallest available antigen binding fragments (15 kDa) derived from Camelid heavy chain-only antibodies (11, 12). Nbs are stable, soluble, have a high affinity, and show an increased tissue penetration, making them particularly suitable for tumor targeting (13, 14). Previous work showed that a Nb-conjugate can efficiently target and kill cancer cells harboring a model antigen (15, 16). In addition, employing pinhole SPECT/Micro-CT technology, ⁹⁹mTc-labeled Nbs have been successfully used as probes for cancer cell markers in tumor imaging (17-20) and for dendritic cell markers to image their in vivo biodistribution (21). Indeed, because of their small size, unbound Nbs are rapidly eliminated by renal clearance, resulting in high signal-to-noise ratios. As a result, imaging can be performed as early as 1 hour post-injection of the nanobody probe, enabling the use of short-lived radio-isotopes with a clear benefit for the patient (22).

Here, we describe the production, selection and characterization of Nbs against MMR. We show that ⁹⁹mTc-labeled anti-MMR Nbs allow a fast and specific targeting of MMR⁺ cells in tissues, including a strong labeling of tumoral stromal cells, as shown by pinhole SPECT/Micro-CT imaging. Importantly, excess administration of unlabeled bivalent anti-MMR Nbs provides a novel strategy for eliminating extratumoral signals while maintaining the targeting of tumor-associated MMR⁺ cells, a major part of which are MMR⁺ TAM. Altogether, we preclinically validated ⁹⁹mTc-labeled MMR targeting tracers suitable for molecular imaging of MMR⁺ stromal cells using SPECT cameras, which might pave the way for novel approaches in diagnostic imaging and therapeutic targeting of the tumor stroma.
MATERIALS AND METHODS

Mice, cell lines

Animal studies followed the guidelines of the institutional review board. Female Balb/c and C57BL/6 mice were from Harlan. C57BL/6 MMR-deficient, CCR2-deficient and MMTV-PyMT mice were provided by Etienne Pays (Université Libre de Bruxelles), Frank Tacke (Aachen University) and Massimiliano Mazzone (KULeuven), respectively. The Balb/c mammary adenocarcinoma TS/A and 3LL-R clone of the C57BL/6 Lewis Lung carcinoma (8) were injected subcutaneously (s.c.) in the flank or in the fat pads (3x10^6 cells). 12-14 days after inoculation, TS/A and 3LL-R tumor-bearing mice were imaged. MMTV-PyMT mice bearing macroscopic tumors were consecutively imaged with distinct tracers 48-72h apart. Tumor dissection and flow cytometry were performed 96h after the last scan.

Tumor preparation, flow cytometry

Preparation of tumor single-cell suspensions has been described (8). Antibodies for stainings were anti-CD11b(M1/70)/PE-Cy7, anti-Ly6G(1A8)/FITC (BD Biosciences), anti-IA/IE(M5/114.15.2)/PerCPCy5.5 (Biolegend), anti-Ly6C(ER-MP20)/AF647, anti-MMR(MR5D3)/PE, anti-F4/80(CI:A3-1)/PE (Serotec). To prevent aspecific binding, rat anti-mouse CD16/CD32 (2.4G2, BD Biosciences) was used. Nanobodies were labeled using the Alexafluor®488 or Alexafluor®647 Protein Labeling kit (Invitrogen) according to the manufacturers’ instructions.

Immunofluorescence stainings

Mice were injected intravenously with 500 μg Alexa-fluor647-labeled Nbs and intraperitoneally with 80 mg/kg pimonidazole [hypoxyprobe-1, HPI, Inc.] for hypoxia stainings. Two hours later, tumors were fixed in 4% paraformaldehyde, rehydrated overnight.
(20% sucrose) and sectioned (5μm). Antibodies were: rat anti-F4/80/alexa-fluor488 (Cl:A3-1, Serotec), F(ab')2 donkey anti-rabbit/Cy3 Jackson Immuno). Pictures were acquired with a Plan-Neofluar 10x/0.30 or 20x/0.50 (Carl Zeiss) objective on a Zeiss Axioplan2 microscope with an Orca-R2 camera (Hamamatsu) and Smartcapture 3 software (Digital Scientific UK).

**Generation of mono- and bivalent Nanobodies.**

The anti-MMR Nanobodies (Nb) clone 1 and 3 were isolated from an immune Nb phage-display library (23, 24). An alpaca (Vicugna pacos) was immunised weekly with 100μg MMR extracellular domain (R&D Systems) for six times. Peripheral blood lymphocyte mRNA was converted to cDNA, from which Nb-coding sequences were amplified and ligated into the pHEN4 phagemid vector (25). Using M13K07 helper phages, the Nb library was expressed on phages and specific Nb-phages were enriched by 3 rounds of selection on microtiter plates (Nunc) coated with recombinant MMR. Individual colonies were screened in ELISA for antigen recognition and sequenced. The Nb genes of clones 1 and 3 were recloned into the vector pHEN6 to encode a C-terminal His6 tag (25). As negative control Nanobody Nb BCII10 (25) was used.

