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γ-Tilmanocept, a New Radiopharmaceutical Tracer for Cancer Sentinel Lymph Nodes, Binds to the Mannose Receptor (CD206)

Abul K. Azad,*,†,1 Murugesan V. S. Rajaram,*,†,1 Wendy L. Metz,‡ Frederick O. Cope,‡ Michael S. Blue,‡ David R. Vera,§ and Larry S. Schlesinger*†

γ-Tilmanocept (99mTc-labeled-tilmanocept or [99mTc]-tilmanocept) is the first mannose-containing, receptor-directed, radiolabeled tracer for the highly sensitive imaging of sentinel lymph nodes in solid tumor staging. To elucidate the mannose-binding receptor that retains tilmanocept in this microenvironment, human macrophages were used that have high expression of the C-type lectin mannose receptor (MR; CD206). Cy3-labeled tilmanocept exhibited high specificity binding to macrophages that was nearly abolished in competitive inhibition experiments. Furthermore, Cy3-tilmanocept binding was markedly reduced on macrophages deficient in the MR by small interfering RNA treatment and was increased on MR-transfected HEK 293 cells. Finally, confocal microscopy revealed colocalization of Cy3-tilmanocept with the macrophage membrane MR and binding of labeled tilmanocept to MR+ cells (macrophages and/or dendritic cells) in human sentinel lymph node tissues. Together these data provide strong evidence that CD206 is a major binding receptor for γ-tilmanocept. Identification of CD206 as the γ-tilmanocept–binding receptor enables opportunities for designing receptor-targeted advanced imaging agents and therapeutics for cancer and other diseases. The Journal of Immunology, 2015, 195: 2019–2029.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AF, Alexa Fluor; A.U., arbitrary unit; DC, dendritic cell; DC-SIGN, DC-specific ICAM-3-grabbing nonintegrin; FFPE, formalin-fixed paraffin-embedded; GalNAc, N-acetyl-D-galactosamine; MDM, monocyte-derived macrophage; MFI, mean fluorescence intensity; MR, mannose receptor; NIH, National Institutes of Health; siRNA, small interfering RNA; SLN, sentinel lymph node; TAM, tumor-associated macrophage.

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The presence of mannose in tilmanocept predicts its binding to mannose-binding lectins, such as the mannose receptor (MR; CD206) (14, 15). The MR, a member of the C-type lectin family, is a type I transmembrane glycoprotein that is expressed on most types of macrophages and on dendritic cell (DC) subsets, but not on monocytes (reviewed in Refs. 16, 17). The MR is considered to be a prototypic and widely used marker for alternatively activated colloid (4). Submucosal administration of γ-tilmanocept into pig colon and stomach disseminated into lymph nodes within 10 min (5, 6) and persisted for 3 h (7). Rapid uptake of γ-tilmanocept by SLNs was also observed in as little as 10 min after direct injection into the porcine prostate gland (8). The clinical use of γ-tilmanocept in SLN mapping and biopsy has shown it to be an excellent tool in the staging of a number of solid tumors including melanoma (9, 10), breast cancer (9–13), and squamous cell carcinoma (3).

γ-Tilmanocept is not a particle; it belongs to a new class of radiotracers called receptor-binding radiopharmaceuticals. It is a synthetic nanomolecule (~18 kDa; 7 nm) consisting of multiple units of mannose and diethylene triamine penta-acetic acid, attached to a 10-kDa dextran backbone (12). The mannose residues serve as a ligand for receptors expressed on myeloid cells for recognition and binding. The diethylene triamine penta-acetic acid serves as an attachment site for labeling the macromolecule with [99mTc] (t 0.5 = 6.02 h; 142 keV; see Fig. 1).

The rapid injection-site clearance and persistent SLN retention of γ-tilmanocept are due to its unique structural and molecular properties. After injection of γ-tilmanocept in or near to primary tumor sites, the molecular nature of this compound allows it to rapidly enter into the lymphatic channels, localize in tumor-draining lymph nodes, and bind to target receptor(s) for longer retention in these SLNs. γ-Tilmanocept was designed to target mannose-binding receptors present on the surface of lymphatic resident reticuloendothelial cells, which are composed mainly of macrophages or histiocytes. However, to date, no assurance of the receptor for tilmanocept relative to its clinical efficacy has been established.

The presence of mannose in tilmanocept predicts its binding to mannose-binding lectins, such as the mannose receptor (MR; CD206) (14, 15). The MR, a member of the C-type lectin family, is a type I transmembrane glycoprotein that is expressed on most types of macrophages and on dendritic cell (DC) subsets, but not on monocytes (reviewed in Refs. 16, 17). The MR is considered to be a prototypic and widely used marker for alternatively activated...
type 2 (M2) macrophages (16). It preferentially recognizes glycococonjugates containing terminal mannose, fucose, GlcNAc, and glucose (18). Apart from its function as a phagocytic receptor for numerous mannose-coated microbes (19–22), the MR functions in endocytosis as a scavenger receptor for clearing unwanted mammalian high mannose N-linked glycoproteins from the blood circulation to maintain homeostasis (23). The MR has been targeted for DNA delivery into human cells for therapeutic purposes (17).

Cancer progression is a complex multistep process, involving tumor growth, invasion, and metastasis. Macrophages that infiltrate into tumor microenvironment are called tumor-associated macrophages (TAMs), which have complex function in their interactions with neoplastic cells, are part of inflammatory circuits, and promote tumor progression (24–26). TAMs are characterized as M2 polarized macrophages through the action of various growth factors, TGF-β and IL-10, produced from tumor cells (27). TAMs express high levels of C-type lectins, particularly the MR (26, 28). Infiltration of TAMs into lymph nodes indicates cancer progression.

In this study, we examined the receptor(s) for tilmanocept. Using complementary approaches, we show for the first time, to our knowledge, that tilmanocept binds predominantly to human macrophages and that the MR is the major receptor for its recognition. Thus, we provide evidence for a potential mechanism underlying the utility of tilmanocept as a sensitive detector of lymph nodes that have the highest likelihood of containing cancer cells if metastasis has occurred.

