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Mol. Pharmaceutics, **Just Accepted Manuscript** • DOI: 10.1021/acs.molpharmaceut.5b00947 • Publication Date (Web): 10 Mar 2016

Isolation and ^{111}In-oxine labeling of murine NK cells for assessment of cell trafficking in orthotopic lung tumor model

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Key Words:
Murine NK cell, ^{111}In-oxine labeling, *in-vivo* cell trafficking, lung tumor

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ABSTRACT

A non-invasive *in vivo* imaging method for NK cell trafficking is essential to gain further understanding of the pathogenesis of NK cell mediated immune response to the novel cancer treatment strategies, and to discover the homing sites and physiological distribution of NK cells. Although human NK cells can be labeled for *in vivo* imaging, a little is known about the murine NK cell labeling and its application in animal models. This study describes the isolation and *ex-vivo* radiolabeling of murine NK cells for the evaluation of cell trafficking in an orthotopic model of human lung cancer in mice.

Scid-Tg(FCGR3A)Blt transgenic SCID mice were used to isolate NK cells from mouse splenocytes using the CD49b (DX5) MicroBeads positive selection method. The purity and viability of the isolated NK cells were confirmed by FACS analysis. Different labeling buffers and incubation times were evaluated to optimize ^{111}In-oxine labeling conditions. Functionality of the radiolabeled NK cell was assessed by ^{51}Cr-release assay. We evaluated physiological distribution of ^{111}In-oxine labeled murine NK cells in normal SCID mice and biodistribution in irradiated and non-irradiated SCID mice with orthotopic A549 human lung tumor lesions. Imaging findings were confirmed by histology.

Results showed that incubation with 0.011 MBq of ^{111}In-oxine per million murine NK cells in PBS (pH 7.4) for 20-min is the best condition that provides optimum labeling efficiency without affecting cell viability and functionality. Physiological distribution in normal SCID mice demonstrated NK cells homing mainly in the spleen, while ^{111}In released from NK cells was excreted via kidneys into urine. Biodistribution studies demonstrated a higher lung uptake in orthotopic lung tumor-bearing mice than control mice. In irradiated mice, lung tumor uptake of radiolabeled murine NK cells decreased between 24h and 72h p.i., which was accompanied by tumor regression, while in non-irradiated mice, radiolabeled NK cells were retained in the lung tumor lesions up to 72h p.i. without tumor regression. In tumor-bearing mice that were only irradiated but did not receive radiolabeled murine NK cells, a high tumor burden was observed at 72h p.i., which indicates that irradiation in combination with murine NK cell allocation, but not irradiation alone, induced a remarkable anti-tumor effect in the orthotopic A549 lung tumor bearing mouse model.

In conclusion, we describe a method to evaluate murine NK cell trafficking and biodistribution, which can be used to determine potential effects of immune-mediated therapeutic agents on NK cell biodistribution.

INTRODUCTION

Different immune cells, including natural killer (NK) cells, execute key role in cancer immunotherapy.[1,2] Therefore, new cancer treatment strategies that activates immune cells and promote infiltration in tumor lesions are being generated. Assessing the *in vivo* efficacy of these immunotherapeutic approaches needs non-invasive and sensitive cell-tracking method. Use of radiolabeled immune cells to follow cell trafficking and discover the homing sites may facilitate the understanding of the pathogenesis of different immune cell mediated responses to the immunotherapeutic strategies.

Many studies have been published using ^{111}In-oxine labeled human lymphocytes[3] and a few studies used labeled human NK cells for *in vivo* biodistribution studies. Meller et al.[4] labeled human NK cells with 0.05 to 0.1 MBq of ^{111}In per million cells, and Brand et al.[5] labeled human NK cells with 0.074 MBq of ^{111}In per million cells, for the evaluation of NK cell kinetics and biodistribution in renal cell carcinoma patients. In both studies, patients received average 5 x 10^8 NK cells labeled with ^{111}In-oxine with tenfold excess of unlabeled NK cells for therapy. Whole-body scans were performed from 0.5h to 144h post-transfusion to follow cell-trafficking, however cells were evidently detectable up to first 72h. Both studies confirmed that allogenic NK cells retain their immune reactivity even after radiolabeling, specifically accumulate in the tumor lesions and showed *in vivo* anti-tumor activity. Matera et al.[6] labeled human adherent NK cells with 0.074 MBq of ^{111}In per million cells for the single-photon emission computed tomography (SPECT) evaluation of migration and homing of NK cells in patients with colon carcinoma; authors also compared their differential localization in normal and neoplastic liver using ^{99m}Tc-phytate. 5 x 10^8 labeled NK cells administered either systemic (intra-venous) or locoregional (intra-arterial) routes to each patient. This study showed that locoregionally injected labeled NK cells predominantly localize in the liver metastases and these results were consistence with ^{99m}Tc-phytate SPECT analysis. Such scintigraphic approaches using radiolabeled NK cells may contribute to the optimization of immunotherapeutic strategies.

In all the above mentioned clinical studies investigators *ex-vivo* enriched and cultivated NK cells for 2 weeks using rIL-2 and other lymphokines[7], which help to obtain a good cell count (about 100 fold expansion) of NK cells to be radiolabeled with 37 MBq of ^{111}In-oxine for scintigraphy studies. However, the main problem with the use of lymphokines activated NK cell is their heterogeneous population due to change in the expression of different cell surface markers during the cultivation process[8]. Because of these changes expanded NK cells are preferably used for adoptive immunotherapy purpose due to its highly activated population. However, the use of expanded NK cells for in-vivo imaging of cell trafficking to investigate the effect of any immunotherapeutic agent on a specific immune cell could be tricky, since the results could be compromised by the change in the expression of cell surface markers during cell expansion procedure.

An interesting study was performed by Frohn et al.[9] to evaluate if adoptive immunotherapy with NK cells could be well tolerated when only highly enriched NK cells is applied without *ex-vivo* expansion. In this feasibility study, 10 cancer patients were repeatedly transfused with average 1.02±0.27 x 10^9 NK cells per transfusion. The transfusion was repeated up to four

times after 5-6 weeks. Authors did not observe any serious acute/delayed reaction or allergic response and patient results demonstrated a good tolerability. The downside of this immunotherapy study was the use of a much higher blood volume (ranged from 10 to 12 L) for apheresis procedure in comparison to the studies where *ex-vivo* expansion of NK cells performed before transfusion (ranged from 3 to 4 L). However, this study demonstrated that we can use immune cells without *ex-vivo* expansion, and fortunately, for *in-vivo* imaging (cell-trafficking) studies we require a significantly lower amount of radiolabeled immune cells.

In preclinical setting, we can isolate about 1 million NK cells from each mouse spleen and only 0.011 MBq of ^{111}In-oxine can be used to label them, since higher ^{111}In dose will affect cell viability. Moreover, if we want to perform a SPECT imaging study in mouse model then relatively higher doses (20-60 MBq) of ^{111}In-labeled murine NK cells are required to achieve reasonable images, due to low sensitivity of microSPECT camera. Therefore, an enormous number of mice would be required to isolate NK cells for a microSPECT imaging study. This is the reason why till now no study demonstrated murine NK cell labeling with ^{111}In-oxine for microSPECT imaging. To overcome this issue, we decided to perform *ex-vivo* biodistribution and gamma counting study, which is a much more sensitive method and requires much less animals.

The ^{111}In-oxine complex is a well-known for human lymphocyte labeling and imaging due to its long half-life and easy labeling procedure.[15-17] However, some other PET imaging isotopes have also been proposed for human lymphocyte, stem cell and mouse cancer cell labeling that includes ^{99m}Tc-HMPAO, ^{18}F-FDG, ^{64}Cu and recently published ^{89}Zr, each one has its own benefits and drawbacks.[17-20] Nevertheless, few studies also attempted to specifically image human NK cells using *indirect* approaches that comprise labeling of genetically modified human NK cell line NK-92-scFv(FRP5)-zeta with ^{18}F-FDG,[21] and use of ^{99m}Tc-anti-CD56 antibody against human NK cells.[22]

In the present study, we evaluated the isolation and *ex-vivo* ^{111}In-oxine labeling of murine NK cells for monitoring physiological distribution and *in-vivo* kinetics in an orthotopic model of A549 human lung cancer in mice. Results were compared with irradiated mice groups, in which autologous NK cells were depleted to avoid the possibility of interference with the adoptively transferred NK cells.

MATERIALS AND METHODS

Mice and cell lines

All animal studies were performed according to Dutch national regulations after approval was received from the Institutional Animal Care and Use Committee (IACUC).

Scid-Tg(FCGR3A)Blt transgenic mice (Charles River Laboratories Inc./ Roche Glycart AG, Switzerland) were used (GGO.Nr: IG 11-104) to isolate NK cells. These mice express human FcgRIIIA (CD16) positive NK cells as effectors (about 80% of murine NK cells express human CD16), allowing a more accurate representation of efficacy via antibody-dependent cell–mediated cytotoxicity.[10]

CB17/Icr-Prkdc^{scid}/IcrlcoCrl mice (Charles River Laboratories Inc.) were used for biodistribution experiments. These mice have T and B-lymphocyte deficiency, but normal NK cell activity and radiation sensitivity, providing an appropriate *in-vivo* microenvironment for NK cell trafficking and irradiation experiments.

Human lung adenocarcinoma A549 cell line and murine lymphoma YAC-1 cell line were used for lung tumor growth and the NK cell functionality assay, respectively. Cells were maintained in a RPMI 1640 culture medium (Sigma-Aldrich) supplemented with 10% heat-inactivated foetal calf serum (Gibco), 2 mM L-Glutamine (Gibco), 100 U/ ml penicillin (Gibco) and 100 µg/ ml streptomycin (Gibco). YAC-1 cells also received 50 mM 2-marcaptoethanol (Sigma) in culture medium. Both cell lines were cultured in 25 cm^2 culture flasks (BD Falcon) at 37 °C in an atmosphere of 5% CO_2 and 95% air. Cell viability was determined by trypan blue dye exclusion assay.