Bivalent Nbs were generated by attaching a linker sequence 3’ of the anti-MMR Nb clone 1 VHH sequence using primer biNbF and primers biNbG4SR, biNb2cR, and biNbIgAR (Supplementary Table 1) which code for a (G4S)3 (GGGGSGGGSGGGGS), llama IgG2 hinge (AHHSEDPSKAPKAPMA) or human IgA hinge (SPSTPPTPSPTPPAS) linker, respectively. PCR fragments were inserted 5’ of the α-MMR cl1 gene or the BCII10 gene in the pHEN6 vector.

Periplasmic expression and purification of mono- and bivalent Nbs was performed as described previously (19).
Nanobodies were labeled with $^{99m}$Tc at their hexahistidine tail and quality assured, as described (17, 19, 21). Mice were intravenously injected with 100-200 μl 45-155 MBq of $^{99m}$Tc-labeled Nb, with or without an excess of bivalent unlabeled Nanobody. 60 or 180 min post-injection, anesthesia, microCT and pinhole SPECT-imaging were performed as described (19). Image viewing was performed using AMIDE Medical Image Data Examiner software. High-resolution image 3D-reconstructions were generated using OsiriX Imaging Software. 30 min after initiating microCT/SPECT acquisition, organs were removed and weighed, and radioactivity was measured using an automated $\gamma$-counter (Cobra II Inspector 5003; Canberra-Packard). Tissue and organ uptake was calculated as percentage of injected activity per gram tissue (%IA/g), corrected for decay.

**Statistics**

Statistical analyses were performed using the student’s t-test assuming unequal variances. Since multiple comparisons are made (9-10 different organs), the p-values of the student’s t-test were adjusted by Holm’s procedure (26). The R environment (27) and the multtest package (28) were used for statistical analyses and figures. The significance of the student t-tests and corrections for multiple testing was set to 0.05.
RESULTS

1. MMR as a potential marker for the differential targeting of TAM subsets in vivo

Previously, we showed that in tumor single-cell suspensions, MMR was differentially expressed between MHC II\textsuperscript{hi} and MHC II\textsuperscript{low} TAMs, as assessed by flow cytometry using anti-MMR monoclonal antibodies (8). Here, we show in TS/A mammary carcinoma and 3LL-R lung carcinoma subcutaneous tumor single-cell suspensions that MMR was not/poorly expressed on CD11b\textsuperscript{−} cells, granulocytes, monocytes and Ly6C\textsuperscript{int} TAMs (Supplementary Figure 1). Next, we investigated MMR expression patterns in TS/A tumor sections triple-stained for MMR, CD11b and MHC II (Supplementary Figure 2). MMR and CD11b staining almost completely co-localized, showing that MMR\textsuperscript{+} cells were indeed TAMs. Interestingly, MMR expression poorly co-localized with CD11b\textsuperscript{+}MHC II\textsuperscript{+} cells (mostly corresponding to MHC II\textsuperscript{hi} TAMs), indicating that MMR staining was mainly restricted to MHC II\textsuperscript{low} TAMs. Therefore, MMR can be used for differentially labeling MHC II\textsuperscript{hi} and MHC II\textsuperscript{low} TAMs on tumor sections. Together, these results suggest that MMR could be an interesting marker for specifically targeting the M2-like/hypoxic MHC II\textsuperscript{low} TAMs in vivo.

2. Generation and characterization of α-MMR nanobodies

Nbs were raised against the recombinant extracellular domain of MMR and after screening of an immune phage library, 2 MMR-specific Nb clones were isolated: Nb cl1 and cl3. The binding characteristics of the anti-MMR Nbs were compared using surface plasmon resonance (SPR) measurements (Supplementary Table 2, Supplementary Figure 3). Nb cl1 demonstrated an 8-fold higher apparent affinity for immobilized recombinant MMR compared to Nb cl3 (K_{D} = 2.31 \times 10^{-8} \text{ M} versus 1.91 \times 10^{-7} \text{ M}, respectively). In addition, SPR competition studies demonstrated that pretreatment with cl1 does not preclude cl3 binding...
and vice versa, suggesting that anti-MMR Nbs cl1 and cl3 bind to non-overlapping epitopes (Supplementary Figure 3).

First, we investigated whether the α-MMR Nb cl1 could bind surface-expressed MMR on TAMs ex vivo. Hereeto, flow cytometric analyses were performed using fluorescently labeled Nb cl1 on subcutaneous TS/A and 3LL-R tumor single-cell suspensions (Figure 1). As a negative control Nb, we consistently used Nb BCII10 (25). The α-MMR Nb cl1 bound to a subset of CD11b+ cells, but not to CD11b- cells (Figure 1A-B). Within the CD11b+ fraction of TS/A tumors, the α-MMR Nb did not bind to monocytes (Figure 1C, gate 1), granulocytes (gate 5) and only very weakly to Ly6Cint TAMs (gate 2). Staining was therefore restricted to MHC IIhi (gate 3) and MHC IIlow TAMs (gate 4), with the latter subset binding α-MMR Nb to a much greater extent. For 3LL-R tumors, α-MMR Nb binding was restricted to MHC IIlow TAMs (Figure 1D, gate 5) and was not recorded on MHC IIhi TAMs (Figure 1D, gate 4) nor CD11b+ MHC IIhi Tumor-Associated DCs (TADCs, Figure 1D, gate 3), expressing high levels of CD11c and costimulatory molecules (data not shown). These results are therefore in line with our observations using α-MMR monoclonal antibodies (Supplementary Figure 1).