Materials and Methods

Reagents and Abs

Unlabeled and [99mTc]- or Cy3- or Alexa Fluor (AF) 488–labeled tilmanocept compounds and their derivatives with different numbers of mannose units were supplied by Navidea Biopharmaceuticals (Dublin, OH). For immunofluorescence, anti-MR (CD206) mouse Abs and rabbit polyclonal Abs were purchased from Serotec (Raleigh, NC) and Biozzi (Woburn, MA), respectively, and for Western blotting anti-MR rabbit polyclonal Ab was purchased from Santa Cruz (Santa Cruz, CA). CD68 mouse monoclonal, CD163 rabbit monoclonal, and DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN; CD209) rabbit Abs, as well as isotype control mouse Ab IgG2a, were purchased from Novus (Littleton, CO). Isotype control rabbit IgG3 was from Jackson Laboratory and mouse IgG1 from Abcam (Cambridge, MA). LXXVE-1 rabbit polyclonal Ab was from LifeSpan Biosciences (Seattle, WA). AF488-conjugated goat anti-mouse, AF555-conjugated donkey anti-mouse, and AF549-conjugated goat anti-secondary Ab were obtained from Molecular Probes (Grand Island, NY). Allophycocyanin-conjugated mouse, and AF549-conjugated goat anti-rabbit secondary Abs were obtained from Biolegend (St. Louis, MO) or with mouse IgG1 from Abcam (Cambridge, MA). MR-specific polyclonal Ab, followed by HRP-conjugated secondary Ab, and developed using ECL (Amersham Biosciences). Band densitometry was measured using Scion image software (National Institutes of Health [NIH]). To quantify the band in each sample, we first subtracted the background, normalized the signal to the amount of β-actin in the lysate, and plotted the values as band intensities.

Confocal microscopy of MDMs

MDMs (1.5 × 10^6) were adhered to glass coverslips in 24-well tissue culture plates for 2 h at 37˚C. The cell monolayers were washed with RPMI 1640, preincubated at 37˚C for 5 min in serum-free RHH (RPMI 1640 + human serum albumin + HEPES buffer) medium with or without an excess amount (100-fold) of unlabeled tilmanocept, followed by an additional incubation with Cy3-labeled tilmanocept (20 μg/ml) for 10 min. The monolayers were fixed with 2% parafomaldehyde (nonpermeabilized) for 10 min at room temperature. After washing with PBS, cell nuclei were stained with 0.1 mg/ml of the DNA stain, DAPI (Molecular Probes), for 5 min at room temperature. The mounted coverslips on glass slides were viewed using a Fluoview 1000 Laser Scanning Confocal microscope (Olympus). The MFI of random confocal images was quantified using a pixel intensity measurement (NIH ImageJ program). The MFI was determined for ∼150 macrophage cells per coverslip, on duplicate slides per experiment. In mannann inhibition experiments, macrophage monolayers were preincubated at 37˚C for 20 min with 2 μg/ml mannan (Sigma, St. Louis, MO) or with N-acetyll-β-D-galactosamine (GalNAc; EY Lab, San Mateo, CA) as a negative control. In MR expression and MR-tilmanocept colocalization experiments, parafomaldehyde-fixed MDM monolayers (nonpermeabilized) were incubated with the MR or isotype control Ab (2.5 μg/ml) for 1 h at 37˚C, washed with blocking buffer (PBS + 0.5% BSA + 10% FBS), and stained with an AF488-conjugated anti-mouse secondary Ab (1:250 dilution) for 1 h at 37˚C.

Transfection of HEK293 cells

HEK293 cells (∼2 × 10^6 cells/well) were cultured until ∼90–95% confluency in DMEM medium containing 10% FBS in a six-well tissue culture plate. A human MR- or DC-SIGN-expressing plasmid or respective control empty vector (20) was transfected into HEK293 cells by using Lipo-pectamine 2000 reagent (Invitrogen, Grand Island, NY) according to the manufacturer’s protocol. After 24-h incubation of transfected cells at 37˚C in a CO2 incubator, the serum-free transfection medium was replaced with DMEM medium containing 10% serum for an additional 24 h. Expression of the MR or DC-SIGN was confirmed by Western blot of cell lysates and/or by flow-cytometry analysis.

Tilmanocept lectin blot

MR plasmid or empty vector–transfected HEK293 cells were lysed, and 20 μg protein from the lysate of each sample was separated by SDS-PAGE and transferred to a nitrocellulose membrane (three sets). One membrane was used to confirm MR expression by Western blot using anti-MR Ab, and the other two membranes were used for lectin blots. The blots were blocked in 5% nonfat milk solution for 30 min, washed with PBS, and preincubated with or without unlabeled tilmanocept (100-fold excess) in PBS with Ca2+, Mg2+ for 2 h with gentle rocking at room temperature. After washing, the blots were incubated with radiolabeled γ-tilmanocept...
(20 μg/ml) for 2 h. The blots were then washed extensively with PBS and exposed overnight to X-ray film (Kodak X-max film).

**Immunohistochemistry**

Formalin-fixed, paraffin-embedded (FFPE) lymph node tissue sections on glass slides were provided through the phase 3 clinical trial arrangements with the Department of Surgery, The Ohio State University (ClinicalTrials.gov registration number NCT00911326). The tissue sections were first deparaffinized with xylene, followed by rehydration with graded alcohols (100, 95%). A heat-induced epitope retrieval procedure was carried out by heating the tissue slides in citrate buffer (pH 6.0) at 95°C for 10 min (31). Each tissue section was rehydrated with PBS buffer, blocked (5% nonfat dry milk in PBS + 0.01% sodium azide) for 3 h at room temperature, and then incubated with specific primary Abs using manufacturer-recommended dilutions in a humidified chamber at 4°C overnight. After extensive washing with PBS, the sections were counterstained with AF488-conjugated anti-rabbit and AF549-conjugated anti-rabbit secondary Abs (double staining) for 1 h at room temperature. The sections were washed again extensively and stained with the nuclear DNA stain DAPI for 10 min at room temperature. After washing and drying at room temperature, the slides were examined by a FlowView 1000 Laser Scanning Confocal microscope (Olympus). The MFI of a randomly selected group of confocal images was quantified using a pixel intensity measurement (NIH ImageJ program).

