Published in final edited form as:
Bioconjug Chem. 2011 August 17; 22(8): 1706–1714. doi:10.1021/bc200309h.

Synthesis and Evaluation of an Anti-MLC1 × Anti-CD90 Bispecific Antibody for Targeting and Retaining Bone-Marrow Derived Multipotent Stromal Cells in Infarcted Myocardium

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Abstract

A key issue regarding the use of stem cells in cardiovascular regenerative medicine is their retention in target tissues. Here, we have generated and assessed a bispecific antibody heterodimer designed to improve the retention of bone marrow–derived multipotent stromal cells (BMMSC) in cardiac tissue damaged by myocardial infarction. The heterodimer comprises an anti-human CD90 monoclonal antibody (mAb) (clone 5E10) and an anti-myosin light chain 1 (MLC1) mAb (clone MLM508) covalently cross-linked by a bis-aryl hydrazone. We modified the anti-CD90 antibody with a pegylated-4-formylbenzamide moiety to a molar substitution ratio (MSR) of 2.6 and the anti-MLC1 antibody with a 6-hydrazinonicotinamide moiety to a MSR of 0.9. The covalent modifications had no significant deleterious effect on mAb epitope binding. Furthermore, the binding of anti-CD90 antibody to BMMSCs did not prevent their differentiation into adipo-, chondro-, or osteogenic lineages. Modified antibodies were combined under mild conditions (RT, pH 6, 1 h) in the presence of a catalyst (aniline) to allow for rapid generation of the covalent bis-aryl hydrazone, which was monitored at A_{354}. We evaluated epitope immunoreactivity for each mAb in the construct. Flow cytometry demonstrated binding of the bispecific construct to BMMSCs that was competed by free anti-CD90 mAb, verifying that modification and cross-linking were not detrimental to the anti-CD90 complementarity-determining region. Similarly, ELISA-based assays demonstrated bispecific antibody binding to plastic-immobilized recombinant MLC1. Excess anti-MLC1 mAb competed for bispecific antibody binding. Finally, the anti-CD90 × anti-MLC1 bispecific antibody construct induced BMMSC adhesion to plastic-immobilized MLC1 that was resistant to shear stress, as measured in parallel-plate flow chamber assays. We used mAbs that bind both human antigens and the respective pig homologues. Thus, the anti-CD90 × anti-MLC1 bispecific antibody may be used in large animal studies of acute myocardial infarction and may provide a starting point for clinical studies.

INTRODUCTION

Stem cell therapy is a promising therapeutic modality for restoring cardiac function in cardiovascular disease.[1, 2] The preclinical findings that bone marrow–derived multipotent stromal cells (BMMSCs) effect tissue repair[3] and have immunomodulatory activity[4] have led to the clinical testing of these cells in the treatment of a variety of diseases.[5] Furthermore, in a pig model of myocardial infarction, BMMSC engraftment and

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differentiation has been correlated with improved heart function.[6] However, the effectiveness of this therapy may be limited by low rates of cellular retention and engraftment in areas of cell delivery, even when cells are applied directly to the myocardium.[7, 8]

One approach to address this issue is to target stem cells to ischemic tissue by creating a bispecific antibody that can bind to antigens specific for the stem cell and to the target tissue. This approach has been used in cancer therapy to target immune effector cells to tumor cells,[9] and catumaxomab, a first-generation bispecific antibody, has been approved recently in Europe for treating malignant ascites.[10] Lee and colleagues[11] have applied this methodology to cardiac stem cell therapy by generating a bispecific antibody construct to direct hematopoietic stem cells to infarcted myocardium in rodents.[11, 12] In this construct, the tissue-targeting arm uses a monoclonal antibody (mAb) specific for myosin light chain 1 (MLC1) (mAb MLM508), which is found within the interstitial tissue of damaged hearts[13] and serves as an antigen marker for injured myocardium.[14-16] The other arm of the construct for binding hematopoietic stem cells is composed of a mAb to CD45,[11, 12] which is a common leukocyte antigen[17] found on $CD34^+$ hematopoietic stem cells.[18]

In the present study, we have created a novel bispecific antibody for targeting BMMSCs to injured myocardium. The tissue-targeting arm of our reagent comprises the mAb MLM508 described above, which reacts with both pig and human MLC1.[19] Because BMMSCs do not express CD45[20] [the antigen used in the construct made by Lee and colleagues],[11, 12] the stem-cell binding arm of our antibody was composed of mAb5E10, which recognizes the phenotypic cell surface marker CD90 (Thy-1)[21] found on BMMSCs. mAb 5E10 recognizes both pig and human BMMSCs.[22]

To generate an anti-CD90 × anti-MLC1 bispecific antibody construct, we used methods that labeled one mAb with a 6-hydrazinonicotinamide moiety *via* a conventional NHS-ester. The second mAb in the bispecific construct was modified with a pegylated 4-formylbenzamide moiety, *via* a pentafluorophenyl ester. Formation of the bis-aryl hydrazone, which is readily monitored by UV-vis spectroscopy, was rapid and precluded formation of homodimeric antibody products, which can occur with the use of other protein cross-linking chemistries. Herein, we describe the methodology involved in creating our bispecific antibody and the evaluation of its potential use as a BMMSC-targeting agent.

EXPERIMENTAL PROCEDURES

S-HyNic Modification of Anti-MLC1 Antibody

Anti-MLC1 (mouse anti-myosin light chain 1 antibody [MLM508, IgG2a]; Abcam, Cambridge, MA) was concentrated to 3.5 mg/mL and exchanged into modification buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) by using a spin column (Zeba, Thermo Fisher Scientific, Rockford, IL) according to the manufacturer's instructions. A portion of the resulting solution (30 μL, 2.7 mg/mL) was mixed with S-HyNic (0.8 μL, 35.5 mM; Solulink [San Diego, CA]) in anhydrous dimethylformamide (DMF) (Sigma-Aldrich, St. Louis, MO). After incubation at room temperature for 2 h, the reaction mixture was applied to a spin column equilibrated with coupling buffer (100 mM sodium phosphate, 150 mM sodium chloride, pH 6.0). HyNic-MLM508 (**1**, 35 μL, 2.0 mg/mL) was stored at 4 °C.