Finally, α-MMR Nb cl1 also bound to myeloid subsets in healthy organs of tumor-bearing mice, an important example being the liver, where in single-cell suspensions distinct macrophage subpopulations were stained (Supplementary Figure 4).

3. Assessment of the biodistribution and specificity of α-MMR nanobodies in naive mice

using Pinhole SPECT/micro-CT analysis and ex vivo dissection

Next, we assessed whether the α-MMR Nbs could be used for targeting and imaging of MMR-expressing cells in vivo. First, this was investigated in naive mice, where MMR can be expressed on tissue macrophages, dendritic cells and subsets of hepatic and lymphatic endothelial cells (29, 30). Hereeto, 99mTc-labeled α-MMR Nb cl1, α-MMR Nb cl3, and control
Nb BCII10 were injected intravenously in naive C57BL/6 mice. 1 hour post injection, total-body scans were acquired using pinhole SPECT/micro-CT (Figure 2A; only shown for cl1). In addition, mice were sacrificed and the biodistribution was assessed by measuring radioactivity in various dissected organs and was expressed as injected activity per gram tissue (%IA/g) (Supplementary Table 3). Besides intense staining of the kidneys and bladder, consistent with the rapid clearance of Nb, an increased retention of the α-MMR Nb, but not the control Nb BCII10, was measured in several organs and tissues, including cardiac muscle, bone marrow, spleen and liver, with the latter two showing the most intense signals (Supplementary Table 3, Figure 2A). In WT mice, α-MMR Nb cl1 showed a clearly higher uptake than α-MMR Nb cl3 (Supplementary Table 3). Importantly, in vivo retention of Nb cl1 and cl3 is receptor-specific as only background-level tracer uptake is seen in organs of MMR-KO mice (Supplementary Table 3, Figure 2A). Hence, the α-MMR Nbs have a high in vivo specificity and can efficiently target organs such as the liver and spleen. Given the higher affinity of Nb cl1 for recombinant MMR and the higher in vivo uptake, we chose this Nb as lead compound for further research.

4. Tumor-targeting potential of α-MMR Nb cl1

Next, we investigated whether α-MMR Nb cl1 could be used for TAM-targeting in vivo. Hereto, 99mTc-labeled α-MMR Nb cl1 was injected intravenously in TS/A and 3LL-R s.c. tumor-bearing mice and SPECT/micro-CT and ex vivo dissection analyses were performed. 99mTc-labeled Nb BCII10 was injected as negative control. Interestingly, both TS/A and 3LL-R tumors showed a clear uptake of α-MMR Nb, which was significantly higher than tumor uptake of Nb BCII10 (Figure 2B-C). These findings were confirmed through ex vivo dissection analysis: TS/A tumor uptake was 3.02 ± 0.10 %IA/g for α-MMR Nb and 0.40 ± 0.03 %IA/g for Nb BCII10; 3LL-R tumor uptake was 3.02 ± 0.19 %IA/g for α-MMR Nb and
0.74 ± 0.03 %IA/g for Nb BCII10 (Table 1). To further ascertain the specificity of tumor uptake, 3LL-R tumors were grown in MMR-KO mice. In these animals, 3LL-R tumors grew progressively and the distinct TAM subsets remained present and were MMR negative, as assessed by flow cytometry (Supplementary Figure 5). Importantly, tumor uptake of α-MMR Nb was reduced by 10-fold (0.33 ± 0.03 %IA/g, Table 1), showing its dependence on MMR expression by host cells.