Mouse lung tumor model

Orthotopic lung tumors were induced by i.v. injection of human non-small cell lung cancer (NSCLC) A549 tumor cells ($5x10^6$ cell/ mouse) in 200 µL of AIM V media (Life Technologies). Mice were maintained in a specific pathogen-free facility and monitored for weight loss or any clinical symptom on daily basis. Lung tumor lesions were allowed to grow for 2 weeks.

After 2 weeks, mice were divided into two groups: (i) mice treated with irradiation (n=15), and (ii) control mice without irradiation (n=15). One day before the administration of radiolabeled murine NK cells, irradiated mice group treated with 2.4 Gy whole-body irradiation using an X-ray source (X-RAD 320, Precision X-Ray, USA).[11] According to the directions of the IACUC and Dutch national regulations, all mice were maintained under '*irradiation care protocol*' from one week prior to irradiation, until the end of the study. Thus, the mice received high energy breeding diet and sterile water with neomycin and sucrose to prevent any severe morbidity and mortality that may occur as any side effect of the irradiation procedure.

Murine NK cell isolation

NK cell isolation was performed by positive selection of murine NK cells using magnetic MicroBeads. Single cell suspension of splenocytes was prepared at 4 ºC by manual grinding of spleens obtained from female Scid-Tg(FCGR3A)Blt transgenic mice (8-10 weeks) followed by filtration using 70 micrometre cell strainer (BD Biosciences) to remove any cell clump in the cell suspension. Splenocytes were labeled with CD49b (DX5) magnetic MicroBeads (Miltenyi Biotec GmbH) and subsequently passed through a MACS® Column in the magnetic field of a MACS® Separator (Miltenyi Biotec GmbH). The cells labeled with CD49b (DX5) magnetic MicroBeads were retained on the column and unlabeled cells ran through the column. Finally, the column was removed from the magnetic field and magnetic MicroBeads labeled CD49b (DX5) positive NK cells were eluted.

Isolated murine NK cells were assessed for viability by the trypan blue exclusion test. Fluorescence-activated cell sorting (FACS, Calibur, BD Biosciences) analysis using different combinations of fluorescent labeled antibodies was performed to evaluate the purity (NKp46

+ CD49b and CD49b + huCD16 positive), maturation (NKp46 + CD11b and NKp46 + CD27 positive), activation (NKp46 + CD107α positive) and exhaustion (NKp46 + PD-1 positive) of murine NK cells after the isolation procedure. 7-amino-actinomycin D (7-AAD, eBiosciences) viability solution was used for the exclusion of non-viable cells in FACS analysis. Kaluza 1.2 flow cytometry analysis software (Beckman Coulter Inc.) was used for FACS data analysis.

The possibility of *ex-vivo* NK cell expansion (obtained from C57BL/6 mice) was investigated in presence of recombinant murine IL-15 (R&D systems). But, we found altered expression levels of various cell receptors; therefore we decided to use freshly purified NK cells, without *ex-vivo* expansion (see supporting information).

Ex-vivo ^{111}In-oxine labeling of murine NK cells

Different labeling buffers, including phosphate buffered saline (PBS, pH 7.4); Tris-HCl; 0.9% saline + 25% human serum albumin (HSA); RPMI + 10% FCS; RPMI; and RPMI + 10% HEPES Buffer were evaluated to optimize the labeling conditions. Purified murine NK cell suspensions were washed 3 times with buffer solution and incubated with ^{111}Indium-8-hydroxyquinoline (^{111}In-oxine, half-life= 67h, gamma energy= 171/245 keV) from a ready-to-use radiopharmaceutical vial (Mallinckrodt Medical B.V., Netherlands) in a final volume of 1 ml at 37 ºC. Various incubation periods (from 5 to 30 min) were tested. During incubation the cell suspension was gently swirled periodically to avoid cell sedimentation. After incubation, centrifugation was performed to obtain a radiolabeled cell pellet that was washed 3 times with 5 ml buffer solution. The cell pellet containing radiolabeled murine NK cells and the supernatant containing unbound ^{111}In-oxine were counted separately in an automated gamma counter (LKB-Wallac, Finland) and the labeling efficiency (%LE, i.e. radioactivity incorporated to the cells) was calculated. After the radiolabeling procedure, cell viability was again assessed by the trypan blue exclusion test.

For ex-vivo stability evaluation, we radiolabeled 1 million murine NK cells with 0.011 MBq of ^{111}In-oxine and incubated in 96 well plates with PBS (pH 7.4) at 37 ºC. After regular time-intervals, cells were harvested by centrifugation, and gamma counting of cell pellet and supernatant was performed to calculate % release of the radiolabel from labeled NK cells.

^{51}Cr release assay for ^{111}In-oxine labeled murine NK cells

A chromium-51 (^{51}Cr) release assay was performed to evaluate whether the cytotoxic activity of the NK cells is maintained after the radiolabeling procedure. In this assay, ^{51}Cr labeled murine lymphoma YAC-1 cells were used as 'target cells' and freshly purified ^{111}In-oxine labeled or unlabeled (control) murine NK cells were used as 'effector cells'. YAC-1 cells were labeled with 3.7 MBq of ^{51}Cr (half-life 27.7 days, gamma energy 320 keV) from a ready-to-use radiopharmaceutical vial (Mallinckrodt Medical B.V., Netherlands) for 1 hour and 30 minute at 37 ºC in an incubator with a 5% CO_2 and 95% air atmosphere. In 96-well 'V' bottom plates, 1 x 10^3 YAC-1 cells (target) per well were plated in triplicate, purified labeled or unlabeled murine NK cells (effector) were added in different ratios of effector and target cells (i.e. 25:1, 6:1, 1.5:1, 0.5:1, 0:1). For spontaneous release, target cells were incubated without effector cells in assay medium alone. Plates were centrifuged at 460 g for 3

min and incubated for 8 hours at 37 ºC in an atmosphere with 5% CO_2 and 95% air.[12] After the incubation period, supernatant and cell pellet were collected separately and counted in an automated gamma counter to determine the specific lysis of ^{51}Cr labeled YAC-1 cells (target). The percentage of specific lysis was calculated as follows:
= 100 x [(experimental release - spontaneous release) / (total release- spontaneous release)].[13]
Results are presented as percent ^{51}Cr release (specific lysis) for the indicated type of murine NK cell (labeled or unlabeled) to tumor cell ratio.
We also evaluated change in CD107α expression by FACS analysis, to correlate the cytotoxic activity levels of NK cells observed in ^{51}Cr release assay with the expression of CD107α, which is activation marker of NK cells.

Physiological distribution of labeled murine NK cells in SCID mice

We performed an *ex-vivo* organ counting assay to evaluate the physiological biodistribution of ^{111}In-oxine labeled murine NK cells. In this experiment, we aim to differentiate between ^{111}In inside NK cells and ^{111}In that released from the cells after labelling. We left the ^{111}In-oxine labelled cells in culture media at room temperature and collected the supernatant after centrifugation at 1500g for 5 mins to avoid any cells or cell debris contamination, i.e. released ^{111}In. However, we did not evaluate the nature of radioactivity present in the supernatant. Male CB17/Icr-Prkdc^{scid}/IcrlcoCrl mice (8-10 weeks) were acclimatised for one week in a pathogen-free facility, without prophylactic antibiotics. Mice had access to sterilized food and water ad libitum.[14] One million freshly isolated, ^{111}In-oxine labeled murine NK cells were intravenously (i.v.) injected in one group of mice (n= 3), while the second group of mice (n=3) received released ^{111}In. At 24 hour post-injection (p.i.), all mice were sacrificed and major organs and tissues were collected (blood, urine, bladder, heart, trachea, lung, thymus, liver, spleen, stomach, pancreas, small bowel, large bowel, kidneys, fat, muscle, bone, and brain) and weighed. Radioactivity in these tissues was measured in an automated gamma counter. Organ radioactivity is represented as standardized uptake value (SUV), using the formula: [(tissue activity concentration (MBq/g)]/ [(injected dose (MBq)/ body weight (g)].

Biodistribution studies in orthotopic A549 human lung tumor mice

Male CB17/Icr-Prkdc^{scid}/IcrlcoCrl mice (8-10 weeks) were housed in individually ventilated cages (IVC) for one week for acclimatisation. To assess murine NK cell trafficking towards lung tumor lesions in the orthotopic A549 human lung tumor mouse model, we performed an *ex-vivo* organ counting assay. To evaluate the possibility of interference with autologous murine NK cells, in the biodistribution of adoptively transferred NK cells, we performed whole-body irradiation and compared the results with non-irradiated mice group.
Both groups (irradiated and non-irradiated) of mice were i.v. injected with about $1x10^6$ freshly isolated ^{111}In-oxine labeled murine NK cells. Mice were sacrificed at 6h, 24h and 72h p.i. (n= 5 per group for each time point), major organs and tissues were collected (as stated above), weighed and radioactivity in the samples was measured in an automated gamma counter. Organ radioactivity was represented as SUV. Based on visual inspection of the lungs, a semi-quantitative score was given to lung tumor burden: i.e. Score 1 for <25% lung covered

with tumor nodules, Score 2 for ~50% lung covered with tumor nodules, and Score 3 for >75% lung covered with tumor nodules. Lung tissue was collected in formalin for further analysis by histology.

To evaluate the effect of irradiation alone an additional group of orthotopic A549 lung tumor-bearing mice without administration of radiolabeled murine NK cells (n=5) was sacrificed at 72h after 2.4 Gy whole-body irradiation. Histology of lung tissue samples was performed.

Histology

To evaluate the status of lung tumor lesions, we performed histology of tissues samples from different groups of mice. In brief, organs were stored in formalin vials and paraffin-embedded. Five micrometre sections of lung tissues were freed of paraffin, stained with haematoxylin and eosin (H&E) and examined by microscopic evaluation.

Statistical analysis

Quantitative variables are expressed as mean ± standard deviation (SD). The Student's t test for independent samples was used to compare the physiological distribution of ^{111}In-oxine labeled murine NK cells versus released ^{111}In, and to compare the ^{111}In labeled murine NK cells uptake in different organs in irradiated versus non-irradiated mice groups. Statistical significance was set at $p<0.05$.