4FB$(PEG)_4$-PFP Modification of Anti-CD90 Antibody

Anti-CD90 (mouse anti-human CD90 antibody [5E10, IgG1]);BD Pharmingen, San Diego, CA) was concentrated to 3.5 mg/mL and subjected to buffer exchange as above. An aliquot of the resulting solution (25 μL, 3.5 mg/mL) was mixed with 4FB$(PEG)_4$-PFP (Solulink)

(0.5 μL, 6.9 mM) in anhydrous DMF. After incubation at room temperature for 2 h, the reaction mixture was applied to a spin column equilibrated with coupling buffer. 4FB(PEG)$_4$-5E10 (**2**, 30 μL, 2.2 mg/mL) was stored at 4 °C.

Measurement of Molar Substitution Ratio

An aliquot of modified antibody (3.3 μL) was mixed with either 2-hydrazinopyridine dihydrochloride (2-HP) or 4-nitrobenzaldehyde (NB) (both from Sigma-Aldrich) (6.7 μL, 0.5 mM in 100 mM MES, pH 6.0). The reaction was incubated at 37 °C for 0.5 h, and the absorbance at either 390 nm (4-NB) or 354 nm (2-HP) was recorded. UV-vis and absorbance spectra were measured with a Nanodrop 1000 (Nanodrop Products, Wilmington, DE). The MSR was calculated by using Beer's Law and an extinction coefficient of 24,000 (4-NB) or 18,000 (2-HP).

ELISA Procedure

ELISA assays were performed to determine the functional activity of HyNic-MLM508 either alone, or as part of the bispecific antibody construct. Briefly, recombinant human MLC1 (Abcam, 20 μg/mL) in TBS was immobilized onto wells of a 96-well plate (BD Falcon 353228) overnight at 4 °C. BSA (1% w/v) was added for 1 h at RT as a blocker. The wells were washed 3 times with PBS-T (0.1 mL, 10 mM sodium phosphate, 150 mM NaCl, 0.1 % Tween-20 (v/v), pH 7.2) containing 1% FBS (v/v), and control antibodies or bispecific reagent were incubated in the wells for 1 h at RT in PBS-T containing 1% FBS (v/v). In competition experiments, wells were incubated with the excess MLM508 for 0.5 h, followed by 0.5 h with the bispecific reagent. The wells were washed with PBS-T containing 1% FBS (v/v), and the secondary antibody-HRP conjugate (Biosource International, Camarillo, CA; 1:500 dilution from commercial stock in PBS-T containing 1% FBS (v/v)) was incubated at RT for 1 h. For measurements of HyNic-MLM508 bound to MLC1, GAM-HRP was utilized. For measurements of the bispecific antibody binding to MLC1, anti-IgG1 (the isotype of mAb 5E10) was used. Wells were washed 4 times with PBS-T containing 1% FBS (v/v), then the 1-Step Turbo TMB ELISA reagent (Thermo Fisher Scientific) was added to the wells. The colorimetric reaction was stopped with 1.5 M H_2SO_4, and the absorbance at 450 nm was recorded on a Safire2 (Tecan, Durham, NC) plate reader.

Pig Bone Marrow–derived Multipotent Stromal Cells

BMMSCs were isolated on the basis of their ability to adhere to plastic surfaces. Bone marrow aspirates from male pigs were collected into 50 mL conical tubes through a cell strainer (70 μm mesh). Cells were counted and diluted in PBS to a final concentration of 10^7/mL. The mononuclear fraction was isolated by density-dependent cell separation (Ficoll, 1.077 g/mL, GE Healthcare, Uppsala, Sweden). After 2 washes with PBS, the mononuclear cells were plated in 175 cm^2 flasks at 0.5-1 × 10^6 cells per cm^2 in α Minimal Essential Medium (αMEM, Invitrogen, Carlsbad, CA) containing ribonucleosides supplemented with 10% FBS (v/v) (Atlanta Biologicals, Lawrenceville, GA). One day later, fresh medium was added, and nonadherent cells were removed. At 70% confluence, cells were harvested with 0.25% trypsin (Invitrogen)/1 mM EDTA and further seeded at 10^3 cells per cm^2. The medium was changed every 3-4 days, and BMMSC were further passaged at 70% confluency. BMMSCs between passages 3-6 were used in this study.

Bone Marrow–derived Multipotent Stromal Cells Differentiation Assays

Chondro-, osteo-, and adipogenesis differentiation assays were performed as previously described.[20, 23] Briefly, monolayer cultures for osteogenesis and adipogenesis assays were initiated by seeding 2 × 10^3 cells in 6-well tissue culture plates in αMEM. At 70%

confluence, adipogenesis was induced by incubation with αMEM containing 10 mg/mL insulin, 10% FBS (v/v), 1 mM dexamethasone, 0.5 mM methyl-isobutylxanthine, and 100 mM indomethacine for 3 d, followed by 3 d in adipogenic maintenance medium (αMEM containing 10 mg/mL insulin and 10% (v/v) FBS). This alternating treatment was repeated until full adipogenic differentiation was obtained (approximately 2 weeks). Oil Red staining of neutral lipids was used to denote adipocytes.

Osteogenesis was induced in 70% confluent monolayers by incubation of BMMSCs with medium containing 50 mg/mL ascorbate-2-phosphate, 0.1 mM dexamethasone, and 10 mM β-glycerol phosphate and 10% FBS (v/v) for 21 d. The presence of osteoblasts was evaluated by the accumulation of intracellular alkaline phosphatase (Vector Red alkaline phosphatase substrate kit; Sigma-Aldrich).