5. α-MMR Nb cl1 targets hypoxic Tumor-Associated Macrophages in vivo

Having established that α-MMR Nb cl1 specifically targeted MMR+ cells in tumors, we wished to ascertain whether this was due to TAM targeting. Previous work showed that CCR2-deficiency can result in a significant decrease in TAM infiltration with only a minimal effect on tumor growth, resulting from the compensatory influx of tumor-promoting neutrophils (31, 32). To investigate whether CCR2-deficiency affected the numbers of TAMs and in particular MHC IIlow TAMs in our model, flow cytometric analyses were performed on single-cell suspensions of equally sized s.c. 3LL-R tumors grown in WT or CCR2-KO mice. This showed that CCR2-deficiency led to a dramatic reduction in the number of MHC IIlow TAMs, while infiltration of Ly6G%MNR+ neutrophils was significantly increased (Figure 3A, Supplementary Figure 6). Next, we compared the tumor-uptake of 99mTc-labeled α-MMR Nb cl1 injected in WT vs CCR2-KO 3LL-R tumor-bearing mice. 99mTc-labeled α-MMR Nb showed a similar biodistribution in the organs/tissues of CCR2-KO vs WT tumor-bearers (Supplementary Table 4). Importantly however, uptake of 99mTc-labeled α-MMR Nb was significantly reduced in CCR2-KO tumors: 2.97 ± 0.22 %IA/g in WT vs 1.83 ± 0.1 %IA/g in CCR2-KO tumors (Figure 3B). This indicates that TAMs residing in solid tumors are indeed targets of α-MMR Nbs in vivo.
Since MHC II$^{low}$ MMR$^+$ TAM were reported to associate with hypoxic regions (8), we next assessed whether α-MMR Nbs preferentially label hypoxic TAM in vivo. Here, AF647-coupled α-MMR Nbs were injected i.v. in s.c. 3LL-R WT or MMR-KO tumor-bearing mice. 2h later tumors were collected, sectioned and stained for the hypoxia marker pimonidazole (hypoxyprobe) and the macrophage marker F4/80. Interestingly, AF647 fluorescence almost completely colocalized with F4/80 staining in WT tumors, but was absent from MMR-KO tumors (Figure 3C). In addition, the majority of AF647(bright) cells were located in hypoxic areas and stained with pimonidazole (Figure 3C-D). These results convincingly show that α-MMR Nbs can target hypoxic tumor regions in vivo, where they bind to the residing MMR$^+$ macrophages.

6. Strategies for increasing the tumor-to-tissue ratio of $^{99m}$Tc-labeled α-MMR Nb cl1

A methodology for the specific in vivo targeting of a tracer to TAMs, but not to other sites in the body, could be of important diagnostic and therapeutic significance. However, both in the TS/A and 3LL-R model, $^{99m}$Tc-labeled anti-MMR Nb accumulates to a higher extent in liver and spleen as compared to tumor. Therefore, we aimed to minimize binding of labeled tracer in these extratumoral sites, while preserving tumor targeting. The efficient tumor targeting potential of nanobodies is thought to be a direct result of their small size. To investigate this, a series of larger bivalent Nbs were created (Figure 4A). First, αMMR-αMMR bivalent Nbs were made by cloning three different peptide linkers with increasing proline content (glycine-serine linker, part of the llama IgG2c hinge or part of the human IgA hinge) between two Nb cl1 sequences. All these bivalent Nbs showed a 5-fold higher avidity compared to the monovalent Nb cl1, which can be largely attributed to a 3-fold increase in $k_d$ (Supplementary Table 2), and displayed a very similar in vivo biodistribution (Supplementary Table 5). In addition, using the llama IgG2c linker, αMMR-BCII10 bispecific Nbs and BCII10-BCII10
bivalent Nbs were generated and their in vivo biodistribution was evaluated in TS/A and 3LL-R tumor-bearing mice. Interestingly, αMMR-BCII10 and especially αMMR-αMMR Nbs showed a significantly enhanced targeting of liver and spleen, but a dramatically reduced targeting of tumor, as compared to monovalent α-MMR Nbs (Figure 4B). Hence, these bivalent Nbs seem to possess desirable features to efficiently block extratumoral binding sites while preserving intratumoral binding sites. To test this, we co-injected 99mTc-labeled monovalent α-MMR Nb with a 20-fold molar excess of unlabeled bivalent αMMR-αMMR Nb and assessed the specific uptake of labeled Nb in distinct organs. While the retention of monovalent 99mTc-labeled α-MMR Nb is reduced in all organs to the aspecific background level seen with Nb BCII10, the uptake in tumors is only slightly diminished (Figure 4C). As a result, the tumor-to-tissue ratio of labeled α-MMR Nb is dramatically increased and tracer uptake is highest in the tumor. This allowed the tumor to be clearly distinguishable in SPECT/micro-CT imaging of mice bearing subcutaneous tumors (Figure 5A,B, Supplementary video 1). Importantly, very similar imaging data were obtained when TS/A tumors were grown orthotopically in the mammary fat pad (Figure 5C-D, Supplementary video 2), for which the presence of the two main TAM subsets was reported before (8). Finally, imaging studies were performed in transgenic MMTV-PyMT mice, which spontaneously develop mammary tumors (33). Hereto, a mouse bearing multiple macroscopic tumors was consecutively imaged (48h intervals to allow complete elimination and decay of the 99mTc tracer) with either 99mTc-labeled α-MMR Nb, 99mTc-labeled BCII10 Nb or 99mTc-labeled α-MMR Nb co-injected with unlabeled bivalent αMMR-αMMR Nb. When 99mTc-labeled α-MMR Nb was injected alone, tumors were not easily distinguishable due to high extratumoral uptake (Figure 6A). However, co-injecting unlabeled bivalent αMMR-αMMR Nb minimalised extratumoral Nb retention and resulted in tracer uptake in the most prominent macroscopic nodules as seen via high-resolution 3D CT reconstructions (Figure
6B, Supplementary Video 3). Notably, FACS analysis showed that for all three selected tumors highlighted in Figure 6B, distinct TAM subpopulations were present, whereby MMR expression was highest on the MHC II\textsuperscript{low} TAMs (Figure 6C).