RESULTS

Murine NK cell isolation

FACS analysis with the 7-AAD viability solution confirmed that a high proportion of viable murine NK cell population (>95%) was isolated from transgenic Scid-Tg(FCGR3A)Blt mice spleens using the anti-CD49b (DX5) micro-beads positive isolation method. FACS analysis demonstrated that isolated murine NK cells were >95% pure (NKp46 + CD49b and CD49b + huCD16 positive), >93% mature (NKp46 + CD11b and NKp46 + CD27 positive), ~70% activated (NKp46 + CD107α positive) and only ~7% exhausted (NKp46 + PD-1 positive).

Ex-vivo ^{111}In-oxine labeling of murine NK cells

The optimal LE (>25%) without affecting the cell viability was achieved using 0.011 MBq of ^{111}In-oxine per million purified murine NK cells. Higher amounts of radioactivity resulted in a substantial loss of viability of NK cells. PBS (pH 7.4) buffer and a 20-min incubation period provided the best labeling conditions (Figure 1a). The *ex-vivo* stability assay demonstrated a release of ~30% label during >5h incubation period in PBS buffer (pH 7.4) at 37 ºC (Figure 1b).

^{51}Cr release assay for ^{111}In-oxine labeled murine NK cells

Both ^{111}In-oxine labeled and unlabeled (control) murine NK cells demonstrated cytotoxic activity in the ^{51}Cr release assay. No significant difference in the cytotoxic activity level ($p>0.1$), i.e. percentage of specific lysis, was observed in ^{111}In-oxine labeled cells in comparison to unlabeled murine NK cells (Figure 2a). The FACS analysis results of murine

NK cells that revealed an up-regulated expression of the activation marker CD107α in ^{111}In-oxine labeled cells in comparison to unlabeled murine NK cells (89.3% ± 2.4% versus 70% ± 1%, p= 0.009, Figure 2b) already before the start of the ^{51}Cr release assay (Figure 2c).

Physiological distribution of labeled murine NK cells in SCID mice

Biodistribution studies in SCID mice demonstrated that at 24h p.i. ^{111}In-oxine labeled murine NK cells mainly accumulate in the spleen, and to a lesser extent in the liver (Figure 3). In contrast, released ^{111}In showed significantly lower uptake in spleen and liver (7.0 ± 3.5 versus 120 ± 40 and 3.0 ± 1.2 versus 21.9 ± 2.0, respectively) and was mainly excreted from the body via the kidneys into the urine (2.13 ± 0.42 versus 0.60 ± 0.14). Low lung uptake of ^{111}In-oxine labeled cells (1.22 ± 0.22) suggested negligible damage to the murine NK cells during the isolation and/or labeling procedure.

Biodistribution studies in orthotopic A549 human lung tumor mouse model

Non-irradiated orthotopic lung tumor-bearing mice demonstrated higher uptake at 6h after injection of ^{111}In-oxine labeled murine NK cells in comparison with irradiated mice (1.93 ± 0.42 versus 1.07 ± 0.6; p=0.06). Both irradiated and non-irradiated mice groups demonstrated higher lung uptake at 24h p.i. in comparison with mice without lung tumors (2.07 ± 0.30, 1.51 ± 0.54 and 1.22 ± 0.22 for irradiated tumor-bearing mice, non-irradiated tumor-bearing mice and control mice, respectively). In irradiated mice, lung tumor uptake of ^{111}In-oxine labeled murine NK cells decreased between 24h and 72h (2.07 ± 0.30 versus 1.15 ± 0.39; p=0.01) (Figure 5a). This decrease in tumor uptake was accompanied by tumor regression (score 3 to score 1) (Figure 4, Table 1). In non-irradiated mice, the accumulation of labeled murine NK cells in lung tumor remained unchanged during same period, i.e. 24h versus 72h (1.51 ± 0.54 versus 1.48 ± 0.12; p=0.9) (Figure 5b). Unlike irradiated mice, the lung tumor in non-irradiated mice did not show major tumor regression (score 3 to score 2/3) (Figure 5c). These results were confirmed by histology of lung tissue samples.

In mice treated with only irradiation without administration of ^{111}In-oxine labeled murine NK cells, a high tumor burden (score 3) was observed at 72h after irradiation (Figure 6).

These results are indicating that irradiation in combination with murine NK cell transfer, but not irradiation alone, induced an anti-tumor effect in A549 lung tumor bearing mice.

Histology

H&E staining of lung tissues showed proper tumor growth in 2 weeks duration and substantial infiltration of ^{111}In-labeled NK cells (Figure 7 a-c).

H&E staining of lung tissue sections of irradiated and non-irradiated tumor bearing mice confirmed that the lung tumor regressed in 'irradiated and NK cells injected' mice group at 72h p.i., while in other groups there was no considerable changes (Figure 8). This data was in accordance with our visual semi-quantitative scores for lung tumor burden (showed in Table-1).

DISCUSSION

Until today, researcher were emphasising only on ^{111}In labeled *human NK cells* in their research, but none of the study was performed to investigate the opportunity of using ^{111}In labeled *murine NK cells*. Therefore, we developed an optimised protocol for ^{111}In-oxine labeling of murine NK cells, including selection of the ideal buffer solution, accurate amount of radioactivity and adequate incubation time. We evaluated six different buffers for radiolabeling and *ex-vivo* stability assay. PBS provided optimal results. A possible explanation of this could be that PBS does not contain any proteins that may cause chelation of ^{111}In-hydroxyquinoline in the reaction mixture. To select the accurate amount of radioactivity, we radiolabeled purified murine NK cells with an increasing amount of ^{111}In-oxine. ^{111}In-oxine is known for its cytotoxic property at higher concentrations. In this study, we also observed a decreasing cell viability pattern with an increasing amount of ^{111}In-oxine. To select the best incubation time, the labeling efficiency was evaluated at different time points. We found that the labeling efficiency increased during the first 20 min. Therefore, this time was selected as an adequate incubation time for the radiolabeling procedure. Because only a very low dose of ^{111}In-oxine was used for the radiolabeling procedure, no loss in the biological activity of the murine NK cells was observed. The ^{51}Cr release assay demonstrated no significant difference in cytotoxic activity between labeled and unlabeled murine NK cells. However labeled murine NK cells seemed to become more activated due to isolation and radiolabeling procedure and showed a higher expression of CD107α than unlabeled NK cells.

Studies demonstrated that the CD16 receptor that presents in human NK cells is responsible for recognizing the Fc domain of therapeutic IgG1 antibodies and mediates Fc related immune effectors functions;[23] therefore we intended to investigate the trafficking of human CD16 receptor positive NK cells. For this purpose, we isolated NK cells from Scid-Tg(FCGR3A)Blt transgenic mice, which are generated to express human CD16 receptor. After ^{111}In-oxine labeling, we adoptively transfer murine NK cells in the orthotopic A549 human lung tumor mouse model for biodistribution study. To deplete autologous murine NK cells that may interfere with the biodistribution of adoptively transferred NK cells, we performed whole-body irradiation using an X-ray source, and compared the results with non-irradiated mice group. A low X-ray radiation dose, i.e. 2.4 Gy, was used to irradiate the mice,[11] because higher irradiation dose, usually >3 Gy, may cause side-effects in mice such as, anaemia, weight loss, infection, intestinal bleeding and incisor damage. We did not observe any such health consequences in mice at this radiation dose. The mouse model used in the study could be considered as an appropriate model to validate the effect of new immune-activating drugs on immune cells, since it provides us a unique opportunity to investigate the trafficking of human CD16 receptor positive NK cells in orthotopic human tumor mouse model.

To reduce the number of transgenic mice for the isolation of NK cells, the possibility of *ex-vivo* cell expansion was evaluated (see supporting information). Murine NK cells (obtained from C57BL/6 mice) expansion in the presence of recombinant murine IL-15 (R&D systems) for one week induced cell proliferation and caused activation of the NK cells (i.e. up regulation of CD107α and CD69). Furthermore, FACS analysis also revealed altered expression levels of various cell receptors that include CD49b, CD27, and PD-1. A similar

phenomenon was also observed by other researchers using human NK cells.[24] We, therefore, decided to use freshly purified NK cells, without *ex-vivo* expansion, for the radiolabeling procedure for all *in-vivo* experiments.

Experiments to evaluate the physiological biodistribution of ^{111}In-oxine labeled murine NK cells and released ^{111}In revealed that released ^{111}In had an entirely different uptake pattern than ^{111}In-oxine labeled murine NK cells. As expected, ^{111}In-oxine labeled murine NK cells accumulated mainly in spleen, which was their home organ from where we purified NK cells. On the other hand, released ^{111}In was mainly excreted via kidneys into urine. Consequently, we can exclude the possibility of accumulation of released ^{111}In in any target organ of interest.