For chondrogenic differentiation, 2×10^5 cells were centrifuged at 450 g for 5 min in 15 mL conical centrifuge tubes. The cell pellets were incubated for 21 d in differentiation medium (40 mg/mL proline, 100 mg/mL sodium pyruvate, 10 ng/mL TGF-β3, 0.1 mM dexamethasone, 50 mg/mL ascorbate-2-phosphate). To demarcate glycosaminoglycans, Alcian blue staining was applied to cell pellets fixed in 4% (w/v) paraformaldehyde.

Flow Cytometry

For analysis of 4FB(PEG)$_4$-5E10 binding to BMMSCs, cells (2×10^5) were incubated with 4FB(PEG)$_4$-5E10 for 0.5 h at 4 °C in PBS. After 2 washes in PBS containing 1% FBS (v/v), cells were incubated with GAM-IgG-FITC conjugate and analyzed on a LSRII (BD Biosciences). For analysis of bispecific antibody binding to BMMSCs, cells were treated as above, except that secondary antibody treatment was GAM-IgG2a-FITC (BD Biosciences) to detect the MLM508 antibody.

Formation of Anti-MLC1 × Anti-CD90 Bispecific Antibody

HyNic-MLM508 (**1**, 27.5 μL) and 4FB(PEG)$_4$-5E10 (**2**, 25 μL) were combined with aniline (200 mM, 2.6 μL) in conjugation buffer to give a final aniline concentration of 10 mM. Reaction progress was monitored by measuring the UV-vis spectrum of 2 μL aliquots of the reaction mixture. After room-temperature incubation for 2.5 h, the reaction mixture was applied to a spin column equilibrated with 100 mM sodium phosphate, 150 mM sodium chloride, pH 7.2. The anti-MLC1 × anti-CD90 bispecific antibody mixture (**3**, 60 μL, 1.3 mg/mL) was stored at 4 °C.

Parallel-Plate Flow Experiments

Recombinant human MLC1 (20 μg/mL in 0.1 M $NaHCO_3$, pH 9.5) was immobilized overnight at 4 °C onto 24 × 50-mm slides cut from 15 × 100 mm polystyrene Petri dishes. The slides were washed with PBS, blocked with 2% (w/v) BSA for 2 h at RT, and assembled into a parallel-plate flow chamber. Control wells were coated with BSA (2%, w/v) only. Pig BMMSCs were either untreated or treated with bispecific antibody (5 μg/mL) for 0.5 h at 4 °C before washing and resuspension in running buffer (10 nM Tris, 103 mM NaCl, 24 mM $NaHCO_3$, 5.5 mM glucose, 5.4 mM KCl, 2 mg/mL BSA , pH 7.4). Then, the BMMSCs (2.0×10^6 cells) were injected into the flow chamber and allowed to settle on the slides for 10 min. An increasing linear gradient of shear flow was pulled over the adherent cells for 300 s with the use of a computer-controlled syringe pump (Harvard Apparatus), and the number of adherent cells remaining was recorded by digital microscopy. Shear stress calculations were determined every 20 s. The shear stress in dynes/cm^2 is defined as $(6\mu Q)/(wh^2)$: μ is the viscosity of the medium (0.007); Q is the flow rate in cm^3/s; w is the width of the chamber (0.3175 cm); and h is the height of the chamber (0.01524 cm). The number of cells attached was recorded by digital microscopy (VI-470 charge-coupled device video

camera; Optronics Engineering) at 20× on an inverted Nikon DIAPHOT-TMD microscope every 20 s and was plotted against time, as previously described.[24]

RESULTS

S-HyNic Modification of Anti-MLC1 mAb MLM508

mAb MLM508 (anti-MLC1) was modified by treatment with S-HyNic, yielding HyNic-MLM508 (**1,** Figure 1A). To determine the molar substitution ratio (MSR), 4-NB was added to a portion of buffer-exchanged HyNic-MLM508 to generate 4NB-HyNic-MLM508 (Figure 1B) with an absorbance at 390 nm. The calculated MSR was 0.9, with a mass recovery of 86%. To determine whether the HyNic modification of MLM508 (**1**) altered epitope recognition of MLC1, we performed an ELISA assay utilizing purified recombinant human MLC1 as a substrate. The binding of HyNic-MLM508 (10 μg/mL) to immobilized MLC1 protein was measured relative to the binding of unmodified MLM508 (10 μg/mL) by ELISA, and the optical density values were within one standard deviation (Figure 1C). Thus, modifying MLM508 with S-HyNic had only a minimal effect on MLM508 interactions with MLC1.

Effects of CD90 mAb on Bone Marrow–derived Multipotent Stromal Cells Differentiation

The anti-CD90 component of the bispecific antibody construct binds BMMSCs. To ensure that the binding of the CD90 antigen on BMMSCs did not affect the differentiation ability of BMMSCs, we performed osteo-, adipo-, and chrondogenic differentiation assays in the presence of the anti-CD90 mAb or an isotype control mAb (Figure 2). Anti-CD90 was used at a concentration of 10 μg/mL, which was the saturating concentration as shown by flow cytometric analysis (data not shown). Under culture conditions for differentiation, BMMSCs showed the ability to differentiate along osteo-, adipo-, and chrondogenic lineages in the presence of the anti-CD90 antibody, indicating that binding of the CD90 surface antigen did not adversely affect cell differentiation (Figure 2).