7. Effect of mono- and bivalent $\alpha$-MMR Nb cl1 on immune cell activation

Monoclonal anti-MMR antibodies are known to potentially activate macrophages and DCs (34). To assess whether mono- or bivalent $\alpha$-MMR Nb cl1 elicits a response, Nbs were added in varying concentrations to bone-marrow derived DCs (BMDCs) or macrophages (BMDMs) in vitro or were injected at a high dose in vivo. Monovalent $\alpha$-MMR Nbs did not alter cytokine/chemokine production by BMDCs nor BMDMs in vitro, with or without LPS stimulation (Supplementary Figure 7). With the highest concentration of bivalent Nb (40 $\mu$g/ml) we observed a small, but significant, increase in TNF production by DCs and TNF and IL1Ra production by macrophages in vitro. Importantly however, the highest in vivo dose of Nb used in this study (5$\mu$g monovalent Nb + 200$\mu$g bivalent Nb) did not induce any significant increase in the serum cytokine levels, both for naive and tumor-bearing mice (Supplementary Figure 8). Overall, we conclude that anti-MMR Nbs are innovative tools for the targeting and imaging of hypoxic MMR\textsuperscript{+} TAMs without the risk of inducing overt innate immune responses in vivo.
DISCUSSION

Until now, antibody-based tumor-targeting approaches have mostly been directed against antigens expressed on cancer cells (10). However, the antigenic profile of cancer cells can be unstable and depends on the cancer type. Tumors also contain a large stromal compartment, which includes myeloid cells such as macrophages (35). Stromal cells might provide a good alternative for tumor-targeting, since their antigenic profile is more stable and might be similar across different cancer types. Our previous work indicated MMR as an interesting marker for targeting the most M2-oriented (and potentially most tumor-promoting) macrophage subset in tumors (8). Here, we describe the production of MMR-specific nanobodies and show they can be used for efficient in vivo targeting and imaging of TAMs in solid tumors.

We describe two α-MMR Nbs which bind to different epitopes and have distinct affinities and in vivo targeting efficiencies. α-MMR Nb cl1 was chosen as lead compound and its tumor-targeting potential was first examined in mice bearing subcutaneous 3LL-R lung or TS/A breast carcinoma tumors. For both models, ex vivo dissection showed that the α-MMR Nb had a tumor uptake of about 3 %IA/g. Previous studies using nanobodies for the targeting of antigens (CEA, HER-2) on tumor xenografts, in which all cancer cells ectopically express the antigen, showed tumor uptake levels in the same range (15, 16). These amounts were furthermore enough to almost completely eradicate tumors in an antibody-dependent enzyme prodrug therapy approach (16). We thus conclude that α-MMR Nb efficiently targeted solid tumors.

To investigate whether nanobody targeting was receptor-specific, we compared the tumor uptake of α-MMR Nb with that of Nb BCII10, and, more stringently, we compared the uptake of α-MMR Nb in tumors from WT vs MMR-KO mice. Together these data
convincingly showed that in vivo targeting of the α-MMR Nb is MMR-specific. In tumor single cell suspensions α-MMR Nbs primarily bound to TAMs, suggesting that this was also their target in vivo. However, we could not exclude the potential targeting of other MMR+ cells in intact tumors. Therefore, we compared the tumor retention of α-MMR Nb injected in WT or CCR2-KO tumor-bearing mice. 3LL-R tumors in CCR2-KO mice contained significantly lower TAM numbers as compared to WT tumors, and showed a significant reduction in α-MMR Nb uptake, arguing for TAM targeting. Moreover, AF647-labeled α-MMR Nb injected in tumor-bearing mice mainly stained MMR+F4/80+ macrophages in hypoxic regions. Together these results indicate that tumor accumulation of α-MMR Nb is mainly due to its penetration in hypoxic environments and targeting of MHC IIlow TAMs residing there. However, CCR2-KO tumors still showed some α-MMR Nb retention. This could suggest that, besides residual targeting of the remaining TAMs, other MMR+ cells are targeted. For example, MMR can be expressed on lymphatic vessels where it affects leukocyte trafficking and contributes to cancer cell metastasis (36), suggesting the potential value of α-MMR Nb for targeting tumor lymphatics. Future investigations will have to address this issue.

