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Abstract

Over the last decades, it has been established that the immune system is crucial for the impediment of cancer development by recognizing and destroying transformed cells. This process has been termed cancer immunosurveillance. Small animal models have significantly facilitated our understanding of it. Dissecting the contribution of any specific immune cell type participating in this process requires the ability to specifically target it while leaving the other immune components as well as the cancer model system unperturbed *in vivo*. Here, we provide a simple and rapid protocol for the generation of transgenic mice expressing Cre recombinase in a cell-type specific manner – in our example we chose cells expressing *Ncr1* that encodes for the surface protein NKp46 -, and the use of those mice to ablate NKp46+ cells in order to study their role in a model of cancer immunosurveillance against experimental pulmonary metastases. This protocol can easily be adapted to target other cell types and other cancer models.

1. Introduction

The term cancer immunosurveillance designates the role of the immune system in the defense against the development of cancers [1]. Many facets of this process are under study regarding the cell types involved, the effector mechanism employed, or elucidating escape mechanisms used by the cancer cells to counter recognition and destruction by the immune system.

A general approach to identify what role a specific component fulfills within a biological process is to remove that component and observe the effect this has on the performance of the process. One elegant way to apply this general approach to the study of cancer immunosurveillance uses conditional gene targeting to ablate a specific cell type *in vivo* [2]. This targeted ablation does neither interfere with other components of the cancer immunosurveillance machinery nor with the cancer model system. This genetic approach results in a high ablation efficiency, which is stable from animal to animal and not subject to inter-experimental variations in contrast to other approaches, like antibody-mediated cell depletion or adoptive cell transfer studies that require extensive manipulations of mice and cells. Therefore, we advocate conditional cell ablation as a method of choice for dissecting the contribution of specific components of the immune system to cancer immunosuveillance in a reproducible manner *in vivo*.

There are several ways to achieve the conditional ablation of a specific cell type. We will describe a system based on conditional gene targeting mediated by the Cre-loxP system. Cre is a recombinase derived from the bacteriophage P1. This recombinase recognizes short DNA sequences called loxP sites and depending on their orientation towards each other either excises the intervening DNA or inverts it [3]. Thus, two components are necessary for conditional cell ablation with this system: a) the expression of Cre specifically in the cell type to be ablated, and b) an appropriate

target DNA sequence flanked by loxP sites that upon Cre-mediated recombination results in the death of the target cell.

This protocol consists of three parts: in part I, we describe the generation of Ncr1-cre transgenic (Ncr1-cre TG) mice, necessary to direct the activity of Cre to Natural Killer (NK) cells; in part II, we describe the strategies that can be used to achieve ablation of the targeted cell type; in part III, we describe the application of this approach to a widely used system of experimental pulmonary metastases.

Importantly, as this protocol involves work with live animals, you have to make sure that all animal procedures have been reviewed and approved by your institutional animal welfare committee before starting the project.

Part I

Generation of transgenic mice expressing Cre recombinase in a cell-type specific manner in NKp46+ cells

1. Introduction

A primary requisite for cell type-specific gene targeting is the identification of a suitable promoter or regulatory elements that can be used to direct the expression of *Cre* to the cell type of choice. In the case of NK cells, it has previously been shown that NKp46, encoded by *Ncr1*, is expressed by all NK cells [4]. Subsequent work then showed that *Ncr1* is also expressed by more recently identified tissue-resident NK cells and other group 1 innate lymphoid cells [ILC] in the mouse as well as a small subset of intestinal ILC3 [5]). There are three principal ways to direct *Cre* expression to NKp46+ cells: generating either i) a transgene, where *Cre* is under the control of the *Ncr1* promoter, ii) a bacterial artifical chromosome (BAC) transgene by inserting *Cre* into the *Ncr1* locus present in the BAC, or iii) by inserting *Cre* into the *Ncr1* genomic locus.

Of note, it has been previously shown that a small promoter fragment is sufficient to direct the expression of *Ncr1* to NK cells [4]. We take advantage of this and use only a 626bp small fragment of the promoter to achieve faithful expression of *Cre* in *Ncr1* positive cells [2]. We will produce a transgene carrying a EGFP-Cre fusion. This allows for simple detection of transgene positive cells using flow cytometry and histology and is highly appreciable when untouched cells need to be isolated via cell sorting.