**Tilmanocept binding analysis on FFPE lymph node tissue sections**

First, deparaffinization and Ag epitope retrieval procedure of FFPE tissue sections were performed as described earlier. AF488-labeled tilmanocept, resuspended in PBS (with Ca²⁺, Mg²⁺), was added at a concentration of 200 μg/ml to tissue sections and incubated at room temperature in a humidified chamber for 24 h. Sections were washed, blocked, and stained with primary Abs (CD206, DC-SIGN, and/or LYVE-1), followed by fluorescence-conjugated secondary Abs (AF555-conjugated donkey anti-mouse and AF549-conjugated goat anti-rabbit) as described earlier. Tilmanocept binding to MR⁺ and DC-SIGN⁺ cells was analyzed by a laser scanning confocal microscope.

**Statistical analysis**

Experiments were carried out two to three times with different donors. Absolute results varied among donors, but the patterns relative to internal controls in each experiment were consistent. Representative results are shown. Prism 5 software (Version 5.04; GraphPad) was used to determine the statistical significance of differences in the means of experimental groups using an unpaired, two-tailed Student t test. The p values <0.05 were considered significant.

**Results**

**Tilmanocept binds to human macrophages, but not to lymphocytes, in a dose-dependent manner**

Because lymph nodes are considered to be garrisons of lymphocytes (especially T lymphocytes), in addition to other cells including macrophages, we first determined whether tilmanocept (Fig. 1) binds to lymphocytes or macrophages using human PBMCs. PBMCs, cultured for 5 d to enable blood monocytes to differentiate into macrophages (MDMs), were pretreated with or without cold (unlabeled) tilmanocept followed by incubation with increasing concentrations of Cy3-labeled tilmanocept. Tilmanocept binding to cell populations was analyzed by flow cytometry by gating separately for MDMs and lymphocytes (Supplemental Fig. 1A) as described previously (32). Our data show that tilmanocept binds specifically to the macrophage population in a dose-dependent manner (Fig. 2A). Moreover, when we pretreated the macrophages with cold tilmanocept (100-fold excess), the binding of Cy3-tilmanocept was nearly abolished even at high concentrations (Fig. 2B). To ensure that the lack of tilmanocept binding to lymphocytes was not as a result of lymphocyte death, we determined that ~92–96% of lymphocytes were viable by trypan blue exclusion. We also measured IL-2 in 5-d culture supernatants of PBMCs from several donors, which were found to contain significant amounts of IL-2 (Supplemental Fig. 1B), thus facilitating lymphocyte viability.

To corroborate these findings, we treated pure MDMs in monolayer culture in a similar way and performed fluorescence confocal microscopy experiments. The binding of Cy3-tilmanocept to macrophages was readily apparent, and this binding was nearly abolished for macrophages that were pretreated with cold tilmanocept (Fig. 2C). These results were consistent with receptor-mediated binding of tilmanocept to macrophages.

Next, we determined the macrophage binding properties of tilmanocept derivatives containing different numbers of mannose residues (7.4, 13.6, and 19.1 mannose moieties/dextran, respectively) by evaluating their abilities to compete for the binding of the tilmanocept reference standard compound that contains 17.2 mannose moieties/dextran (Supplemental Fig. 2). The inhibition profiles for derivatives with 13.6 and 19.1 mannose moieties/dextran were not significantly different from those using the reference standard tilmanocept (p > 0.05 for all cases, Tukey’s test). However, the derivative with 7.4 mannose moieties/dextran exhibited a significantly reduced inhibition profile compared with the other three derivatives (p < 0.001, Tukey’s test). A spline regression analysis showed that tilmanocept with 11.7 mannose moieties/dextran was the break point for maximal binding inhibition. Based on a reduction in competitiveness of the 7.4 mannose tilmanocept species, this analysis indicated a theoretical reduction of 10% binding compared with the reference standard, an in vitro performance level that provided an appropriate cutoff point for the clinical drug construct specification. From similar plots using [⁹⁹mTc]-tilmanocept ([⁹⁹mTc] being substituted for Cy3 as the reporter molecule), we additionally derived Scatchard assessments from both the fluorescence and radioactive reporter molecules. We determined the primary Kd of γ-tilmanocept and Cy3-tilmanocept for the MR to be 3.0 × 10⁻¹⁰ M; we also determined that at least two molecules of tilmanocept were bound/molecule of the MR; there was no indication of positive or negative cooperativity in this binding (primary site = 3.0 × 10⁻¹⁰ M; secondary site = 2.6 × 10⁻¹⁰ M). We believe this is consistent with the multiple carbohydrate recognition domains in the extra-

![FIGURE 1. [⁹⁹mTc]-γ-tilmanocept chemical structure.](image-url)
cellular portion of each molecule) on the MR (containing up to three higher- and five lesser-affinity sites for mannose per receptor molecule) (33).

Tilmanocept binding to macrophages is nearly abolished after pretreatment with mannan

The tilmanocept dextran backbone is linked to mannose subunits; therefore, we next sought to determine whether tilmanocept binds to macrophages via a mannose-binding receptor by performing a mannan-inhibition experiment. Macrophage monolayers were preincubated with a saturating amount of mannan (19) followed by the addition of Cy3-tilmanocept and then tilmanocept binding was examined by confocal microscopy. Tilmanocept binding to macrophages was nearly abolished in mannan-pretreated cells (Fig. 3A, lower left panel). In contrast, pretreatment of macrophages with GalNAc, a carbohydrate that does not bind to the MR, did not inhibit tilmanocept binding to macrophages compared with untreated (-GalNAc) cells (Fig. 3C). The specificity of tilmanocept binding was calculated by measuring an MFI for imaged cells with and without mannan or GalNAc pretreatment and calculating the percent decrease in binding in the presence of the soluble carbohydrate. Compared with control macrophages, the mean MFI values for tilmanocept binding to mannan-pretreated macrophages were reduced by 59.17 ± 4.73% (*p < 0.005; Fig. 3B), whereas GalNAc pretreatment had a negligible effect (Fig. 3D). Thus, pretreatment of macrophages with mannan results in significant inhibition of tilmanocept binding, which indicates that a mannose-binding receptor such as the MR is involved.

