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A ^{99m}Tc-Labeled dual-domain cytokine ligand for imaging of inflammation

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Abstract

Introduction—Interleukin-1 (IL-1) and interleukin-18 (IL-18) are potent pro-inflammatory cytokines in inflammation-related diseases. Their actions are regulated by IL-1 receptor antagonist (IL-1ra) and IL-18 binding protein (IL-18bp). This study was designed to ^{99m}Tc-radiolabel an IL-1ra and IL-18bp dual-domain cytokine ligand, IL-18bp-Fc-IL-1ra, for specific inflammation targeting.

Methods—^{99m}Tc-IL-18bp-Fc-IL-1ra was obtained by direct labeling via 2-iminothiolane reduction. Competitive binding of ^{99m}Tc-labeled and unlabeled IL-18bp-Fc-IL-1ra to rat polymorphonuclear leukocytes was assessed *in vitro*. A mouse ear edema model was used to evaluate specific targeting properties of ^{99m}Tc-IL-18bp-Fc-IL-1ra *in vivo*. The correlation between ^{99m}Tc-IL-18bp-Fc-IL-1ra uptake and ¹¹¹In-labeled polymorphonuclear neutrophil infiltration was studied using ischemic-reperfused rat hearts.

Results—Direct ^{99m}Tc-labeling yielded a stable dual-domain cytokine radioligand with radiochemical purity greater than 95% after gel filtration. Competitive binding studies showed specific targeting of ^{99m}Tc-IL-18bp-Fc-IL-1ra to inflammatory cells. ^{99m}Tc-IL-18bp-Fc-IL-1ra uptake was 1.80 ± 0.17 % injected dose per gram (%ID/g) in the inflamed ear without blocking, whereas uptake in the presence of IL-18bp-Fc-IL-1ra was 1.09 ± 0.08 %ID/g (*P* < 0.05). The amounts of IL-1β and IL-18 were significantly increased in the inflamed ears compared to the vehicle controls. A significant correlation of ^{99m}Tc-IL-18bp-Fc-IL-1ra with ¹¹¹In-labeled neutrophil distribution was observed in the ischemic-reperfused hearts (*P* < 0.001).

Conclusion—Targeting pro-inflammatory cytokines with ^{99m}Tc-IL-18bp-Fc-IL-1ra may provide a suitable approach for specific detection of inflammatory sites.

Keywords

^{99m}Tc; Interleukin-1; Interleukin-18; Inflammation; SPECT

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1. Introduction

Pro-inflammatory cytokines are associated with many systemic diseases, such as arthritis, atherosclerosis, and myocardial ischemia-reperfusion injury. Interleukin (IL) 1 (IL-1) is one of the most pleiotropic cytokines and causes a wide variety of biological effects related to inflammation, infection and autoimmune processes [1, 2]. The action of IL-1 is regulated by the structurally-related IL-1 receptor antagonist (IL-1ra), which is primarily produced by activated monocytes and tissue macrophages, as well as a variety of other cell types [3, 4]. IL-1ra is a specific receptor antagonist for two IL-1 gene family members (IL-1 α and IL-1 β); it binds to cell membrane IL-1 type I receptor and prevents IL-1 from binding to the same IL-1 receptor [4, 5]. IL-1ra has no agonist activity and does not initiate signal transduction. A recombinant, nonglycosylated form of the human IL-1ra, anakinra (Kineret[®]), has been approved by FDA for the treatment of rheumatoid arthritis (RA) [6]. IL-1ra has been shown great potential as a probe for experimental investigation and as a treatment for IL-1 mediated inflammatory disease [6, 7].

IL-18 is another pro-inflammatory cytokine that possesses significant inflammatory activity [2, 8, 9]. It is also known as interferon-gamma (IFN- γ) inducing factor and is produced by a wide range of human cell types, including macrophages, dendritic cells, keratinocytes, Kupffer cells and epithelial cells [2, 10–12]. IL-18 induces production of tumor necrosis factor alpha (TNF- α) and IL-1 β in mononuclear cells [13] and initiates a cytokine cascade with concomitant expression of pro-inflammatory markers. Interestingly, IL-18 is a pro-inflammatory cytokine closely related to IL-1 and shares numerous properties with IL-1. For example, both inactive precursor forms of IL-1 and IL-18 are cleaved by caspase-1 to mature biologically active forms; IL-1 and IL-18 regulate each other by stimulating the production of caspase-1; and the IL-18 receptor chains (IL-18R α and IL-18R β) are also members of the IL-1 receptor family [14, 15].

The action of IL-18 is regulated by IL-18 binding protein (IL-18bp), which is a secreted extracellular protein that acts as a decoy receptor. IL-18bp has a strong binding affinity to IL-18 and thereby inactivates IL-18, thus preventing activation of the cell surface receptors [16–18]. Binding of IL-18bp to IL-18 further forms an inactive complex with IL-18R β , which prevents the IL-18R α chain from activating the cell [2].

