

RESEARCH ARTICLE

Synthesis and Evaluation of ^{99m}Tc-Labelled Monoclonal Antibody 1D09C3 for Molecular Imaging of Major Histocompatibility Complex Class II Protein Expression

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Abstract

Purpose: It is known that major histocompatibility complex class II protein HLA-DR is highly expressed in B-cell lymphomas and in a variety of autoimmune and inflammatory diseases. Therefore, a radiolabelled fully humanized IgG4 monoclonal antibody (mAb) can provide useful prognostic and diagnostic information. Aims of the present study were to radiolabel an anti-HLA-DR mAb with technetium-99m and to evaluate its binding specificity, tissue distribution and targeting potential.

Procedures: For labelling, we compared a direct method, after 2-mercaptoethanol (2-ME) reduction of disulphide bonds, with a two-step labelling method, using a heterobifunctional succinimidyl-6-hydrazinonicotinate hydrochloride chelator. Several *in vitro* quality controls and *in vivo* experiments in mice were performed.

Results: We obtained highest labelling efficiency (LE, >98%) and specific activity (SA; 5,550 MBq/mg) via the direct method. *In vitro* quality control showed good stability, structural integrity and retention of the binding properties of the labelled mAb. The biodistribution in mice showed high and persistent uptake in spleen and suggests kidney and liver-mediated clearance pathways. In tumour targeting experiments, we observed high uptake in HLA-DR-positive xenografts compared to controls. *In vivo* binding was proportional to the number of injected cells. In the *in vivo* blocking assay, uptake of radiolabelled mAb was significantly decreased in mice pre-injected with 100-fold molar excess of unlabelled mAb.

Conclusion: We efficiently labelled a humanized anti-HLA-DR mAb with ^{99m}Tc using a direct labelling method. Radiolabelled mAb binds to human HLA-DR antigens and therefore warrants further evaluation as a prognostic and diagnostic tool for patients with lymphoma or autoimmune diseases.

Key Words: Anti-HLA-DR monoclonal antibody, 1D09C3, Lymphoma, Autoimmune disease, Radiolabelling, Molecular imaging

Introduction

The major histocompatibility complex (MHC) consists of membrane-bound glycoproteins that are involved in different immunological and non-immunological phenomena

[1]. In humans, MHC class I genes, consisting of the three loci HLA-A, B and C, are expressed on almost all cells. In contrast, MHC class II genes, which encode for HLA-DR, DQ and DP antigens, are expressed mainly on B lymphocytes, activated T lymphocytes, macrophages, monocytes, dendritic cells, activated NK cells and progenitor haemopoietic cells. HLA-DR molecules are composed of α (35kD)

and β (28kD) subunits. Each subunit contains two extracellular domains, a membrane-spanning domain and a cytoplasmic tail. In mice, two subclasses, H2-A (HLA-DQ homologue) and H2-E (HLA-DR homologue), are known, and both are functional [2–4]. Delovitch *et al.* demonstrated that mouse anti-Ia alloantisera apparently react more strongly with human Ia antigens than do human alloantisera, therefore it is reasonable that a mice model could be used to study the functional properties of human Ia antigens, which are coded by MHC-II [5, 6].

The HLA-DR antigens play important roles in the cellular interaction involved in immune response. The HLA-DR protein is an intermediate activation antigen that is expressed on the surface of CD4 and CD8-positive T cells in the course of lymphocyte activation. In the resting state of T lymphocytes, HLA-DR is not expressed and is therefore a specific biomarker for T cell activation. This activation antigen is expressed on a high percentage of tissue infiltrating lymphocytes for a longer time span than other activation markers, such as CD25 (the IL-2 receptor), VLA antigens and 4F2 antigens. It is therefore a suitable target for nuclear imaging with a radioactive probe for the *in vivo* detection of T cell-mediated inflammation, including autoimmune diseases.

It is also known that other cells, such as vessel endothelium, may express HLA-DR following the release of local inflammatory molecules. Isobe and co-workers found the expression of MHC class II antigens in an animal model of heart rejection and also in kidney allograft rejection using an ^{111}In -labelled anti-MHC class II antigen monoclonal antibody (mAb) [7, 8]. Indeed, scintigraphy and histological examination revealed the presence of MHC class II antigen (HLA-DR) molecules on both the graft endothelium and the infiltrating mononuclear cells.

As far as malignancies are concerned, an abnormal HLA-DR expression has been demonstrated on the cell surface of several cancer types, mainly on leukemia and lymphoma cells. Loss of MHC-II molecules on diffuse large B-cell lymphoma (DLBCL) has been associated with poor survival. Recently, Rimsza *et al.* (2007) demonstrated that the HLA-DR protein status can predict the survival in patients with DLBCL treated with the MACOP-B chemotherapy regimen and therefore can be used as a prognostic marker [9]. Other authors outlined the effect of tumour cell MHC antigens on cytotoxic T lymphocytes (CTL) induction. Both MHC class I and class II antigens on the head and neck tumour cells were shown to play a critical role in inducing CTL [10].

A fully humanized IgG4 mAb named 1D09C3 discovered by screening the Morphosys Human Combinatorial Antibody Library, a diverse library of single-chain antibody variable fragments, was constructed by combining highly variable complementarity determining regions [11]. IgG4 antibodies differ functionally from other IgG subclasses by their poor ability to stimulate the complement system thus minimizing side effects due to Fc-portion-mediated effector functions [12, 13]. Studies showed its *in vivo* as well as *in vitro* tumouricidal activity and described that it acts

selectively on tumour-transformed and activated cells via a non-apoptotic mechanism [14, 15]. It has been shown that the HLA-DR protein status predicts survival in patients with B-cell lymphoma, but little is known whether it is possible to obtain this information *in vivo* by non-invasive imaging modalities. Moreover, patient variability in HLA-DR expression on both cancer cells and inflammatory cells is unclear. These facts highlight the opportunity to use a radiolabelled anti-HLA-DR monoclonal antibody probe for *in vivo* studying of the tumour selectivity of the mAb and patient variability in HLA-DR expression on tumour cells. Such a probe would also allow non-invasive evaluation of disease extent and severity in both B-cell lymphoma patients and patients affected by autoimmune diseases. This method could be useful for monitoring the efficacy of several therapies in autoimmune diseases and in cancer patients, particularly in those patients that are treated with unlabelled anti-HLA-DR antibodies.