2. Material

2.1 Reagents

2.1.1 Plasmids/BAC clones used:

1. RPCI23-458I16: BAC clone carrying a 185kb fragment of chromosome 7 containing *Ncr1*. This BAC was derived from a female C57BL/6J mouse. It serves as template for the generation of the *Ncr1* promoter fragment. It is available from the *Children's Hospital Oakland Research Institute* (CHORI).

2. pGCIN21: plasmid carrying an enhanced green fluorescent protein (EGFP) fused to Cre, provided by Neal Copeland **[1]**.

3. pL425: plasmid carrying the bovine growth hormone poly A site (bGHpA), provided by Neal Copeland **[2]**.

2.1.2 Primers used: **[3]**

1. P1 5'- GATTGAGAGACCCTGCCTCAGTG-3'.
2. P2 5'-CAGCTCCTCGCCCTTGCTCACCATAC CAGTGCCAGACCAGTGCTGAAC-3'.
   Primer p2 carries at 3' 21-bp overlap complementary to the 5' sequence of *EGFP-Cre* from the pIGCN21 vector.
3. Pinv2 5'-GTTCAGCACTGGTCTGGCCACTGGTA TGGTGACAAGGGCGAGGAGCTG-3'.
   Primer pinv2 is the inverted version of p2, thus it carries at 5' a 28-bp overlap to the 3' sequence of the Ncr1 promoter fragment.

4. P3 5’- CTAGAGAATTGATCCCCTCAAAGCTG ATCAGTATCTAGATCC-3’. Primer 3 carries at 3’ a 20bp overlap to the bGHpA fragment.
5. Pinv3 5’- GGATCTAGATAACTGATCAGCTTTGA GGGGATCAATTCTCTAG-3’. Primer pinv3 carries at 5’ a 23-bp overlap to the to the 3’ region of the *EGFP-Cre.*
6. P4 5’- TAAGGGTTCCGCAAGCTCTAGTCG-3’.

2.1.3 Enzymes and buffers

1. Phusion® DNA polymerase
2. 5X PCR Phusion HF buffer
3. dNTPs
4. EcoRI restriction enzyme and the appropriate reaction buffer
5. NotI restriction enzyme and the appropriate reaction buffer
6. TE buffer (10mM Tris-HCl, 1mM EDTA)

2.1.4 Media

1. LB medium
2. Ampicillin
3. Chloramphenicol
4. LB agar plates containing 100µg/mL ampicillin
5. Deionized sterile water

2.2 Equipment

1. Thermal cycler

2. Agarose gel electrophoresis system

3. Table top microcentrifuge

4. Centrifuge for 15ml and 50ml tubes (for plasmid maxi preps)

5. Eppendorf 1.5ml reaction tubes

6. 37°C shaker for bacterial cultures

7. 15 ml tubes and 50 ml tubes

8. ZeroBlunt Topo PCR Cloning kit (ThermoFisher) **[4]**

9. EndoFree Plasmid Maxi Kit (Qiagen) **[4]**

10. QIAGEN Plasmid Maxi Kit **[4]**

11. QIAprep Spin Miniprep **[4]**

12. QIAquick Gel Extraction Kit **[4]**

13. Slide-a-lyzer dialysis device (ThermoFisher) **[4]**

3. Methods for part I

4 steps are necessary to generate the *Ncr1-EGFP-Cre-bGHpA* transgene by PCR-overlap-extension cloning.

General PCR protocol (50µl reaction volume):

1ng of template DNA (plasmid or BAC)

250nM primer A (final concentration)

250nM primer B (final concentration)

200µM dNTPs (final concentration each)

10µl 5X Phusion HF buffer

1 unit Phusion polymerase

add H_2O to reach 50µl final volume

General PCR program [5]

Number of cycles	PCR step	Temperature (C°)	Time
1	Initial denaturation	98	1 min
30-32	Denaturation	98	20s
	Annealing	60	20s
	Extension	72	30s/kb
1	Final extension	72	10 min

Step 1: Generation of the *Ncr1* promoter fragment

1. BAC clones are propagated as stab cultures of DH10B bacteria in LB agar containing 12.5mg/L chloramphenicol. Streak the culture on a LB agar plate containing 12.5mg/L chloramphenicol and incubate over night at 37°C.
2. Next day, pick a single colony into 20µl of sterile water.
3. Use 1µl as template for the PCR reaction.
4. Add buffer, primers P1 and P2, dNTPs and polymerase enzyme according to the general PCR protocol (see above) and run the general PCR program (see above).
5. Purify PCR reaction to remove remaining template, primers, dNTPs, enzyme and buffer. **[6]** For that, separate it on a 1% agarose-gel, cut out the desired DNA band and isolate the DNA using the Qiagen Gel Extraction kit according to the manufacturer's instructions. **[7]**
6. Keep the purified DNA at 4°C for short-term storage and at -20°C for long-term storage.