Tilmanocept binding is markedly reduced in MR (CD206)-deficient human macrophages

The MR (CD206) and DC-SIGN (CD209) are two well-known cell-surface–associated mannose-binding receptors. However, DC-SIGN is not expressed on unstimulated human macrophages [(34) and our unpublished observations]. Thus, the MR is the predominant mannose-binding, surface-associated receptor on human macrophages. To determine whether the MR is involved in binding tilmanocept, we performed a tilmanocept binding experiment using MR knockdown MDMs (29). MR-specific siRNA-mediated knockdown was confirmed by Western blot (Fig. 4A). When Cy3-tilmanocept was added to these cells, there was a marked reduction in the binding of tilmanocept to macrophages deficient in the MR compared with control siRNA-treated macrophages (Fig. 4B). Next, we calculated the MFI of Cy3 on macrophages, and the results showed that there was an ~2.5-fold reduction in tilmanocept binding to MR-siRNA transfected cells compared with control cells (Fig. 4C). Together, these results provide strong evidence that the MR (CD206) is the binding receptor for tilmanocept on human macrophages.

Tilmanocept binding to the MR expressed in a human cell line HEK293

To confirm that the macrophage MR is the tilmanocept-binding receptor, we performed a ligand blot experiment using protein lysates from control and MR-expressing transfected HEK293 cells (20) in which MR protein transferred to a nitrocellulose membrane was overlaid with radioactive γ-tilmanocept. The binding of
tilmanocept to the MR protein band (∼175 kDa) was analyzed by autoradiography. As predicted, γ-tilmanocept bound specifically to the MR from MR-expressing HEK293 cells (Fig. 5A, left panel, lane 2). Tilmanocept binding was abrogated when the membrane was pretreated with excess cold tilmanocept (Fig. 5A, right panel, lane 2). Western blot shows the location of the MR protein from MR-expressing HEK293 cells (Fig. 5B, upper panel). The nitrocellulose membrane was reprobed with β-actin Ab to ensure dilution.

FIGURE 3. Binding of tilmanocept to human macrophages is nearly abolished after treatment with mannan. MDMs were incubated without or with mannan or GalNAc followed by incubation with Cy3-tilmanocept. Tilmanocept binding was evaluated by confocal microscopy. (A) Representative confocal images (original magnification ×1200) showing binding of Cy3-tilmanocept to mannan-untreated macrophages (upper left panel) and inhibition of its binding after mannan pretreatment (lower left panel). (B) Bar graph showing the difference in MFI A.U. for tilmanocept binding between untreated and mannan-pretreated macrophages. (C) Representative confocal images (original magnification ×1200) showing binding of Cy3-tilmanocept to GalNAc-untreated macrophages (upper left panel) and after GalNAc pretreatment (lower left panel). (D) Bar graph showing mean MFI values for tilmanocept binding between untreated and GalNAc-pretreated macrophages. Blue represents DAPI staining of macrophage nuclei; red represents Cy3-tilmanocept. The MFI was determined for ∼350–400 macrophages per coverslip, with duplicate slides per experiment of at least two independent experiments of each category (**p < 0.005). DIC, differential interference contrast.

FIGURE 4. Reduced binding of tilmanocept to MR-deficient human macrophages. MDMs were transfected with MR-siRNA or scramble control siRNA by nucleofection. Treated MDMs were then incubated with Cy3-tilmanocept, and the binding was analyzed by confocal microscopy. (A) Western blot showing siRNA-mediated knockdown of the MR protein; β-actin was used as the loading control. (B) Representative confocal images (original magnification ×1200) showing binding of tilmanocept to control macrophages (upper left panel) and markedly reduced binding of tilmanocept to MR-deficient macrophages (lower left panel). Blue represents DAPI staining of macrophage nuclei; red represents Cy3-tilmanocept. (C) Bar graph showing the difference in MFI (A.U.) between the control and MR-deficient macrophages. The MFI was determined for ∼150 macrophage cells per coverslip, using duplicate slides per experiment (n = 2; **p < 0.005). DIC, differential interference contrast.
and exposed to autoradiography. (A) Autoradiograms showing binding of $^{99m}$Tc-tilmanocept to the MR protein (arrow) on an untreated blot (left panel) and lack of $^{99m}$Tc-tilmanocept binding to a blot that was pretreated with cold tilmanocept (right panel). (A and B) Lane 1, cell lysate of HEK293 cells transfected with empty vector; lane 2, cell lysate of HEK293 cells transfected with the MR expression plasmid. Molecular weight markers are shown on the left of each blot. (B) Western blot showing expression of the MR protein in transfected HEK293 cells (upper panel). The same blot was reprobed with anti-actin Ab to show β-actin as an internal protein control (lower panel). Results shown are representative of two independent experiments.

![Figure 5. Binding of tilmanocept to the MR protein by lectin blot. HEK293 cells, transfected with empty vector or an MR expression plasmid, were lysed, and total proteins (20 μg from each sample) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was preincubated with or without 100-fold excess cold tilmanocept, followed by incubation with $^{99m}$Tc-tilmanocept (γ-tilmanocept, 20 μg/ml) for 2 h, washed and exposed to autoradiography.](image)

**FIGURE 5.** Binding of tilmanocept to the MR protein by lectin blot. HEK293 cells, transfected with empty vector or an MR expression plasmid, were lysed, and total proteins (20 μg from each sample) were separated by SDS-PAGE and transferred to nitrocellulose membranes. The membrane was preincubated with or without 100-fold excess cold tilmanocept, followed by incubation with $^{99m}$Tc-tilmanocept (γ-tilmanocept, 20 μg/ml) for 2 h, washed and exposed to autoradiography. (A) Autoradiograms showing binding of $^{99m}$Tc-tilmanocept to the MR protein (arrow) on an untreated blot (left panel) and lack of $^{99m}$Tc-tilmanocept binding to a blot that was pretreated with cold tilmanocept (right panel). (A and B) Lane 1, cell lysate of HEK293 cells transfected with empty vector; lane 2, cell lysate of HEK293 cells transfected with the MR expression plasmid. Molecular weight markers are shown on the left of each blot. (B) Western blot showing expression of the MR protein in transfected HEK293 cells (upper panel). The same blot was reprobed with anti-actin Ab to show β-actin as an internal protein control (lower panel). Results shown are representative of two independent experiments.