Materials and Methods

Antibodies

Anti-HLA-DR mAb (1D09C3) was kindly provided by GPC Biotech, Germany. We tried both, direct and indirect, radiolabelling methods to label anti-HLA-DR mAb with ^{99m}Tc in order to obtain a high labelling efficiency (LE) and specific activity (SA) without any modification in biological specificity of the antibody.

Labelling of Anti-HLA-DR mAb with ^{99m}Tc by the Indirect Heterobifunctional Linker Method

Indirect labelling of anti-HLA-DR mAb was performed by conjugation of the mAb with the heterobifunctional linker succinimidyl-6-hydrazinonicotinate hydrochloride (SHNH). In brief, of SHNH (SoluLink, USA; 100 mM in DMF) was added dropwise at different molar ratios to a stirred solution of antibody (20 mM) in 100 mM of sodium phosphate/150 mM of NaCl buffer solution pH 7.6–8.0. The mixture was purified by G-25 Sephadex PD10 column chromatography (GE Healthcare, Sweden) using nitrogen-purged cold phosphate-buffered saline (pH 7.4) as eluent. To label the mAb–SHNH complex efficiently with ^{99m}Tc , to minimize the percentage of colloid formation and to optimize the influence of the amount of co-ligand on the LE, titrations of tricaine (100 mg/mL, Sigma-Aldrich Chemicals, UK) and SnCl_2 (2 mg/mL in 0.1 M HCl, Sigma-Aldrich Chemicals, UK) were performed with mAb–SHNH complex (100 μg) in 1 M sodium acetate (pH 5.5) using 520–555 MBq of freshly eluted $^{99m}\text{TcO}_4^-$ (100 μL) while keeping the volume of the reaction constant.

Labelling of Anti-HLA-DR mAb with ^{99m}Tc by the Direct 2-Mercaptoethanol Reduction Method

The anti-HLA-DR mAb was also tested for labelling with ^{99m}Tc using the 2-mercaptoethanol (2-ME) reduction method as described by Mather and Ellison [16]. Briefly, the disulfide bridges of the

mAb were reduced with 2-ME using various 2-ME to mAb molar ratios (1,000: 1; 2,000: 1 and 4,000: 1) in order to achieve the best activation of the antibody and consequently the highest LE. The activated antibody was then purified using G-25 Sephadex PD10 column chromatography and nitrogen-purged cold phosphate-buffered saline (pH 7.4) as eluent. Methylene diphosphonic acid (MDP) was used as a weak competitive ligand. The bone scan kit (Amersham, UK), containing 10 mg of methylene diphosphonic acid, 0.17 mg of SnCl_2 and 2 mg of ascorbic acid, was reconstituted with 1 mL of nitrogen-purged saline. Different amounts (from 1 to 7 μL) of the methylene diphosphonate solution were tested with 100 μg of activated antibodies, using 520–555 MBq of $^{99m}\text{TcO}_4^-$ freshly eluted from a $^{99}\text{Mo}/^{99m}\text{Tc}$ generator in order to achieve the highest LE.

Radiochemical Purity of ^{99m}Tc -anti-HLA-DR mAb

Quality controls were performed using instant thin layer chromatography-silica gel (ITLC-SG) strips (VWR International, Italy). The strips were analyzed with a radio scanner (Bioscan Inc., USA) to quantitate the percentage of activity bound to the antibody. When 0.9% NaCl was used as the solvent (with normal ITLC-SG strips), retention factors (Rf) were ^{99m}Tc -labelled antibody=0.0, ^{99m}Tc -tricine, ^{99m}Tc -MDP and free $^{99m}\text{TcO}_4^-$ =0.9–1.0. When NH_3 to H_2O to EtOH (1: 5: 2) was used as the solvent and albumin absorbed ITLC-SG strips, Rf values were ^{99m}Tc -colloids=0.0, ^{99m}Tc -labelled antibody=1.0, ^{99m}Tc -tricine and free $^{99m}\text{TcO}_4^-$ =0.9–1.0.

Stability and Structural Integrity of ^{99m}Tc -anti-HLA-DR mAb

The stability of the labelled antibody was measured in human serum and in normal saline at 37°C up to 20 h. To obtain human blood serum, 5 mL of blood was collected in a vial without any anticoagulant and was left at room temperature for 30 min in a vertical position for clotting. Then the vial was centrifuged at 20°C, 1,500 g for 10 min, and the serum was removed gently from cell pellet. One hundred microlitres of ^{99m}Tc -anti-HLA-DR mAb was added to 900 μL of fresh human blood serum or normal saline and was incubated at 37°C. The percentages of free ^{99m}Tc and antibody-bound radioactivity were measured at different time points (1, 3, 6 and 20 h) by ITLC-SG.

In addition, a cysteine challenge assay was performed to check the *in vitro* stability of the radiolabelled antibody. The ^{99m}Tc -anti-HLA-DR mAb was incubated at 37°C for 60 min at different cysteine to mAb molar ratios, which ranged from 500:1 at the highest cysteine concentration to zero in the absence of cysteine. At the end of the incubation time, each reaction mixture was evaluated by ITLC-SG as described above. All known chemical forms of ^{99m}Tc -cysteine have Rf values between 0.5 and 1.0, when normal saline is used as the eluent.

Possible modifications induced by the 2-ME reduction procedure on anti-HLA-DR mAb were tested by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in non-reducing conditions. Equal amounts of native mAb and radiolabelled mAb (60 μg) and a protein molecular weight marker (17–250 kD, Fermentas Life Sciences) were applied to separate slots of a 8% SDS polyacrylamide gel. The gel was run at 45 mA constant voltage and subsequently stained with Coomassie Brilliant Blue G250 (Sigma) for 30 min and destained with a methanol to H_2O to acetic acid (4.5: 4.5: 1) solution. Autoradiography of ^{99m}Tc -anti-

HLA-DR mAb was also performed to check the incorporation of radioactivity in the mAb after radiolabelling. For this purpose, radiolabelled mAb (11.1 MBq) was run in a separate lane of the SDS-PAGE. The gel was exposed to a photographic plate for 20 min in a cassette (Kodak Biomax) and the photographic plate was developed subsequently according to standard procedures.

Immunoreactive Fraction Assay

The assay for determination of the fraction of immunoreactive antibody by linear extrapolation to conditions representing infinite antigen excess has been adapted with slight modifications from the method described by Lindmo *et al.* [17].