Knowledge of cytokine pathways associated with inflammation may identify opportunities to detect the inflammatory response using radioligands. A specific cytokine radioligand that serves as a biomarker is clinically desirable in inflammatory diseases to noninvasively detect an inappropriate level of inflammation and promote timely therapeutic intervention. Because key cytokine combinations can work together in an organ or tissue to produce coordinated effects [19–23], strategies that target multiple proinflammatory pathways simultaneously, may be more effective than those targeting a single pathway. IL-1 and IL-18 share numerous properties in terms of targeting receptors and signal transduction pathways. The effects of IL-1 and IL-18 are additive or synergistic in promoting pathophysiological processes observed in many inflammation-related diseases, such as depressing myocardial function in ischemic myocardium and mediating endotoxemic myocardial dysfunction. The potential for using the synergistic effects of cytokines motivated us to develop radiolabeled dual-domain cytokine ligands for detecting inflammatory sites, and for predicting and evaluating therapies in inflammatory and autoimmune diseases. This study was designed to validate a technetium-99m-labeled dual-domain cytokine ligand, ^{99m}Tc-IL-18bp-Fc-IL-1ra, for inflammation imaging. IL-18bp-Fc-IL-1ra is a recombinant human fusion protein. It contains an amino-terminal segment that specifically binds to IL-18 and a carboxy-terminal sequence of IL-1ra that binds to the IL-1 receptor. The Fc portion of human IgG1 in the fusion protein links the two segments and is capable of dimerizing.

2. Materials and Methods

2.1. Production of IL-18bp-Fc-IL-1ra

The dual-domain protein IL-18bp-Fc-IL-1ra, with a sequence of 576 amino acids, was developed by AmProtein Corporation (Camarillo, CA). It is a recombinant fusion protein that is derived from recombinant DNA. In brief, the fusion protein was created through the joining of three genes which originally coded for human IL-18bp, IL-1ra, and IgG1 Fc fragment. Translation of this fusion gene resulted in a single polypeptide with functional properties derived from each of the original proteins. Production of IL-18bp-Fc-IL-1ra was accomplished by the large-scale culturing of Chinese hamster ovary (CHO) cells that have been "cloned" to express the recombinant DNA construct. The cells were cultured in a serum-free suspension system with CHO-CD4 medium (Irvine Scientific, Santa Ana, CA) and in-house feed medium, and scaled up in a 3-liter bioreactor (Applikon Biotechnology, Foster City, CA). The protein was purified by Protein-A direct capture, followed by ion-exchange and hydrophobic chromatography. The purified protein was further processed and analyzed by size-exclusion and high-performance liquid chromatography (SEC-HPLC).

2.2. Radiolabeling IL-18bp-Fc-IL-1ra with ^{99m}Tc

IL-18bp-Fc-IL-1ra was radiolabeled with ^{99m}Tc using a modified non-specific labeling protocol as previously described [24–26] in which glucoheptonic acid serves as a transfer agent to chelate tin-reduced $^{99m}\text{TcO}_4^-$ to thiolated proteins. Glucoheptonic acid and 2-iminothiolane were purchased from Sigma Aldrich, Inc. (St Louis, MO). A total of 100 μg protein in 200 μL PBS was incubated with 20 μL 2-iminothiolane (10 mg/mL in saline) at 37°C for 30 minutes. A freshly prepared 5 μL SnCl_2 solution (1 mg/mL in nitrogen-purged 0.1 N HCl) was mixed with 200 μL of glucoheptonic acid solution (2 mg/mL in nitrogen-purged saline). Three hundred μL of $^{99m}\text{TcO}_4^-$ (30 mCi) in 0.9% NaCl was added to the stannous glucoheptonate mixture and incubated at room temperature for 5 minutes to produce ^{99m}Tc -glucoheptonate. The thiolated protein was added into the vial of ^{99m}Tc -glucoheptonate and incubated at room temperature for 30 minutes. The reaction mixture was purified using a disposable PD-10 Desalting Column pre-packed with Sephadex G-25 Medium (GE Healthcare, Piscataway, NJ). The column was pre-equilibrated and eluted with PBS, pH 7.4. Protein concentrations of collected fractions were determined using a Genesys 10 Bio spectrophotometer and BCATM Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL).

Radiolabeling efficiency (radiochemical purity, RCP) and stability were determined by instant thin-layer chromatography (ITLC-SG), ascending paper chromatography on Whatman 3MM paper, SG-81 paper, and size-exclusion high performance liquid chromatography (SEC-HPLC). The mobile phases for ITLC and paper chromatography were 0.9% saline and acetone. In this saline/acetone solvent system, free $^{99m}\text{TcO}_4^-$ moves with the solvent front, leaving labeled and reduced/hydrolyzed (R/H) ^{99m}Tc at the point of application. R/H ^{99m}Tc was separated by using 5% bovine serum albumin (BSA)-impregnated ITLC-SG strips as the stationary phase and solvent mixture ethanol:ammonia:water = 2:1:5 (v/v) as the mobile phase. In this system, free ^{99m}Tc and labeled proteins move with the solvent front, whereas R/H ^{99m}Tc remains at the point of application. Radiochromatograms of the ITLC-SG and paper strips were obtained using digital autoradiograph imaging. The strips were exposed to Fujifilm phosphor imaging plates for 1–5 minutes. A Fujifilm BAS-5000 Bio-Imaging Analysis System (Fujifilm Medical Systems USA, Stamford, CT) was used to scan the plates for digital autoradiograph collection. The strips were cut into 1-cm pieces and counted by a CRC-15W Dose Calibrator/Well Counter (Capintec, Ramsey, NJ).

Additional quality control was performed by SEC-HPLC using a Shodex KW 802.5 column (7.8 × 300 mm, 5 µm particle size) (Thomson Instrument Co., Oceanside, CA) and a Waters Breeze system (Waters Technologies Corp., Milford, MA) equipped with a Waters 1525 Binary HPLC Pump and a Waters 2489 dual absorbance detector (280 nm) in line with a Flow-Count™ Radio HPLC Flow Through Detector (Bioscan Inc., Washington, DC). The radiolabeled protein was eluted with 0.1 mol/L KH₂PO₄ at pH 7.0 at a flow rate of 0.8 mL/min.