Finding tumor-specific markers for antibody-based targeting remains a daunting task. This is especially true when targeting the tumor stroma, since stromal antigens are typically not restricted to tumors. In this regard, α-MMR Nbs also targeted to a higher extent the liver and spleen of mice bearing subcutaneous tumors. This may hamper the usefulness of these tools both for diagnostic and therapeutic applications. Importantly however, we describe a novel approach to reduce the targeting of tracers to healthy organs to background levels, while preserving an efficient targeting of the tumor. Indeed, co-injecting an excess of unlabeled bivalent α-MMR Nb blocked all extratumoral sites, while only slightly affecting tumor-specific tracer uptake. This is a result of a bivalent Nb's higher uptake in extratumoral organs.
such as liver and spleen (probably explained by a higher avidity for αMMR-αMMR Nb and/or increased in vivo retention due to larger dimensions for αMMR-αMMR and αMMR-BCII10), coupled to a low accumulation in the tumor (probably due to poor tumor penetration). Interestingly, modelling studies have suggested that intermediate-sized targeting agents (~25-30 kDa) have the lowest tumor uptake levels among a spectrum of tumor-targeting polypeptides of various sizes (37). Bivalent Nbs, which are 30 kDa, therefore seem to follow this rule and have a low tumor uptake. Similar observations were made with DARPins, which are similar in size to nanobodies (15 kDa), and for which fusion of two DARPins results in a significantly lower tumor uptake (38).

We believe that the strategy of co-injecting bivalent cold α-MMR Nb to reduce extratumoral tracer uptake might be translatable to the clinic. Preloading therapies, where an excess of cold antibody is injected in patients are already performed. In antibody-based radioimmunotherapy of non-Hodgkin lymphoma, excess amounts of unlabeled anti-CD20 antibody is predosed to patients prior to injection of 90Y- or 131I-conjugated anti-CD20 antibody (39), resulting in increased tracer uptake in tumors and reduced uptake in extratumoral organs such as the spleen. However, determining the optimal cold dose for individual patients is not straightforward since the cold antibody can compete with labeled antibody for free antigen sites in the tumor (40, 41). The monovalent-labeled – bivalent-cold Nb approach described here seems an attractive alternative, since bivalent Nbs do not efficiently compete for free binding sites in the tumor, while they block extratumoral sites much more efficiently.

Since TAMs are found to be a major stromal component in many cancer types, α-MMR Nbs could potentially be used for targeting a variety of unrelated tumors. We have successfully used this approach for the subcutaneous 3LL-R lung carcinoma model, the subcutaneous and orthotopic TS/A breast carcinoma model and for the spontaneous MMTV-PyMT breast
carcinoma model. Coupled to our methodology of restricting extratumoral tracer uptake, this could now provide novel and attractive diagnostic or therapeutic opportunities. Clear examples would be diagnostic tumor imaging and the non-invasive quantification of TAMs or specific TAM subsets inside any given tumor, which could be of prognostic value. Furthermore, since $\alpha$-MMR Nbs can penetrate hypoxic areas where the majority of MHC II$^\text{low}$/MMR$^+$ TAMs reside, this might provide a new avenue for visualizing hypoxic regions within the tumor, potentially relevant for guided radiotherapy (42). In addition, radioimmunotherapy might be the most promising therapeutic application for these Nbs, since coupling of Nbs to proteins (e.g., toxins or prodrug converting enzymes (43)), might reduce the tumor targeting efficiency due to a size increase. As a cautionary note, engagement of MMR could potentially trigger cytokine/chemokine release by DCs and macrophages (34, 44). However, our results did not show an overt cytokine/chemokine response after in vivo administration of high doses of mono- and bivalent $\alpha$-MMR Nb.

MMR is also a widely used marker for human M2 macrophages (45-47), which is expressed on TAMs from human tumors. Coculture of human macrophages and ovarian cancer cells induces a strong upregulation of MMR expression (48). Furthermore, Allavena et al have shown that MMR is widely expressed on TAMs isolated from ovarian cancer patients, and that its engagement by tumor mucins can induce an immune suppressive phenotype (44). In addition, our ongoing preliminary studies show that in human breast cancer samples, MMR$^+$ TAMs are clearly detected and, interestingly, are enriched in fibrotic foci which are known to be a marker for intratumoral hypoxia and correlate with a poor prognosis (data not shown) (49). However, it remains to be tested whether MRC1$^+$ TAMs perform the tumor-promoting, pro-angiogenic functions in human tumors as reported previously in murine tumors.

In conclusion, our work indicates that in pre-clinical models TAM subsets can be efficiently targeted in vivo using nanobodies against MMR. In addition, we provide a methodology to
restrict tracer uptake to the tumor. This could form the basis for developing novel imaging and therapeutic applications for the diagnosis and treatment of cancer.

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REFERENCES


Table 1. Uptake values of $^{99m}$Tc-labeled α-MMR or BCII10 Nb in TS/A and 3LL-R tumor-bearing WT mice, based on dissection at 3h post injection. Tracer uptake is expressed as injected activity per gram (%IA/g). Data are mean±SEM (n=6). Significance was tested between α-MMR Nb vs Nb BCII10 and α-MMR Nb injected in WT vs MMR-KO (3LL-R), for the indicated organ. *p<0.05, **p<0.01, ***p<0.0001