The immunoreactive fraction assay was performed using a constant concentration of radiolabelled mAb and serial dilutions of DAUDI cells, a human lymphocytic Burkitt's lymphoma cell line. The cells were washed three times in phosphate-buffered saline (pH 7.4) and suspended in a cold PBS with 1% BSA. Radiolabelled mAb at a constant concentration of 50 ng/mL in PBS with 1% BSA was added to different amounts of cells (concentration ranging from 2.6×10^6 to 0.08×10^6 cells/mL). The cells were incubated for 2 h at 4°C and then washed twice with 500 μL of cold PBS with 1% BSA before counting cell-associated radioactivity in a single-well gamma counter (Gammatom s.p.a., Italy). The data were plotted as a double inverse plot of the applied radiolabelled antibody over the specific binding, as a function of the inverse cell concentration. In this plot, the origin of the abscissa represents infinite cell concentration, *i.e.*, conditions of infinite antigen excess. All experiments were performed in duplicate.

In Vitro Competitive Binding Assay

HLA-DR positive DAUDI cells were maintained in a RPMI 1640 culture medium (Sigma-Aldrich Chemical, UK) supplemented with 10% foetal calf serum, 2 mM of glutamine, 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ of streptomycin. Cells were cultured at $4\text{--}6 \times 10^5$ cells/mL at 37°C in a 5% CO_2 incubator for 1 week.

A binding assay was performed on DAUDI cells in order to test the specific binding of labelled anti-HLA-DR mAb. Briefly, 10^6 DAUDI cells were incubated in triplicate with different concentrations (from 50 nM to 0.05 nM) of radiolabelled anti-HLA-DR mAb at 4°C for 90 min. Specific binding was determined by performing the assay in the presence and absence of a 100-fold molar excess of unlabelled anti-HLA-DR mAb (500 nM). At the end of the incubation time, cells were harvested by centrifugation (5,000 g for 3 min). Each supernatant was collected in separate vials, then cells and supernatants were counted separately for radioactivity in a single-well gamma counter. The curve of specific binding was generated as the difference between total binding and non-specific binding. A Scatchard analysis was performed using GraphPad Prism version 5.00 software (GraphPad Software, Inc.) to determine the dissociation constant (K_d).

Retention Assay (LigandTracer™ Assay)

Real-time measurements of cellular uptake and retention were performed using a rotating radioimmuno assay (RIA) in a LigandTracer™ instrument (Ridgeview Instruments AB, Uppsala,

Sweden) [18–20]. HLA-DR positive DAUDI cells were cultured in complete RPMI medium, as described above. Approximately 10×10^6 cells were spun down twice in PBS and were resuspended in approximately 1 mL of PBS. Fibronectin-coated circular plastic Petri plates (BD BioCoat™, BD Biosciences) were rinsed twice with Milli Q water and were left in a tilted position. To activate part of the dish, 1 mL of 0.20 M N-ethyl-N'-(dimethylaminopropyl) carbodiimide (Sigma-Aldrich Chemicals, UK) and 0.05 M N-hydroxysuccinimide (Sigma-Aldrich Chemicals, UK) was added to the lower part of the dish and incubated at room temperature for 30 min. Then, the dish was rinsed and the cell suspension was carefully dispensed onto the activated section of the dish. The dish was put in a CO₂ incubator for 1 h, after which 4 mL of complete RPMI medium was added to the dish and incubated for another hour. The dish was then placed in the LigandTracer apparatus and rotated continuously for 1 h to allow release of weakly attached cells. After one gentle wash, the Petri plate was ready for measurement. Radiolabelled mAb (0.7 nM) in PBS pH 7.4 supplemented with 7% cell culture medium devoid of FCS was added. When the radiolabelled antibody binds to the cells, a detector placed over the elevated part of the dish registers the cell-bound activity each time the cells pass through the detector. By following the (peak) activity over time, a real-time binding curve was obtained using a LigandTracer software 1.0 (Ridgeview Instruments AB, Uppsala, Sweden). Retention studies were performed in two steps. First, radiolabelled anti-HLA-DR mAb was added to the Petri plate followed by incubation for 2–4 h. Second, the assay buffer containing radiolabelled mAb was replaced with an assay buffer without radiolabelled mAb, after which LigandTracer was run several hours to follow the retention of the bound material.

We also repeated the experiment with the control cell line TPC1 to compare the uptake and retention properties of the radiolabelled anti-HLA-DR mAb.

Biodistribution of ^{99m}Tc -Labelled Anti-HLA-DR mAb in Balb/c Mice

The biodistribution of ^{99m}Tc -labelled anti-HLA-DR mAb was studied in nine female Balb/c nude mice of 10–12 weeks (Charles River Laboratories, USA). The mice were injected i.v. with 10.5–11.5 MBq (approximately 2 μg) of ^{99m}Tc -labelled anti-HLA-DR mAb. Animals were sacrificed by cervical dislocation after 1 h ($n=3$), 3 h ($n=3$) or 24 h ($n=3$) and immediately perfused with normal saline by heart puncture and cutting of the abdominal vein to wash out all blood from organs and tissues. Major organs (heart, lungs, liver, spleen, kidney, stomach, small bowel, large bowel, muscle and bone) were excised, weighted and counted in a single-well gamma counter. To correct for radioactive decay and permit calculation of the uptake of the radiopharmaceuticals in each organ as fraction of the injected dose, an aliquot of the injected dose was counted simultaneously. Results were expressed as percentage of the injected dose per gram of tissue \pm standard deviation (SD). All animal studies were performed in compliance with the local ethical committee and according to national regulations.

High Resolution Gamma Camera

The high-resolution gamma camera (HRC) (Li-tech S.r.l., Italy) is composed of a 30-mm long collimator consisting of 200- μ thick

tungsten blades which form $3 \times 3 \text{ mm}^2$ large square holes, in which $3 \times 3 \times 5 \text{ mm}^3$ CsI(Tl) position sensitive crystals (Spectra Physics-Hilger Analytical, UK) are inserted. Thus, the crystal-free length of the holes is 25 mm. The crystal collimator structure is coupled to a Hamamatsu H8500 (Hamamatsu, Japan) position sensitive photo multiplier tube (PSPMT), charge readout electronics and a data acquisition system [21, 22]. The system allows performing real-time acquisitions with a refresh time of 0.5 s. The HRC energy resolution is about 20% at 140 keV (^{99m}Tc). The sensitivity is 210 cps/MBq and the uniformity is $\pm 5\%$ while it provides a 2.2-mm intrinsic resolution suitable for our *in vivo* imaging experiments in small animals.