Tilmanocept colocalizes with the MR on the cell surface of human macrophages

MDMs were exposed to Cy3-tilmanocept and, after fixation, stained with either isotype control Ab or anti-MR Ab and examined by confocal microscopy to determine whether tilmanocept colocalizes with the MR on the surface of macrophages. The MR is abundantly expressed on MDMs (Fig. 6A, middle panel). No nonspecific staining was evident when macrophages were stained with the isotype control Ab (Fig. 6A, left panel). Analysis of confocal images from tilmanocept binding experiments demonstrated robust binding of Cy3-tilmanocept to the macrophage surface and colocalization of tilmanocept with the macrophage MR (Fig. 6B). These data are consistent with MR-specific binding of tilmanocept to the macrophage surface.

Lymph nodes from cancer patients harbor resident macrophages

Because SLNs are the primary organ for cancer staging using the tracer agent γ-tilmanocept, we sought to identify the presence of macrophages in SLNs from head and neck cancer patients in this study (nodes collected in phase 3 study NCT00911326). CD163 and CD68 are considered to be two prominent macrophage-specific markers with variable expression depending on macrophage activation (35). We used these two markers in an immunohistochemistry experiment to identify the presence of macrophages within the lymph node microenvironment. FFPE SLN tissue sections from head and neck cancer patients who had a primary diagnosis of squamous cell carcinoma (T1-4, N0, M0) were processed and subjected to double staining with Abs against CD163 and CD68, and sections were analyzed by confocal microscopy. Isotype control Abs were used in a different tissue section that showed the absence of staining for both CD163 and CD68 (data not shown). The tissue section contained a large population of cells (Supplemental Fig. 3, left panel) within which a subset of cells was stained with CD163, CD68, or both (Supplemental Fig. 3, middle two panels and merged image), confirming the presence of macrophages in these lymph nodes.

**FIGURE 6.** MR expression and colocalization with tilmanocept on human macrophages. (A) MDM monolayers were fixed with paraformaldehyde (without permeabilization), incubated with either the isotype control Ab or the anti-MR Ab, and stained with AF488-conjugated secondary Ab. The monolayers were then analyzed by confocal microscopy. Representative confocal images (original magnification ×1200) showing MR expression (green in middle panel) and no staining in the case of the isotype control Ab (left panel). Right panel shows differential interference contrast (DIC, phase-contrast microscopy). Data are representative of n = 2 in duplicate. (B) MDM monolayers were incubated with Cy3-tilmanocept for 10 min, fixed with paraformaldehyde, incubated with anti-MR Ab, and stained with AF488-conjugated secondary Ab. The monolayers were then analyzed by confocal microscopy. Representative confocal images (original magnification ×1600) showing MR expression (green in first panel), tilmanocept binding by the macrophage (red in second panel), and colocalization of the MR and tilmanocept in both confocal and phase-contrast images (yellow in third and fourth panels, arrow shows an example of intense colocalization). Results shown are representative of three independent experiments.

Macrophages in SLNs from cancer patients express the MR

TAMs constitute a major subset of tumor-infiltrating leukocytes in SLNs, and their infiltration is associated with rapid tumor progression (36). They are characterized to be M2-like polarized macrophages, which abundantly express C-type lectins, especially the MR (27). To determine whether macrophages present in SLNs of cancer patients express the MR that would enable the binding of tilmanocept, we used tissue sections from pathologically proved tumor-negative and -positive SLNs from head and neck cancer patients who had a primary diagnosis of squamous cell carcinoma (T1-4, N0, M0) were processed and subjected to double staining with Abs against CD163 and CD68, and sections were analyzed by confocal microscopy. Representative confocal images (original magnification ×1200) showing MR expression (green in middle panel) and no staining in the case of the isotype control Ab (left panel). Right panel shows differential interference contrast (DIC, phase-contrast microscopy). Data are representative of n = 2 in duplicate. (B) MDM monolayers were incubated with Cy3-tilmanocept for 10 min, fixed with paraformaldehyde, incubated with anti-MR Ab, and stained with AF488-conjugated secondary Ab. The monolayers were then analyzed by confocal microscopy. Representative confocal images (original magnification ×1600) showing MR expression (green in first panel), tilmanocept binding by the macrophage (red in second panel), and colocalization of the MR and tilmanocept in both confocal and phase-contrast images (yellow in third and fourth panels, arrow shows an example of intense colocalization). Results shown are representative of three independent experiments.

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patients for immunohistochemical analysis. We analyzed a total of 12 tissue sections (4 sections from tumor-positive and -negative SLNs from each patient, n = 3 patients). Tissue sections were stained (double staining) with anti-MR Ab and anti-CD163 Ab and analyzed by confocal microscopy. Isotype control Abs showed the absence of staining for both the MR and CD163 (data not shown). Tissue sections from both tumor-negative (Fig. 7A) and tumor-positive (Fig. 7B) SLNs showed expression of the MR by a subset of CD163+ macrophages. Tumor-positive tissue sections showed a robust recruitment of macrophages, which indicates the infiltration of TAMs into the tumor microenvironment with high MR expression. For a given confocal image, macrophages present in the tumor-positive tissue sections expressed approximately twice the amount of MR compared with those in tumor-negative lymph nodes from the cancer patients [5.11 ± 1.05 for tumor-negative versus 10.33 ± 1.49 arbitrary units (A.U.) for tumor-positive; Fig. 7C]. These findings are consistent with the ability of tilmanocept to bind to a greater extent to tumor-positive SLNs in cancer patients.

