INTRODUCTION

Immunotherapy has been an area of great interest and strong research efforts in the last decades. The anti-CD20 monoclonal antibody rituximab has been used in combination treatment regimens with chemotherapy as first line, maintenance and salvage therapies for non-Hodgkin's lymphoma (NHL).\textsuperscript{1-5} This has resulted in significantly improved response rate and survival in patients with CD20 positive B-cell lymphoproliferative disease. However, not every patient responds to rituximab and many relapse after an initial response.\textsuperscript{6,7} Therefore, it is necessary to develop new strategies that will enhance the biological activity of rituximab in these patients.

Combination of $^{177}$Lu-lilotomab with rituximab significantly improves the therapeutic outcome in preclinical models of non-Hodgkin’s lymphoma

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Abstract

Objectives: To investigate the therapeutic potential of the next-generation anti-CD37 radioimmunoconjugate $^{177}$Lu-lilotomab satetraxetan ($^{177}$Lu-lilotomab) in combination with the anti-CD20 antibody rituximab for treatment of mice with non-Hodgkin’s lymphoma (NHL) xenografts.

Methods: Nude mice with subcutaneous (s.c.) Burkitt’s lymphoma Daudi xenografts and SCID mice intravenously (i.v.) injected with Mantle cell lymphoma Rec-1 cells were treated with either $^{177}$Lu-lilotomab or rituximab alone or with the combination of both treatments. Tumour volume, body weight, blood counts and clinical status were monitored. CD20 expression was measured using flow cytometry with fluorescence-labelled rituximab.

Results: The combination of $^{177}$Lu-lilotomab and rituximab was synergistic for treatment of nude mice with s.c. Daudi xenografts while it was additive for treatment of SCID mice with i.v. injected Rec-1 cells. Binding of rituximab to NHL cells in-vitro was increased by pretreatment with $^{177}$Lu-lilotomab.

Conclusions: Treatment of mice with NHL xenografts with $^{177}$Lu-lilotomab synergistically increased tumour suppression of subsequent anti-CD20 immunotherapy and improved survival. If the same effect is confirmed in a recently started clinical study, it could change the way radioimmunotherapy and CD20 immunotherapy would be used in the future.

KEYWORDS
beta-lutin, CD37, lilotomab, lutetium, lymphoma, non-Hodgkin’s, preclinical, radioimmunotherapy, rituximab, synergy
We have recently developed a next-generation radioimmunoconjugate (RIC) based on the beta-emitting radionuclide $^{177}$Lu chelated to p-SCN-Bn-DOTA (satetraxetan) conjugated to the anti-CD37 antibody liliotomab ($^{177}$Lu-lilotomab satetraxetan, referred also as $^{177}$Lu-lilotomab, trade name Betalutin®). $^{177}$Lu has a half-life of 6.7 days, and the beta particles emitted have a maximum value in tissue of 1.76 mm $^8$ which allows for cross-irradiation, i.e. untargeted cells can be killed by $^{177}$Lu-lilotomab bound to neighbouring cells. CD37 is an internalising transmembrane glycoprotein strongly expressed on mature B lymphocytes, including normal and neoplastic cells. $^9$-$^{12}$ $^{177}$Lu-lilotomab has shown strong anti-tumour effect in preclinical models $^9$, $^{13}$ and in a completed phase 1/2a clinical trial. $^{14}$

To be effective, rituximab depends on selective expression of a sufficient number of CD20 antigens per cell. $^1$-$^4$ Treatment with high-dose External Beam Radiation (EBR) upregulates antigens such as HER2, EGFR and CD20 in cancer cells, $^{15}$-$^{17}$ and an increase in the antigen expression is correlated with an increase in anti-tumour activity of immunotherapies targeting these antigens. $^{15}$-$^{18}$-$^{19}$ Patients treated with low-dose EBR immediately prior to anti-CD20 radioimmunotherapy (RIT) with ibritumomab tiuxetan conjugated to Yttrium-90 had longer freedom from progression (FFP) than patients only treated with RIT with no additional toxicity. $^{20}$ The authors hypothesised that the superior therapeutic effect of anti-CD20 RIT after EBR was due to surface upregulation of CD20 after EBR. We wanted to study if the selectively delivered low-dose rate radiation from $^{177}$Lu-lilotomab affected the CD20 expression of NHL cells and subsequently altered the efficacy of rituximab. Indeed, we found that the combination of $^{177}$Lu-lilotomab with rituximab synergistically increased the therapeutic effect in nude mice with NHL xenografts and rituximab bound to a higher extent to NHL cells treated with $^{177}$Lu-lilotomab than to un-treated cells.