### TS/A Tumor-bearing mice

<table>
<thead>
<tr>
<th>Organs/Tissues</th>
<th>α-MMR Nb in WT (%IA/g)</th>
<th>Nb BcII10 in WT (%IA/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>1.45 ± 0.12</td>
<td>0.10 ± 0.01 ***</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.55 ± 0.36</td>
<td>0.98 ± 0.12</td>
</tr>
<tr>
<td>Liver</td>
<td>12.6 ± 0.54</td>
<td>0.59 ± 0.02 ***</td>
</tr>
<tr>
<td>Spleen</td>
<td>8.95 ± 0.60</td>
<td>0.24 ± 0.01 ***</td>
</tr>
<tr>
<td>Kidney Left</td>
<td>79.7 ± 2.32</td>
<td>273 ± 14.8 ***</td>
</tr>
<tr>
<td>Kidney Right</td>
<td>80.8 ± 3.62</td>
<td>261 ± 11.4 ***</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.52 ± 0.03</td>
<td>0.05 ± 0.01 ***</td>
</tr>
<tr>
<td>Bone</td>
<td>1.33 ± 0.10</td>
<td>0.08 ± 0.01 ***</td>
</tr>
<tr>
<td>Blood</td>
<td>0.13 ± 0.02</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>Tumor</td>
<td>3.02 ± 0.10</td>
<td>0.40 ± 0.03 ***</td>
</tr>
</tbody>
</table>

### 3LL-R Tumor-bearing mice

<table>
<thead>
<tr>
<th>Organs/Tissues</th>
<th>α-MMR Nb in WT (%IA/g)</th>
<th>Nb BcII10 in WT (%IA/g)</th>
<th>α-MMR Nb in MMR-KO (%IA/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart</td>
<td>2.02 ± 0.11</td>
<td>0.17 ± 0.01 ***</td>
<td>0.06 ± 0.01 ***</td>
</tr>
<tr>
<td>Lungs</td>
<td>1.46 ± 0.05</td>
<td>0.58 ± 0.04 ***</td>
<td>1.02 ± 0.70</td>
</tr>
<tr>
<td>Liver</td>
<td>9.55 ± 1.02</td>
<td>1.03 ± 0.06 **</td>
<td>1.36 ± 1.06 **</td>
</tr>
<tr>
<td>Spleen</td>
<td>4.61 ± 0.50</td>
<td>0.41 ± 0.03 **</td>
<td>0.17 ± 0.02 **</td>
</tr>
<tr>
<td>Kidney Left</td>
<td>108 ± 16.1</td>
<td>368 ± 10.10 ***</td>
<td>153 ± 27.2</td>
</tr>
<tr>
<td>Kidney Right</td>
<td>88.6 ± 21.7</td>
<td>305 ± 54.7 **</td>
<td>155 ± 20.7</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.61 ± 0.05</td>
<td>0.08 ± 0.02 ***</td>
<td>0.05 ± 0.02 ***</td>
</tr>
<tr>
<td>Bone</td>
<td>1.69 ± 0.10</td>
<td>0.13 ± 0.01 ***</td>
<td>0.06 ± 0.01 ***</td>
</tr>
<tr>
<td>Blood</td>
<td>0.10 ± 0.01</td>
<td>0.24 ± 0.01 ***</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td>Tumor</td>
<td>3.02 ± 0.19</td>
<td>0.74 ± 0.03 ***</td>
<td>0.33 ± 0.03 ***</td>
</tr>
</tbody>
</table>
**FIGURE LEGENDS**

Figure 1. α-MMR Nb cl1 differentially binds to TAM subsets in tumor single-cell suspensions. Single-cell suspensions of TS/A (A) or 3LL-R tumors (B) were stained with the indicated markers. Staining of α-MMR Nb was examined on gated myeloid subsets of TS/A (C) or 3LL-R tumors (D). Shaded histograms represent Nb BCII10 staining.

Figure 2. Fused Pinhole SPECT/Micro-CT images of mice injected with 99mTc-labeled α-MMR Nb cl1. (A) Naive WT or MMR-KO mice were injected with 99mTc-labeled α-MMR Nb cl1 and 1h post-injection images were acquired. Coronal and sagittal views are shown. (B) Coronal and transverse views of WT s.c. TS/A-bearing mice 3h after 99mTc-labeled Nb BCII10 or α-MMR Nb injection. (C) idem as (B) for s.c. 3LL-R-bearing mice.

Figure 3. α-MMR Nb targeting in WT and CCR2-KO tumor-bearing mice. (A) Percentages of MHC II<sub>low</sub> TAMs and Ly6G<sup>+</sup> neutrophils in tumor single-cell suspensions of WT and CCR2-KO tumors. Mean±SEM (n=4) (B) Uptake values of 99mTc-labeled α-MMR Nb cl1 or Nb BCII10 in WT or CCR2-KO mice 12-days post 3LL-R injection. *** p<0.001 (C) AF647-labeled α-MMR Nb cl1 and pimonidazole were injected i.v. in 3LL-R WT or MMR-KO tumor-bearers. 2h later tumors were collected and stained for F4/80 and hypoxyprobe. (D) Overlays of α-MMR Nb-AF647, hypoxyprobe and F4/80 signals in WT 3LL-R tumors.