Biodistribution data revealed a significantly higher uptake of ^{111}In-oxine labeled murine NK cells in the lungs of mice with orthotopic lung tumors in comparison with control mice, which suggests specific migration of the labeled NK cells to the tumor lesions. According to the '*EANM guidelines for the labeling of leucocytes with ^{111}In-oxine*'[16] diffuse retention of ^{111}In labeled cells in lungs is an indication of cell damage as a result of the labeling procedure. We did not find such uptake in the lungs of control animals, indicating that the labeled murine NK cells had remained intact during labeling. Moreover, our data demonstrated a higher activity in spleen than in liver, which is another measure to demonstrate cell integrity after labeling. According to the same EANM guidelines, liver activity equal to or higher than spleen activity indicates cell damage.[16]

Biodistribution experiment in irradiated versus non-irradiated mice groups injected with ^{111}In-labeled murine NK cells demonstrated a significant decrease in uptake between 24h and 72h p.i. in irradiated mice group that was accompanied by tumor regression. Histology data of lung tissue sample and visual semi-quantitative scores also confirmed the considerably lower lung tumor burden. We did not evaluate the possibility if there is any effect of irradiation on normal lung tissue that may contribute in increased uptake. The higher uptake in irradiated group could be associated with the fact that the irradiation process could have provoked some stress or damage to tumour or normal lung tissue cells, which might have triggered the migration of the adoptively transferred labeled NK cells. In contrast, non-irradiated mice injected with murine NK cells and irradiated mice that were not injected with NK cells did not demonstrated such decrease in uptake and/or tumor regression at 72h p.i., which was confirmed by histological data. This could be because of the negative effect of the residual NK cells against the adoptive infused labeled NK cells in the non-irradiated mice group; and due to negligible circulating immune cells in irradiated mice group that were not injected with labeled NK cells. Therefore, our data indicates that irradiation in combination with murine NK cell administration, but not irradiation or NK cell administration alone, induced a remarkable anti-tumor effect. This study demonstrated application of murine ^{111}In-labelled NK cells for *ex-vivo* biodistribution studies, however this could also be associated with therapeutic effects when combined with irradiation. Other indirect imaging approaches, including tracer dose of radiolabeled antibody or peptide, may not need irradiation and have negligible coincidental therapeutic effects. Nevertheless, while extrapolating mice data to humans, we should also consider that cancer induced in mice could be in a different

microenvironment than those we observe in human patients and that the host response to cancer could be different in humans.

CONCLUSIONS

This study demonstrated that isolation and radiolabeling of murine NK cells with ^{111}In-oxine is feasible without affecting their viability and functionality, provided that only low doses of radioactivity are used. We also describe a method to evaluate the biodistribution and trafficking of murine NK cells, which might be useful for the determination of potential effects of immune mediated drugs on NK cell trafficking. Nevertheless, these data also demonstrated that care should be taken when applying labeled NK cells in intervention studies as they can potentially augment the therapeutic effect of the intervention.

ASSOCIATED CONTENT

Supporting Information

Data of *ex-vivo* expansion of NK cells isolated from the spleens of C57BL/6 black mice and receptor expression on expanded cells in comparison to freshly isolated murine NK cells. This material is available free of charge via the Internet at http://pubs.acs.org.

ACKNOWLEDGEMENT

We thank Sara Colombetti and Claire Dunn for helping in cell isolation experiments, Filippo Galli for useful advices on NK labeling and Nuclear Medicine Discovery for support in project development. The research work performed in the manuscript was funded by the Roche Postdoctoral Fellowship Program, Pharma Research and Early Development, Roche Innovation Center, Basel, Switzerland. Contribution of AS was partially supported by AIRC research grant IG-14151.

REFERENCE

1. Gao, J. Q.; Okada, N.; Mayumi, T.; Nakagawa S. Immune cell recruitment and cell-based system for cancer therapy. *Pharm. Res*. **2008**, 25, 752–768.
2. Malviya, G.; Galli, F.; Sonni, I.; Pacilio, M.; Signore, A. Targeting T and B lymphocytes with radiolabelled antibodies for diagnostic and therapeutic applications. *Q. J. Nucl. Med. Mol. Imaging* **2010**, 54(6), 654-676.
3. Malviya, G.; Galli, F.; Sonni, I.; Signore, A. Imaging T-lymphocytes in inflammatory diseases: a nuclear medicine approach. *Q. J. Nucl. Med. Mol. Imaging* **2014**, 58(3), 237-257.
4. Meller, B.; Frohn, C.; Brand, J. M.; Lauer, I.; Schelper, L. F.; von Hof, K.; Kirchner, H.; Richter, E.; Baehre, M. Monitoring of a new approach of immunotherapy with allogenic (111)In-labelled NK cells in patients with renal cell carcinoma. *Eur. J. Nucl. Med. Mol. Imaging* **2004**, 31(3), 403-407.
5. Brand, J. M.; Meller, B.; Von Hof, K.; Luhm, J.; Bähre, M.; Kirchner, H.; Frohn, C. Kinetics and organ distribution of allogeneic natural killer lymphocytes transfused into patients suffering from renal cell carcinoma. *Stem Cells Dev*. **2004**, 13(3), 307-314.
6. Matera, L.; Galetto, A.; Bello, M.; Baiocco, C.; Chiappino, I.; Castellano, G.; Stacchini, A.; Satolli, M. A.; Mele, M.; Sandrucci, S.; Mussa, A.; Bisi, G.; Whiteside, T. L. In vivo migration of labeled autologous natural killer cells to liver metastases in patients with colon carcinoma. *J. Transl. Med*. **2006**, 4, 49.
7. Luhm, J.; Brand, J. M.; Koritke, P.; Höppner, M.; Kirchner, H.; Frohn, C. Large-scale generation of natural killer lymphocytes for clinical application. *J. Hematother. Stem Cell Res*. **2002**, 11(4), 651-657.
8. Carlens, S.; Gilljam, M.; Chambers, B. J.; Aschan, J.; Guven, H.; Ljunggren, H. G.; Christensson, B.; Dilber, M. S. A new method for in vitro expansion of cytotoxic human CD3- CD561 natural killer cells. *Hum. Immunol*. **2001**; 62, 1092–1098.
9. Frohn, C.; Doehn, C.; Durek, C.; Böhle, A.; Schlenke, P.; Jocham, D.; Kirchner, H. Feasibility of the adoptive transfusion of allogenic HLA-matched NK-cells in patients with renal cell carcinoma. *J. Immunother*. **2000**, 23, 499–504.
10. Gerdes, C. A.; Nicolini, V. G.; Herter, S.; van Puijenbroek, E.; Lang, S.; Roemmele, M.; Moessner, E.; Freytag, O.; Friess, T.; Ries, C. H.; Bossenmaier, B.; Mueller, H. J.; Umaña, P. GA201 (RG7160): a novel, humanized, glycoengineered anti-EGFR antibody with enhanced ADCC and superior in vivo efficacy compared with cetuximab. *Clin. Cancer Res*. **2013**, 19(5), 1126-1138.
11. Miyakawa, Y.; Ohnishi, Y.; Tomisawa, M.; Monnai, M.; Kohmura, K.; Ueyama, Y.; Ito, M.; Ikeda, Y.; Kizaki, M.; Nakamura, M. Establishment of a new model of human multiple myeloma using NOD/SCID/gammac(null) (NOG) mice. *Biochem. Biophys. Res. Commun*. **2004**, 313(2), 258-262.
12. Bhat, R.; Watzl, C. Serial killing of tumor cells by human natural killer cells- enhancement by therapeutic antibodies. *PLoS. One*. **2007**, 2(3), e326.
13. De Colvenaer, V.; Taveirne, S.; Hamann, J.; de Bruin, A. M.; De Smedt, M.; Taghon, T.; Vandekerckhove, B.; Plum, J.; van Lier, R.; Leclercq, G. Continuous CD27 triggering in vivo strongly reduces NK cell numbers. *Eur. J. Immunol*. **2010**, 40(4), 1107-1117.
14. Malviya, G.; D'Alessandria, C.; Bonanno, E.; Vexler, V.; Massari, R.; Trotta, C.; Scopinaro, F.; Dierckx, R. A.; Signore, A. Radiolabeled Humanized Anti-CD3 monoclonal antibody visilizumab for imaging human T-lymphocytes. *J. Nucl. Med*. **2009**, 50(10), 1683-1691.

15. Welling, M. M.; Duijvestein, M.; Signore, A.; van der Weerd, L. In vivo biodistribution of stem cells using molecular nuclear medicine imaging. *J. Cell Physiol.* **2011**, 226(6), 1444-1452.
16. Roca, M.; de Vries, E. F.; Jamar, F.; Israel, O.; Signore, A. Guidelines for the labelling of leucocytes with (111)In-oxine. Inflammation/Infection Taskgroup of the European Association of Nuclear Medicine. *Eur. J. Nucl. Med. Mol. Imaging.* **2010**, 37(4), 835-841.
17. Botti, C.; Negri, D. R.; Seregni, E.; Ramakrishna, V.; Arienti, F.; Maffioli, L.; Lombardo, C.; Bogni, A.; Pascali, C.; Crippa, F.; Massaron, S.; Remonti, F.; Nerini-Molteni, S.; Canevari, S.; Bombardieri, E. Comparison of three different methods for radiolabelling human activated T lymphocytes. *Eur. J. Nucl. Med.* **1997**, 24(5), 497-504.
18. Stojanov, K.; de Vries, E. F.; Hoekstra, D.; van Waarde, A.; Dierckx, R. A.; Zuhorn, I. S. [18F]FDG labeling of neural stem cells for in vivo cell tracking with positron emission tomography: inhibition of tracer release by phloretin. *Mol. Imaging.* **2012**, 11, 1–12.
19. Adonai, N.; Nguyen, K. N.; Walsh, J.; Iyer, M.; Toyokuni, T.; Phelps, M. E.; McCarthy, T.; McCarthy, D. W.; Gambhir, S. S. Ex vivo cell labeling with 64Cu-pyruvaldehyde-bis(N4-methylthiosemicarbazone) for imaging cell trafficking in mice with positron-emission tomography. *Proc. Nat. Acad. Sci.* **2002**, 99, 3030–3035.
20. Bansal, A.; Pandey, M. K.; Demirhan, Y. E.; Nesbitt, J. J.; Crespo-Diaz, R. J.; Terzic, A.; Behfar, A.; DeGrado, T. R. Novel (89)Zr cell labeling approach for PET-based cell trafficking studies. *EJNMMI Res.* **2015**, 5, 19.
21. Meier, R.; Piert, M.; Piontek, G.; Rudelius, M.; Oostendorp, R. A.; Senekowitsch-Schmidtke, R.; Henning, T. D.; Wels, W. S.; Uherek, C.; Rummeny, E. J.; Daldrup-Link, H. E. Tracking of [18F]FDG-labeled natural killer cells to HER2/neu-positive tumors. *Nucl. Med. Biol.* **2008**, 35(5), 579-588.
22. Galli, F.; Rapisarda, A. S.; Stabile, H.; Manni, I.; Bonanno, E.; Piaggio, G.; Gismondi, A.; Santoni, A.; Signore, A. In vivo imaging of NK cell trafficking in tumors. *J. Nucl. Med.* **2015**, 56(10), 1575-1580.
23. Ravetch, J. V.; Perussia, B. Alternative membrane forms of Fc gamma RIII (CD16) on human natural killer cells and neutrophils. Cell type-specific expression of two genes that differ in single nucleotide substitutions. *J. Exp. Med.* **1989**, 170, 481–497.
24. Vitale, M.; Bassini, A.; Secchiero, P.; Mirandola, P.; Ponti, C.; Zamai, L.; Mariani, A. R.; Falconi, M.; Azzali, G. NK-active cytokines IL-2, IL-12, and IL-15 selectively modulate specific protein kinase C (PKC) isoforms in primary human NK cells. *Anat. Rec.* **2002**, 266(2), 87-92.