2 | MATERIALS AND METHODS

2.1 | Labelling of liliotomab with $^{177}$Lu

The chelator (p-SCN-Bn-DOTA, Macroyclics, TX, USA) was dissolved in 0.005 M HCl, added to the antibody in a 6:1 ratio and pH-adjusted to approximately 8.5 using carbonate buffer. After 45 minutes of incubation at 37°C, the reaction was stopped by the addition of 50 μL per mg of Ab of 0.2 mol/L glycine solution. To remove free p-SCN-Bn-DOTA, the conjugated antibody was washed using Vivaspin 20 centrifuge tubes (Sartorius Stedim Biotech, Göttingen Germany) 4-5 times with NaCl 0.9%. Before labelling with $^{177}$Lu, the pH was adjusted to 5.3 ± 0.3 using 0.25 mol/L ammonium acetate buffer. Between 120 and 220 MBq of $^{177}$Lu (ITG, Garching, Germany) was added to 1 mg of satetraxetan-Ab and incubated for 15-30 minutes at 37°C. The radiochemical purity (RCP) of the conjugate was evaluated using instant thin-layer chromatography. If RCP was below 95% the conjugate was purified by elution through a Sephadex G-25 PD-10 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

2.2 | Cell lines

Cell suspensions of lymphoma cell lines Daudi (Burkitt’s lymphoma), and Rec-1 (Mantle Cell Lymphoma, both acquired from ATCC) were grown in RPMI 1640 medium (PAA, Linz, Austria) supplemented with 10% heat-inactivated FCS (PAA), 1% L-glutamine (PAA) and 1% penicillin-streptomycin (PAA) in a humid atmosphere with 95% air/5% CO$_2$ and maintained in exponential growth phase through sub-culturing every 2-4 days.

2.3 | Immunoreactive fraction of $^{177}$Lu-lilotomab

The immunoreactivity of the radioimmunoconjugates was measured using NHL Ramos cells and a one point modified Lindmo method. $^{21}$-$^{22}$ The cell concentration used was 75 million cells/mL. The immunoreactivity of the conjugates was between 60% and 82%.

2.4 | Animal models

A subcutaneous Daudi model was established in the Institute for Comparative Medicine, Radium Hospital, Oslo, Norway. Institutionally bred female athymic nude Foxn1nu mice that were between 6 and 8 weeks old and had body weights between 18 and 24 g at the start of the study were used. All mice had 1-2 weeks for acclimation before the studies began. The animals were maintained under pathogen-free conditions with a 12-hour lighting cycle at a room temperature of 23°C and air relative humidity of 55% in plastic cages. Food and water were supplied ad libitum, and bedding was changed regularly. All procedures and experiments involving animals in this study were approved by The Norwegian Animal Research Authority (NARA). The Department of Comparative Medicine institutional veterinarian has established the rules for feeding, monitoring, handling and sacrifice of animals in compliance with regulations set by the Ministry of Agriculture of Norway and “The European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes.” The institutional veterinarian has delegated authority from the Norwegian Animal Research Authority (NARA). The laboratory animal facilities are subject to a routine health-monitoring programme and tested for infectious organisms according to a modification of Federation of European Laboratory Animal Science Associations (FELASA) recommendations. Mice were injected subcutaneously in both flanks with 100 μL of 100 million Daudi cells/mL using a 1:1 Matrigel dilution.