4FB(PEG)$_4$-PFP Modification of anti-CD90 mAb 5E10

Anti-CD90 mAb 5E10 was modified by treatment with 4FB(PEG)$_4$-PFP, yielding 4FB(PEG)$_4$-5E10 (**2,** Figure 3A). To determine the MSR, 2-HP was incubated with a portion of 4FB(PEG)$_4$-5E10 to form 2HP-4FB(PEG)$_4$-5E10 (Figure 3B). On the basis of absorbance at 354 nm, we calculated an MSR of 2.6, and mass recovery was 75%. To determine whether 4FB(PEG)$_4$ modification of 5E10 affected its ability to bind to CD90 on the surface of pig BMMSCs, we compared the binding of 4FB(PEG)$_4$-5E10 to BMMSCs with that of unmodified 5E10 by flow cytometric analysis (Figure 3C). The mean fluorescent intensity of 4FB(PEG)$_4$-5E10 (10 μg/mL) binding to BMMSCs *in vitro* was similar to that of unmodified 5E10 (10 μg/mL) (19722 vs 22029, respectively). This finding is in contrast to previous attempts to label mAb 5E10 with 4FB lacking the (PEG)$_4$ spacer, which inhibited 5E10 interactions with CD90 (Figure 3C).

Generation of Bispecific Antibody

To generate the bispecific antibody, HyNic-MLM508 (**1**) was combined with 4FB(PEG$_4$)-5E10 (**2**) in a 1:1 molar ratio in the presence of 10 mM aniline[25] as a catalyst (Figure 4). The progress of the reaction was monitored over time by UV-vis spectroscopy, measuring A_{354} from the bis-aryl hydrazone (Figure 5A, rectangle). The reaction reached a maximum A_{354} within 1 h, indicating that the reaction was complete (Figure 5B). Aniline was removed from the reaction mixture by buffer exchange of the bispecific antibody into PBS (pH 7.2) for storage. We analyzed a sample of the product (**3**) by nonreducing SDS-

PAGE (Figure 6). As shown on densitometry, the reaction product comprised 61% mAb monomers, 25% dimers, and 14% multimers.

Antigen Specificity of the Bispecific Antibody

Individual modification of MLM508 and 5E10 did not result in the loss of antigen-binding activity (Figures 1C and 3C). However, to target BMMSCs to ischemic tissue, each arm of the bispecific antibody must remain functional after heterodimer formation. Thus, we tested the bispecific antibody reagent (**3**, Figure 7) to verify that the immunoreactivity of each half of the heterodimer was not reduced in the cross-linked species. First, we used an ELISA to verify that the bispecific antibody mixture could bind purified recombinant MLC1. We coated the wells with BSA (0.1% w/v) as a control or with MLC1 (10 μg/mL); unbound sites were blocked with BSA (0.1% w/v). HRP-conjugated goat anti-mouse IgG1 (the isotype of anti-CD90 mAb 5E10) was used as the detection antibody. Several control assays were performed to rule out nonspecific binding of the bispecific antibody to immobilized MLC1. No binding was observed when the bispecific antibody was incubated in wells coated with BSA only (Figure 7B). In addition, IgG2a (the isotype of MLM508) did not bind to immobilized MLC1, serving as a control for the MLM508 arm of the bispecific antibody. Furthermore, nonspecific binding of mAb 5E10 to immobilized MLC1 was not observed. Importantly, we saw significant binding of the bispecific antibody to immobilized MLC1, and this binding was inhibited by preincubation of the ELISA wells with free, unmodified MLM508 (Figure 7B).

We used flow cytometry to test the anti-CD90 portion of the bispecific construct. Pig BMMSCs expressing CD90 were treated with individual unmodified antibodies, the bispecific antibody mixture, and excess unmodified anti-CD90 followed by the bispecific antibody mixture. No nonspecific binding of MLM508 to BMMSCs was observed (data not shown). Bispecific antibody binding to BMMSC was detected with goat-anti-mouse IgG2a-FITC (Figure 7C). Excess unmodified anti-CD90 inhibited the bispecific antibody from binding to BMMSCs by more than 90% (Figure 7C).

Functional Activity of the Bispecific Antibody

Parallel-plate flow chambers were used to test whether the bispecific agent we generated could increase the binding of pig BMMSCs to immobilized MLC1. MLC1 was immobilized in the chamber, and BMMSCs pre-treated with bispecific antibody or left untreated (as controls) were perfused into the flow chamber. At 2.5 dynes/cm^2, 40% of the bispecific antibody–treated BMMSCs remained adherent to immobilized MLC1 substrate, whereas the percent of cells adhered in the control chambers was negligible (Figure 8). Even at high shear levels (15 dynes/cm^2), 10% of the bispecific antibody–treated BMMSCs were still attached (data not shown).

DISCUSSION

Developing an effective method for increasing the retention of transplanted stem cells in the injured myocardium would improve the benefits of cell therapy after myocardial infarction. Here, we describe the generation of a novel bispecific antibody designed to target BMMSCs to infarct tissue. The arms of the bispecific antibody are composed of the anti-CD90 mAb 5E10 and the anti-MLC1 mAb MLM508. The bis-aryl hydrazone cross-linking chemistry we used in generating the construct allows for rapid quantification of antibody modifications, real-time nondestructive monitoring of bispecific antibody generation, and the assurance of heterodimer formation. Finally, our bispecific antibody product retained immunoreactivity of both arms of the construct (anti-CD90 and anti-MLC1) and mediated the retention of BMMSCs to MLC1 under stringent adhesion conditions.