Step 2: Generation of the *EGFP-Cre* fragment

1. Use pIGCN21 plasmid DNA as template and primers Pinv2 and P3 for the PCR reaction.
2. Add Phusion polymerase, buffer and dNTPs according to the general PCR protocol above.
3. Run the general PCR program.
4. Purify the PCR fragment as described in the previous step. **[7, 8]**

Step 3: Generation of the *bGHpA* fragment

1. Use pL452 plasmid DNA as template and primers Pinv3 and P4 for the PCR reaction.
2. Add Phusion polymerase, buffer and dNTPs according to the general PCR protocol above.
3. Run the general PCR program.
4. Purify the PCR fragment as described in step 1. **[7, 9]**

Step 4: Generation of the final *Ncr1-EGFP-Cre-bGHpA* fragment.

This reaction requires a specific PCR protocol as well as a specific PCR program. At first, the purified PCR products from steps 1 to 3 will be subjected to 5 cycles of PCR without addition of any primers in order to generate the full length *Ncr1-EGFP-Cre-bGHpA* fragment by extension of the respective overlaps produced.

Secondly, to amplify the full-length fragment simply add the external primers P1 and P4 and perform a PCR reaction.

Extension PCR protocol

Number of cycles	PCR step	Temperature (C°)	Time
Part I			
1	Initial denaturation	98	1 min
5	Denaturation	98	20s
	Annealing	60	1 min
	Extension	72	30s/kb

Part II			
Pause reaction at 10°C to allow for addition of primers, buffer and H2O			
30-32	Denaturation	98	20s
	Annealing	60	20s
	Extension	72	30s/kb
1	Final extension	72	10 min

1. Use equimolar amounts of the PCR fragments generated in steps 1to 3.
2. Add Phusion polymerase, buffer and dNTPs according to the general PCR protocol above.
3. Run part I of the PCR extension program.
4. Add primers P1 and P4 at final concentration of 250nM and adjust volume and buffer concentration as necessary.
5. Run part II of the extension PCR program.
6. Purify the PCR fragment as described in step 1. **[7, 10]**
7. Use 1µl of the purified PCR fragment to subclone the PCR product into the ZeroBlunt-Topo-PCR plasmid (ThermoFisher) according to the manufacturer's instructions.
8. Transform the competent bacteria provided with the ZeroBlunt-Topo-PCR cloning kit with the subcloning reaction according to the manufacturer's instructions.
9. Plate the transformation reaction on LB agar plates containing 100µg/L ampicillin and incubated at 37°C over night.

10. Pick bacteria colonies the following day into 15ml tubes containing 3ml LB medium with 100µg/mL ampicillin and incubate mini cultures over night at 37°C in a shaker at 200rpm.
11. The next day isolate plasmid DNA using 2ml of the mini cultures using the QiaPrep Spin Miniprep kit according to the manufacturer's instructions. Keep the remaining 1ml of each mini culture at 4°C for potential subsequent use.
12. The presence of the PCR insert is tested digesting the plasmid DNA with the restriction enzyme NotI, which linearizes the plasmid and in a second digest with the restriction enzyme EcoRI, which releases the insert and cuts it once.
13. Choose at least 4 bacterial clones carrying a plasmid with the correct PCR insert and sequence it.
14. Choose a plasmid carrying an error-free PCR insert and inoculate 500mL LB medium containing 100µg/mL ampicillin with the remaining volume of the respective mini culture.
15. The following day isolate the plasmid using the Qiagen EndoFree plasmid maxi prep kit.
16. Linearize 10µg of the plasmid DNA with NotI.
17. Purify the restriction digestion as described in step 1. **[7]**
18. Dialyse purified DNA against TE buffer for 18 hours using the slide-a-lyzer device according to the manufacturer's instructions.