An intravenous Rec-1 model was established in ArctiCLAS, Reykjavik, Iceland. Female CB17-SCID mice, 6 weeks of age, weighing no less than 15 g, were ordered from Taconic in Denmark and allowed for one week of acclimation prior to study start. SCID mice were chosen because it was not possible to establish a disseminated model in nude mice. In this type of models, tumours are of microscopic dimensions at injection of treatment. Due to the SCID mutation, this mouse strain tolerates less radiation than nude mice. $^{23}$ The mice were weighed and earmarked in the acclimation week. The animals were housed in an IVC-rack (individually ventilated cages). Five mice
were housed per cage (Euro-standard Type-II). Cages, water bottles, nesting material and hideaways were autoclaved at ArcticLAS prior to use. Cages were changed once a week. The mice were provided with autoclaved drinking water. Water bottles were changed every other day. The mice were fed with irradiated rodent diet (Altromin NIH®31 M - from Brogarden, Denmark). The animals were provided with irradiated-Tapevei aspen bedding from Brogarden, Denmark. Mice were injected intravenously with 100 μL containing 10 million Rec-1 cells via their lateral tail vein. The animal studies were approved by the national committee for animal experiments prior to study start. ArcticLAS animal facility works under authorisation and approval from the Food and Veterinary authority in Iceland (MAST). The facility is inspected regularly by the District veterinary office.

### 2.5 Therapy studies

The therapeutic effect of the combination of $^{177}$Lu-lilotomab and rituximab in the s.c. Daudi model was studied using one injection of 250 MBq/kg $^{177}$Lu-lilotomab, one dose of 40 mg/kg rituximab, four doses of 10 mg/kg rituximab or NaCl (Table 1). The activity of $^{177}$Lu-lilotomab used was approximately 50% of the maximum tolerated dosage (MTD) $^{24}$ and was chosen so that the therapeutic effect of the single treatment was suboptimal in order to be able to detect an increased effect of the combination with rituximab. The same thinking guided the choice of rituximab dosage which was chosen based on published data on similar animal models.$^{31,32}$ Nine to ten mice were used per group. Mice were administered with the first treatment on day 8; study day 0 was set at injection of tumour cells. The second treatment was given 5 days later. The mice were weighed at least twice a week, and they were inspected at least once daily for clinical signs of disease. When clinical signs of the disease were apparent, an abdominal palpation for tumours was performed once/twice a week. At termination, all mice were necropsied and key organs (lungs, liver, spleen, kidneys, stomach, ovaries, brain, femur and skull) were harvested for histopathological evaluation. Mice that were still alive at the end of the study (day 222) were necropsied, and key organs (lungs, liver, spleen, kidneys, stomach, ovaries, brain, femur and skull) were harvested for histopathological evaluation.

The therapeutic effect of the combination in the Rec-1 i.v. model was performed in a blinded study using one injection of 40 MBq/kg $^{177}$Lu-lilotomab, one injection of 100 μg of rituximab per mouse (around 5 mg/kg for a 20 g mouse) or NaCl (Table 2). The activity of $^{177}$Lu-lilotomab used was approximately 50% of the maximum tolerated dosage (MTD) $^{26}$ and was chosen so that the therapeutic effect of the single treatment was suboptimal in order to be able to detect an increased effect of the combination with rituximab. The same thinking guided the choice of rituximab dosage which was chosen based on published data on similar animal models.$^{31,32}$ Nine to ten mice were used per group. Mice were administered with the first treatment on day 8; study day 0 was set at injection of tumour cells. The second treatment was given 5 days later. The mice were weighed at least twice a week, and they were inspected at least once daily for clinical signs of disease. When clinical signs of the disease were apparent, an abdominal palpation for tumours was performed once/twice a week. At termination, all mice were necropsied and the following organs were collected for histopathological evaluation: skull, brain, femur, liver, spleen, easily accessible lymph nodes, uterus, ovaries, the whole vertebrae and tumours. The organs were fixed in 10% buffered formalin and further processed for histopathological evaluation. Blood was drawn from the animals every 3 weeks until week 9 for haematology analysis. Blood samples (no more than 10% of the total blood volume) were drawn from vena facialis and collected into 100 μL EDTA-coated tubes (Microvette®100 K3E, Sarsted). The tubes were turned/swirled for around 1 minute to ensure all EDTA was mixed well with the blood. White blood cells, platelets and red blood cells were counted on an automated haematology analyzer (MS4 analyzer from Melet Schloeing Laboratories, France).