Figure 4. Increasing tumor-to-tissue ratios of 99mTc-α-MMR Nb tracer uptake by excess unlabeled bivalent α-MMR Nb cl1. (A) Overview of different Nb constructs. (B) Mono-
and bivalent $^{99m}$Tc-labeled Nbs were injected in s.c. TS/A or 3LL-R tumor-bearing mice and uptake values were calculated 3h post injection via organ dissection (C) s.c. TS/A tumor-bearing mice were injected with $^{99m}$Tc-labeled Nb BCII10, $^{99m}$Tc-labeled α-MMR Nb cl1 or $^{99m}$Tc-labeled α-MMR Nb + 20-fold molar excess of unlabeled bivalent α-MMR Nb cl1. C1: uptake values of $^{99m}$Tc-α-MMR Nb (expressed as injected activity per gram (%IA/g)) at 3h post injection. Mean±SEM (n=6). C2: α-MMR Nb-to-background ratio, calculated as $^{99m}$Tc-α-MMR Nb uptake values / $^{99m}$Tc-Nb BCII10. C3: tumor-to-tissue ratio of $^{99m}$Tc-α-MMR Nb, calculated as ‘tracer uptake in the tumor’ / ‘tracer uptake in the organ’. Statistical significance was tested between $^{99m}$Tc-α-MMR Nb and $^{99m}$Tc-α-MMN Nb + cold Nb *p<0.05, **p<0.01, ***p<0.001

Figure 5. Fused Pinhole SPECT/Micro-CT images of mice co-injected with $^{99m}$Tc-labeled α-MMR Nb with excess unlabeled bivalent α-MMR Nb. (A) Coronal views of subcutaneous TS/A-bearing mice 3h after injection of $^{99m}$Tc-labeled α-MMR Nb cl1, $^{99m}$Tc-labeled α-MMR Nb cl1 + 20-fold molar excess of unlabeled bivalent α-MMR Nb cl1 or $^{99m}$Tc-labeled Nb BCII10. (B) 3D reconstruction of SPECT/CT images of a subcutaneous TS/A-bearing mouse injected with indicated tracer, 3h p.i. (planar view, Supplementary Video 1 for 3D view) (C) Coronal and sagital views of mice bearing orthotopic TS/A tumors in the mammary gland 3h after injection with indicated tracers. (D) High-resolution 3D reconstruction of CT and SPECT/CT images of an orthotopic TS/A-bearing mouse injected with indicated tracer, 3h p.i. (planar view, Supplementary Video 2 for 3D view).

Figure 6. α-MMR Nb-based imaging and TAM targeting in MMTV-PyMT mice. (A) A MMTV-PyMT mouse with multiple macroscopic nodules was consecutively (48-76 hours intervals) injected with indicated tracers; images were taken 3h p.i. Coronal views are shown.
n=3 (B) High-resolution 3D reconstruction of CT and SPECT/CT images of the same mouse after injection of $^{99m}$Tc-labeled α-MMR Nb and blocking bivalent α-MMR Nb. Out of multiple nodules, the numbers indicate those tumors that were chosen for dissection (C) FACS analysis of single-cell suspensions from the tumors indicated in (B).
Figure 1

(A) CD11b

Control Nb BCII10

MMR Nb

(B) CD11b

Control Nb BCII10

MMR Nb

(C) TS/A

1. Ly6C^hi mono
2. Ly6C^int TAM
3. MHC II^hi TAM
4. MHC II^low TAM
5. Granulocytes

(D) 3LL-R

1. Ly6C^hi mono
2. Ly6C^int TAM
3. MHC II^hi TADC
4. MHC II^hi TAM
5. MHC II^low TAM
6. Granulocytes

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Figure 2

A. Naive WT mouse vs. Naive MMR-KO mouse
- Liver
- Spleen
- Kidney
- Bladder
- Bone

B. BCⅡ10 Nb in WT vs. α-MMR Nb in WT
- TS/A Tumor

C. BCⅡ10 Nb in WT vs. α-MMR Nb in MMR+/− vs. α-MMR Nb in WT
- 3LL Tumor

Coronal and Sagittal views.
Figure 3

C

WT 3LL-R

MMR-KO 3LL-R

25 μm

D

α-MMR Nb-AF647

Hypoxynprobe

α-MMR Nb-AF647

Hypoxynprobe

F4/80

α-MMR Nb-AF647

F4/80

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Figure 5
Figure 6

A

$^{99m}$Tc α-MMR Nb

$^{99m}$Tc α-MMR Nb + 20x biv. α-MMR Nb

$^{99m}$Tc Nb BCI10

B

$^{99m}$Tc α-MMR Nb + 20x biv. α-MMR Nb

SPECT/CT

C

Tumor 1

Tumor 2

Tumor 3

CD11b

Ly6C

SSC

MHC II

MMR

1. Ly6C$^\text{Hi}$ mono
2. Ly6C$^\text{Int}$ TAM
3. MHC II$^\text{Hi}$ TAM
4. MHC II$^\text{Low}$ TAM
Nanobody-based targeting of the Macrophage Mannose Receptor for effective in vivo imaging of tumor-associated macrophages

Kiavash Movahedi, Steve Schoonooghe, Damya Laoui, et al.

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