The resulting DNA can now be used for pronuclear microinjections into C57BL/6J donor zygotes. This requires specific capabilities and equipment and should be performed by trained personal only. **[11]**

Part II

Generating compound mice for the conditional ablation of the target cell population

1. Introduction

A strategy for efficient ablation of a target cell types requires, besides the availability of a transgenic mouse line having the required cell-type specific Cre-expression, a loxP flanked target sequence whose deletion results in the loss of the cell in which the deletion occurs. There are multiple strategies to achieve this: either the Cre-mediated recombination i) removes an essential survival signal by targeting an indispensable component of a an essential growth factor-signaling pathway, or ii) it induces cell death via expression of a toxin (e.g. diphtheria toxin) or a toxin receptor (e.g., diphtheria toxin receptor that upon injection of diphtheria toxin A results in the death of the receptor expressing cell) normally not present in the mouse genome and whose expression is controlled by a loxP-flanked stop cassette.

For our purpose to ablate NK cells, we chose to target γ_c, encoded by *Il2rg*, which is an essential subunit of the receptors for the cytokines IL-2, -4, -7, -9, -15, and -21. It has been previously shown that IL-15 signaling is essential for the homeostasis of NK cells [6] and that conditional targeting of γ_c leads to rapid loss of NK cells carrying the mutation [7]. Given the fact that γ_c is located on the X chromosome the breeding strategy is straightforward:

2. Material

2.1 Mice

1. Male Ncr1-cre TG^{+} generated in part I
2. Female $\gamma_c^{flox/wt}$ mice (The Jackson laboratories)

3. Methods

3.1 Generating Ncr1-cre TG^{+} $\gamma_c^{flox/y}$ mice

Set-up crosses of male Ncr1-cre TG^{+} with female $\gamma_c^{flox/wt}$ mice. This represents the optimal breeding scheme. **[12, 13, 14]**

Part III

Using conditional ablation of *Ncr1* expressing cells to study their role in the defense against experimentally pulmonary metastases *in vivo*.

1. Introduction

One of the most common and effective models of metastatic cancer in the mouse is the B16 melanoma cell line [8]. B16 cells are derived from a naturally occurring melanoma in the C57BL/6 background [9]. Several sublines of B16 have been generated that possess different metastatic potential: B16-F1cells are poorly metastatic, while B16-F10 and B16-BL6 cells are both highly metastatic when injected intravenously. Moreover, B16-BL6 cells display an increased capacity to form metastasis from primary tumors [10–13]. Interestingly, B16F10 cells preferentially colonize the lung, thus representing a powerful tool to study pulmonary metastasis and the immune response against it.

However, many studies utilizing this metastasis model represent snapshot- or endpoint-analyses of those immune responses lacking information on the fourth dimension – time. The *in vivo* bioluminescence imaging technique represents one approach to investigate biological processes in whole animals over time. It is based on a naturally occurring biochemical reaction between the luciferase enzyme and its substrate, luciferin. The bioluminescent signal generated by this reaction can be detected even in deep tissues and through the skin of small animals so that the development of the signal strength in space and time can be monitored simply by providing at the appropriate times the substrate to the enzyme.

In part III, we provide a protocol using B16 melanoma cells expressing firefly luciferase (B16F10-luc2) together with the compound mice generated in parts I and II to study the role of NK cells in the immune response against the development of pulmonary metastases in the same cohort of mice in a non-invasive fashion over time [2].

1. Materials

1.1. Mice and cells

1. Tumor cells: B16F10-luc2 melanoma cells (PerkinElmer) represent B16F10 cells infected with pGL4 luc2 Lentivirus containing the firefly luciferase gene (luc2) under the control of the human ubiquitin C promoter.
2. Ncr1-cre TG^{+} $\gamma_c^{flox/y}$ mice and Ncr1-cre TG^{+} $\gamma_c^{+/y}$ littermate control mice generated in part I and part II.

1.2. Reagents:

1. B16F10-luc2 culture medium: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated FCS, 100 U/mL of penicillin streptomycin, 2 mM L-glutamine, and 5 mM mercaptoethanol.
2. 1X Dulbecco's Phosphate Buffered Saline (DPBS) without Mg^{2+} and Ca^{2+}.
3. 0.05% Trypsin-EDTA (Gibco).
4. D-Luciferin (Beetle luciferin, Promega).

1.3. Equipment:

1. T75 culture flasks.
2. Sterile 40 μm Cell strainer (BD Biosciences).
3. Cell-counting equipment.
4. 0,3 mL insulin syringe (U100- Insulin syringe, BD Micro-Fine + Demi; needle 30G) or other syringes with fine needles (25G-30G).
5. 0.2 μm filter (Millipore).