In Vivo Targeting Experiment on DAUDI Cells Xenografted Balb/c Mice

To evaluate the ability of the radiolabelled anti-HLA-DR mAb to specifically bind to DAUDI cells *in vivo*, we performed a targeting experiment in nine nude Balb/c nu/nu mice. The mice were divided in three groups and subcutaneously implanted with an increasing number of DAUDI cells in the left shoulder, *i.e.*, 10×10^6 , 20×10^6 or 30×10^6 cells, in Matrigel™ (BD Biosciences, USA). In the right shoulder, the mice were implanted with same number of a control HLA-DR-negative cell line. For this control cell line, we used TPC1 cells, derived from human thyroid papillary cancer [23]. After 2 h, the mice were injected in the tail vein with 10.5–11.5 MBq (approximately 2 μg) of radiolabelled anti-HLA-DR mAb and high-resolution gamma-camera images were acquired after 1, 3, 6 and 24 h. Regions of interest were drawn over the left (target) and right shoulder (background) and target to background (T/B) ratios were calculated.

In Vivo Blocking of ^{99m}Tc -anti-HLA-DR mAb Binding with Cold Anti-HLA-DR mAb

Eight nude Balb/c nu/nu mice underwent a competition study to assess to what extent the uptake of ^{99m}Tc -labelled anti-HLA-DR mAb to HLA-DR positive cells could be blocked by an excess of unlabelled antibody. To this aim, the mice were subcutaneously implanted with 30×10^6 DAUDI cells in Matrigel™ in the right shoulder. As control, the mice were implanted with the same volume of Matrigel™ without cells in the left shoulder. Four mice were pre-injected i.v. with a 100-fold excess of unlabelled anti-HLA-DR mAb immediately before the injection of a tracer dose of 10.5–11.5 MBq (approximately 2 μg) of ^{99m}Tc -anti-HLA-DR mAb in the tail vein. Another group of four mice received only ^{99m}Tc -anti-HLA-DR mAb. High-resolution gamma-camera images were acquired after 1, 3, 6 and 24 h. Regions of interest were drawn over the right (target) and left shoulder (background) and T/B ratios were calculated.

Results

Labelling of Anti-HLA-DR mAb with ^{99m}Tc by the Indirect Heterobifunctional Linker Method

Highest LE efficiency could be obtained when the mAb was conjugated with SHNH at a 1:20 ratio. Optimization of the coupling of the mAb–SHNH conjugate (100 μg) with ^{99m}Tc

showed that the use of 10 μL of tricine (100 mg/mL) and 10 μL of SnCl_2 (2 mg/mL) gave the highest LE and the lowest amount of colloids. After 60 min of incubation under these optimized conditions, we obtained an LE of only $60 \pm 5\%$ with a high percentage of colloids ($\geq 25\%$). Moreover, the ^{99m}Tc -SHNH-anti-HLA-DR mAb complex also had a low SA (3,000 MBq/mg). Due to the low LE and high colloid formation of the anti-HLA-DR mAb, we did not proceed with the indirect labelling method as the method of choice.

Labelling of Anti-HLA-DR mAb with ^{99m}Tc by the Direct 2-Mercaptoethanol Reduction Method

With the direct labelling method, the best results were obtained when sulphide bridges of the antibody were reduced using a 2,000-fold excess of 2-ME. We obtained the highest LE with negligible amount of colloids when the activated mAb was labelled with only 3 μL of methylene diphosphonic acid solution (from the bone scan kit). Using this radiolabelling method, ^{99m}Tc -labelled anti-HLA-DR mAb could be obtained with a very high LE ($>98\%$), negligible percentage of colloids ($<2\%$) and high SA (5,550 MBq/mg). Thus, a post-labelling purification step could be avoided. Indeed, we used this labelling method as a method of choice for *in vitro* and *in vivo* studies.

Stability and Structural Integrity of ^{99m}Tc -anti-HLA-DR mAb

The ^{99m}Tc -anti-HLA-DR mAb was stable when incubated in human serum or in normal saline at 37°C for at least 6 h, as shown in Fig. 1a. After 20 h, still more than 60% of the radioactivity was bound to the antibody in both media. The cysteine challenge assay also demonstrated a very high stability when the labelled antibody was exposed to up to a 50-fold excess of cysteine, whereas exposure to a 500-fold excess of cysteine resulted in only a 30% release of mAb-associated radioactivity (Fig. 1b).

SDS-PAGE analysis of the radiolabelled anti-HLA-DR mAb showed no significant differences with the native anti-HLA-DR mAb (Fig. 1c). Native and labelled mAb showed a band of 166 kDa (*i.e.*, molecular weight of complete mAb); however, a band of approximately 66 kDa was also present in both lanes and could be ascribed to one half of the complete mAb (*i.e.*, heavy-light chain). No other band was seen in radiolabelled mAb lane. Autoradiographic analysis of ^{99m}Tc -anti-HLA-DR mAb, however, showed that the radioactivity (^{99m}Tc) was associated with the band of approximately 166 kDa, which corresponds to the molecular weight of native anti-HLA-DR mAb (Fig. 1c).

Immunoreactive Fraction Assay

The data demonstrate a very closely linear relationship of 'total applied/specific binding' as a function of the inverse cell concentration. Fitting of a straight line to the data by means of