Table 1

Visual semi-quantitative scores for lung tumor burden in different mice groups

Time point	Average Score		
	Non-irradiated/ NK cell injected	Irradiated/ NK cell injected	Irradiated Only/ without NK cell injection
6 hour	3	3	N/A
24 hour	3	3	N/A
72 hour	2/3	1	3

For Score 1 <25% lung covered with tumor nodules; Score 2 ~50% lung covered with tumor nodules; and Score 3 >75% lung covered with tumor nodules.

Figure 1 (a)

Different buffers evaluated for *ex-vivo* ^{111}In-oxine labeling (mean ± SD) of murine NK cells

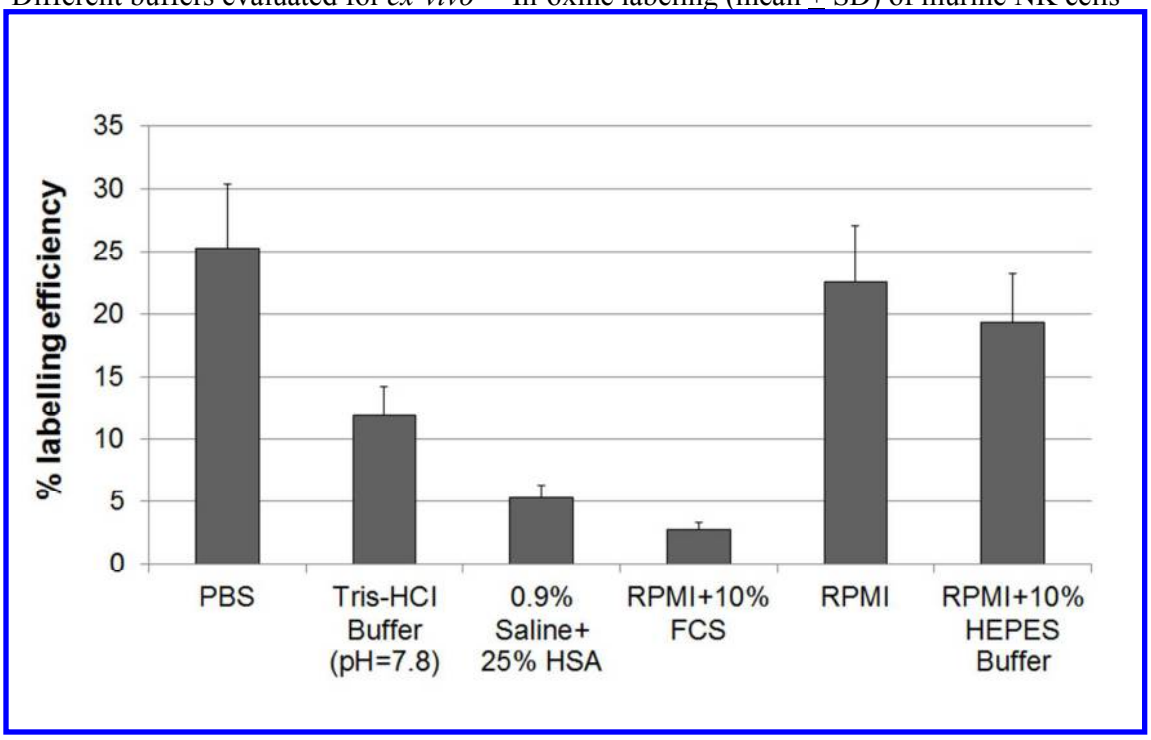


Figure 1 (b)

Percentage of retention of ^{111}In-label inside murine NK cells during incubation in PBS (pH 7.4) at 37ºC

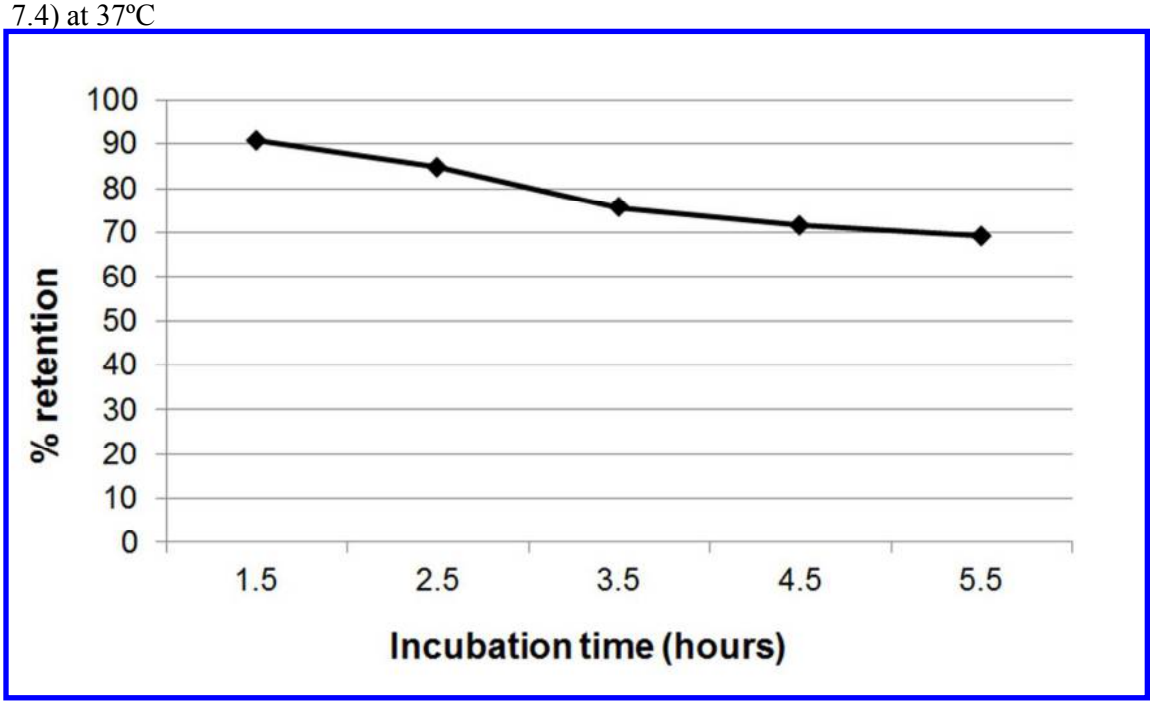


Figure 2 (a)

^{51}Cr release assay for ^{111}In-oxine labeled and unlabeled (control) murine NK cells: a higher but non-significant ($p > 0.1$, mean $\pm$ SD) cytotoxic activity level was observed in ^{111}In-oxine labeled cells.

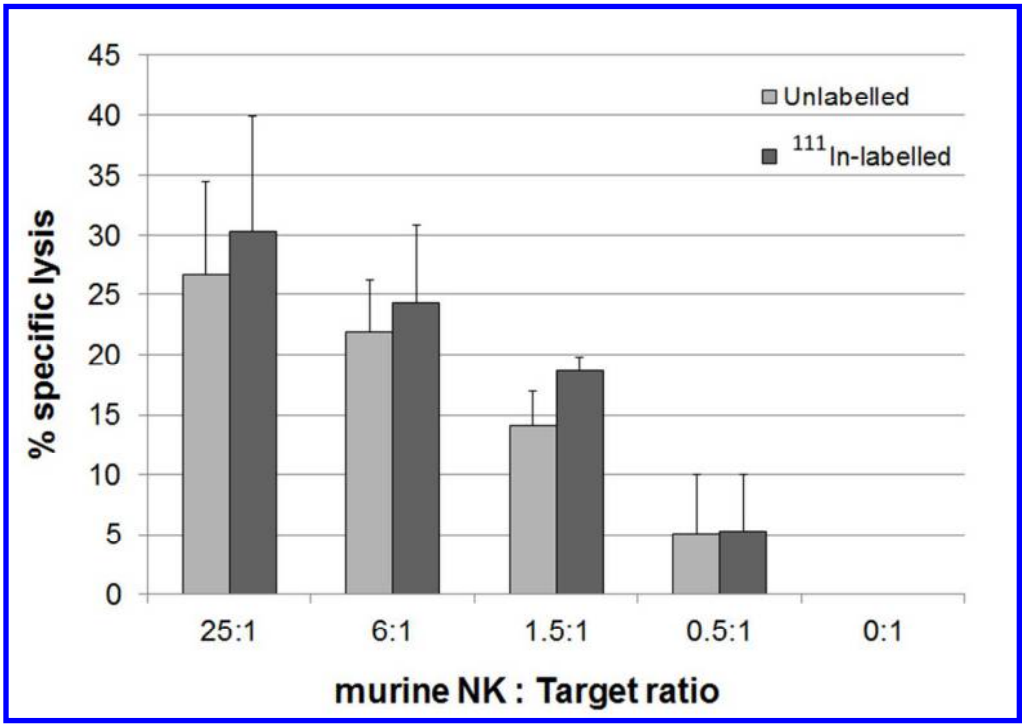


Figure 2 (b)

Expression of CD107α (mean $\pm$ SD) in murine NK cells determined by FACS analysis (* p-value <0.01)

