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

Gaurav Malviya,1,2 E. F. J. de Vries,1 Rudi A. Dierckx,1 Alberto Signore1,2

1Department of Nuclear Medicine and Molecular Imaging, University Medical Centre Groningen, University of Groningen, Groningen, The Netherlands
2Nuclear Medicine Unit, Second Faculty of Medicine and Surgery, “Sapienza” University of Rome, Rome, Italy

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)
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 tricine (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 $^{99m}$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 SnCl₂ 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 μCi of 99mTc-tricine and free 99mTcO₄⁻ and was incubated at 37°C. The percentages of free 99mTc and antibody-bound radioactivity were measured at different time points (1, 3, 6 and 20 h) by ITLC-SG.

Radiochemical Purity of ⁹⁹ᵐTc-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 99mTc-labelled antibody = 0.0, 99mTc-tricine, 99mTc-MDP and free 99mTcO₄⁻ = 0.9–1.0. When NH₃·H₂O to EtOH (1: 5: 2) was used as the solvent and albumin absorbed ITLC-SG strips, Rf values were 99mTc-colloids = 0.0, 99mTc-labelled antibody = 1.0, 99mTc-tricine and free 99mTcO₄⁻ = 0.9–1.0.

Stability and Structural Integrity of ⁹⁹ᵐTc-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 the cell pellet. One hundred microlitres of ⁹⁹ᵐTc-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 ⁹⁹ᵐTc 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 ⁹⁹ᵐTc-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 ⁹⁹ᵐTc-cysteine have Rf values between 0.5 and 1.0, when normal saline is used as the eluent. 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 (Gammatorr 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 µg/mL of streptomycin. Cells were cultured 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 (K₀).

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,
Swedish) [18–20]. HLA-DR positive DAUDI cells were cultured in complete RPMI medium, as described above. Approximately 10×10^6 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. Radio-labelled 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 99mTc-Labelled Anti-HLA-DR mAb in Balb/c Mice**

The biodistribution of 99mTc-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 99mTc-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, 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±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 3×3 mm² large square holes, in which 3×3×5 mm³ 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 (99mTc). The sensitivity is 210 cps/MBq and the uniformity is ±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 99mTc-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 99mTc-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 99mTc-anti-HLA-DR mAb in the tail vein. Another group of four mice received only 99mTc-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 99mTc 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 99mTc
showed that the use of 10 μL of tricine (100 mg/mL) and 10 μL of SnCl₂ (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±5% with a high percentage of colloids (≥25%). Moreover, the ⁹⁹ᵐTc-SHNH₂ 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 ⁹⁹ᵐTc 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, ⁹⁹ᵐTc-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 ⁹⁹ᵐTc-anti-HLA-DR mAb**

The ⁹⁹ᵐTc-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 ⁹⁹ᵐTc-anti-HLA-DR mAb, however, showed that the radioactivity (⁹⁹ᵐTc) was associated with the band of approximately 166 kD, 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
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 \(^{99m}\text{Tc}-1D09C3 \) 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., \( 5 \times 10^6 \) 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 \(^{99m}\text{Tc}-\text{anti-HLA-DR} \) 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 \(^{99m}\text{Tc}-\text{anti-HLA-DR} \) mAb during the 10-h time duration (Fig. 4, insert).

Biodistribution of \(^{99m}\text{Tc}-\text{Labelled Anti-HLA-DR mAb in Balb/c Mice}
The biodistribution study of \(^{99m}\text{Tc}-\text{labelled anti-HLA-DR mAb} \) performed in nine Balb/c nude mice revealed a high and persistent uptake in spleen, which could be because of binding of \(^{99m}\text{Tc}-\text{labelled anti-HLA-DR mAb} \) 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 \(^{99m}\text{Tc}-\text{labelled anti-HLA-DR} \) 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 \(^{99m}\text{Tc}-\text{anti-HLA-DR} \) 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, \( 10 \times 10^6 \text{vs.} \ 20 \times 10^6 \) injected cells at 1 h (\( p=0.03 \)) and at 3 h (\( p=0.01 \)); \( 20 \times 10^6 \text{vs.} \ 30 \times 10^6 \) injected cells at 3 h (\( p=0.003 \)) and at 6 h (\( p=0.01 \)); \( 10 \times 10^6 \text{vs.} \ 30 \times 10^6 \) 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}\text{Tc}-\text{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

\[ y = 1.561x + 1.158 \]
\[ R^2 = 0.997 \]