The bispecific antibody approach has been used successfully to target human $CD34^+$ hematopoietic stem cells to ischemic myocardial tissue.[11, 12] In these studies, the bispecific reagent comprised an anti-CD45 × anti-MLC1 antibody. This approach is not suitable for use in targeting BMMSCs because CD45 is not expressed on this cell population.[20] We used the anti-CD90 (Thy-1) mAb 5E10 in the heteroconjugate, as CD90 is a glycophosphatidylinositol-linked cell surface glycoprotein expressed on the surface of BMMSCs.[20] Although primarily used in the past as a phenotypic cell marker, CD90 has now been shown to regulate several cell surface signaling receptors.[21] Before using the anti-CD90 mAb 5E10 as one arm of the bispecific antibody, we showed that the binding of mAb 5E10 did not adversely affect BMMSC differentiation into adipogenic, chondrogenic, or osteogenic lineages (Figure 2). Furthermore, modification of mAb 5E10 with the extended linker $4FB(PEG)_4$-PFP, at an MSR of 2.2, did not affect the binding of the mAB to CD90 expressed on the cell surface, thus maintaining antibody functionality (Figure 3). Previously, we had modified mAb 5E10 with S-4FB (the same moiety without the PEG extension, introduced *via* a NHS-ester), with an MSR of 3.0, but this modification inhibited mAb 5E10 from binding CD90 as indicated by flow cytometry experiments (Figure 3C). This indicates the extended linker ($4FB(PEG)_4$) was superior in maintaining functionality after modification. Notably, in addition to human CD90, mAb 5E10 binds pig CD90 and, thus, is suitable for use in large animal studies.

In the present study, an antibody to MLC1 was used as the arm that binds antigens exposed within ischemic tissue. Because ischemia damages the myocyte cell membrane[26] and exposes intracellular contents to the extracellular environment,[27] MLC1 can be found within the interstitial tissue of damaged hearts.[13] Futhermore, MLC1 is not cleaved by caspases[28] that are active after myocardial apoptosis,[29] and circulating MLC1 can be detected as early as 6 h after an infarction.[14-16] We used S-HyNic to modify MLM508 (anti-MLC1). This modification allowed for the rapid determination of the MSR of MLM508 by its incubation with 4-nitrobenzaldehyde, as the resulting bis-arylhydrazone adsorbs at 390 nm. Modifying MLM508 with S-HyNic at an MSR of 0.9 did not affect the binding of MLM508 to MLC1 (Figure 1).

Many methods are available for generating bispecific antibodies, including the method of chemical cross-linking of purified mAbs to generate a heteroconjugate.[30] In the method used in our study, a covalent cross-link was formed between partners when a 6-hydrazinonicotinamide moiety reacted with a 4-formylbenzamide moiety, yielding a bis-aryl hydrazone. These groups are readily introduced into proteins by using conventional NHS-ester or pentafluorophenyl ester reaction with free amines, presumably lysine side chains. We chose this approach because of its advantages in cross-linking specificity and the ability to readily quantify both mAb modifications and the extent of cross-link formation.

One common protein-protein cross-linking approach is to modify one partner with a thiol-reactive group, such as a malemide or pyridyl disulfide, and introduce free thiols into the other partner. When the two modified proteins are mixed, covalent intermolecular disulfide bonds are formed. A significant disadvantage of this method is the formation of homodimers. The thiol modified proteins can form disulfide dimers and because of this, thiol-modified proteins typically must be used immediately after production. Our approach of using two functional groups that do not naturally occur in proteins and are not self-reactive allows for storage of modified proteins before use and avoids homodimer formation. Additionally, the distinct chromophore of the bis-aryl hydrazone cross-link allows for rapid monitoring of reactions by UV-vis spectroscopy. When a low-volume UV-vis spectrometer is used, small cross-linking reactions (10 μL) may be conducted and monitored in a nondestructive and nondilutive manner, making this approach suitable for using difficult-to-obtain proteins.

Several elegant strategies are now available to generate bispecific antibodies for clinical development, involving both whole IgG and Ig fragments such as Fab's and scFv's.[31] However, the majority of these strategies are recombinant protein based, which requires mAb sequence information and long lead times for development. An approach to generating bispecific antibodies that can be utilized even for antibodies that are only available commercially is advantageous, because several different mAb can be tested before a candidate for clinical development can be developed and further evaluated. For *in vivo* studies, the bispecific antibody reaction product will be purified on an anti-IgG2a affinity column. This will result in the removal of free monomeric IgG1 (the anti-CD90 arm). Then, the reagent will be incubated with BMMSCs, which will bind only the antibodies in the mixture that contain anti-CD90. Free antibody (eg, monomeric anti-MLC1) will be washed away from the cells, leaving only bispecific reagent bound to cells before transplantation.

We have shown that each arm of the bispecific construct is functional, and that the reagent can tether BMMSCs to immobilized MLC1 under conditions of shear stress. BMMSCs treated with the bispecific antibody resisted shear of up to 7.5 dynes/cm^2 (Figure 8), and continued to resist shear at levels of 15 dynes/cm^2 (data not shown). During inflammation, cell migration usually occurs at post-capillary venules, where vessel wall shear stress ranges from 1 to 10 dynes/cm^2.[32] These findings indicate that the bispecific antibody can bind BMMSCs to MLC1 during periods of physiologic shear stress. Thus, in addition to use in the direct administration of BMMSC by transendocardial injection, this feature would facilitate the administration of bispecific mAb–armed BMMSCs by intravenous injection.

In the present study, we describe the generation of a functional bispecific antibody comprising anti-CD90 and anti-MLC1 arms. Future studies will quantitatively assess whether this antibody can target human BMMSCs to injured myocardium in an NOD/SCID murine model. Moreover, because of its species cross-reactivity, this bispecific antibody can also be used in pig models of myocardial infarction.

Acknowledgments

The authors would like to thank Stephen N. Palmer, Ph.D., E.L.S., and Rebecca Bartow, Ph.D., of The Texas Heart Institute at St. Luke's Episcopal Hospital for their editorial assistance in the preparation of the manuscript.