6. Bioluminescence imaging instrument, for example: IVIS (PerkinElmer) **[15].**
7. Isoflurane gas anesthesia system **[16]**.
8. Mouse restraining device (for the injections).
9. Computer with analysis software, for example *Living Image* software for IVIS Imaging Systems.

2. Methods for part III

2.1. Tumor cell culture

1. Culture tumor cells in appropriate culture medium (see Materials), in T75 culture flasks in a humid incubator, at 37°C, 5% CO_2 **[17]**.
2. Change medium frequently according to cell density every 2 to 3 days. To split cells, aspirate medium, wash cells 3 times with 5ml of pre-warmed 1X DPBS. Then add 1ml of trypsin-EDTA and place flask back in the incubator for 3 minutes **[18]**. Check the detachment of cells under the microscope. If no cell aggregates remain, add 10 ml of pre-warmed culture medium and pellet the cells (300g, 5', room temperature (RT)). Resuspend the cells in 9 ml of pre-warmed culture medium and seed 3ml in a new flask; add 7 ml of pre-warmed culture medium, mix carefully and place the new flask in the incubator.
3. Cells should be in logarithmic growth phase at day of injection, that is, the density should be around 50-70%. Check cell density the day before transplantation of the cells and add fresh culture medium to the flask. Do not split the cells at this time point. To harvest cells, trypsinize the cells as described (see above) but instead of resuspending the cells in culture medium wash the cells once with 10 ml of 1X DPBS at RT. Resuspend cells in 1ml of ice-cold 1X DPBS per flask used for the harvest and filter them through a 40 µm sterile filter**.**
4. Count cells.
5. Adjust the volume to reach the intended cell density and place the cells on ice. For example, for the injection of 10^6 cells **[19]** resuspend the cells at a density of $5*10^6$ cells/ml and inject 200 µL per mouse **[20].**

2.2. Tumor cell injection

It is not necessary to anesthetize the mice prior to the tumor cell transplantation. However, a mouse restraining device should be used **[21]**. This procedure should only be practiced by an experienced person as the precise injection of the required volumes/cell numbers consistently is crucial for the reproducibility and thus interpretability of the results.

1. It is common practice to dilate the mouse veins to ease the injection process **[22]**.
2. Using a 300 µL syringe with a 25-30G needle, slowly inject 200 µL of tumor cell suspension into the lateral caudal vein of the mice **[20, 23]**.

2.3. *In vivo* bioluminescence imaging

At each time point of measurement:

1. Prepare a fresh D-Luciferin solution at 30 mg/mL in 1X DPBS and filter it through a 0.2µm sterile filter. Keep it on ice and protected from light **[24]**.
2. Turn on the computer and bioluminescence imager and follow the instruction applicable to your imaging system **[15]**.
3. Shave the ventral side of the mice **[25]**.
4. Inject 150mg/kg of the D-Luciferin solution per mouse intraperitoneally. This represents 3mg for a 20g mouse and corresponds to 100 µL of the 30 mg/ml solution prepared **[26]**.
5. Transfer mice into the anesthesia induction chamber **[27]**.
6. Place the mice dorsally in the imaging chamber of the Bioluminescence Imager so that the ventral view positions the chest closest to the CCD

camera and connect the nose cones from the gas anesthesia system to assure that the mice do not move during image acquisition.

7. Adjust the position of the platform to best fit the field of view of the camera. This depends on the number of animals imaged in parallel. Adjust focus if necessary.
8. Take a black-and-white photo before acquisition to allow for localization of the bioluminescent signal once the photon image is superimposed over it.
9. Acquire data 15 minutes post D-Luciferin injection **[28]**, and during 15 to 20 minutes. The specific settings for acquisition depend on the imaging system used, number of mice imaged in parallel and the strength of the bioluminescent signal and have to be determined empirically by the experimenter for the model under study.

   The detected luminescent signal is expressed in photons/seconds/cm^2/steradian, and is a measure of the tumor load. Average radiance and area (cm^2) can be calculated using the system software.

2.4. *Ex vivo* bioluminescence imaging

Alternatively, *ex vivo* analysis of explanted tissues can be performed.

1. 5 minutes prior to euthanasia, inject 75mg/kg of D-Luciferin per mouse. This represents 1.5 mg for a 20g mouse and corresponds to 50 µL of the 30 mg/ml solution prepared **[26]**.
2. Sacrifice the mice and harvest the lungs **[29]**.