In vitro stability study was carried out in saline at room temperature and rat serum at 37°C, respectively. In vivo stability study was performed in two Sprague-Dawley rats that received intravenous injection of ^{99m}Tc-labeled protein purified by the PD-10 gel filtration (5–7 mCi). Blood samples were collected at 30, 60, and 180 minutes post-injection. The samples were centrifuged at 5000 rpm for 5 min to separate plasma and cellular fractions, and individual fractions were measured with the CRC-15W Dose Calibrator/Well Counter. The plasma was analyzed by SEC-HPLC.

2.3. *In vitro* cell binding studies

^{99m}Tc-IL-18bp-Fc-IL-1ra competitive binding assays were carried out using isolated rat polymorphonuclear leukocytes to determine the ligand concentration that inhibits 50% of the maximum specific binding (IC₅₀) to the cells. The polymorphonuclear leukocytes were collected by blood sampling from three healthy male Sprague-Dawley rats (350–450 grams) using the hydroxyethyl-starch exchange transfusion method described previously [27–29]. The rats were anesthetized by 1.2% isoflurane and heparinized with intra-peritoneal injection of 1 mL heparin (1000 units). The right carotid artery and tail vein were catheterized. A total of 80–90 mL of blood was exchanged using 6% hydroxyethyl-starch-saline (DuPont Chemical Co., Dorval, Quebec, Canada). The leukocyte-rich plasma was separated from the blood by 40-minute sedimentation of red blood cells at room temperature. The leukocytes were recovered by centrifugation at 3,500g for 5 min and resuspended in 5 mL calcium-free and magnesium-free Tyrode's solution with 10% plasma and 25 units heparin per milliliter. The polymorphonuclear leukocytes were separated using sequential centrifugation and suspended in Roswell Park Memorial Institute (RPMI) medium with 10% fetal bovine serum (FBS) and 25 units heparin/mL and stored overnight at 2–8°C.

A total of 5×10^5 cells suspended in Eppendorf tubes with 1 mL of binding solution (cell culture medium containing 0.1% bovine serum albumin) were incubated with 2 nM ^{99m}Tc-IL-18bp-Fc-IL-1ra at varying concentrations of unlabelled IL-18bp-Fc-IL-1ra for 2 hours at room temperature. The cells were centrifuged at 3,500g for 5 min, the supernatant was removed, and the pellet was washed twice with 1 mL of ice cold PBS. After removing supernatant, the radioactivity bound to the cell pellet and the free radioactivity in the supernatant were counted using the CRC-15W Dose Calibrator/Well Counter. The results were calculated using the averages of triplicate determinations. Data were analyzed with non-linear regression analyses using SigmaPlot 11.0 Software (Systat Software Inc., Chicago, IL).

2.4. Biodistribution measurements

Biodistribution studies of ^{99m}Tc-IL-18bp-Fc-IL-1ra were performed in five healthy male Sprague-Dawley rats (250–300 g) and three healthy ICR mice. Three hours after intravenous injection of the radioligands (3.5–5.5 mCi for rats, 2.0–2.5 mCi for mice), the animals were euthanized with an overdose of sodium pentobarbital. Blood, skeletal muscle, heart, lung, stomach, intestine, liver, spleen, and kidneys were harvested, weighed, and measured using the CRC-15W Dose Calibrator/Well Counter.

2.5. Blood clearance

^{99m}Tc -IL-18bp-Fc-IL-1ra (3.5–5.5 mCi, 0.2–0.3 mL) was intravenously injected in four male Sprague-Dawley rats (250–300 g). Serial blood samples (200 μL) were collected from a carotid artery catheter at 0, 1, 3, 6, 10, 30, 60, 120, and 180 minutes after injection. Each volume of sample withdrawn was replaced with an equal volume of warm physiological saline solution. The samples were weighed and measured with the CRC-15W Dose Calibrator/Well Counter to determine percent injected dose per gram (%ID/g) at each time point. Blood-clearance curves were modeled using a non-linear Curve-Fit Kinetic Equation by TableCurve 2D[®] software (Systat Software Inc., Chicago, IL).

2.6. Accumulations of ^{99m}Tc -IL-18bp-Fc-IL-1ra in TPA-induced inflammation in mouse ear

We assessed the ability of ^{99m}Tc -IL-18bp-Fc-IL-1ra to target inflammation *in vivo* in a mouse model of skin inflammation [30–32]. Ear edema was induced in ICR mice (10–15 weeks old) by topical application of 2.0 μg 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma Aldrich, Inc., St Louis, MO) dissolved in 20 μL of acetone administered through a micropipette to the right ear (10 μL on both the inner and outer surface of the ear. The TPA treatment was repeated at 24 hours. The left ear was treated with 20 μL of acetone (vehicle) topically as a negative control. The thickness of each ear was measured using a digital caliper before each TPA administration and radiotracer injection. Three hours after the second TPA (or vehicle) treatment, ^{99m}Tc -IL-18bp-Fc-IL-1ra (2.0–2.5 mCi, 0.2 mL) was injected intravenously in 6 mice. To determine the specificity of ^{99m}Tc -IL-18bp-Fc-IL-1ra uptake in the inflamed area, blocking tests were carried out in 5 mice by intravenous injection of 500 μg (0.2 mL) non-radiolabeled IL-18bp-Fc-IL-1ra 30 minutes before radioligand administration. The animals were imaged using a small-animal SPECT imager, FastSPECT II, at 2–3 hours after radiotracer injection. The ears were localized in the center of the field of view. The animals were sacrificed at the end of the imaging session. The ears were excised for radioactive measurement using the CRC-15W Dose Calibrator/Well Counter and digital autoradiograph imaging using the Fujifilm phosphor imaging plates and the BAS-5000 system. Major organs and tissues in three TPA-treated mice were harvested and measured to determine the biodistribution of ^{99m}Tc -IL-18bp-Fc-IL-1ra.