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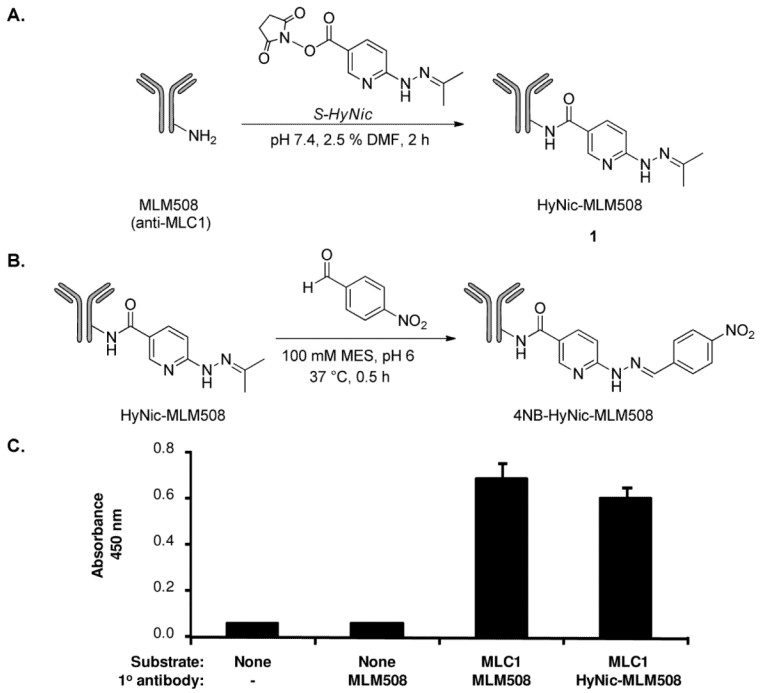

Figure 1.

S-HyNic modification of anti-MLC1 antibody and retention of epitope specificity. (A) Reaction of anti-MLC1 antibody (mAb MLM508) with S-HyNic. The reaction products not shown include excess S-HyNic, N-hydroxysuccinimide (NHS), and unreacted mAb. S-HyNic and NHS were removed by buffer exchange before further testing and generation of the bispecific reagent. Modified antibodies are represented with one modification per molecule for simplicity. DMF - dimethylformamide; MES - 2-(*N*-morpholino)ethanesulfonic acid. (B) Reaction scheme followed to measure the molar substitution ratio (MSR). A portion of HyNic-MLM508 was incubated with 4-nitrobenzaldehyde (4-NB) to generate 4NB-HyNic-MLM508, which absorbs at 390 nm. During incubation at pH 6, the alkyl hydrazone hydrolyzes *in situ* to produce the free hydrazine. The calculated MSR was 0.9. (C) ELISA analysis of HyNic-MLM508 binding to immobilized substrate recombinant human MLC1. Modified MLM508 binding was detected with GAM-HRP. All antibodies were used at a concentration of 10 μg/mL. Results are presented as average A_{450} ± SD from triplicate wells. One of three experiments performed is shown.

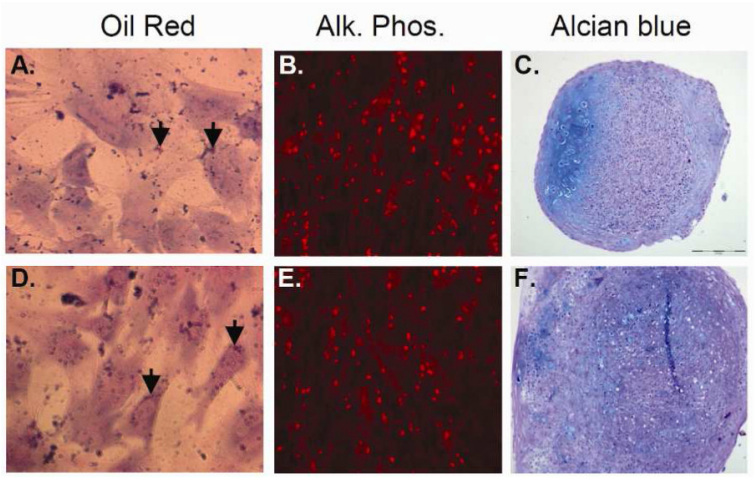

Figure 2.

Differentiation capacity of BMMSCs treated with anti-CD90 antibody (mAb 5E10). BMMSCs were treated with anti-CD90 mAb 5E10 (A-C) or isotype control mAb (D-E). Differentiation of cells into adipogenic (A, D; arrows indicate neutral lipid accumulation), osteogenic (B, E), and chondrogenic (C, F) lineages was examined.

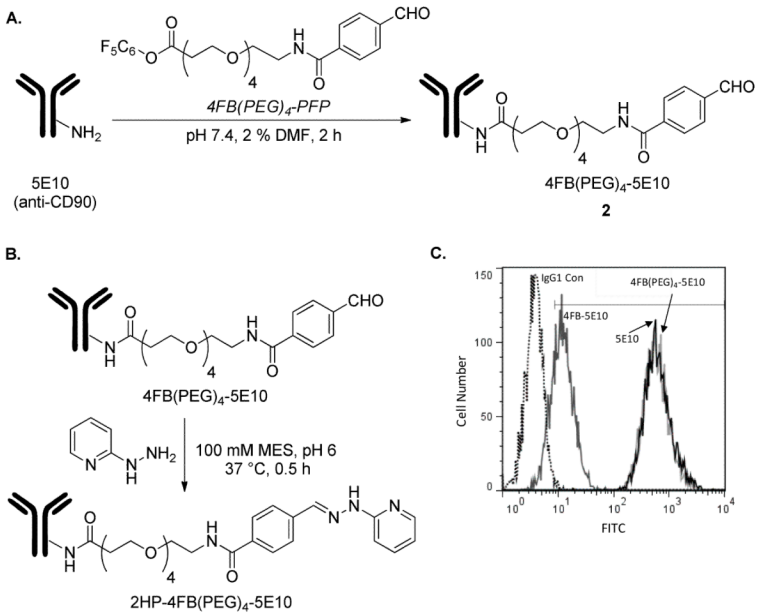

Figure 3.