3. Place the lungs in a 24-well plate containing enough of a 30 µg/mL D-Luciferin solution per well to cover the tissue and incubate them for 10 minutes at RT. When imaging more than one lung at the same time avoid placing the lungs in neighboring wells.
4. Image the plate without removing the solution.
5. Acquire data according to the sections 3.3.2 and 3.3.8-9 of the Methods.

4. Notes

[1] If this plasmid is not available, other plasmids carrying an *EGFP-Cre* fusion are available at *addgene.org*. If you are using another plasmid than the one mentioned here, modify the sequence of the primer accordingly.

[2] If this plasmid is not available, other plasmids carrying the bovine growth hormone poly A site (bGHpA) are available at *addgene.org*. If you are using another plasmid than the one mentioned here, modify the sequence of the primer accordingly.

[3] The use of high quality cloning oligos is strongly recommended to assure sequence and thus cloning accuracy.

[4] Corresponding kits or equipment from other suppliers can be used.

[5] The program should be tested and adjusted on the specific thermal cycler that you will be using. Ideally, only the expected DNA fragments should be amplified by the described PCR reactions.

[6] The expected fragment size is 647bp.

[7] Avoid exposure of the DNA to UV light. For that, pore a 1% agarose gel without ethidium bromide. Prepare a gel with one oversized well (200µl volume) flanked by two normal sized wells (25µl volume). Load the DNA ladder into the left well, a small aliquot of the digest into the right well and the rest of the digest into the well in the middle. Run the DNA at low voltage (50V) and take care not to let it run too long. After the run, cut-off the gel slice containing the right well with the corresponding DNA lane and incubate it in gel running buffer with ethidium bromide for 15 minutes at RT. Wash briefly with clean gel running buffer and place gel slice on UV screen. Mark the boundaries of the wanted DNA fragment with

drops of loading buffer in the corner surrounding the fragment. Put slice back to original position at the gel and report the markings to the part of the gel containing the wanted DNA fragment of the remaining plasmid digest. Cut out the corresponding agarose gel piece and continue with the isolation of the DNA.

[8] The expected fragment size is 880bp.

[9] The expected fragment size is 350bp.

[10] The expected fragment size is 2777bp.

[11] A genotyping strategy should be developed beforehand. This can be based on detection of EGFP or Cre by PCR. In addition, PCR positive animals should be bled to verify expression of EGFP by NKp46+ cells in the blood and subsequently in all organs containing NKp46+ cells. Moreover, also the activity of Cre needs to be verified as well as its cell-type specificity. For this, transgenic animal whose NKp46+ cells in the peripheral blood express EGFP should be crossed to Cre-reporter mice. Those mice carry a reporter gene, whose expression is controlled by a 5' loxP-flanked stop cassette. In presence of Cre, this stop cassette will be removed leading to the expression of the reporter gene. If the reporter gene is a fluorescent protein its expression can be measured by FACS in peripheral blood NKp46+ cells at the same time as the expression of EGFP. The frequencies of EGFP+ reporter gene+, EGFP+ reporter gene-, EGFP- reporter gene+, should be determined in all organs containing NKp46+ cells.

[12] Half of the male offspring will carry $\gamma_c^{flox/y}$ and half of these will carry the Ncr1-cre TG in addition. This provides a good frequency of mice of interest (Ncr1-cre TG^+ $\gamma_c^{flox/y}$) and littermate controls (Ncr1-cre TG^+ $\gamma_c^{wt/y}$).

[13] Infidel Cre expression has been reported in several mouse models [14], including germline recombination and transient expression of Cre during

development [15]. This phenomenon can confound any experimental analysis and has to be controlled for. The crossing of the Cre-transgenic mice to Cre-reporter mice is therefore also helpful in detecting aberrant/infidel Cre activity, which is identified by the presence of cells expressing the reporter gene but that are unrelated to NKp46+ cells, like, for example, B cells or T cells.

Importantly, any genotyping strategy aiming at detecting the presence of Cre as well of a loxP flanked target gene should also include the detection of the deleted target gene allele [15].

[14] Mice expressing the cre transgene without any loxP flanked target gene allele should be used as controls. This is necessary because Cre can be toxic for cells. This might be due to DNA damage induced by Cre via recognition of cryptic target recognitions sites in the mouse genome [14].