2.7. Measurements of IL-1 β and IL-18 cytokines in homogenized mouse ear skin

The mouse ears treated with TPA or vehicle was harvested, snap frozen in liquid nitrogen, stored in a -80°C freezer. Ear tissues were homogenized in a cell extraction buffer (Invitrogen Corporation, Camarillo, CA) containing 10 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM, $\text{Na}_4\text{P}_2\text{O}_7$, 2 mM, Na_3VO_4 , 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, 0.5% deoxycholate. This cell extraction buffer was supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich Corp., St. Louis, MO) just prior to use. The tissue mixture was placed on ice, sonicated for 30 seconds, and incubated at 4°C for 10 minutes. The final homogenate was centrifuged at 20,000g at 4°C for 15 minutes. The supernatant fraction was aliquoted and frozen at -80°C until assays were performed for determination of cytokine protein levels.

The levels of IL-1 β and IL-18 were quantified using a solid-phase sandwich enzyme-linked immunosorbent assay (ELISA). Mouse IL-1 β ELISA kits were purchased from Invitrogen Corp. (Camarillo, CA) and mouse IL-18 ELISA kits were obtained from MBL (Medial & Biological Laboratories Co., Ltd., Nagoya, Japan). The IL-1 β and IL-18 ELISA assays were performed according to the manufacturer's recommended protocol. Briefly, diluted samples and standards at 50–100 μL were transferred to the antibody-coated wells in a 96-well polyvinyl plate. The plates were incubated for 60–120 minutes at room temperature and the wells were washed with wash solution. A solution of enzyme-conjugated detection antibody

was added. After a second incubation and washing, a substrate solution containing tetramethylbenzidine/H₂O₂ was added. After 30-min incubation, the stop solution (0.5 mol/L H₂SO₄) was added and the plates were read using a DTX 880 Multimode Detector (Beckman Coulter, Inc., Fullerton, CA) at a wavelength of 450 nm.

2.8. Correlation of ^{99m}Tc-IL-18bp-Fc-IL-1ra distribution with polymorphonuclear leukocyte accumulation

An ischemic-reperfused rat heart model was used to determine the correlation between ^{99m}Tc-IL-18bp-Fc-IL-1ra distribution and ¹¹¹In-oxine labeled neutrophil infiltration. Three Sprague-Dawley rats (male, 250–300 g) were intubated and anesthetized with 1.2% isoflurane. The animals were ventilated using a volume-controlled Inspira Advanced Safety Ventilator (Harvard Apparatus, Holliston, MA) with a mixture of oxygen and room air. A left thoracotomy incision was made at the fifth intercostal space and a ligature was placed around the left coronary artery (LCA), which was occluded for 45 minutes, followed by 2-hour reperfusion after ligature release. After ischemia-reperfusion treatment, the chest was closed in layers with sutures. The surgical procedures were performed under sterile conditions.

Polymorphonuclear leukocytes were isolated from 3 healthy male Sprague-Dawley rats (350–450 grams) using the hydroxyethyl-starch exchange transfusion technique described above. The cells were suspended in RPMI medium with 10% FBS and 25 units heparin/mL and stored overnight at 2–8°C. The purified leukocytes were labeled with ¹¹¹In-oxine (GE HealthCare, Phoenix, AZ). In brief, the cells were washed with PBS, mixed and suspended with ¹¹¹In-oxine (100 µCi per million cells), followed by 20-minute incubation at room temperature. Subsequently, the cells were washed twice, ¹¹¹In-oxine labeling efficiency was measured, and cell viability was determined by trypan blue exclusion. The ¹¹¹In-labeled leukocytes (250 µCi, 0.2 mL) were injected intravenously in three rats treated with ischemia-reperfusion. Twenty-four hours after the administration of ¹¹¹In-labeled leukocytes, ^{99m}Tc-IL-18bp-Fc-IL-1ra (3.0 mCi, 0.3 mL) was intravenously injected. The animals were euthanized 3 hours after the injection of ^{99m}Tc-IL-18bp-Fc-IL-1ra and the hearts were excised for tissue radioactivity measurement. After the right ventricle and great vessels were removed, the left ventricle was sliced into 1-cm-thick rings from apex to base and further cut into 20–24 tissue samples (46.8 ± 2.7 mg). All tissue samples were assayed in a Captus 3000 Well System with 1024 Multichannel Analyzer (Capintec, Ramsey, NJ) for ^{99m}Tc and ¹¹¹In activity. Results were expressed as counts per minute per gram (cpm/g) for each radionuclide. These counts were corrected for isotope decay and cross-talk (downscatter) of ¹¹¹In counts into the ^{99m}Tc window.

2.9. Data analysis

All quantitative results were expressed as mean ± S.E.M. Comparisons between two variables were performed with one-way analysis of variance. Probability values less than 0.05 were considered significant.

2.10. Ethics

The animal experiments were performed in accordance with Principles of Laboratory Animal Care from the National Institutes of Health (NIH Publication 85-23, revised 1985) and were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Arizona.