### TABLE 1  Study groups and treatment schedule in the study of $^{177}$Lu-lilotomab and rituximab combination in nude mice with s.c. Daudi xenografts

| Group name          | Study day$^a$ | Tumour volume$^b$ (mm$^3$) |
|---------------------|--------------|---------------------------|
|                     | 0  | 3  | 6  | 10 | 13 |
| NaCl × 5            | NaCl | NaCl | NaCl | NaCl | NaCl |
| $^{177}$Lu-lilotomab + 4 × NaCl | $^{177}$Lu-lilotomab (250 MBq/kg) | NaCl | NaCl | NaCl | NaCl |
| $^{177}$Lu-lilotomab + 4 × Rituximab | $^{177}$Lu-lilotomab (250 MBq/kg) | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | 302 ± 101 |
| $^{177}$Lu-lilotomab + 1 × Rituximab | $^{177}$Lu-lilotomab (250 MBq/kg) | Rituximab (40 mg/kg) | - | - | - | 313 ± 120 |
| NaCl + 4 × Rituximab | NaCl | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | Rituximab (10 mg/kg) | 305 ± 89 |
| NaCl + 1 × Rituximab | NaCl | Rituximab (40 mg/kg) | - | - | - | 264 ± 119 |

$^a$Mice were inoculated at day -15.

$^b$Average ± SD at injection of first treatment (day 0).
TABLE 2  Study groups and treatment schedule in the study of 177Lu-lilotomab and rituximab combination in SCID mice with i.v. injected Rec-1 cells

| Group name | Study day* | Day 8 | Day 13 |
|------------|------------|-------|--------|
| 177Lu-lilotomab + Rituximab | 177Lu-lilotomab (40 MBq/kg) | Rituximab (100 μg) |
| NaCl + NaCl | NaCl | NaCl |
| 177Lu-lilotomab + NaCl | 177Lu-lilotomab (40 MBq/kg) | NaCl |
| NaCl + Rituximab | NaCl | Rituximab (100 μg) |

*Days after cell inoculation.

The end-points in the i.v. Rec-1 model and in the s.c. Daudi model were weight loss of more than 10% over a period of one week or of 20% from highest recorded weight, respectively, signs of substantial discomfort or tumour size equal to 20 mm in diameter.

2.6  Statistical analysis

Survival analysis was performed using log-rank test and the Holm-Sidak method for all multiple pairwise comparisons (Sigma Plot 12.5, Systat Software Inc., San Jose, California, USA). In addition, the Cox proportional hazards regression model was used for the analysis of the s.c. Daudi survival data. The hazard ratio HR of the combination and of the interaction (Equation 1) was used to assess if there was a synergistic effect of the combination treatment. A HR of 1 indicates no effect and a value <1 indicates lower risk for event.

\[ HR_T = HR_{BvsN} \cdot HR_{RvsN} \cdot HR_{Int} \]  

In equation 1, \( HR_T \) is the HR of the combination treatment, \( HR_{BvsN} \) and \( HR_{RvsN} \) are the HR of 177Lu-lilotomab and rituximab alone vs NaCl, respectively, and \( HR_{Int} \) is the HR of the interaction between 177Lu-lilotomab and rituximab. The multiplication of \( HR_{BvsN} \) by \( HR_{RvsN} \) gives the HR of the combination when both treatments are additive (no interaction; \( HR_{Int} = 1 \)). The lower the \( HR_{Int} \) value, the stronger the interaction effect and thus the synergy between the treatments. The threshold for statistical significance for the synergy was set at a \( P \)-value associated with \( HR_{Int} \) lower than 0.05.