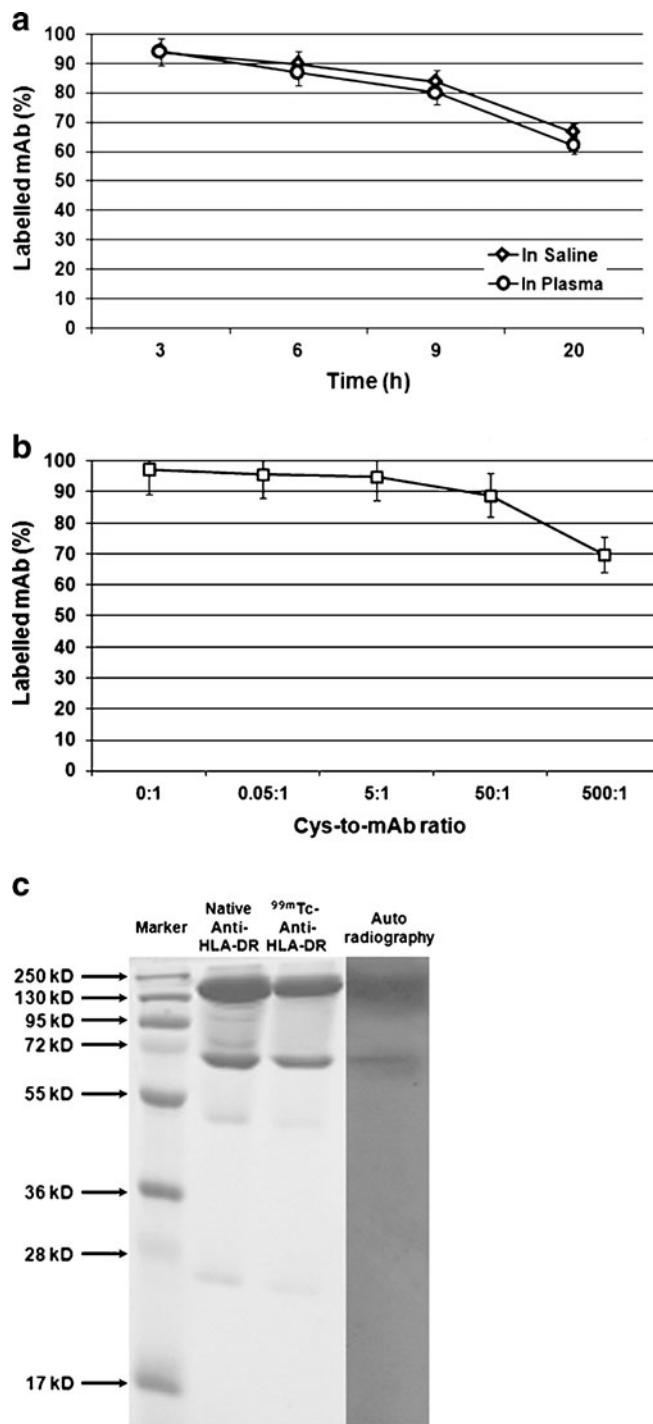


Fig. 1. **a** Stability of ^{99m}Tc -anti-HLA-DR mAb in saline and in plasma assessed by ITLC-SG at different time points. **b** Cysteine challenge assay of ^{99m}Tc -anti-HLA-DR mAb assessed by ITLC-SG at an increasing ratio between cysteine and mAb. **c** SDS-PAGE of native and radiolabelled anti-HLA-DR mAb and autoradiographic analysis of ^{99m}Tc -anti-HLA-DR mAb.

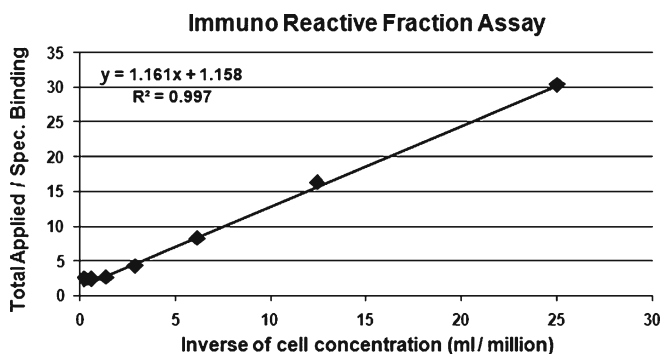


Fig. 2. A double inverse plot of the immunoreactivity fraction assay.

linear regression analysis allows an easy and precise determination of the intercept value at the ordinate. This value equals 1/immunoreactive fraction; thus in this case, the percent immunoreactive fraction equals 86%, as shown in the Fig. 2.

In Vitro Competitive Binding Assay

The saturation binding curve was plotted as specifically bound radioactivity against the molar concentration of radiolabelled mAb. This curve showed a plateau (Fig. 3), indicating that the antibody specifically binds to a target that can be saturated. A 100-fold molar excess of unlabelled antibody saturates the receptors present on the cells and consequently prevented the specific binding of the radiolabelled 1D09C3. This shows that 1D09C3 retained its specific binding to HLA-DR receptors expressed on DAUDI cells, even after the radiolabelling with technetium-99m. The K_d for ^{99m}Tc -1D09C3 was 6.7 nM, which is comparable to the K_d of native 1D09C3, *i.e.*, 2.9 nM.

Retention Assay (LigandTracer™ Assay)

Approximately 50% of the total suspended DAUDI cells, *i.e.*, 5×10^6 cells, were covalently adhered to the fibronectin-coated plastic Petri plate. The adhesion was persistent for at least 25 h. The curve for cellular uptake and retention of ^{99m}Tc -anti-HLA-DR mAb is shown in Fig. 4. When the radiolabelled mAb was removed from the medium, about 34% of the radioactivity is released from the cells with a half-life of 0.53 h, whereas approximately two thirds of the radioactivity remains attached to the target cells even after 25 h.

When we repeated the experiment with the control cell line TPC1, we did not find any uptake of ^{99m}Tc -labelled anti-HLA-DR mAb during the 10-h time duration (Fig. 4, insert).

Biodistribution of ^{99m}Tc -Labelled Anti-HLA-DR mAb in Balb/c Mice

The biodistribution study of ^{99m}Tc -labelled anti-HLA-DR mAb performed in nine Balb/c nude mice revealed a high and persistent uptake in spleen, which could be because of binding of ^{99m}Tc -labelled anti-HLA-DR mAb to splenic B lymphocytes.

A significant uptake of radiolabelled mAb was also observed in the liver and kidney, suggesting a mixed hepatic/renal clearance mechanism of the radiolabelled mAb. The ^{99m}Tc -labelled anti-HLA-DR mAb also shows substantial uptake in the bones and lungs. Moreover, negligible accumulation was observed in other organs and tissues over a period of 24 h (Fig. 5).

In Vivo Targeting Experiment in DAUDI Cells Xenografted Balb/c Mice

HLA-DR positive cells were detected by high-resolution gamma-camera imaging in Balb/c mice implanted with an increasing number of DAUDI cells (Fig. 6a). Quantification of ^{99m}Tc -anti-HLA-DR mAb uptake showed a significant increase in T/B ratio over time ($p < 0.05$) (Table 1).