3. Results

3.1. ^{99m}Tc -labeling of IL-18bp-Fc-IL-1ra

The radiolabeling yield of ^{99m}Tc -IL-18bp-Fc-IL-1ra was typically greater than 75%. After gel filtration purification, RCP was greater than 95%. ITLC showed that ^{99m}Tc -labeled protein remained at the loading position when saline/acetone was used as the mobile phase. There was no significant movement of free ^{99m}Tc with the solvent front. When ethanol:ammonia:water was used as the mobile phase, labeled protein moved with the solvent front, and no significant reduced/hydrolyzed ^{99m}Tc was found at the loading point. A representative HPLC chromatogram of ^{99m}Tc -IL-18bp-Fc-IL-1ra is shown in Fig. 1. The specific activity of ^{99m}Tc -IL-18bp-Fc-IL-1ra was 7.4–8.3 MBq/ μg .

Stability tests of ^{99m}Tc -IL-18bp-Fc-IL-1ra using thin-layer chromatography and HPLC showed no significant degradation of the product up to 5 hours in either saline at room temperature or serum at 37°C. After 6 hours, more than 90% of the radioactivity was still associated with IL-18bp-Fc-IL-1ra. At 24 hours, RCP was 86.9% in saline and 92.2% in serum.

The *inv vivo* stability study showed that that 87.4%, 84.1%, and 86.4% of the radioactivity in blood was found in the plasma at 30, 60, and 180 minutes post-injection, respectively, with the remaining amounts associated with the cell fraction. HPLC analysis of plasma showed that the radioactivity associated with IL-18bp-Fc-IL-1ra was 91.7% at 3 hours post-injection. There was no significant amount of free ^{99m}Tc in the plasma.

3.2. *In vitro* binding assays

Binding of ^{99m}Tc -IL-18bp-Fc-IL-1ra to leukocytes gradually decreased with addition of increasing amounts of non-radiolabeled IL-18bp-Fc-IL-1ra, as shown in Fig. 2. The 50% inhibitory concentration (IC_{50}) for ^{99m}Tc -IL-18bp-Fc-IL-1ra was 73.56 nM ($n=3$).

3.3. Blood clearance and biodistribution measurements

Blood radioactivity (%ID/gm) and normalized clearance (% peak) over time are shown in Fig. 3. After rapid early clearance within ten minutes post-injection, the radioactive washout became relatively slower. ^{99m}Tc fractional retention at 180 minutes post-injection was $11.0 \pm 0.7\%$ of peak activity. The blood clearance was best fit to a Two Second Order Independent Decay (Non-linear Equation 8153) with calculated half-lives of 6.0 minutes. The equation was $Y=a+b/(1+b*c*X)+d/(1+d*e*X)$, where $a = -0.145$, $b = 6.04$, $c = 0.221$, $d = 1.772$, $e = 0.007$ ($\gamma = 0.999$).

Tissue radioactive distributions in rats and mice are summarized in Table 1. The organs with greatest activity at 180 minutes post-injection include liver, spleen, and kidneys. There was low radioactive uptake in heart, skeletal muscle, and skin. In terms of %ID/g, the results of biodistribution measurements in the blood and other major organs/tissues of ICR mice were significantly higher than that of rats.

3.4. TPA-induced skin inflammation

Ear edema was observed in all animals that received TPA treatments. Ear thickness increased to 0.54 ± 0.08 mm from 0.21 ± 0.01 ($P < 0.01$) after TPA treatments. In the ears treated only with vehicle, ear thickness remained unchanged in comparison with the initial thickness (0.21 ± 0.01 mm vs. 0.23 ± 0.01 , $P > 0.05$).

The inflamed ears in the TPA-treated animals exhibited high accumulation of ^{99m}Tc -IL-18bp-Fc-IL-1ra, which could be clearly detected by *in vivo* SPECT imaging and *ex vivo*

autoradiograph imaging. The radioactive uptake in the control ear was minimal and typically invisible by SPECT imaging. The *in vivo* specificity of ^{99m}Tc -IL-18bp-Fc-IL-1ra binding was established by blocking of radioactive uptake in the inflamed ears with non-radiolabeled IL-18bp-Fc-IL-1ra. Representative FastSPECT II and autoradiograph images of the ears in animals with and without a blocking dose of unlabeled IL-18bp-Fc-IL-1ra are shown in Fig. 4. The ears with TPA-induced edema exhibited a significant reduction of radiotracer uptake on both *in vivo* and *ex vivo* images after the blocking dose.

Biodistribution results of ^{99m}Tc -IL-18bp-Fc-IL-1ra in the mice with TPA-induced inflammatory ears are presented in Table 1. There was no difference in biodistribution between TPA-treated mice and healthy control mice. Based on postmortem gamma well counting at 3 hours post injection, the uptake ratio of inflamed ears to contralateral vehicle-treated ears was 8.75 ± 2.20 in the animals that received saline and 3.84 ± 0.61 in the animals that received unlabeled IL-18bp-Fc-IL-1ra ($P < 0.05$). The average ear uptake of the radiopharmaceutical (%ID/g) was 1.80 ± 0.17 (right ears) vs. 0.48 ± 0.07 (left ears) ($P < 0.001$) in the animals without blocking, compared to 1.09 ± 0.08 (right ears) vs. 0.53 ± 0.13 (left ears) ($P < 0.05$) with blocking. The difference between the radioactivity of inflamed ears in the animals with and without blocking was significant ($P < 0.05$).