Modification of anti-CD90 antibody (mAb 5E10) with 4FB(PEG)$_4$-PFP and retention of antibody-binding specificity. (A) Reaction of mAb 5E10 with 4FB(PEG)$_4$-PFP. Reaction products not shown include excess 4FB(PEG)$_4$-PFP, pentafluorophenol, and unreacted antibody. Modified antibodies are represented with one modification per molecule for simplicity. 4FB(PEG)$_4$-PFP and pentafluorophenol were removed by buffer exchange before generation of the bispecific reagent and further use. DMF, dimethylformamide. (B) Reaction scheme to determine the molar substitution ratio (MSR). A portion of 4FB(PEG)$_4$-5E10 was incubated with 2-hydrazinopyridine dihydrochloride (2HP) to form 2HP-4FB(PEG)$_4$-5E10, which absorbs at 354 nm. The calculated MSR was 2.2. (C) Flow cytometric analysis of 4FB-5E10 and 4FB(PEG)$_4$-5E10 binding to pig BMMSCs. Unmodified 5E10 (solid, black), 4FB(PEG)$_4$-5E10 (solid, light grey), 4FB-5E10 (solid, dark grey), and IgG1 control antibody (dotted, black) binding to BMMSCs was detected with GAM-FITC (10 μg/mL). 4FB-5E10 was synthesized in the same fashion as 2 (MSR = 3.0).The histograms are representative of three experiments.

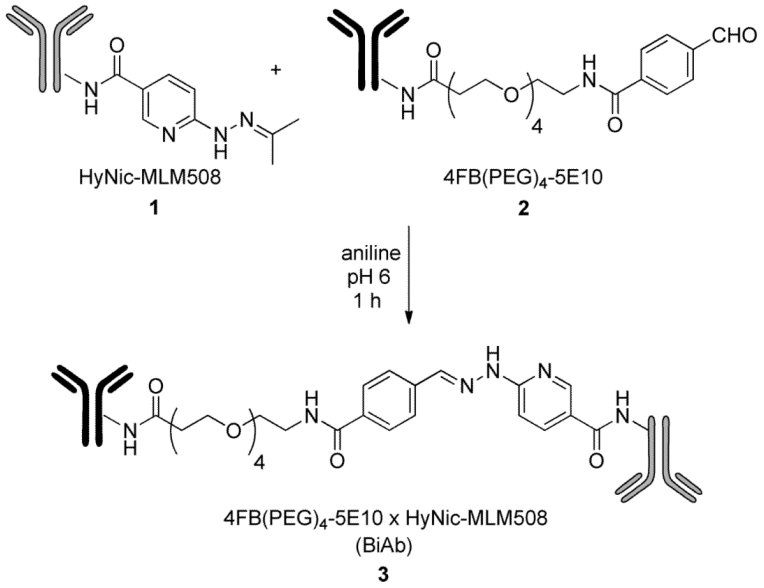

Figure 4.

Reaction of two independently modified antibodies (HyNic-MLM508 and $4FB(PEG)_4$-5E10) to form a bispecific construct mixture. The reactant-modified antibody mixtures are represented with one modification per molecule and without unmodified antibody for simplicity. Reaction products not shown include unmodified antibodies, **1**, **2**, heterodimers bearing multiple cross-links, and multimers.

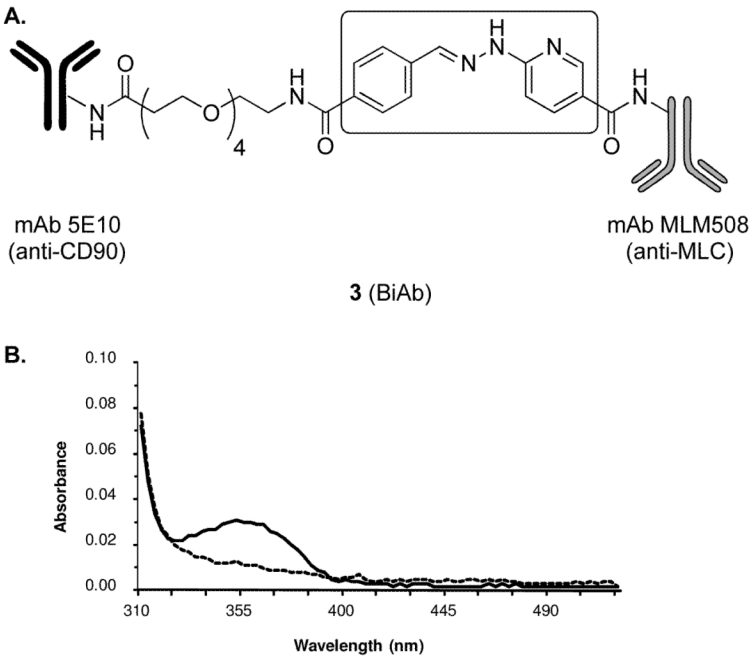

Figure 5.

Generation of the bispecific antibody as monitored by UV-vis spectroscopy. (A) Bis-aryl hydrazone (rectangle) generated in the reaction of 4FB(PEG)$_4$-5E10 with HyNic-MLM508 (see Figure 4). (B) The progress of the cross-linking reaction shown in Figure 4 was monitored by the absorbance of the bis-aryl hydrazone at 354 nm. (dashed, t = 0; solid, t = 1 h). A_{354} plateaued 1 h after initiation.