**Tilmanocept binds to MR-expressing cells in SLN tissues**

To demonstrate the binding of tilmanocept directly to MR-expressing cells present in the lymph node tissues, we subjected FFPE tissue sections to the Ag retrieval procedure (see immunohistochemistry method) followed by incubation with AF488-labeled tilmanocept and staining with anti-MR Ab. Tilmanocept (green) was found to specifically bind to the majority of the MR+ (red) population of cells in the tissue sections, colocalizing (yellow) with MR staining (Fig. 8). This binding to MR-expressing cells was inhibitable by mannan pretreatment (data not shown). Some MR+ cells (open arrow in the merged image in Fig. 8) did not bind tilmanocept, whereas some MR- cells (solid white arrow in the merged image in Fig. 8) did.

**FIGURE 7.** Expression of the MR (CD206) and CD163 by macrophages present in tumor-negative and tumor-positive lymph nodes from cancer patients. FFPE tissue sections from tumor-negative (A) and tumor-positive (B) lymph nodes from cancer patients were deparaffinized, followed by an Ag retrieval procedure. Sections were then subjected to double staining with Abs against the MR and CD163, and analyzed by confocal microscopy. Representative confocal images (original magnification ×400) showing the total number of cells (blue, nuclear staining) and expression of the MR (green) and CD163 (red) in tissue sections from tumor-negative (A) and tumor-positive (B) lymph nodes from cancer patients. Results shown are representative of 3 independent experiments using a total of 12 tissue sections (4 sections from tumor-positive and tumor-negative SLNs from each patient, n = 3 patients). (C) MR expression was quantified in 20 randomly selected confocal images using the NIH ImageJ program, 10 each from tumor-negative and tumor-positive tissue sections (*p < 0.05). The MFI in A.U. shown represents an average value (± SD) per image section.

**DC-SIGN contributes to binding of tilmanocept in the lymph node tissue microenvironment**

Because DCs coexist with macrophages in lymph nodes, and DC-SIGN expressed by DCs is another mannan-binding receptor (37, 38), we examined whether lymph nodes from cancer patients contain DCs along with macrophages by confocal microscopy after staining the processed FFPE lymph nodes with anti-MR Ab (AF488) and anti–DC-SIGN Ab (AF549). Our results indicate that lymph nodes from cancer patients contain both MR+ and DC-SIGN+ cells, representing macrophages and DCs (Supplemental Fig. 4A). Next, we determined whether DCs can bind tilmanocept in the lymph node region. We processed FFPE lymph node tissue sections as described earlier before incubating them with AF488-labeled tilmanocept and staining with anti–DC-SIGN Ab. Tilmanocept (green) was found to bind and colocalize with a subset of DC-SIGN+ cells (red) in the tissue sections (Supplemental Fig. 4B). To verify tilmanocept binding to DC-SIGN, we transfected HEK293 cells with a DC-SIGN expression construct and incubated cells with AF488-labeled tilmanocept. Flow-cytometric analysis showed that DC-SIGN-expressing cells (DCSIGN-HEK293) bind tilmanocept (Supplemental Fig. 4C). Tilmanocept binding by both DC-SIGN and the MR on this cell line was inhibitable by mannan (Supplemental Fig. 4C); however, the level of inhibition for DCSIGN-HEK293 cells was less than on MR-HEK293 cells (29 versus 46%; Supplemental Fig. 4D).

**LYVE-1+ lymphatic endothelial cells do not express the MR or bind to tilmanocept in SLN tissue**

Based on reports of MR expression on lymphatic endothelium (39–42), we further tested whether lymphatic vessel endothelial cells in SLNs express the MR and interact with tilmanocept. SLN tissue
sections were stained with the lymphatic endothelial marker LYVE-1 and either costained with CD206 to assess for colocalization or incubated with tilmanocept to assess its binding (Fig. 9). Our results indicate that LYVE-1+ endothelial cells, which appeared to be lining a lymphatic vessel, do not express the MR, although several LYVE-1− cells outside the vascular lining do show MR expression (Fig. 9A, arrow as an example). We also observed that LYVE-1− endothelial cells in an apparent lymphatic vessel do not bind to tilmanocept, although a few LYVE-1− cells outside the vasculature lining in this section did show some tilmanocept binding (Fig. 9B, arrow as an example). In other regions of the SLN tissue, MR expression (Fig. 9C, green) or tilmanocept binding (Fig. 9D, green) did not colocalize with LYVE-1+ cells (red).

Discussion

SLN mapping is a clinical procedure used worldwide for prognosticating cancer progression. Tilmanocept was designed as a receptor-targeted, 99mTc-radiolabeled, intraoperative lymphatic mapping agent (1), effectively being used for identifying SLNs that have the highest potential for containing metastases in patients with breast cancer, melanoma, and head and neck squamous cell carcinoma. Some of the advantages of the use of γ-tilmanocept include its enhanced specificity and sensitivity for reliable SLN mapping, and its ability to identify unpredictable lymphatic drainage patterns in cancer patients with solid tumors. Many clinical studies, including two complete phase 3 trials for product registration (pending), were performed in cancer patients, as well as in animal models on the efficacy and safety of tilmanocept (1, 5, 6, 8–11, 13). However, no molecular study has yet been done to elucidate the mechanism of tilmanocept binding to SLN constituents, including the exact binding partner(s) involved (e.g., mannose-binding receptors) of tilmanocept. Lack of information regarding the binding partner(s) or binding sites is a critical limitation in the usage of the compound as a clinical diagnostic or prognostic agent. In this work, we demonstrate that macrophages are a major target cell and the MR (CD206) is the major binding receptor for tilmanocept.