2.7  Expression of cell surface CD20 in-vitro

The expression of the CD20 antigen was measured in the cell lines Daudi and Rec-1 at various time-points after treatment with naked lilotomab, 177Lu-lilotomab or external beam radiation (EBR). An X-ray machine Faxitron CP160 was used as source of External Beam Radiation (maximum energy of 160 keV with a current of 6.3 mA and a linear energy distribution). To avoid the lowest energies, 2 filters were used: 0.5 mm Cu and 0.8 mm Be. Dose rate was 1 Gy/min. Non-irradiated cells were used as controls. Treatment with radio-immunotherapy was given by incubating cells for 18 hours with either 0.44 or 0.88 MBq/mL of 177Lu-lilotomab. Control cells were incubated for the same period of time with an equivalent amount of naked lilotomab. After the incubation time, cells were washed 3 times and resuspended in fresh medium. The surface expression of CD20 was estimated by incubating the cells with Alexa 488- or Alexa 647-labelled rituximab and measuring Mean Fluorescence Intensity (MFI) with a Guava EasyCyte 12HT (Merck KGaA, Darmstadt, Germany). Rituximab was labelled with Alexa 488 or Alexa 647 using labelling kits and protocols supplied by Invitrogen, Oregon, US. Binding to CD20 was measured every 1-5 days and up to 3 days in Rec-1 and 13 days in Daudi (duplicates). The absorbed dose by 177Lu-lilotomab to the cells was estimated by assuming a homogeneous distribution of the RIC in the cell suspension during the 18 hours of incubation time, which gives an estimated absorbed dose to the cells of 0.5 Gy for 0.44 MBq/mL 177Lu-lilotomab and 1.5 Gy for 0.88 MBq/mL 177Lu-lilotomab treatments. This might underestimate the actual dose received by the cells. Based on the results reported by Marcatili et al. for Ramos cells incubated with 177Lu-lilotomab for 18 hours and maintained in culture for 5 days, the absorbed doses to the cells were around 0.8 Gy for 0.44 Mq/mL 177Lu-lilotomab and 1.5 Gy for 0.88 MBq/mL 177Lu-lilotomab.

3  RESULTS

3.1  Synergistic effect of 177Lu-lilotomab and rituximab in nude mice with s.c. Daudi xenografts

The expression of the CD20 antigen was measured in the cell lines Daudi and Rec-1 at various time-points after treatment with naked lilotomab, 177Lu-lilotomab or external beam radiation (EBR). An X-ray machine Faxitron CP160 was used as source of External Beam Radiation (maximum energy of 160 keV with a current of 6.3 mA and a linear energy distribution). To avoid the lowest energies, 2 filters were used: 0.5 mm Cu and 0.8 mm Be. Dose rate was 1 Gy/min. Non-irradiated cells were used as controls. Treatment with radio-immunotherapy was given by incubating cells for 18 hours with either 0.44 or 0.88 MBq/mL of 177Lu-lilotomab. Control cells were incubated for the same period of time with an equivalent amount of naked lilotomab. After the incubation time, cells were washed 3 times and resuspended in fresh medium. The surface expression of CD20 was estimated by incubating the cells with Alexa 488- or Alexa 647-labelled rituximab and measuring Mean Fluorescence Intensity (MFI) with a Guava EasyCyte 12HT (Merck KGaA, Darmstadt, Germany). Rituximab was labelled with Alexa 488 or Alexa 647 using labelling kits and protocols supplied by Invitrogen, Oregon, US. Binding to CD20 was measured every 1-5 days and up to 3 days in Rec-1 and 13 days in Daudi (duplicates). The absorbed dose by 177Lu-lilotomab to the cells was estimated by assuming a homogeneous distribution of the RIC in the cell suspension during the 18 hours of incubation time, which gives an estimated absorbed dose to the cells of 0.5 Gy for 0.44 MBq/mL 177Lu-lilotomab and 1.5 Gy for 0.88 MBq/mL 177Lu-lilotomab treatments. This might underestimate the actual dose received by the cells. Based on the results reported by Marcatili et al. for Ramos cells incubated with 177Lu-lilotomab for 18 hours and maintained in culture for 5 days, the absorbed doses to the cells were around 0.8 Gy for 0.44 Mq/mL 177Lu-lilotomab and 1.5 Gy for 0.88 MBq/mL 177Lu-lilotomab.
receiving $^{177}$Lu-lilotomab in combination with one or four doses of rituximab). The expected HR of the combination group given an additive effect was 0.186 ($HR_{B\text{vs}N} \cdot HR_{R\text{vs}N}$, Table 4) vs NaCl, whereas $HR_{R}$ for the combination group was 0.024 ($HR_{B\text{vs}N} \cdot HR_{R\text{vs}N} \cdot HR_{Int}$) vs NaCl. Due to the low number of events (ie mice never reaching tumour diameter $>20$ mm and thus being censored at the end of the study) in the combination group, the test for interaction did not reach the threshold for significance ($P = 0.078$). In addition, the spread in the survival of mice treated with only rituximab and the dependence of HR with time might have also contributed to the lack of statistical significance.