3.5. Levels of IL-1 β and IL-18 in the inflamed mouse ears

ELISA results of cytokine measurements in homogenized ear tissue are shown in Fig. 5. The amounts of IL-1 β and IL-18 (pg/ml) were significantly increased in the ears with TPA-induced inflammation compared with vehicle-treated ears (IL-1 β : 3986.7 ± 40.6 vs. 555.4 ± 196.3 , $P < 0.001$; IL-18: 223.1 ± 19.3 vs. 138.6 ± 12.1 , $P < 0.05$). The ratio of inflamed ears to contralateral vehicle-treated ears was 12.87 ± 4.65 in IL-1 β and 1.65 ± 0.18 in IL-18 ($P < 0.05$).

3.6. Correlation between ^{99m}Tc -IL-18bp-Fc-IL-1ra distribution and polymorphonuclear leukocyte accumulation

Cell-labeling efficiency of the donor rat leukocytes was 70–82%. The viability of labeled cells, determined by trypan blue exclusion, was greater than 96% at 3 hours. Fig. 6 shows a linear relationship between ^{99m}Tc -IL-18bp-Fc-IL-1ra uptake and ^{111}In -labeled leukocyte infiltration in the ischemic-reperfused rat heart model. The correlation between the two was significant ($\gamma = 0.921$, $P < 0.001$).

4. Discussion

Conventional imaging methods typically rely on anatomical, physiological or metabolic changes to provide an indirect or non-specific demonstration of inflammation [33–35]. Scintigraphic imaging with radiolabeled leukocytes is a standard clinical procedure for detection of occult infectious and inflammatory diseases [36–39]. However, *in vitro* radiolabeling of leukocytes requires a complex procedure with potential risk of infection or misadministration. Nonspecific activation of leukocytes may interfere with targeted localization *in vivo* [37]. Fluorine-18 labeled 2-deoxyglucose, [^{18}F]FDG, can also be used to image inflammation. [^{18}F]FDG competes with glucose for uptake into metabolically active cells, where it accumulates in proportion to metabolic activity. [^{18}F]FDG PET imaging can demonstrate the increased metabolic activity that accompanies inflammation, but the procedure offers low specificity for inflammation.

Molecular imaging using cytokines may hold promise for detection of infection and inflammation [40–42]. An ideal cytokine-based radiolabeled molecular probe should bind specifically to pro-inflammatory cytokines or receptors with high affinity and should have

sufficient residence time at sites of inflammation to allow for imaging. The radiotracer should be sufficiently cleared from the circulation to provide adequate contrast between inflammatory lesions and normal surrounding tissues or blood pool. Factors affecting the behavior of a cytokine radioligand for imaging inflammation include choice of the molecular ligand, membrane receptor composition in the target cells, and biological availability of the radioligand. In this study, we investigated a dual-domain cytokine radioligand to specifically localize sites of inflammation in animal models.

IL-18bp is a constitutively secreted inhibitor of the biological activity of IL-18 with high-affinity and specificity. IL-18bp resembles a soluble receptor for IL-18, but it lacks a transmembrane and cytosolic domain [2, 16]. A soluble recombinant IL-18bp-Fc can be produced by attaching a sequence encoding an isoform-a of human IL-18bp to a sequence encoding human IgG1 Fc [43–45]. This hybrid IL-18bp-Fc has many advantages, including specificity, low immunogenicity and high affinity. Using recombinant DNA technology, IL-18bp-Fc can be secondarily fused with IL-1ra to produce a single two-domain polypeptide for potential inflammation targeting via IL-1 and IL-18 pathways bi-specifically. First, like anakinra, the carboxy-terminal sequence of IL-18bp-Fc-IL-1ra may act as a competitive receptor antagonist for binding of IL-1 to cell-membrane-bound IL-1 type I receptors on numerous cell types. The amino-terminal segment of IL-18bp-Fc-IL-1ra may specifically bind to IL-18 and function independently as a decoy to prevent the initiation of signal transduction at the cell membrane IL-18 receptor. When IL-18bp-Fc-IL-1ra is bound on the cell surface in inflammatory tissues, the IL-18bp domain may retain the ability to bind IL-18. Theoretically, when IL-18bp-Fc-IL-1ra is systemically administered, the IL-18bp domain can target circulating IL-18. However, because exogenous IL-1ra exhibits a rapid pharmacokinetic profile of blood clearance in humans and other species [46–48], the recombinant IL-1ra domain may guide the fusion protein to IL-1 receptor-rich inflammatory sites quickly.

IL-18bp-Fc-IL-1ra was radiolabeled with ^{99m}Tc using the 2-step thiolation protocol in this study. The direct ^{99m}Tc -labeling approach is simple and efficient. It involves the initial reduction of endogenous disulfide bonds to generate thiol binding sites. Using this approach, the dual-domain protein was ^{99m}Tc -labeled with high radiochemical purity and without significant ^{99m}Tc -colloid formation. We have also implemented an indirect labeling approach using 6-hydrazinopyridine-3-carboxylic acid (HYNIC) as a bifunctional chelator (data not shown). Indirect labeling is versatile and can be site-specific but is more complicated than direct labeling. Further studies are needed to compare inflammation targeting of fusion proteins labeled using thiolation and HYNIC methods.