Tilmanocept was designed to target mannose-binding receptors such as the MR, DC-SIGN, and mannose binding lectin. Mannose binding lectin is a soluble protein produced by liver cells of both human and animal origin (43). DC-SIGN is not expressed by unstimulated human macrophages (34). Thus, the MR is the predominant mannose-binding, surface-associated receptor on human macrophages. We used human MDMs in our studies that abundantly express the MR on their surface (Fig. 6A, 6B).
competitive inhibition of Cy3-tilmanocept binding to macrophages by mannose-containing compounds such as cold tilmanocept (Fig. 2B, 2C) and mannan (Fig. 3A, 3B) gave the first indication that the MR could be the binding receptor for tilmanocept. Further confirmation of the MR as the binding receptor was achieved using several complementary assays (Figs. 4, 5, 6B). Although MR-transfected HEK293 experiments provide strong evidence that the MR is sufficient for tilmanocept binding, we cannot exclude the possibility that the MR acts as a coreceptor, or that optimal tilmanocept binding requires MR binding in conjunction with another receptor. In this regard, we found that DC-SIGN could act as an additional contributory receptor for tilmanocept binding (Supplemental Fig. 4). Because the MR is a recycling receptor through the endosome (44), it is predicted that MR-bound tilmanocept would accumulate in macrophages over time. In our studies, we expressed macrophages to tilmanocept for only a short period (10–15 min).

To relate our studies using MR-expressing MDMs to the TAMs present in the SLN microenvironment, we immunohistochemically analyzed SLN tissue sections by using Abs directed against CD68 and CD163, both of which are accepted to be macrophage-associated Ags (Supplemental Fig. 3). CD68 is widely used as a pan-macrophage marker and has been shown to correlate with an adverse prognosis in a variety of malignancies, for example, follicular lymphoma (45). CD163 is a glycoprotein belonging to the cysteine-rich scavenger receptor super family (46) and has been suggested to be a more specific marker for macrophages (especially for the M2 type) than CD68 (35, 47). We found that CD163 expression was relatively higher than that of CD68 in SLN macrophages from head and neck cancer patients (Supplemental Fig. 3). This finding is in keeping with observations in previous studies in which higher numbers of tumor-infiltrating CD163 (compared with CD68) macrophages were found in malignant melanoma (48), leiomyosarcomas (49), and classical Hodgkin’s lymphoma (50).

We also examined MR and CD163 expression by macrophages present in SLNs (Fig. 7). We compared tissue sections from both pathologically proved tumor-negative and -positive SLNs. Greater numbers of macrophages with higher levels of MR expression were observed in tumor-positive compared with tumor-negative samples. Macrophages are often found to infiltrate malignant lesions in solid tumors, for example, breast cancer (51, 52). These infiltrating macrophages are usually TAMs belonging to M2-type macrophage category. TAMs are known to express more MR than other types of tissue macrophages (36). A high number of CD163+ TAMs was found to be significantly associated with an adverse prognosis in a variety of cancers such as pancreatic cancer (53), malignant melanoma (48), mantle cell lymphoma (54), and classical Hodgkin’s lymphoma (50). In the MR-tilmanocept binding experiments using SLN tissues, we observed binding of tilmanocept to some MR+ cells, and variability with little to no binding of tilmanocept to some MR+ cells (solid and open arrows, respectively, in the merged image in Fig. 8). The former observation could be due to only DC-SIGN expression by those cells and the latter to partial destruction of the MR structural binding domains after harsh Ag retrieval treatment of the tissue section. In addition, observed differences in staining pattern and tilmanocept-CD206 colocalization may in part reflect differences in PBMC-derived macrophages (Fig. 6) compared with SLN-associated macrophages (Fig. 8).

DCs, especially immature DCs, are known to express the MR and DC-SIGN; the latter is also a C-type lectin mannan-binding receptor (37, 38). In this study, we found a subpopulation of DCs expressing both C-type lectins in the SLN tissue (Supplemental Fig. 4A). However, unlike MR+ macrophages, the presence of DC-SIGN+ cells was not seen consistently in all tissue sections we analyzed (data not shown) and the number of these cells was always less than the cells that are only MR positive. We determined that DC-SIGN also binds tilmanocept (Supplemental Fig. 4), and more frequently in a cluster form in SLNs, which might be because of the contribution of both the MR and DC-SIGN on these DCs (Supplemental Fig. 4A). Overall, our studies indicate that expression of the MR by macrophages and DCs (if present) in SLNs is the major mechanism by which tilmanocept binds and is retained in this microenvironment. DC-SIGN contributes to the binding of γ-tilmanocept, at least on a subset of cells, which may also express the MR. Based on reports that the MR is also expressed on lymphatic endothelium (39–42), and thus the possibility that tilmanocept may also bind to the tumor-draining lymphatic vessels, we examined for MR expression and tilmanocept binding by lymphatic endothelial cells expressing LYVE-1, a commonly used marker (41, 42, 55–62). Neither MR expression nor tilmanocept binding was observed by LYVE-1+ cells in the SLN tissue samples (Fig. 9). However, our studies do not rule out the possibility that tilmanocept binds to subsets of lymphatic endothelial cells because distinct populations have been described using different markers (e.g., PROX1, STAB2) (63, 64). Other possibilities for our negative results include the use of fixed and embedded tissue sections versus frozen tissue, potential differences in MR expression on peritumoral and intratumoral lymphatic vessels (40) versus SLNs, which were used in our study, the latter are distal to the tumor bed, or differences in posttranslational modifications of the MR (or DC-SIGN, which has also been described on subsets of lymphatic endothelial cells) that do not allow for tilmanocept to bind. Overall, the ability of tilmanocept to bind to MR-expressing cells leads to its enhanced tumor-draining SLN identification, making it suitable for both preoperative and intra-operative imaging of lymph nodes.

In a preliminary evaluation of intraoperative findings from >3000 lymph nodes excised from 80 patients with head and neck squamous cell carcinoma (cutaneous and intraoral) participating in a phase 3 study of [18F]-tilmanocept, the tumor-positive SLNs had mean gamma counts of 3235 cpm (95% CI, 208–6259) versus tumor-negative nodes of 379 cpm (95% CI, 113–1890; p < 0.0001; counts normalized per 2 s; full data not shown), showing increased accumulation of tilmanocept in tumor-positive lymph nodes. In this study, we demonstrate specific binding of tilmanocept to MR+ cells in the FFPE lymph node tissue sections (Fig. 8).