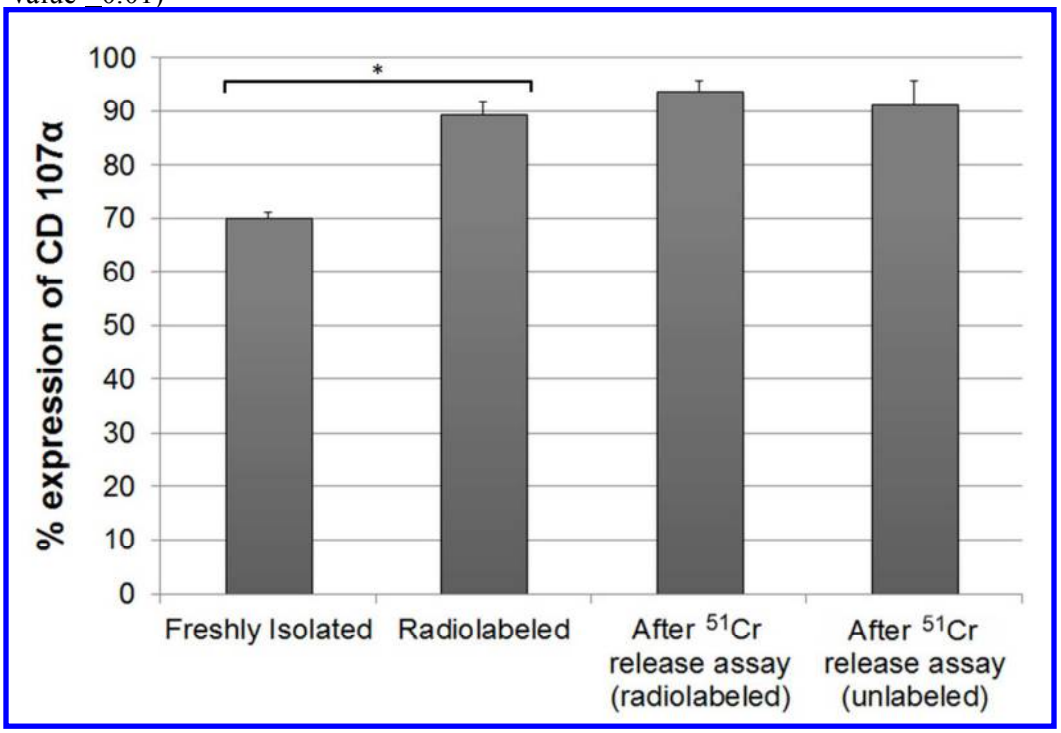


Figure 2 (c)

Representative dot plots indicating expression of CD107α (mean ± SD) in murine NK cells (NKp46$^+$ CD107α$^+$ cells) determined by FACS analysis

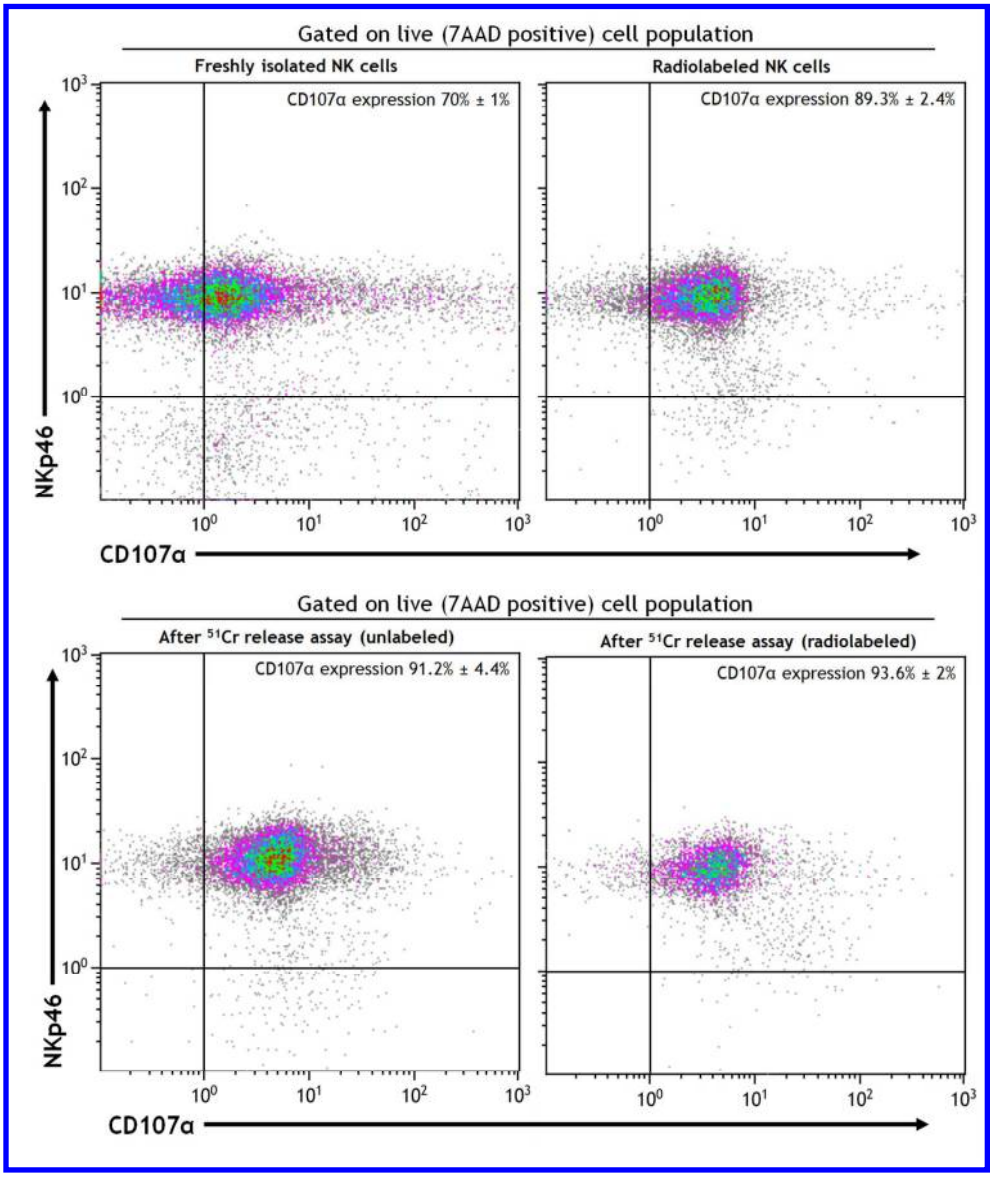


Figure 3

Biodistribution study in SCID mice injected with either ^{111}In-oxine labeled murine NK cells or ^{111}In released from labeled murine NK cells at 24h p.i. (* p-value ≤0.02, Avg SUV ± SD)