We also found a statistically significant increase in T/B ratio with an increasing number of cells xenografted into shoulders of mice, 10×10^6 vs. 20×10^6 injected cells at 1 h ($p = 0.03$) and at 3 h ($p = 0.01$); 20×10^6 vs. 30×10^6 injected cells at 3 h ($p = 0.003$) and at 6 h ($p = 0.01$); 10×10^6 vs. 30×10^6 injected cells at 1 h ($p = 0.008$), at 3 h ($p = 0.001$), at 6 h ($p = 0.01$) and at 24 h ($p = 0.02$) (Fig. 6b).

In Vivo Blocking of ^{99m}Tc -anti-HLA-DR mAb Binding with Cold Anti-HLA-DR Mab

As shown in Fig. 7a, the uptake of radiolabelled anti-HLA-DR mAb was significantly reduced after pre-injection of a 100-fold excess of unlabelled anti-HLA-DR mAb. In mice pre-treated with cold mAb, the radioactivity uptake in the HLA-DR expressing xenograft was 34% ($p = 0.34$), 90% ($p = 0.01$), 94% ($p = 0.02$) and 96% ($p = 0.03$) lower than in control animals at 1, 3, 6 and 24 h after injection of the radiolabelled antibody, respectively (Fig. 7b).

Discussion

Several studies demonstrated the therapeutic potential of various anti-HLA-DR mAb, such as Lym-1 [24, 25] and

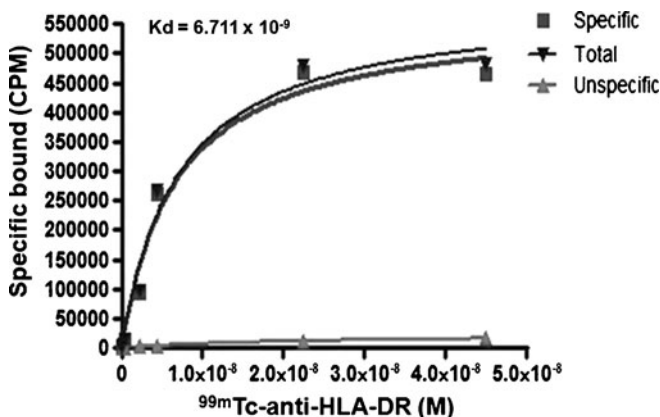


Fig. 3. Saturation binding curve of ^{99m}Tc labelled anti-HLA-DR mAb to DAUDI cells. Curve fitting was performed using GraphPad software.

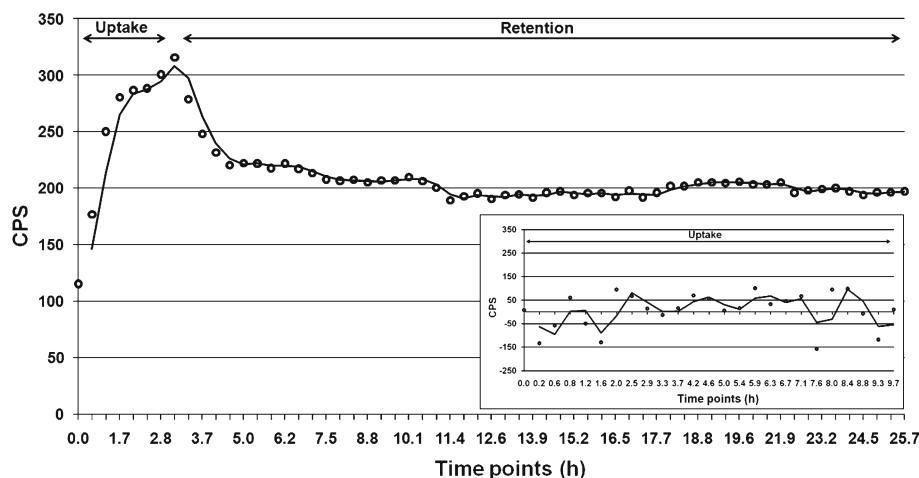


Fig. 4. Binding trace graph (decay-corrected) for uptake and retention of ^{99m}Tc -anti-HLA-DR mAb bound to DAUDI cells. Binding studies were performed in two steps: addition of radiolabelled anti-HLA-DR mAb (*Uptake*), followed by replacing radiolabelled mAb with assay buffer without radiopharmaceutical (*Retention*). Results of the same experiment with TPC1 control cells (*inset*) did not show any peak for specific uptake of ^{99m}Tc -labelled anti-HLA-DR mAb in the uptake phase.

1D10 [26]; however, previous mAbs were differing in many aspects from the anti-HLA-DR mAb 1D09C3 that we used here. The former two antibodies recognize what seem to be post-translational modifications on HLA-DR molecules that occur preferentially in B-cell-derived tumours, but also to some extent in normal B cells [24, 27]. Moreover, Lym-1 is a murine antibody with substantial immunogenicity for humans, and 1D10 is a humanized antibody. The anti-HLA-DR antibody that we have selected is a fully human antibody with selectivity for activated and/or tumour-transformed cells. This antibody demonstrates a substantially different binding profile and mechanism of action as compared to previous anti-HLA-DR antibodies. If radio-labelled, this anti-HLA-DR mAb may provide a valuable novel diagnostic and prognostic tool to image the infiltration

of HLA-DR positive cells in autoimmune and lymphoma/leukemia patients.

We therefore attempt to label anti-HLA-DR mAb with technetium-99m. We tried both direct and indirect labelling procedures in order to develop a reliable and simple method that allows the formation of a stable ^{99m}Tc -conjugate with no modification in its biological activity. The labelling procedure based on 2-ME activation of the mAb gave the best results, allowing us to achieve a very high LE (>98%) and a high SA (5,550 MBq/mg) with a negligible presence of colloids and therefore no need for post-labelling purification. The labelled product was stable in normal saline and serum up to 6 h at 37°C, whereas degradation at 20 h was still less than 40%, which is sufficient for imaging purposes in patients. The K_d value of the labelled mAb is in the same