Although our study was not aimed at determining the prognosis of cancer patients, our laboratory results are consistent with the findings in this phase 3 study. These findings are also consistent with the theoretical (65) and observed behavior (66) of receptor-targeted radiopharmaceuticals. These imaging agents obey a bimolecular rate law, which predicts that the rate of receptor binding, and hence tissue localization, is governed by the concentration of the radiopharmaceutical, as well as the receptor tissue density.

The MR has been exploited as a vehicle for DNA-based vaccine delivery, mostly through mannosylated liposomes, into both human and animal cells for therapeutic purposes (17, 67–70). The recent use of mannosylated nanoparticles for high-efficiency delivery of MR-targeted siRNA into macrophages indicates its potential for small-molecule delivery to target tissues (71). Demonstration of manobody- and mannosylated liposome-based targeting of the MR on TAMs for in vivo imaging purposes has provided future potential of drug delivery to tumor sites by using the TAM-MR pathway (72, 73). There is also a recent report on MR targeting of a radiolabeled mannosylated compound for advanced imaging in atherosclerosis (74). However, little is known about the direct binding and delivery of drugs using this receptor. We provide evidence in this work that tilmanocept is the first receptor-targeted...
cancer prognostic agent that is bound by the MR. This discovery provides a novel and valuable approach to designing MR-targeted imaging agents for magnetic resonance imaging, positron emission tomography, and single-photon emission computed tomography/computed tomography used in clinical diagnosis and prognosis. In addition, design of drug molecules targeting the MR has future potential for the delivery of drugs to the sites of metastasized cancer cells, for example, in lymph nodes, for therapeutic purposes.

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Disclosures

The authors have no financial conflicts of interest.

References


**Supplemental Fig. S1 (Azad et al.)**

(A) **Dot plots of representative flow cytometry analysis showing gated MDM and lymphocyte populations.** Day 5 PBMCs (6-8 x 10^6) isolated from Teflon wells were washed, placed in FACS buffer and incubated in the presence of Cy3-tilmanocept for 15 min at 37°C. Cells were washed with FACS buffer and fixed in 2% paraformaldehyde. Binding of Cy3-tilmanocept to PBMCs was analyzed by BD FACSCalibur System with gating on the macrophage (large gate, M) and lymphocyte (small gate, L) cell populations separately. (B) **IL-2 levels in day 5 PBMC cultures.** Day 5 PBMCs (2x10^6 cells/ml) were isolated from Teflon wells, centrifuged and cell-free culture supernatants were used to measure the IL-2 levels by ELISA using R&D system ELISA antibodies. The graph shown is data from 5 different donors (each bar represents one donor ± SD).
Supplemental Fig. S2 (Azad et al.)

*Binding profiles of reference standard tilmanocept compared to its derivative compounds containing different numbers of mannose units.* Five day old PBMCs containing monocyte-derived macrophages and lymphocytes were pre-incubated with increasing amounts of cold reference standard tilmanocept (17.2 mannose residues/dextran core) and its derivatives containing different numbers of mannose units (7.4, 13.6 and 19.1 mannose residues/dextran, respectively) and then incubated with 20 µg/ml of Cy3-labeled reference standard tilmanocept in duplicate. Binding of tilmanocept to macrophages was assessed using MFI values after gating for fluorescence specific to the macrophage population. Reduction in MFI was plotted relative to the increasing concentration of unlabeled tilmanocept added to the cell suspensions. (A) Inhibition of Cy3-tilmanocept binding to macrophages versus increasing concentration of unlabeled tilmanocept and its derivatives with varying mannose units (direct binding). (B) Graph plotted with the same data from (A) with non-specific binding removed. Date are representative of N = 3.
Identification of macrophages in lymph nodes from cancer patients. Formalin-fixed and paraffin-embedded SLN tissue sections (pathologically tumor-negative) from cancer patients were deparaffinized, followed by an antigen retrieval procedure. Sections were then subjected to double staining with Abs against CD163 and CD68, and analyzed by confocal microscopy. Representative confocal images (original magnification: 1200x) show nuclear staining by DAPI of the total cell populations (blue), as well as, staining for CD163 (red) and CD68 (green). The merged image (right most panel) shows that a subset of the cell population (arrow heads, as examples) is stained with both CD163 and CD68 which represent resident macrophages. The results shown are representative of 2 independent experiments using 2 SLN tissue sections from 2 patients in each experiment.
Supplemental Fig. S4 (Azad et al.)

(A) Expression of DC-SIGN and MR by DCs and macrophages, respectively, and their co-localization on a subset of DCs in SLN tissue. FFPE tissue sections were processed for antigen retrieval (see immunohistochemistry method) and then stained with DAPI, anti-DC-SIGN- and anti-MR Abs. Sections were analyzed by confocal microscopy. Representative confocal images (original magnification: 800x) showing the total number of cells (blue, nuclear staining by DAPI), DC-SIGN (red)- and MR (green)-positive cells. A subset of DCs expresses both DC-SIGN and the MR as evidenced by their co-localization (yellow; arrow heads show 2 examples).

(B) Binding of tilmanocept to DC-SIGN-expressing cells in SLN tissue. FFPE tissue sections were processed as described above and then incubated with AF488-labeled tilmanocept (green) for 24 h at room temperature. Sections were then stained with DAPI and anti-DC-SIGN Ab, and analyzed by confocal microscopy. A representative merged confocal image (original magnification: 2400x) showing binding and co-localization of tilmanocept (yellow) with some of the DC-SIGN-positive cells (red).

(C, D) Binding of tilmanocept to a human cell line transfected with DC-SIGN. HEK293 cells were transfected with either an MR- or DC-SIGN...
expression plasmid and pre-treated or not with mannan before incubating cells with AF488-tilmanocept. The binding of tilmanocept was analyzed by flow cytometry. The graph in C is representative of 2 independent experiments and shows the level of tilmanocept binding to the MR- or DC-SIGN-expressing cells with or without mannan pre-treatment. The graph in D shows the percentage of inhibition of tilmanocept binding by mannan pre-treatment of the MR- or DC-SIGN-expressing cells, as calculated from the inhibition results in C.