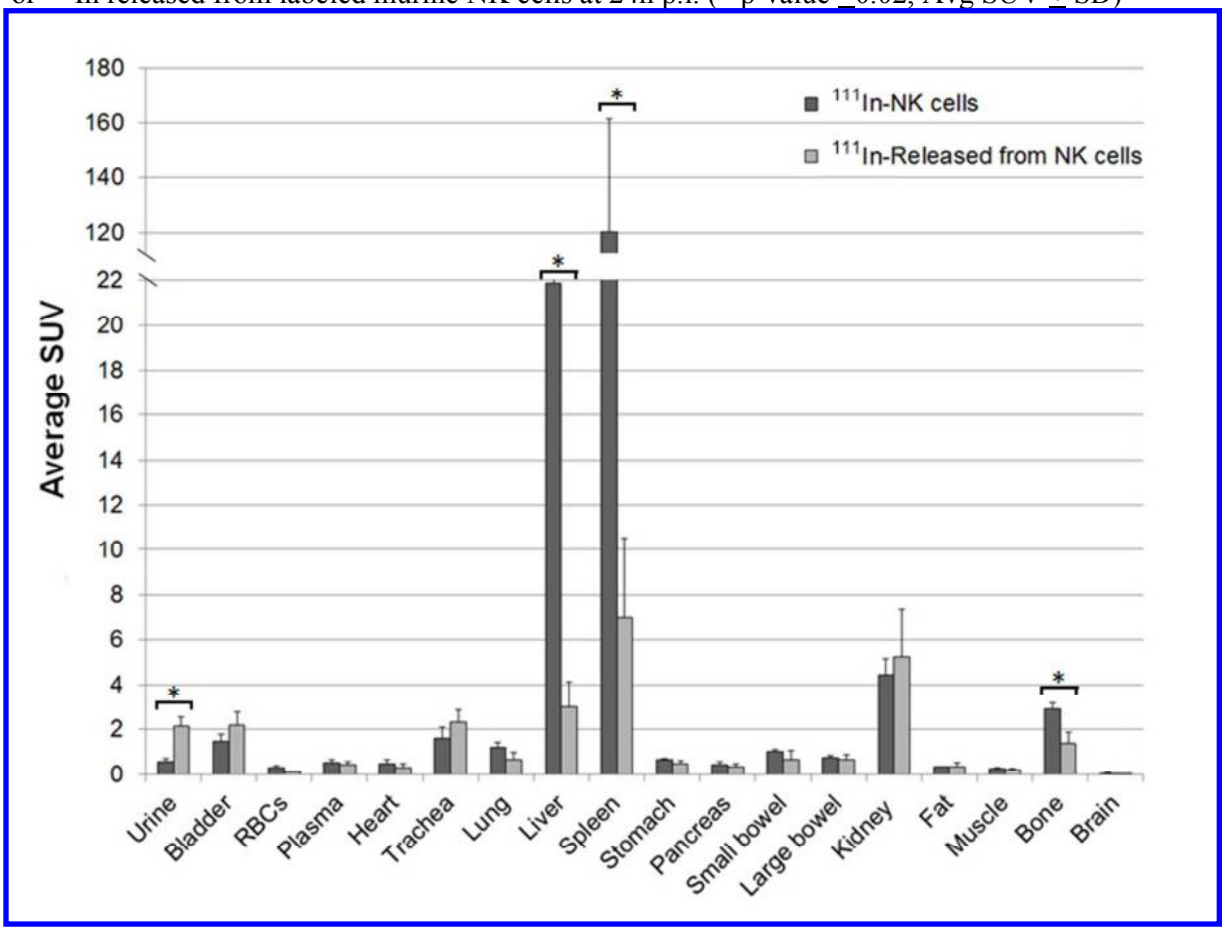


Figure 4

Representative images of visual semi-quantitative scores for lung tumor burden

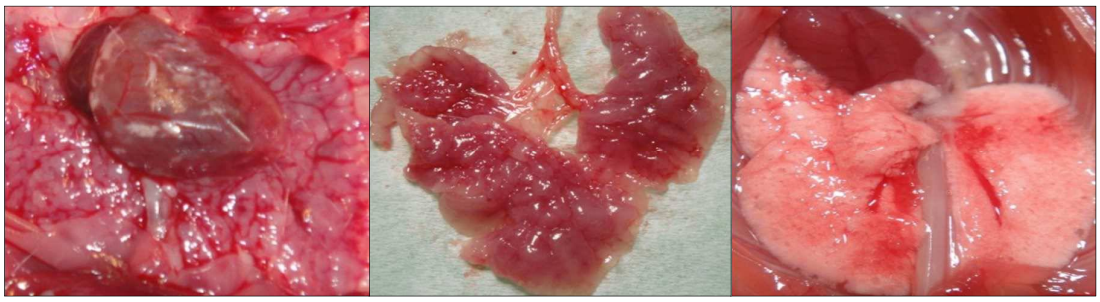
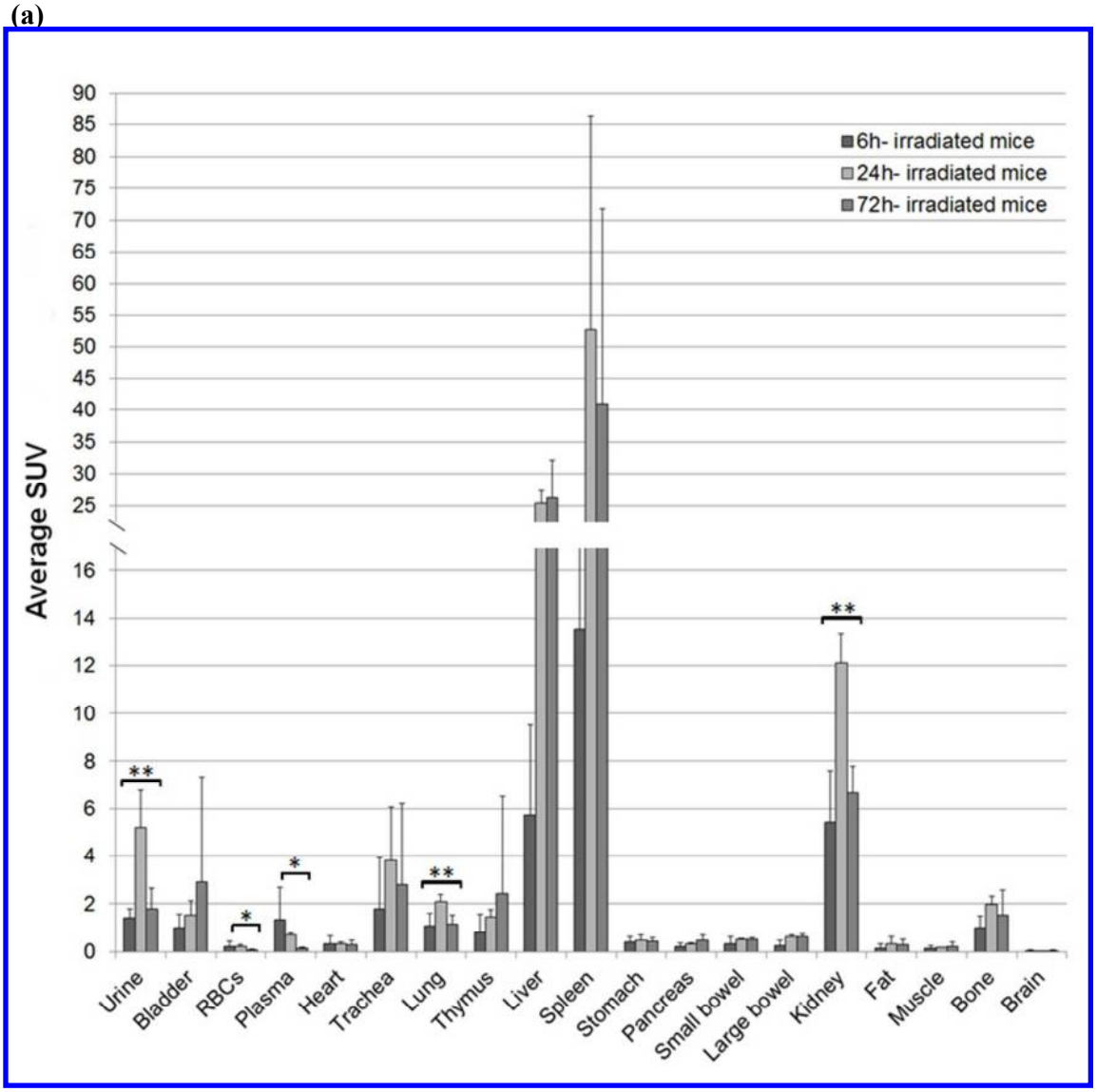


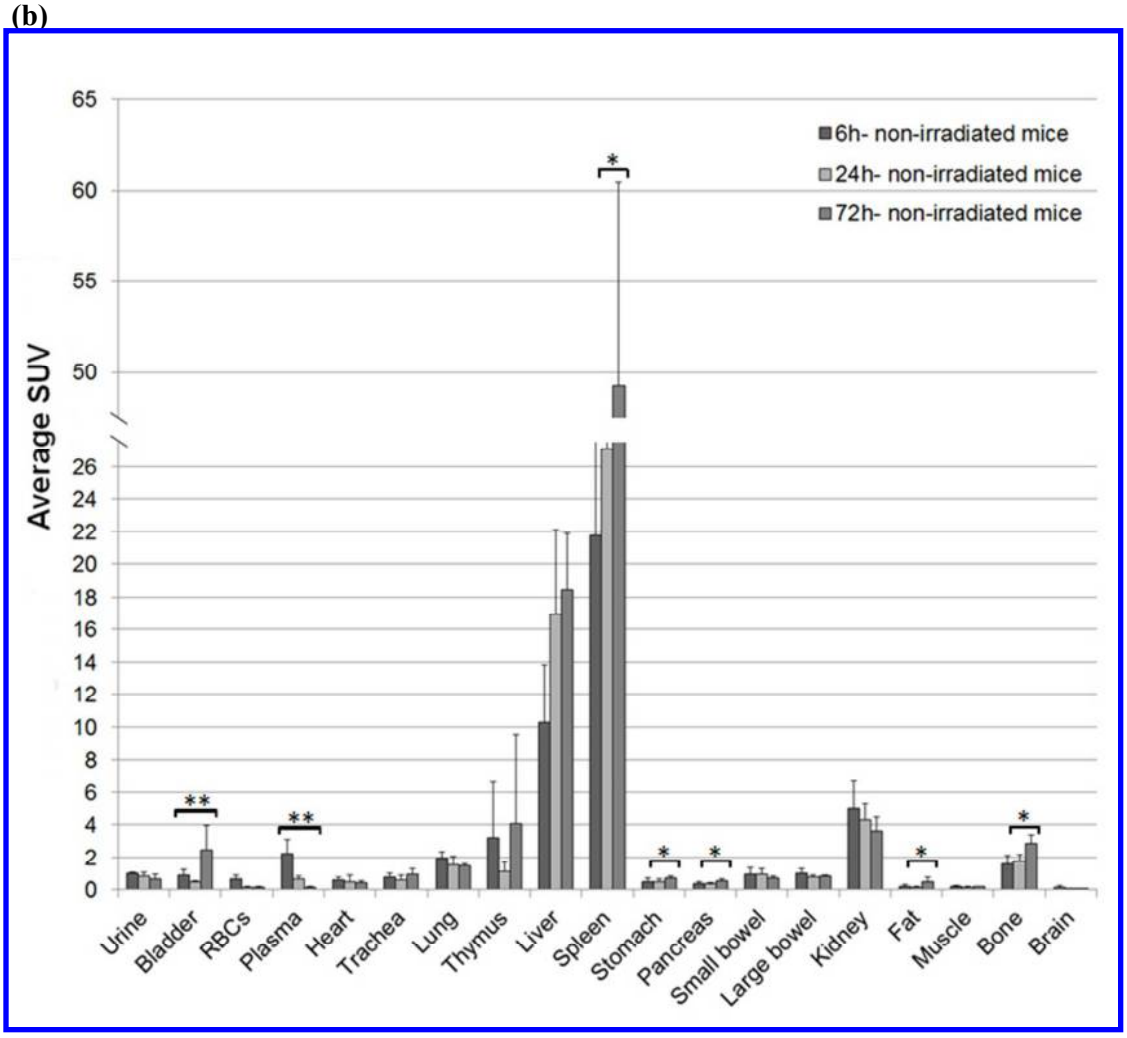
Figure 5

Biodistribution study in **(a)** irradiated and **(b)** non-irradiated SCID mice injected with ^{111}In-oxine labeled murine NK cells **(c)** comparison only in lung tissue (* p value = <0.05 - 24h Vs 72h; ** p value= <0.05 - 6h Vs 24h Vs 72h; Avg SUV ± SD)

(a)



(b)


Average SUV
65
60
55
50
26
24
22
20
18
16
14
12
10
8
6
4
2
0
6h- non-irradiated mice
24h- non-irradiated mice
72h- non-irradiated mice
**
**
*
*
*
*
*
Urine
Bladder
RBCs
Plasma
Heart
Trachea
Lung
Thymus
Liver
Spleen
Stomach
Pancreas
Small bowel
Large bowel
Kidney
Fat
Muscle
Bone
Brain

(c)