Fig. 3. Saturation binding curve of \(^{99m}\text{Tc} \) labelled anti-HLA-DR mAb to DAUDI cells. Curve fitting was performed using GraphPad software.
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 99mTc-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. 4. Binding trace graph (decay-corrected) for uptake and retention of 99mTc-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 99mTc-labelled anti-HLA-DR mAb in the uptake phase.](image)

![Fig. 5. Biodistribution of 99mTc-labelled anti-DR mAb in Balb/c nude mice at 1, 6 and 24 h post injection.](image)
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 radio-labelled 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

![Fig. 6. a Dorsal scintigraphic images obtained with a high-resolution camera at 6 h in mice xenografted with $10 \times 10^6$, $20 \times 10^6$ or $30 \times 10^6$ 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 ± 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, $10 \times 10^6$ and $20 \times 10^6$ (*), $20 \times 10^6$ and $30 \times 10^6$ (**), $10 \times 10^6$ and $30 \times 10^6$ (**), are indicated with asterisk.

### Table 1. Increase in T/B ratio over time

| No. of injected cells | T/B ratio±SD |
|-----------------------|--------------|
|                       | 1 h          | 3 h          | 6 h          | 24 h         |
| $10 \times 10^6$      | 1.17±0.04    | 1.61±0.05 ($p=0.005$) | 2.33±0.22 ($p=0.02$) | 3.25±0.35 ($p=0.04$) |
| $20 \times 10^6$      | 1.42±0.09    | 1.95±0.07 ($p=0.01$)  | 2.72±0.12 ($p=0.008$) | 3.92±0.59 ($p=0.05$) |
| $30 \times 10^6$      | 1.59±0.06    | 2.90±0.08 ($p=0.001$) | 3.68±0.17 ($p=0.01$)  | 4.80±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.
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 99mTc-anti-HLA-DR mAb with a 100-fold excess of unlabelled 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 99mTc-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 99mTc-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 99mTc-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.

Open Access. This article is distributed under the terms of the Creative Commons Attribution Noncommercial License which permits any noncommercial use, distribution, and reproduction in any medium, provided the original author(s) and source are credited.