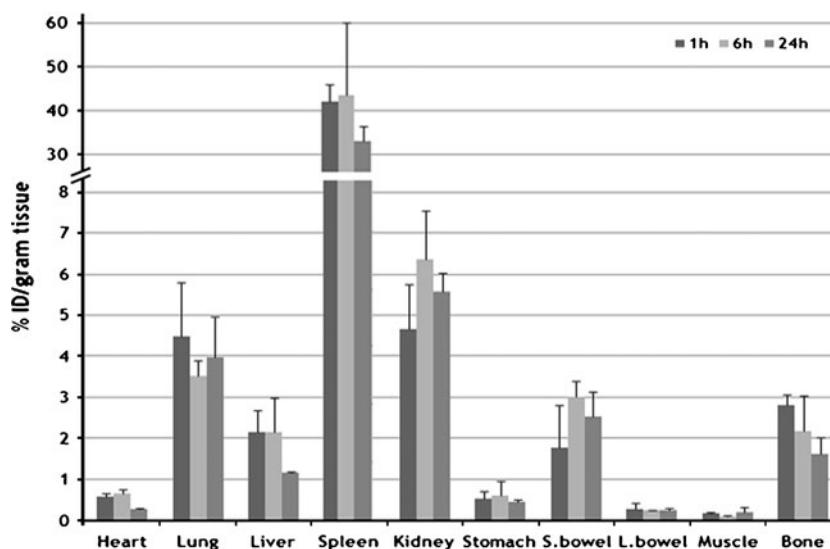


Fig. 5. Biodistribution of ^{99m}Tc -labelled anti-DR mAb in Balb/c nude mice at 1, 6 and 24 h post injection.

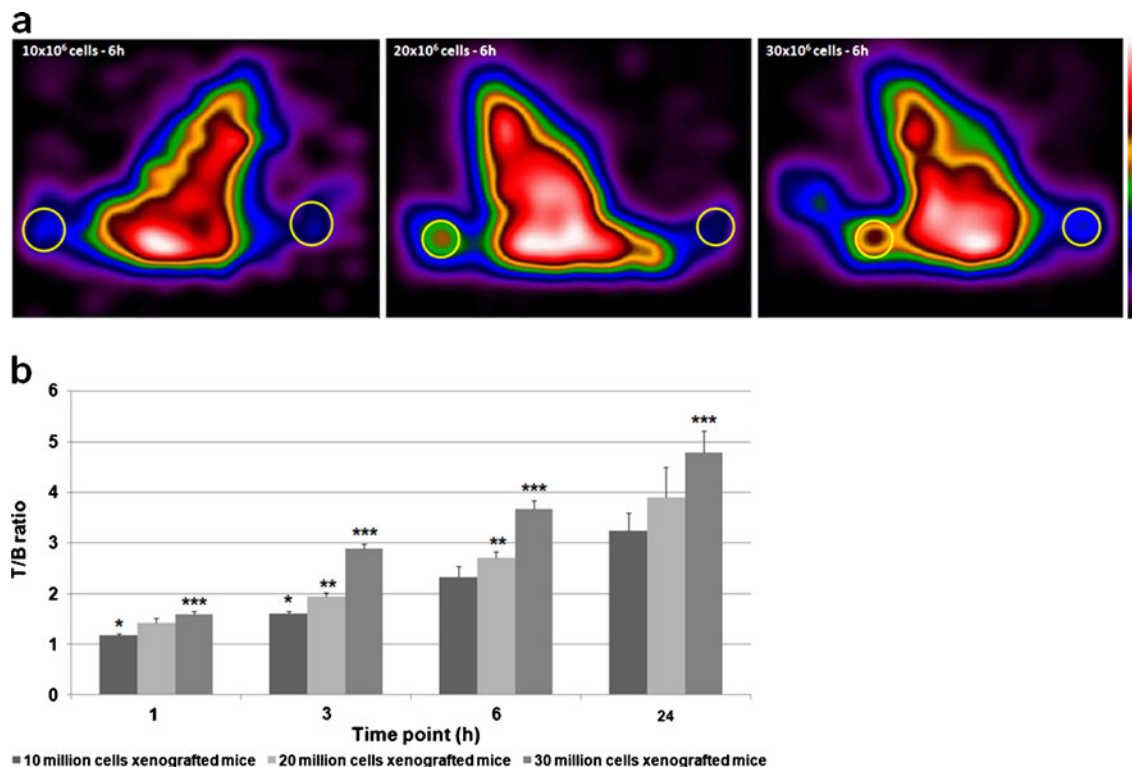


Fig. 6. **a** Dorsal scintigraphic images obtained with a high-resolution camera at 6 h in mice xenografted with 10×10^6 , 20×10^6 or 30×10^6 DAUDI tumour cells in the left shoulder and same number of TPC1 control cells in the right shoulder. Mice were injected with 10.5–11.5 MBq of ^{99m}Tc -anti-HLA-DR mAb. Higher uptake in DAUDI cell xenografts is clearly visible as compared to DR negative cell xenografts. Regions of interests for T/B ratio calculation are also shown. On the right, a radioactivity scale has been shown from high to low (*top to bottom*). **b** Mean \pm SD of T/B ratios at different time points calculated for three groups of mice xenografted with increasing number of DAUDI cells. Statistically significant differences ($p < 0.05$) in T/B ratio between sequential amounts of cells, 10×10^6 and 20×10^6 (*), 20×10^6 and 30×10^6 (**), 10×10^6 and 30×10^6 (***), are indicated with asterisk.

nanomolar range as the K_d of native anti-HLA-DR mAb, which indicates that the biological activity of mAb was not significantly deteriorated by the labelling procedure. Our data show that *in vitro*, the majority of radiolabelled mAb remains irreversibly bound to the target cells, which is consistent with the gradual increase in tracer uptake over time that was observed *in vivo*.

In the biodistribution experiments, the spleen showed high and persistent uptake, which was not unexpected because MHC-II DR antigens that are constitutively expressed on splenic B lymphocytes; however, we also found high bone uptake, which could be due to the fact that bone marrow lymphocytes are primarily B cells [28].