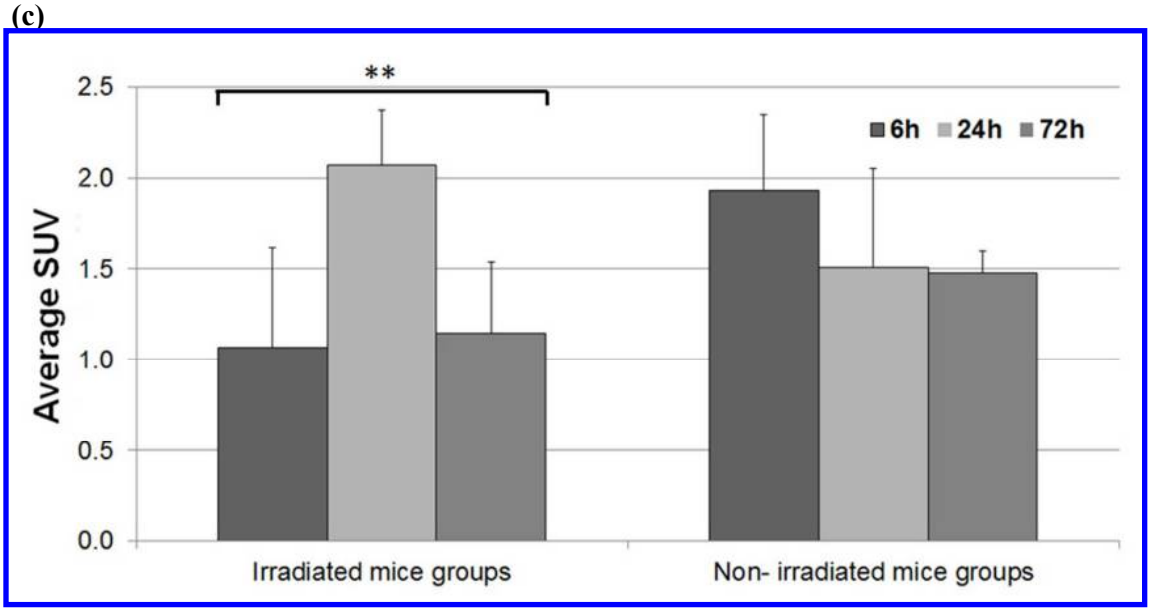


Figure 6

Representative images of lung of same mouse of only irradiation (without ^{111}In-oxine labeled NK cells administration) group showing high tumor burden (score 3).

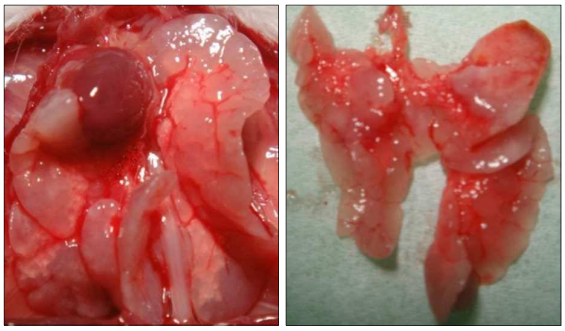


Figure 7

(a) Orthotopic lung tumor and their H&E staining at **(b)** 5x, and **(c)** 40x magnification, red arrow represents the infiltrating lymphocytes (red arrows) in tumor regions.

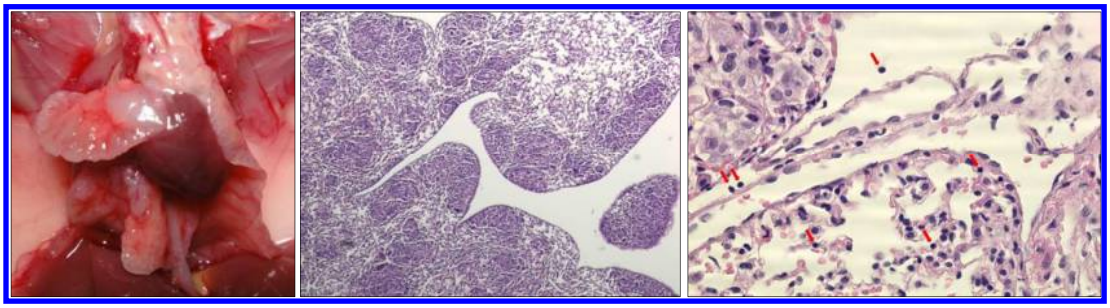
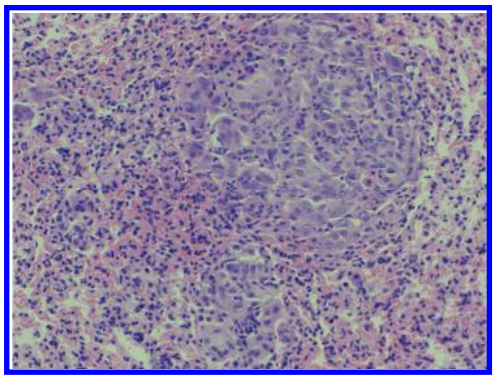
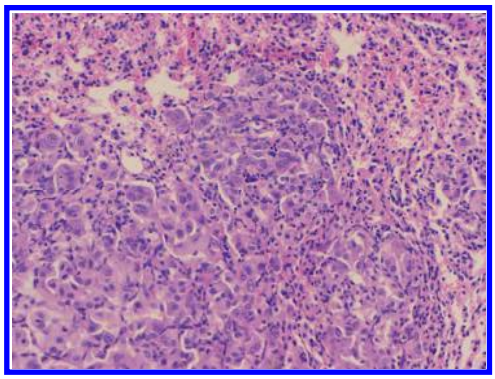
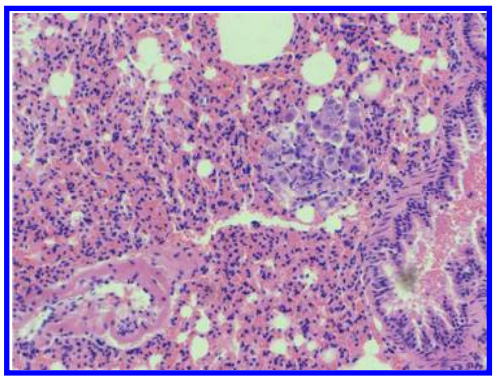
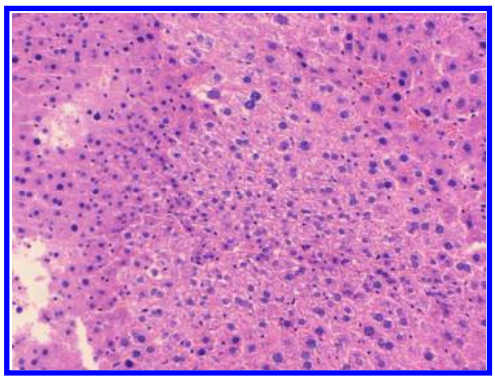
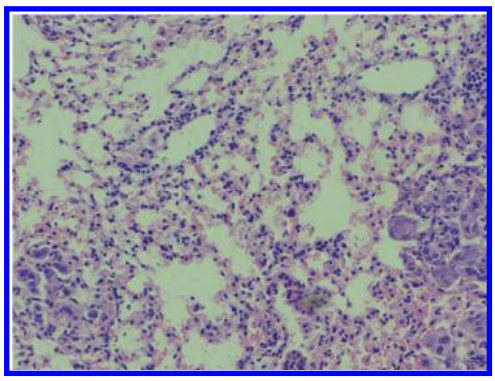
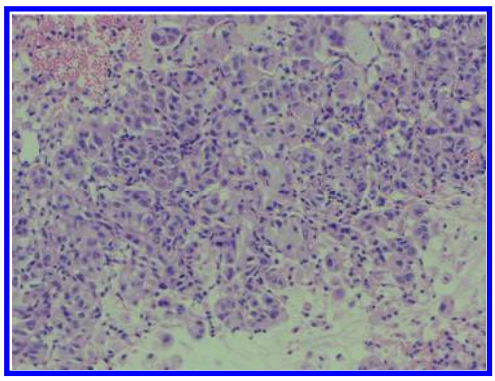
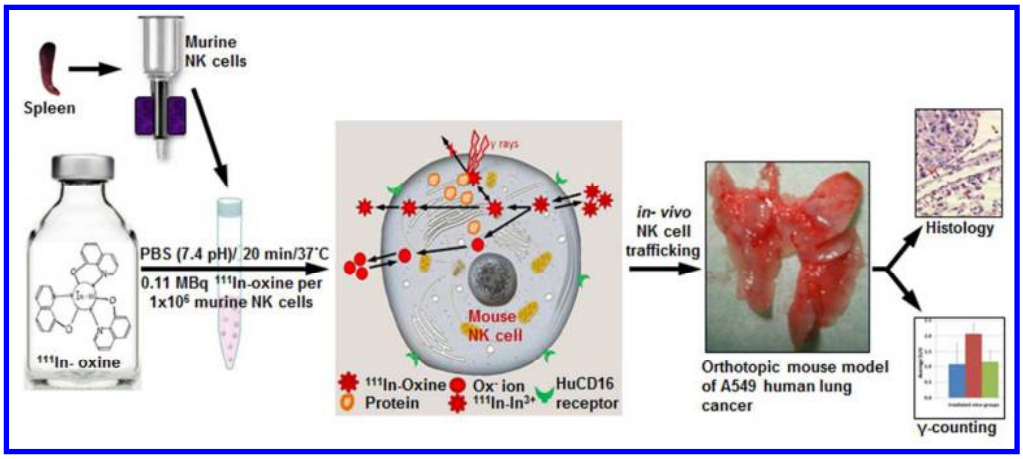


Figure 8

H&E staining of lung tissue sections of irradiated and non-irradiated tumor bearing mice confirmed that the lung tumor was regressed in irradiated mice group at 72h p.i. while in other mice groups it was unaffected.

	Irradiated mice group (20x)	Non-irradiated mice group (20x)
6h		
24h		
72h		



298x129mm (72 x 72 DPI)