Average body weight was similar in all treatment groups (data not shown). There were, however, some mice in the combination groups that experienced body weight loss and other clinical symptoms of sickness and discomfort (Table 5). Histopathological analysis of mice euthanised at the end of the study showed no evidence of long-term toxicity associated with the combination treatments. The body weight loss observed after day 100 was probably due to normal aging of the mice.

### 3.2 Additive effect of $^{177}$Lu-lilotomab and rituximab in SCID mice with i.v. injected Rec-1 cells

There was an increased survival of SCID mice with Rec-1 i.v. injected cells treated with the combination of $^{177}$Lu-lilotomab and rituximab as compared with mice treated with either treatment alone or with NaCl (Figure 3). However, the differences were only statistically significant for the comparison with NaCl (Table 6). The median survival

| Treatment | Median survival ± SE (days) | Doubling time of average tumour volume (days) |
|-----------|-----------------------------|---------------------------------------------|
| 5 × NaCl  | 24 ± 5                      | 4                                           |
| $^{177}$Lu-lilotomab + 4 × NaCl | 60 ± 9                        | 42                                          |
| $^{177}$Lu-lilotomab + 4 × Rituximab | $>222^a$                     | Not Reached                                 |
| $^{177}$Lu-lilotomab + 1 × Rituximab | $>222^a$                     | Not Reached                                 |
| NaCl + 4 × Rituximab | 31 ± 5                      | 15                                          |
| NaCl + 1 × Rituximab | 40 ± 11                      | 15                                          |

$a$Significantly different from 5 × NaCl and NaCl + 4 × Rituximab ($P < 0.05$, Log-Rank).
It was more than 132 days (106% increase as compared to NaCl control) for the combination while it was 92 days (44% increase) for \(^{177}\)Lu-lilotomab alone and 75 days (15% increase) for rituximab alone (Table 6). The HR found using the Cox proportional hazards regression model was 0.104 (Table 4) which was close to the HR of the combination if only an additive effect is considered (HR \(_{\text{BvsN}} \cdot \text{HR}_{\text{RvsN}} = 0.138\)). In addition, the \(P\)-value associated to HR \(_{\text{Int}}\) was substantially higher than the statistical threshold, which makes us conclude that the combination of \(^{177}\)Lu-lilotomab and rituximab in this study was additive.

**TABLE 4** Output from cox regression model from study in nude mice with s.c. Daudi xenografts and tumour diameter equal or larger than 20 mm as end-point and from study in SCID mice with i.v. injected Rec-1 cells and euthanasia due to sickness, discomfort or palpable tumour diameter equal to 20 mm as end-point

| Treatment                                      | s.c. Daudi Hazard ratio | s.c. Daudi 95% CI | s.c. Daudi \(P\)-value | i.v. Rec-1 Hazard Ratio | i.v. Rec-1 95% CI | i.v. Rec-1 \(P\)-value |
|------------------------------------------------|------------------------|-------------------|------------------------|------------------------|-------------------|------------------------|
| \(^{177}\)Lu-lilotomab vs NaCl (HR\(_{\text{BvsN}}\)) | 0.30                   | (0.10-0.90)       | 0.032                  | 0.30                   | (0.11-0.87)       | 0.027                  |
| Rituximab \(^{4}\) vs NaCl (HR\(_{\text{RvsN}}\))       | 0.62                   | (0.25-1.53)       | 0.298                  | 0.46                   | (0.16-1.31)       | 0.147                  |
| Interaction of \(^{177}\)Lu-lilotomab and Rituximab \(^{5}\) (HR\(_{\text{Int}}\)) | 0.13                   | (0.01-1.26)       | 0.078                  | 0.75                   | (0.14-4.09)       | 0.736                  |

\(^{4}\)Pooled data from both groups receiving rituximab alone.