[15] Several different Bioluminescence Imaging systems exist. It is important to acquire the necessary technical skills to operate the system in advance of the project or to seek help at a technical platform. Given the variety of different systems with their proprietary analysis software on the market we do neither provide information about the specific settings of the imaging system we used nor for the use of the respective software in order not to confuse the experimenter.

[16] Several different gas anesthesia systems exist. It is important to acquire the necessary technical skills to operate the system in advance of the project or to seek help at a technical platform. This system should only be used by trained personal.

[17] Make sure cells do not reach a confluent state.

[18] When most cells are detached, but float in clumps, take a 1 ml pipette and pipette cells carefully up and down to dislodge the clumps. Make sure to reach all

areas of the flask bottom. Check again under the microscope and continue the pipetting until you obtained a single cell suspension.

[19] The number of cells to inject depends, among other parameters, on the sensitivity of the Bioluminescent Imaging system, the strength of the bioluminescent signal, and the target organ. It is easier to detect bioluminescent signals from a subcutaneous region than from the liver or lung. For the latter, more events and thus more cells might be necessary to detect the presence of the tumor cells. Alternatively, tissues can be explanted for a more sensitive measurement. However, this procedure should be reserved, if possible, for the final acquisition time point. To determine the sensitivity of the system or – in other words - the minimal cell number necessary to detect a bioluminescent signal, a serial dilution of the luciferase expressing cells is prepared in a black 96-well plate and measured 5 minutes after addition of the substrate.

[20] B16 cells sediment rapidly at the bottom of the tube. To bring them back into suspension invert the tube gently avoiding bubbles. This has to be done frequently in order to prevent cells from aggregating and forming clumps: cell concentration would change and cell clumps could cause a pulmonary embolism killing the mice.

[21] Make sure that the device's size is suitable for the animal. If too small the mouse is put under unnecessary stress, if too big the mouse might be able to move and thus get injured during the injection.

[22] There are several ways to dilate the tail veins. One can place the whole animal in a warm environment (often under an overhead heat lamp) or by dipping the mouse tail into warm water. With the former method, it is difficult to control the temperature the mouse is exposed to and it represents additional stress to the animal if the whole body is exposed to an elevated temperature. We favor dipping the tails

of the mice in warm water (43-45°C) for a short period of time (30 – 60 seconds). Care needs to be taken not to use too warm water, which would harm the mice.

[23] After vasodilation place mice in restraining device. Hold tail firmly with index and thumb of non-dominant hand. Rotate tail slightly such that the lateral vein faces up. The vein is superficial and should well visible after the dilatation. Wipe injection site (at around half to 2/3 of the length of the tail) with alcohol. Bend tail slightly over index at site of injection and insert needle with bevel up into the vein towards the head. Keep the needle and syringe parallel to the tail. The needle should move smoothly into the vein without resistance. Inject the cells slowly. The injected volume should displace the blood temporarily such that the vein will appear clear. If there is resistance during injection and the tissue around the injection site dilates, stop injecting and remove the needle. Re-insert needle above the first site and continue the procedure. Remove needle after completion of injection and apply pressure with a clean, 70% ethanol-soaked gauze to stop bleeding. Place animal carefully back into its cage.

[24] Luciferin solution can be stored at -20°C.

[25] The dark fur of the C57BL/6 mice prevents the detection of the luminescent signal resulting from the luciferase-luciferin reaction. Nude or light haired mice do not require shaving [16]. If long-term experiments are undertaken, special attention has to be paid to the phenomenon of temporal variations of skin pigmentation during the re-growth of hair subsequent to hair removal as these variations might affect the detection of bioluminescent signals differentially [17].

[26] The optimal concentration and amount of luciferin to be injected has to be determined beforehand by the experimenter.

[27] The handling of the gas anesthesia system and the settings to be used depend on the type and version of equipment available. Please, contact the local person in charge of the system for details and advice.

[28] This delay time is necessary as the enzyme needs to diffuse through the body to reach the target organ and is influenced by the target tissue to be imaged, route of injection, metabolic state of the animal or if the animal is anesthetized or not. It thus needs to be empirically determined by the experimenter. For this, the required number of cells **[5]** is injected into the mouse and at the appropriate time point the substrate is injected via the route defined in the experimental protocol. The mice are imaged immediately every 2 minutes during 1 hour. The time required until the strongest signal stemming from the target organ is observed represents the delay time.

[29] Do not flush the lungs.

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