The radiopharmaceutical kinetic profile and inflammation targeting properties of ^{99m}Tc -IL-18bp-Fc-IL-1ra were investigated in rat and mouse models in this study. Human IL-1ra can bind to murine IL-1 receptors and inhibit IL-1-mediated responses with similar avidity [49]. Unlike antibody to IL-18, IL-18bp does not exhibit species specificity, and human IL-18bp neutralizes both human and murine IL-18 [16]. Thus, the *in vivo* data of ^{99m}Tc -IL-18bp-Fc-IL-1ra in rats and mice should provide clinically valuable information regarding the usefulness of this dual-domain cytokine ligand in targeting inflammation sites. In rats, ^{99m}Tc -IL-18bp-Fc-IL-1ra showed improved blood-clearance kinetics compared to other radiolabeled recombinant proteins reported previously [48, 50]. The moderately fast blood-clearance kinetics of ^{99m}Tc -IL-18bp-Fc-IL-1ra may enhance the delivery of the radiotracer to target sites and produce a favorable target to non-target ratio for inflammation imaging. Biodistribution results in rats and mice indicate that ^{99m}Tc -IL-18bp-Fc-IL-1ra was excreted via the kidneys and liver, similar to results reported in previous studies with ^{125}I -labeled recombinant IL-1ra in Swiss mice [48]. Higher radioactive distribution of blood and organs in the mice relative to rats may be related to a kinetic difference between species. Overall,

the biodistribution of ^{99m}Tc -IL-18bp-Fc-IL-1ra in this study is similar to the data of ^{125}I -labeled recombinant IL-1ra [48]. This observation suggests that the IL-1ra domain of ^{99m}Tc -IL-18bp-Fc-IL-1ra may serve as ligand carrier and guide the dual-domain radioligand to tissue sites. Because the surface-bound IL-1ra does not undergo receptor-mediated internalization [51], the domain of IL-18bp in ^{99m}Tc -IL-18bp-Fc-IL-1ra anchored on the membrane can still function as an inhibitor of IL18. Reduction of ^{99m}Tc -IL-18bp-Fc-IL-1ra uptake following the blocking agent in the inflammatory ears could theoretically reflect the simultaneous blockade of two domains.

Our experimental results indicate that ^{99m}Tc -IL-18bp-Fc-IL-1ra may be a suitable candidate for specific imaging of inflammation. The specific binding of ^{99m}Tc -IL-18bp-Fc-IL-1ra to leukocytes was demonstrated in an *in vitro* competitive binding assay. This cell-based *in vitro* binding result indicates that the IL-1ra domain of ^{99m}Tc -IL-18bp-Fc-IL-1ra can target cell-membrane-bound IL-1 receptors because IL-18bp binds to IL-18, not IL-18 receptors. The binding specificity of ^{99m}Tc -IL-18bp-Fc-IL-1ra was further documented *in vivo* by blocking experiments in the TPA-induced skin-inflammation model. TPA-induced skin inflammation is a good model for investigating cytokine targeting of inflammation since it is characterized by high production of cytokines, including IL-1 β , IL-6, IL-12, and TNF α , as well as epidermal hyperproliferation, T-cell infiltration, and increased angiogenesis [52, 53]. However, based on our results of cytokine quantification, IL-18 may not play a significant role in this acute mouse ear inflammation model. The increased radioactive uptake in the inflamed ears might result predominantly from the high expression of IL-1 receptors. Because we only used IL-18bp-Fc-IL-1ra as a blocker, it is unclear whether a single domain or two domains are responsible for ^{99m}Tc -IL-18bp-Fc-IL-1ra binding. Additional studies using unlabeled IL-1ra and IL-18bp are required to determine the specificities of two domains in targeting IL-1 receptors and IL-18.

The correlation between ^{99m}Tc -IL-18bp-Fc-IL-1ra and ^{111}In -labeled leukocyte uptake in the ischemic-reperfused rat heart model provides further support for ^{99m}Tc -IL-18bp-Fc-IL-1ra as an inflammation-imaging agent. We selected the ischemic-reperfused rat heart model to explore the inflammation targeting properties of ^{99m}Tc -IL-18bp-Fc-IL-1ra because proinflammatory cytokines, such as tumor necrosis factor (TNF- α), interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-18 (IL-18), and interferon γ (IFN- γ), are induced at the site of myocardial injury [22, 54–58].

^{99m}Tc -IL-18bp-Fc-IL-1ra may have clinical implications in cardiovascular diseases. In acute myocardial infarction, there is robust upregulation of intramyocardial cytokines in the infarct zone as well as in non-infarcted myocardium within the first hours to one day [54, 55]. The elaborated cytokines then regulate myocytes and non-myocytes to trigger additional cellular inflammatory response. ^{99m}Tc -IL-18bp-Fc-IL-1ra may be useful to assess myocardial ischemia-reperfusion injury and evaluate the intensity of inflammation via IL-1 and IL-18 pathways. This dual-domain cytokine radioligand may also be used to evaluate the inflammation accompanying other cardiovascular disorders such as atherosclerosis, cardiomyopathy, and myocarditis.

In RA, a plethora of pro-inflammatory cytokines, including TNF- α , IL-1, IL-6, IL-7, IL-17, IL-18, and interferon- γ (IFN- γ), are involved in synovial inflammation and the resulting joint destruction and cartilage degradation [59, 60]. Elevated expression of IL-18 increases production of TNF- α and IL-1 in the RA synovial membrane and correlates significantly with local inflammation [61]. A variety of cytokine-based strategies are being explored in RA patients. Recombinant human IL-1ra (anakinra) was the first biologic agent approved for RA treatment [6]. IL-18bp is currently in clinical trials for the treatment of RA and severe psoriasis [62]. An objective approach is needed to evaluate treatment response and disease

activity because the presentation of the disease may vary considerably [60]. Targeting IL-1 and IL-18, as well as other cytokines, using cytokine radioligands may have a significant clinical impact on prediction and stratification in the individual patient [63].