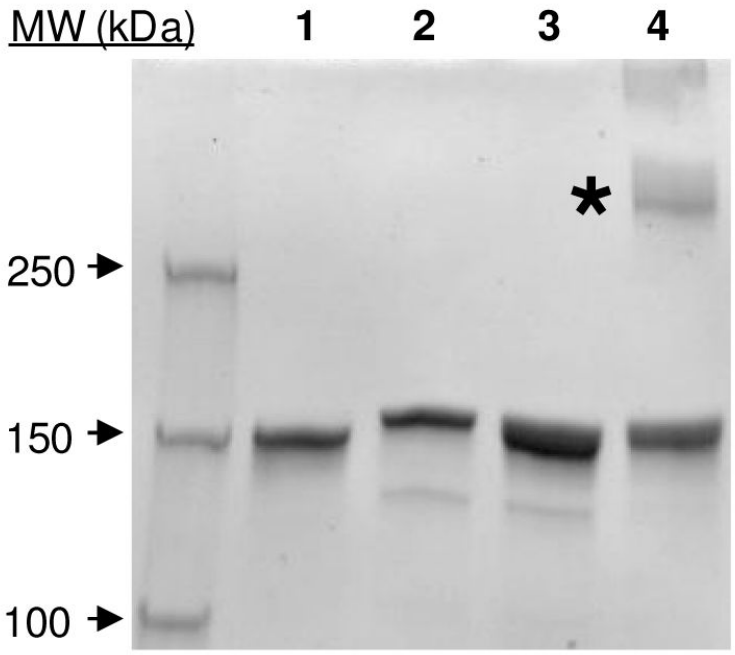

Figure 6.
Analysis of reaction products by nonreducing SDS-PAGE. Lane 1, unmodified MLM508 (2 μg); lane 2, unmodified mAb 5E10 (2 μg); lane 3, unmodified MLM508 and 5E10 combined (2 μg each, 4 μg total); lane 4, bispecific antibody reactant products (4 μg). Asterisk indicates bispecific antibody.

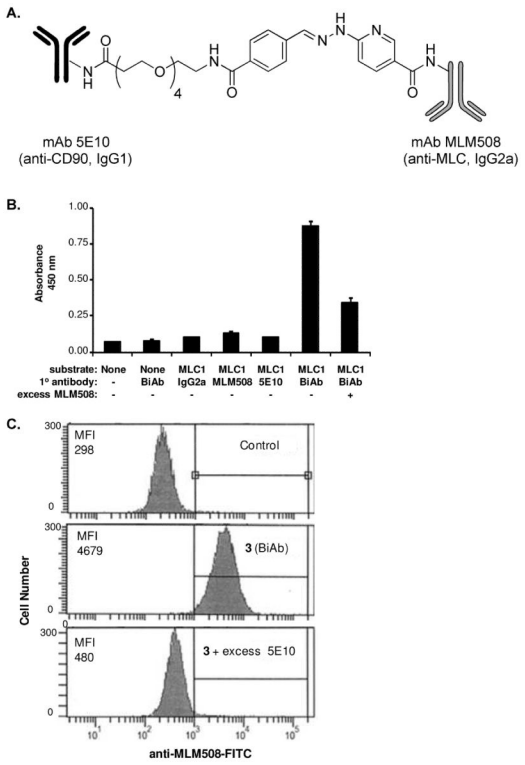

Figure 7.

Antigen specificity of the bispecific antibody product (**3**). (A) Bispecific antibody (BiAb) highlighting the isotypes of the mAb of each respective arm. (B) ELISA analysis of BiAb binding to plastic immobilized recombinant human MLC1. MLC1 was immobilized at 10 μg/mL. After blocking with BSA, primary antibody was added at a concentration of 10 μg/mL. In the case of "excess MLM508," unmodified MLM508 was added to the ELISA wells (20 μg/mL) 0.5 h before adding primary antibody. HRP-conjugated secondary antibody specific for IgG1 (the isotype of 5E10) was used for detection. Data are presented as average A_{450} ± SD from triplicate wells. One of three representative experiments is shown. (C) BiAb binding to pig BMMSCs as detected with anti IgG2a-FITC. To demonstrate specific BiAb binding, BMMSCs were pretreated with saturating levels of unlabeled mAb 5E10 (10 μg/mL) for 1 h before adding BiAb. Histograms are representative of three experiments.

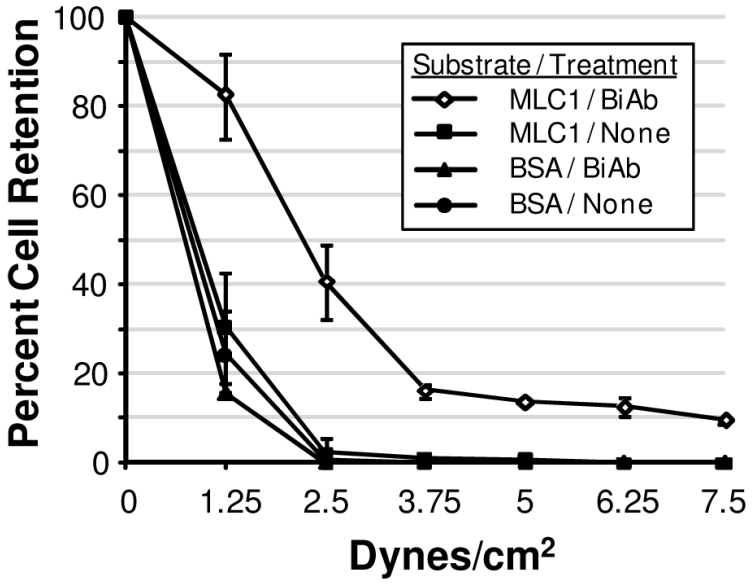

Figure 8.

Bispecific antibody–induced retention of BMMSCs on MLC1 under conditions of shear stress. Parallel-plate flow chambers were coated with MLC1 or BSA as a control. Cells were treated either with the bispecific antibody (BiAb; 5 μg/mL) or untreated (controls) and then loaded onto the substrate and incubated for 0.5 h at room temperature. Cell detachment was measured as shear stress increased. Data represent the average percent of cell adhesion. Error bars indicate the data range (n=2).