References
1. Concha A, Esteban F, Cabrera T et al (1991) Tumor aggressiveness and MHC class I and II antigens in laryngeal and breast cancer. Semin Cancer Biol 2:47–54
2. Arimura Y, Koda T, Kishi M, Kakimura M (1996) Mouse HLA-DPA homologue H2-Pu: a pseudogene that maps between H2-Pb and H2-Ou. Immunogenetics 43:152–155
3. Sønderstrup G, McDevitt HO (2001) DR, DQ, and you: MHC alleles and autoimmunity. J Clin Invest 107:795–796
4. Cosgrove D, Bodmer H, Bogue M, Benoist C, Mathis D (1992) Evaluation of the functional equivalence of major histocompatibility complex class II A and E complexes. J Exp Med 176:629–634
5. Delovitch TL, Falk JA (1979) Evidence for structural homology between murine and human Ia antigens. Immunogenetics 8:405–418
6. Carlo-Stella C, Di Nicola M, Turco MC et al (2006) The anti-human leukocyte antigen-DR monoclonal antibody 1D09C3 activates the mitochondrial cell death pathway and exerts a potent antitumor activity in lymphoma-bearing nonobese diabetic/severe combined immunodeficient mice. Cancer Res 66:1799–1808
7. Isobe M, Narula J, Southern JF, Strauss HW, Khaw BA, Haber E (1992) Imaging the rejecting heart. In vivo detection of major histocompatibility complex class II antigen induction. Circulation 85:738–746
8. Isobe M (1993) Scintigraphic imaging of MHC class II antigen induction in mouse kidney allografts: a new approach to non-invasive detection of early rejection. Transpl Int 6:263–269
9. Rimba LM, Farinha P, Fuchs DA, Masoudi H, Connors JM, Gascoyne RD (2007) HLA-DR protein status predicts survival in patients with diffuse large B-cell lymphoma treated on the MACOP-B chemotherapy regimen. Leuk Lymphoma 48:542–546
10. Chikamatsu K, Eura M, Matsuoka H, Murakami H, Fukiage T, Ishikawa T (1994) The role of major histocompatibility complex expression on head and neck cancer cells in the induction of autologous cytotoxic T lymphocytes. Cancer Immunol Immunother 38:358–364
11. Zola H, Beare A (2008) 1D09C3, an mAb specific for MHC-II. Curr Opin Mol Ther 10:68–74
12. Billing R, Chatterjee S (1983) Prolongation of skin allograft survival in monkeys treated with anti-la and anti-blast/monocyte monoclonal antibodies. Transplant Proc 15:649–650
13. Jonker M, Nosuij FJM, den Butter G, van Lambalgen R, Fuccello AJ (1988) Side effects and immunogenicity of murine lymphocyte-specific monoclonal antibodies in subhuman primates. Transplantation 45:677–682
14. Nagy ZA, Hubner B, Löhning C et al (2002) Fully human, HLA-DR-specific monoclonal antibodies efficiently induce programmed death of malignant lymphoid cells. Nat Med 8:801–807
15. Carlo-Stella C, Guidetti A, Di Nicola M et al (2007) IFN-gamma enhances the antymyeloma activity of the fully human anti-human leukocyte antigen-DR monoclonal antibody 1D09C3. Cancer Res 67:3269–3275
16. Mather SJ, Ellison D (1990) Reduction-mediated technetium-99m labelling of monoclonal antibodies. J Nucl Med 31:692–697
17. Lindmo T, Boven E, Cuttitta F et al (1984) Determination of the immunoreactive fraction of radiolabelled monoclonal antibodies by linear extrapolation to binding at infinite antigen excess. J Immunol Meth 72:77–89
18. Björke H, Andersson K (2006) Automated, high-resolution cellular retention and uptake studies in vitro. Appl Radiat Isot 64:901–905
19. Björke H, Andersson K (2006) Measuring the affinity of a radioligand with its receptor using a rotating cell dish with in situ reference area. Appl Radiat Isot 64:32–37
20. Nestora M, Andersson K, Lundqvist H (2008) Characterization of 111In and 177Lu-labeled antibodies binding to CD44v6 using a novel automated radioimmunoassay. J Mol Recognit 21:179–183
21. Solarli A, Scopinaro F, De Vincentis G et al (2003) 99mTc [13LEU] bombesin and a new gamma camera, the imaging probe, are able to guide mammotome breast biopsy. Anticancer Res 23:2139–2142
22. Scopinaro F, Massari R, Varvarigou AD et al (2007) High resolution small animal single photon emission computed tomography: uptake of [199 mTc]bombesin and [123I]ioflupane by rat brain. J Nucl Mol Imaging 51:204–210
23. Ishizaka Y, Itoh F, Tahira T et al (1989) Presence of aberrant transcripts of ret proto-oncogene in a human papillary thyroid carcinoma cell line. Jpn J Cancer Res 80:1149–1152
24. Epstein AL, Marder RJ, Winter JN et al (1987) Two new monoclonal antibodies, Lym-1 and Lym-2, reactive with human B-lymphocytes and derived tumors, with immunodiagnostic and immunotherapeutic potential. Cancer Res 47:830–840
25. DeNardo SJ, DeNardo GL, O’Grady LF et al (1988) Treatment of B-cell malignancies with 131I Lym-1 monoclonal antibodies. Int J Cancer Suppl 3:96–101
26. Gingrich RD, Dahlle CE, Hoskins KF, Sennett MJ (1990) Identification and characterization of a new surface membrane antigen found predominantly on malignant B lymphocytes. Blood 75:2375–2387
27. Link BK, Kostelnky SA, Cole MS, Fusselman WP, Tso JY, Weiner GJ (1998) Anti-CD3-based bispecific antibody designed for the therapy of human B-cell malignancies can induce T-cell activation by antigen-dependent and antigen-independent mechanisms. Int J Cancer 77:251–256
28. Osmond DG (1986) Population dynamics of bone marrow B lymphocytes. Immunol Rev 93:103–124
29. Taylor PM, Rose ML, Yacoob MH (1989) Expression of MHC antigens in normal human lungs and transplanted lungs with obliterative bronchiolitis. Transplantation 48:506–510
30. Nagy ZA, Leyer S, Lobenwein K, Obermayr F, Ivanov I, Vlock D (2005) 1D09C3, a novel apoptotic human monoclonal antibody: mode of action affects dosing schedules. Ann Oncol 16:134
31. D’Alessandria C, Malviya G, Viscido A et al (2007) Use of a 99 m-Technetium labelled anti-TNFα monoclonal antibody in Crohn’s disease: in vitro and in vivo studies. Q J Nucl Med Mol Imaging 51:1–9