There are several limitations in this study. As mentioned above, we only assessed *in vitro* competitive binding of ^{99m}Tc -IL-18bp-Fc-IL-1ra to rat polymorphonuclear leukocytes with unlabeled IL-18bp-Fc-IL-1ra. Although the specific targeting of ^{99m}Tc -IL-18bp-Fc-IL-1ra to inflammatory cells gives a clue that the IL-1ra domain may be able to target the cell membrane-bound IL-1 receptors, it is unclear if the IL-18bp domain is biologically involved in the *in vivo* binding to IL-18. Further *in vitro* studies are warranted to investigate the bi-specificity of this dual-domain cytokine radioligand. We have demonstrated that ^{99m}Tc -IL-18bp-Fc-IL-1ra has the ability to target inflammatory sites specifically. However, we have not yet obtained *in vivo* data to determine whether an individual cytokine radioligand, ^{99m}Tc -labeled IL-1ra-Fc or IL-18bp-Fc, would achieve the same uptake level (%ID/g) and binding specificity as the dual-domain radioligand. There are some shortcomings in the animal models used in this study. The mouse ear with TPA-induced acute inflammation might not be an optimal animal model for studies of inflammation targeting via the IL-18 pathway because it does not have high IL-18 expression. Increased capillary permeability at sites of inflammation in the mouse ear edema model may contribute to accumulation of radiolabeled proteins. Similarly, the pathophysiological changes in the ischemic-reperfused myocardium increase vascular permeability and also increase interstitial space [64]. As a result, the radioactivity detected in the inflamed lesions might be a combination of specific cytokine binding and nonspecific leakage of the tracer. The contribution of nonspecific binding and leakage needs to be quantitatively clarified in further studies using radiolabeled non-specific antibodies or proteins with similar molecular weight. Finally, it needs to be explored whether an elevated blood level of individual cytokines, such as IL-18, could prevent the dual-domain radioligand from reaching its target site.

5. Conclusions

IL-18bp-Fc-IL-1ra was successfully radiolabeled with ^{99m}Tc via 2-iminothiolane thiolation. The dual-domain radioligand, ^{99m}Tc -IL-18bp-Fc-IL-1ra, exhibited a favorable radiopharmaceutical kinetic profile. Targeting pro-inflammatory cytokines with the novel dual-domain ^{99m}Tc -IL-18bp-Fc-IL-1ra appears to be a promising approach for specific detection of inflammatory sites and assessment of inflammatory response.

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Fig. 1.

A representative HPLC-chromatogram of $^{99\text{m}}\text{Tc}$ -labeled IL-18bp-Fc-IL-1ra product using a 2-step thiolation protocol immediately after gel purification.

Fig. 2.
Results of in vitro cell binding competitive experiments of ^{99m}Tc -IL-18bp-Fc-IL-1ra using isolated rat leukocytes.

Fig. 3. Blood-clearance curves in healthy rats. The animals were anesthetized with 1.5% isoflurane. ^{99m}Tc -IL-18bp-Fc-IL-1ra (3.5–5.5 mCi, 0.2–0.3 mL) was intravenously injected. Left panel: Original blood clearance data in terms of %ID/g; Right panel: Normalized blood-clearance curves, in which radioactivity at each time point is given as percentage of peak activity at 2 minutes post-injection.

Fig. 4.

A and C: Representative SPECT and autoradiograph images in a mouse with TPA-induced right ear edema. The SPECT images were acquired for 15 minutes at 3 hours post-injection of 2.21 mCi radiotracer (0.2 ml). The animal was anesthetized with 1.5% isoflurane. B and D: SPECT and autoradiograph images in a mouse with ear inflammation received unlabeled IL-18bp-Fc-IL-1ra blocking. The animal received 2.24 mCi radiotracer (0.2 ml) and 15-minute SPECT image acquisition. It is noted that the ears on C and D were exposed on the same phosphor imaging plate simultaneously and the scale was set at the same quantitative value. Based on computerized analysis, the ratio of inflamed ear to controlled ear on SPECT and autoradiograph images was 2.06 and 2.41 in the mouse with unlabeled IL-18bp-Fc-IL-1ra blocking and 4.61 and 6.45 without blocking, respectively.

Fig. 5. Expression of IL-1 β and IL-18 in the mouse ear lesions of TPA-induced acute inflammation determined by measuring their concentration in the supernatant of ear homogenate by ELISA. Data are mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.001$ compared with corresponding vehicle-treated ears.

Fig. 6.
Regression analysis of $^{99\text{m}}\text{Tc}$ -IL-18bp-Fc-IL-1ra versus ^{111}In -oxine labeled leukocytes in ischemic-reperfused rat hearts.

Table 1Biodistribution measurements of ^{99}mTc -IL-18bp-Fc-IL-1ra (%ID/gm)

Tissues	Rats (n=5)	Mice (n=3)	Mice/TPA* (n=3)
Blood	0.36±0.03	1.15±0.07	1.07±0.02
Heart	0.11±0.02	0.41±0.05	0.38±0.06
Lung	0.20±0.02	0.80±0.13	0.79±0.14
Liver	3.47±0.24	23.12±1.51	23.08±1.55
Stomach	0.14±0.01	0.66±0.10	0.52±0.08
Spleen	1.12±0.16	3.91±0.43	3.30±0.46
Intestine	0.64±0.13	14.29±4.05	16.51±1.84
Kidneys	5.84±0.34	10.03±1.19	9.07±0.85
Skin	0.03±0.01	0.38±0.04	0.33±0.05
Muscle	0.02±0.01	0.10±0.02	0.09±0.02

Mice/TPA*: mice with TPA-induced ear edema