\(^{5}\)Pooled data from both groups receiving \(^{177}\)Lu-lilotomab in combination with 4 doses of 10 mg/kg rituximab and one dose of 40 mg/kg rituximab.

It is worth noticing that at necropsy, 2 of the 7 mice surviving until the end of the study in the combination treatment group showed pathological signs of tumours and therefore a total of 5 animals of 10 in this group had clear pathological signs of tumours. No sign of treatment toxicity was observed in the mice. Platelet (THR), and red blood cell (RBC) counts were within or close to the established reference interval and similar to the control group during the whole study (data not shown). Average body weight of all treatment groups followed that of the control group, showing severe body weight loss close to euthanasia due to the disease (data not shown).
It took 40 days or more for tumours to be palpable, or for animals to start showing signs of disease in this animal model. Macroscopic tumours found at euthanasia were commonly located in the skull, ventral vertebral muscles of the thorax or abdomen, in the mediastinum, ovaries or mesovarium, uterus or mesometrium, in skeletal muscles and superficial lymph nodes. Other less common locations were in the abdominal cavity where tumours were seen around the kidneys or stomach, or as large tumours pendulating from the dorsal abdomen.

### 3.3 Expression of cell surface CD20 in-vitro

The cell lines showed increased binding of rituximab after treatment with either EBR or $^{177}$Lu-lilotomab as compared to untreated cells or cells treated with naked lilotomab (Figure 4). This may indicate an increased expression or upregulation of the CD20 antigen. Daudi cells showed the highest increase, increasing up to 356% compared to control, 5 days after treatment. The upregulation lasted up to 13 days which was the last time-point measured. The amount of increased binding and the length of the increase when treating with the RIC $^{177}$Lu-lilotomab was superior to that found when the same cells were treated with similar doses of EBR.

### 4 DISCUSSION

The use of radioimmunotherapy has been approved as a therapeutic option in cancer therapy for several years. The radioimmunotherapy is mainly used in those patients experiencing relapse from chemotherapy and immunotherapy. We have previously shown that $^{177}$Lu-lilotomab can target and deliver radiation selectively to lymphoma tumour cells and xenografts, and the treatment is currently being tested clinically for treatment of both aggressive and indolent NHL. In this study, we showed that $^{177}$Lu-lilotomab can interact synergistically with rituximab to give an increased anti-tumour effect, prolonging the survival of mice with s.c. NHL xenografts. There are several mechanisms that can lead to the observed synergy. Among them is the upregulation of CD20, which would lead to increased binding of rituximab. It has previously been shown that high-dose rate External Beam Radiation (EBR) can cause a reactive oxygen species (ROS) mediated increase of CD20 in B cells and that the effect can last for up to 2-3 days.

#### TABLE 6 Median survival of SCID mice intravenously injected with Rec-1 cells after treatment with $^{177}$Lu-lilotomab, rituximab or the combination

| Treatment                      | Median Survival (days) ± SE | % Increased Survival vs control |
|-------------------------------|-----------------------------|--------------------------------|
| NaCl + NaCl                   | 64 ± 2                      | NA                             |
| NaCl + Rituximab              | 75 ± 11                     | 14.5                           |
| $^{177}$Lu-lilotomab + NaCl   | $^{92} ± 13$                | 43.8                           |
| $^{177}$Lu-lilotomab + Rituximab | $^{>132}$                  | $^{>106.3}$                    |

*Statistically significant different from the NaCl + NaCl control group ($P < 0.05$, log-rank).