Moreover, uptake in the lung was also relatively high as a result of the HLA class II antigens that are constitutively expressed in lung tissue [29]. We did not collect blood samples in mice and we did not calculate the blood half-life, but it has been reported that 1D09C3 has a fast (<3 h) blood clearance in mice [30]. This characteristic of ^{99m}Tc -anti-HLA-DR mAb makes it distinct from other radiolabelled mAbs since complete mAbs have predominantly slow blood clearance from the body [31]. Results of the *in vivo* targeting experiments in mice showed that accumulation of radiolabelled anti-HLA-DR mAb in DAUDI cells was proportional to the number of implanted cells. We also observed an increase in T/B ratio over time, indicating that imaging at

Table 1. Increase in T/B ratio over time

No. of injected cells	T/B ratio \pm SD			
	1 h	3 h	6 h	24 h
10×10^6	1.17 \pm 0.04	1.61 \pm 0.05 ($p=0.005$)	2.33 \pm 0.22 ($p=0.02$)	3.25 \pm 0.35 ($p=0.04$)
20×10^6	1.42 \pm 0.09	1.95 \pm 0.07 ($p=0.01$)	2.72 \pm 0.12 ($p=0.008$)	3.92 \pm 0.59 ($p=0.05$)
30×10^6	1.59 \pm 0.06	2.90 \pm 0.08 ($p=0.001$)	3.68 \pm 0.17 ($p=0.01$)	4.80 \pm 0.42 ($p=0.03$)

The p value in the brackets indicates a significant difference in T/B ratio, as compared to the previous time point

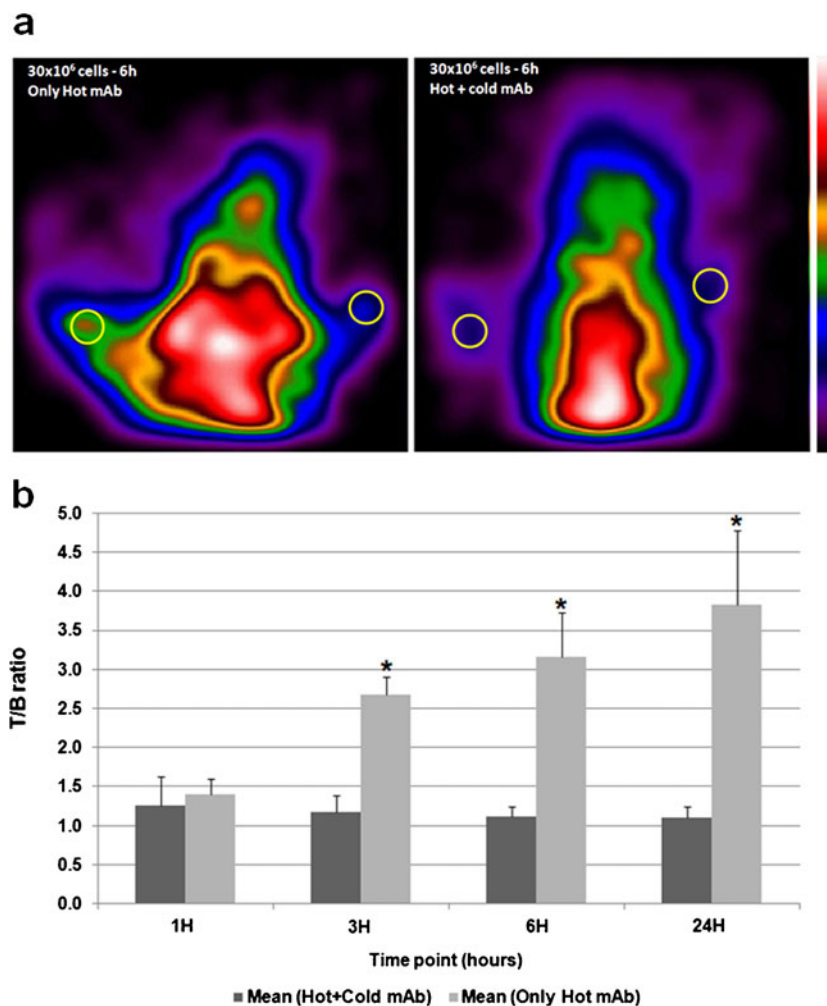


Fig. 7. a Dorsal scintigraphic images at 6 h of mice xenografted with 30×10^6 DAUDI tumour cells mixed with Matrigel in the left shoulder and with Matrigel only in the right shoulder. Mice were injected with 10.5–11.5 MBq of ^{99m}Tc -anti-HLA-DR mAb, after administration of a 100-fold excess of unlabelled anti-HLA-DR mAb. Regions of interests for T/B calculation are shown. The left image shows a mouse injected with only labelled mAb (control) and the right image shows a mouse pre-injected with a 100-fold excess of unlabelled anti-DR mAb immediately before the injection of ^{99m}Tc -anti-HLA-DR mAb, in the tail vein. The right image shows almost complete blocking of radiolabelled antibody uptake after pre-injection of cold antibody. On the right radioactivity scale has been shown from high to low (*top to bottom*). **b** Mean \pm SD of T/B ratios calculated for the two groups of mice xenografted with 30×10^6 DAUDI cells and injected either with a 100-fold excess of unlabelled anti-HLA-DR mAb immediately before a tracer dose of 10.5–11.5 MBq of ^{99m}Tc -anti-DR mAb or only with a tracer dose of ^{99m}Tc -anti-HLA-DR mAb (control). (* $P < 0.03$).

later time points may be preferred and thus, in the future, isotopes with a longer half-life than technetium-99m may be applied to radiolabel this mAb for imaging of lymphoma patients. Interestingly, *in vivo* blocking of ^{99m}Tc -anti-HLA-DR mAb with a 100-fold molar excess of cold anti-HLA-DR mAb demonstrated up to 96% specific binding of the labelled mAb. These experiments show that the newly radiolabelled mAb is highly specific for HLA-DR *in vivo* as well.

Our results are important for possible clinical use of ^{99m}Tc -anti-HLA-DR mAb for molecular imaging of major histocompatibility complex class II protein HLA-DR expression for diagnostic and prognostic purposes presumably without any side effects because of its high specific activity.

Indeed, only 67 μg (*i.e.*, 370 MBq) of radiolabelled anti-HLA-DR mAb should be sufficient for a diagnostic scan in humans as compared to the very high dose given for therapy (up to 10 mg/kg/day in an ongoing clinical trial).

Future studies should be focused on the further evaluation of ^{99m}Tc -anti-HLA-DR mAb as a prognostic/diagnostic tool for imaging HLA-DR positive cell infiltration in patients affected by B-cell lymphoma/leukemia and autoimmune inflammatory diseases.

Conclusions

The anti-HLA-DR mAb, 1D09C3, can be efficiently labelled with technetium-99m for imaging HLA-DR positive cells.

The labelling method is simple, rapid, reliable and effective, yielding high specific activity without loss of immune reactivity. In particular, we were able to demonstrate that ^{99m}Tc-anti-HLA-DR mAb targets HLA-DR positive cells *in vivo* in Balb/c mice model, and therefore warrants further evaluation as a prognostic/diagnostic tool in lymphoma patients or in patients with autoimmune diseases.

Conflict of Interest Disclosure. The authors declare that they have no conflict of interest.

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