#### FIGURE 4

Percentage increase in CD20 expression for A, Daudi, B, Rec-1 cells as compared to naked lilotomab (control), 0.4 or 0.9 MBq/mL $^{177}$Lu-lilotomab or 0.5 or 1.5 Gy external beam radiation for different time-points after start of treatment. C, Example of gating used to acquire the FC histograms presented in (D). D, Example of histograms acquired through Flow Cytometry using Daudi cells 2 days after treatment with naked lilotomab or $^{177}$Lu-lilotomab stained with fluorescently-labelled rituximab or without staining (blank).
stronger and more prolonged increase in rituximab binding by radioimmunotherapy with $^{177}$Lu-lilotomab as compared to EBR. It could be speculated that the prolonged and stronger effect could be related to increased formation of ROS induced by the continuous, low-dose rate beta-irradiation from $^{177}$Lu-lilotomab in contrast to the short, high-dose rate gamma-irradiation from the EBR treatment. Consequently, anti-CD37 radioimmunotherapy with $^{177}$Lu-lilotomab can be used to both deliver short-range beta-radiation selectively and continuously to tumour cells, minimising irradiation of healthy tissues and increase CD20 binding in those cells that are not killed by the radiation dose delivered and make these cells more susceptible for subsequent rituximab treatment.

There might be other mechanisms behind the synergistic effect. Radioimmunotherapy has been shown to increase the permeability of tumour vasculature which might lead to better tumour uptake of antibodies. Moreover, CD20 binding of rituximab has shown to improve internalisation of an anti-CD37 antibody drug conjugate (ADC) thereby enhancing its efficacy. The efficacy of $^{177}$Lu-lilotomab might therefore be also increased if the same enhanced internalisation occurs. In addition, it has been shown that radiation induces immunogenic modulation of tumour cells. There might be several reasons for the lower effect of the combination of $^{177}$Lu-lilotomab and rituximab in the model using SCID mice with i.v. injected Rec-1 cells: (a) Rec-1 cells showed around 10 times lower CD20 upregulation than Daudi cells (b) SCID mice were treated with a lower dose of $^{177}$Lu-lilotomab than the nude mice which might further decrease the CD20 upregulation since the degree of upregulation has shown to be dose dependent in our in vitro studies and (c) the effect of increased vascularity would be negligible in a disseminated model where tumours would be of microscopic size at treatment injection compared to the bulky s.c. model which may further decrease the synergistic effect of the combination treatment.

It is important to notice that $^{177}$Lu-lilotomab does not bind to murine CD37 (data not shown) and rituximab does not bind to murine CD20. Therefore, in the mouse models, there is not non-specific binding to normal B cells, which is not the case in human patients. The lack of binding to normal B cells in the mouse models represents a perfect scenario in human patients where all normal B cells have been either blocked by pre-dosing with unlabelled lilotomab, or depleted by previous treatment with rituximab, which would decrease treatment associated toxicity and increase treatment efficacy.

CD20 downregulation after treatment with rituximab has been repeatedly observed in rituximab resistant patients although the exact mechanism for development of rituximab resistance is not yet known. It could be potentially mediated by alterations in CD20 expression or signalling, both by genetic or epigenetic changes, elevated apoptotic threshold, modulation of complement activity or diminished cellular cytotoxicity. In addition treatment with molecular targeting therapeutics such as ibrutinib, lenalidomide and bortezomib downregulate CD20. Given that $^{177}$Lu-lilotomab has been shown to increase rituximab binding to NHL cells, we hypothesise that treatment with $^{177}$Lu-lilotomab could be potentially used to revert downregulation of CD20 and resistance to rituximab. Further studies using rituximab resistant cell lines will be performed to explore this hypothesis.

5 | CONCLUSION

We have shown that treatment of NHL in vivo with $^{177}$Lu-lilotomab results in an increased tumour suppression of anti-CD20 immunotherapy and improved survival. We have also shown that the interaction can be synergistic. If the same effect is confirmed in clinical studies, it could change the way RIT and CD20 immunotherapy would be used in the future.

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